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ROS, MAPK/ERK and PKC play distinct roles in EGF-stimulated human corneal cell proliferation and migration

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Abstract

Cornea is at the outermost surface of eye globe, and it easily receives damage from ultraviolet light exposure, physiology wounding, and infections. It is essential to understand the mechanisms controlling human corneal epithelial (HCE) cell proliferation and wound healing. Epidermal growth factor (EGF) could stimulate cell proliferation and migration in various cell types. Therefore, we investigated the roles and mechanisms of EGF on HCE cell proliferation and migration. CCK-8 kit and wound healing experiment were used to investigate HCE cell proliferation and cell migration, respectively. ROS activity was quantified by DCFDA and flow cytometry. Western blot and Q-PCR were performed to examine protein and RNA levels. EGF could promote HCE cell proliferation and migration in both physiology status and UV irradiation conditions, which is used to mimic the disease condition in human corneal epithelial cells. Interestingly, the promotion effect of EGF on HCE cell proliferation is mainly mediated by activated ROS signaling under disease condition. However, the EGF function is mediated by ROS and MAPK/ERK pathway in EGF-treated corneal epithelial cells in physiology status, in which ROS and MAPK/ERK pathway have no mutual influence on the other signaling pathway in EGF-stimulated corneal epithelial cells. We also revealed that MAPK/ERK pathway instead of ROS mediates EGF-stimulated HCE cell migration. Interestingly, we found that PKC proteins were downregulated by EGF in HCE cells that is partially mediated by ROS signaling, while PKC pathway was not involved in EGF-stimulated corneal cell proliferation and migration. EGF promotes human corneal cell proliferation and migration both in physiology and disease conditions, and ROS, MAPK/ERK and PKC pathways play different roles in these processes.

Key words: EGF, HCE cell, cell proliferation, migration, ROS, MAPK/ERK, PKC.

Introduction

Visual impairment and blindness caused by corneal disease or corneal scarring affect millions of people worldwide. Cornea locates at the outermost surface of the eye globe (1). It may receive damage from ultraviolet light exposure (UV) and may be damaged by physiology wounding or bacterial infections. Maintaining a healthy cornea, or repairing a wounded cornea, is of vital importance for normal vision. Therefore, it is essential to understand the mechanisms underlying corneal cell proliferation and wound healing. Moreover, corneal transplantation is also frequently used for corneal disease or scarring patients (2). Complete epithelialization of implanted tissue will maintain a tear film, provide a natural barrier against bacterial infection, and protect the stroma from collagenase and proteinase enzymatic activity for longer implant stability (3). Therefore, developing strategies to promote corneal epithelial cell proliferation and wound healing is highly desired for clinical applications.

The important steps of corneal epithelial cell wound healing are cell proliferation from the healthy area and cell migration to the damaged area (4, 5). Previous studies have shown that epidermal growth factor (EGF) could stimulate cell proliferation and migration in various cell types, such as endometrial carcinoma cells, retinal neruoepithelial cells, and neural precursor cells of mouse embryonic mesencephalon (6-9). EGF is a single polypeptide molecule with three intermolecular disulfide bonds required for biologic activity. It binds to a tyrosine kinase receptor (EGFR) and activates several downstream pathways, such as MAPK/ERK (10, 11) and PKC (12), to promote cell growth and differentiation (13). It has been reported that EGF could also activate reactive oxygen species (ROS) and that the released ROS are second messengers for growth factor signaling required for cellular functions in many cell types (14-16). ROS, including superoxide anion ($O_2^{\cdot-}$), hydroxyl radical ($^{\cdot}OH$), and hydrogen peroxide (H_2O_2), which is known be harmful to cells and tissues, have been reported to be associated with aging (17), cancer, and other diseases (18). It has been recently reported that ROS could promote cell growth, adhesion, and migration under EGF stimulation (19). However, the relationship between MAPK/ERK, PKC and ROS signaling pathways in the EGF-stimulated cell proliferation and migration has not been systematically elucidated.

In this study, we found EGF could promote human corneal epithelial cell proliferation in both physiology and disease conditions, which mainly by stimulating HCE cell to secrete ROS. While under physiology condition, ERK1/2 may also play a partial role in EGF-stimulated proliferation. EGF could also promote HCE cell migration by activating ERK1/2 signaling pathway. We also found that PKC pathway was not involved in EGF-elicited HCE cell proliferation and migration. Thus, ROS and MAPK/ERK play distinct roles in EGF-

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promoted HCE cell proliferation and migration. These results provide a novel concept for the molecular mechanisms of EGF-induced cellular functions in cornea under physiology and disease conditions.

