ウズラ卵黄膜の精子レセプターの同定
および生合成機構について

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はしがき

本報告書は平成９・１０年度の２年間にわたる科学研究費補助金（基盤研究 C）『ウズラ卵黄膜の精子レセプターの同定および生合成機構について』の研究成果をまとめたものである。

鳥類の卵黄膜は、哺乳類の透明帯と相同の膜であり、精子レセプターとしての機能を備えているが、これに関する研究は今まで皆無であった。本研究代表者は、卵黄膜の分子量 33000 の糖タンパク（３３ｋタンパクと命名したが後に ZPC と改名）が精子レセプターの本体であることをつきとめ、科学研究費補助金によって研究を推進し、これが卵胞顆粒膜細胞で合成分泌されていることを発見した。

本報告書はその研究内容をまとめたものであるが、第１章では顆粒膜細胞の増殖に MAP キナーゼが深く関与していること、そして第２章では３３ｋタンパクの生合成にタンパクキナーゼ C が関与していることを中心とした。これが我々のあとに続く研究者にとって参考になれば幸いである。

研究組織

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Chapter 1

INVOLVEMENT OF MITOGEN-ACTIVATED PROTEIN (MAP) KINASE IN TGFα-STIMULATED CELL PROLIFERATION IN THE CULTURED GRANULOSA CELLS OF THE JAPANESE QUAIL

INTRODUCTION

In comparison with that of other species, granulosa cells of avian follicles are unique in that the homogeneous cells can be isolated as a sheet of monolayer. The number of granulosa cells increases about 5-fold during the rapid growth phase of follicles (1). The granulosa cells also produce perivitelline membrane glycoproteins (2), one of which is suggested to be the candidate of sperm receptor in quail oocyte (3). In addition, when the follicle becomes the largest, the granulosa cells differentiate to produce progesterone in response to luteinizing hormone (LH) (4).

It is well known that the function of granulosa cells is regulated not only by classical endocrine factors such as pituitary gonadotropins, but also under autocrine and paracrine controls by various growth factors (5). Growth factor is thought to mediate its mitogenic effects in cells through a complex signal transduction pathway, including the rapid phosphorylation of mitogen-activated protein (MAP) kinase, also termed extracellular signal-regulated protein kinase (ERK) (6).

In order to study the involvement of MAP kinase in proliferation of the granulosa cells in avian species, we have examined the presence of MAP kinase in quail granulosa cells and have studied the in-vitro effects of various hormones and growth factors on the activated state of the kinase.

MATERIALS AND METHODS

Animals. Female Japanese quail were obtained from Tokai Kigyo (Toyohashi, Japan) at 6 weeks of age. They were individually caged under a 14L:10D lighting schedule and provided water and a commercial diet *ad libitum*.

Reagents. Medium 199 with Earle's salts (M199), Dulbecco's modified Eagle medium, fetal calf serum (FCS) were purchased from GIBCO (Grand Island, NY). Ovine follicle stimulating hormone (FSH; NIDDK-oFSH-17) and ovine LH (NIDDK-oLH-25) were generous gifts from National Hormone and Pituitary Program (Baltimore, MD). Recombinant human transforming growth factor α (TGFα) was obtained from Bachem (Torrance, CA), and recombinant human insulin-like growth factor I and II (IGF-I and IGF-II) were obtained from Genzyme (Cambridge, MA). Mouse epidermal growth factor (EGF) was purchased from Sigma Chemical Co. (St. Louis, MO). Genistein was obtained from Wako chemical (Osaka, Japan). Mouse anti-pan-ERK antibody, generated against a 16 kDa fragment of rat ERK was obtained from Transduction Laboratories (Lexington, KY). Enhanced chemiluminescence (ECL) reagents were purchased from Amersham (Arlington Heights, IL). All other reagents were of analytical grade from Sigma Chemical Co. (St. Louis, MO).

Culture of Granulosa Cells. Approximately 8-10 hr before the expected time of ovulation, quail at 15-30 weeks of age were killed by cervical dislocation. Three largest follicles were removed and placed in 0.9% NaCl solution. Granulosa layers were isolated from theca layers according to the procedure described previously (4). Granulosa layers obtained from at least three quail were pooled and incubated for 10 min at 37 C with 500 U/ml collagenase (Type I; Sigma Chemical Co., St. Louis, MO) in Hanks' balanced salt solution (Hanks' BSS). Dispersed cells were washed three times with Hanks' BSS by repeated centrifugation at 80 x *g* for 3 min and finally suspended in M199 containing 10% FCS, 25 mM HEPES and antibiotics (100 U/ml

penicillin and 75 U/ml streptomycin). Cell numbers were counted by hematocytometer and cell viability, determined by the trypan blue dye exclusion test, was greater than 95%. The granulosa cells were inoculated in 24-well culture plates at a concentration of 1 x 10^5 viable cells per well for the cell number counting and [^{3}H]thymidine incorporation studies and at a concentration of 4 x 10^5 viable cells per well for the immunodetection of MAP kinase. The cells were cultured in a humidified atmosphere of 5% CO_2 and 95% air at 41 C. After the initial plating period of 6 hr, the medium was changed to M199 supplemented with 5% FCS, 25 mM HEPES and antibiotics, with or without various hormones, growth factors or inhibitor. To examine the effects on cell proliferation, the cultured granulosa cells were detached by adding trypsin-EDTA and their number was counted by hematocytometer.

Western Immunoblotting. In order to detect the immunoreactive MAP kinase, the culture medium was removed and the cells were treated with ice-cold lysis buffer (10 mM Tris-HCl pH 7.4, 150 mM NaCl, 1% Triton X-100, 0.5% Nonidet P-40, 1 mM EDTA, 1 mM EGTA, 0.2 mM sodium vanadate, 0.2 mM phenylmethane sulphonyl fluoride) with constant agitation at 4 C. Insoluble materials were precipitated by centrifugation at 25,000 x *g* for 15 min. The supernatants were served as total cell lysates and stored at -80 C until electrophoresis. Thawed cell lysates were subjected to electrophoresis on a 12% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and the separated protein was transferred onto nitrocellulose membrane. Western immunoblotting was performed using anti-pan-ERK antibody (final dilution, 1:2000), and the blots were visualized using ECL reagents. Multiple exposure times (1 to 10 min) was performed to obtain the maximum visualization of MAP kinase.

Thymidine Incorporation. For determination of [^{3}H]thymidine incorporation to the cells, the medium was removed, and cells were incubated in Dulbecco's modified Eagle medium containing 18.5 kBq [6-^{3}H]thymidine (SA; 603.1 GBq/mmole, NEN Life Science, Boston, MA). After 0.5-2 hr of incubation, cells were fixed with methanol for 10 min and air dried. After washing with two change of Hanks' BSS, the cells were treated with ice-cold 10% trichloroacetic acid and solubilized with 1% SDS in 0.3 N NaOH. The radioactivity in the portion of cell lysates was counted by a liquid scintillation counter.

