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Effect of epidermal growth factor on follicle-stimulating hormone-induced proliferation of granulosa cells from chicken prehierarchical follicles*

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Abstract: The development of ovarian follicular cells is controlled by multiple circulating and local hormones and factors, including follicle-stimulating hormone (FSH) and epidermal growth factor (EGF). In this study, the stage-specific effect of EGF on FSH-induced proliferation of granulosa cells was evaluated in the ovarian follicles of egg-laying chickens. Results showed that *EGF* and its receptor (*EGFR*) mRNAs displayed a high expression in granulosa cells from the prehierarchical follicles, including the large white follicle (LWF) and small yellow follicle (SYF), and thereafter the expression decreased markedly to the stage of the largest preovulatory follicle. SYF represents a turning point of *EGF*/*EGFR* mRNA expression during follicle selection. Subsequently the granulosa cells from SYF were cultured to reveal the mediation of EGF in FSH action. Cell proliferation was remarkably increased by treatment with either EGF or FSH (0.1–100 ng/ml). This result was confirmed by elevated proliferating cell nuclear antigen (PCNA) expression and decreased cell apoptosis. Furthermore, EGF-induced cell proliferation was accompanied by increased mRNA expressions of EGFR, FSH receptor, and the cell cycle-regulating genes (cyclins D1 and E1, cyclin-dependent kinases 2 and 6) as well as decreased expression of luteinizing hormone receptor mRNA. However, the EGF or FSH-elicited effect was reversed by simultaneous treatment with an EGFR inhibitor AG1478. In conclusion, EGF and EGFR expressions manifested stage-specific changes during follicular development and EGF mediated FSH-induced cell proliferation and retarded cell differentiation in the prehierarchical follicles. These expressions thus stimulated follicular growth before selection in the egg-laying chicken.

Key words: Epidermal growth factor, Follicle-stimulating hormone, Granulosa cell, Proliferation, Chicken
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1 Introduction

Avian follicular development depends on a complex interaction of complex biochemical and physiological processes. Reproductive axis hormones, such as follicle-stimulating hormone (FSH), play a pivotal role in the process of follicular development. As an indispensable gonadotropin secreted by the anterior pituitary gland, FSH regulates follicular growth in females (Uhm *et al*., 2010), and stimulates 17β estradiol and progesterone production in follicle granulosa cells (Shanmugam *et al*., 2010). Circulating levels of FSH and its receptor (FSHR) expressions on granulosa cells play a decisive role in avian follicular selection. In the avian ovary, prior to follicle selection, the granulosa cells from prehierarchical follicles remain undifferentiated, as defined by high *FSHR* mRNA expression, as well as the low expression of

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luteinizing hormone receptor (*LHR*) mRNA (Haugen and Johnson, 2010).

Folliculogenesis in mammals is a complex process involving a series of sequential steps in which a growing follicle either develops to the ovulation stage or undergoes atresia (Wang *et al*., 2010). The normal maturation process of the chicken oocyte within the ovarian follicle depends on the coordinated development of follicular wall inducing granulosa and/or theca cells under the influences of various hormones and growth factors. As endocrine cells, contiguous assembly of granulosa cells around the oocyte regulates the follicular growth and development via paracrine signals. There are numerous reports concerning the selection of avian ovarian follicles (Johnson and Woods, 2009; Schneider, 2009). However, because of the complexity of regulation by endocrine, autocrine, and paracrine factors on avian follicular development, the exact mechanisms of interaction between hormones and growth factors are still far from clear. Meanwhile, there is little information about the effect of epidermal growth factor (EGF) on FSH action in chicken ovarian granulosa cells.

It is now generally recognized that the control of vertebrate ovarian follicular development involves not only pituitary gonadotropins, but also various local paracrine and autocrine growth factors (Tse and Ge, 2010). Recently, members of EGF family have received increasing attention among numerous growth factors reported. It has been demonstrated that the EGF ligand/receptor system plays important crucial physiologic roles in cell proliferation, survival, adhesion, motility, invasion, and angiogenesis (Lafky *et al*., 2008). EGF receptor (EGFR), as a member of ErbB family of receptor tyrosine kinases, has been investigated extensively (Normanno *et al*., 2006). Differentiation of the selected follicles is associated with granulosa-specific changes in the availability of one or more EGF family ligands, and the expression of ErbB receptors (Woods and Johnson, 2006).

