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Epidermal Growth Factor-Induced Proliferation of Chicken Primordial Germ Cells: Involvement of Calcium/Protein Kinase C and NFKB1[1]

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ABSTRACT

Epidermal growth factor (EGF) has been shown to stimulate survival in diverse cells in vitro. In the present study, the effects of EGF and the EGF-related signaling pathway on proliferation of chicken primordial germ cells (PGCs) were investigated. Results showed that EGF (10–100 ng/ml) increased the number and area of PGC colonies in a time- and dose-dependent manner. EGF also activated PKC, a process that was inhibited by AG1478 (an EGFR tyrosine kinase inhibitor) and ethyleneglycol-*bis*-(beta-aminoethyl ether)-*N*,*N*′-tetraacetic acid (EGTA; an intracellular Ca^{2+} chelator). In addition, the degradation of NFKBIA and NFKB1 (p65) translocation was observed after EGF treatment, which was significantly blocked by pretreatment with AG1478, EGTA, H_7, or SN50 (NFKB1-specific inhibitor). Furthermore, we found that EGF-induced cell proliferation was significantly attenuated by AG1478, EGTA, H_7, and SN50, respectively. On the other hand, inhibition of EGFR, Ca^{2+}/PKC, or NFKB1 abolished the EGF-stimulated increase in the expression of cyclins *CCND1* and *CCNE1*, cyclin-dependent kinase 6 (*CDK6*), *CDK2*, and BCL2, and restored the EGF-induced inhibition of BAX expression and caspase 3/9 activity, indicating that EGFR, PKC, and NFKB1 signaling cascades were involved in EGF-stimulated DNA synthesis and antiapoptosis action. In conclusion, EGF stimulated proliferation of chicken PGCs via activation of Ca^{2+}/PKC involving NFKB1 signaling pathway. These observations suggest that EGF signaling is important in regulating germ cell proliferation in the chicken embryonic gonad.

apoptosis, Ca^{2+}/PKC, embryo, epidermal growth factor, NFKB1, polypeptide receptors, primordial germ cell, proliferation, signal transduction

INTRODUCTION

In vertebrates, primordial germ cells (PGCs) are embryonic precursor cells to ova and spermatozoa, and are the target cell type for modification of the vertebrate genome [1, 2]. Over the last 15 yr, the study of avian PGCs has been difficult because the number of PGCs obtained during the early stages of embryonic development is small. This has stimulated research into methods to culture PGCs to gain a large number with the view of producing transgenic animals [1, 3]. Nevertheless, the signaling pathways involved in avian PGC proliferation are relatively unknown. Epidermal growth factor (EGF) has been characterized in many cell types, and is known to participate in a wide variety of biological responses, including cellular proliferation, migration, survival, and differentiation [4]. It has been known that EGF exerts the mitogenic effect through activation of the EGF receptor (EGFR) signaling pathway. EGF interacts with transmembrane EGFR, leading to the receptor dimerization, activation of its kinase activity, and autophosphorylation of EGFR on tyrosine residues. Signaling proteins inside the cell, such as phospholipase C, then bind to these new phosphorylated tyrosine residues, initiating the signaling cascade that ultimately elicits DNA synthesis and cellular proliferation in a variety of cell types [5, 6]. It has been reported that EGF and EGFR are highly expressed in preimplantation embryos, suggesting that EGFR signaling is involved in early embryo development in an autocrine and/or paracrine manner [7, 8]. Moreover, Lee and Fukui [9] reported that treatment with EGF increased the total cell number of blastocysts in vitro. Subsequently, Heo et al. [10] provided evidence that the EGFR pathway appeared to be a factor in embryonic stem cell proliferation.

As the critical mediator of signal transduction, the Ca^{2+}-dependent protein kinase C (PKC) family plays an important role in cellular survival and apoptosis signal cascades. It's well known that the PKC family, downstream of signaling by PKC, is activated by EGF or EGFR in various types of cells [11]. A previous report showed that EGF-dependent mitogenesis was associated with the activation of PKC in normal mammary epithelial cells [12]. Recently, the involvement of PKC in EGF-induced cell proliferation in embryonic stem cells was verified [10]. Likewise, several reports indicate that nuclear factor (NF)-KB1, a family of dimeric transcription factors that regulates cell division, apoptosis, and inflammation, could be activated through EGF or EGFR system [13–15]. In addition, the Ca^{2+}/PKC, NFKB1 signal pathway was found to mediate DNA synthesis in mouse embryonic stem cells [16]. Furthermore, Ge et al. [17] demonstrated that PKC-linked NFKB1 activation modulated ginsenoside-stimulated proliferation of chicken PGCs. All these reports suggest that the EGF/EGFR, Ca^{2+}/PKC, NFKB1 signal cascade performs a crucial role in cell survival, proliferation, or target gene expression. In regard to early germ cells, it has been shown that the EGFR pathway is involved in regulating PGC numbers in the ovary of *Drosophila* larvae [18]. However, no reports exist on the activity of the EGF system in vertebrate PGCs. Therefore, the purpose of this study was to investigate the effect of EGF on

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TABLE 1. Primers for PCR analysis.

Gene	Accession no.	Primer sequence (5′ to 3′)	Product length (bp)
POU5F1	DQ867024	GTT GTC CGG GTC TGG TTC T GTG GAA AGG TGG CAT GTA GAC	189
NANOG	DQ867025	CAG CAG ACC TCT CCT TGA CC TTC CTT GTC CCA CTC TCA CC	187
SOX2	U12532	GCA GAG AAA AGG GAA AAA GGA TTT CCT AGG GAG GGG TAT GAA	171
EGFR	NM_205497	AGG AGT ATC ACG CAG AGG G TCA TCC AGC ATT TGA CCA T	258
CCND1	NM_205381	CTG CTC AAT GAC AGG GTG C TCG GGT CTG ATG GAG TTG T	341
CCNE1	NM_00103158	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
CDK6	NM_001007892	CCG ACC AAC AGT ATG AGT GCG GAA AAT CCA GTC CCC GAA ACA	381
ACTB	NM_205518	ACG TCG CAC TGG ATT TCG AG TGT CAG CAA TGC CAG GGT AC	282

cell proliferation and its related signaling pathways in chicken PGCs.