Materials and methods

Human corneal epithelial cells

SV40-immortalized human corneal epithelial (HCE) cells were a gift from Dr. Peter Reinach of SUNY College of Optometry. HCE cells were cultured in SHEM medium supplemented with 100 units/ml penicillin, 100 mg/ml streptomycin, 10% FBS, 5 ng/ml EGF (PproTech, USA) and 5 mg/ml insulin in a humidified 5% CO_2 incubator at 37°C. ROS inhibitor NAC (Sigma) or ERK1/2 inhibitor (Selleck) was add when needed based on experiment design.

Cell viability analysis

Cell viability was examined with Cell counting Kit-8 (CCK-8, Dojindo, Japan) according to the manual. Briefly, add 10 ul CCK-8 solutions to HCE cell culture plate wells, and incubate for 1-4 hours before measuring the absorbance at 450 nm using microplate reader.

Wound healing

To test the effect of ROS on EGF-induced cell migration, HCE cells (0.2 $X10^6$) were cultured in 12-well plates and the confluent cells were used for the in vitro scratch-wound assay (20). The cells were serum-starved for a minimum of 16 hours to establish quiescence. A uniform scratch wound was made across the culture dish using a 200 ml plastic pipette tip and washed with medium to remove detached cells. These cells were fixed and photographed after 0, 24 and 48 h incubation.

ROS activity measuring

ROS activity was measured with 5-(and-6)-chloromethyl-2', 7'-dichlorodihydro- fluorescein diacetate (acetyl ester CM-H2DCFDA, Invitrogen) as previous report (21). Briefly, HCE cells were washed with PBS, and incubate in pre-warmed DCFDA probe loading buffer for 40 minutes. Wash out loading buffer and incubate in medium for recovery. Examine the intensity of fluorescence by flow cytometry, which is excited at 485 nm.

Western blot

Proteins in cell lysates were separated by 12% to SDS-PAGE and immunoblotted as previously described (19). Hybond™nitrocellulose membrane (TransBlot, Bio-Rad) were probed against with specific antibodies, p-ERK1/2, total ERK1/2, JNK p-JNK, p-p38, total p38, PKCi, PKCmu, PKCd, PKCe, PKCa (Cell Signaling Technology). Immunodetection were analyzed with an imaging system of Fluor-S MAX MultImager (Bio-Rad, Richmond, CA).

RNA extraction and Q-PCR analysis

Total RNA was extracted from cells using Trizol reagent (Invitrogen) and reverse transcribed using SuperScript III reverse transcriptase (Invitrogen). Q-PCR analysis was carried out on the Eppendorf Realplex2. Each sample was carried out in triplicate, with the Ct values averaged and then normalized to beta-actin control. Primer sequences are available upon request.

Statistical analysis

All data are tested for statistical significance by statistical analysis with unpaired Student's T test using SPSS software. Differences were considered significant when $P < 0.05$.

Results

EGF promoted human corneal epithelial cell proliferation and migration in both physiology and disease conditions

It has been reported that EGF could stimulate cell proliferation and migration in various cell types (6-9). Previous studies showed that EGF at the range of 0.1–10 ng/ml is optimal for cell proliferation within 2–4 days (4), and 5 ng/ml is best for short-term study (19). Because corneal cells often get hurt from ultraviolet light (UV), we want to investigate the specific roles of EGF on human corneal epithelial (HCE) cells in both physiology and disease conditions and we treated human corneal epithelial cells with ultraviolet light to mimic the disease conditions of corneal epithelial cells. We treated HCE cells with 5 ng/ml EGF in physiology and disease conditions, and detected cell viability with Cell Counting Kit-8 (CCK8). We found that compared with control groups without EGF treatment, EGF could increase HCE cell viability in both physiology and disease conditions (Fig. 1A). Moreover, EDU staining was also performed to determine HCE cell proliferation in physiology and UV conditions with or without EGF treatment. Similarly, EGF increased the percentage of EDU positive cells in both physiology and UV conditions (Figure 1B).

