·Basic Research·

PI3K-mediated glioprotective effect of epidermal growth factor under oxidative stress conditions

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Abstract

• **AIM: To determine the effects of epidermal growth factor (EGF) on the proliferation and migration of Müller cell line Moorfields/Institute of Ophthalmology-Müller 1 (MIO-M1), and its related molecular mechanisms under normal and oxidative stress conditions.**

• **METHODS: Müller cells were cultured with different concentrations of EGF in the presence or absence of varied amounts of H_2O_2 and glucose oxidase (GO) which induced oxidative stress. The proliferation and migration of Müller cells were examined by 5-Bromo-2-deoxyUridine (BrdU), MTT assay, Transwell assay and scratch wound healing assays. The cell viability was determined with the MTT assay. The secretion of EGF by Müller cells was evaluated by ELISA. Western blot was performed to detect the activation of extracellular regulated protein kinases (ERK)1/2 and Akt signal pathways.**

• **RESULTS: EGF stimulated the proliferation and migration of Müller cells in a concentration-dependent manner *in vitro* Under oxidative damage condition, 2h of pretreatment with 10-100 ng/mL EGF can mostly inhibit 50% lethal dose of 0.08 mmol/L H_2O_2-induced cell damage. The Western blot results showed that after Müller cells were exposed to varying EGF for 24h, Akt and ERK1/2 were phosphorylated in a dose-dependent manner. In the presence of the LY294002, the potent PI3K inhibitor, the p-Akt was significantly attenuated.**

• **CONCLUSION: EGF may induce the proliferation and migration of human Müller cells through the Akt and the ERK1/2 signal pathways, and induce PI3K-mediated glioprotective effect under oxidative stress.**

• **KEYWORDS:** epidermal growth factor; human Müller cells; PI3K
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INTRODUCTION

The Müller cells are the main glial cells in the retina which span the entire thickness of the retina and wrap all retinal neurons and processes in which play a great many of crucial roles [1]. They coordinate with retinal capillary endothelial cells to form inner blood-retinal barrier and to secrete several growth factors and other tropic factors to play an important role in the homeostatic regulation of the microenvironment in the retina[1,2]. And it plays a crucial role in many pathogenesis of proliferative retinopathies, *e.g.* proliferative diabetic retinopathy, proliferative vitreoretinopathy and epiretinal membrane [3-5]. The cellular processes and mechanisms of Müller cells under hypoxic condition involve in those diseases have got great attention these years. However, the changes of Müller cells under oxidative stress, have a little understanding. Müller cell plays an important role in surviving neurons and transmitting excitation. Oxidative stress occurred in diabetes mellitus is involved in dysfunction and structural abnormalities of Müller cell [6]. The extravasated, modified lipoproteins may be implicated in apoptotic Müller cell death, acting at least partially *via* enhanced levels of oxidative and endoplasmic reticulum (ER) stresses[7]. Oxidative stress is also thought to induce an antioxidant response with cooperation between the mitochondria, which made mitochondria are vulnerable to oxidative stress despite a protective protein during early phases of aging-related disease [8,9]. Previous investigators have proposed that the mechanisms of oxidative stress damage and inflammatory and immune responses have important roles in the development of age-related macular degeneration (AMD) [10-15]. The apoptosis of the retinal pigment epithelium (RPE) due to oxidative lesions in the development of AMD has been the hot issue [16-18]. H_2O_2-induced cell lesions in human ocular tissues have been

demonstrated, the non-radical forming of reactive oxygen species of H_2O_2 can easily penetrate the cell membrane and damage cells in the electron transport chain, which stimulate the process of programmed cell death, apoptosis [19-21]. Müller cells are also an important component of the blood-retinal barrier. They have a critical role in maintaining the inner blood-retinal barrier (iBRB) function which include support for neuronal activity and maintaining the homeostasis of the retina [20]. There have been several reports released the ultrastructural and physiological transformation of Müller cells in AMD patients and animal models[22-26].

Several studies reported that epidermal growth factor (EGF) can induce the proliferation and migration of animal Müller cells [27-29]. The effect of EGF on human Müller cells and whether EGF protect Müller cells from oxidant injury by a model oxidant, H_2O_2 and glucose oxidase (GO) which generates very low concentrations of H_2O_2 in the presence of glucose, and the associate mechanisms have not been well described [30,31]. In this study, we observed the human Müller cell proliferation and migration under optimal concentration EGF, and detected mechanisms of the activation of signal transduction pathway under normal and oxidative stress conditions *in vitro*.