MATERIALS AND METHODS

Materials

EGF was obtained from CytoLab Ltd. (Rehovot, Israel). Human leukemia inhibitory factor (LIF) and fetal calf serum (FCS) were from Stem Cell Tech Inc. (Vancouver, Canada). AG1478, phorbol-12-myristate-13-acetate (PMA), H_7, bromodeoxyuridine (BrdU), and mouse monoclonal anti-BrdU antibody were purchased from Sigma (St. Louis, MO), while SN50 was from Alexis (San Diego, CA). Antibodies against EGF, EGFR, pan-PKC, NFKB1, NFKBIA, BAX, BCL2, caspase 3, caspase 9, biotin-conjugated goat anti-mouse IgG or anti-rabbit IgG, and streptavidin-biotin-peroxidase complex (SABC) immunoreaction kit were from Boster Inc. (Wuhan, China). Phospho-EGFR antibody was from Cell Signaling Technology (Beverly, MA). Mouse monoclonal antibody for stage-specific embryonic antigens-1 (official symbol, FUT4) was obtained from Chemicon (Temecula).

Isolation and Culture of PGCs

All procedures described here were reviewed and approved by the Zhejiang University Animal Care and Use Committee, and were performed in accordance with the *Guiding Principles for the Care and Use of Laboratory Animals*. Fertilized Arbor Acres broiler chicken (*Gallus gallus*) eggs were obtained from a commercial hatchery and incubated in an egg incubator at 38.5°C and 60% humidity for 3.5–4 days. PGCs were prepared as follows. Briefly, genital ridges were carefully dissected from the mesonephrose and dissociated in 0.05% trypsin/EDTA (E. Merck, Darmstadt, Germany) solution at room temperature for about 5 min. The dissociated cells were cultured in Medium 199 supplemented with 5% FCS and 10 ng/ml LIF. After a 24-h primary culture, PGC colonies were teased from the dish using a glass needle under a microsurgery microscope, then dissociated with 0.05% trypsin/EDTA and seeded onto a mitotically inactivated (by mytomycin C) chicken embryonic fibroblast feeder layer at a density of 2×10^4 PGCs/well in serum-free Medium 199 supplemented with 10 μg/ml insulin, 5 μg/ml transferrin, 3×10^{-8} M selenite (Sigma), and 10 ng/ml LIF. PGCs were incubated at 38.5°C in an air atmosphere containing 5% CO_2. Cultures were maintained for 24 h before use.

Treatment of Cultured PGCs

For experiments involving PGC proliferation and cell cycle regulatory and apoptosis-related proteins, PGCs were treated with EGF at 1–100 ng/ml in the presence of feeder layer for 1–7 days. In those experiments involving EGFR, PKC, and NFKB1, PGCs were pretreated with various inhibitors for 30 min without a feeder layer prior to exposure to 10 ng/ml EGF for 0–120 min. Controls received the vehicle only, with a final ethanol concentration of $\leq$0.1%. PGC proliferation was assessed after staining for endogenous alkaline phosphatase activity or immunohistochemistry for FUT4.

Immunocytochemical Staining

After two washes with PBS, PGCs with different treatments were fixed in 4% neutral paraformaldehyde (in PBS) for 30 min and then incubated with mouse monoclonal antibodies for FUT4 (1:50), rabbit anti-NFKB1 (1:400), and anti-EGF or EGFR polyclonal antibodies (1:400) as the first antibody overnight at 4°C. The secondary antibody was biotin-conjugated goat anti-mouse IgG or anti-rabbit IgG. Immunoreaction was detected by using SABC system, as described in the manufacturer's protocol.

BrdU Incorporation

PGCs were treated with EGF for 24 h, as described above, after which 15 μg/ml of BrdU labeling reagent was added, and incubation continued for an additional 2 h. The BrdU staining was conducted according to a previously published method [19]. The number of BrdU-labeled cells relative to the total number of cells per field of vision was determined as the percentage of BrdU-positive cells.

RNA Isolation and RT-PCR

The total RNA was extracted from PGCs with different treatments by Trizol reagent (GIBCO-BRL). Reverse transcription (RT) was performed using 2 μg of RNA and oligo $(dT)_{18}$ primer (BioSynthesis, Lewisville, TX) according to a reverse transcriptase kit (Sangon, Shanghai, China). Four microliters of RT products were then used for PCR amplification under the following conditions: denaturation at 95°C for 3 min followed by 30 cycles at 94°C for 30 sec, 58°C for 40 sec, and 72°C for 1 min, followed by 7 min of extension at 72°C. The primer sequence and PCR product length are listed in Table 1. Amplified products were verified by 1.5% agarose gel electrophoresis.

Real-Time RT-PCR

A two-step, real-time RT-PCR was used to measure the expression of *POU5F1*, *NANOG*, and *SOX2*. Briefly, total RNA was extracted from PGC cultures by Trizol reagent (GIBCO-BRL) and treated with DNase (Promega). RT of 2 μg total RNA was carried out using the SuperScript First-Strand Synthesis System (Promega) based upon the manufacturer's protocol. The primer pairs are listed in Table 1. The relative abundance of transcripts was assayed using a Bio-Rad iCycler. The previously synthesized cDNA was used as template. The PCR reactions contained approximately 15–30 ng of cDNA, 1× iQ SYBR Green Supermix, and 0.25 μM of each reverse and forward primer specific for the tested genes, in triplicate. Reactions were run for 50 cycles (95°C for 30 sec, 58°C for 30 sec, 72°C for 30 sec) following an initial 2-min step at 50°C for enzyme activation and 7-min incubation at 95°C. β-actin (*ACTB*) was used as a reference gene.

