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Follicle-stimulating hormone promotes proliferation of cultured chicken ovarian germ cells through protein kinases A and C activation*

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Abstract: The study was conducted to investigate the effects of follicle-stimulating hormone (FSH) on embryonic chicken ovarian germ cell proliferation and its possible involvements of protein kinases A (PKA) and C (PKC) pathways. Ovarian cells were treated with FSH alone or in the presence of forskolin (FRSK), PKA inhibitor (H_{89}), PKC activator (PMA) or inhibitor (H_7). The germ cell number was counted from micropictures. The immunocytochemistry of proliferating cell nuclear antigen (PCNA) was applied to identify the proliferating cells. The germ cell labeling index (LI) was determined for cell proliferation. The FSH treatment increased the germ cell number, and this stimulating effect was enhanced by FRSK or PMA, but inhibited by H_{89} or H_7 in a dose-dependent manner. Moreover, the PCNA-LI showed parallel changes with germ cell numbers. This study suggests that FSH may stimulate proliferation of cultured chicken ovarian germ cells by activation of both the PKA and PKC signaling pathways.

Key words: Follicle-stimulating hormone, Protein kinase A, Protein kinase C, Germ cell, Proliferation
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1 Introduction

Follicle-stimulating hormone (FSH) plays a critical role in gonad development. FSH stimulates cell proliferation, growth, and DNA synthesis, enhances the secretion of steroids in the embryonic ovary, provokes structural changes of the ovary, and increases the cell numbers of different ovarian cell subpopulations of hatched chicks (Velázquez *et al*., 1997; Méndez-Herrera *et al*., 1998; Ohtani *et al*., 2001). FSH-binding cells have been detected on Day 6.5 of incubation in the left embryonic ovarian cells. With embryo development, the FSH-positive cells in the left ovary increased, which further stimulated the sex hormone synthesis and accelerated the germ cell development (Woods *et al*., 1991). The effects of FSH on ovarian granulosa cell proliferation, differentiation, and hormone secretion have been extensively investigated in vitro (Dorrington *et al*., 1988; Pedernera *et al*., 1999; Yogo *et al*., 2002). In our previous study, it was shown that gonadotropins, especially FSH, stimulated the germ cell proliferation (Xie *et al*., 2004). These findings suggest that FSH plays a promoter role in regulating growth and proliferation of ovarian cells. However, the biological function of FSH and its signal transduction in ovarian germ cells are still unknown.

The FSH signal transduction has been considered to be mainly mediated through cyclic adenosine 3′,5′-monophosphate (cAMP)-dependent protein kinase A

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(PKA) pathway (Yu *et al*., 2003; Hunzicker-Dunn and Maizels, 2006), which has been proposed as a first reaction tier by which FSH controls cellular proliferation, differentiation, and apoptosis. However, some recent studies reported that the diacylglycerol (DG)-protein kinase C (PKC) also plays an equal role in FSH action (Lu *et al*., 2001; Ali and Sirard, 2005; Jin *et al*., 2006). PKC signaling pathway was included in the desensitization of FSH-induced adenylate cyclase (AC) activity in rat sertoli cells (Eskola *et al*., 1998) and chick embryo testis cells (Peralta *et al*., 2009). Lu *et al*. (2001) reported that PKC, rather than PKA, was involved in FSH-mediated resumption of mouse cumulus cell-enclosed oocytes. In addition, FSH increases PKC activity in human ovarian cancer cells (Ohtani *et al*., 2001). These results suggest that the PKC signaling pathway, at least in part, is involved in FSH-stimulated cell proliferation and growth. Determining whether the PKC-mediated system is involved in the FSH-stimulated cell proliferation in cultured chicken ovarian germ cells still requires further investigation.

This study was carried out to investigate the underlying signal transduction mechanism of FSH on germ cell proliferation through the PKA and PKC-mediated systems by activation of PKA and PKC or blockade of PKA and PKC.

