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ROLE OF ERK1/2 IN FSH-INDUCED PCNA EXPRESSION AND STEROIDOGENESIS IN GRANULOSA CELLS

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1. ABSTRACT

Follicular development is characterized by both proliferation and differentiation of granulosa cells (GCs) under the control of FSH. However, the cellular mechanism by FSH is not known. Using cultured GCs, we examined whether FSH-activated ERK1/2 was involved in the regulation of the proliferation-related gene proliferating cell nuclear antigen (PCNA) and steroidogenesis. GCs were obtained from the ovaries of DES-treated immature rats and cultured in serum-free medium. The results showed that FSH activated ERK1/2 in a time-dependent manner, with a peak at 20 min. Such activation was PKA-dependent as was inhibited by specific inhibitors. FSH induced PCNA expression in a time-dependent manner, with a maximum stimulation at 2 h. Similarly, StAR and steroid levels increased as FSH treatment time extended, with a maximum progesterone and StAR production at 48 h. ERK1/2 inactivation by UO126 inhibited the stimulatory effects of FSH on both PCNA and StAR expression and steroid synthesis in the GCs (p less than 0.01). Immunocytochemical studies further revealed that ERK1/2 inhibition led to a reduction of mitochondrial StAR in the GCs by FSH. These observations suggested that the stimulation of FSH on PCNA expression and steroidogenesis in GCs was mediated at least partially by ERK1/2.

2. INTRODUCTION

The organization of primordial follicles and the initiation of follicular growth occur in the absence of pituitary gonadotropins. However, small growing follicles are responsive to FSH, and the final stage growth of preovulatory follicles is strictly dependent on FSH action. In another word, follicular development beyond the early stages is absolutely FSH-dependent (1). Gene knockout experiments have demonstrated that follicular development is arrested in mice lacking FSH beta or FSH receptor in granulosa cells (GCs) (2-4). On one hand, FSH induces GC proliferation through promoting the expression and activity of proliferation-associated proteins such as cyclin D2 and cyclin dependent kinase 4 (CDK4) (5-7). Moreover, FSH and activin synergistically act to increase proliferating cell nuclear antigen (PCNA) and cyclin D2 in GCs (8). On the other hand, FSH also stimulates GC differentiation. For example, FSH promotes the expression of differentiation-related genes including receptors for FSH, LH and PRL, as well as steroidogenic enzymes such as cholesterol side-chain cleavage cytochrome P450, aromatase cytochrome P450 (P450arom) and 3beta-hydroxysteroid dehydrogenase (9). Despite of these observations been made, the molecular mechanism underlying FSH's regulation of GC proliferation and differentiation is still not well understood.

In the classical model, FSH binds to its cognate G-protein-coupled receptor and activates the membrane-associated adenylyl cyclase, resulting in the elevation of intracellular cAMP level. cAMP subsequently activates cAMP-dependent protein kinase A (PKA) leading to the phosphorylation or expression of cellular proteins controlling GC proliferation and differentiation (10-11). However, the intracellular signaling events occurring downstream of PKA activation in GCs remain uncharacterized. It is unclear whether the activation of the cAMP-PKA signaling pathway alone is sufficient to

account for the expression pattern of many genes during FSH-dependent follicular development. Recent studies have shown that in addition to PKA, FSH also activates multiple mitogen-activated protein kinases (MAPKs), such as extracellular signal-regulated kinases-1and -2 (ERK1/2) and p38 MAPK (12-16). ERK1/2 activation in a number of cells undergoing proliferation and differentiation in response to a variety of extracellular signals has well been documented (17-18). Members of MAPK super-family regulate acute cellular responses and transcriptional events via the phosphorylation of target enzymes and transcription factors (19-20)**.** FSH-activated ERK-2 induced the expression of proliferation-related gene cyclin D2 in rat GCs (5). Therefore, it is possible that FSH promotes the proliferation and differentiation of ovarian GCs through ERK1/2 activation.

It has been demonstrated that PCNA is a highly conserved auxiliary protein of DNA polymerase which is required for DNA synthesis and repair (21-22). Moreover it can be used as a molecular marker for cell proliferation (23). In ovarian GCs, the expression of PCNA begins upon the formation of primary follicle, and the expression level seems to increase during gonadotropin-dependent stages of preovulatory follicular development (24). Whether FSH-activated ERK1/2 plays a role in PCNA expression in cultured primary GCs is not known. Steroidogenesis is one of the marks of GC differentiation. Studies have shown that steroidogenic acute regulatory protein (StAR) is a rate-limiting protein in the process of steroidogenesis, and participates in the transport of cholesterol from mitochondrial outer membrane to inner membrane (25-26). To date, the observations on the role of ERK1/2 cascade on steroidogenesis were contradictory. For example, it was reported that the MAPK cascade inhibited gonadotropin-stimulated StAR expression in two granulosa-derived cell lines and human granulosa-luteal cells (27-28). However, Gyles et al (29) made the contrary conclusion with mouse adrenocortical Y1 cells that ERK1/2 stimulated cyclic AMP-increased the transcription of StAR gene. The role of gondatropins-activated ERK1/2 signaling pathway in steroidogenesis in cultured primary GCs is not clear.

Basing the above questions, we initiated the current study aiming at the investigation of the role of FSH-activated ERK1/2 in PCNA expression and steroidogenesis in GCs. Using GCs from the ovaries of DES-treated immature rat, we demonstrated that ERK1/2 activation by FSH stimulated PCNA and StAR expression and steroids synthesis.

3. MATERIALS AND METHODS

3.1. Materials and reagents

Culture materials were purchased from Corning, Inc. (Corning, NY). FSH (NIADDK-hFSH-I-3) was obtained from the National Hormone and Pituitary Distribution Program, National Institute of Arthritis, Diabetes, Digestive and Kidney Diseases. McCoy's 5a medium, specific PKA inhibitor H89, specific ERK1/2 inhibitor UO126, forskolin, monoclonal anti-beta-actin antibody, diethylstilbestrol (DES), 4-androstene-3,17-dione (androstenedione), soybean trypsin inhibitor, aprotinin and leupeptin were all purchased from Sigma-Aldrich Co. (St. Louis, MO). Antibodies for ERK1/2 and phospho ERK1/2 were from New England Biolabs, Inc. anti-PCNA antibody was from Santa Cruz Biotechnology, Inc. Antibody against StAR was generously provided by Dr. D. M. Stocco.