Preparation of Cell Homogenates and Western Blot Analysis

PGCs were harvested after treatment with each designated agent. Preparation of membrane, cytosolic, and nuclear fractions was performed as

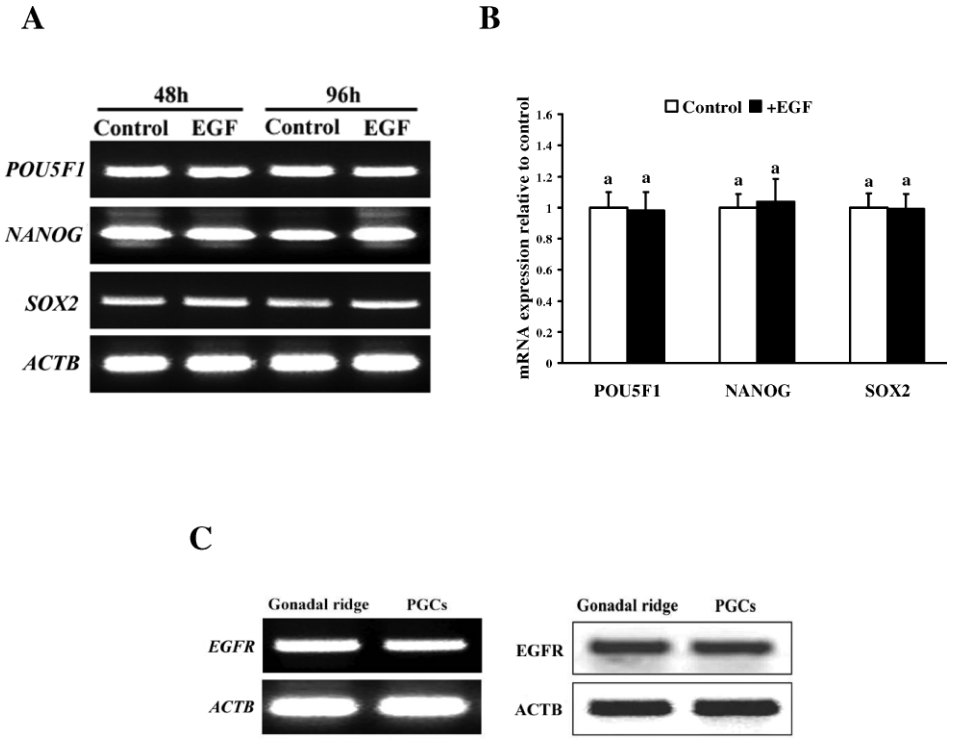

FIG. 1. Characterization of chicken PGCs. **A**) Expression of *POU5F1*, *NANOG*, and *SOX2* mRNAs in chicken PGCs treated with or without 10 ng/ml EGF for 48 or 96 h. **B**) Real-time RT-PCR analysis of *POU5F1*, *NANOG*, and *SOX2* mRNA expression relative to *ACTB* in PGCs treated with or without 10 ng/ml EGF after 96 h. **C**) Expression of EGFR in PGCs was measured by RT-PCR (left) and Western blot (right) analysis. Whole gonadal ridge (3.5 days of incubation) was used as positive control. Values are means ± SD of three experiments. Bars with the same letters are not statistically different.

described by Hsieh et al. [20]. The same amount of cell lysates and membrane, cytosolic, and nuclear fractions (20 μg protein) were separated on 10% SDS-PAGE and transferred onto nitrocellulose membrane. The membrane was incubated in 5% dry milk at room temperature for 1 h, and subsequently with appropriate primary antibodies at dilutions recommended by the supplier at room temperature for 2 h. Antibody recognition was detected with the respective secondary antibody linked to horseradish peroxidase at room temperature for 60 min. The ACTB bands were adopted as an internal control. The immunoreactive bands were visualized by enhanced chemiluminescence kit (Amersham) and exposed to BAS 3000 (Fuji Film, Japan).

Statistical Analysis

Each experiment was repeated three times. Analysis of PGC colonies was achieved by using Simple PCI Advanced Imaging Software (Compix Inc.) from the images captured with a digital video camera (Pixera Pro 150ES). All data were expressed as the mean ± SD and analyzed by ANOVA and Duncan multiple range tests using the SAS 8.0 software. $P < 0.05$ was considered as significantly different.

RESULTS

Effect of EGF on PGC Proliferation

To confirm the undifferentiated state of PGCs used in the experiments, PGCs were characterized by expression of markers of undifferentiated germ line stem cells, including the carbohydrate epitope FUT4, and transcription factors *POU5F1*, *NANOG*, and *SOX2*. After 48 h or 96 h culture in the presence of 10 ng/ml EGF, PGC mRNA expression of *POU5F1*, *NANOG*, and *SOX2* relative to *ACTB* were equivalent to those in the control group (Fig. 1, A and B). In addition, FUT4 expression was maintained (data not shown). To investigate whether EGF can act directly on PGC proliferation, we sought to determine whether PGCs express the EGFR. Using RT-PCR and Western blot, expression of EGFR was detected in cultured PGCs as well as in whole gonadal ridge (Fig. 1C).

To examine the effect of EGF on PGC proliferation, measurements were made of the changes in area and number of PGC colonies with different doses of EGF (0–100 ng/ml) for 24 h and with 10 ng/ml EGF for varying periods of time (1–7 days). As shown in Figure 2A, EGF increased the number and total area of PGC colonies in a time- and dose-dependent manner, which was consistent with BrdU incorporation results (Fig. 2B). Treatment with 10 ng/ml EGF for 24 h significantly increased the number of PGC colonies (46.2%) and the colony area (62.0%) over that observed in the controls. Likewise, 100 ng/ml EGF significantly augmented the number and area of the PGC colonies (54.2% and 78.5%, respectively, vs. control, $P < 0.05$). During 7 days of culture, treatment of EGF at 10 ng/ml resulted in significantly enhanced PGC colony numbers and area (Fig. 2, C and D) by the fifth day of culture compared with the control (41% and 54.5% higher in number and area vs. control, $P < 0.05$).

Involvement of EGFR in EGF-Induced Cell Proliferation

Changes in the expression of phosphorylated EGFR and total EGFR after incubation with EGF were investigated. Western blot analysis revealed that 10–100 ng/ml EGF significantly accelerated the phosphorylation of EGFR, without altering the total EGFR content (Fig. 3A). Furthermore, the time course of the response is transient, reaching a maximum at 15–30 min, and declining by 60 min (Fig. 3B). In addition, pretreatment of PGCs with the selective EGFR tyrosine kinase inhibitor, AG1478, markedly attenuated the EGF-stimulated increases in number and area of PGC colonies (Fig. 4).