Data Analyses. All experiments in this study were repeated at least twice, and the data were expressed as means ± S.D. and subjected to ANOVA (7). The means were separated by Duncan's multiple range tests. All statements of significance were based on a probability level of <0.05.

RESULTS

Effects on Cell Proliferation. In order to evaluate the effect of various hormones and growth factors on cell proliferation, quail granulosa cells isolated from the three largest follicles were cultured for 66 hr with TGFα (10 ng/ml), EGF (10 ng/ml), FSH (100 ng/ml), LH (100 ng/ml), IGF-I (10 ng/ml), IGF-II (10 ng/ml), progesterone (1 μg/ml), estradiol-17β (1 μg/ml) or testosterone (1 μg/ml). As shown in Figure 1, only TGFα had the prominent effect on increase in number of the granulosa cells. No significant effects were observed in the culture with other hormones or growth factors.

Next, we measured the [^{3}H]thymidine incorporation to the granulosa cells cultured for 42 hr with TGFα (10 ng/ml), EGF (10 ng/ml), FSH (100 ng/ml) or LH (100 ng/ml). As shown in Figure 2, [^{3}H]thymidine incorporation was more than 20-fold high in the granulosa cells cultured with TGFα compared with the control, while other hormones and growth factors had no effect. The time-course study of [^{3}H]thymidine incorporation to the cells was also examined after 42 hr of culture with or without TGFα. [^{3}H]Thymidine incorporation in the

cells cultured without TGF α was low throughout 2 hr of incubation, while the cells that had been stimulated with TGF α for 42 hr incorporated more [^{3}H]thymidine in a time-related manner (Figure 3).

Retarded Mobility of MAP Kinase by TGF α. To investigate the effect of various hormones and growth factors on MAP kinase in granulosa cells, we performed the Western immunoblotting analysis using anti-pan-ERK antibody. The lysates of quail granulosa cells were shown to contain immunodetectable protein with 42 kDa suggesting that the presence of MAP kinase (Figure 4; Lane CONT). By the stimulation of the cells with TGF α for 10 min, a retarded mobility of MAP kinase in the gel of SDS-PAGE was observed by close inspection of the gel (Figure 4; Lane TGF α). Other hormones, such as EGF, FSH or LH did not affect the mobility shift in the gel.

To investigate the dose response of TGF α on the mobility shift of the immunodetectable MAP kinase, the granulosa cells were stimulated with various concentration of TGF α for 10 min. Result in Figure 5 indicates that as low as 1 ng/ml TGF α can cause the mobility shift of MAP kinase (Figure 5; Lane 1.0 ng/ml), and this became more pronounced by the stimulation with 10 ng/ml of TGF α (Figure 5; Lane 10 ng/ml). We also studied the time-course response of TGF α treatment on MAP kinase. The lysates prepared from the granulosa cells treated with 10 ng/ml of TGF α for 1, 2.5, 5, 10 and 30 min were probed using anti-pan-ERK antibody (Figure 6). The 1-min of exposure to TGF α caused an apparent mobility shift of MAP kinase in the gel, and it continued to be observed until 30 min of incubation without no change of relative mobility shift.

Effects of Genistein. In the next set of experiments, we examined the effect of genistein, a potent inhibitor of tyrosine kinase (8), on the [^{3}H]thymidine incorporation and the mobility shift of the MAP kinase on the gel. Results shown in Figure 7 demonstrates that TGF α-stimulated [^{3}H]thymidine incorporation was completely abolished by the addition of genistein, and this inhibitor also blocked the retarded mobility of the MAP kinase on the gel induced by the addition of TGF α (Figure 8).

Figure legends

Figure 1. Effects of various hormones and growth factors on granulosa cell proliferation. Granulosa cells were inoculated on 24-well culture plates at a concentration of 1 x 10^5 viable cells per well in Medium 199 containing 10% (v/v) FCS and allowed to attach for 6 hr. The cells were then cultured for a further 66 hr in Medium 199 containing 5% (v/v) FCS in the absence (CONT) or presence of TGFα (10 ng/ml), EGF (10 ng/ml), FSH (100 ng/ml), LH (100 ng/ml), IGF-I (10 ng/ml), IGF-II (10 ng/ml), progesterone, (P4; 1 μg/ml), estradiol-17β (E2; 1 μg/ml) or testosterone (T; 1 μg/ml). Cell numbers were counted on hematocytometer. Values are means ± S.D. of six independent experiments.

Figure 2. Effects of TGFα, EGF, FSH and LH on [^{3}H]thymidine incorporation to the granulosa cells. Granulosa cells were inoculated on 24-well culture plates at a concentration of 1 x 10^5 viable cells per well in Medium 199 containing 10% (v/v) FCS, and allowed to attach for 6 hr. Cells were then cultured for a further 42 hr in Medium 199 containing 5% (v/v) FCS in the absence (CONT) or presence of TGFα (10 ng/ml), EGF (10 ng/ml), FSH (100 ng/ml) or LH (100 ng/ml). After the culture, the cells were incubated with 18.5 kBq [^{3}H]thymidine, and incorporation of radioactivity was measured for 1 hr as descried in Materials and Methods. Values are means ± S.D. of triplicated assay of single experiment. Similar results were obtained from the repeated experiments, in which the raioactivities of incorporated thymidine were lower than this experiment.

Figure 3. Time course of [^{3}H]thymidine incorporation to the granulosa cells. Granulosa cells were inoculated on 24-well culture plates at a concentration of 1 x 10^5 viable cells per well in Medium 199 containing 10% (v/v) FCS, and allowed to attach for 6 hr. The cells were then cultured for a further 42 hr in Medium 199 containing 5% (v/v) FCS in the absence (■) or presence (●) of TGFα (10 ng/ml). After the culture, [^{3}H]thymidine incorporation to the cells was measured for 0, 0.5, 1, 1.5 and 2 hr. Values are means ± S.D. of triplicated assay of a single experiment.

Figure 4. Effects of various hormones or growth factors on the immunodetection of MAP kinase in the granulosa cells. Granulosa cells were plated on 24 multi-well plates in a concentration of 4 x 10^5 cells/well in Medium 199 containing 10% (v/v) FCS, and allowed to attach for 6 hr. The cells were then treated with TGFα (10 ng/ml), EGF (10 ng/ml), FSH (100 ng/ml) or LH (100 ng/ml) in Medium 199 containing 5% (v/v) FCS for 10 min. A portion of the cell lysates was subjected to SDS-PAGE and the bands were detected by Western immunoblotting using anti-pan-ERK antibody.