SUBJECTS AND METHODS

Subjects The spontaneously immortalized human cell line Moorfields/Institute of Ophthalmology-Müller 1 (MIO-M1) from Institute of Ophthalmology and Moorfields Eye Hospital, London. Human EGF, MTT, 5-Bromo-2-deoxyuridine (BrdU), trypsin, Dulbecco's modified essential medium (DMEM), and goat anti-rabbit secondary antibody were purchased from Sigma Chemical (St. Louis, MO, USA). Antibodies to Akt, phosphor-Akt, phosphorylated extracellular regulated protein kinases (ERK), ERK1/2, and LY294002, were obtained from Cell Signaling Technology (Beverly, MA, USA). Human EGF ELISA kit was purchased from R&D Systems (Minneapolis, Minnesota, USA). Transwell chambers were obtained from Costar, Corning, Inc. (Corning, NY, USA).

Human Müller Cell Culture The spontaneously immortalized human cell line MIO-M1 from Institute of Ophthalmology and Moorfields Eye Hospital, London, were grown in high glucose Dulbecco's modified Eagle's medium, and containing 100 μg/mL each of penicillin and streptomycin at 37℃ in a humidified environment containing 5% CO_2 to confluence[32]. When the cells became confluent, they were detached using a 0.05% trypsin.

Proliferation Assay

BrdU labeling and detection assay Cells were seeded in 24-well plates at a density of 5.0×10^4/well and were grown in DMEM (Sigma, St. Louis. Mo., USA) containing 10% fetal bovine serum (FBS) in a humidified environment containing 5% CO_2 at 37℃. Twenty-four hours later, cells were starved in serum-free DMEM for 12h and then were incubated with serum-free DMEM containing varying dosages of EGF (0, 1, 10, 30, 100 ng/mL), and the medium only with serum-free was left as control. The cells were incubated for 24h before evaluation. BrdU was added to the cells and they were re-incubated for 4h. After the culture medium was removed, the cells were fixed and added anti-BrdU I and anti-BrdU II and following the manufacturer's protocol.

Colorimetric assay for cellular growth and survival (MTT assay) The Müller cells were seeded into 96-well plates at a density of 1.0×10^4/well. After incubation at 37℃ for 24h, the medium was removed from each well and the cells were incubated with serum-free DMEM with varying dosages of EGF, H_2O_2, and GO. It was divided into six groups: 1) varying dosages of EGF (0, 1, 10, 30, 100 ng/mL), and the medium only with serum-free was left as control，was added to each well and cultured in an incubator that contained 5% CO_2 at 37℃ for 24-, 48-, 72-h periods; 2) varying concentration of H_2O_2 (0, 0.01, 0.02, 0.04, 0.06, 0.08, 0.1, 0.2, 0.4 mmol/L), was added to each well and cultured in an incubator that contained 5% CO_2 at 37℃ for 12-, 24-h periods; 3) different dosages of EGF was added to each well before 2h median lethal dose H_2O_2 was added; 4) varying doses of GO (0, 0.5, 1, 5, 6, 7, 8 ,9, 10 mU/mL), was added to each well and cultured in an incubator that contained 5% CO_2 at 37℃ for 12-, 24-h periods; 5) different dosages of EGF was added to each well before 2h median lethal dose GO was added; 6)and different dosages of EGF and LY294002 were added before 2h median lethal dose H_2O_2 was added, then 20 μL of tetrazolium salt MTT was added to each well and the cells were incubated for 4h, then dimethyl sulfoxide was added to each well, the plates were then agitated on a plate shaker and the optical density (OD) was read. OD measurements were carried out with an enzyme-linked immunosorbent assay reader at 570 nm with dimethyl sulfoxide as the blank. The proliferation rate of cells was calculated by OD.

Migration Assay

***In vitro* wound assay** Müller cells were plated at a density of 1.5×10^5/well in 12-well plates. Cells were incubated in DMEM containing 10% FBS for 24h to confluence and then starved in serum-free DMEM for 12h. The confluent monolayer of each well was scratched with a 200 μL pipette tip to create a wound. The Müller cells were then incubated with serum-free DMEM or DMEM containing 2% FBS supplemented with different concentrations of EGF, respectively. The cells were cultured in an incubator that contained 5% CO_2 at 37℃ for 12, 24, 48, and 72h periods. Detection of cell migration trends by measuring the migration distance.