Involvement of PKC in EGF-Induced Cell Proliferation

Ethyleneglycol-*bis*-(β-aminoethyl ether)-*N*,*N*′-tetraacetic acid (EGTA; intracellular Ca^{2+} chelator, 10^{-4} M), H_7 (PKC inhibitor, 1 μM), and PMA (PKC activator, 10^{-8} M) were used to address the possible involvement of Ca^{2+}/PKC in EGF-induced cell proliferation. Pretreatment with EGTA and H_7 caused significantly attenuated EGF-induced increases in number and area of PGC colonies (Fig. 4). In contrast, the

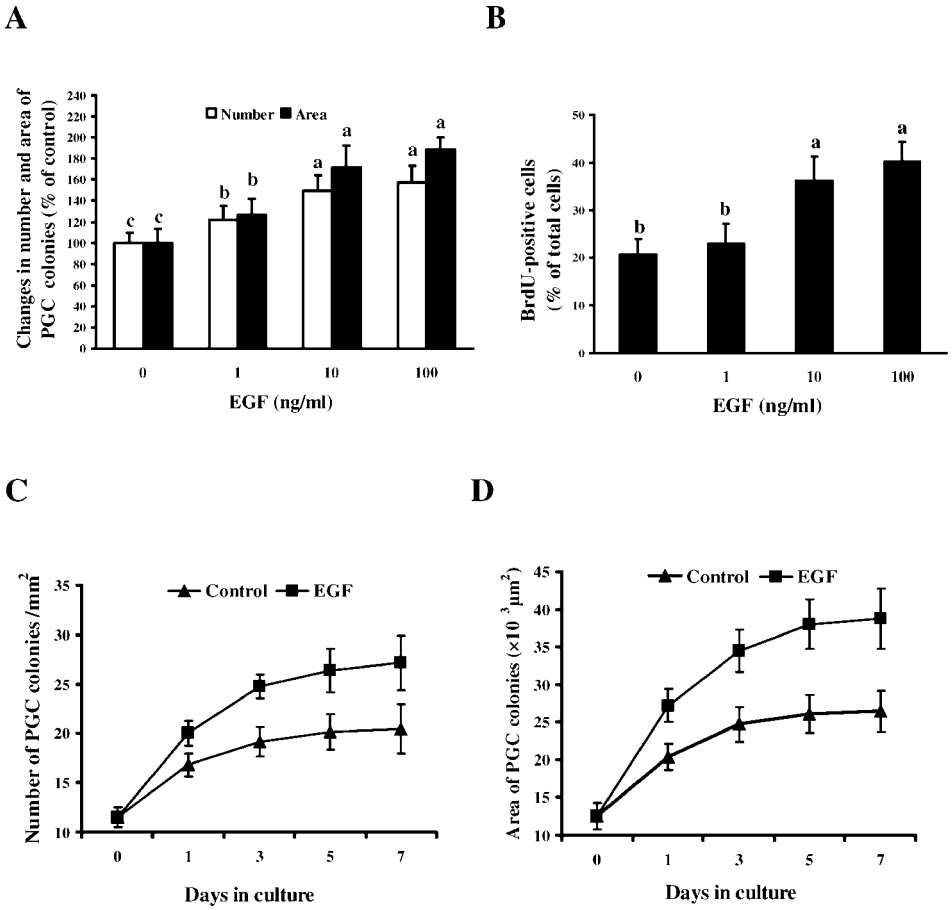

FIG. 2. Effect of EGF on PGC proliferation. **A**) Changes in number and area of PGC colonies after incubation with EGF (1–100 ng/ml) for 24 h. **B**) The proportion of BrdU-labeled PGCs in response to EGF (1–100 ng/ml) after 24 h. PGCs were incubated in the presence of EGF (10 ng/ml) for various periods of time (1–7 days), and the number (**C**) and area (**D**) of PGC colonies was measured. Values are means ± SD of four independent experiments with triplicate dishes. **A** and **B**) Bars associated with different letters are statistically different ($P < 0.05$).

combined administration of PMA enhanced the proliferative effect of EGF on PGCs (data not shown). Meanwhile, we also determined whether PKC translocation was involved in EGF-induced cell proliferation. As shown in Figure 5A, PKC was rapidly translocated to plasma membrane as early as 15 min upon EGF stimulation, maintained at higher levels at 30 and 60 min, and then declined by 120 min. Furthermore, preincubation with AG1478, H_7, or EGTA significantly blocked EGF-stimulated PKC translocation in PGCs, whereas PMA exerted the opposite effect (Fig. 5B).

Involvement of NFKB1 in EGF-Induced Cell Proliferation

As shown in Figure 4, SN50 (NFKB1 nucleus translocation inhibitor, 500 ng/ml) sharply inhibited the EGF-induced increases in number and area of PGC colonies. On the other hand, Western blot analysis revealed that EGF obviously increased NFKB1 translocation and NFKBIA degradation in a time-dependent manner, reaching a maximum at 60 min, and declining to basal levels within 4 h (Fig. 6A). However, these effects were significantly inhibited by AG1478, EGTA, H_7, or SN50 (Fig. 6B). Furthermore, to ensure the involvement of NFKB1 in the EGF-stimulated cell proliferation, immunocytochemical staining of NFKB1 was employed, and verified that EGF treatment stimulated NFKB1 translocation from cytosol to the nucleus (Fig. 6C).

Effect of EGF on Cell Cycle Regulatory and Apoptosis-Related Proteins

In support of the effect of EGF on proliferation of PGCs, we observed the effect of EGF on mRNA abundance of *CCND1*, *CCNE1*, *CDK6*, and *CDK2*, which are considered to be critical factors in G1/S progression. Treatment with 10 ng/ml EGF augmented the mRNA expression of *CCND1*, *CCNE1*, *CDK6*, and *CDK2*. Pretreatment with AG1478, EGTA, H_7, or SN50 imposed a vigorous inhibitory response to EGF (Fig. 7). We also addressed the effect of EGF on apoptosis-related protein expression. Western blot analysis showed that treatment with 10 ng/ml EGF for 24 h increased BCL2 expression and inhibited BAX expression (Fig. 8A). Meanwhile, EGF inhibited the activation of caspase 3 and caspase 9 (Fig. 8, B and C), determined by monitoring the decreases in the level of the precursor and increases in the cleaved fragment subunits. All these EGF responses on apoptosis-related protein were attenuated by pretreatment with AG1478, H_7, or SN50 (Fig. 8, B and C).

DISCUSSION

PGCs are the embryonic progenitors of mature germ cells. Normally, PGCs can be characterized by special cell surface and nuclear markers. In a first series of experiments, alkaline phosphatase enzyme activity and FUT4 expression were still maintained after 48 h culture with EGF (data not shown). Moreover, it was confirmed that the mRNA expression levels of the transcription factors *POU5F1*, *NANOG*, and *SOX2* were not altered after EGF treatment for 96 h. Therefore, these results indicate that PGCs maintain an undifferentiated status under EGF treatment for 4 days.