3.2. Experimental animals

Immature female Sprage-Dawley rats (23 days old) were obtained from the Experiment Animal Center, Chinese Academy of Sciences and housed under 16-h light, 8-h dark schedule with food and water *ad libitum*. The animals were treated in accordance with the NIH Guide for the care and Use of Laboratory Animals. All the protocols had the approval of the Institutional Committee on Animal Care and Use.

3.3. Primary cell culture

Female SD rats (23 days old) were injected with 0.5 mg DES /day for consecutive 3 days to increase follicular GC numbers. Then the GCs were harvested by puncturing the individual ovarian follicles with 25 gauge needles and collected by centrifugation (500×g, 5 min). The cells were washed three times with culture medium, and an aliquot of the cells was mixed with trypan blue stain for determining the cell number and viability. The cells were cultured overnight for adhesion in serum-free McCoy's 5a medium supplemented with 2 mM L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin sulfate at 37^0C in an atmosphere of 5% CO_2 and 95% air, and further incubated in the fresh medium with the presence or absence of the various reagents for the indicated times.

3.4. Steroid radioimmunoassay (RIA)

GCs (5×10^5 vial cells) were cultured overnight in a 24-well plate with 500 μl medium. Then the cells were treated with various reagents for the indicated time. For the assessment of estradiol production, 100 nM androstenedione, a substrate for P450arom, was added to the medium. By the end of culture, the conditioned media were collected and stored at -20^0C until assay. The levels of progesterone and estradiol in the media were measured by the standard RIA procedures.

3.5. Western blotting analysis

GCs (2×10^6 viable cells) were cultured for the indicated times in a six-well plate with 1.5 ml McCoy's 5a alone (control) or supplemented with FSH (50 ng/ml), H89 (10 μM) and/or UO126 (10 μM). The protein sample in each culture was prepared as described by Seger et al (27). Protein extract (20 μg) from each sample was separated by SDS-polyacrylamide gel electrophoresis, and transferred to nitrocellulose membranes, then probed with an antibody that recognizes both the non-phosphorylated and phosphorylated (total) isoforms of ERK1/2 and with an antibody that recognizes only the phosphorylated (active) isoforms. PCNA and StAR immunoreactivities were detected by their respective specific antibodies. The antibody binding was detected by enhanced chemiluminescence.

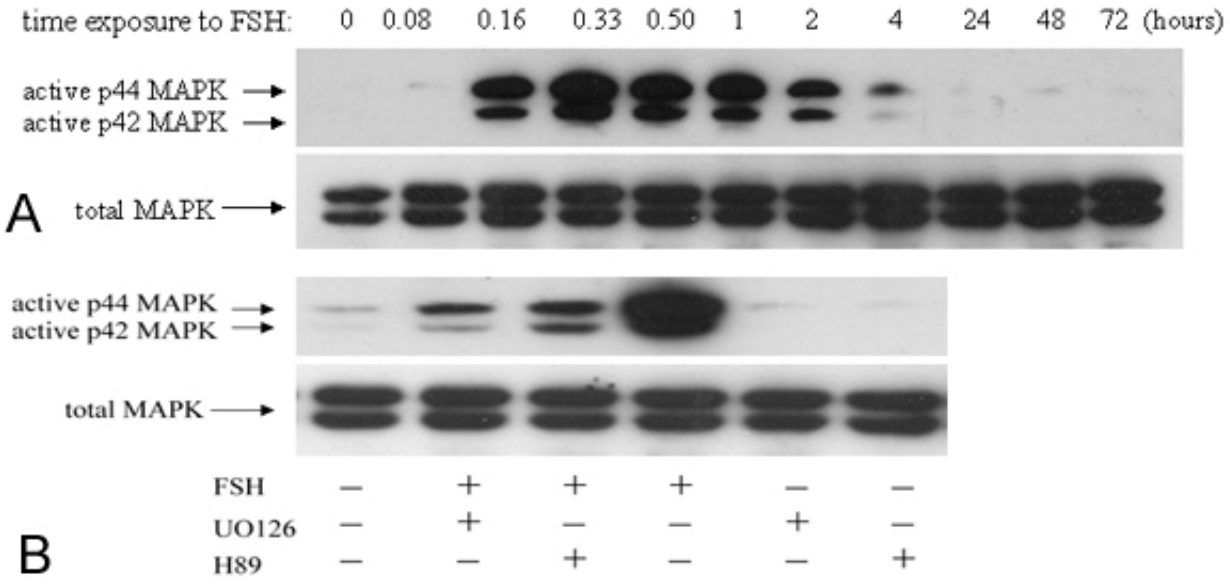

Figure 1. FSH induced ERK1/2 activation in a time- and PKA-dependent manner. Granulosa cells (GCs) were harvested from the ovaries of DES-primed immature female rats and cultured overnight at 37^0C in serum-free McCoy's 5a media. A, GCs (2×10^6 viable cells) were treated with 50 ng/ml FSH for different time spans as indicated; B, GCs were cultured with PKA inhibitor H89 (10 μM) or ERK1/2 inhibitor UO126 (10 μM) for 20 min, followed by treatment with 50 ng/ml FSH for 20 min. Subsequently, the cell lysates were made and subjected to immunoblotting analysis using antibodies recognizing either phosphorylated ERK1/2 or total ERK1/2. Immunoreactive bands were visualized by enhanced chemiluminescence (ECL).