Transwell assay Müller cells were plated at 2.0×10^5/well in

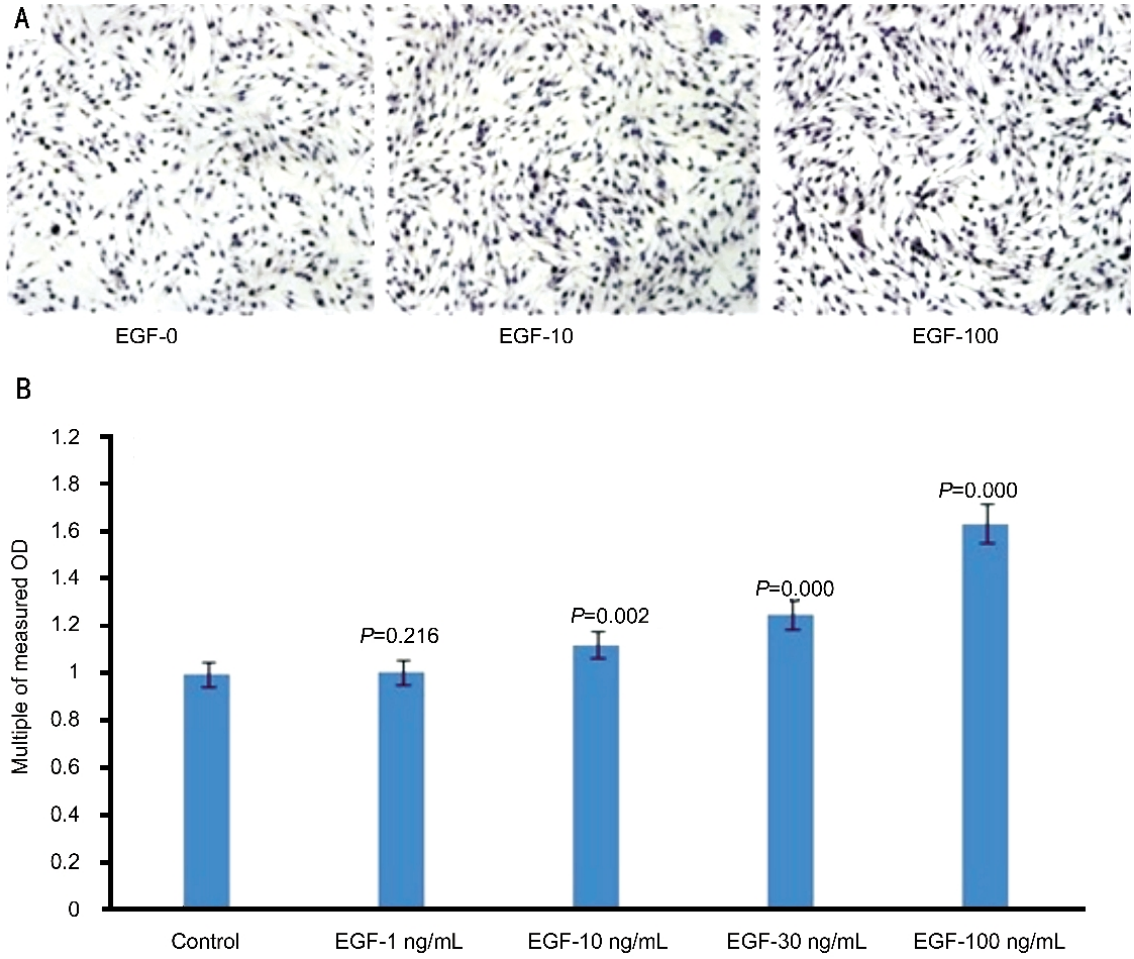

Figure 1 Effect of EGF on cultured human Müller cell proliferation A: Photo-micrographs show the proliferation effect of EGF on human Müller cells (BrdU); B: After incubated with EGF (1-100 ng/mL) 48h later, the cell viability was assessed using MTT assay. The proliferation of Müller cells in a concentration-dependent manner. P<0.01 compared to control group (One-way ANOVA).

8 μm transwell plates (Corning Inc., Corning, NY, USA) with serum-free DMEM containing varying dosages of EGF. After incubation for 24h, cells were fixed in polyoxymethylene solution and stained with hematoxylin. The upper layer cells were scratched with a 100 μL pipette tip, and 5 randomly selected visual fields were photographed and the number of cells was counted.

Enzyme –linked immunosorbent assay Cells were cultured at 5.0×10^5/well in 6 well-plates (100 μL culture medium per well) for 24h. Subsequently, the medium was removed and the cells were stimulated with median and 80% lethal dose of H_2O_2 and GO respectively. Supernatants were collected after 24h or 48h and the levels of EGF in the cultured media were determined by ELISA (R&D Systems).

