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Migration of retinal pigment epithelial cells is EGFR/PI3K/AKT dependent

Lei Zhang[1], Fenghua Wang[2], Yuan Jiang[1,2], Shan Xu[1,2],Fengqing Lu[1,2],Wenqiu Wang[1,2], Xiangjun Sun[3], Xiaodong Sun[1,2]

[1]Department of Ophthalmology, Shanghai First People's Hospital Affiliated Shanghai Jiao Tong University, 100 Haining road, Shanghai, PR China, 200080, [2]Shanghai Eye Research Institute, 100 Haining road, Shanghai, PR China, 200080, [3]School of Agriculture and Biology, Shanghai Jiao Tong University, 800 Dongchuan RD. Minhang District, Shanghai, PR China, 200080

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1. ABSTRACT

Abnormal migration of retinal pigment epithelium (RPE) contributes to a variety of disorders such as proliferative vitreoretinopathy. Here, the effect of epidermal growth factor (EGF), and signaling by its receptor (ERGR)/phosphatidylinositol 3-kinase (PI3K)/protein kinase B (AKT) on RPE cell migration was studied. The *in vitro* wound healing and migration of the human RPE cell line, ARPE19 cell was accelerated, in a dose dependent manner, in response to EGF stimulation, while pretreatment with EGFR, PI3K or AKT inhibitor, inhibited both events. Exposure of cells to EGF activated the AKT phosphorylation, whereas EGFR and PI3K inhibitors blocked EGF-induced AKT phosphorylation in a dose-dependent manner. These data suggest that EGF mediate ARPE-19 cell migration through EGFR/PI3K/AKT signaling pathway.

2. INTRODUCTION

Proliferative vitreoretinopathy (PVR) is not only an important complication of rhegmatogenous retinal detachment and ocular trauma, but also a leading cause of failed surgery repair. Although the vitreoretinal surgical techniques have improved dramatically in the past 20 years, the incidence of PVR following the primary retinal detachment and vitreoretinal surgery is still significant, ranging from 5.1-11.7% [1]. The pathological migration of retinal pigment epithelial (RPE) cells serves as an initial step in the development of PVR. In response to microenvironmental pathologic changes, the activated RPE cells migrate to the vitreous cavity and retinal surface followed by the formation of PVR proliferative membrane [2, 3]. The cell proliferation and membrane contraction in PVR may lead to recurrent retinal detachment [4]. Therefore, it is very essential to investigate the exact

mechanism of RPE cell migration for preventing the formation of PVR proliferative membrane.

It is reported that a number of cytokines and growth factors secreted by the wounded RPE cells and inflammatory cells can promote the migration of RPE cells [5-8]. Among these factors, epidermal growth factor (EGF) and its receptor (EGFR) are most widely investigated and confirmed to have an important role in the regulation of cell growth, differentiation, and migration [9-10]. It has been reported that the gene and protein expression of EGF are remarkably enhanced in the vitreous cavity and subretinal fluid in patients with PVR[6,11]. EGFR was also found to be highly expressed in early stage of PVR membranes [12-14], indicating that EGF/ EGFR might be involved in the formation of PVR proliferative membrane. In addition, EGF was also found to enhance *in vitro* cultured RPE cell wound closure [15-16] via activating downstream signaling cascades to regulate cytoskeleton remodeling, influence integrin expression[17] and induce epithelial-to-mesenchymal transition[18].However, the underlying molecular mechanism is still not fully understood.

EGF can stimulate EGFR to promote cell migration via both mitogen-activated protein kinase (MAPK) and phosphoinositol-3-kinase (PI3K) intracellular pathways in different cell types [19-21]. Yan and co-workers [12] identified MAPK nuclear translocation in RPE cell migration in the presence of EGF, which suggested that MAPK signal pathway was involved in EGF-induced RPE migration. However, the fact that inhibition of MAPK pathway could only partly impede RPE migration indicates the involvement of other signaling pathways in RPE migration. Indeed, it is recently reported that PI3K/protein kinase B (AKT) signaling pathway could be triggered by the activation of EGF/EGFR or other growth factors/receptors to mediate the cell migratory signal in cancer cells [20, 22]. However, whether PI3K/AKT contributes to EGF-induced RPE cell migration remains to be determined.

In the present study, we employed a widely used human RPE cell line, ARPE19[23], to investigate: 1) the effect of EGF on the migration of RPE cells; 2) if EGFR/PI3K/AKT signaling pathway mediates the migratory signal in RPE cells; 3) which AKT isozyme is mainly involved in EGF-induced RPE cell migration.

3. MATERIALS AND METHODS

3.1. Reagents

Dulbecco modified essential medium (DMEM), penicillin/streptomycin, fetal bovine serum (FBS) and trypsin were purchased from Invitrogen (Camarillo, CA). Human recombinant EGF was from R&D Systems (Minneapolis, MN). AG1478 (an EGFR activation inhibitor) and LY294002 (a PI3-kinase inhibitor) was from Cell signaling (Beverly, MA). AKT inhibitor III (a pan-AKT inhibitor) was from Invitrogen (Camarillo, CA). Primary antibodies against total-AKT, phospho-total-AKT, AKT1, phospho-AKT1, AKT2 and phospho-AKT2 were from abCam (Boston,MA). 3-(4,5-dimethyl-2-yl)-5-(3-carboxymeth-oxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) was purchsased from Promega (Madison,WI), The Transwell Chamber (24wells, polycarbonate membranes with 8-μm pores) was from Corning (NYC,NY).

3.2. Cell culture

The ARPE-19 cell line was purchased from American Type Culture Collection, (Manassas, VA). Cells were grown in DMEM with 10% FBS and 1% penicillin/ streptomycin, at 37°C in a humidified incubator containing 5% CO_2 and 95% air. Culture medium was changed every second day. Cells were dissociated using 0.25% trypsin when they were grown to 80% confluence. To minimize the effect of serum on cell migration and proliferation, cells were starved in serum-free DMEM overnight before any experiments were done.

