Original Article
EGFL6 regulates hypoxia-induced angiogenic factors in retinal pigment epithelial cells

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Abstract: Epidermal growth factor like domain 6 (EGFL6), which is a member of the epidermal growth factor repeat superfamily of proteins, has been demonstrated to be important in angiogenesis. However, the expression and role of EGFL6 in the retinal pigment epithelial (RPE) cells remains to be elucidated. The aim of the present study was to investigate the role of EGFL6 in cultured human RPE cells. ARPE-19 cells were pretreated by 200 μM CoCl2 for 24 h, then transfected with siRNA-EGFL6 or control siRNA. Cell proliferation was measured using the MTT assay. The expression protein levels of EGFL6, VEGF, pigment epithelium-derived factor (PEDF), stromal cell-derived factor (SDF)-1 and IL-1β were detected using western blotting. The expression of EGFL6 was significantly elevated under hypoxic conditions. EGFL6 knockdown inhibited hypoxia-induced RPE cell proliferation. In addition, knockdown of EGFL6 inhibited hypoxia-induced pro-angiogenic factor expression and the expression of inflammatory factors in RPE cells. These results suggested that the knockdown of EGFL6 suppressed pro-angiogenic factors induced by hypoxia in RPE cells. Therefore, EGFL6 may be a potential therapeutic target for the treatment of age-related macular degeneration.

Keywords: Epidermal growth factor like domain 6, retinal pigment epithelial, hypoxia, angiogenic factors

Introduction
Age-related macular degeneration (AMD) is one of the major causes of visual impairment in the elderly population worldwide [1]. A severe form of AMD is choroidal neovascularization (CNV), in which new blood vessels grow from the choroids, through the Bruch's membrane into the subretinal space [2]. The retinal pigment epithelium (RPE), which forms a blood-retinal barrier between the neural retina and choriocapillaris, is important in maintaining the ocular angiogenic homeostasis [3]. RPE cells are the primary cells involved in CNV, and vascular endothelial growth factor (VEGF) secretion by RPE cells leads to neovascularization in the posterior segment of the eye [4]. In addition, hypoxia could contribute to the development of retinal and CNV formation [5-7]. Under hypoxic conditions, increased production of angiogenic factors, result in hypoxia-induced retinal angiogenesis [8].

The epidermal growth factor (EGF) repeat motif defines a superfamily of diverse proteins involved in mediating a variety of cellular processes, including cell adhesion, proliferation, growth, survival and differentiation [9]. EGF-like domain 6 (EGFL6), a member of the EGF repeat protein superfamily expressed in various tissues [10]. Furthermore, it was reported that EGFL6 promotes the endothelial cell tube-like structure formation and angiogenesis in a chick embryo chorioallantoic membrane [11]. However, the role of EGFL6 in RPE remains to be elucidated. The present studies, aimed to examine the effects of EGFL6 during hypoxia-induced angiogenesis in RPE cells.

Materials and methods
Cell culture and hypoxic treatment
Human RPE cells (ARPE-19) were obtained from the American Type Culture Collection (Manass-
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as, VA, USA) and cultured in Dulbecco’s Modified Eagle’s Medium/F-12 (Gibco, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (FBS; Invitrogen, Carlsbad, CA, USA), 100 U/ml penicillin, 100 μg/ml streptomycin and 4 mmol/l glutamine at 37°C in an incubator with an atmosphere containing 5% CO₂. For the hypoxia treatment, 200 μM cobalt chloride (CoCl₂; Sigma Chemical Co., St. Louis, MO) was added to the culture medium. The controls were cultured at 37°C in a humidified atmosphere containing 5% CO₂ for the same time period.

Small interfering RNA (siRNA) transfection

Human-specific EGFL6 siRNA (siRNA-EGFL6, 5'-GCTTCTCTCTACAACTTT-3') and non-targeting negative control siRNA (mock) were purchased from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA). For siRNA transfection, ARPE-19 cells were seeded onto 6-well plates at a density of 3×10³ cells/well and cultured to 60-80% confluence, then siRNAs were transfected into ARPE-19 cells at a final concentration of 30 nM with siRNA Transfection Reagent (Santa Cruz Biotechnology, Inc.) according to the manufacturer’s protocol.

