

Inhibition of EGFR attenuates EGF-induced activation of retinal pigment epithelium cell *via* EGFR/AKT signaling pathway

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Abstract

• **AIM:** To explore the effect of epidermal growth factor receptor (EGFR) inhibition by erlotinib and EGFR siRNA on epidermal growth factor (EGF)-induced activation of retinal pigment epithelium (RPE) cells.

• **METHODS:** Human RPE cell line (ARPE-19 cells) was activated by 100 ng/mL EGF. Erlotinib and EGFR siRNA were used to intervene EGF treatment. Cellular viability, proliferation, and migration were detected by methyl thiazolyl tetrazolium (MTT) assay, bromodeoxyuridine (BrdU) staining assay and wound healing assay, respectively. EGFR/protein kinase B (AKT) pathway proteins and N-cadherin, α -smooth muscle actin (α -SMA), and vimentin were tested by Western blot assay. EGFR was also determined by immunofluorescence staining.

• **RESULTS:** EGF treatment for 24h induced a significant increase of ARPE-19 cells' viability, proliferation and migration, phosphorylation of EGFR/AKT proteins, and decreased total EGFR expression. Erlotinib suppressed ARPE-19 cells' viability, proliferation and migration through down regulating total EGFR and AKT protein expressions. Erlotinib also inhibited EGF-induced an increase of proliferative and migrative ability in ARPE-19 cells and clearly suppressed EGF-induced EGFR/AKT proteins

phosphorylation and decreased expression of N-cadherin, α -SMA, and vimentin proteins. Similarly, EGFR inhibition by EGFR siRNA significantly affected EGF-induced an increase of cell proliferation, viability, and migration, phosphorylation of EGFR/AKT proteins, and up-regulation of N-cadherin, α -SMA, and vimentin proteins.

• **CONCLUSION:** Erlotinib and EGFR-knockdown suppress EGF-induced cell viability, proliferation, and migration *via* EGFR/AKT pathway in RPE cells. EGFR inhibition may be a possible therapeutic approach for proliferative vitreoretinopathy (PVR).

• **KEYWORDS:** erlotinib; epidermal growth factor receptor; protein kinase B; epithelial-mesenchymal transition; retinal pigment epithelium cell

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INTRODUCTION

Proliferative vitreoretinopathy (PVR) is a severe fibrosis retinopathy that usually lead to blindness and is hard to effectively treat by existing approaches^[1]. The early fibrotic appearance of cell is an important part in pathological process of PVR^[2]. Some studies have showed that abnormal activation of retinal pigment epithelium (RPE) cells play significant roles in the development of fibrosis during of PVR^[3-5]. The conversion of RPE cells into fibroblast-like cells accelerates the development of PVR^[6]. When the extracellular environment out-of-balance condition in the eye, RPE cells exposed to abnormal cytokines and occurred uncontrolled migration and proliferation, which led to further acceleration of PVR^[7]. It has been reported that during the occurrence of PVR, the proliferation and migration ability of RPE cells are up-regulated^[8-9]. Several kinds of growth factors and cytokines were believed to be linked with the process of PVR, including transforming growth factor, epidermal growth factor (EGF),

interleukin 6, *etc*^[10-11]. Some data have indicated that EGF can promote RPE cell proliferation and migration, and accelerate the progress of PVR^[12-13]. However, whether it can attenuate the evolution of PVR by inhibiting the EGF-induced activation of RPE cells is not fully understood.

EGF receptor (EGFR) is a multifunctional glycoprotein widely distributed on the cell membrane and plays a significant part in cellular proliferation and differentiation^[14-16]. Some scholars have found EGFR is closely related to fibrosis diseases^[17-19]. Another study showed that EGFR degradation can suppress epithelial-mesenchymal transition (EMT) of cells^[20]. Our previous studies showed that EGFR were linked to RPE cells survival^[21-22]. Therefore, targeting EGFR might be one approach to avoid the abnormal activation of RPE cells and PVR. Erlotinib is an effective EGFR inhibitor, which can significantly down regulate EGFR expression^[23]. Some Studies have shown that erlotinib or EGFR knock-down can inhibit the evolution of PVR through Yes-associated protein signaling pathways^[24-25]. However, whether EGFR inhibition can attenuate EGF-induced activation of RPE cells is not fully clear. Therefore, it is necessary to investigate the precise mechanism of EGFR inhibition in RPE cells for searching novel treatment approaches for PVR.

In the present study, we discussed the effect of EGFR inhibition on EGF-caused activation of the RPE cells *in vitro*.

MATERIALS AND METHODS

Reagents Erlotinib hydrochloride and 5-Bromodeoxyuridine (BrdU) came from Selleck Chemicals LLC (Houston, TX, USA). EGF was supplied by Peprotech Inc. (Rocky Hill, NJ, USA). Methyl thiazolyl tetrazolium (MTT) and fetal bovine serum (FBS) were from Sigma-Aldrich Inc (St. Louis, MO, USA). EGFR-small interfering RNA (EGFR-siRNA) was from Santa Cruz Biotechnology Inc. (Dallas, Texas, USA). The EGFR, phosphorylated-EGFR (p-EGFR), AKT, phosphorylated-AKT (p-AKT), N-cadherin, α -smooth muscle actin (α -SMA), vimentin primary antibodies were from Cell Signaling Technology (Danvers, MA, USA).