As an effective mitogen, EGF is known for promoting cell survival and proliferation in diverse somatic cell types. However, few studies have examined its effect on germ or stem cells. In the mouse, supplementation with EGF, IGF1, and

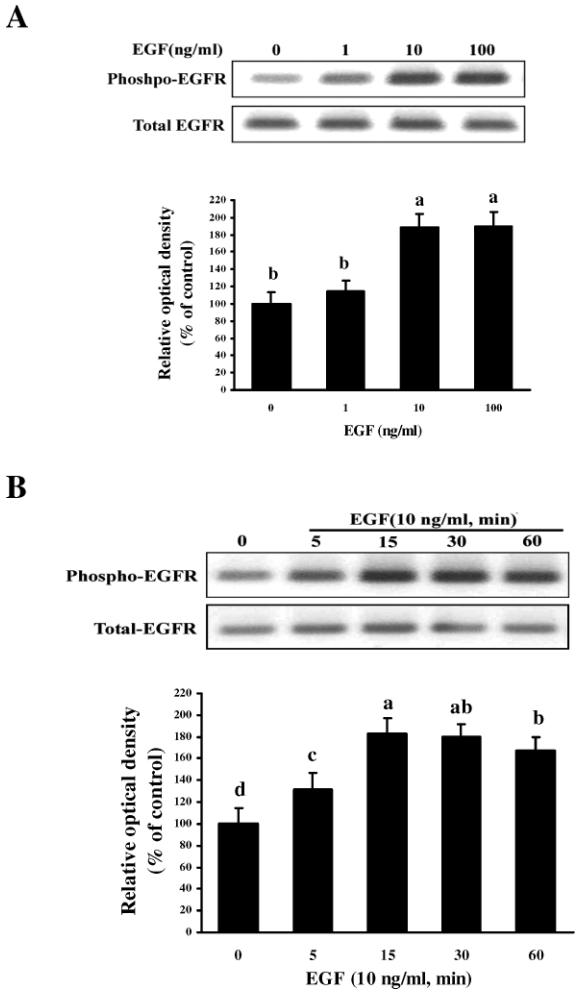

FIG. 3. Involvement of EGFR in EGF-induced proliferation of PGCs. Dose (**A**) and time (**B**) dependence of EGF-stimulated phosphorylation of EGFR. PGCs in serum-free suspension culture were treated with various concentrations of EGF for the indicated times. Each figure is representative of three independent experiments. **A** and **B**) Bottom: means ± SD of three experiments for each condition determined from densitometry (top) relative to total EGFR. Bars with different letters are statistically different ($P < 0.05$).

IGF2 (1–1000 ng/ml) resulted in a significant increase in cell number of the blastocyst inner cell mass compared with controls [21]. Moreover, it has been reported that EGF helps to maintain neural stem cell self-renewal and multilineage potential [22]. In addition, EGF could be used for in vitro expansion of mesenchymal and mouse embryonic stem cells [23, 10]. In the present study, a significant increase in the number and area of PGC colonies was observed after treatment with EGF (10–100 ng/ml). At the same time, EGF enhanced the BrdU incorporation of PGCs, indicating that EGF elicited DNA synthesis in PGCs. Although EGF has different effects through different signaling cascades, the intracellular molecular mechanism responsible for the stimulatory effect of EGF on PGCs was largely unknown. In the present work, EGFR expression was revealed in PGCs, which confirms that EGF could act directly on chicken PGCs.

The activation of EGFR tyrosine kinase by binding of EGF leads to intracellular signaling and usually cell proliferation. In this experiment, EGF treatment of PGCs elicited a rapid, transient increase in EGFR tyrosine phosphate that was blocked by the EGFR tyrosine kinase inhibitor, AG-1478. Furthermore, AG1478 attenuated the EGF-induced stimulation of cell proliferation. This suggests that the onset of the cell growth signal required an increase of tyrosine kinase activity linked to EGFR. This observation was consistent with those of a previous report that genistein, a nonspecific tyrosine kinase inhibitor, was able to induce apoptosis in rat RPE-J cells [24]. It was reported that PKC isozymes play a crucial role in the control of cell proliferation and apoptosis in many cell types [25]. For example, EGF was demonstrated to protect the intestinal monolayer barrier against oxidant damage via activation of PKC signal transduction and normalization of Ca^{2+} [26]. Recently, Heo et al. [10] found that EGF-induced mitogenesis requires the activation of Ca^{2+}-dependent PKC in mouse embryonic stem cells. The results of the present study of PKC translocation from the cytosolic compartment to the membrane compartment in chicken PGCs was consistent with those from that report. To clarify whether PKC pathway is involved in EGF/EGFR-mediated proliferation of chicken PGCs, we investigated the inhibitory effects of H_7, AG1478, and EGTA. EGF-stimulated cell proliferation was obviously inhibited by combined treatment with H_7, AG1478, or EGTA. In addition, pretreatment with AG1478 or EGTA completely inhibited PKC translocation, indicating that Ca^{2+} influx was required for the EGF-induced activation of PKC. All these results suggest that Ca^{2+}/PKC are involved in EGF-induced cell proliferation. To further explore the molecular mechanism, we attempted to understand the underlying modulator of Ca^{2+}/PKC-involved EGF-stimulated proliferation of chicken PGCs.

NFKB1 belongs to the REL family of transcription factors that regulate genes involved in inflammatory responses, cell growth control, and protection against apoptosis [27]. Activation of NFKB1 classically depends on degradation of NFKBIA [28]. In our previous study, it was shown that a PKC-dependent NFKB1 signaling pathway was involved in ginsenoside-induced proliferation of chicken PGCs [17]. Hence, we predicted that a crosstalk exists between NFKB1 and Ca^{2+}/PKC in the course of EGF-induced cell proliferation. To prove this hypothesis, we analyzed the nuclear translocation of