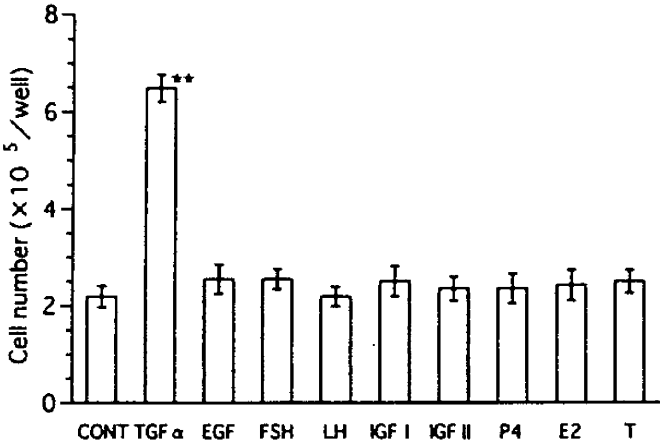
Figure 5. Concentration dependence of TGFα treatment on the immunodetection of MAP kinase in cultured granulosa cells. The granulosa cells were treated with 0-10 ng/ml TGFα for 10 min as described in Figure 4. A portion of the cell lysates was subjected to SDS-PAGE and the bands were detected by Western immunoblotting using anti-pan-ERK antibody.

Figure 6. Temporal response of TGFα treatment on the immunodetection of MAP kinase in cultured granulosa cells. The granulosa cells were treated with (TGFα) or without (CONT) 10 ng/ml TGFα for 1 - 30 min as described in Figure 4. A portion of the cell lysates was subjected to SDS-PAGE and the bands were detected by Western immunoblotting using anti-pan-ERK antibody.

Figure 7. Effects of genistein on TGFα-stimulated [^{3}H]thymidine incorporation to the granulosa cells. The granulosa cells were plated on 24 multi-well plates in a concentration of 1 x 10^5 cells/well in Medium 199 containing 10% (v/v) FCS, and allowed to attach for 6 hr. Cells were then cultured for a further 42 hr in Medium 199 containing 5%

(v/v) FCS with or without TGFα (10 ng/ml) and/or genistein (100 μ g/ml). After washing, the cells were incubated with 18.5 kBq [^{3}H]thymidine for 1 hr. Radioactivity incorporated to the cell was measured as described in Materials and methods. Values are means $\pm$ S.D. of triplicated well of single experiment. Similar results were obtained from the repeated experiments.