Our previous studies showed that EGF, prostaglandin, and gonadotropin promoted the proliferation of chicken follicular cells or ovarian germ cells, and there were interactions in prostaglandin with EGF and FSH (Jin *et al*., 2007; Jia *et al*., 2010; Liu *et al*., 2010). Based on the crucial functions of EGF/EGFR in follicular development, the present study was designed to evaluate the effect of EGF on granulosa cell proliferation through experiments on stage-specific changes of EGF/EGFR in chicken follicles and the proliferating action of EGF on granulosa cells from prehierarchical follicles.

2 Materials and methods

2.1 Animals

Buff egg-laying chickens (40 to 50 weeks old) were obtained from the Ningbo Zhenning Animal Husbandry Co., Ltd., China. All hens had a normal clutch for at least 5 d. The hens were sacrificed by cervical bleeding after anesthesia with pentobarbital sodium.

2.2 Classification of follicles and separation of granulosa cells

According to the diameter, the follicles were classified as small white follicles (SWFs, <2 mm), large white follicles (LWFs, 3–5 mm), small yellow follicles (SYFs, 6–8 mm), large yellow follicles (LYFs, 9–12 mm), and large yellow preovulatory follicles (13–40 mm, named F5, F4, F3, F2, and F1, respectively). The granulosa cells from different stage follicles (except SWFs) were separated from theca layers for reverse transcription polymerase chain reaction (RT-PCR).

2.3 Dispersion and culture of granulosa cells

A pool of SYFs was removed from the laying hens and placed in ice-cold M199 medium (Hyclone Laboratories Inc., Logan, UT, USA). The granulosa cells were separated from theca layers and minced by a mechanical method before digestion with 12.5 μg/ml type II collagenase (Gibco-BRL Co., Ltd., Grand Island, NY, USA). The dispersed cells were filtered through a 200-mesh sieve and centrifuged for 8 min at 1000 r/min. Cell viability was >95% by trypan blue extrusion test. The cell suspension was seeded in 96-well culture plates (Nunc, Roskilde, Denmark) at a density of 5×10^4 cells/well in 100 μl medium and incubated at 38.5 °C in a water-saturated atmosphere of 95% air and 5% CO_2.

2.4 Treatment of cultured granulosa cells

At the beginning of culture, granulosa cells were seeded in M199 medium supplemented with 0.5%

fetal calf serum (FCS; Gibco-BRL, USA) for 12 h, and the cells attached to the plate bottom. Then the serum-containing medium was replaced with serum-free medium which was supplemented with 10 μg/ml insulin, 5 μg/ml transferrin, and 0.03 μmol/L selenite (ITS medium, Sigma, St. Louis, MO, USA). To study the effect of EGF on cell proliferation, the cultured cells were treated with either 0.1 to 100 ng/ml EGF (StemCell Technologies, Inc., Vancouver, Canada) or 0.1 to 100 ng/ml FSH (Sigma, USA) alone or in combinations with 10 ng/ml EGF for 24 h. The signal transduction of EGF was studied by treatment of cultured cells with 0.01 to 1.00 μmol/L AG1478 (EGFR inhibitor, Calbiochem, La Jolla, CA, USA) with 10 ng/ml EGF or 10 ng/ml FSH for 24 h.

The chemicals were dissolved in ethanol or dimethylsulfoxide and diluted with ITS medium. The highest concentration of ethanol or dimethylsulfoxide in the medium was 0.1%.