Cell Culture ARPE-19 cell line was purchased from American Type Culture Collection (Manassas, VA, USA). ARPE-19 cells were cultured with DMEM/F12 medium (Thermo Fisher Scientific, Waltham, Massachusetts, USA) contained 10% FBS at 37°C in a incubator with 5% carbon dioxide. ARPE-19 cells were treated with different concentrations of erlotinib (0, 5, 10, 20, 50 μ mol/L) and 100 ng/mL EGF for different time.

siRNA Transfection EGFR-siRNA and control-siRNA were transfected in ARPE-19 cells through Lipofectamine 2000 for 12h. DMEM/F12 medium contained 10% FBS was then used to cultivate ARPE-19 cells for 6h. Transfection efficacy was measured by Western blot through assessing expression of EGFR protein.

MTT Assay ARPE-19 cells were treated by EGF (100 ng/mL) or erlotinib for different time in a 96-well plate. After treatment, MTT was added to each well to change cultural medium. MTT solution were removed after incubation for 4h, and dimethyl sulfoxide was add into the 96-well plate. The absorbance was determined at 570 nm by micro plate absorbance reader (ELx808IU, Gene company limited, HK, China).

Cell Proliferation Assay After treatment with reagents, ARPE-19 cells were incubated with BrdU for 4h. Next, the cells were fixated by 4% paraformaldehyde (PFA) for 10min. ARPE-19 cells were washed thrice and hatched with 2 mol/L hydrochloric acid (HCl) for 5min. After washed twice, ARPE-19 cells were in the BrdU primary antibody (1:1000) was incubated for 2h, rinsed thrice, and incubated in secondary antibody diluent (1:1000) for 1.5h. Finally, cells were mounted by antifade solution with diaminido-2-phenylindole (DAPI) and were detected by fluorescence microscope (IX73, Olympus, Japan).

Wound Healing Assay ARPE-19 cells were cultured in 12-well plates for 12-24h. When the cells were full, a wound injury was made. The changes of wound size were recorded at different time. The relative migration distance [(the beginning distance–the distance after migration)/the beginning distance] \times 100% was analyzed.

Western Blotting ARPE-19 cells were dissociated by 1.5 \times sodium dodecyl sulfate buffer after different treatments. The protein samples were separated and transferred on a polyvinylidene fluoride membranes. Next, the membranes were blocked in 5% skimmed milk at room temperature for 1h, washed three times, and incubated in p-EGFR, EGFR, p-AKT, AKT, N-cadherin, vimentin, α -SMA primary antibodies solutions at 4°C overnight. After washing thrice, the membranes were combined with secondary antibodies solutions for 1h. At last, the membranes were immersed in a chemiluminescent solution and exposed by a G:Box Chemi-XR (Gene company limited, HK, China).

Immunofluorescence Staining After treatments, ARPE-19 cells were fixated by 4% PFA for 20min, permeabilized by 0.5% Triton X-100 solutions for 15min, blocked in 5% bovine serum albumin for 1h, incubated in primary antibodies solutions for 2h at room temperature. Then, cells were combined with fluorescent secondary antibodies for 1h. Finally, cells were sealed by antifade solution with DAPI and were detected by the fluorescence microscope (IX73, Olympus, Japan).

Statistical Analysis All data from at least three times independent experiments were presented as mean \pm standard error of mean (SEM). Statistical evaluation was performed by both Bonferroni test and Student's unpaired *t*-test provided by GraphPad Prism 8.0 software (GraphPad Software, La Jolla, CA, USA) to compare the various treatments.