Western Blot Following incubations under oxidative stress conditions, the medium was removed from each well and cells were washed twice in phosphate buffer saline twice. Each well was added 150 μL 1×SDS-loading buffer protein lysates. And the protein was collected to EP tube stored at -20℃. For immunoblotting, protein samples were electrophoresed on polyacrylamide gel and transferred to nitrocellulose. Blots were blocked with either 5% non-fat dry milk in 1% bovine serum albumin (BSA) in Tris-buffered saline (TBS) for probing with anti-ERK and anti-phospho-Akt antibodies or 5% BSA in butylene succinate-co-butylene terephthalate (PBST) for probing with anti-phosphotyrosine antibodies. Akt, p-Akt, ERK, and p-ERK were detected using 1:1000 dilutions of the respective anti-Akt and anti-ERK antibodies. After extensive washes in PBST, blots were probed with peroxidase-conjugated goat anti-mouse or goat anti-rabbit IgG (1:2000 dilution) and developed using enhanced chemiluminescence (ECL).

Statistical Analysis To determine whether the differences in groups of Müller cells were statistically significant, we performed one-way analysis of variance (MTT and Transwell assay). P <0.05 was considered statistically significant. Each test was done in triplicate.

RESULTS

Epidermal Growth Factor Stimulation of Cultured Human Müller Cell Proliferation Under normal culture conditions, EGF stimulated the proliferation of cultured human Müller cells in a concentration-dependent manner *in vitro* (Figure 1),10 ng/mL of EGF began to stimulate Müller cell proliferation (P<0.001) and the maximal proliferation

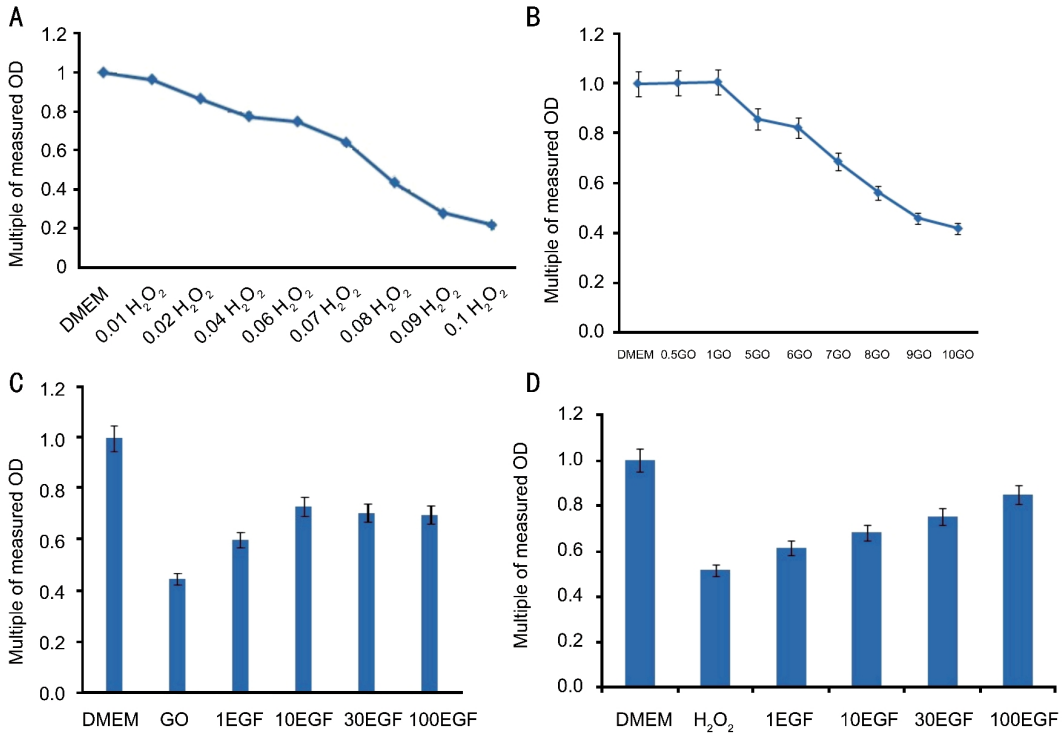

Figure 2 EGF prevented the decrease in Müller cells viability induced by oxidative damage A: Concentration-dependent decrease in Müller cell viability with increasing H_2O_2 after 24h of incubation. $P<0.05$ compared to control; B: Concentration-dependent decrease in Müller cells viability with increasing GO after 24h of incubation. $P<0.05$ compared to control; C: Inhibition of H_2O_2-induced apoptosis of Müller cell by EGF. $P<0.05$ compared to H_2O_2; D: Inhibition of GO-induced decrease in cell viability by EGF in Müller cells. $P<0.05$ compared to GO.

rate of the Müller cells was 160% at 100 ng/mL of EGF.