2.5 RNA isolation and RT-PCR

After 24-h treatment, the cultured granulosa cells were collected by digestion with 0.25% trypsin. Total RNA was extracted by TRIzol reagent (Invitrogen Co., Carlsbad, CA, USA) and 2 μg total RNA was reverse-transcribed by the RevertAid first cDNA synthesis kit (MBI Fermentas, Burlington, ON, Canada). A total of 2 μl of RT products were used for PCR amplification in 25 μl volume. The number of cycles used to amplify each cDNA was chosen to ensure the PCR to proceed in a linear range. The sequences of the primers were listed in Table 1. PCR cycling condition was 5 min 94 °C for an initial denaturation; 32 cycles of 30 s at 94 °C, 40 s at 57 °C, 1 min at 72 °C; and a final extension of 10 min at 72 °C. PCR products were visualized on 1.5% agarose gel with ethidium bromide after electrophoresis, and quantified with Tanon Gel Imaging system (Tanon Biotech Inc., Shanghai, China). The *β-actin* was used for normalization of the target gene mRNA abundance.

2.6 Proliferating cell nuclear antigen (PCNA) immunocytochemistry

The cultured cells were fixed in 0.04 g/ml neutral paraformaldehyde and rinsed with 0.01 mol/L phosphate-buffered saline (PBS; pH 7.4). The PCNA labeling was conducted according to a previous method (Jin *et al*., 2006) with minor modification. Non-specific binding was blocked with 10% goat serum for 20 min at 37 °C. The primary antibody was mouse anti-PCNA polyclonal antibody (1:200 dilution, Boster Bioengineering Co., Ltd., Wuhan, China). Subsequently the cells were stained with the secondary antibody (fluorescein isothiocyanate (FITC)-labeled goat anti-mouse IgG; KPL Inc., Gaithersburg, MD, USA) at 1:200 dilution for 45 min. Cells were counterstained with 4′,6-diamidino-2-phenylindole (DAPI; Sigma, USA) for 5 min. PCNA-labeling index (LI) was determined as the percentage of PCNA-positive cells to the total cells in the same field.

Table 1 Primers for PCR analysis

Gene	Accession No.	Primer sequence (5′ to 3′)	Product length (bp)
EGF	NM_001001292	CAG ATG GGA GTA GTC TTA GCG CCC TCG GTC TGT CCA ATA C	198
EGFR	NM_205497	GAC GCA CAC CAT GGG TGT AC CGC GAT TAT GCT CCA CGT AG	260
FSHR	NM_205079	AAG AGC GAG GTC TAC ATA CA GTG GTG TTC CCA GTG ATA G	414
LHR	AB009283	GCT GCT CAT TGC TTC GG GCT CTG CTC GGC TCT TAC	713
CCND1	NM_205381	CTG CTC AAT GAC AGG GTG C TCG GGT CTG ATG GAG TTG T	341
CDK6	NM_001007892	CCG ACC AAC AGT ATG AGT GCG GAA AAT CCA GTC CCC GAA ACA	381
CCNE1	NM_001031358	ACC TAA AAT GAG AAC AAT CC GGC AAC AAT ACC TCG TAA A	381
CDK2	EF182713	ACT GCT GTG GAC ATC TGG A CTT GTT GGG ATC GTA GTG C	276
β-actin	NM_205518	ACG TCG CAC TGG ATT TCG AG TGT CAG CAA TGC CAG GGT AC	282

2.7 Terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling (TUNEL) assay

Apoptotic cells were identified by TUNEL method to label 3′-end of fragmented DNA. After 24-h treatment, the cells were fixed in 0.04 g/ml paraformaldehyde for 45 min, then permeabilized by 0.01 mol/L PBS (pH 7.4) containing 0.1% Triton X-100. The apoptotic cells were detected with FITC end-labeling fragmented DNA by the TUNEL cell apoptosis detection kit (Beyotime Institute of Biotechnology, Haimen, China). Cells were counterstained with DAPI for 5 min. Images of apoptotic cells were captured with IX71 microscope (Olympus, Tokyo, Japan). Percentage of apoptotic cells was denoted as the ratio of FITC-labeled TUNEL-positive cells to the total cells in the same field.

2.8 Statistical analysis

The experiment was repeated at least three times with quadruplicate for statistical analysis. Morphological change of the granulosa cells was observed under IX70 phase contrast microscope (Olympus, Tokyo, Japan). Five different regions were selected randomly in each well and the cell number was counted in each image. Analysis was achieved by using Simple PCI advanced imaging software (Compix Inc., Cranberry Twp, PA, USA).