2 Materials and methods

2.1 Animals

Fertilized chicken eggs were purchased from a commercial hatchery and incubated at 38.5 °C with 60% relative humidity until Day 18.

2.2 Culture of ovarian cells

The dispersion and culture procedures of ovarian cells were described as elsewhere (Liu *et al*., 2006). Briefly, left ovary fragments were minced and digested. The dispersed cells were seeded at 5×10^4 cells/well in McCoy's 5A medium (GIBCO BRL, CA, USA) containing 1.75 mmol/L 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 2 mmol/L glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin (Sangon, Shanghai, China), 3×10^{-8} mol/L selenite, 5 μg/ml transferrin and 10 μg/ml insulin (Sigma, St. Louis, MO, USA). Cells were incubated at 39 °C under a water-saturated atmosphere containing 95% air and 5% CO_2.

2.3 Treatment of cultured cells with chemicals

At the beginning of culture, cells were treated with different doses of FSH (1–1000 ng/ml, OVAGEN™, New Zealand), and then were incubated with forskolin (FRSK, 10^{-7}–10^{-5} mol/L, activator of the adenylate cyclase, Sigma), H_{89} (10^{-7}–10^{-5} mol/L, PKA inhibitor, Sigma), PMA (10^{-8}–10^{-6} mol/L, PKC activator, Sigma), or H_7 (10^{-7}–10^{-5} mol/L, PKC inhibitor, Sigma) alone or in combination with an optimal dose of FSH.

2.4 Analysis of morphological changes

Phase-contrast images were captured with a CCD camera (Pixera Pro 150ES, USA) and Simple PCI advanced imaging software (Compix Inc., USA). The images were used to analyze the cell morphological changes. The germ cell number was counted in each image.

2.5 Immunocytochemistry of proliferating cell nuclear antigen (PCNA)

The cells were fixed and incubated overnight at 4 °C with mouse anti-PCNA antibody (Boster Co., Wuhan, China), followed by biotin-goat anti-mouse IgG as secondary antibody. Cells with nuclei that were brown to black were considered as positive. The labeling index (LI) was shown as the percentage of the germ cell number of PCNA-labeled nuclei to the total germ cell number.

2.6 Statistical analysis

Data were analyzed by analysis of variance (ANOVA) and Duncan's multiple range tests using the SAS 8.0 software system and were expressed as the mean±standard error of mean (SEM). A significant difference was considered at $P<0.05$.

3 Results

3.1 Effect of FSH on germ cell proliferation

FSH (10–1000 ng/ml) treatment increased the germ cell number after 48-h culture ($P<0.05$), but lower concentrations of FSH (1 ng/ml) failed to increase the germ cell number (Fig. 1). The germ cells

in the FSH-treated groups were more definite and had higher cuboidal form. The PCNA-staining was deeper after FSH treatment (Figs. 2a and 2b) and the germ cell LI was markedly higher, relative to the control (P<0.05, Fig. 3).

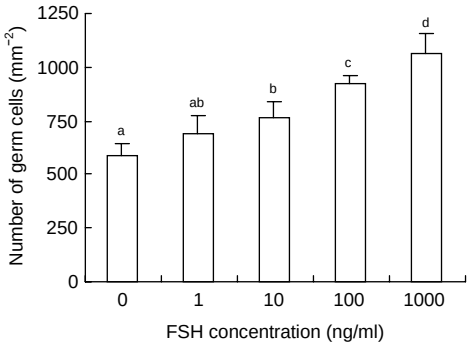

Fig. 1 Effects of FSH on proliferation of ovarian germ cells from embryonic chickens after 48-h culture
Values are expressed as mean±SEM (n=4). Bars with different superscripts are statistically different at P<0.05