3.6. Confocal immunohistochemistry

GCs were seeded onto 24×24-mm coverslips placed in 6-well plates at a density of 2×10^5 cells/coverslip. The cells were cultured overnight for adhesion in McCoy's 5a medium at 37°C and then treated with 10 μM H89 or 10 μM UO126 for 20 min, followed by the incubation with 50 ng/ml FSH for 48 h. By the end of incubation, the media were removed and the cells were fixated with 4% paraformaldehyde for 30 min, followed by incubation for 1 h with 2% goat serum in phosphate-buffered saline (pH 7.4), 0.01% Triton-100× (PBS-Triton buffer). Then the cells were incubated overnight with an antibody to StAR (1:200) at 4□. The cells were washed with PBS-Triton buffer three times and further incubated with goat anti-rabbit antibody conjugated fluorescein (1:100) at room temperature for 1.5 h. The antibody binding was visualized using Leica confocal microscope. For negative controls, the cells were incubated with non-immune rabbit serum, followed by the second antibodies.

3.7. Statistical analysis

All the experiments were repeated three times with GC preparations obtained from separate groups. All values were expressed as the mean ± SD (n=3). Differences between groups were analyzed for statistical significance using analysis of variance (SPSS Standard Version 10.0.1. SPSS Inc. Chicago, IL). P value<0.05 were accepted as statistical significance. For the immunofluorescence data, one representative was shown from three similar independent experiments.

4. RESULTS

4.1. FSH induced ERK 1 / 2 activation in a time- and PKA-dependent manner

GCs were collected from ovaries from DES-treated immature rats and incubated with FSH for 72 h. Activation of ERK1/2 was assessed by immunoblotting with a phospho-specific, anti-active ERK antibody that detects dually phosphorylated ERK1 and ERK2. As shown in Figure 1A, treatment of GCs with FSH (50 ng/ml) resulted in a transient activation of ERK1/2 kinases. Phosphorylated ERK1/2 were detected at 5 min, peaked at 20 min, and then slowly decreased to undetectable level by 48 h. Reprobing the membranes using a antibody recognizing both the phosphorylated and nonphosphorylated forms of ERK1/2 indicated that FSH specifically altered MAPK activity but not its expression levels. Subsequently, a 20-min incubation period was chosen for experiments. To further confirm the specificity of FSH-induced ERK1/2 activation, the cells were pretreated with PKA inhibitor H89 (10 μM) or ERK1/2 inhibitor UO126 (10 μM) for 20 min, followed by treatment with FSH for 20 min. Incubation of GCs with H89 or UO126 alone did not influence the level of phosphorylated-ERK1/2. However, they respectively inhibited the phosphorylation of ERK1/2 stimulated by FSH (Figure 1B). Collectively, these results indicated that FSH stimulated the phosphorylation of ERK1/2 in a time-dependent manner, and that FSH-induced ERK1/2 activation was predominately mediated by PKA.

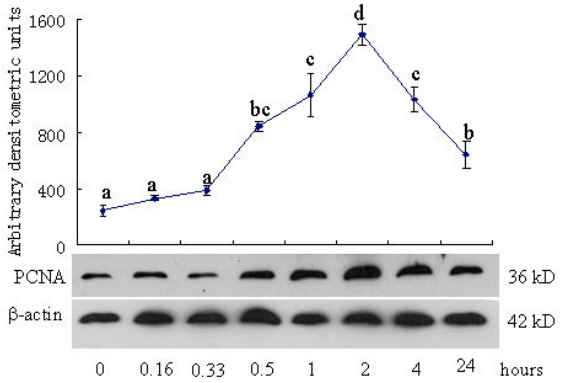

Figure 2. FSH stimulated PCNA expression in a time-dependent manner. GCs were cultured as described in Fig. 1. The cells (2×10^6 viable cells) were treated with 50 ng/ml FSH for different time spans as indicated. PCNA protein level was detected by Western blotting. Densitometric values of PCNA were normalized by beta-actin content. These experiments were repeated three times. Data were shown as means ± SD (n=3). Different letters labeled values that were significantly different at $p < 0.05$.

4.2. FSH stimulated PCNA expression in a time-dependent manner

The effect of FSH on PCNA expression in cultured primary GCs was assessed by Western blotting. As shown in Figure 2, PCNA protein was low in GCs in the absence of FSH. The low protein level was consistent with the low proliferation rate of early antral follicular in the absence of extracellular signals. PCNA did not significantly increase as incubation time extended (data not shown). In contrast, PCNA protein level increased in a time-dependent manner after exposure to FSH. PCNA protein was significantly induced at 30 min ($p<0.05$), reached a peak at 2 h, and decreased thereafter. Thus, a 2-hour incubation period was chosen for the following experiments.

4.3. FSH increased progesterone and estradiol production and StAR protein expression

Steroidogenesis in the GCs in response to FSH was studied. As shown in Fig. 3, progesterone (Figure 3A) and StAR (Figure 3C) production were stimulated by FSH in a time-dependent manner (0-72 h). The magnitude of progesterone production was significant only by 24 h ($p<0.01$), reaching the peak level at 48 h, and then starting to decline. StAR protein was detected at 6 h, the magnitude became significant at 12 h ($p<0.01$), increased gradually up to 48 h, and then dropped slightly by 72 h. The time course of StAR expression was similar to that of progesterone secretion. At the same time, estradiol level increased as FSH treatment time prolonged, but no decrease in the steroid production was observed by 72 h. (Figure 3B).

4.4. ERK1/2 inactivation inhibited FSH-induced PCNA expression and steroid synthesis

In the following experiments, we sought to determine whether FSH-activated ERK1/2 signaling pathway played a role in PCNA expression and steroid production. The GCs were pretreated with 10 μM UO126 or 10 μM H89 for 20 min, followed by incubation with 50 ng/ml FSH for 2 h. As shown in Figure 4, UO126 used alone had little influence on the level of PCNA protein, however it significantly inhibited FSH-stimulated PCNA expression. Parallel studies were carried out to examine the effect of the inhibitors on progesterone and estradiol synthesis. Inhibition of ERK1/2 activity by UO126 caused a marked decrease in FSH-increased progesterone and estrodial production ($p<0.01$) (Figure 5). Similarly, PKA inactivation by H89 also significantly inhibited FSH-promoted PCNA expression and steroids synthesis.