Next, we studied the effect of EGF on corneal epithelial cell migration using wound-healing experiment. HCE cells were grown to confluence and then the plate was gently scraped across the monolayer cells with a pipette tip to generate an even gap (the scratch wound). The wound healing or the cell migration was examined after culture with or without EGF for 24 h or 48 h. Our result showed that the wound of HCE cells cultured with EGF almost completely healed after 48 h, much faster than that of the control cells without EGF treatment (Fig. 1C). These results demonstrate that EGF could promote cell proliferation and migration in both physiology and disease conditions.

EGF activated ERK1/2 and ROS pathway, but repressed the protein levels in PKC pathway

Next, the underlying mechanisms of EGF-promoted HCE cell proliferation and migration were investigated. ERK1/2, PKC, and ROS have been reported to be activated by EGF, and these pathways play roles in cell growth and migration. Therefore, we want to know whether they mediated the roles of EGF in HCE cells (10-12, 19). Consistent with our previous report (19), western blot analysis showed that the level of phosphorylated ERK1/2 (p-ERK1/2) were upregulated dramatically as early as 5 minutes after EGF treatment in HCE cells (Fig. 2A, left panel), suggesting that ERK1/2 pathway was activated upon EGF treatment. Moreover, flow cytometry analysis showed that EGF also induced

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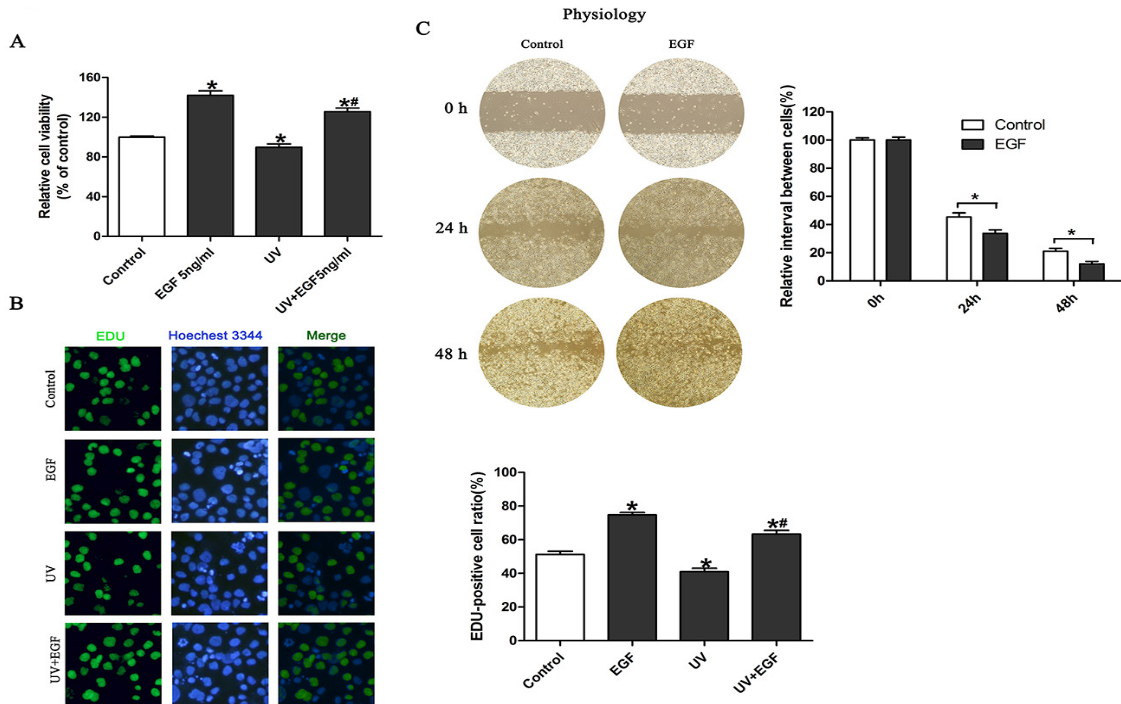

Figure 1. EGF promoted HCE cell proliferation and migration in physiology and disease conditions. A. EGF promotes HCE cell proliferation in both physiology and disease conditions. HCE cells were treated without or with EGF (5 ng/ml) in the absence or presence of ultraviolet light (UV) for 48 h. CCK8 assays were performed to examine the HCE cell viability. B. EDU staining was performed to determine the HEC cell proliferation in physiology and UV conditions. Hoechest 3344 was used to label the cell nucleus. The percentage of EDU positive cells was calculated. C. Wound healing results showed that EGF promoted HCE cell migration in physiology condition. The percentage of wound closure was calculated.