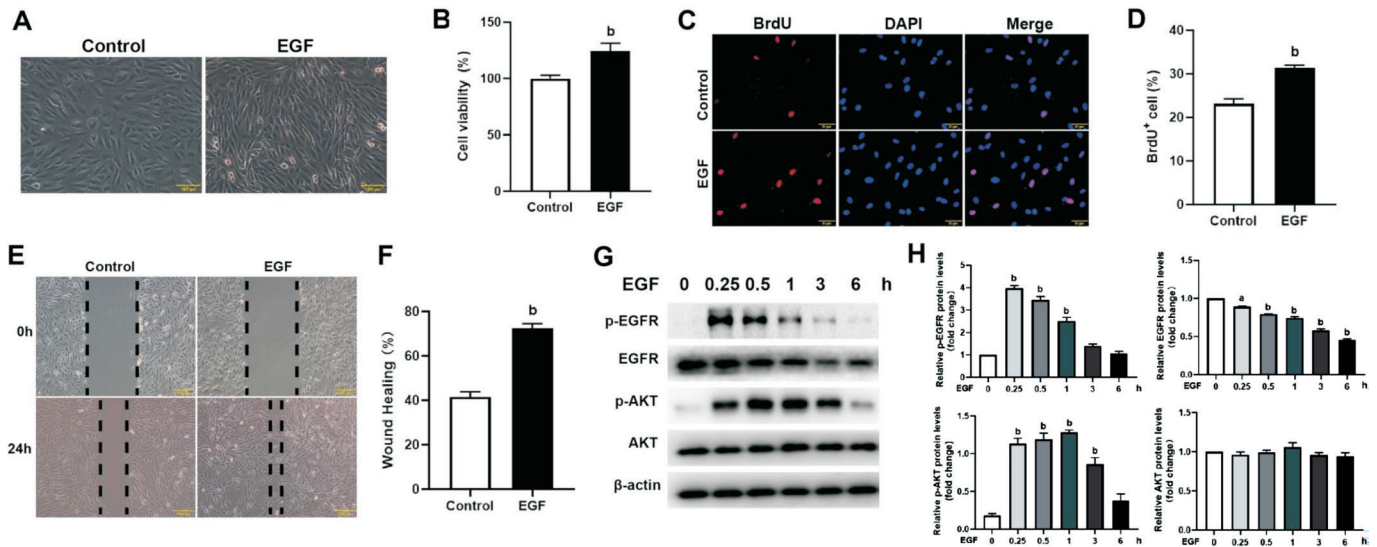


Figure 1 EGF induced activation of ARPE-19 cells via EGFR/AKT signaling pathway ARPE-19 cells were stimulated by 100 ng/mL of EGF for 24h. A: The images of cells morphology were obtained after treatment, scale bar: 100 μ m. B: Cellular viability was tested by the MTT assay, $n=6$. C, D: Cellular proliferation was analyzed by the BrdU labeling assay, scale bar: 50 μ m, $n=3$. E, F: Cellular migration was measured by the wound healing assay, scale bar: 200 μ m, $n=3$. G, H: After treatment with EGF (100 ng/mL) for 0, 0.25, 0.5, 1.0, 3.0, and 6.0h, EGFR, p-EGFR, AKT and p-AKT proteins in RPE cells were detected by Western blot assay. ^a $P<0.05$, ^b $P<0.01$. EGF: Epidermal growth factor; APRE-19: Adult retinalpigment epithelial cell line-19; EGFR: Epithelial growth factor receptor; AKT: Protein kinase B; MTT: Methyl thiazolyl tetrazolium; BrdU: 5-Bromodeoxyuridine; DAPI: Diamidino-2-phenylindole.

RESULTS

Activation of ARPE-19 Cells Induced by EGF Through EGFR/AKT Signaling Pathway

Our results indicated that 100 ng/mL EGF induced the ARPE-19 cell morphology transformed from pebble shape into fusiform-shaped (Figure 1A). After treatment with EGF for 24h, ARPE-19 cell viability increased by 24% (Figure 1B). BrdU staining assay showed the percentage of BrdU staining positive cells was 23% in the control group, 31% in the EGF treatment group. The BrdU staining positive cells in the EGF treatment group was up-regulated 8% compared to the control group (Figure 1C, 1D). After EGF treatment for 24h, the relative migration distance of cells was 44% in the control group, 69% in the EGF treated group (Figure 1E, 1F). Western blot results indicated that EGF induced phosphorylation of EGFR/AKT proteins in ARPE-19 cells. The phosphorylated EGFR increased and reached maximal at 15min, phosphorylated AKT reached the top at 60min. Total EGFR proteins reduced immediately after treatment and EGF did not influence total AKT expression (Figure 1G, 1H).

Activity of ARPE-19 Cells Suppressed by Erlotinib Through EGFR/AKT Pathway

Our data indicated that 20 and 50 μ mol/L erlotinib changed the ARPE-19 cells' morphology (Figure 2A). The MTT assay showed that treatment with 10, 20, and 50 μ mol/L of erlotinib for 24h induced obviously a down-regulation of cell viability (Figure 2B). BrdU staining assay indicated that 10, 20, and 50 μ mol/L of erlotinib treatment for 24h induced significantly a decrease

of BrdU staining positive cells (Figure 2C, 2D). Wound healing assay showed that 5, 10, 20, and 50 μ mol/L of erlotinib inhibited migration of ARPE-19 cells (Figure 2E, 2F). The result from Western blot assay revealed that erlotinib inhibited expression of total EGFR and AKT proteins after treatment for 24h (Figure 3A, 3B). Immunofluorescence staining assay indicated that 50 μ mol/L erlotinib reduced the expression of EGFR proteins and disrupted cell cytoskeleton in ARPE-19 cells (Figure 3C).

EGF-induced Activation of ARPE-19 Cells Inhibited by Erlotinib Through EGFR/AKT Signaling Pathway

EGF significantly promoted the capacity of proliferation, viability and migration in ARPE-19 cells. Increased of proliferation and migration were inhibited by pretreatment with 20 μ mol/L of erlotinib (Figure 4A-4E). Western blot results showed that pretreatment by erlotinib significantly suppressed p-EGFR and p-AKT proteins activated by EGF for 15min (Figure 4F, 4G). Additionally, the results from Western bolt showed that EGF-induced up-regulation of N-cadherin, α -SMA, and vimentin proteins were suppressed by pretreatment with erlotinib (Figure 4H, 4I).