4.5. Inhibition of ERK1/2 activity attenuated FSH-stimulated StAR expression

Due to StAR being a rate-limiting protein in steroidogenesis, we further examined the role of the MAPK on the expression and localization of StAR protein by Western blotting and immunocytochemistry techniques. As shown in Figure 6, FSH strongly stimulated StAR protein expression ($p<0.01$) as compared with control groups. ERK1/2 inactivation by UO126 significantly attenuated FSH-induced StAR expression ($p<0.01$), correlated well with the action of UO126 on FSH-stimulated steroid production. To verify whether inhibition of ERK1/2 activity leads to a decrease in the mitochondrial steroidogenic protein, GCs were incubated with FSH in the presence or absence of UO126 for 48 h. Immunocytochemical data using specific antibody revealed that StAR protein was localized in the mitochondria of GCs treated with FSH (27) and the staining was reduced by the addition of UO126 (Figure 7). The immunocytochemical observations further confirmed the results obtained by Western blotting. Inhibition of PKA activity by H89 also caused a marked reduction in StAR protein level in the GCs in response to FSH.

5. DISCUSSION

It is well known that FSH is the primary stimulus for ovarian follicular growth and differentiation. However, the intracellular signaling circuits utilized by FSH are not well characterized. Previous studies have mainly focused on cAMP-PKA pathway. FSH receptor occupancy results in the activation of adenyl cyclase, followed by the elevation of intracellular cAMP and subsequent activation of PKA (10-11). cAMP analogs or agents can mimic the differentiating actions of FSH, showing that the cAMP-PKA pathway may be the key second messenger-activated pathway in FSH's action (9). The cellular signaling events occurring downstream of PKA activation in immature rat ovarian cells remain undetermined. Using cultured primary GCs, we demonstrated that FSH rapidly induced the activation of ERK1/2 kinases in a time-dependent manner, reaching the peak level at 20 min and decreasing to the undetected level after 48 h. Time course of ERK1/2 activation by FSH was similar to that of LH-activated ERK1/2 in the GCs from preovulatory follicles (30). In the present work, we showed that FSH-induced ERK1/2 activation was PKA-dependent as indicated by the results using specific inhibitors. The similar conclusion was recently reported for immature and preovulatory GCs (13, 30).

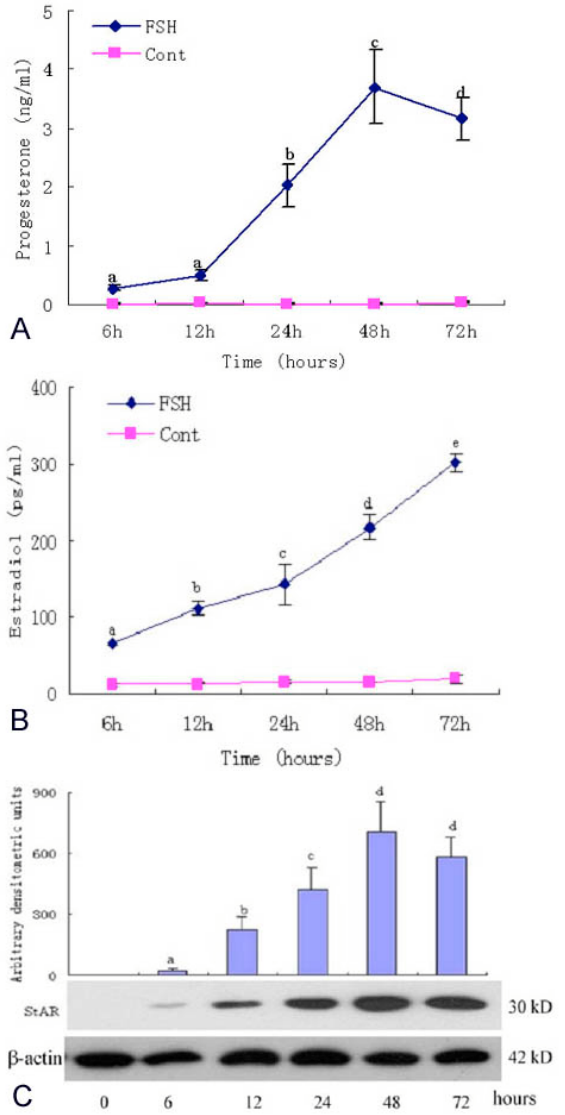

Figure 3. FSH increased progesterone and estradiol production and StAR protein expression. GCs were cultured as described in Fig. 1. 5×10^5 vial cells were incubated with FSH (50ng/ml) for RIA analysis of progesterone and estradiol production. 2×10^6 viable cells were treated with FSH (50ng/ml) for Western blotting analysis of StAR protein. These experiments were repeated three times. Data were shown as means ± SD (n=3). Different letters labeled values that were significantly different at $p < 0.05$.

PCNA is a 35 kD nuclear protein and plays a role in eukaryotic cell cycle progression, DNA synthesis, and DNA repair. Experiments blocking PCNA expression using antisense oligonucleotides suggest it is essential for DNA replication (31). It can be used as a molecular maker for proliferating cells (23). In addition to its function as an accessory factor for DNA polymerase delta, PCNA may also influence the G1 and S phase of the cell cycle by its ability to form complexes with cyclin D, cyclin-dependent kinases as well as the cyclin-dependent kinase inhibitor p21 (32). In the current study, we investigated the effect of FSH on the expression of PCNA gene in cultured primary GCs. The results showed that FSH increased PCNA protein in a time-dependent manner with the peak at 2 h (Fig. 2). The FSH-induced PCNA expression in cultured GCs was similar with the results by previous studies, which reported that PCNA expression in ovarian GCs was correlated with follicular growth, and eliminated in the atretic follicles or non-proliferating corpus luteum (33). In addition, we found that there was also low PCNA protein level in the control groups. The reason may be that intraovarian factors may contribute to the expression of PCNA protein. It has been demonstrated that before GCs become competent to response to FSH, PCNA protein appeared in the GCs of primary and preantral follicles (24). However, our result was inconsistent with the report by El-Hefnawy et al (8), in which the up-regulation of PCNA expression by FSH was not observed. The reason for this discrepancy could be due to different culture conditions used: the GCs used in our experiment were obtained from the ovaries of DES-treated immature rats, while the GCs by El-Hefnawy et al were from untreated immature rats. Secondly, we did use serum-free McCoy's 5a medium during the experiments, while they cultured the cells first with M199 containing 10% FBS overnight and then with serum-free M199 for further culture.