Cell proliferation assay

Cell proliferation was measured using the MTT assay. Briefly, ARPE-19 cells transfected with siRNA-EGFL6 or mock were seeded in a 96-well plate at a density of 1×10⁴ cells/well and then cultured for 24 and 48 h. Then, the initial culture medium was replaced with fresh medium containing MTT (5 mg/ml; Sigma-Aldrich, Germany) and incubated for an additional 4 h. The formazan was dissolved in dimethylsulfoxide (150 μl/well; Sigma-Aldrich, Germany) for 10 min. The absorbance was subsequently measured on a Bio-Rad Microplate Reader (Bio-Rad Laboratories, Inc., Hercules, CA, USA) using a test wavelength of 490 nm. Each experiment was performed in triplicate.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) analysis

Total cellular RNA was isolated from cultured cells using RNeasy Mini kit (Qiagen GmbH, Hilden, Germany) in accordance with the manufacturer’s protocol. For RT, single-stranded cDNA was reverse transcribed from 2 μg total RNA using reverse transcriptase (Invitrogen, Carlsbad, CA, USA) with an oligo (dT) primer. The qRT-PCR was performed in a final volume of 20 μl, containing 50 ng of total RNA, 10 μl 2×SYBR Green I reagent, 6.25 U Multi-Scribe reverse transcriptase, 10 μl RNase inhibitor and 0.1 mM primers. The primer sequences used were as follows: Forward 5′-TCTGTGCTGCTGCTGATTG-3′ and reverse 5′-ACGGTAACAACTT-3′ for β-actin. Relative mRNA levels were evaluated by qPCR (LightCycler; Roche Applied Science, Penzburg, Germany) using Absolute Blue qPCR kit (Thermo Fisher Scientific, Inc., Waltham, MA, USA). The PCR conditions comprised 40 cycles of denaturation at 94°C for 30 sec, annealing at 54°C for 30 sec and polymerization at 72°C for 30 sec. The PCR products obtained were resolved on a 1.5% agarose gel and visualized with ethidium bromide under a transilluminator. Relative levels of gene expression were expressed relative to β-actin and calculated using the 2⁻ΔΔCq method [12].

Western blotting

Total protein was extracted from ARPE-19 cells using RIPA Cell Lysis Buffer (Bio-Rad, Hercules, CA, USA), containing a phosphatase inhibitor and the protease inhibitor cocktail (Sigma-Aldrich), by incubation on ice for 30 min. The concentration of the protein was measured using the BCA Protein Assay Kit (Pierce; Thermo Fisher Scientific, Inc.). Equal amounts of protein (30 μg per lane) were boiled for 10 min in sample buffer, separated by 10% SDS-PAGE and transferred to a polyvinylidene membrane. The blots were blocked in 5% nonfat milk in TBS-Tween (5 mM Tris-HCl (pH 7.4) 136 mM NaCl, 0.1% Tween 20) for 1 h at room temperature and then probed with mouse anti-human EGF-L6 polyclonal IgG (1:2500; ab-167281; Abcam, Cambridge, UK), mouse anti-human VEGF monoclonal IgG (1:2500; sc-57496), mouse anti-human pigment epithelium-derived factor (PEDF) monoclonal IgG (1:2500; sc-57496), mouse anti-human stromal cell-derived factor (SDF)-1 monoclonal IgG (1:3000; sc-390172), mouse anti-human interleukin (IL)-1β monoclonal IgG (1:3000; sc-520172) and mouse anti-human β-actin monoclonal IgG (1:2000; sc-
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8432) (Santa Cruz Biotechnology Inc.) over-night at 4°C. Subsequently, the membranes were incubated with goat anti-mouse horseradish peroxidase-conjugated secondary antibody (1:3000; sc-2031) for 1 h at room temperature. Proteins were visualized via enhanced chemiluminescence detection (Pierce; Thermo Fisher Scientific, Inc.) according to the manufacturer’s protocol.