Figure 8. Effect of genistein on the immunodetection of MAP kinase in cultured granulosa cells. The granulosa cells were treated for 10 min with media alone (CONT) or 10 ng/ml TGFα in the presence or absence of 100 μg/ml genistein (GEN). After treatment, a portion of the cell lysates was subjected to SDS-PAGE and the bands were detected by Western immunoblotting using anti-pan-ERK antibody.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6
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2
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CONT
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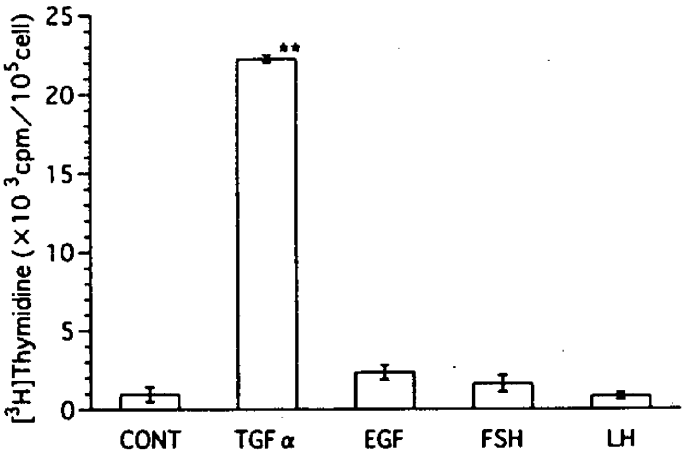
EGF
FSH
LH
IGF I
IGF II
P4
E2
T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15
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5
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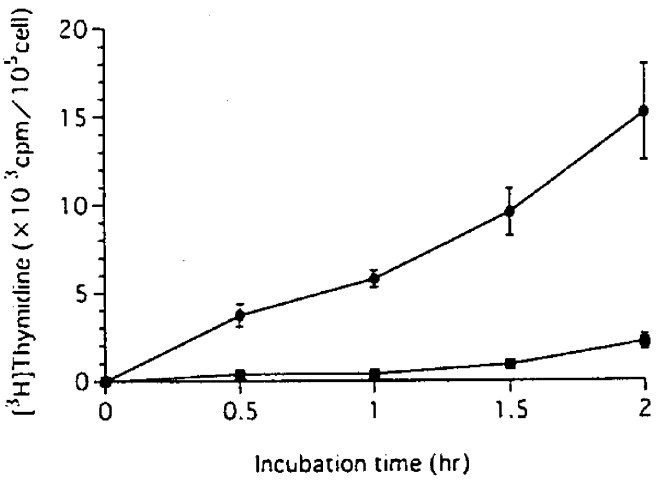
CONT
TGF α
EGF
FSH
LH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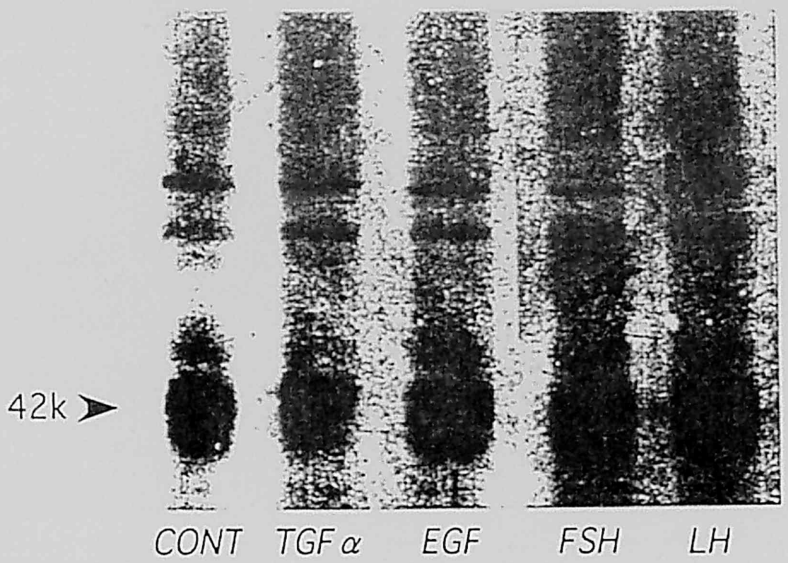
1
1.5
2
Incubation time (hr)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TGF α
EGF
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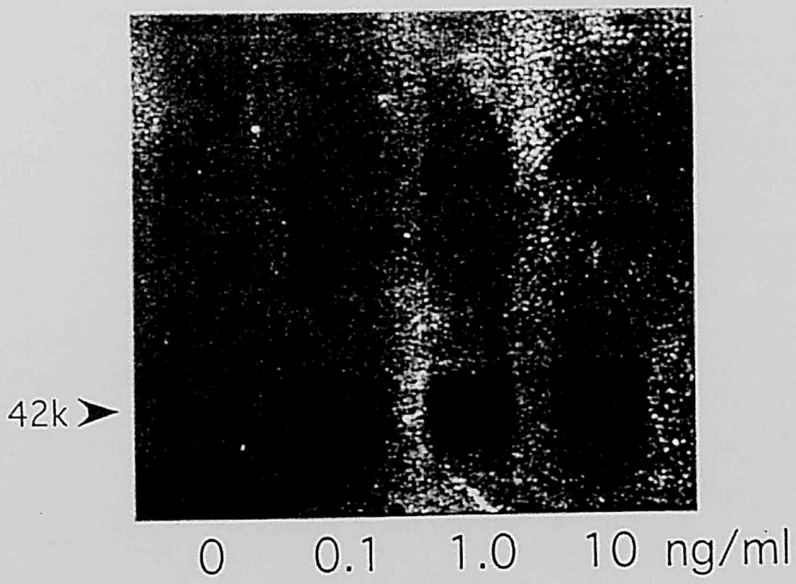
LH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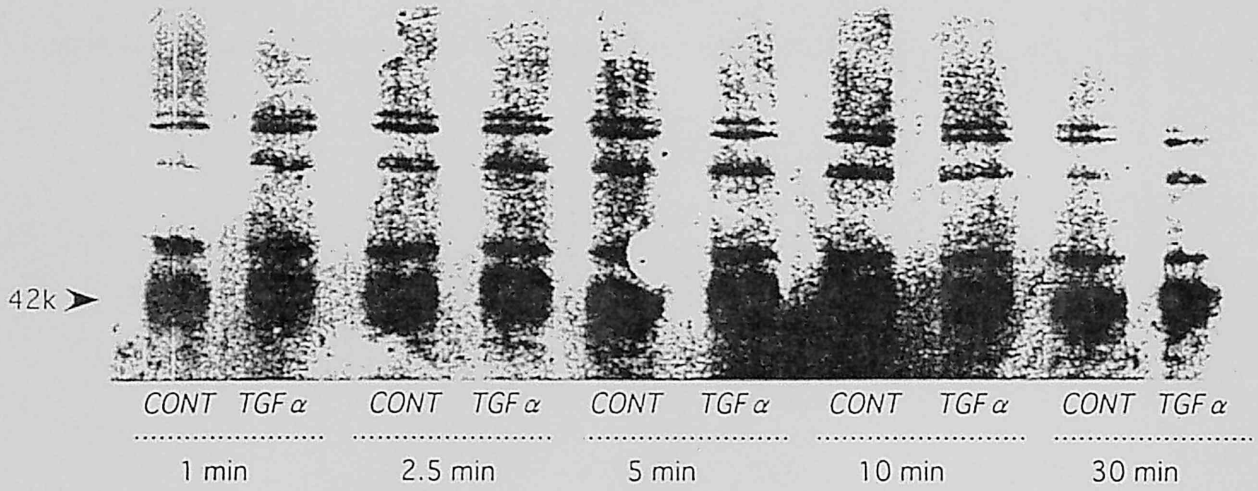
1.0
10 ng/ml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CONT TGF α
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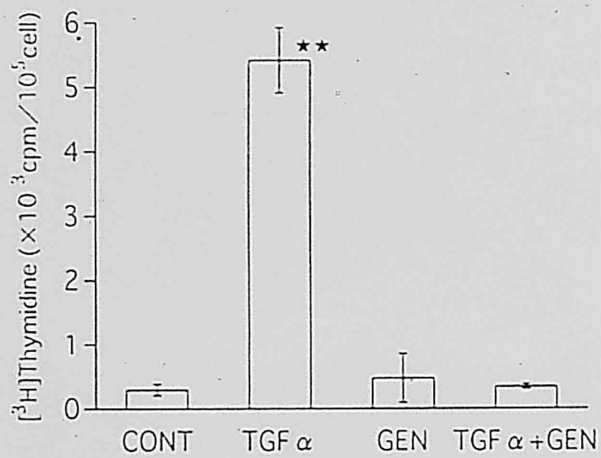
CONT TGF α
1 min
2.5 min
5 min
10 min
30 min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TGF α
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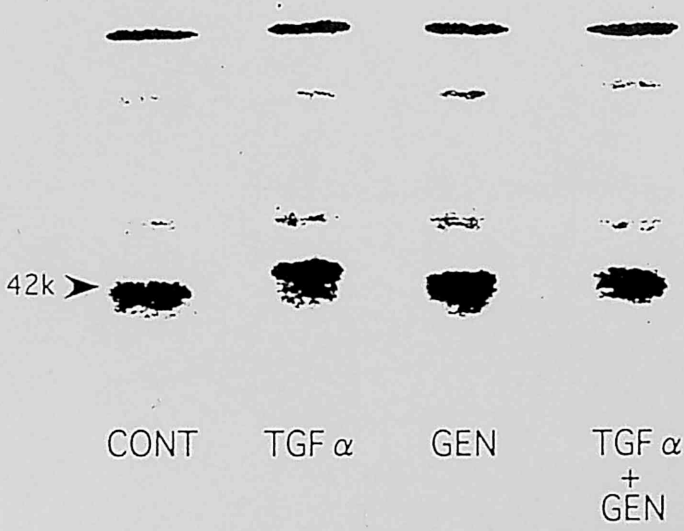
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DISCUSSION

The present study provides the first evidence of an involvement of MAP kinase in the regulation of cell proliferation by TGF α in quail granulosa cells.

It is reported that several growth factors such as EGF and TGF α have a potent mitogenic effect on mammalian and avian granulosa cells (9, 10). In these species, ovarian theca and granulosa cells produce EGF or TGF α (11, 12). Moreover, Onagbesan *et al*. (12) detected the EGF receptor (EGF-R) in chicken granulosa cells by the immunohistochemical analysis using polyclonal antibody against EGF-R, and showed that the cells obtained from small follicles tended to exhibit more intense staining than those from mature follicles. Taken together, it is suggested that EGF or TGF α might control the granulosa cell proliferation during follicular growth in paracrine or autocrine manner.

In our results presented in Figures 1 and 2, TGF α stimulated the granulosa cell proliferation, while EGF failed to exert its mitogenic action. EGF and TGF α are structurally- and functionally-related growth factors (13) and both are believed to bind to EGF-R (14). However, the binding affinity of these growth factors to EGF-R is different. For example, mouse EGF bound to chicken EGF-R, expressed in NIH-3T3 cells, with approximately 100 folds lower affinity than that of human TGF α (15). In chicken granulosa cells, human TGF α bound to EGF-R with 300 times higher affinity than human EGF (16). Although there is no information concerning the ligand specificity of quail EGF-R, the effectiveness of TGF α on cell division demonstrated in the present study could be attributed to its higher binding affinity to EGF-R than EGF itself.