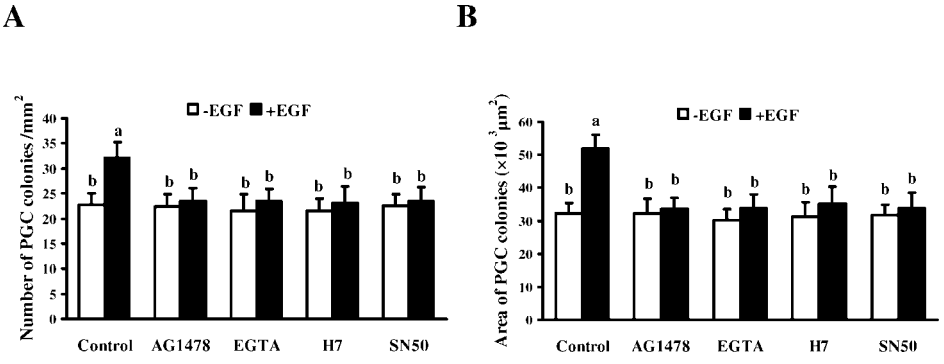

FIG. 4. Effect of inhibitors on EGF-stimulated cell proliferation. PGCs were pretreated with AG1478 (10^{-6} M), EGTA (10^{-4} M), H_7 (10^{-6} M), or SN50 (500 ng/ml) for 30 min, then cultured in the presence or absence of 10 ng/ml EGF for 24 h. The number (**A**) and area (**B**) of PGC colonies were assessed at 24 h. FUT4-positive PGC colonies were identified and counted. Values are the means ± SD (n = 4). Bars with different letters are statistically different ($P < 0.05$).

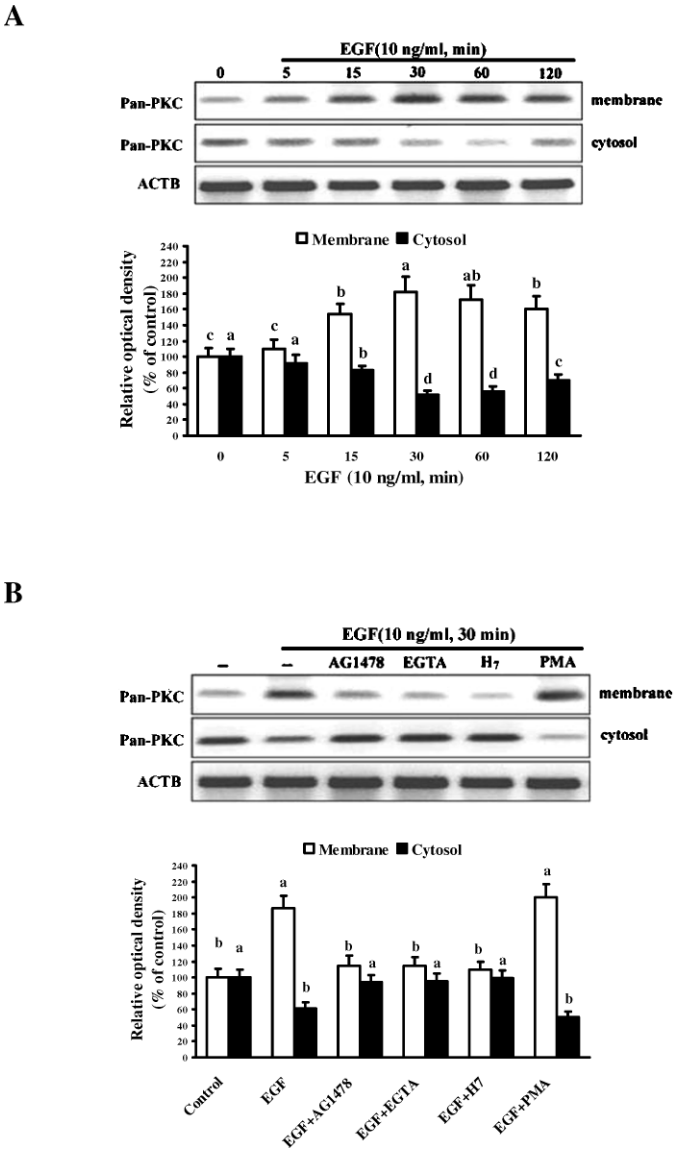

FIG. 5. Effect of EGF on PKC activation. **A**) Time dependence of EGF-stimulated PKC translocation from cytosol to membrane. PGCs were incubated with 10 ng/ml EGF for 0–120 min. **B**) PGCs were pretreated with AG1478 (10^{-6} M), EGTA (10^{-4} M), H_7 (10^{-6} M), or PMA (10^{-8} M) for 30 min before EGF (10 ng/ml) treatment for 60 min. Each figure represents one of three similar experiments. **A** and **B**) Bottom: means ± SD of three experiments for each condition determined from densitometry (top) relative to ACTB. Bars with different letters are statistically different ($P < 0.05$).

NFKB1 (p65) and the degradation level of NFKBIA. The nuclear translocation of NFKB1 often serves as a fundamental index of transcriptional activation. In addition, NFKB1 activation requires sequential activation, phosphorylation, ubiquitination, and degradation of NFKBIA (NFKB1 inhibitory subunit), as well as consequent exposure of the nuclear localization signal on NFKB1. Therefore, the measurement of NFKBIA levels offers information regarding the mechanism of NFKB1 activation. Our data show that 10 ng/ml EGF stimulated the translocation of NFKB1, as well as degradation of NFKBIA, as determined by Western blot analysis. However, these responses to EGF were inhibited by pretreatment with AG1478, EGTA, H_7, or SN50, and were further verified by NFKB1 immunochemistry. Moreover, the increases in number and area of PGC colonies were obviously attenuated by the