The result of the Müller cell viability shows that either the H_2O_2 or GO induced the cell damage in a concentration-dependent manner after 24h of incubation (Figure 2A, B). However, when varying concentrations of EGF were added, EGF prevented the decrease in Müller cells viability caused by oxidative damage. 10-100 ng/mL EGF notably induced protection of Müller cells against oxidative lesions by GO and H_2O_2 (Figure 2C, D).

Effects of Epidermal Growth Factor on Müller Cell Migration The maximal migration rate of Müller cells migration was at 10 ng/mL of EGF (*in vitro* wound assay), the results are consistent with the Transwell assay (Figures 3, 4). The migration rate under different culture conditions (Transwell assay) are as follows: control (1.000 ±0.172), EGF-1 ng/mL (3.211±0.439), EGF-10 ng/mL (4.500±0.630), EGF-30 ng/mL (2.105 ±0.208), EGF-100 ng/mL (0.768 ± 0.171).

Result of Enzyme-Linked Immunosorbent Assay After the cells were stimulated with median and 80% lethal dose of H_2O_2 and GO respectively, the result of the OD read under different culture conditions are as follows: control (0.076± 0.023), H_2O_2-0.08 nmol/L (0.061 ±0.003), H_2O_2-0.4 nmol/L (0.073±0.008), GO-6 mU/mL (0.052±0.004), GO-10 mU/mL (0.064 ±0.007). The result of ELISA did not indicate the human Müller cells secrete EGF, and the decrease is likely caused by the decrease of the cell viability.

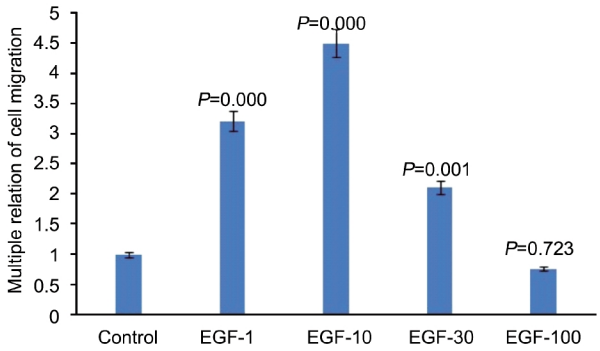

Figure 3 The migration effect of different concentrations of EGF on Müller cells after incubation for 24h (Transwell assay) The number of cells that had migrated through the pores was quantified by counting five independent visual fields using a 20× microscope objective. Three independent assays were performed. $P<0.01$ (One-way ANOVA).

Epidermal Growth Factor-induced Protein Kinase B (Akt) and Extracellular Signal-regulated Kinase (ERK1/2) Phosphorylation When Müller cells were exposed to varying EGF for 24h, Akt and ERK1/2 were phosphorylated in a dose-dependent manner (Figure 5). In contrast, total Akt and ERK1/2 levels were not greatly affected by any of the tested concentrations. GAPDH was not affected by EGF treatment.

ERK1/2 and Akt signaling pathways are involved in Müller cell proliferation induced by EGF and cell survival under oxidative stress.