The precise signal transduction cascade on cell proliferation and the participation of MAP kinase to this process is not well understood in avian granulosa cells. In mammalian system, it is widely accepted that the ligand binding to the EGF-R leads to autophosphorylation of itself, and it activates Ras, a small GTP-binding protein (17). Activated Ras is then able to associate with and activate Raf-1 (18), a member of MAP kinase kinase kinases that activate MAP kinase kinases, also referred to as MEKs (19). Activated MEKs are able to activate MAP kinase by phosphorylating both threonine and tyrosine residues (20). The activation of MAP kinase occurs within a few minutes of stimulation by growth factor in porcine granulosa cells (21) and dog thyroid epithelial cells (22). Furthermore, a direct relationship between MAP kinase activation and cell proliferation was suggested (6).

Keel *et al*. (21) showed that porcine granulosa cells contain several MAP kinase isoforms; ERK 1 (44 kDa), ERK 2 (42 kDa) and two kinds of MAP kinase-related proteins (54 kDa and 87 kDa). EGF stimuli caused a retarded mobility of ERK 2 in the gel of SDS-PAGE, whereas other proteins were not affected. It is reported that the retardation of mobility is related to the activation of MAP kinase (22, 23), due to the phosphorylation of tyrosine and threonine residues (24). Thus, ERK 1 and 2 are regarded as key enzymes for the signal transduction of cell proliferation in porcine granulosa cells. Immunodetection of MAP kinase in quail granulosa cells using anti-pan-ERK antibody indicated that the cells contain 42 kDa protein (Figure 4; Lane CONT), and the treatment of the cells with TGF α resulted in a mobility shift of this protein, electrophoretically from fast to slow migration (Figure 4; Lane TGF α). These results indicated that MAP kinase in quail granulosa cells was phosphorylated and activated by the stimulation of TGF α.

To investigate the participation of tyrosine phosphorylation in quail granulosa cell proliferation, we evaluated the effect of genistein, specific inhibitor of tyrosine kinase (8), on [^{3}H]thymidine

incorporation and the mobility shift of MAP kinase. As expected, genistein inhibited not only TGFα-stimulated [^{3}H]thymidine incorporation but also the mobility shift of MAP kinase (Figures 7 and 8). These data indicated that TGFα promotes granulosa cell proliferation via signal transduction cascade involved with tyrosine kinase activation located upstream of MAP kinase.

In contrast, Das *et al*. (25) reported that FSH stimulation leads to activation of both ERK 1 and 2 in immature rat granulosa cells via cyclic AMP and protein kinase A dependent pathway. Cameron *et al*. (26) using porcine granulosa cells demonstrated that not only gonadotropins but also cyclic AMP increase enzyme activities of ERK 1 and ERK 2 in nuclear fractions in a different manner from that of EGF stimulation. In our results, FSH and LH had no effect on MAP kinase activation suggesting that there are no MAP kinases, which are activated by these gonadotropins in quail granulosa cells. Yet, another possibility is that there is other MAP kinases, which can not be detected by the antibody raised against mammalian antigen.

In conclusion, our results comprise the first report that quail granulosa cells contain MAP kinase, and it was immediately activated by TGFα stimulation.

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Chapter 2

FOLLICLE-STIMULATING HORMONE ENHANCED PRODUCTION OF 33-KDA PROTEIN OF INNER LAYER OF VITELLINE MEMBRANE BY QUAIL GRANULOSA CELLS

INTRODUCTION

Mature oocytes of vertebrates are surrounded by extracellular matrix generally termed the egg envelope, though the different names were adopted in different classes [1]. For most mammalian species, egg envelope called zona pellucida (ZP) comprises three glycoproteins; ZPA, ZPB and ZPC [2]. These components are synthesized and secreted to form the ZP during the process of oocyte growth. There is clear evidence showing that all the ZP glycoproteins in mouse are synthesized by the oocytes itself [3] whereas the granulosa cells in the follicles also participate in the formation of the ZP in rabbit [4]. While the vitelline membrane, one of the egg envelope of amphibia, is evidently synthesized in the oocytes [5], a glycoprotein component of the chorion, teleost egg envelope, is produced in the liver and transported to the ovary by the blood circulation, like vitellogenin [6].

The avian oocytes are surrounded by the several layers of egg envelope, called vitelline membrane, albumen, shell membrane and eggshell. The vitelline membrane consists of three layers, the inner and outer layers with the continuous membrane in between [7]. The outer layer, which is composed of a varying number of sub-layers of a latticework of fine fibrils, is formed in the infundibulum part of the oviduct [8]. The continuous membrane, which is a thin granular membrane, is probably formed in the infundibulum because the oocyte is surrounded only by the inner layer at the time of ovulation [8]. The inner layer is a three-dimensional network of fibers and is observed between the granulosa cells and the oocyte in follicles before ovulation [9]. Two glycoproteins were identified as components of the inner layer in avian oocytes; 33- and 175-kDa glycoproteins in quail [10] and 32- and 183-kDa glycoproteins in hen [11]. The presence of 33-kDa glycoprotein was demonstrated in quail granulosa cells [12], and mRNA for 32-kDa glycoprotein was detected in chicken granulosa cells [13], indicating that the inner layer of avian vitelline membrane is originated in the granulosa cells of preovulatory follicles.

The cDNA encoding for 32-kDa protein in hen was cloned (D89097, Takeuchi Y. et al.) and a high degree of homology of nucleotide sequence and primary polypeptide structure with mammalian, amphibian and fish ZPC homologues was pointed out [13]. Although a common ancestor would be suggested for ZPC homologues of various species, expression of ZPC appears to be regulated differently in different tissue. While the regulation of mammalian ZP production in oocytes has not been studied, gene expression of fish ZP homologue in liver was clarified to be regulated by estrogens [6, 14, 15]. Moreover, down-regulation of ZP homologue by human chorionic gonadotropin and 11-ketotestosterone was reported in the testes of Japanese eel [16].

The aim of present study was conducted to evaluate the hormonal regulation in production of inner layer of vitelline membrane by granulosa cells in Japanese quail. We investigated in-vitro effects of various hormones, growth factors and protein kinase inhibitors on 33-kDa protein production during culture.

MATERIALS AND METHODS

Animals Female Japanese quail were obtained from Tokai Kigyo (Toyohashi, Japan) at 6 weeks of age. They were individually caged under a 14L:10D lighting schedule with light on at 0500, and provided water and a commercial quail diet *ad libitum*. Almost all birds laid

eggs regularly at the same time (between 1600 and 1800) every day.

Hormones and Chemicals Medium 199 with Earle's salts (M199), Dulbecco's minimum essential medium (D-MEM), leucine-free D-MEM, fetal calf serum (FCS) and formalin-fixed *Staphylococcus aureus Cowan I* (Immunoprecipitin) were purchased from GIBCO-BRL (Grand Island, NY). Ovine FSH (NIDDK-oFSH-17) and ovine LH (NIDDK-oLH-25) were generous gifts from National Hormone and Pituitary Program (Baltimore, MD). Recombinant human transforming growth factor α (TGFα) was obtained from Bachem (Torrance, CA) and recombinant human insulin-like growth factor I and II (IGF-I and -II) were obtained from Genzyme (Cambridge, MA). Mouse epidermal growth factor (EGF) was purchased from Sigma Chemical Company (St. Louis, MO). N-[2-(*p*-bromocinnamylamino)ethyl]-5-isoquinolinesulfonamide (H-89) was obtained from Seikagaku Kogyo (Tokyo, Japan) and staurosporine was purchased from Biomol Research Lab. (Plymouth Meeting, PA).