3.3. MTS cell proliferation assay

Monolayer ARPE-19 cells were pretreated with series concentrations of EGF or inhibitors mixed with 10ng/ml EGF, then incubated at 37^{o}C, 5% CO_2 for 6hrs. Cells treated with DMEM were set as control. A stock solution of MTS was prepared in PBS and added to the cell suspension. The cell suspension was then cultured for 2hr at 37^{o}C, 5% CO_2. The absorbance was recorded at 490nm using a microplate reader. The cell proliferation rate was calculated as following formula: (the absorbance of cells in EGF or inhibitor group/ the absorbance of cells in DMEM control group) x 100 %.

3.4. *In vitro* wound healing assay

ARPE-19 cells were seeded in 12-well plates containing DMEM with 10% FBS for two days to grow a uniform monolayer and starved in serum-free DMEM for 24 hrs. Then cells were lined out with a sterile 20-μl pipette tip to remove cells by two perpendicular linear scrapes which crossed in the middle of the cell monolayer. Floating cells were removed by washing twice with 0.01M PBS. ARPE-19 cells in control group were treated with serum-free DMEM. Cells in the EGF-stimulating group were treated with 10ng/ml EGF without any inhibitors. Cells in the inhibitory group were treated with 10ng/ml EGF plus indicated inhibitors. All the treated cells were incubated at 37˚C in 5%CO_2 for appropriate time and the distance of wounds was evaluated under an inverted microscope. Each group contains triplicate wells. Each experiment was repeated for 3 times.

3.5. Transwell cell migration assay

The migration of ARPE-19 cells in the presence of indicated reagents was detected by 24-well transwell chamber as described by Sun *et al* [24]. Briefly, ARPE-19 cells were suspended to a cell concentration of 2×10^6 /ml in serum-free DMEM. For each well of transwell chamber, 100 μl cell suspension was loaded into the upper compartment, while EGF was loaded into the lower compartment as a migration inducer. The upper and lower compartments were divided by a porous membrane, which allows cells moving into the lower compartment. For inhibitory assays, suspended cells were pretreated with inhibitors at indicated concentration for 1 hour at 37°C, and then loaded into the upper compartment, 10ng/ml EGF was

loaded into the lower compartment. After incubation in transwell chamber for 18 hrs, cells on top of the membrane were fully scraped by tampons. Then the membrane was washed by 0.01M PBS, fixed in 4% paraformaldehyde (PFA), and stained with haematoxylin. The number of migrated cells (attached in the lower side of membrane) was counted in three random fields under 200×magnification. Each experimental group contains triplicate wells and each experiment was performed for 3 times. The migratory index was calculated as the following formula: the number of migrated cells responding to specific reagent / the number of migrated cells in a DMEM control group. In inhibition test, we used inhibition rate to evaluate the inhibitory effect of indicate inhibitor on RPE cell migration: inhibition rate= (1 − migrated cell number in inhibitor group / migrated cell number in EGF treatment group) X 100%.

3.6. Western blot

For EGF stimulation assays, the cells were pretreated with 10ng/ml EGF at 37°C for 5, 15 or 30 minutes. For inhibitory assays, cells were pretreated with EGFR inhibitor AG1478 or PI3K inhibitor LY294002, respectively, for 2 hrs at 37°C and then stimulated with 10ng/ml EGF for 15min. Treated cells were washed with cold PBS and subjected to lysis in phosphosafe extraction reagent (Merck, Darmstadt, Germany) suspended with protease inhibitors(Merck, Darmstadt, Germany). Bradford dye assay was used to determine the concentration of protein in collected cell lysates. Equal amount of cell lysates from the same samples were loaded onto two separate SDS-PAGE systems. One was used for detection of total proteins and the other for phosphorylated proteins. Cell lysates containing equal amount of proteins were loaded and separated by 10% SDS-PAGE. The proteins were transferred onto a PVDF membrane (Milipore, Billerica, MA). After blocked with 5% nonfat milk for 2 hrs at room temperature, the membrane was incubated with corresponding primary antibodies overnight at 4°C. Then the membrane was washed using TBS with 0.05% Tween 20, and incubated with peroxidase-conjugated secondary antibodies (Santa Cruz biotechnology, Santa Cruz, CA). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a loading control.

3.7. Immunofluorescence

Expression of phospho-AKT in ARPE-19 cells was detected by indirect immunofluorescence. ARPE-19 cells were seeded on 5μg/ml fibronectin coated glass coverslips and cultured with serum-free DMEM overnight to achieve 80% confluence. After treatment in the presence or absence of EGF (10ng/ml) for 15 minutes at 37°C, cells were fixed with methanol and acetone (1:1 mixture) for 10minutes at room temperature and incubated with primary antibody against phospho-AKT at 37°C for 2 hrs. After washes with PBS, cells were incubated with FITC-conjugated secondary antibody for 1hour at 37°C in a light-protect box. Cells were then washed with PBS, and stained for 5 minutes with propidium iodide (PI) to display the nuclei. Mounted in mounting medium, cells were observed and pictured under a spinning disc confocal microscope (DSU; Olympus, Tokyo, Japan).

3.8. Statistical analysis

Statistical analysis was carried out to determine the significance of cell viability and migratory response using SPSS 16.0. Difference between the experimental and control group was analyzed by Dunnet-t test. One-way ANOVA or two-way ANOVA was used to test for differences among multiple comparisons. Statistical significance was set at P less than 0.05.

4. RESULTS

4.1. ARPE-19 cell proliferation assay

An MTS cell proliferation assay was employed to determine the effect of EGF on ARPE19 cell proliferation. As indicated in Table 1, cell proliferation rate was increased after 6hr treatment of EGF. In addition, MTS cell survival assay was performed to test the effect of indicated inhibitors on ARPE-19 cell viability. In the presence of 10ng/ml EGF, AG1478, an EGFR activation inhibitor and LY294002, a PI3K inhibitor showed no cytocytoxicity, since the cell proliferation rate did not have significant difference compared to the control group. Although LY294002 was normally used at the concentration of 20ng/ml, 1-1000ng/ml LY294002 were used in our current study since 100ng/ml or 1000ng/ml LY294002 did not induce massive cell death as the cell proliferation rate more than 97%. AKT inhibitor III, an AKT inhibitor, exhibited slight cytotoxicity at concentration of 10μM and 15μM, as the cell proliferation rates were 74% and 70% respectively. All the enzyme inhibitor dosages chosen in the current study were in consistent with previous study [24], and/or showed no or low cell cytotoxicity in MTS assay.