All data were expressed as the mean±standard error of the mean (SEM) and analyzed by analysis of variance (ANOVA) and Duncan's multiple-range tests using the SAS 8.0 software. $P<0.05$ was considered to be statistically different.

3 Results

3.1 Expressions of *EGF* and *EGFR* mRNAs in granulosa cells

The RT-PCR analysis showed stage-specific changes in the *EGF* and its receptor (*EGFR*) mRNAs in granulosa cells through prehierarchical LWF to preovulatory F1 follicle in the egg-laying chickens (Fig. 1a). *EGF* mRNA expression displayed a similar tendency with *EGFR* mRNA in follicles of different developmental stages. High expression was demonstrated in the granulosa cells from the prehierarchical follicles including LWF and SYF, and its highest level occurred in SYF. Subsequently the mRNA expression decreased markedly to F1 (Fig. 1b).

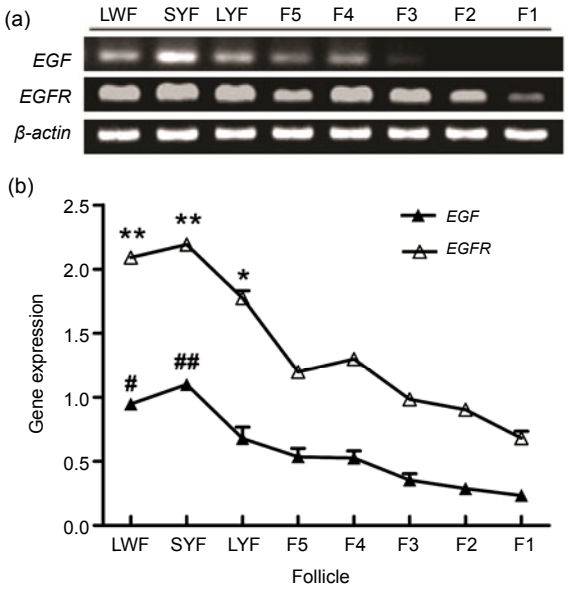

Fig. 1 Developmental changes of *EGF* and *EGFR* mRNA expressions in granulosa cells from ovarian follicles at different stages

(a) Electrophoresis of PCR products. LWF, SYF, and LYF indicate large white follicle, small yellow follicle, and large yellow follicle, respectively. (b) Means±SEM of four experiments for each condition determined from densitometry, relative to *β-actin*. Statistically different: $^{*}P<0.05$, $^{**}P<0.01$, $^{\#}P<0.05$, $^{\#\#}P<0.01$, compared to F5

3.2 Effect of EGF on cultured granulosa cell proliferation

After treatment with EGF (0.1 to 100 ng/ml), a significant change in granulosa cell proliferation was observed (Fig. 2). The cells appeared clear three-dimensional (3D) oval appearance after EGF treatment, whereas the cells of the control displayed flat form. According to cell number, EGF-stimulated cell proliferation manifested a dose-dependent manner from 0.1 to 100 ng/ml (Fig. 2). Besides the morphological observation, the proliferating effect of EGF was confirmed by PCNA immunocytochemistry. Cell proliferation was significantly enhanced after 1 ng/ml EGF treatment (Fig. 2, $P<0.05$), with the maximal stimulating effect at 10 ng/ml, but decreased slightly at 100 ng/ml. In addition, the TUNEL assay showed that the number of apoptotic granulosa cells in the EGF-treated group (10 ng/ml) was lower than that in

the control group (Fig. 5c; $P<0.05$). Taking cell morphology, proliferation, and apoptosis-inhibition into account, the treatment of granulosa cells with 10 ng/ml EGF for 24 h was adopted for subsequent studies.