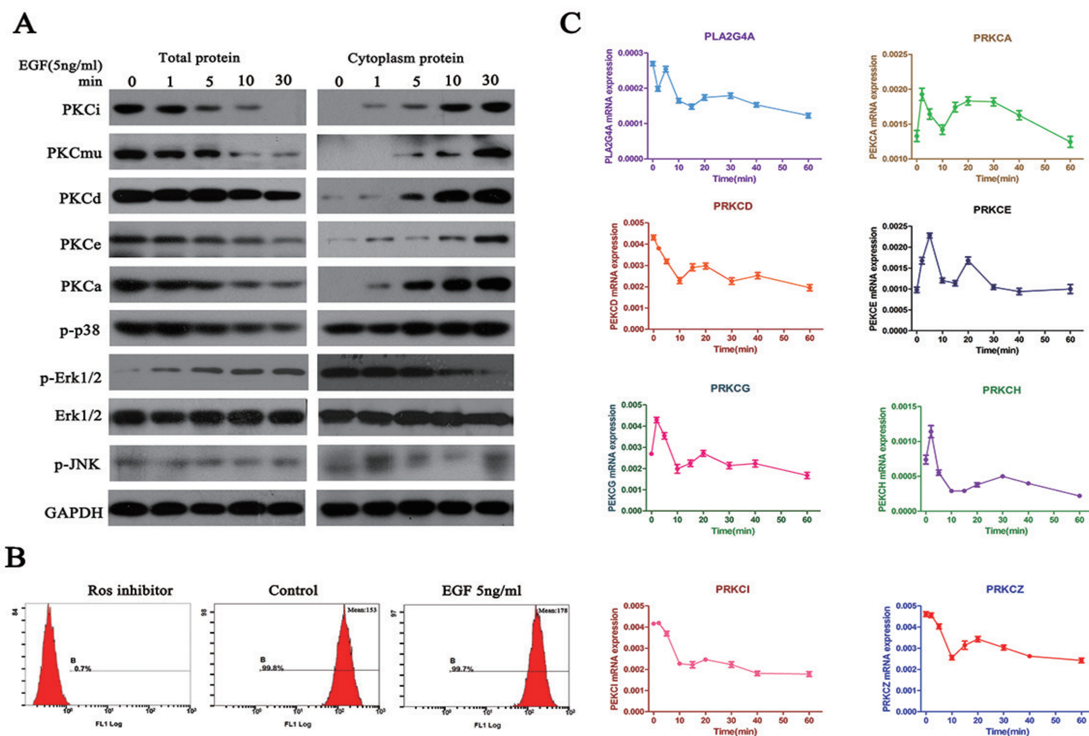

Figure 2. EGF activated ERK1/2 and ROS pathway, but repressed the protein level in PKC pathway. A. Western blot analysis showed that EGF increased the levels of p-ERK1/2 but decreased the PKC proteins in HCE cells. B. Flow cytometry showed that EGF could increase the level of ROS in HCE cells. C. Time-course Q-PCR analysis of the downregulation of EGF on PKC proteins.

the upregulation of ROS in HCE cells (Fig. 2B).

Surprisingly, unlike in other cell lines (22), PKC pathway-related proteins, such as PKCi, PKCmu, PKCd, PKCe, PKCa, p-p38, and other PKC proteins were decreased after EGF treatment in HCE cells (Fig. 2A), which was confirmed by real-time PCR analysis (Fig. 2C). Furthermore, the cytoplasmic PKC protein levels were increased (Fig. 2A, right panel), indicating that the activity of PKC pathway was decreased in EGF-treated HCE cells. These results suggest that ROS and ERK pathway but not PKC pathway might be involved in EGF-induced HCE cell proliferation and migration.