4.2. EGF Enhances the migration of ARPE-19 cells via EGFR

In response to damage, *in vivo* activated RPE cells would initiate a wound-healing process, which is to secret a variety of growth factors to promote RPE cells to migrate towards the wound and proliferate. In our current study, to study the effect of EGF on the wound-healing of ARPE-19 cells, *in vitro* wound-healing assay was used to mimic the *in vivo* wound-healing process. The mobility of ARPE-19 cells was increased when exposed to 10ng/ml EGF. As shown in Figure 1B, ARPE-19 cells migration initiated at 6h after the wound (data not shown), and at 24 h, the wounds were almost closed by migrated cells. By contrast, ARPE-19 cells without exogenously growth factor showed a weaker mobility, and there are only a few cells migrated over the wound at 24h after wound (Figure 1A). To test if EGF enhanced the mobility of ARPE-19 cells through EGFR activation, AG1478, a specific inhibitor was applied to block EGFR activation. We observed that fewer cells migrated towards the wound after pretreated with AG1478 by compared to those stimulated only by EGF (Figure 1C).

Given that EGF may promote both cell migration and proliferation in *in vitro* wound healing assay, a transwell assay was employed to focus our study on the effect of EGF on ARPE-19 cell migration. Briefly, EGF (0, 1, 10, 100, or 1000ng/ml) was applied in the lower

Table 1. Cell proliferation rate when ARPE-19 cells were treated with indicated reagent for 6 hours.

EGF ng/ml	**cell proliferation rate**	**P value (compared to control group)**
1	110.76%	Less than 0.0001
10	106.78%	Less than 0.0001
100	108.62%	Less than 0.0001
1000	110.90%	Less than 0.0001
AG1478 nM + 10ng/ml EGF		
50	107.21%	0.002
100	101.37%	0.827
200	97.31%	0.13
500	96%	0.094
LY194002 ng/ml + 10ng/ml EGF		
1	99.85%	1.000
10	99.27%	0.989
100	102.35%	0.499
1000	97.33%	0.767
AKT inhibitorIII μM + 10ng/ml EGF		
1	97.93%	0.279
5	91.85%	Less than 0.0001
10	74%	Less than 0.0001
15	70%	Less than 0.0001

EGF: epidermal growth factor

compartments while the ARPE-19 cells were suspended in the upper compartments. A porous membrane separated the lower and upper compartments only allowing cells to migrate through the pores. The migratory capability of ARPE-19 cells was determined by the number of cells migrating to the lower side of the membrane. We found that in the presence of EGF (1-1000ng/ml), the number of migrated ARPE-19 cells was accelerated in a bell-shaped dose-response manner (Figure 2A). The migratory index was in proportional to EGF concentration when EGF supplied at 1-10ng/ml, while the migratory index was in reverse proportional to EGF concentration when 10-1000ng/ml EGF was added to the culture medium. EGF with a concentration of 10ng/ml showed the most significant effect on promoting ARPE-19 cell migration with a migratory index of 104.8. The cells treated with 100ng/ml or 1000ng/ml EGF showed less dramatic migratory ability.

To study the effect of EGFR on EGF-mediated ARPE-19 cell migration, we pretreated ARPE-19 cells with EGFR activation inhibitor AG1478 for 1 hr before the supplement of 10ng/ml EGF. The migratory index decreased when the concentration of AG1478 increased (Figure 2B), indicating that AG1478 blocked the cell migration in a dose-dependent manner. This result suggested that EGFR activation was required for EGF-mediated ARPE-19 cell migration.

4.3. PI3-Kinase activation is required for EGF-mediated ARPE-19 cell migration

We further studied whether PI3K intracellular pathway was involved in EGF- mediated ARPE-19 cell wound-healing and migration. We applied a specific PI3K inhibitor LY294002 to study the role of PI3K in EGF-mediated ARPE-19 cell wound-healing and migration. Cells treated with 1000ng/ml LY294002 plus 10ng/ml EGF showed a remarkably delayed and milder wound closure in the *in vitro* wound-healing assay (Fig1 D). In the transwell assay, we found that the migratory index was in reverse proportion to the dosage of LY294002 in the presence of 10ng/ml EGF (Figure 3A, B). In 1000ng/ml LY294002 group, few cells have migrated toward the lower compartment of the transwell chamber, suggesting that cell migration was almost completely abolished (Figure 3B). It indicated that PI3K intracellular pathway was crucial for EGF-mediated ARPE-19 cell wound-healing and migration.

4.4. AKT is involved in EGF-mediated ARPE-19 cell migration

AKT is a downstream target of PI3K that participates in a variety of biological responses, including cell migration and proliferation. To assess whether AKT is involved in transducing signals of EGF-mediated ARPE-19 cell wound-healing and migration, we inhibited AKT molecule and then observe the ARPE-19 cell migration in response to EGF. The application of AKT inhibitor III, an inhibitor blocked all AKT isotypes, exhibited dramatic inhibition effects on EGF-mediated ARPE-19 cell wound-healing and migration. When treated with15μM AKT inhibitor III plus 10ng/ml EGF, the wound was barely closed even at 24hr (Fig1E). In the transwell assay, at AKT inhibitor III concentrations of 10μM and 15μM, the migration indexes were significantly decreased than that in the 10ng/ml EGF group. (Figure 4A, B). These results indicate that AKT is required in EGF-mediated ARPE-19 cell migration.