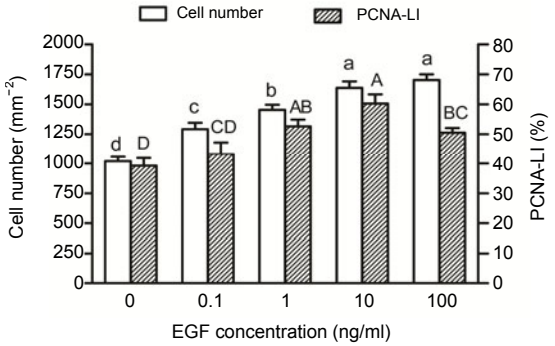

Fig. 2 Effect of EGF (0.1–100 ng/ml) on granulosa cell proliferation, including changes in cell number and PCNA-LI after treatment for 24 h

Values represent the means±SEM (n=4). Bars with different letters are statistically different ($P<0.05$)

3.3 Effect of EGF on expression of cell cycle-regulating genes

In support of the promoting effect of EGF on cell proliferation, the mRNA abundances of cell cycle-regulating genes, including cyclins D1 and E1 (*CCND1* and *CCNE1*), cyclin-dependent kinases (CDKs) 2 and 6 were examined. These genes are considered to be critical for G_0/G_1 and G_1/S progressions. After treatment with EGF (10 ng/ml) for 24 h, the expressions of all four mRNAs were increased significantly in the cultured cells (Fig. 3, $P<0.05$).

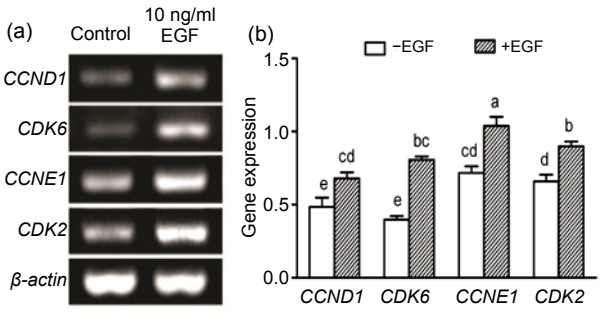

Fig. 3 Effect of EGF (10 ng/ml) on expression of cell cycle-regulating genes in cultured granulosa cells after treatment for 24 h

(a) PCR product electrophoresis of *CCND1*/*CDK6* and *CCNE1*/*CDK2* mRNA expressions; (b) Means±SEM of four experiments for each condition determined from densitometry, relative to *β-actin*. Bars with different letters are statistically different ($P<0.05$)

3.4 Effect of EGF on *EGFR* and gonadotropin receptor mRNA expressions

Treatment with EGF at 10 ng/ml resulted in a higher expression of *EGFR* mRNA in granulosa cells compared with the control (126.12% increase, Fig. 4, $P<0.05$). In addition, treatment with EGF (10 ng/ml) also increased *FSHR* mRNA expression (92.18% increase, $P<0.05$), but decreased luteinizing hormone receptor (*LHR*) mRNA expression (35.51% decrease, $P<0.05$).

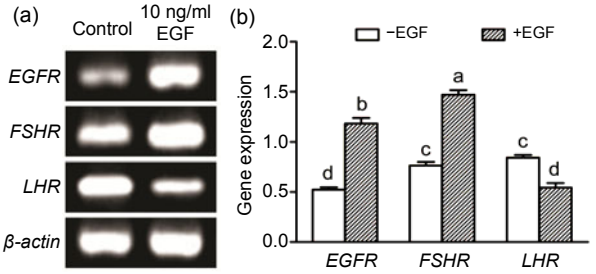

Fig. 4 Expressions of *EGFR*, *FSHR*, and *LHR* mRNAs in EGF-induced proliferation of granulosa cells

Cells were treated with EGF (10 ng/ml) for 24 h. (a) PCR product electrophoresis; (b) Means±SEM of four experiments for each condition determined from densitometry, relative to *β-actin*. Bars with different letters are statistically different ($P<0.05$)

3.5 Effect of EGF on FSH-induced proliferation of granulosa cells

Treatment with FSH for 24 h significantly stimulated the proliferation of cultured granulosa cells in a dose-dependent manner from 0.1 to 100 ng/ml (Fig. 5a). Compared with EGF (10 ng/ml) treatment alone, the cell number was further increased after combined treatment with 10–100 ng/ml FSH (Fig. 5a, $P<0.05$). In addition to the cell number increase, PCNA-LI was also significantly increased (Figs. 5b and 5c, $P<0.05$), and the percentage of apoptotic cells was remarkably decreased (Figs. 5b and 5c, $P<0.05$) after simultaneous treatment with EGF and FSH.