EGF-induced Activation of ARPE-19 Cells Suppressed by EGFR Knockdown

Western blot results indicated that EGFR-siRNA obviously decrease the levels of EGFR/AKT proteins in ARPE-19 cells (Figure 5A, 5B). MTT results revealed that EGFR-knockdown down-regulate the viability induced by EGF (Figure 5C). The BrdU staining assay indicated that the percentage of positive staining of cells was 20%

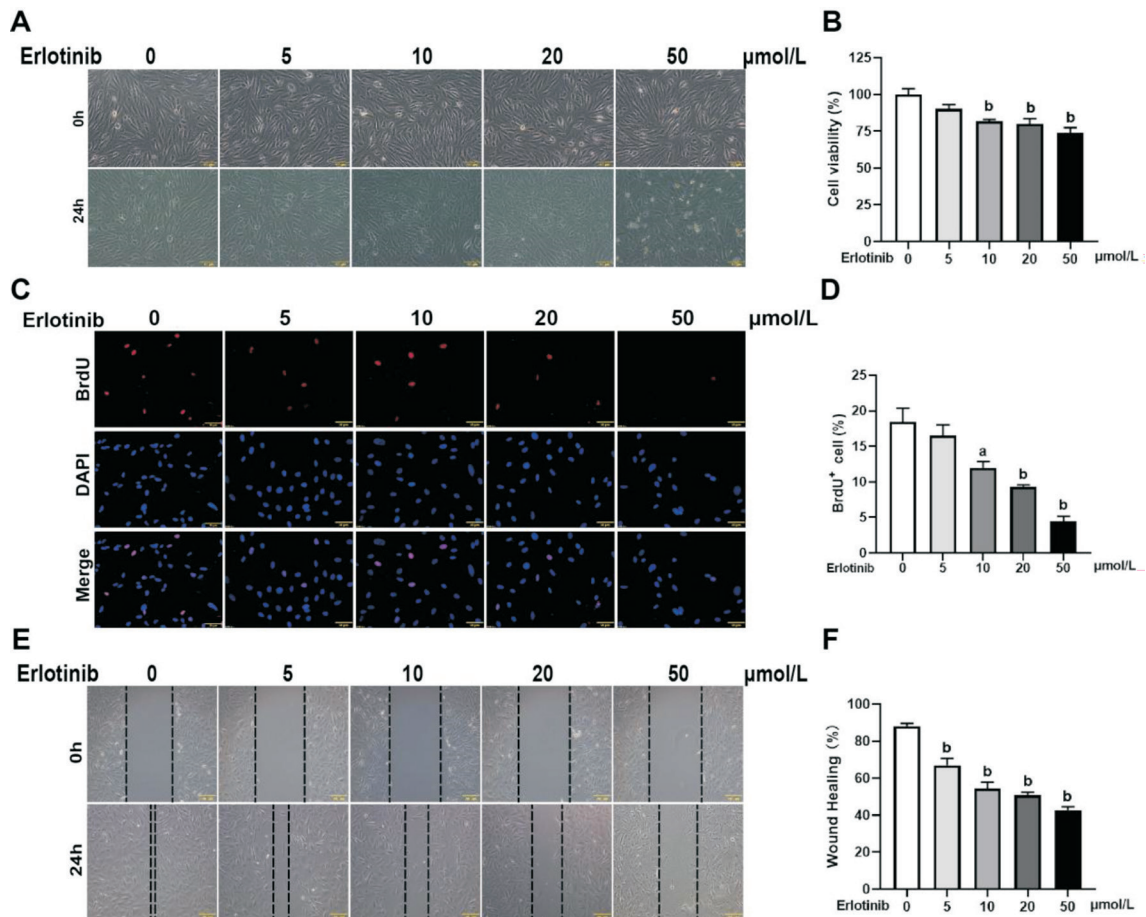


Figure 2 Erlotinib suppressed ARPE-19 cell activity A: The images of cells morphology were obtained after erlotinib treatment for 24h, scale bar: 100 μm. B: Cellular viability was tested by the MTT assay after erlotinib treatment for 24h. C, D: Cellular proliferation was measured by the BrdU labeling assay after erlotinib treatment for 24h, scale bar: 50 μm, $n=3$. E, F: Cellular migration was tested by the wound healing assay after erlotinib treatment for 24h, scale bar: 200 μm, $n=3$. ^a $P<0.05$, ^b $P<0.01$. MTT: Methyl thiazolyl tetrazolium; BrdU: 5-Bromodeoxyuridine; DAPI: Diamidino-2-phenylindole.