Analysis of the PCNA promoter has shown that cAMP response element is essential for stimulus-induced transcription (34-35). Accordingly, cAMP-PKA plays an important role in PCNA expression. Indeed, PKA inactivation significantly inhibited the promotion of FSH on PCNA expression (Fig. 4), indicating that PKA is an important signal transducing element in the regulation. In addition to PKA, we demonstrated that activated ERK1/2 by FSH also stimulated PCNA expression in cultured primary GCs. ERK1/2 inactivation by UO126 resulted in a significant decrease in PCNA protein level. Similarly, the mitogenic action of TSH has been reported to be associated with an induction of PCNA expression in a cAMP-dependent manner in thyroid epithelial cells (36). ERK1/2 activation has been implicated in the mitogenic responses of human H295R or mouse Y1 cells to angiotensin II or ACTH respectively (37-38). These data suggest that FSH-activated ERK1/2 may stimulate GC proliferation through inducing PCNA expression.

It is unequivocal that steroidogenesis is an important hallmark for GC differentiation. Abundant evidences have shown that StAR is a rate-limiting protein in biosynthesis of steroid hormones and plays a role in mediating the intra-mitochondrial transport of cholesterol to the cholesterol side chain cleavage cytochrome P450 enzyme (25-26). Rapid expression of StAR in response to extracellular signals is required to initiate and maintain steroid hormone biosynthesis. With steroidogenesis as a marker for GC differentiation, we studied the effect of FSH on GC differentiation through examining StAR protein and steroid levels, examining the role of FSH-activated ERK1/2 in the intracellular signaling transduction. We demonstrated that FSH induced StAR protein expression in GCs in a time-dependent manner, similar to that of FSH-increased

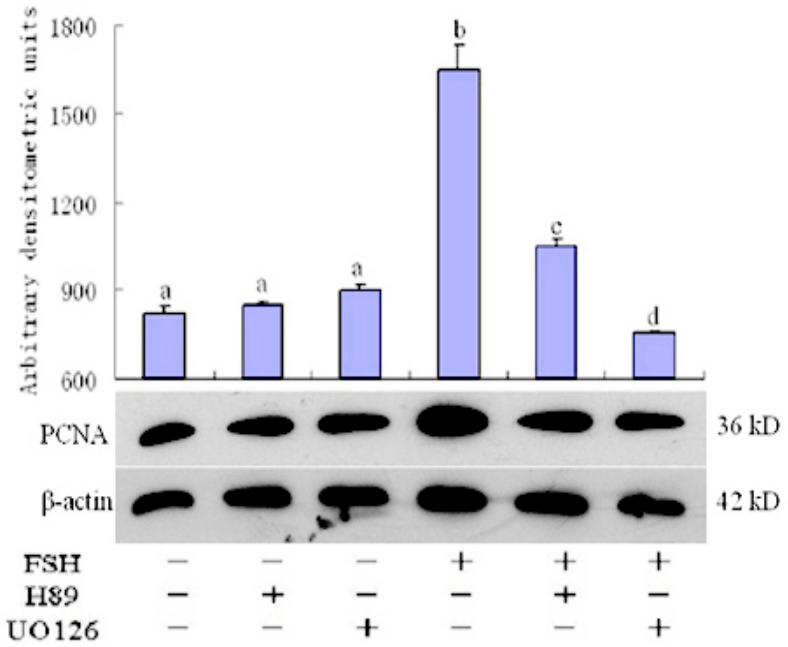

Figure 4. ERK1/2 inactivation inhibited FSH-induced PCNA expression. 2×10^6 viable GCs were cultured and incubated with H89 (10 μM) or UO126 (10 μM) for 20 min, followed by the treatment with FSH (50ng/ml) for 2 h. The cell lysates were subjected to Western blotting using anti-PCNA or anti-beta-actin antibodies. Densitometric values of PCNA protein were normalized by beta-actin content. The experiments were repeated three times. Data were represented as mean ± SD (n=3). Bars with different letters indicated means that were significantly different at $p < 0.05$.

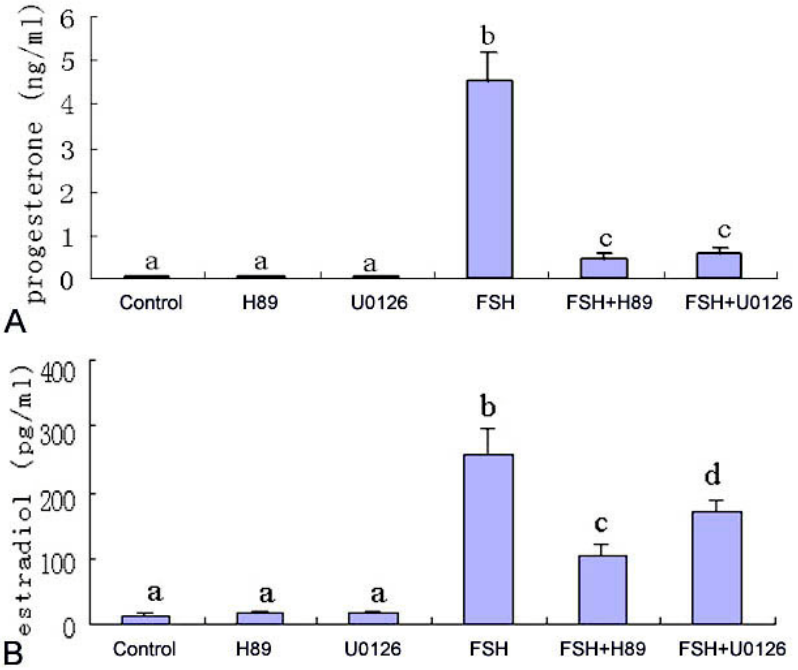

Figure 5. ERK1/2 inactivation blocked FSH-promoted steroid synthesis. GCs (5×10^5 vial cells) were cultured in serum-free media and treated with H89 (10 μM) or UO126 (10 μM) for 20 min, followed by the incubation with FSH (50 ng/ml) for 48 h . Progesterone (A) and estradiol (B) level in the media was measured by RIA. These experiments were repeated three times. Data were shown as means ± SD (n=3); Bars with different letters indicated means that were significantly different at $p < 0.05$.