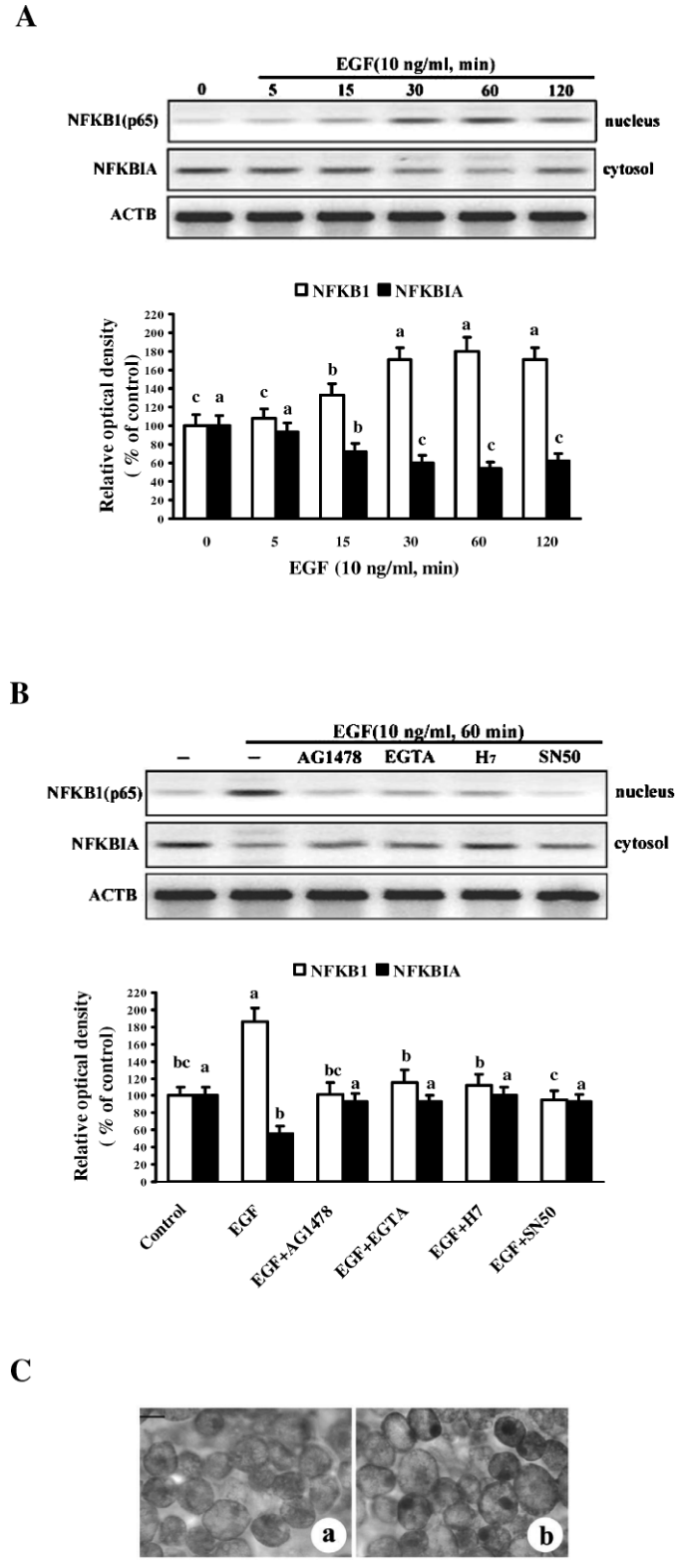

FIG. 6. Effect of EGF on NFKB1 (p65) activation. **A**) Time dependence of EGF-induced NFKB1 (p65) translocation; PGCs were incubated with 10 ng/ml EGF for 0–120 min. **B**) PGCs were pretreated with AG1478 (10^{-6} M), EGTA (10^{-4} M), H_7 (10^{-6} M), or SN50 (500 ng/ml) for 30 min before EGF (10 ng/ml) treatment for 120 min. **C**) The immunochemistry of NFKB1 (p65) showed that EGF stimulated NFKB1 translocation from cytosol to nucleus. PGCs were cultured in the absence (a) or presence (b) of 10 ng/ml EGF for 1 h; bar = 20 μm. Each figure represents one of three similar experiments. **A** and **B**) Bottom: means ± SD of three experiments for each condition determined from densitometry (top) relative to ACTB. Bars with different letters are statistically different ($P < 0.05$).

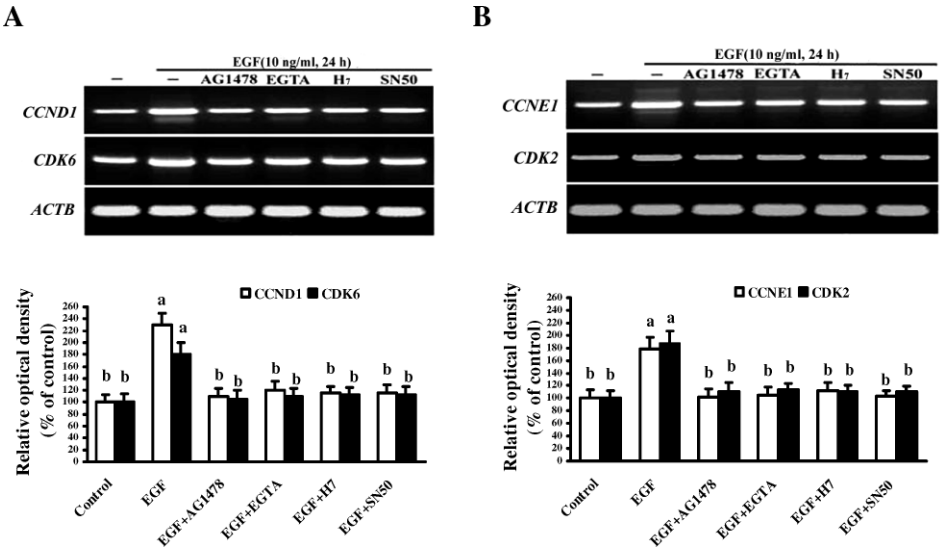

FIG. 7. Effect of EGF on expression of cell cycle regulatory genes. PGCs were pretreated with AG1478 (10^{-6} M), EGTA (10^{-4} M), H_7 (10^{-6} M), or SN50 (500 ng/ml) for 30 min and then cultured in the presence or absence of 10 ng/ml EGF for 24 h. **A** and **B**) Bottom: means ± SD of three experiments for each condition determined from densitometry (top) relative to *ACTB*. Bars with different letters are statistically different ($P < 0.05$). Each figure represents one of three similar experiments.

