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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

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

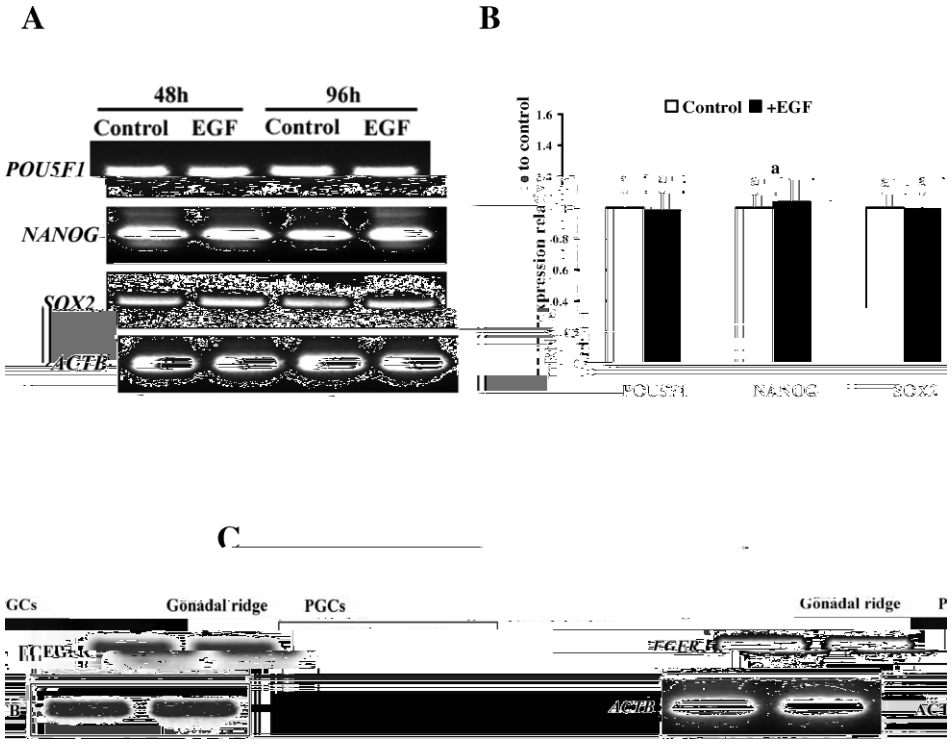
All procedures described here were reviewed and approved by the Zhejiang University Animal Care and Use Committee, and were performed in accordance with the *Guiding Principles for the Care and Use of Laboratory Animals*. Fertilized Arbor Acres broiler chicken (*Gallus gallus*) eggs were obtained from 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 μ

A *POU5F1* *NANOG*
SOX2 A

B
POU5F1
NANOG *SOX2* A
ACTB

C

±



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).