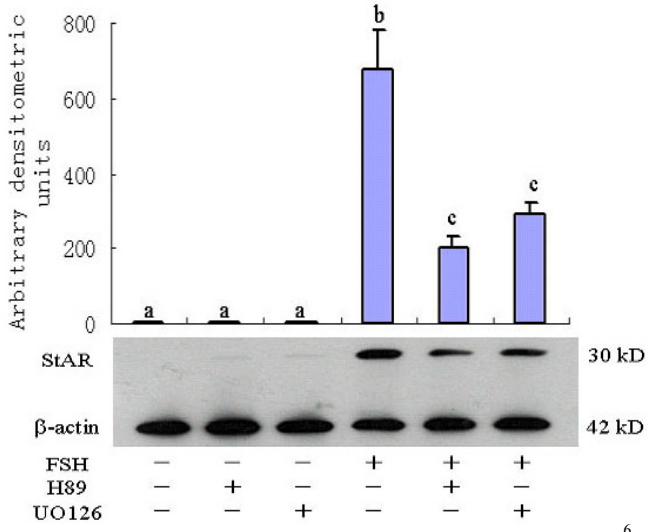

Figure 6. Inhibition of ERK1/2 activity attenuated FSH-stimulated StAR expression. 2×10^6 viable GCs were cultured and incubated with H89 (10 μM) or UO126 (10 μM) for 20 min, followed by the treatment with FSH (50ng/ml) for 48 h. The cell lysates were subjected to Western blotting using anti-StAR or anti-beta-actin antibodies. Densitometric values of StAR protein were normalized by beta-actin content. The experiments were repeated three times. Data were represented as mean ± SD (n=3). Bars with different letters indicated means that were significantly different at $p < 0.01$.

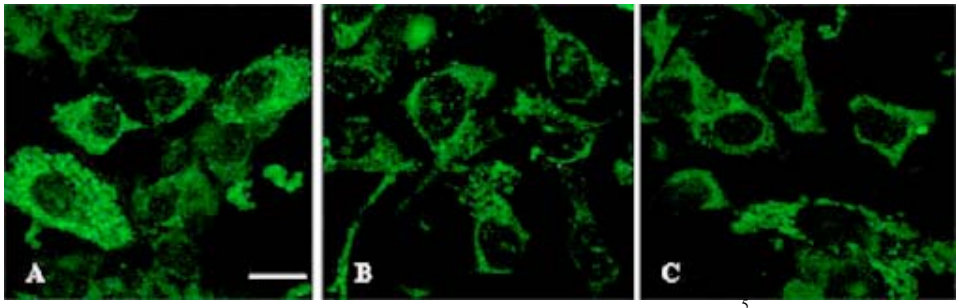

Figure 7. Subcellular localization of StAR upon incubation with FSH and inhibitors. 2×10^5 viable GCs were cultured overnight for adhesion and treated with H89 or UO126 for 20 min, followed by incubation with FSH for 48 h. Concentration of each agent was as previously described. The sub-confluent GCs were further incubated with the anti-StAR antibodies, and then probed with goat anti-rabbit IgG conjugated to fluorescein. The localization of StAR in GC mitochondria was manifested by dotted green fluorescence. Magnification is 1000 fold. The length of the horizontal bar represents 10 μm.

steroid synthesis. The stimulation of FSH on steroidogenesis was dramatically blocked by UO126. The confocal data further showed that ERK1/2 inactivation resulted in a decrease in StAR content in the mitochondria of GCs treated by FSH. These data indicate that ERK1/2 activation is very important in the initiation of GC steroidogenesis by FSH.

A number of recent studies have suggested that the MAPK cascades are involved in the regulation of steroidogenesis, although many of the observations reported to date appear to be contradictory. In the present work, we verified that ERK1/2 activation mediated FSH-stimulated steroidogenesis in the cultured primary GCs. Similarly, LH and FSH were reported to activate ERK1/2 and enhanced steroid production in ovarian cells (12, 14). In Y1 cell line (29), the activation of ERK1/2 by forskolin was linked to increase steroid production, and the cAMP-induced steroidogenesis was inhibited by the inhibitors of ERK1/2. However, there were also several studies showing that ERK1/2 activation inhibited gonadotropin-induced steroid synthesis in granulosa-derived cell lines and human granulosa-luteal cells (27-28). A likely explanation may be that there is the existence of multiple transduction pathways coupled to cell surface receptors and the difference in receptor-effector coupling between cell types, cell lines and cell development stages.

To date, several downstream targets of MAPK kinases have been identified including the 90-kDa ribosomal S6 protein kinase (RSK), cytoplasmic phospholipase A2, MAPK-activated protein kinase-2 and transcriptional factors such as c-myc and Elk-1 (13, 18, 39). Therefore, it can be speculated that activated ERK1/2 may activate uncharacterized signaling molecules leading to GC specific genes expression. Analysis of gene sequence has shown that StAR gene promoter contains a few sites for transcriptional factors (26, 40-41), among which steroidogenic factor-1 (SF-1) has been demonstrated to promote StAR gene transcription (42). In Y1 cell, ERK1/2

activation by forskolin resulted in enhanced phosphorylation of SF-1 and increase in StAR expression and steroid production (29). It was not surprising that PKA was involved in FSH-induced PCNA expression. Because the promoter of PCNA contains a cAMP response element (34-35) and PKA can regulate PCNA expression by activation of CREB. In addition to PKA, ERK1/2 activation by FSH was also demonstrated to stimulate PCNA expression in the current work. It has been reported that RSK-2 is capable of phosphorylating CREB at the serine 133 residue (43), which is obligatory for transactivation (44). It is likely that FSH-activated ERK1/2 may induce CREB phosporylation and subsequent PCNA expression through RSK activation in the primary GCs.