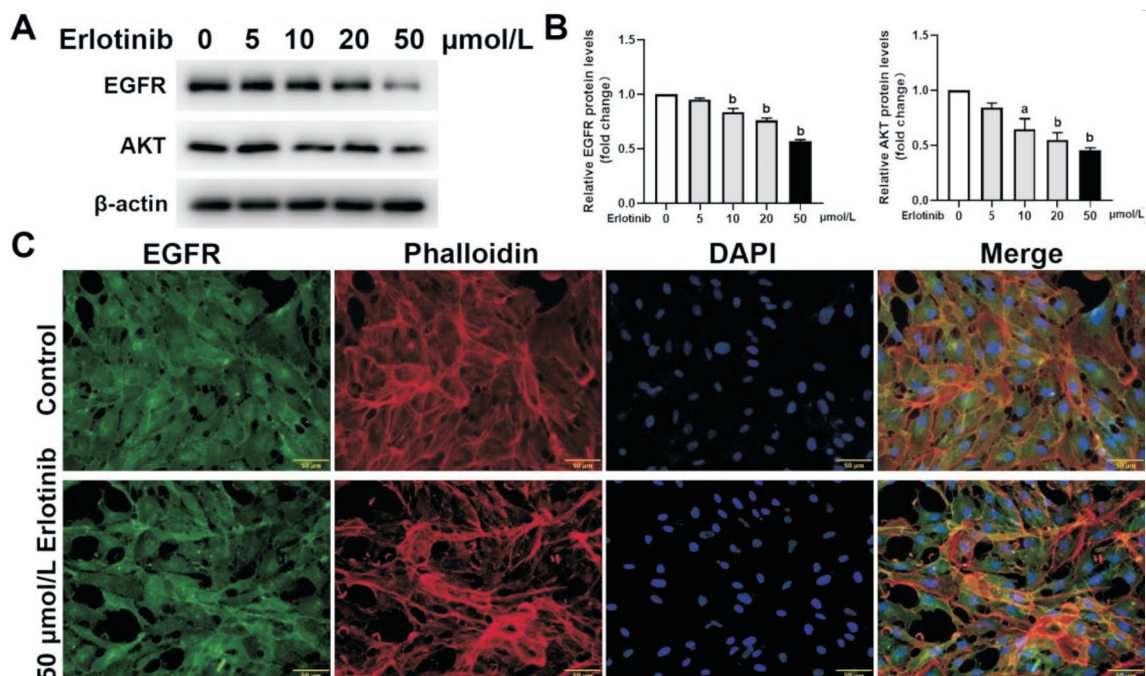


Figure 3 Erlotinib down-regulated the level of EGFR/AKT proteins in ARPE-19 cells A, B: ARPE-19 cells were treated with erlotinib (0, 5, 10, 20, and 50 μmol/L) for 24h. The expression of total EGFR and AKT proteins were measured by Western blot. ^a $P<0.05$, ^b $P<0.01$. C: After treatment with 50 μmol/L erlotinib for 24h, EGFR, F-actin, and nucleus in ARPE-19 cells were measured by immunofluorescence staining, scale bar: 50 μm. ARPE-19: Adult retinal pigment epithelial cell line-19; EGFR: Epidermal growth factor receptor; AKT: Protein kinase B; DAPI: Diamidino-2-phenylindole.