Isolation of granulosa layers Approximately 8 to 10 h before the expected time of ovulation, quail at 15 to 30 weeks of age were killed by cervical dislocation. Ovarian follicles were removed and placed in 0.9% NaCl solution. Granulosa layers (granulosa cells, inner layer of vitelline membrane and basal lamina) were isolated from theca layers according to the procedure of Gilbert and coworkers [17].

Western immunoblotting Granulosa layers obtained from five largest follicles and small white follicles (SWF) were solubilized in 1% SDS buffered at pH 6.8 with 70 mM Tris-HCl (SDS-Tris) overnight with constant shaking and the supernatant was obtained by centrifugation at 10,000 x *g* for 10 min. Protein concentration in each sample was determined by the method of Lowry et al. [18]. Aliquots (3 μg) of the solubilized materials were subjected to SDS-PAGE with 12 % separating gel as described previously [19]. The protein was transferred onto polyvinylidene difluoride (PVDF) membrane (Immobilon, Millipore, Bedford, MA). PVDF membrane was blocked with 0.1% gelatin in saline buffered at pH 7.4 with 10 mM Tris-HCl (TBS) and incubated overnight with anti-33-kDa protein antiserum [12] diluted in TBS (1:2,000). The membrane was washed twice with TBS containing 0.1% Tween 20 (TBS-T) and incubated for 2.5 h with horseradish peroxidase conjugated anti-rabbit IgG (Cappel, Durham, NC) diluted with TBS-T (1:1,000). Finally, membrane was immersed in 50 mM Tris-HCl (pH 7.6) containing 0.06% 3, 3'-diaminobenzidine tetrahydrochloride and 0.03% $NiCl_2$. Visualized blots were quantitated using Macintosh-based image analyzing system (NIH image v.1.55).

Culture of granulosa cells Granulosa layers were incubated for 10 min at 37 ℃ with 500 U/ml collagenase (Type I, Sigma Chemical Company, St. Louis, MO) in Hanks' balanced salt solution (Hanks' BSS). Dispersed cells were washed three times with M199 containing 25 mM Hepes and antibiotics (100 U/ml penicillin and 75 U/ml streptomycin) by repeated centrifugation at 80 x *g* for 3 min and finally suspended in M199 containing 10% FCS, 25 mM Hepes and antibiotics. After counting the cell numbers by hematocytometer, the granulosa cells were inoculated in 24-well culture plates at a concentration of 1 x 10^5 viable cells per well and cultured in a humidified atmosphere of 5% CO_2 and 95% air at 41 ℃. After the initial plating period of 6 h, the medium was changed to M199 supplemented with 5% FCS, 25 mM Hepes and antibiotics, with or without FSH, LH, EGF, IGF-I, IGF-II or TGFα. The cells were further cultured for 66 h (6 to 72 h after inoculation). Total volume of culture medium was adjusted to 0.8 ml/well, and the granulosa cells used in all experiments were obtained from at least two birds.

In order to examine the effects of protein kinase inhibitors, the granulosa cells were cultured for 66 h (6 to 72 h after inoculation) with or without FSH in the presence or absence of 10 nM staurosporine or 1 μM H-89 dissolved in dimethylsulfoxide (final

concentration: 0.1%).

Measurement of 33-kDa protein production After 66 h of culture, medium was discarded and cells were washed with 3 changes of Hanks' BSS. Cells were then incubated for 6 h with 0.2 ml of [4, 5-^{3}H]leucine (229.4 kBq, SA; 2.55 TBq/mmole, Amersham Life Science, Arlington Heights, IL) in leucine-free D-MEM at a constant agitation in a humidified atmosphere of 5% CO_2 and 95% air at 41 ℃. After 6 h of incubation, medium was collected and stored at -20 ℃ until for measurement of 33-kDa protein in the medium. The residual cells were washed 3 times with ice-cold PBS and lysed with 0.2 ml of ice-cold RIPA buffer containing 150 mM NaCl, 1% Nonidet P-40, 0.5% deoxycholate, 0.1% SDS and 50mM Tris-HCl (pH 7.5). After removal of insoluble materials by centrifugation at 10,000 x *g* for 1 min, the supernant was served as total cell lysate and stored at -20 ℃ until measurement of 33-kDa protein in the cells.

Fifty μl of medium or cell lysate were incubated with 12.5 μl of anti-33-kDa protein antiserum [12] (1:10 dilution) for 1 h at 4 ℃ and were further incubated with 25 μl of Immunoprecipitin for 30 min at 4 ℃. Immune complex were washed with PBS by repeated centrifugation at 10,000 x *g* for 1 min and finally suspended in 50 μl of Laemmli's sample buffer [19] and solubilized by heating at 95 ℃ for 5 min. The supernatant obtained by centrifugation at 10,000 x *g* for 1 min was counted for their radioactivities by a liquid scintillation spectrophotometer. Radioactivities derived from the medium and cells were summed and data were expressed as means ± SEM per 10^5 cells.

Statistical analysis The data were subjected to one-way or two-way ANOVA [20]. The means were separated by Duncan's multiple range test. All statements of significance were based on a probability level of < 0.05.

RESULTS

33-kDa Protein Contents in Maturing Follicles The SDS-solubilized granulosa layer was subjected to western immunoblotting to assess the change of 33-kDa protein contents in preovulatory follicles. As shown in Figure 1, SWF contains very small amount of immunoreactive proteins, and the intensity of 33-kDa protein band increase as corresponding to follicular maturation. It reaches maximum when the granulosa layer was isolated from the second largest follicle. In the largest follicle, intensity of the band was comparable to that of F2.

Effects of Various Hormones and Growth Factors on 33-KDa Protein Production in Culture The granulosa cells isolated from the three largest follicles were pooled, and cultured with or without FSH (100 ng/ml), LH (100 ng/ml), EGF (10 ng/ml). IGF-I (10 ng/ml), IGF-II (10 ng/ml) or TGFα (10 ng/ml). The amount of 33-kDa protein produced by granulosa cells was significantly increased ($p < 0.01$) by the addition of FSH compared with the control culture (Fig. 2). In contrast, TGF α reduced ($p < 0.05$) the 33-kDa protein production (Fig. 2). Other hormones or growth factors had no effects. The dose-response of FSH on 33-kDa protein production was also examined. Results in Figure 3 shows that as low as 10 ng/ml of FSH causes significant increase in 33-kDa protein production and this effect reaches maximum when the granulosa cells were cultured with 100 ng/ml of FSH. However, 300 or 1,000 ng/ml of FSH was less effective than 100 ng/ml.