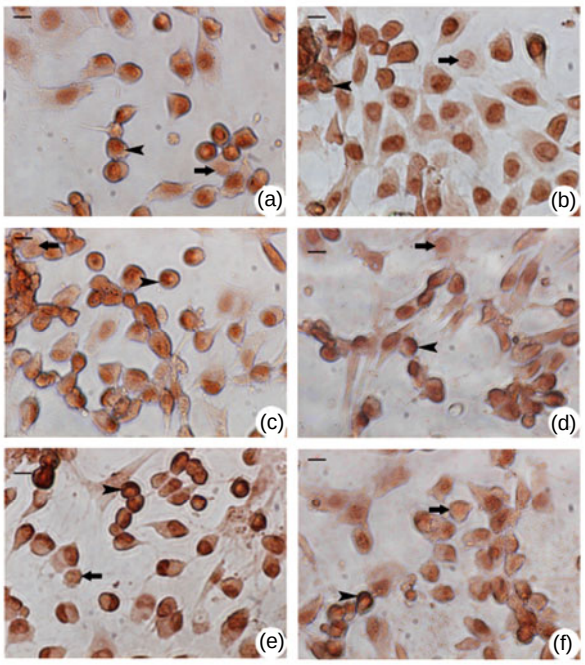

Fig. 2 Immunocytochemical staining of PCNA in embryonic chicken ovarian cells treated with FSH alone or combined with PKA/PKC activators/inhibitors after 48-h culture
(a) Control; (b) FSH (10 ng/ml); (c) FSH+FRSK (10^{-5} mol/L); (d) FSH+H_{89} (10^{-5} mol/L); (e) FSH+PMA (10^{-6} mol/L); (f) FSH+H_7 (10^{-5} mol/L). Arrowheads: PCNA-positive germ cells; Arrow: PCNA-negative germ cells. Scale bar=10 μm

3.2 Effects of FRSK and H_{89} on FSH-stimulated germ cell proliferation

FRSK (10^{-7}–10^{-5} mol/L) augmented the germ cell number (P<0.05, Fig. 4a). Treatment with FRSK and FSH resulted in a visible increase in germ cell number (P<0.05, Fig. 4a) and PCNA expression in all groups (Figs. 2b and 2c). The LI showed an obvious synergic effect of FSH and FRSK on germ cell proliferation (P<0.05, Fig. 3). Morphological analysis demonstrated that FSH-stimulated proliferation of the germ cells was visibly repressed by the treatment of H_{89}. However, no significant changes were found among all groups of H_{89} (10^{-7}–10^{-5} mol/L) alone, relative to the control (Fig. 4b). H_{89} significantly depressed PCNA expression in the united groups compared with FSH alone after 48-h culture (Figs. 2b and 2d), and the germ cell LI was visibly lower in the united groups of H_{89} and FSH than in the FSH group (P<0.05, Fig. 3).

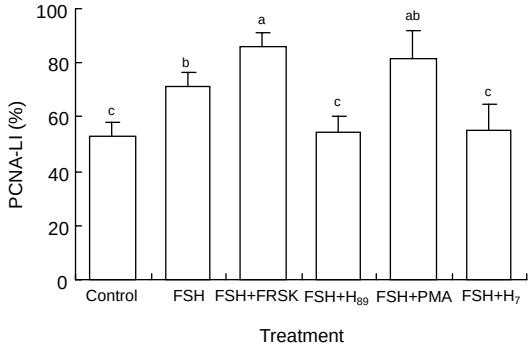

Fig. 3 Changes of PCNA-LI in chicken ovarian germ cells after treatment with FSH alone or combined with PKA/PKC activators/inhibitors after 48-h culture
Values are expressed as mean±SEM (n=4). Bars with different superscripts are statistically different at P<0.05

3.3 Effects of PMA and H_7 on FSH-stimulated germ cell proliferation

No significant change was shown after the treatment with PMA (10^{-8} and 10^{-7} mol/L) alone, but PMA at 10^{-6} mol/L augmented the germ cell number (P<0.05, Fig. 4c). Combination of PMA and FSH induced a visible increase in germ cell number (P<0.05, Fig. 4c) and PCNA expression in all groups (Figs. 2b and 2e). The germ cell LI also displayed a marked synergistic effect of FSH and PMA on germ cell proliferation (P<0.05, Fig. 3). FSH-stimulated