3.6 Effect of AG1478 on EGF and FSH-induced cell proliferation

EGF and FSH-induced cell proliferation was inhibited by treatment with an EGFR inhibitor AG1478 at 0.10 to 1.00 μmol/L (Fig. 6a, $P<0.05$). The EGF or FSH-elicited increase in PCNA-LI was

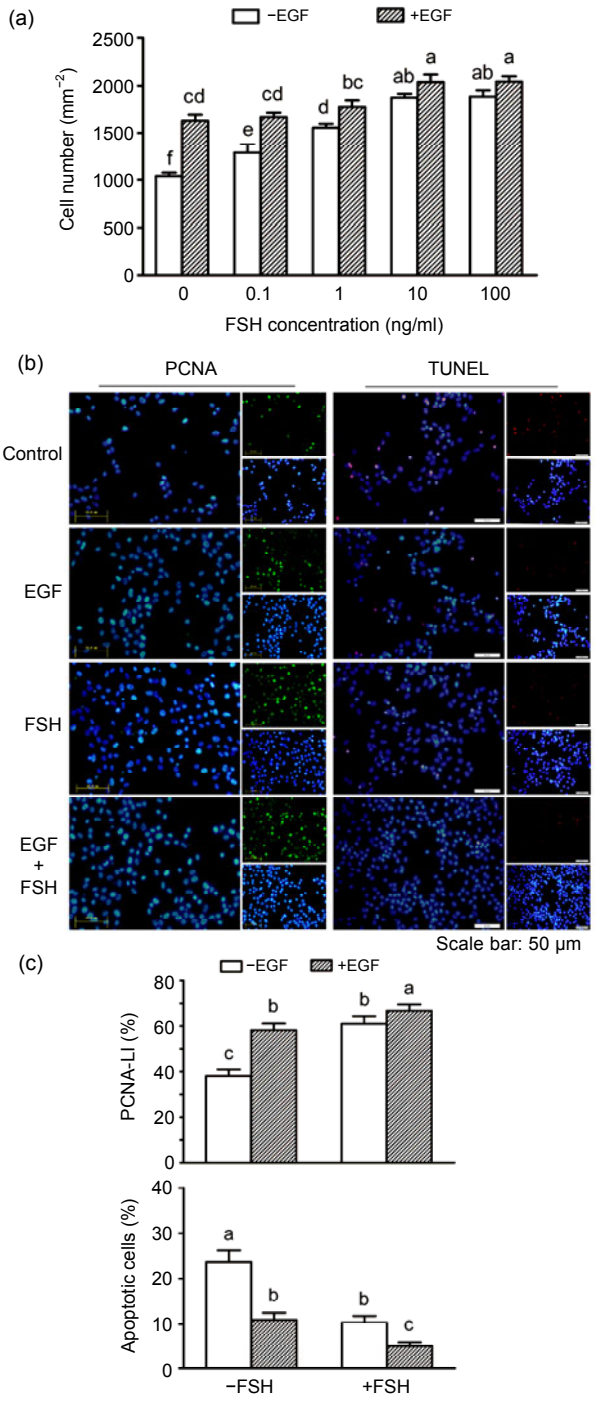

Fig. 5 Effect of EGF on FSH-stimulated proliferation of granulosa cells after treatment for 24 h

(a) Changes in cell number after treatment with FSH (0.1–100 ng/ml) alone or in combination with EGF (10 ng/ml); (b) PCNA immunocytochemistry and TUNEL staining; (c) PCNA-LI and percentage of apoptotic cells after treatment with FSH (10 ng/ml), EGF (10 ng/ml), and FSH (10 ng/ml)+EGF (10 ng/ml). Values are presented as mean±SEM (n=4). Bars with different letters are statistically different (P<0.05)