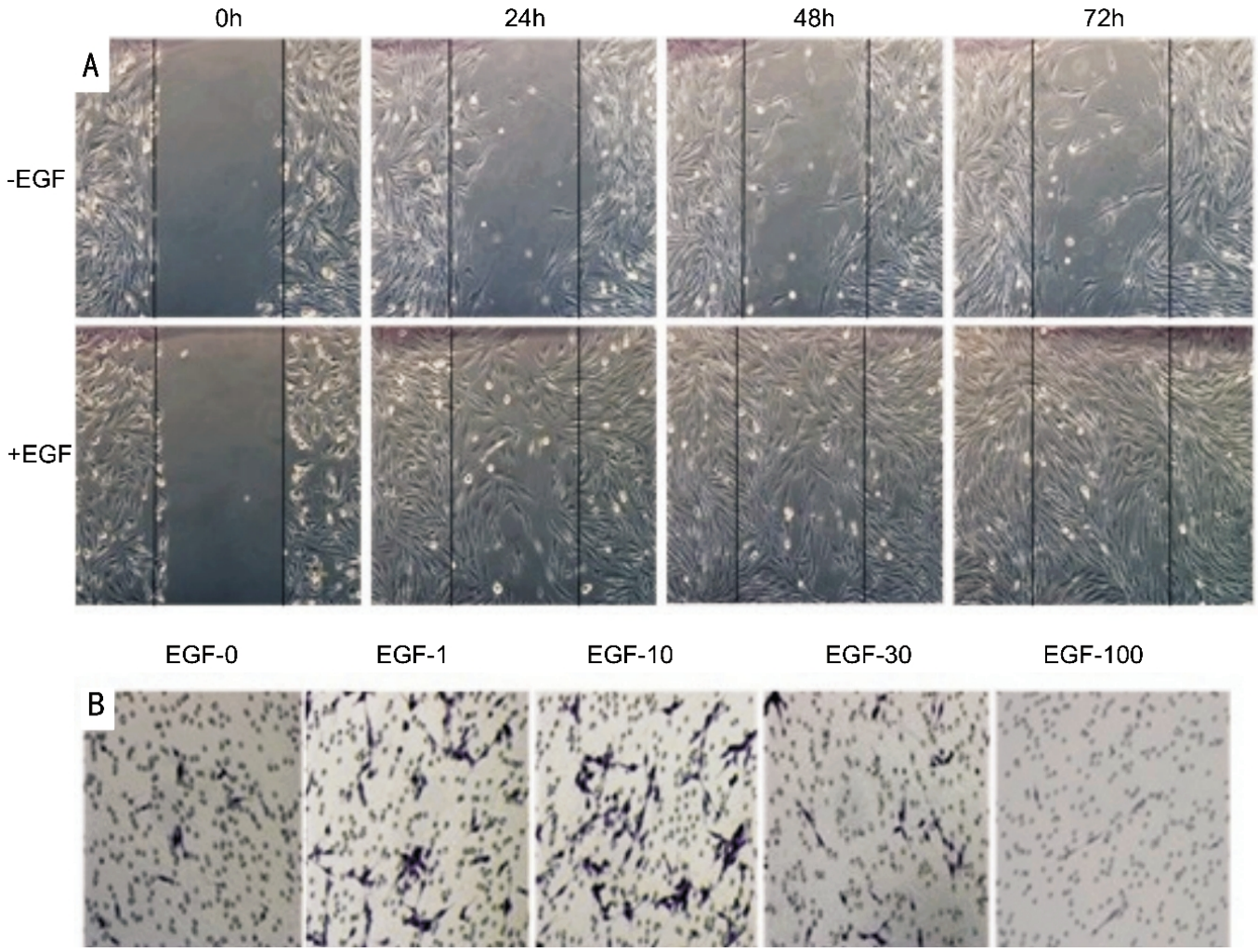

Figure 4 Effect of EGF on the migration of human Müller cells A: *In vitro* wound-healing assay of Müller cell. Scratches were made in the monolayer, and the cells were cultured for 0, 24, 48 or 72h in DMEM without serum (upper pictures) or in DMEM containing 10 ng/mL EGF (lower pictures). The scratched regions were photographed under a phase-contrast microscope (×100). Representative areas from triplicate experiments are presented; B: Transwell migration assay of human Müller cells. Cell morphology was observed by staining with hematoxylin and eosin. The Transwell assay and wound healing assay show the maximal migration rate of Müller cells migration was at 10 ng/mL of EGF.

Akt Phosphorylation Under Different Conditions PI3K activation induced by EGF plays an important role in many cell systems [33]. When PI3K is inhibited, Akt activation is blocked in a great many cells [34,35]. LY294002, a potent inhibitor of the catalytic subunit of PI3K, was used to evaluate whether PI3K signaling pathway was involved in EGF-stimulated Müller cell survival[36].

Under oxidative stress culture conditions, 2h in advance median lethal dose H_2O_2 concentration of 0.08 mmol/L addition, varying dosages of EGF and LY294002 were added. After 24h of incubation, the protection effect induced by EGF was weakened (Figure 6A), there was a significant difference compared to adding EGF alone. As shown in Figure 6B, under oxidative stress condition and in the presence of EGF, p-Akt was enhanced. In the presence of the inhibitor, the p-Akt was significantly attenuated (LY294002+EGF *vs* EGF). The similar result was obtained between EGF+H_2O_2 and EGF+H_2O_2+LY294002.

DISCUSSION

Most research regarding Müller cells was expanded on animal glial cells. In this study, we chose the cell line MIO-M1 that retains the characteristics of primary isolated cells in culture. This human cell line indicates that it expresses Müller cell markers like CRALBP, EGF-R, glutamine synthetase, and α-SMA and the phenotypic features and electrogenic response to L-glutamate of this cell line paralleled those of freshly isolated cells [33,37]. The use of the immortalized Müller cell lines eliminated the disadvantages of cells obtained from post-mortem human or rodent retina.