To compare the FSH-response of the cells during follicular maturation, the granulosa cells isolated from F1, F2 and F3 were separately cultured with FSH (100 ng/ml) and the amounts of 33-kDa protein were measured. As shown in Figure 4, the amount of 33-kDa protein produced without the stimulation of FSH decreased with the stage of follicular maturation. Addition of FSH caused a

significant increase in 33-kDa protein production by the cells irrespective of the maturational stage of follicles. But FSH effect was more pronounced in the cells obtained from the immature follicles than the mature one (F3 > F2 > F1).

Effects of Protein Kinase Inhibitors Next, we examined the effects of protein kinase inhibitors, staurosporine and H-89, on FSH-stimulated 33-kDa protein production. By the addition of staurosporine to the media during the culture with FSH, the amount of 33-kDa protein production decreased (Table 1). No such effect was observed by the addition of H-89 (Table 1).

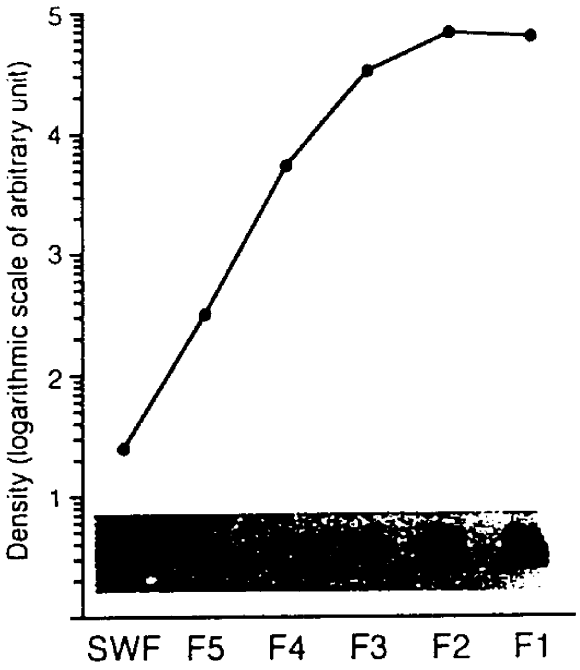
LEGENDS OF FIGURES

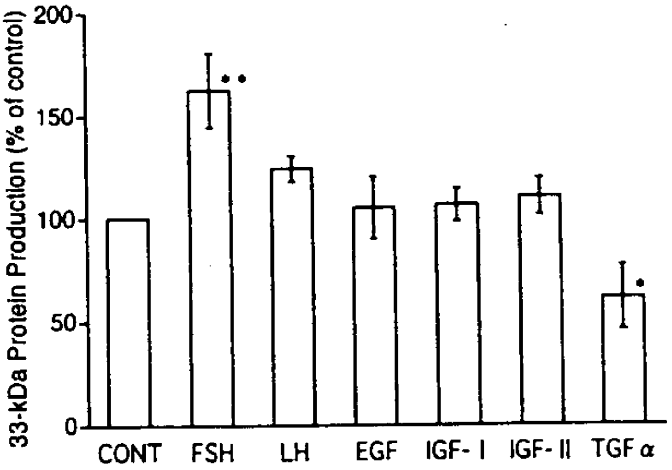
FIG. 1. Changes in the 33-kDa protein content in the granulosa layer of preovulatory follicles during maturation. F1: the largest follicle, F2: the second largest follicle, F3: the third largest follicle, F4 the fourth largest follicle, F5: the fifth largest follicle, SWF; small white follicles.

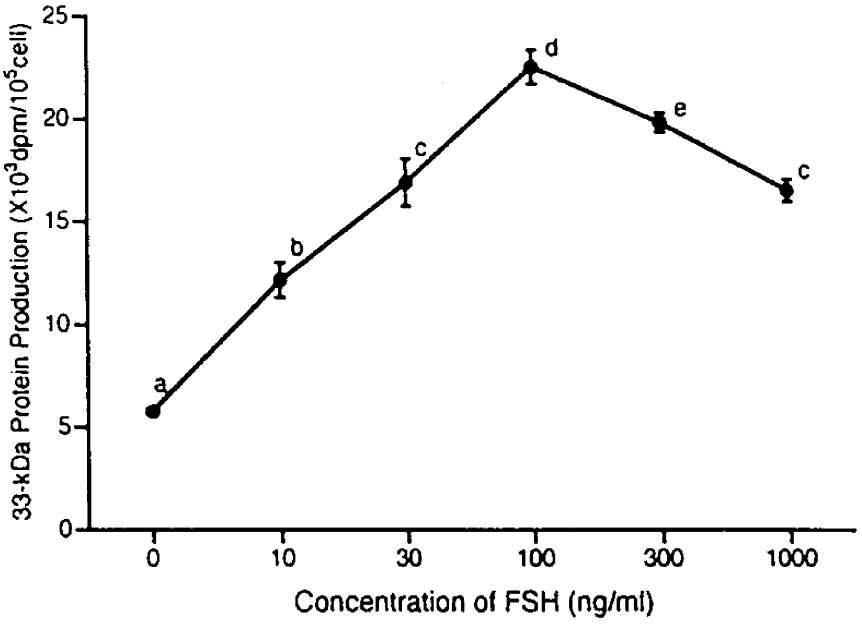
FIG. 2. Effects of FSH (100 ng/ml), LH (100 ng/ml), EGF (10 ng/ml), IGF-Ⅰ (10 ng/ml), IGF-Ⅱ (10 ng/ml) or TGFα (10 ng/ml) on 33-kDa protein production by the cultured granulosa cells. The data were expressed as a percentage of the culture without hormone (CONT). Values are means ± SEM of six independent experiments. * $P < 0.05$ and ** $P < 0.01$ against control.

FIG. 3. Concentration-dependent stimulation by FSH of 33-kDa protein production in the cultured granulosa cells. Values are means ± SEM of triplicate culture wells of single experiment. Values with different superscripts are significantly different ($P < 0.05$).

FIG. 4. Effect of FSH on 33-kDa protein production in the cultured granulosa cells isolated from follicles of different maturation stages. Values are means ± SEM ($n = 3$) and the values with different superscripts are significantly different ($P < 0.05$).