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EGF promoted HCE cell proliferation mainly through ROS and promoted cell migration through MAPK/ERK pathway

The above results showed that EGF promoted the expression levels of ERK1/2, p-ERK1/2 and ROS in HCE cells, indicating that the EGF functions in HCE cell proliferation and migration might be mediated by MAPK/ERK pathway or ROS activation. Therefore, we investigated corneal epithelial cell viability and migration with a ROS inhibitor (N-acetylcysteine, NAC) or ERK1/2 inhibitor, respectively. EGF could promote cell viability in both physiology and disease conditions. However, 40 mM antioxidant NAC treatment inhibited the cell viability for 65% compared with that of control cells, and NAC also blocked EGF-induced cell viability increase in HCE cells in both physiology and disease conditions (Fig. 3A and B). However, the ERK1/2 inhibitor could not affect the cell viability as dramatically as ROS inhibitor did. Interestingly, EGF could promote cell proliferation for the HCE cells cultured with ERK1/2 inhibitor in disease condition but not in physiology condition (Fig. 3A and B). Furthermore, we found that p47 siRNA had no effect in both physiology and disease conditions with or without EGF (Fig. 3A and B). These results suggest that EGF-stimulated cell proliferation was mainly mediated by ROS in both disease and physiology conditions. And, EGF-induced cell proliferation may be also partially mediated by ERK1/2 in physiology condition.

In the wound healing experiments, we found that EGF could promote HCE cell migration even in the presence of NAC and p47 siRNA. However, the effect disappeared when the cells were treated with an ERK1/2 inhibitor or EGF inhibitor (Fig. 3C), which suggests that it is MAPK/ERK pathway instead of ROS that mediates EGF signaling to promote HCE cell migration.

The interactions of ROS, ERK1/2, and PKC in EGF-treated HCE cells

ROS, ERK1/2, and PKC have different functions in EGF-induced HCE cell proliferation and migration. We then examined the relationship of ROS, ERK1/2 and PKC downstream of EGF signaling in HCE cells. Flow