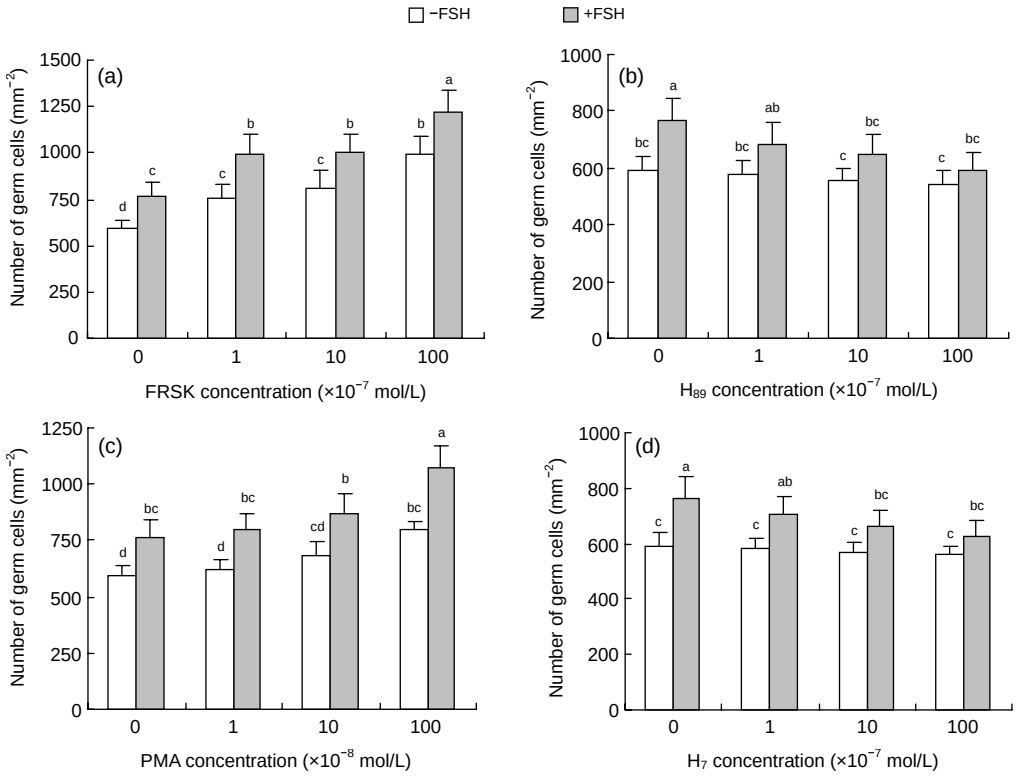

Fig. 4 Effects of FRSK (a), H_{89} (b), PMA (c), and H_7 (d) on FSH-stimulated increase in germ cell number of embryonic chicken ovary after 48-h culture

Values are expressed as mean±SEM (n=4). Bars with different superscripts are statistically different at P<0.05

germ cell proliferation was blocked by treatments with H_7, but there were no evident changes among all groups of H_7 (10^{-7}–10^{-5} mol/L) alone, relative to the control (Fig. 4d). The H_7 treatment reduced PCNA expression of germ cells in the united groups relative to FSH alone after 48-h culture (Figs. 2b and 2f), and the germ cell LI was distinctly lower in the united groups of H_7 and FSH than in the FSH group (P<0.05, Fig. 3).