To further explore whether AKT can be activated by EGF in ARPE-19 cells, immunofluorescence assay was used to detect the phospho-AKT expression. In the presence of 10ng/ml EGF, phospho-AKT was detected in ARPE-19 cells whereas it was absent in the cells cultured without EGF supplement (Figure 5A). Western blot assay was employed to confirm the result of immunofluorescence. We found that AKT phosphorylation was identified from 5min to 30min after EGF treatment, whereas total-AKT protein expression remained the same during the observation (Figure 5B).

To determine whether activation of EGFR was related to AKT phosphorylation, EGFR inhibitor AG1478 was applied before 10ng/ml EGF was added to RPE cell culture medium. The expression of phosphor-AKT was relatively high in the presence of EGF while it was decreased when AG1478 was supplied to the culture medium and this decrease was in a dose-response manner (Figure 5C). These data suggested that EGFR activation is required for AKT activation.

To further assess whether AKT is a downstream molecule of PI3K intracellular pathway that is activated by EGF, western blot assay was performed to detect AKT phosphorylation in the presence or absence of PI3K inhibitor LY294002 when ARPE-19 cells were exposed to EGF. The expression of phospho-AKT was decreased in the presence of LY294002 at high concentrations of 100-1000ng/ml. (Figure 5D).

To further examine which isotype of AKT is involved in EGF-mediated ARPE-19 cell migration, we identified the activation of AKT1 and AKT2 in ARPE-19 cells in the presence of 10ng/ml EGF. In EGF-treated cells,

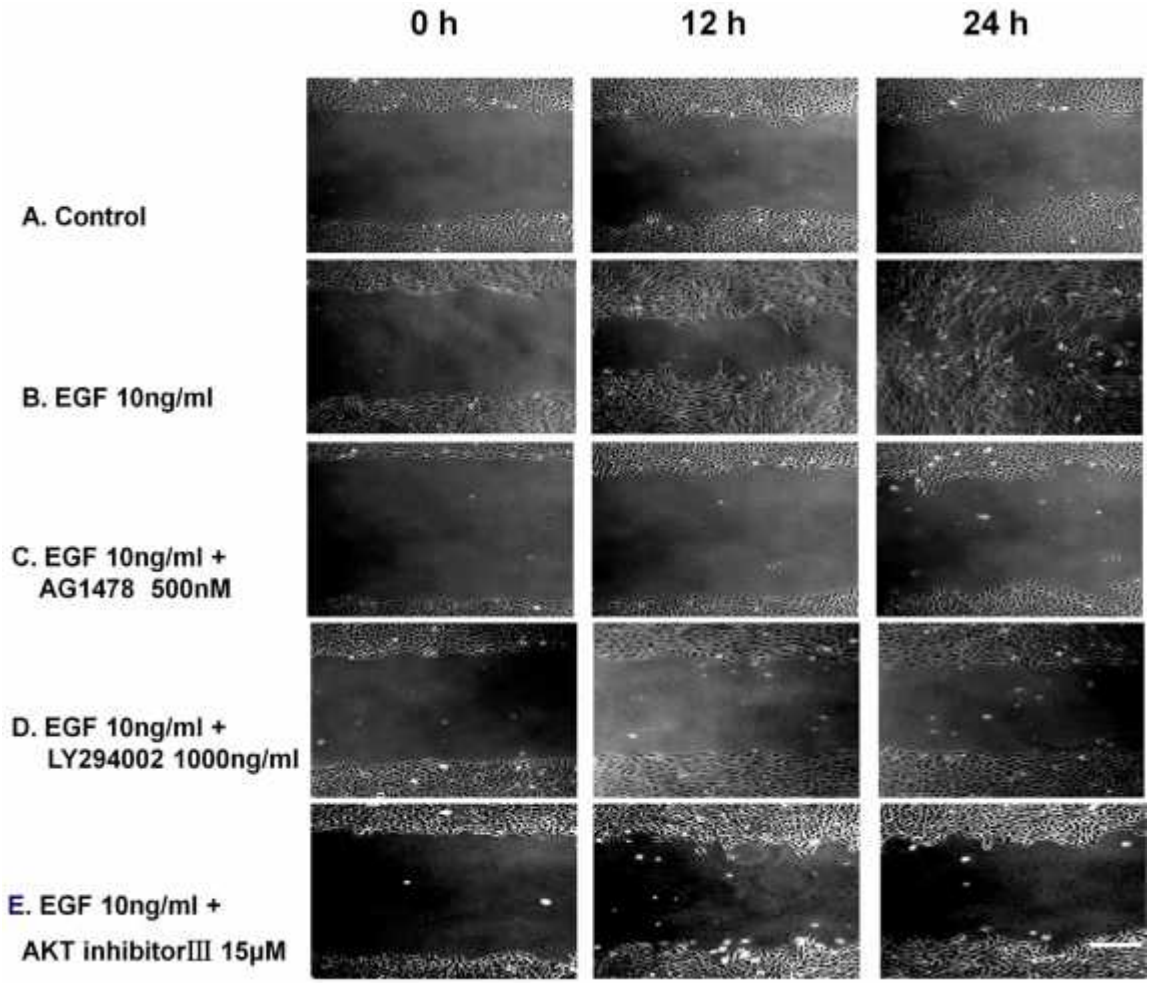

Figure 1. EGF/EGFR/PI3K/AKT promotes RPE cells *in vitro* wound-healing. Overnight starved monolayer ARPE-19 cells were scratched with a sterile 20-μl pipette tip. Rinsed with PBS to remove cell debris, cells were cultured with A- serum-free DMEM; B- DMEM containing EGF(10ng/ml); C- DMEM containing EGF(10ng/ml) and 500nM AG1478 (a inhibitor of EGFR activation); D- DMEM containing EGF(10ng/ml) and 1000ng/mlLY294002 (a specific inhibitor of PI3knase) and E- DMEM containing EGF(10ng/ml) and 15μM AKT inhibitor III (a specific inhibitor of AKT). Wound healing was observed and pictured immediately or at 12hr, 24hr after wounding under a phase–contract microscope (10×). Each experimental group contains triplicate wells and each experiment was performed for 3 times. (Scale bar: 100μm).

the expression of phospho-AKT2 was significantly enhanced whereas phopho-AKT1 expression was not identified (Figure 6A and B). Our data suggested that AKT2 may be a major isotype of AKT involved in EGFR/PI3K/AKT signaling pathway. Further experiments should be performed to detail the function of AKT2 in EGF-mediated ARPE-19 cell migration.