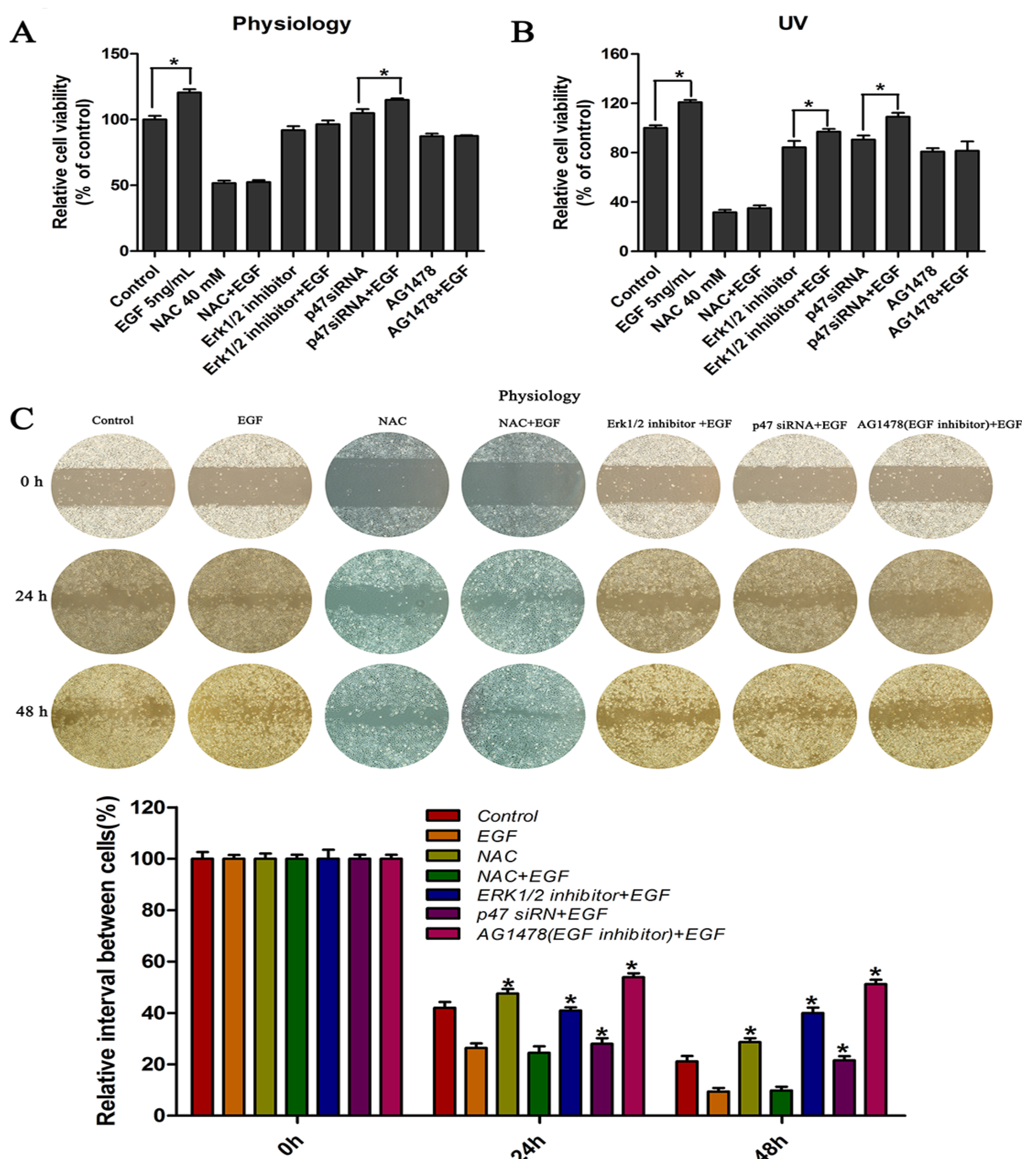

Figure 3. EGF promoted HCE cell proliferation mainly through ROS and promoted cell migration through ERK1/2 pathway. A. In the presence of ROS inhibitor or ERK1/2 inhibitor, EGF could not promote cell proliferation in physiology condition. B. ROS inhibitor repressed HCE cell proliferation in UV condition with or without EGF, but ERK1/2 inhibitor and p47 siRNA had no such function. C. EGF promoted cell proliferation even in the presence of NAC and p47 siRNA, but in the presence of ERK1/2 inhibitor or EGF inhibitor (AG1478), the migration stimulated by EGF disappeared. The percentage of wound closure was calculated.

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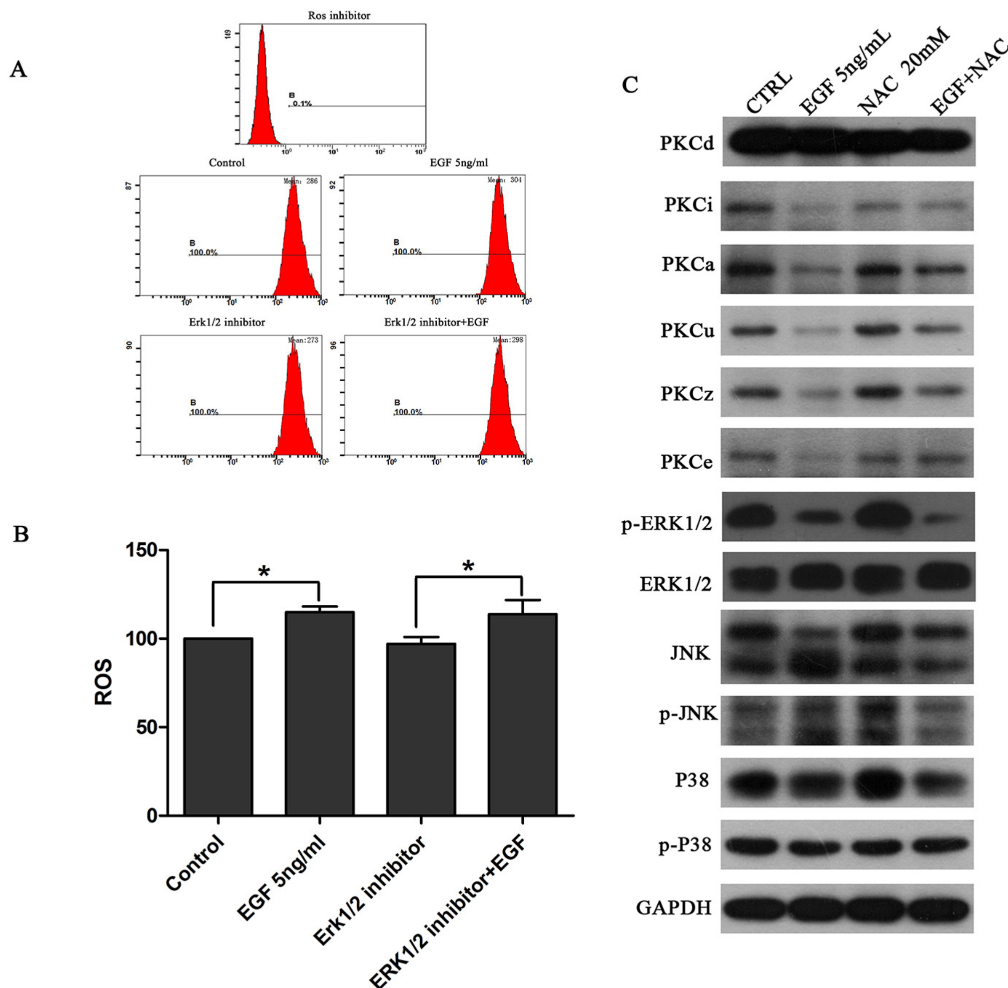