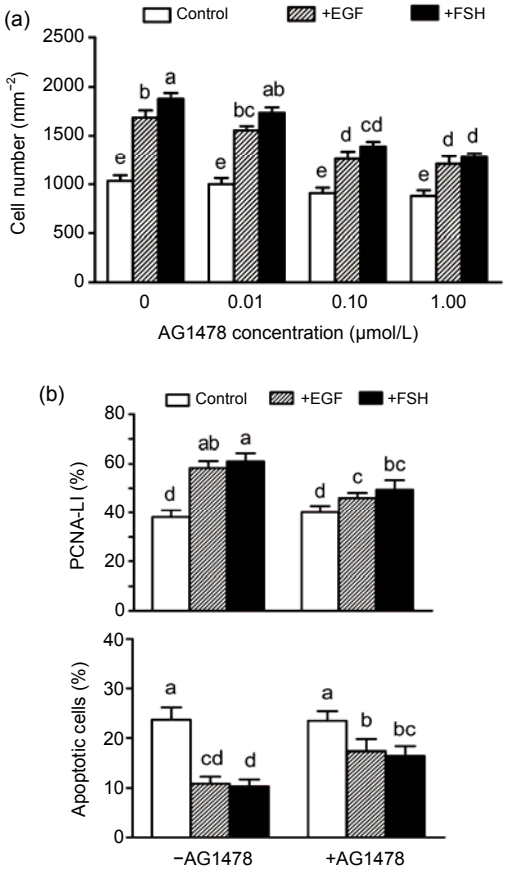

Fig. 6 Effect of EGFR antagonist AG1478 on EGF and FSH-induced proliferation of granulosa cells

(a) Inhibitive effect of AG1478 (0.01–1.00 μmol/L) on EGF (10 ng/ml) or FSH (10 ng/ml)-stimulated cell number increase; (b) PCNA-LI and percentage of apoptotic cells after the treatment of FSH (10 ng/ml) and EGF (10 ng/ml) alone or in combination with AG1478 (0.10 μmol/L), respectively. Values represent the mean±SEM (n=4). Bars with different letters are statistically different (P<0.05)

decreased by AG1478 at 0.10 μmol/L (Fig. 6b, P<0.05). Meanwhile, TUNEL assay showed that AG1478 inhibited either EGF or FSH-reduced granulosa cell apoptosis significantly (Fig. 6b, P<0.05).

4 Discussion

Different growth factors and their respective receptors have been shown to be involved in the autonomous growth of ovarian follicular cells. All EGF family ligands (egf, tgfa, btc, and hbegf) examined were mostly or exclusively expressed in the oocyte of zebrafish (Tse and Ge, 2010). The mRNAs of some growth factors and their receptors have been

reported in avian species (Onagbesan *et al*., 2009). Yao and Bahr (2001a) reported that the germinal disc and granulosa cells are the sources of EGF in chicken preovulatory follicles. However, dynamic expressions of EGF and its receptor in the chicken ovarian follicles have not yet been reported. In this study, the mRNA expressions of *EGF* and *EGFR* were demonstrated in granulosa cells from ovarian follicles at different stages by RT-PCR. The result showed that the mRNA expressions of *EGF* and *EGFR* in the granulosa cells displayed a stage-specific change during follicular development, which maintained a high expression in LWF and SYF, but declined from SYF to F1. This result indicates that SYF represents the turning point of *EGF* and *EGFR* mRNA expressions during follicular development. Interestingly, mRNA expressions of *EGF* and *EGFR* manifested a simultaneous trend of changes with FSHR in chicken follicles, as reported by Woods and Johnson (2005). Hunzicker-Dunn and Maizels (2006) proved that FSH promoted the proliferation of granulosa cells through FSHR, hence to accelerate follicular development. Therefore, we considered that EGF and its receptor (EGFR) play an important role in selection of prehierarchical follicles to hierarchical development that is destined to ovulation, and EGF action is likely related with FSH.