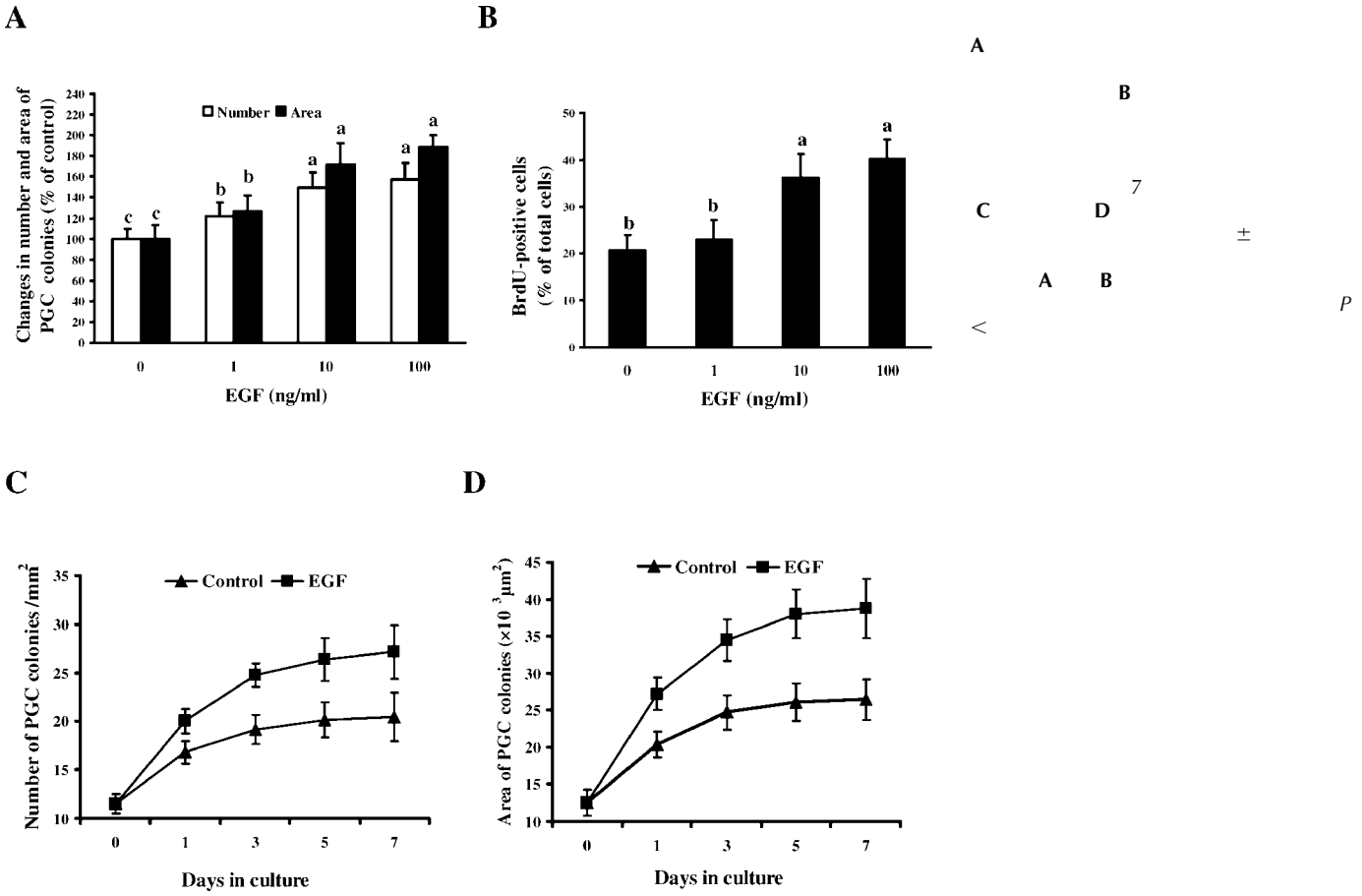
To examine the effect of EGF on PGC proliferation, measurements were made of the changes in area and number of PGC colonies with different doses of EGF (0 100 ng/ml) for 24 h and with 10 ng/ml EGF for varying periods of time (1 7 days). As shown in Figure 2A, EGF increased the number and total area of 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



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

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

7

A 7 – A –

– 7

A B

± ACTB P

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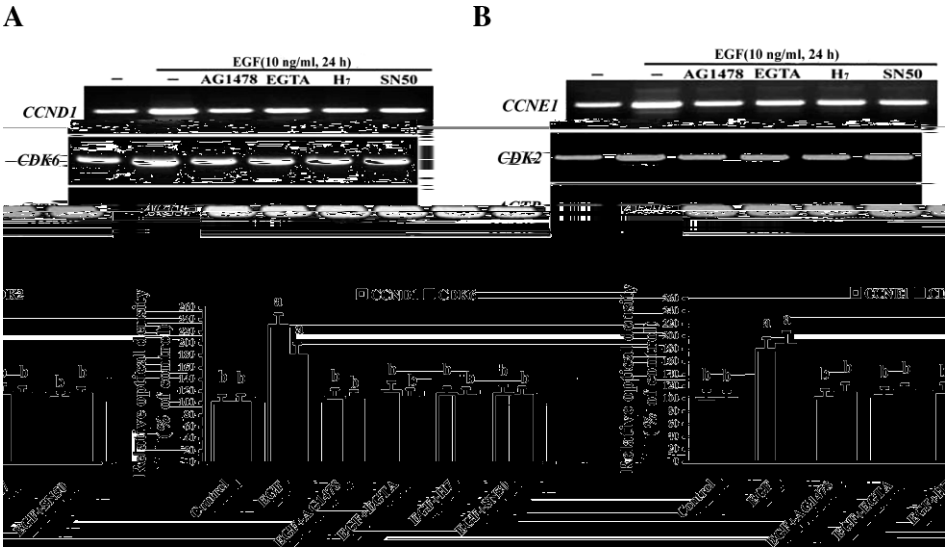