In summary, the current study provided the evidence to show that FSH promoted PCNA expression, and then increased the cell ability of steroidogenesis in the cultured primary GCs. It is suggested that the activated ERK1/2 may be involved in the regulation of ovarian GC proliferation and differentiation through mediating FSH-induced PCNA expression and steroidogenesis.

6. ACKNOWLEDGEMENT

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7. REFERENCES

1. Zeleznik, A. K. and D. F. Benyo: Control of follicular development, corpus luteum function, and recognition of pregnancy in higher primates. In: Physiology of reproduction 2nd ed. Eds: Knobil, E., Neill, J.D., Raven Press, NY vol 2, 751-782 (1994)
2. Abel, M. H., A. N. Wooton, V. Wilkin, I. Huhtaniemi, P. G. Knignt and H. M. Charlton: The effect of a null mutation in the follicle stimulating hormone receptor gene on mouse reproduction. *Endocrinology* 14, 1795-1803 (2000)
3. Dierich, A., M. R. Sairam, L. Monaco, G. M. Fimia, A. Gansmuller, M. Le Meur and P. Sassone-Corsi: Impairing follicle-stimulating hormone (FSH) signaling in vivo: targeted disruption of the FSH receptor leads to aberrant gametogenesis and hormone imbalance. *Proc Natl Acad Sci USA* 95, 13612-13617 (1998)
4. Kumar, T. R., Y. Wang, N. Lu and M. M. Matzuk: Follicle stimulating hormone is required for ovarian follicle maturation but not male fertility. *Nat Genet* 15, 201-204 (1997)
5. Kayampilly, P. P. and K. M. J. Menon: Inhibition of Extra cellular signal-regulated protein kinase-2 phosphorylation by dihydrotesterone reduces FSH mediated cyclin D2 mRNA expression in rat granulosa cells. *Endocrinology* 145, 1786-1793 (2004)
6. Rober, R. L. and J. S. Richards: Hormone-induced proliferation and differentiation of granulosa cells: a coordinated balance of the cell cycle regulators cyclin D2 and p27Kip1. *Mol Endocrinol* 12, 924-940 (1998)
7. Yang, P. and S. Roy: FSH-induced DNA synthesis in the granulosa cells of hamster preantral follicles involves activation of CDK4 rather than cyclin D2 synthesis. Biol Reprod 70, 509-517 (2004)
8. El-Hefnawy, T. and A. J. Zeleznik: Synergism between FSH and activin in the regulation of proliferating cell nuclear antigen (PCNA) and cyclin D2 expression in rat granulosa cells. *Endocrinology* 142, 4357-4362 (2001)
9. Hsueh, A. W. J., E. Y. Adashi, P. B. C. Jones and Jr. T. H. Welsh: Hormonal regulation of the differentiation of cultured ovarian granulosa cells. *Endocr Rev* 5, 76-110 (1984)
10. Gonzalez-Robayna, I. J., T. N. Alliston, P. Buse, G. L. Firestone and J. S. Richards: Functional and subcellular changes in the A-kinase-signaling pathway: relation to aromatase and sgk expression during the transition of granulosa cells to luteal cells. *Mol Endocrinol* 13, 1318-1337 (1999)
11. Richards, J. S.: Hormonal control of gene expression in the ovary. Endocr Rev 15, 725-751 (1994)
12. Cameron, M. R., J. S. Foster, A. Bukovsky and J. Wimalasena: Activation of mitogen-activated protein kianses by gonadotropins and cyclic adenosine 5'-monophosphates in porcine granulosa cells. *Biol Reprod* 55, 111-119 (1996)
13. Das, S., E. T. Maizels, D. DeManno, St. Clair E. Adam S A. and M. Hunzicker-Dunn: A stimulatory role of cyclic adenosine 3',5'-monophosphorylate in follicle stimulating hormone-activated mitogen-activated protein kinase signaling pathway in rat ovarian granulosa cells. *Endocrinology* 137, 967-974 (1996)
14. Dewi, D. A., D. R. Abayasekara and C. P. Wheeler-Jones: Requirement for ERK2/1 activation in the regulation of progesterone production in human granulosa-luteal cells is stimulus specific. *Endocrinology* 143, 877-888 (2002)
15. Gonzalez-Robayna, I. J., and A. E. Falender, S. Ochsner, G. L. Firestone, J. S. Richards: Follicle-stimulating hormone (FSH) stimulates phosphorylation and activation of protein kinase B (PKB/Akt) and serum and glucocorticoid-induced kinase (Sgk): evidence for a kinase-independent signaling by FSH in granulosa cells. *Mol Endocrinol* 14, 1283-1300 (2000)
16. Maizels, E. T., J. Cottom, J. C. R. Jones and M. Hunzicker-Dunn: Follicle stimulating hormone (FSH) activates the p38 mitogenic-activated protein kinase pathway, inducing small heat shock protein phosphorylation and cell rounding in immature rat ovarian granulosa cells. *Endocrinology* 139, 3353-3356 (1998)
17. Johnson, G. L. and R. R. Vaillancourt: Sequential protein kinase reactions controlling cell growth and differentiation. *Curr Opin Cell Biol* 6, 230-238 (1994)
18. Marshall, C. J.: MAP kinase kinase kinase, MAP kinase kinase and MAP kinase. *Curr Opin Gen Dev* 4, 82-89 (1994)
19. Denhardt, D. T.: Signal-transducing protein phosphorylation cascades mediated by Ras/Rho proteins in the mammalian cell: the potential for multiplex signaling. *Biochem J* 318, 729-747 (1996)
20. Kyriakis, J. M. and J. Avruch: Sounding the alarm: protein kinase cascades activated by stress and inflammation. *J Biol Chem* 271, 24313-24316 (1996)
21. Jaskulski, D., J. K. DeRiel, W. E. Mercer, B. Calabretta and R. Baserga: Inhibition of cell proliferation by antisense oligodeoxynucleotides to PCNA cyclin. *Science* 240, 1544-1546 (1988)