4.5. Comparison of the roles of EGFR/PI3K/AKT on ARPE-19 cell migration

To evaluate the role of EGFR/PI3K/AKT on ARPE-19 cell migration, we compared inhibition rates when EGFR, PI3K, AKT inhibitors were respectively applied. In EGFR inhibition tests, 500nM AG1478 exhibited maximal effects on cell migration of which the inhibition rate was 80.8%. In PI3K inhibition tests, the maximal inhibition rate was 94.4% when 1000ng/ml LY294002 was added to the culture medium. In AKT inhibition tests, 15μM AKT inhibitor III had maximal inhibition effects with the inhibition rate at 88.3%. These results suggested that PI3K inhibitor significantly abolished ARPE-19 cell migration, suggesting that PI3K intracellular pathway may be one of the key regulators of EGF-mediated ARPE-19 cell migration.

5. DISCUSSION

In the current study, we found that EGF is able to promote ARPE-19 cell migration in a concentration-dependent pattern. We also found that EGFR/PI3K/ AKT signaling pathway might attribute to the ARPE-19 migratory signal transduction by inhibition tests.

EGFR is a multifunctional glycoprotein belonging to the HER/ErbB family of receptor tyrosine kinase (RTKs). As a membrane-bound protein widely

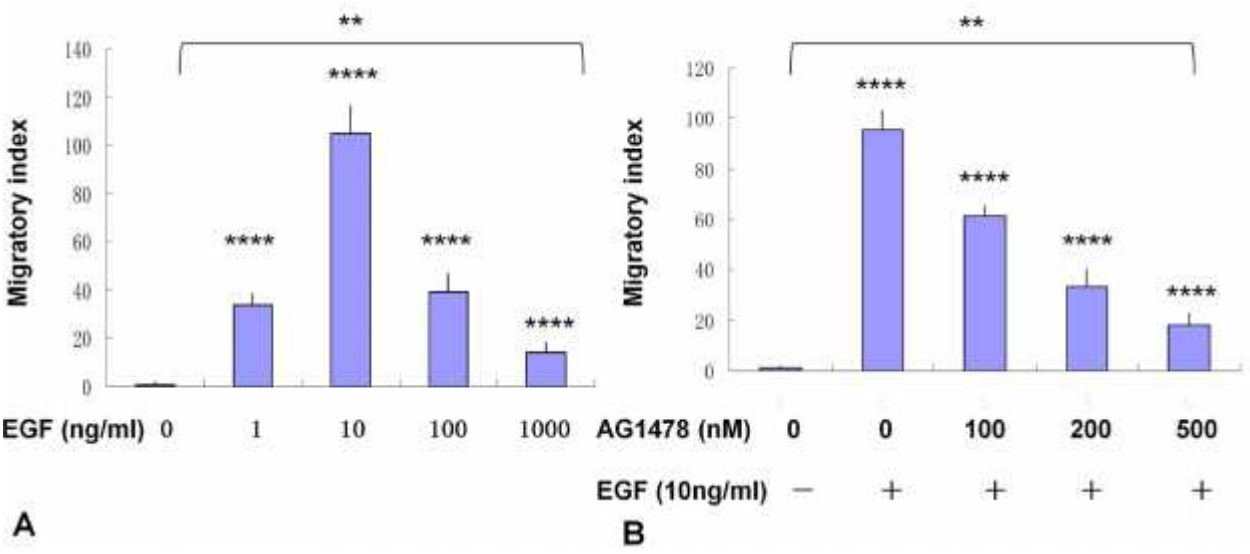

Figure 2. EGF/EGFR induces ARPE-19 cells migration. Transwell assays were performed to assess ARPE-19 cell migration. Cells were suspended in the upper chambers and migrated in response to EGF in the lower chamber. After 18h incubation, migrated cells in the lower side of porous membrane were stained. Data were analyzed by one-way or two-way ANOVA (SPSS 16.0, **P less than 0.001, ****P less than 0.00001). A- Cell number of migrated ARPE-19 cells in response to different concentration of EGF. EGF (1, 10, 100, or 1000ng/ml) was loaded in the lower chamber to attract ARPE-19 cell migration. Serum-free DMEM without EGF was loaded as control. B- Cell number of migrated ARPE-19 cells which were pretreated with EGFR inhibitor AG1478 (0, 100, 200, or 500nM) for 2 hours followed by the treatment of EGF (10ng/ml). The cultures without inhibitor or EGF serve as control.