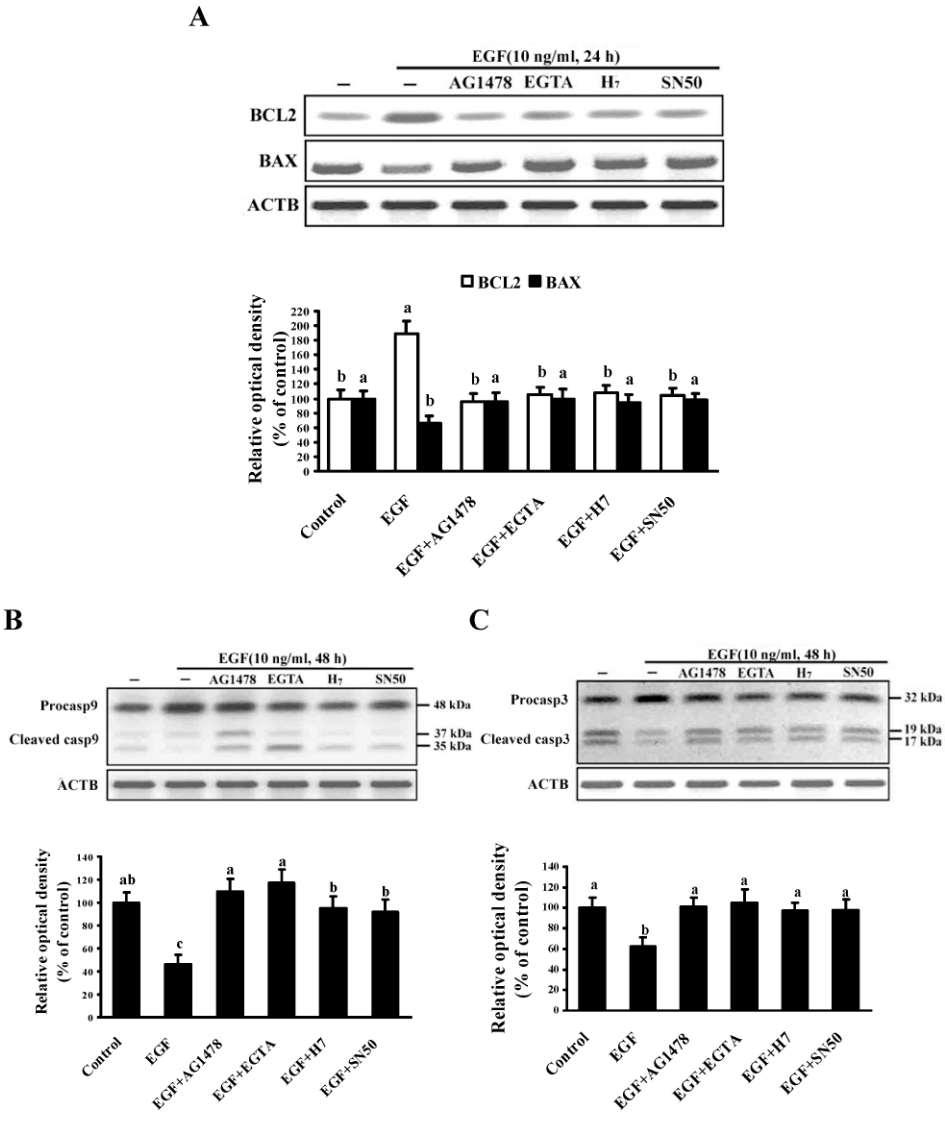

FIG. 8. Effect of EGF on expression of apoptosis-related proteins. PGCs were pretreated with AG1478 (10^{-6} M), EGTA (10^{-4} M), H_7 (10^{-6} M), or SN50 (500 ng/ml) for 30 min and then cultured in the presence or absence of 10 ng/ml EGF for 24 or 48 h. The total protein was extracted and blotted with antibodies against BCL2 and BAX (**A**), caspase 9 (**B**), and caspase 3 (**C**). Each figure represents one of three similar experiments. **A** and **B**) Bottom: means ± SD of three experiments for each condition determined from densitometry (top) relative to ACTB. Bars with different letters are statistically different ($P < 0.05$).

NFKB1-specific inhibitor, SN50. Taken together, the present results suggest that EGF stimulates NFKB1 activation as a downstream target modulator of PKC and EGFR signaling.

EGF is reported to modulate the expression of cell cycle regulatory proteins, some of which contain NFKB1 motifs [29]. These regulators include the cyclins and their catalytic partners, the CDKs, which are essential for the progression of cells through each phase of the cell cycle and various cell cycle checkpoints [30]. The *CCNE1*/*CDK2* formed at the time of G1/S transition in lymphocytes has been shown to be associated with NFKB1 activation. Moreover, Häussler et al. [15] identified a molecular link between stimulation of EGF via activation of NFKB1 and *CCND1* expression in human proximal tubular cells. In addition, PKC is required for the EGF-induced increases in expression levels of *CCND1*/*CDK4* and *CCNE1*/*CDK2* in mouse embryonic stem cells [10]. In the present study, inhibition of EGFR, Ca^{2+}/PKC, NFKB1 pathway blocked the EGF-stimulated expression of cyclins and CDKs, which was consistent with a previous finding that up-regulation of the cell cycle regulatory *CCND1* gene expression by EGF is initiated by interaction with the EGFR, transmitted via activation of PKC-mediated and IKK-dependent activation of NFKB1 [31]. In addition to cell cycle regulator proteins, EGF also modulates apoptosis-related proteins [32, 33], such as BAX/BCL2 and caspase 3/9. A recent study provided evidence that the balance of BAX (proapoptosis protein) and BCLX (antiapoptosis protein) control mouse PGC survival and apoptosis [34]. Initiation of apoptosis in response to many stimuli involves a cysteine protease, caspase 9. Once activated, caspase 9 cleaves and activates related caspases, such as caspase 3, that target a variety of cellular components to dismantle a cell and present the fragments for phagocytosis [35]. It is becoming clear that EGF is able to antagonize apoptosis through the inhibition of caspase 3 activation [36, 37]. In this study, inhibition of EGFR, Ca^{2+}/PKC, or NFKB1 sharply blocked the EGF-induced increases in the expression of *CCND1*, *CDK6*, *CCNE1*, *CDK2*, and BCL2, and restored the EGF-induced inhibition of BAX, activated caspase 3 and caspase 9 expression, indicating that EGFR, Ca^{2+}/PKC, and NFKB1 signal cascades are involved in EGF protection against apoptosis.

Based on all these results, it appears that various downstream signaling molecules of EGFR tyrosine kinase are required for cell cycle and antiapoptosis control, and that these cascades lead to chicken PGC proliferation. In conclusion, EGF treatment stimulated the proliferation of chicken PGCs through changes in *CDK2*/*6*, *CCND1, CCNE1*, BAX/BCL2, and caspase 3/9 expression via the Ca^{2+}/PKC and NFKB1 signaling cascades. Therefore, EGF may be a significant regulator of germ cell proliferation in the chicken, and may regulate germ cell proliferation in the embryonic gonads.

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