Density (logarithmic scale of arbitrary unit)
5
4
3
2
1
SWF
F5
F4
F3
F2
F1


33-kDa Protein Production (% of control)
200
150
100
50
0
CONT
FSH
LH
EGF
IGF-I
IGF-II
TGF α


33-kDa Protein Production (X10³dpm/10⁵cell)
25
20
15
10
5
0
a
b
c
d
e
c
0
10
30
100
300
1000
Concentration of FSH (ng/ml)

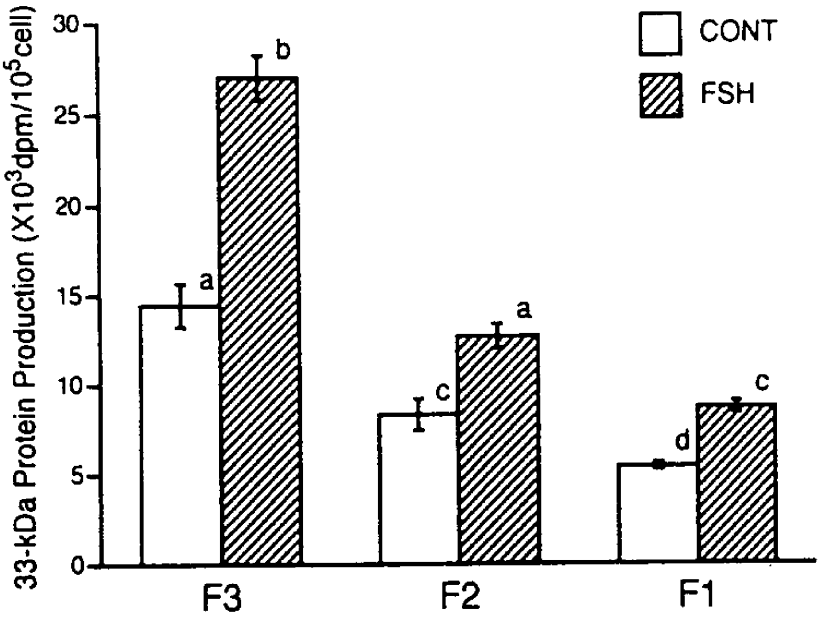

33-kDa Protein Production (X10³dpm/10⁵cell)
30
25
20
15
10
5
0
CONT
FSH
a
b
c
a
d
c
F3
F2
F1

TABLE 1. Effects of protein kinase inhibitors on FSH-stimulated 33-kDa protein production in the granulosa cells.

Treatment	No inhibitor	Staurosporine (10 nM)	H-89 (1 μM)
Control	14.31 ± 3.69 [a]	-	-
FSH (100 ng/ml)	23.20 ± 6.71 [b]	6.22 ± 1.70 [c]	22.79 ± 7.07 [b]

Data are the radioactivities (mean ± SEM) in the immunoprecipitates expressed in 10^3 dpm/10^5 cells.

The values with different superscripts are significantly different ($p < 0.01$).

DISCUSSION

Results of the present study demonstrate that quail granulosa cells in culture produce 33-kDa protein, which is one of the components of the inner layer of vitelline membrane. The data also provided the evidence that FSH stimulates 33-kDa protein production *in vitro*.

It is reported that avian granulosa cells express FSH receptor on cell surface and it plays an important role in the function of granulosa cells [21]. FSH was thought to interact with granulosa cells in maturing follicles rather than matured follicle, because only granulosa cells obtained from less mature follicles can produce progesterone in response to FSH [22]. Indeed, Ritzhaupt and Bahr [23] reported that the granulosa cells obtained from small yellow follicles were shown to contain FSH receptors and the number of binding sites decrease during follicular maturation. In our results, the magnitude of FSH stimulation of 33-kDa protein production decreased with follicular maturation (see Fig. 4). The reduced effect of FSH on 33-kDa protein production in F1 granulosa cells might be due to reduced binding sites for FSH in the granulosa cells of matured follicle. This in-vitro observation was also confirmed by the fact that the immunoreactive materials with anti-33-kDa protein antiserum were not changed in F1 and F2 granulosa layers (Fig. 1).

In mammals, the egg envelope is called zona pellucida, a homologue of inner layer of vitelline membrane, and plays an essential role for species-specific gamete recognition (reviewed in [24, 25]). It is reported that zona pellucida in mouse oocyte consists of three glycoprotein (ZP1, ZP2 and ZP3), of which ZP3 with 83,000 in molecular weight has been identified as sperm receptor [26]. Kuroki and Mori [27] previously demonstrated that the inner layer of vitelline membrane obtained from preovulatory follicle contains the binding site for spermatozoa and suggested that the protein composed inner layer of vitelline membrane might be responsible for oocyte-sperm recognition in quail. It is possible to postulate that one of the important functions of 33-kDa protein might be the sperm receptor in quail.

Other proteins known to be secreted from avian granulosa cells are fibronectin and plasminogen activator. Fibronectin, an insoluble adhesive glycoprotein composed basal lamina, which is situated on the opposite side of granulosa cells to inner layer of vitelline membrane [28]. In chicken granulosa cells, FSH enhances fibronectin production and stimulatory effect of FSH decreases with advancing follicular development [29]. Moreover, EGF and TGFα also stimulate fibronectin production [30].

Plasminogen activator, a neutral soluble serine protease that catalyzes the conversion of plasminogen to plasmin, is thought to be implicated in the process of follicular rupture at the time of ovulation [31] and in the process of cumulus cell expansion in mammals [32]. However, in chicken, the function of plasminogen activator is still obscure because its activity decreases in the few hours before ovulation suggesting that the enzyme may not be important for the rupture of follicle [33]. It is reported that LH markedly suppresses the activity of plasminogen activator [34]. By contrast, prostaglandins E_1 and E_2 and several growth factors have been shown to stimulate chicken granulosa cell plasminogen activator [34, 35].

Thus, hormonal regulation of 33-kDa protein and fibronectin production as well as control of activity of plasminogen activator are likely different. It is unknown whether single population of the granulosa cells assorts proteins to secret to either side or several populations of the cells assign for secretion of different proteins. Although the nature of the cellular mechanisms responsible for the polarity of protein secretion remains to be solved, staggered arrangement of position of nuclei in the granulosa cells

either apical or basal sides was observed [36].

In the study conducted to fibronectin production in domestic fowl showed that 8-bromo-cAMP, an analog of cAMP stimulates fibronectin production [37]. This suggested that fibronectin biosynthesis is mediated by protein kinase A (PKA)-dependent pathway because elevation of intracellular cAMP level leads to PKA activation. Tilly and Johnson [38] reported that the stimulation of plasminogen activator by EGF was completely abolished by the addition of H-7. H-7 inhibits the effects of phorbol ester-induced protein kinase C (PKC) activation in the hen [39]. Therefore, the stimulation of plasminogen activator by EGF might be mediated via PKC activation.

In the present study, FSH-stimulated 33-kDa protein production was significantly suppressed by the addition of staurosporine, while H-89 had no effect. Staurosporine is known to inhibit PKC activity with Ki = 3 nM [40] as well as PKA activity with Ki = 8 nM [41], whereas H-89 is a selective inhibitor for PKA (Ki = 50 nM for PKA and 30 μM for PKC [42]). Therefore, we suggest that FSH-stimulated 33-kDa protein production might be mediated by PKC-dependent pathway.

In summary, present study demonstrates that granulosa cells of Japanese quail produce 33-kDa protein, which is major component of inner layer of vitelline membrane and FSH stimulates 33-kDa protein synthesis *in vitro*. This process might be, at least in part, mediated by staurosporine-sensitive PKC-dependant pathway.

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