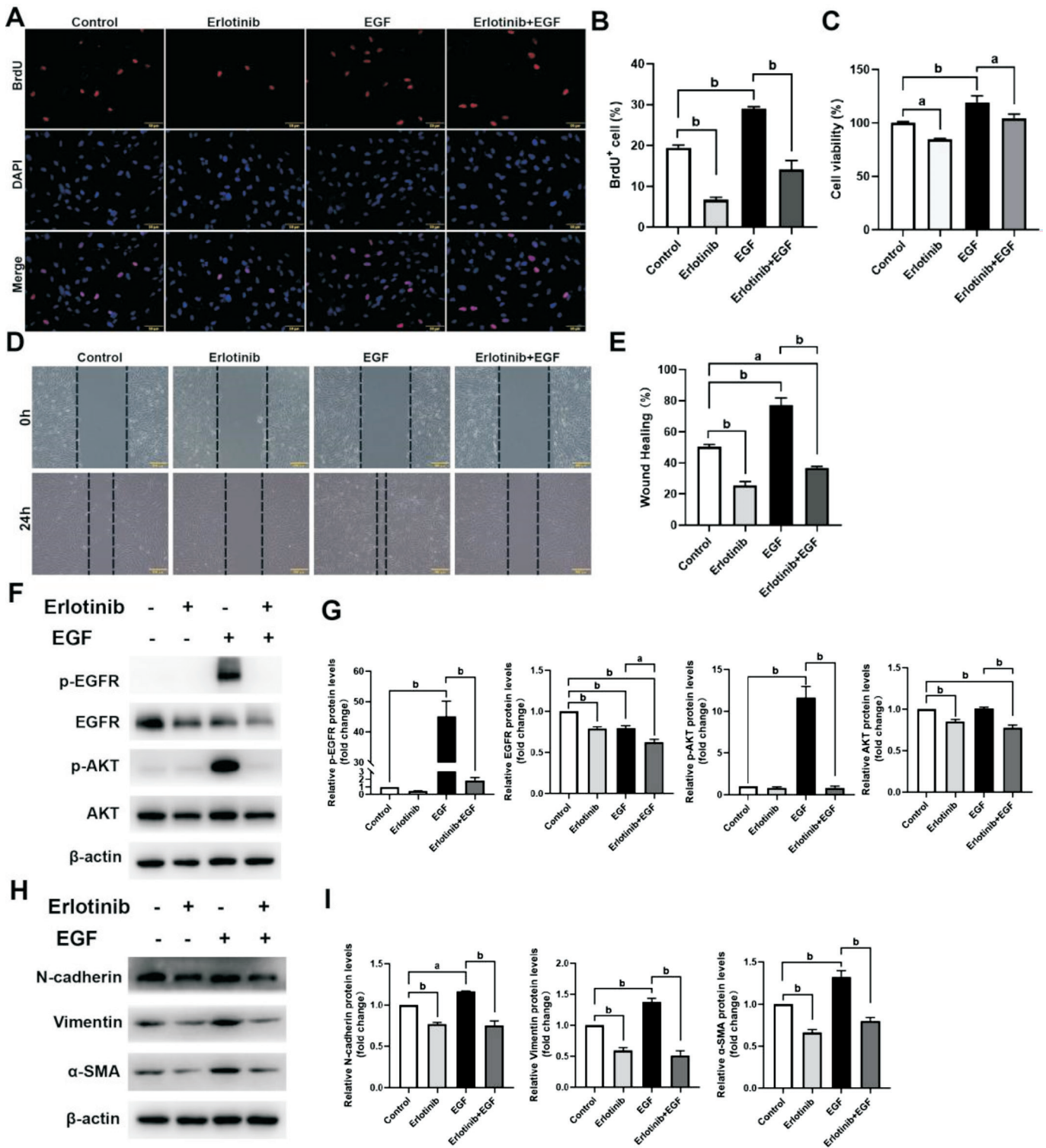


Figure 4 Erlotinib inhibited EGF-induced activation of ARPE-19 cells ARPE-19 cells were pretreated by erlotinib (20 μmol/L) for 12h and then stimulated with 100 ng/mL of EGF for 24h. A, B: BrdU staining assay was used to test cellular proliferation, scale bar: 50 μm, n=3. C: Cellular viability was sized by MTT assay, n=8. D, E: Cellular migration was tested by the wound healing assay, scale bar: 200 μm, n=3. F, G: ARPE-19 cells were pretreated with 20 μmol/L erlotinib for 12h and then treated with EGF (100 ng/mL) for 15min. Western blotting was used to detected EGFR/AKT signaling pathway proteins expressions. H, I: After pretreatment with 20 μmol/L erlotinib for 12h, ARPE-19 cells were treated with EGF (100 ng/mL) for 24h. N-cadherin, α-SMA and vimentin proteins were determined by Western blot. ^aP<0.05, ^bP<0.01. EGF: Epidermal growth factor; MTT: Methyl thiazolyl tetrazolium; BrdU: 5-Bromodeoxyuridinc; APRE-19: Adult retinal pigment epithelial cell line-19; EGFR: Epidermal growth factor receptor; AKT: Protein kinase B; α-SMA: α-smooth muscle actin; DAPI: Diamidino-2-phenylindole.

in the control group, 9% in the EGFR-siRNA knockdown group, 27% in the EGF treatment group, and 13% in the

EGFR-siRNA knockdown combined with EGF treatment group (Figure 5D, 5E). Similarly, wound healing assay also