The role of EGF family in the ovary has received increasing attention recently. It has been shown that there is a paracrine action within the follicle and the oocyte-derived EGF family ligands may mediate mutual communications between oocytes and follicle cells (Tse and Ge, 2010). Our previous studies revealed that EGF stimulated proliferation of chicken primordial germ cells directly (Ge *et al*., 2009). In this study, PCNA immunocytochemistry and TUNEL assay demonstrated that EGF promoted the proliferation of granulosa cells from chicken SYF in a dose-dependent manner, and inhibited cell apoptosis. The regulation of cell proliferation is tightly dependent upon numerous endocrine and autocrine/paracrine factors that converge on the central components of cell cycle (Cannon *et al*., 2007). Here the mRNA expressions of *CCND1*, *CCNE1*, *CDK2*, and *CDK6* increased significantly after administration of EGF on the cultured granulosa cells. Amplification of these genes could shorten the G_1 interval, and thereby accelerate cell proliferation. These results demonstrate that up-regulation of *CCND1/CDK6* and *CCNE1/CDK2* mRNA expressions is important in EGF-induced proliferation of chicken granulosa cells from the prehierarchical follicles.

It is known that EGF exerts the mitogenic effect through activation of the EGFR signaling pathway. EGF has been found to inhibit granulosa cell apoptosis, whereas blockage of EGF receptor by EGFR inhibition has been found to induce apoptosis, as demonstrated by the activation of caspase-3 and by an increased percentage of subdiploid apoptotic nuclei in human luteinized granulosa cells (Khan *et al*., 2005). As is known, FSH signal transduction through its receptor (FSHR) is critical for the maintenance of normal gametogenesis in females. In this study, the expressions of both *EGFR* and *FSHR* mRNAs were elevated significantly in the chicken granulosa cells after treatment with EGF, but *LHR* mRNA expression was decreased. This result is consistent with a previous study that germinal disc-derived EGF stimulates cell proliferation and decreases *LHR* mRNA expression in proximal granulosa cells (Yao and Bahr, 2001b). To combine with the promoting effect of EGF on proliferation of chicken granulosa cells, this result suggests that EGF has an analogous function like FSH on follicular growth before selection, and retards cell differentiation in the prehierarchical follicles.

As a specific inhibitor of EGFR, tyrphostin AG1478 can effectively block EGFR signaling by inhibiting EGFR autophosphorylation without affecting the level of *EGFR* expression (Huang *et al*., 2010). In this study, the pro-proliferation action of EGF on granulosa cells from chicken SYFs was remarkably inhibited by AG1478. The inhibition of EGF-stimulated proliferation indicated that EGF carried out its proliferating action by binding with the cell surface receptor, which led to receptor phosphorylation in the prehierarchical follicles. Simultaneously, EGFR activation plays a physiological role in gonadotropin-induced oocyte maturation (Chen *et al*., 2008). Many reports suggested that AG1478 attenuated FSH-induced actions in ovary from various species. For example, AG1478 augmented *FSHR* mRNA expression and initiated progesterone production in undifferentiated granulosa cells of chicken ovary (Woods *et al*., 2007), and blocked meiotic resumption induced by FSH and amphiregulin in mouse cumulus cell-enclosed oocytes (Downs and Chen,

2008). We showed that AG1478 blocked FSH-stimulated granulosa cell proliferation. These results suggest that EGFR signaling pathway is critical in both EGF and FSH-induced granulosa cell proliferation.

In summary, this study revealed that *EGF* and *EGFR* mRNAs displayed a stage-specific change that manifested a high expression in granulosa cells from the prehierarchical follicles and thereafter decreased markedly till the largest preovulatory follicle. SYF represents a turning point of *EGF*/*EGFR* mRNA expression during follicle selection. EGF promoted proliferation of cultured granulosa cells through binding with EGFR, and was involved in FSH-stimulated cell proliferation. In addition, EGF-stimulated cell proliferation was accompanied with up-regulation of the cell cycle-regulating genes and decreased cell apoptosis. Therefore, the locally produced EGF may stimulate granulosa cell proliferation and retard cell differentiation in the prehierarchical follicles before selection in the egg-laying chicken.

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