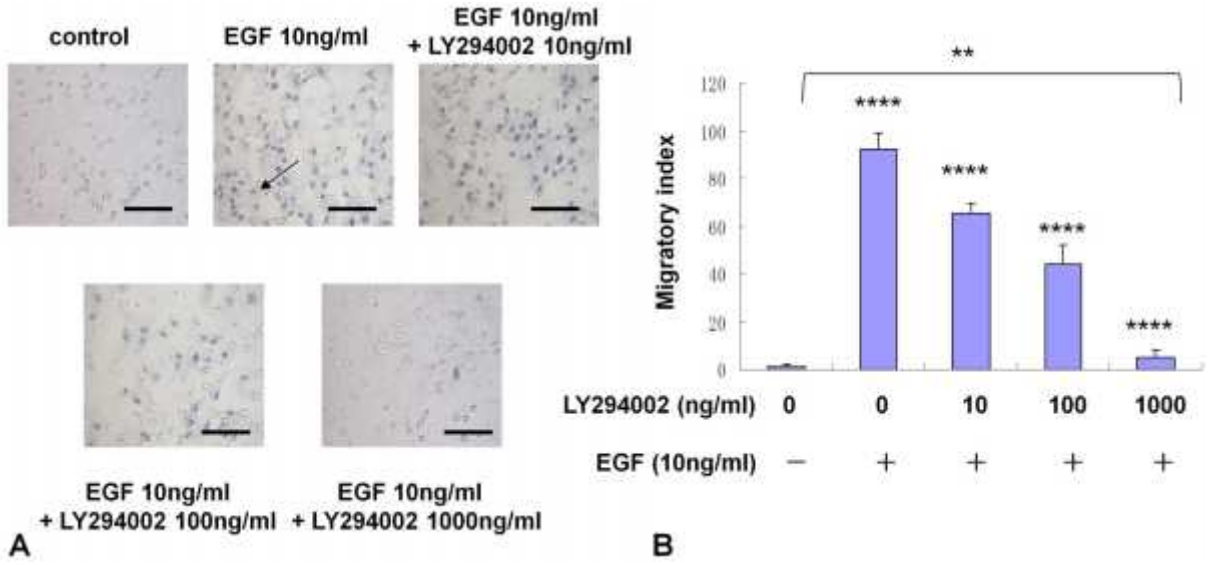

Figure 3. PI3K is required for EGF-mediated ARPE-19 migration. A- Transwell assay was performed to assess the effect of PI3K inhibitor LY294002 on EGF-mediated ARPE-19 cells migration. Cells were pretreated for 2 hours with different concentrations of LY294002 (0, 10, 100, or 1000ng/ml), and allowed to migrate in response to 10ng/ml EGF. Cells that had migrated through the transwell membrane were fixed and stained (arrow). Scale bar: 100μm. B- The migratory index decreased as LY294002 dose increased. Our data were analyzed by Two-way ANOVA (SPSS16.0, ** P less than 0.001,**** P less than 0.00001).

located in human tissues [10], EGFR can be activated by ligands such as EGF, HB-EGF and HGF, and can stimulate downstream signaling pathway such as PI3K/AKT, Ras/Raf/MAKP/ERK, PLC-γ, JAK/STAT to transmit biological signals [25]. In mammalian retina, four members of EGFR family were reported to express in two human RPE cell lines ARPE-19 and hTERT [12, 26].

EGF was reported to play an important role in the pathology of PVR by promoting RPE cell proliferation

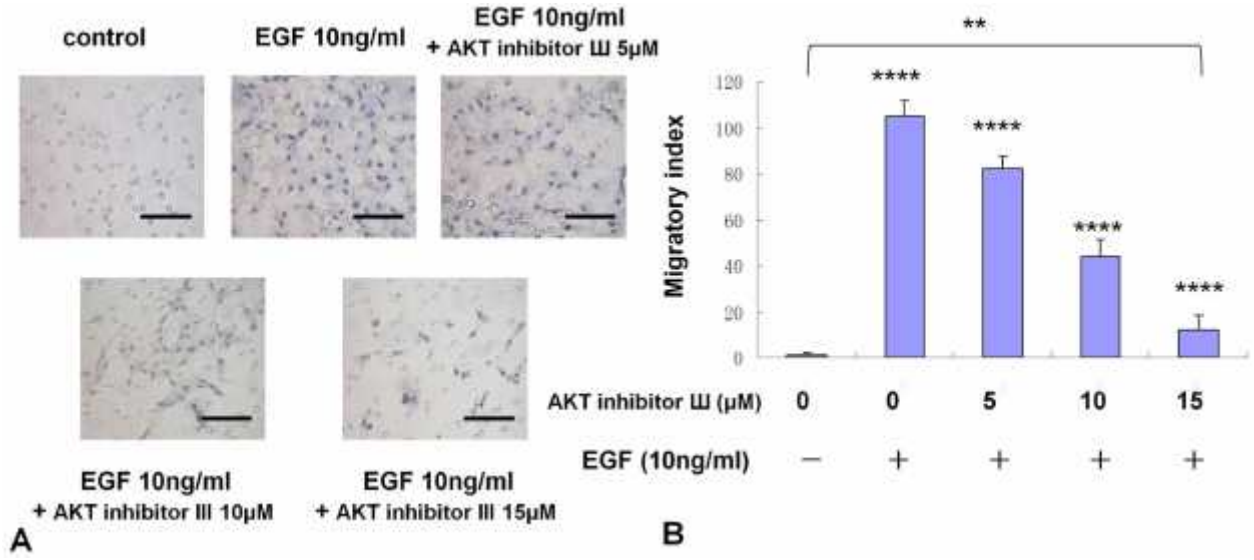

Figure 4. AKT is required for EGF-mediated ARPE-19 cells migration. A- ARPE-19 cells were pretreated for 2 hours with different concentrations of AKT inhibitor III (0, 5, 10, or 15μM) followed by the incubation in the upper transwell chamber. 10ng/ml EGF was supplied to the lower chamber. The cultures without inhibitor pretreatment serve as control. (Scale bar: 100μm). B- The migratory index decreased when AKT concentration increased. This part of data were analyzed by Two-way ANOVA (SPSS16.0, ** P less than 0.001,**** P less than 0.00001).

and migration. Recent studies found that EGF is capable to initiate proliferation and epithelial-mesenchymal transition of RPE cells through Wnt pathway. [27-28] Our MTS data indicated that 6hr treatment of 10ng/ml EGF could promote ARPE-19 cell proliferation. Xu and Yu [12] reported that EGFR activation played an important role in ARPE-19 cell wound-closure. Our current data also indicated EGF is able to promote the would-healing of ARPE19-cells. Although the *in vitro* wound healing assay can well mimic intraocular injury which is normally the cause of PVR, we cannot exclude the possibility that EGF-induced the wound-healing is partly due to the increased cell proliferation, since both cell proliferation and migration were involved in the wound-healing process, To focus our study on the ARPE-19 cell migration, we employed the transwell assay.