A 7 – A –

– 7

A A

B C

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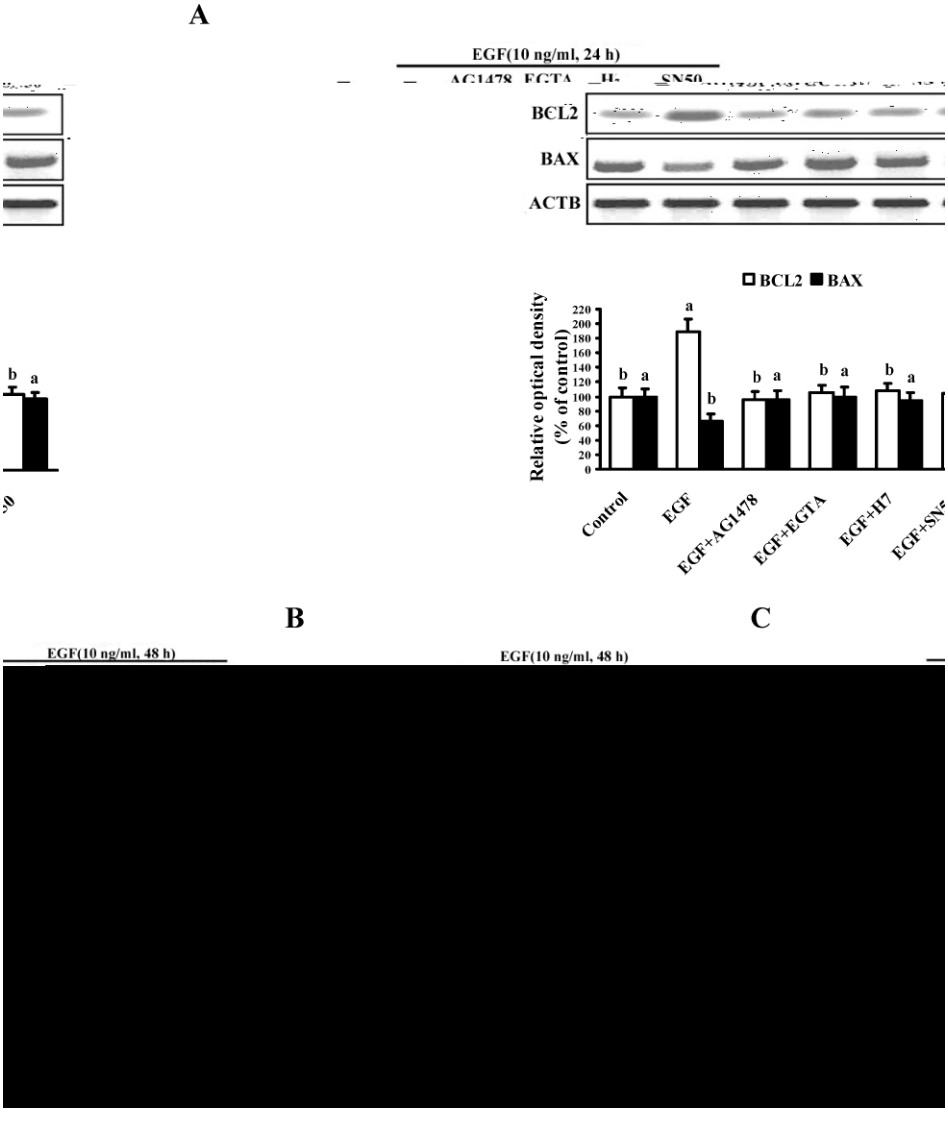

NFKB1-specific inhibitor, SN50. Taken together, the present results suggest that EGF stimulates NFKB1 activation as a downstream target modulator of PKC and EGFR signaling.

EGF is reported to modulate the expression of cell cycle

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