4 Discussion

The FSH exerts its action by binding to its receptor FSHR in target cells. Akazome *et al.* (2002) have detected the expression of FSHR mRNA in undifferentiated gonads of 4-d chick embryos. Our previous study showed that the FSHR mRNA level in embryonic chicken gonads began to increase at 18 d of incubation, and then reached a plateau around hatching (Mao *et al.*, 2000). The expression of FSHR mRNA was mostly located in somatic cells, but partly in the germ cells of the ovary (Méndez *et al.*, 2003). The AC-cAMP pathway has long been accepted as the sole second messenger when FSH regulates follicular development in the ovary (Hunzicker-Dunn and Maizels, 2006). To investigate this point, we examined the proliferating effect of FSH on cultured ovarian germ cells and further investigated the underlying signal transduction mechanism. We confirmed that the germ cell number was increased by FSH treatment and this stimulating effect was confirmed by increased germ cell LI. The results agreed with the findings that FSH induced the proliferation of 14-d chicken embryonic ovarian cells (Méndez *et al.*, 2003). The FSH-stimulated proliferation of the germ cells was markedly increased by united treatment with a specific activator of the adenylate cyclase, FRSK, and there were noticeable changes in the germ cell number after the treatment with FRSK. To further support that the PKA-mediated system was involved in the FSH-stimulated cell proliferation in cultured ovarian germ cells, a specific inhibitor of PKA pathway, H_{89}, was applied. The results showed that,

H_{89} visibly blocked the FSH-stimulated proliferation of the germ cells. The conclusions agreed with the reports that H_{89} inhibited the promoting effects of FSH in sertoli cells (Crépieux *et al*., 2002). In summary, our results indicated that PKA plays an important role on the germ cell proliferation and was stimulated by FSH, since the treatment with H_{89} suppressed the FSH-stimulated cell proliferation while FRSK increased the FSH-stimulated cell proliferation.

As mentioned above, the PKC signaling pathway, at least in part, was involved in FSH-stimulated cell proliferation. The underlying signal transduction mechanism was studied by regulating the PKC-mediated system with PKC inhibitor (H_7) or activator (PMA). H_7 markedly suppressed the FSH-stimulated cell proliferation in cultured ovarian germ cells. The results are consistent with the findings that H_7 inhibited the promoting effects of FSH in human epithelial ovarian cancer cells through PKC-mediated system (Ohtani *et al*., 2001). We also found that there was no evident change after the treatment with lower PMA alone, but an augmentation in the germ cell number with higher PMA. Combined administration of PMA and FSH resulted in a visible increase in the germ cell number and LI. These conclusions were consistent with the viewpoints that the treatment of ovarian germ cells and mouse spermatogonia with PMA and ginsenosides promoted the cell proliferation involving the PKC-mediated system (Liu and Zhang, 2006; Zhang *et al*., 2009). Hence, we propose that PKC-mediated system is involved in the FSH-stimulated cell proliferation in cultured chicken ovarian germ cells.

Though the PKA and PKC pathways are involved in the signal transduction of FSH, the mechanism may also be through other intracellular signaling cascades, including the phosphatidylinositol 3-kinase (PI3-K)/Akt and mitogen-activated protein kinase (MAPK) pathways (Pasapera *et al*., 2005; Alam *et al*., 2009). In granulosa cells, FSH mimicked some actions of insulin-like growth factor 1 (IGF-1) through phosphorylation and activation of protein kinase B (PKB)/Akt. Activation of these alternate pathways perceptibly leads to a more precise control of FSH-stimulated cell proliferation, steroidogenesis, and apoptosis in ovaries (Richards *et al*., 2002; Alam *et al*., 2009). Based on these hypotheses, FSH-induced germ cell proliferation might be mainly involving PKA and PKC-mediated pathways. However, in addition to PKA and PKC-mediated pathways, other signal transduction pathways that involve in the FSH-stimulated proliferation of germ cells require further studies.

In conclusion, this study reveals that FSH promotes proliferation of cultured chicken ovarian germ cells, and the enhancement of PCNA expression further confirms its stimulatory effect. In addition, we demonstrate that PKA and PKC-mediated signal transduction system regulates the FSH-induced germ cell proliferation. Further work will be needed to identify other signaling pathways and the precise molecular mechanism of FSH action in germ cells.

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