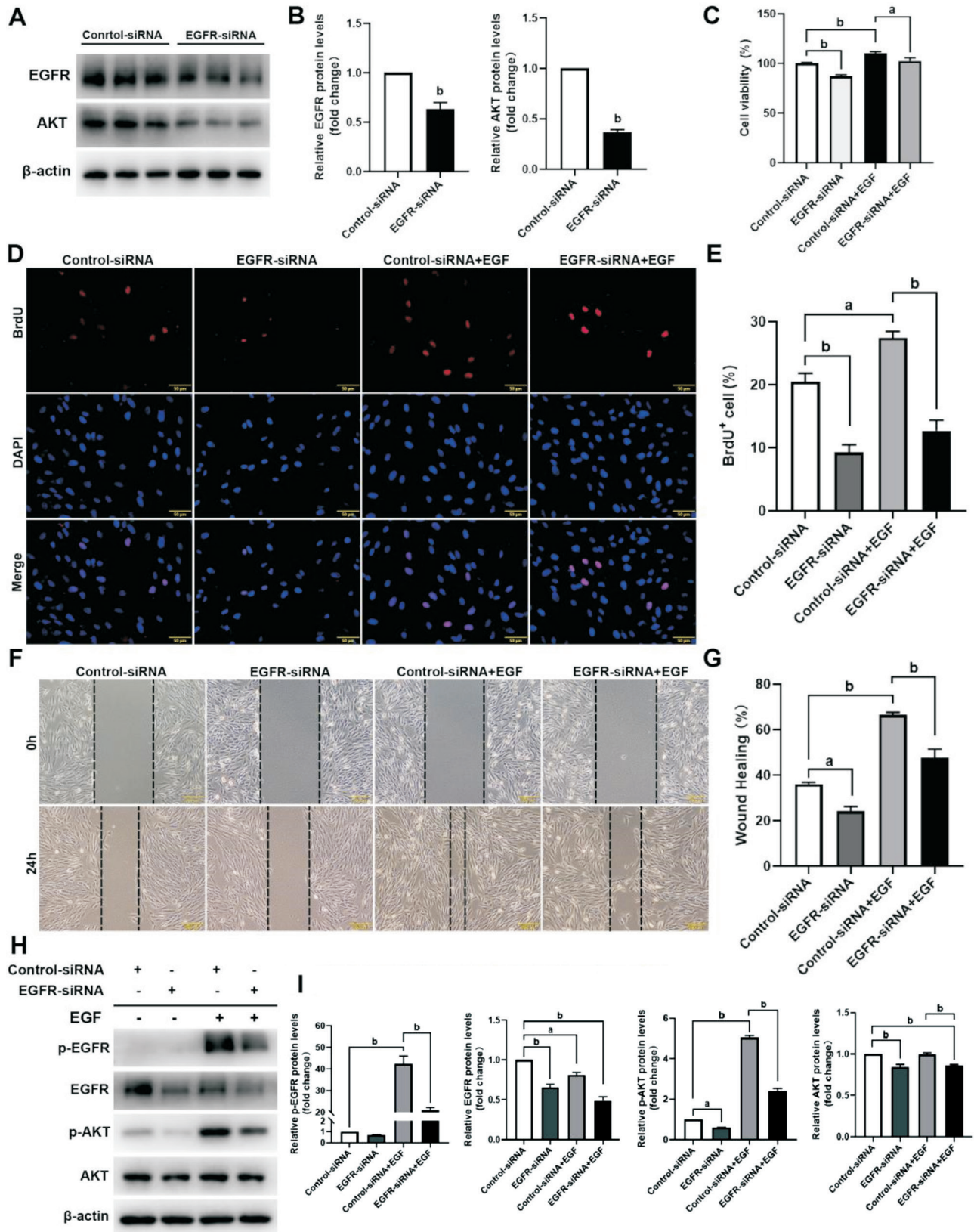


Figure 5 EGFR knockdown suppressed EGF-induced activation of ARPE-19 cells. A, B: After EGFR-siRNA or control-siRNA were transfected into ARPE-19 cells, EGFR and AKT proteins were tested and analyzed by Western blotting. After EGFR-siRNA transfection, ARPE-19 cells were treated without or with 100 ng/mL EGF for 24h. C: Cellular viability was tested by MTT assay, $n=8$. D, E: Cellular proliferation was tested by the BrdU labeling assay, scale bar: 50 μm , $n=3$. F, G: Cellular migration was detected by the wound healing assay, scale bar: 200 μm , $n=3$. H, I: After transfected by control-siRNA or EGFR-siRNA, ARPE-19 cells were stimulated without or with 100 ng/mL of EGF for 15min. EGFR, p-EGFR, AKT and p-AKT protein were detected by Western blot. ^a $P<0.05$, ^b $P<0.01$. siRNA: Small interfering RNA; EGF: Epidermal growth factor; EGFR: Epidermal growth factor receptor; AKT: Protein kinase B; APRE-19: Adult retinal pigment epithelial cell line-19; MTT: Methyl thiazolyl tetrazolium; BrdU: 5-Bromodeoxyuridinc; DAPI: Diamidino-2-phenylindole.

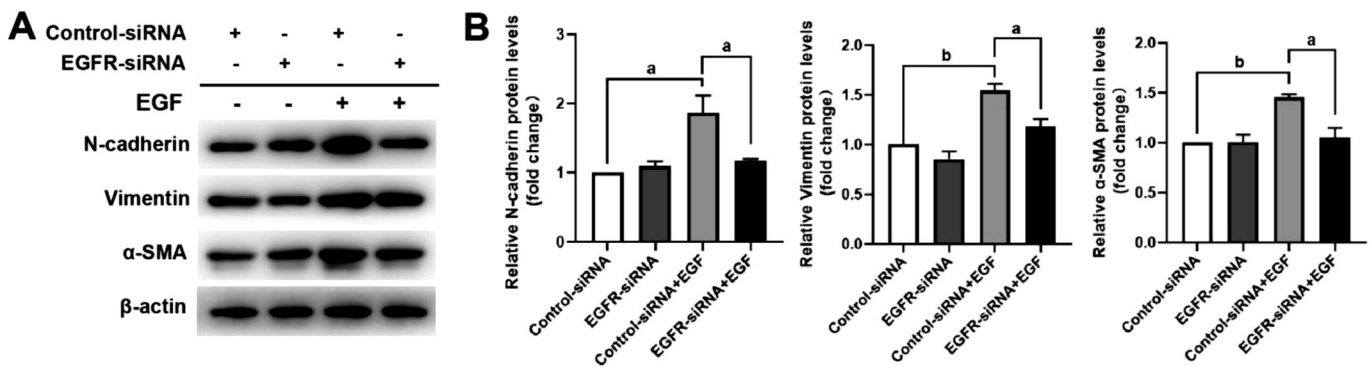


Figure 6 EGFR knockdown suppressed EGF-induced up-regulation of EMT-related proteins in ARPE-19 cells. A, B: After EGFR-siRNA transfections, ARPE-19 cells were treated without or with 100 ng/mL of EGF for 24h. N-cadherin, α -SMA and vimentin proteins were detected by Western blot. ^a $P < 0.05$, ^b $P < 0.01$. EGF: Epidermal growth factor; EGFR: Epidermal growth factor receptor; EMT: Epithelial-mesenchymal transition; APRE-19: Adult retinal pigment epithelial cell line-19; siRNA: Small interfering RNA; α -SMA: α -smooth muscle actin.

indicated the migration caused by EGF was also suppressed by EGFR knockdown (Figure 5F, 5G). The data from Western blotting indicated that EGF treatment enhanced the p-EGFR and p-AKT expressions, which were reduced by EGFR knockdown (Figure 5H, 5I). In addition, EGF-induced increase of N-cadherin, α -SMA and vimentin proteins were attenuated by EGFR knockdown (Figure 6A, 6B).