Figure 4. The interactions of ROS, ERK1/2, and PKC in the EGF treated HCE cells. A and B. Flow Cytometry analysis showed ERK1/2 inhibitor did not affect ROSactivation stimulated by EGF in HCE cells. C. ROS inhibitor did not affect ERK1/2 pathway, but partially rescued the downregulation of PKC proteins by EGF. HCE cells were treated with EGF (5 ng/mL) with or without NAC (20 mM) for 60 min, and total protein samples were subjected to western blot analysis.

cytometry analysis showed that EGF could increase the level of ROS in HCE cells even in the presence of ERK1/2 inhibitor (Fig. 4A), and western blot results showed that ROS inhibitor could not affect the level of ERK1/2 or p-ERK1/2 in control and EGF pre-loaded HCE cells (Fig. 4B). These results suggest that ROS and ERK1/2 pathway have no mutual interactions in HCE cells, although both pathways mediate EGF-induced HCE cell proliferation in physiology condition.

EGF could induce the downregulation of PKC pathway-related proteins in corneal epithelial cells, but ROS inhibitor could partially rescue this downregulation (Fig. 4C), indicating that the downregulation of PKC proteins induced by EGF was partially mediated through the activation of ROS in HCE cells. Taken together, these data suggest that ROS, ERK1/2, and PKC pathways have cross talk with each other downstream of EGF pathway in physiology or disease conditions, and the ROS, ERK1/2, and PKC pathways play distinct roles in HCE cell proliferation and migration.

Discussion

It has been well known that EGF could promote cell proliferation and migration (6-9), which may act through activation of PKC pathway, MAPK/ERK pathway, or ROS (10, 12, 13, 19). However, it remains unclear which pathway plays main roles in cell proliferation or migration. In this study, we found different downstream pathways paly distinct roles in EGF-induced cell proliferation and migration, and the underlying mechanisms in physiology and disease conditions may also be different.

Our results showed that EGF promoted human corneal cell proliferation mainly through activation of ROS in both physiology and disease conditions. ROS inhibitor significantly deceased EGF-stimulated HCE cell vitality, suggesting that ROS is the main downstream of EGF signaling in HCE cell proliferation (Fig. 2A and B). MAPK/ERK pathway only partially mediated the promotion function of EGF in HCE cells in physiology condition (Fig. 3B), instead of in disease condition (Fig. 3A). However, ROS was not involved in EGF-stimulated HCE cell migration, which is mainly mediated by activation of MAPK/ERK pathway (Fig. 3C). Moreover, no mutual interactions between ROS and ERK were found (Fig. 4A and B). Therefore, EGF-promoted cell proliferation and migration might be two independent processes, which are mediated by different EGF downstream signaling pathways.

PKC did not seem to have any function in HCE cell proliferation and migration (Fig. 3). It is known in other cell types that EGF could activate PKC pathway (22). However, EGF indeed repressed the activities of PKC pathway, partially through ROS in HCE cells (Fig. 2A and C, Fig. 4C). These results suggested that the mecha-

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nisms underlying EGF-induced processes in HCE cells are distinct with other cell types.

In summary, we systematically analyzed the roles of EGF in HCE cells and related mechanisms in both physiology and disease conditions. Our findings provide a novel concept for the molecular mechanisms of EGF-induced functions in cornea in both physiology and disease conditions.

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