Here we provided new evidences that EGFR activation promoted the ARPE-19 cell migration. We first detected a high inhibition rate of AG1478 on ARPE-19 cell migration, and then found a potential role of desensitization of EGFR in EGF-elicited bell-shaped chemotactic dose-response of ARPE-19 cells. The dose-dependent effect of EGF on ARPE-19 cell migration with a maximal promoting effect at 10ng/ml is consistent with the study of EGF-mediated human breast cancer cell chemotaxis [24]. EGFR is reported to locate on the cell surface and undergo recycling and shuttling between the plasma membrane and endosomal compartment. Binding with the ligands, EGFR would be internalized to the endosomal compartment of the cells. Once internalized, EGFR would be under destruction or recycling to the plasma membrane [29]. Although the internalized EGFR is able to transmit signal, it is unable to bind ligands presented at the cell surface. In that case, low-dosage EGF (10ng/ml) has the most inducible effect on RPE migration. On the other hand, a portion of the EGF-EGFR complex may be dissociated in the endosomal compartment, which may lead to desensitization of the EGFR and attenuation of EGF-induced signaling [30]. High-dosage EGF would trigger EGFR internalization and desensitization, therefore reduce the EGF signaling transduction.

According to our observation, inhibition of PI3K exerted the most significant inhibitory effect on ARPE-19 cell migration. PI3K with a concentration of 1000ng/ml efficiently block ARPE-19 cell migration. As an essential downstream signaling molecule of EGFR, PI3K was recruited close to the cell membrane by activated growth factor receptors. Activated PI3K then acted on its downstream protein kinases including AKT, SGK, AGC and PKC [31]. The dramatic inhibitory effect of PI3K inhibitor on ARPE-19 cell migration may be due to a variety of factors: 1) Since PI3K can be activated by both G protein-coupled receptors and tyrosine kinase receptors, there occurs possibility that in addition to EGF, some other growth factors autocrined by wounded RPE cells may also activate PI3K intracellular pathway; 2) Besides AKT, activated PI3K can activate other protein kinases such as PKC to transmit migrating signal. It is therefore reasonable to speculate that PI3K may be a critical molecule among the crosstalk network to mediate the EGF-induced RPE cell migration. Drugs targeting at PI3K may effectively inhibit RPE cell migration. Indeed, a number of PI3K inhibitions have been developed by pharmacy companies. And the first generation inhibitors such as LY294002 are now entering mid-phase clinical trials in anti-tumor research. However, although PI3K targeting components are promising in cancer therapy, inhibition of PI3K may affect some other signaling pathways, which are responsible for normal cellular functions such as cell growth, proliferation and