22. Li, S.: Relationship between cellular DNA synthesis, PCNA expression and sex steroid hormone receptor status in the developing mouse ovary, uterus and oviduct. *Histochemistry* 102, 405-413 (1994)
23. Hall, P. A. and D. A. Levison: Review: assessment of cell proliferation in histological material. *J Clin Pathol* 43, 184-192 (1990)
24. Oktay, K., R. S. Schenken and J. F. Nelson: Proliferating cell nuclear antigen marks the initiation of follicular growth in the rat. *Biol Reprod* 53, 295-301 (1995)
25. Christenson, L. K. and J. F. Strauss: Steroidogenic acute regulatory protein (StAR) and the intramitochondrial translocation of cholesterol. *Biochem Biophys Acta* 529, 175-187 (2001)
26. Stocco, D. M.: Intramitochondrial cholesterol transfer. *Biochem Biophys Acta* 1486, 184-197 (2000)
27. Seger, R., T. Hanoch, R. Rosenberg, A. Dantes, W. E. Merz, J. F. Strauss III and A. Amsterdam: The ERK signaling cascade inhibits gonadotropin-stimulated steroidogenesis. *J Biol Chem* 276, 13957-13964 (2001)
28. Tajima, K., A. Dantes, Z. Yao, K. Sorokina, F. Kotsuji, R. Seger and A. Amsterdam: Down-regulation of steroidogenic response to gonadotropins in human rat preovulatory granulosa cell involves mitogen-activated protein kinase activation and modulation of DAX-1 and steroidogenic factor-1. *J Clin Endocrinol Metab* 88, 2288-2299 (2003)
29. Gyles, S. L., C. J. Burns, B. J. Whitehouse, D. Sugden, P. J. Marsh, S. J. Persaud and P. M. Jones: ERKs regulate cyclic AMP-induced steroid synthesis through transcription of the steroidogenic acute regulatory protein (StAR) gene. *J Biol Chem* 276, 34888-34895 (2001)
30. Salvador, L. M., E. Maizels, D. B. Hales, H. E. Miyamoto, H. Yamamoto and M. Hunzicker-Dunn: Acute signaling by the LH receptor is independent of protein kinase C activation. *Endocrinology* 143, 2986-2994 (2002)
31. Liu, Y. C., R. L. Marraccino, P. C. Keng, R. A. Bambara, E. M. Lord, W. G. Chou and S. B. Zain: Requirement for proliferation cell nuclear antigen expression during stages of the Chinese hamster ovary cell cycle. *Biochemistry* 28, 2967-2974 (1989)
32. Tsurimoto, T.: PCNA binding proteins. *Front Biosci* 4, 849-858 (1999)
33. Somers, J. P., D. F. Benyo, L. L. Little-Ihrig and A. J. Zeleznik: Luteinization in primates is accompanied by a loss of a 43-kilodalton adenosine 3',5'-monophosphate response element-binding protein isoform. *Endocrinology* 136, 4762-4768 (1995)
34. Huang, D., P. M. Shippmen-Appsasmy, D. J. Ortenm, S. H. Hinrichsom and M. B. Prystowsky: Promoter activity of the proliferating-cell nuclear antigen gene is associated with inducible CRE-binding proteins in interleukin 2-stimulated T lymphocytes. *Mol Cell Biol* 14, 4233-4243 (1994)
35. Lee, B. H. and M. B. Matthews: Transcriptional coactivator cAMP response element binding protein mediates induction of the human proliferating cell nuclear antigen promoter by the adenovirus E1A oncoprotein. *Proc Natl Acad Sci USA* 94, 4481-4486 (1997)
36. Baptist, M., J. E. Dumont and P. P. Roger: Demonstration of cell cycle kinetics in thyroid primary culture by immunostaining of proliferating cell nuclear antigen: differences in cyclic AMP-dependent and – independent mitogenic stimulation. *J Cell Sci* 105, 69-80 (1993)
37. Lotif, C. F., Z. Todorovic, H. A. Armelin and B. P. Schimmer: Unmasking a growth-promoting effect of the adrenocorticotropic hormone in Y1 mouse adrenocortical tumor cells. *J Biol Chem* 272, 29886-29891 (1997)
38. Watanabe, G., R. J. Lee, C. Albanese, W. E. Rainey, D. Batle and R. G. Pestell: Angiotensin II activation of cyclin D1-dependent kinase activity. *J Biol Chem* 271, 22570-22577 (1996)
39. Davis, R. J.: The mitogenic-activated protein kinase signal transduction pathway. *J Biol Chem* 268, 14553-14556 (1993)
40. Manna, P. R., D. W. Eubank and D. M. Stocco: Assessment of the role of activator protein-1 on transcription of the mouse steroidogenic acute regulatory protein gene. *Mol Endocrinol* 18, 558-573 (2004)
41. Wooton-Kee, C. R. and B. J. Clark: Steroidogenic factor-1 influences protein-deoxyribonucleic acid interactions within the cyclic adenosine 3',5'-monophosphate-responsive regions of the murine steroidogenic acute regulatory protein gene. *Endocrinology* 141, 1345-1355 (2000)
42. Morohashi, K. I. and T. Omura: Ad4bp/SF-1, a transcription factor essential for the transcription of steroidogenic cytochrome P450 genes and for the establishment of the reproductive function. *FASEB J* 10, 1569-1577 (1996)
43. Xing, J., D. D. Ginty and M. E. Greenberg: Coupling of the RAS-MAPK pathway to gene activation by RSK2, a growth factor-regulated CREB kinase. *Science* 273, 959-963 (1996)
44. Gonzalez, G. A. and M. R. Montminy: Cyclic AMP stimulates somatostatin gene transcription by phosphorylation of CREB at serine 133. *Cell* 59, 675-680 (1989)

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