We investigated the effects of EGF on the human Müller cells cultured in normal and model oxidants, H_2O_2 or GO which generates very low concentrations of H_2O_2 in the presence of glucose[30,31].

Müller cells express epidermal growth factor receptor itself, and our data indicates that EGF had proliferation and

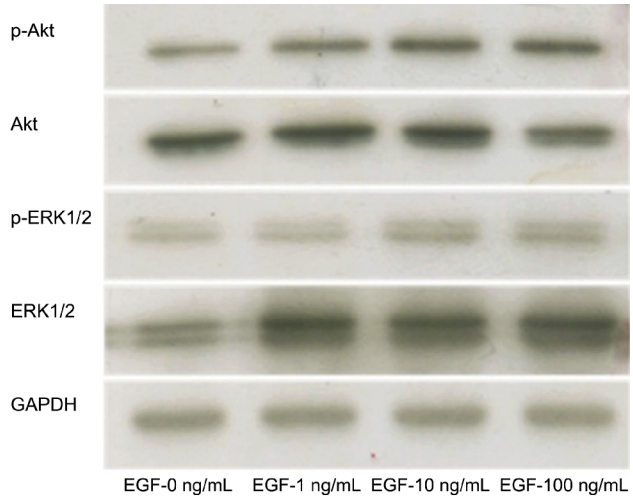

Figure 5 EGF–induced Akt and ERK1/2 phosphorylation in a concentration–dependent manner. Müller cells were treated with medium alone or EGF at different doses for 24h Cytoplasmic proteins were separated by SDS-PAGE and transferred to a nitrocellulose membrane for Western blot analysis. Western blot probed with antibody to p-Akt and p-ERK1/2. GAPDH was not affected by EGF treatment. p-Akt and p-ERK1/2 antibodies were rarely detectable in the absence of EGF, but a series labeled bands appeared aggressively after incubated with EGF.

migration effects on Müller cells [33,38,39]. Previous research reported EGF has protection against ozone-induced airway epithelial cell injury [40]. Under normal culture conditions, EGF stimulated the proliferation of cultured human Müller cells in a concentration-dependent manner *in vitro*, 10 ng/mL of EGF began to stimulate Müller cell proliferation (P<0.001) and the maximal proliferation rate of the Müller cells was 160% at 100 ng/mL of EGF. EGF has a potent, stable cell proliferation effect and has been a research focus in recent years[41]. Emmanuelle Meuillet *et al* [28] reported that EGF can induce the proliferation and migration of animal Müller cells [21]. Cao *et al* [42] by comparing a variety of growth factors that promote proliferation of RPE cells, found that the strongest stimulator was EGF. Our data is accordance with the effect of EGF on the Müller cells of guinea pig and other growth factor on human Müller cells [30,43]. Yan *et al*[44] reported that EGF on cultured human RPE cells in a dose-dependent proliferation and migration. This effect may similar to a hypoxic condition, like retinal detachment or proliferative diabetic retinopathy, activates the Müller cells to proliferation and migration.

Under oxidative stress culture conditions, 2h before median lethal dose H_2O_2 concentration of 0.08 mmol/L was added, varying concentrations of EGF were added. Incubation for 24h, 10-100 ng/mL EGF notably induced protection of Müller cells against oxidative lesions by H_2O_2. On the other hand, 2h in advance added different concentrations of EGF and Akt signal pathway inhibitor LY294002, EGF against MIO-M1 cell damage caused by oxidative damage weakens.