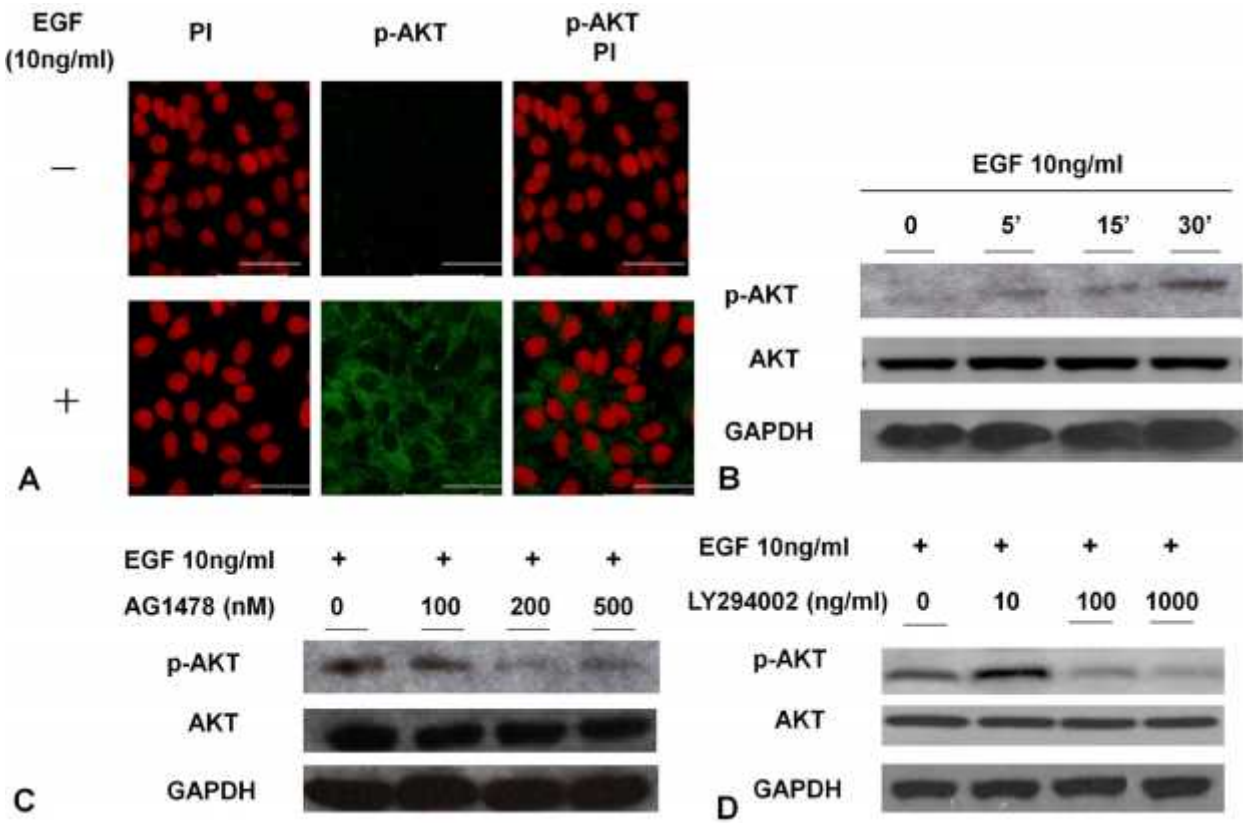

Figure 5. EGF mediates AKT activation. A- ARPE-19 cells were allowed to grow on glass coverslips overnight. After treatment with EGF (10 ng/ml) for 15 minutes, cells were fixed using methanol and acetone (1:1 mixture) followed by the staining of phospho-AKT (*green*) using immunofluorescence. Cells without pretreatment of EGF serve as control. Phosphatidylinositol (PI) was used for nuclear staining (*red*). Scale bar: 50μm. B- ARPE-19 cells were stimulated with 10ng/ml EGF for 0, 5, 15 or 30 minutes. Cell lysates were collected. Half of the cell lysates were used for western blot analysis to detect phospho-AKT (p473) while the other half used for total-AKT. GADPH serves as a loading control. C- ARPE-19 cells were pretreated with AG1478 (0, 100, 200, or 500 nM) for 2 hours followed by the treatment of 10ng/ml EGF for 15 min. Cell lysates were prepared and used for western blot analysis of phospho-AKT and total-AKT to study the effect of AG1478 on EGF-mediated AKT activation. D- ARPE -19 cells were pretreated with LY294002 (0,10,100, or 1000 ng/ml) for 2 hours followed by the treatment of 10ng/ml EGF for 15min. Cell lysates were prepared and used for western blot analysis of phospo-AKT and total-AKT to study the effect of LY294002 on EGF-mediated AKT activation.

survival. Therefore, to avoid affecting normal cell functions, it is important to identify the downstream molecule for drug targeting.

As a well-known downstream signaling molecule of PI3K, AKT regulates cell migration, proliferation and apoptosis by phosphorylating a large number of substrates or regulating gene expression [32]. Our observation of phospho-AKT immunofluorescence and western blot as well as AKT inhibition test suggested that AKT participated in the signal transduction of EGF-induced ARPE-19 cells migration. The inhibition of AKT can efficiently impair ARPE-19 cell migration, suggesting AKT might be a good candidate molecule for drug targeting. To target more specifically, we further studied which isotype of AKT is mainly involved in ARPE-19 migration. The AKT family is composed of three isotypes, AKT1, AKT2 and AKT3. They can be activated by growth factors and regulate pleiotropic cellular activities. Actually, the effect of AKT isotypes on cell migration is cell-type specific. AKT1 is found to promote the migration of embryonic fibroblast cells while AKT2 inhibits it [33]. It is unclear which AKT isotype is involved in the RPE cell migration. We found that AKT2 was significantly phosphorylated in the presence of EGF, indicating that AKT2 might be required in the signaling pathway in regulating ARPE-19 cells migration. In the future study, we will focus on testing if inhibition of AKT2 could effectively impair RPE migration.

Our current findings suggested EGFR/PI3K/AKT pathway played an important role in mediating migratory signals in the process of ARPE-19 cell migration. Recently, a variety of drugs targeting at PI3K/AKT pathway have been developed and used in clinic anticancer trials. Our findings may provide a theoretic supporting for the application of anti-PI3K/AKT drugs on disorders mainly induced by RPE cell pathologically

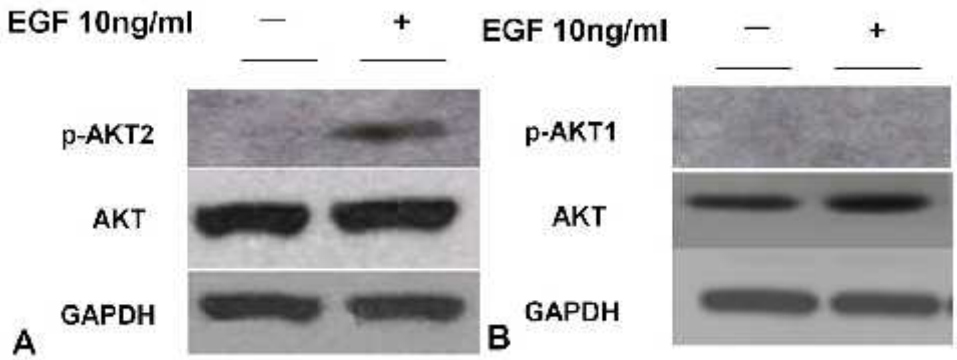

Figure 6. EGF Activates AKT2 but not AKT1. A- ARPE-19 cells were treated with 10ng/ml EGF for 15 min. Cell lysates were prepared and used for western blot analysis of phospho-AKT2 and total-AKT2. B.ARPE-19 cells were treated with 10ng/ml EGF for 15 min. phospho-AKT1 was not detected by western blotting while total-AKT1 was detected.

migration, such as PVR [34]. To further verify the effect of EGFR/PI3K/AKT pathway on RPE cell migration, we will use primary cultured RPE cells to repeat our experiments in the future. We will also try to find out the appropriate downstream targeting components with low cytotoxicity.

In conclusion, we demonstrated that EGF could induce ARPE-19 cell migration via EGFR/PI3K/AKT signaling pathway. ARPE19-cell migration was blocked when EGFR/PI3K/AKT signaling pathway was inhibited. PI3K played an important role in EGF-induced ARPE19-cell migration. As a downstream molecule of PI3K, AKT2 isotype may contribute to EGF-induced RPE cell migration. Our current results provided the promise that molecular targeting treatment for inhibiting EGFR/PI3K/AKT pathway might be a potential therapeutics in PVR.

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Abbreviations: EGF: epidermal growth factor; EGFR: epidermal growth factor receptor; RPE: retinal pigment epithelium; PVR: proliferative vitreoretinopathy; PI3K: phosphatidylinositol 3-kinase; AKT: protein kinase B; DMEM: Dulbecco modified essential medium; FBS: fetal bovine serum; MTS: 3-(4,5-dimethyl-2-yl)-5-(3–carboxymeth-oxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium; PFA: paraformaldehyde; RTK: receptor tyrosine kinase

Key Words: ARPE-19 cell, Migration, EGF, EGFR, PI3K, AKT

Send correspondence to: Xiaodong Sun, Department of Ophthalmology, Shanghai First People's Hospital Affiliated Shanghai Jiao Tong University, Shanghai Eye Research Institute, 100 Haining road, Shanghai, PR China, Tel: 86-21-63240090-6822, Fax: 86-21-63240090-6822, E-mail: xdsun@sjtu.edu.cn