DISCUSSION

Abnormal activation and EMT of RPE cells is an important pathological feature of PVR^[26-27]. When the retina is destroyed or intraocular homeostasis is abnormal, RPE cells transdifferentiate from epithelial cells to fibroblast phenotypic cells, migrate to retinal surface or subretinal space, and form fibrous proliferation membranes^[28]. Recent studies have indicated that multiple growth factors or cytokines were proved to play roles in the progression of PVR^[10]. Therefore, exploring the inducing factor and molecular mechanism of RPE activation might be an interesting topic in the research of PVR.

EGFR on the cell membrane, a multifunctional glycoprotein can promote cell proliferation and migration^[29]. Some scholars pointed out that EGFR was closely related to fibrosis in various diseases^[19,30-32]. It has been confirmed that EGFR has two downstream signaling pathways: EGFR/mitogen-activated protein kinase (MAPK) pathway and EGFR/AKT/mechanistic target of rapamycin (mTOR) pathway^[33]. Recently, AKT has been reported to be closely linked to various fibrosis^[34-36]. AKT protein is also involved in the EMT of RPE cells^[37]. Some data have indicated that EGF contributed to the progression of PVR^[38]. Our previous study showed that EGFR/AKT plays a vital part in RPE cell survival^[39]. In the present study, when we used EGF to induce activation of ARPE-19 cells, EGF changed cell morphology, and enhanced cell viability, proliferation, and migration capacity. Meanwhile, EGF activated phosphorylation of EGFR/AKT signaling pathway. Some early studies have

shown that EGF could be found in 53% of intravitreal cells and 69% of subretinal fluid cells from patients with PVR^[40], and EGFR exists in the early stage of PVR^[41]. A recent study has shown the production of EGFR increased in the retinas of the PVR mouse model at 2 and 4 weeks^[24]. Taken together, these studies suggested that EGF might be a significant inducer of activation of RPE cells, and EGFR/AKT proteins might be potential targets for treating abnormal activation of RPE cells in PVR.

Erlotinib, a well-known EGFR inhibitor, was widely used to suppress the expression of EGFR and was reported to attenuate fibrotic process in various cells^[24,42-43]. In previous studies, we have found that erlotinib inhibited the activation of EGFR/AKT signaling pathway^[39]. Erlotinib was also proved to inhibit cell proliferation^[44]. In the present study, our data indicated that erlotinib significantly affected cell viability, proliferation, and migration. The data from Western blot revealed that erlotinib suppressed the total expression levels of EGFR and AKT proteins. Furthermore, erlotinib attenuated an EGF-induced increase of cell viability, proliferation and migration through blocking EGFR/AKT phosphorylation. Some researchers reported EGFR-siRNA and specific inhibitors induced the silence and inactivation of EGFR and inhibited the activation of AKT pathway^[45]. Similarly, our data indicated that EGFR-siRNA not only suppressed the expression of EGFR and AKT proteins. In addition, we found that EGFR-siRNA transfection significantly blocked the EGF-activated phosphorylation of EGFR/AKT signaling pathway. These data suggested that EGFR inhibition might be a novel idea for preventing abnormal activation of RPE cells.

N-cadherin, α -SMA, and vimentin, as EMT-related markers, were well-known fibrosis-related proteins in RPE cells^[46-48]. N-cadherin, a sign of ongoing EMT, is related to the process of some fibrosis disease and carcinoma^[49]. The increased α -SMA protein, a famous and vital EMT marker, has been known as

the key phenotype of RPE cell transdifferentiation^[50]. Vimentin is closely related to stabilize the structure of migrating cells^[51]. Some studies have reported that erlotinib can be used as a treatment for fibrotic diseases^[52-53]. Our present data showed that erlotinib pretreatment inhibited EGF-induced an increase of N-cadherin, vimentin and α -SMA proteins. Additionally, we explore the effect of EGFR knockdown on the RPE cells. Our results indicated that EGFR knockdown not only suppressed RPE cells' viability, proliferation and migration through down-regulating expression EGFR/AKT proteins, but also inhibited EGF-induced activation of EGFR/AKT signaling pathway and an increase of N-cadherin, α -SMA and vimentin proteins. These results suggested that EGFR inhibition might be a prospective approach for prevention of PVR. Certainly, it is still not entirely clear whether this function of EGFR inhibition is valid *in vivo*. Therefore, it is essential to determine the specific mechanism of EGFR through carrying out some animal models of PVR.

In conclusion, EGFR inhibitions suppressed EGF-induced activation in RPE cell *via* EGFR/AKT signaling pathway, which might supply a novel theoretical basis for preventing and treating abnormal activation of RPE cells in PVR.

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