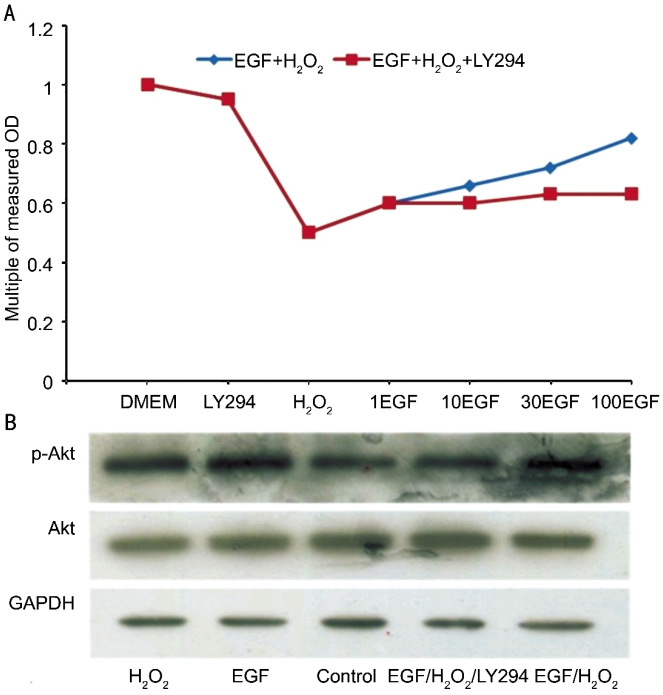

Figure 6 Akt phosphorylation under different conditions A: 2h in advance median lethal dose H_2O_2 concentration of 0.08 mmol/L, varying dosages of EGF and EGF combined LY294002 were added. The protection effect induced by EGF was weakened (P<0.05) after LY294002, a potent inhibitor of phosphoinositide 3-kinases (PI3Ks), was added. However, there is no change in cell viability after adding LY294002 alone (P >0.05); B: Inhibition effects of PI3K on EGF and H_2O_2 stimulated Müller cell Akt phosphorylation. Equal amounts of Müller cell protein extracts (5-8 μg/lane) were immunoblotted with anti-p-Akt antibody. Exposure to EGF (100 ng/mL) or 0.08 mmol/L H_2O_2 significantly increased p-Akt expression. Application of the PI3K inhibitor, LY294002 (50 μm/L LY294002), attenuated p-Akt expression in the presence of EGF and was sufficient to block expression of p-Akt in the presence of EGF under oxidative condition. GAPDH was not affected by EGF treatment.

Research also reported erythropoietin can protect RPE cells from H_2O_2 oxidative damage [17]. Our research showed that H_2O_2 can damage the Müller cells, which means H_2O_2 may induce the Müller cell lesions *in vivo* and destroy the blood-retinal barrier and leading to increased vascular permeability that may lead to the development of AMD. Exogenous EGF played a protection role in H_2O_2-mediated oxidative stress damage of the Müller cells. This effect may maintain the blood-retinal barrier and play a protective role.

Although many signaling pathways, including p44/p42 mitogen-activated protein kinase (MAPK), PI3K, Wnt and Shh, p38 MAPK and ERK1/2 *et al*/can be activated by EGF, whereas ERK1/2 pathway plays a leading role in cell proliferation and resistance to apoptosis [23,24]. It had been reported that PDGF-stimulated Müller cell proliferation occurs *via* activation of the c-JNK and PI3K/Akt signaling pathways[45]. Studies have released oxidative damage induced

by H_2O_2 led to PI3K reaction (phosphatidylinositol 2, 3-phosphate) and the accumulation of its products activated Akt signaling pathway, and the EGFR becomes phosphorylated in a PI3K-dependent manner in response to H_2O_2 [25,46,47,34]. Therefore, we mainly focused on PI3K/PDK1/PKB (Akt) signal transduction pathway. The results of our assay indicated that EGF possibly through ERK1/2 and Akt signaling pathway induced Müller cell proliferation and migration. With different concentrations of the H_2O_2 added to Müller cells 2h before adding exogenous EGF, Akt signaling expression increased, while adding exogenous EGF 2h in advance and Akt signal pathway inhibitor LY294002, Akt signaling pathway protection weakened. This indicates that EGF through this signal transduction may play the protective effect. MAPK signaling pathway consists of four members, ERK1/2 (also known as MAPK), Jun N-terminal kinase/stress-activated protein kinase (JNK/SAPK), P38, and ERK5 in which ERK1/2 and ERK5 is the main intracellular signaling pathway promoting cell proliferation. ERK1/2 signaling pathway as a main pathway promotes RPE proliferation by EGF has been confirmed [48,49]. RPE cells under H_2O_2 oxidative stress condition are protected by Akt signal transduction pathway has been reported, which is similar to our results[18].

In conclusion, our data suggest that EGF can mediate human Müller cell proliferation and migration with different culture conditions *in vitro*. The results of our assay indicated that EGF possibly through ERK1/2 and Akt signaling pathway induced human Müller cell proliferation and migration and EGF protects Müller cells from H_2O_2-induced lesions may through PI3K/PDK1/PKB (Akt) signal transduction pathway. Based on this investigation, further signal pathway detection assay and animal models of AMD should be performed to determine the mechanisms of blood-retinal barrier damage caused by oxidative mediated Müller cell lesions. The protection of Müller cells from injury and promotion of proliferation and migration by EGF during the early stage of AMD may play a prevention or therapeutic role.

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