Statistical analysis

Statistical analyses were performed using the SPSS 17.0 (SPSS, Inc., Chicago, USA). Data were expressed as the mean ± standard deviation. Statistical analysis of data was performed by one-way analysis of variance followed by a Student-Newman-Keuls test, and P<0.05 was considered to indicate a statistically significant difference.
EGFL6 was highly expressed in RPE cells under conditions of hypoxia

To explore the alteration of EGFL6 expression in RPE cells in response to a hypoxic stimulus, the present study examined EGFL6 mRNA and protein expression levels. As presented in Figure 1A, EGFL6 mRNA expression levels were significantly upregulated following 6 h incubation under hypoxic conditions compared with the 0 h group (P<0.05), and reached a maximum at 24 h. Western blotting results additionally demonstrated that hypoxia induced the expression of EGFL6 protein compared with 0 h group (Figure 1B).

Knockdown of EGFL6 inhibits RPE cell proliferation under hypoxic conditions

To investigate the effect of EGFL6 on RPE cell proliferation under hypoxic conditions, siRNA-EGFL6 was transfected into ARPE-19 cells. Following transfection, the expression of EGFL6 was inhibited at the RNA (Figure 2A) and protein (Figure 2B) levels under hypoxic conditions, as compared with the mock group. Subsequently, the MTT assay was used to determine the RPE cell proliferation in vitro. The results demonstrated that hypoxia significantly induced RPE cell proliferation, as compared with the control groups. However, knockdown of EGFL6 suppressed the hypoxia-induced RPE proliferation, compared with the hypoxia control groups (P<0.05; Figure 2C).

Effect of EGFL6 knockdown the expression of angiogenic factors in RPE cells under hypoxia

Angiogenic factors are important in vascular formation and progression. Therefore, the present study evaluated the effects of EGFL6 on the expression levels of pro-angiogenic factor, VEGF, and anti-angiogenic factor, PEDF, in ARPE-19 cells under hypoxic conditions. Western blotting results demonstrated that hypoxia significantly upregulated VEGF expression whereas it downregulated PEDF expression compared with cells in normoxia (P<0.05). However, knockdown of EGFL6 partially rescued the imbalance of pro- and anti-angiogenesis factors caused by hypoxia in ARPE-19 cells (Figure 3).
Knockdown of EGFL6 decreases the expression of inflammatory cytokines in RPE cells under hypoxia

Inflammatory cytokines, including SDF-1 and IL-1β, have been demonstrated to be involved in angiogenesis. Therefore, the present study evaluated the effects of EGFL6 on the expression levels of SDF-1 and IL-1β in ARPE-19 cells under hypoxic conditions. As presented in Figure 4, hypoxia treatment markedly induced the expression of SDF-1 and IL-1β, compared with the control group (P<0.05), whereas knockdown of EGFL6 inhibited the expression of SDF-1 and IL-1β induced by hypoxia in ARPE-19 cells (P<0.05).

Discussion

The present study, to the best of our knowledge, is the first to describe the expression and role of EGFL6 in human RPE cells. The expression of EGFL6 was increased by hypoxia, and EGFL6 knockdown suppressed hypoxia-induced RPE cell proliferation. In addition, knockdown of EGFL6 restored the imbalance of pro- and anti-angiogenic factors, and inhibited the expression of SDF-1 and IL-1β in hypoxia-stimulated ARPE-19 cells.

The role of EGFL6 in angiogenesis has previously been reported. Chim et al. [11] demonstrated that EGFL6 is highly expressed in osteoblastic-like cells, and these express EGFL6 that is capable of promoting endothelial cell migration and angiogenesis via extracellular signal-related kinase activation. Another study showed that recombinant EGFL6 enhanced proliferation of human adipose tissue-derived stromal vascular cells [13]. Similarly, herein, we observed that expression of EGFL6 was increased following hypoxic insult in a time-dependent manner and knockdown of EGFL6 restored the imbalance of pro- and anti-angiogenic factors, and inhibited the expression of SDF-1 and IL-1β in hypoxia-stimulated ARPE-19 cells.

The increased production of VEGF has been identified as essential in the development and progression of CNV. It is well established that hypoxia is important in the development and progression of retinal vascular diseases. It has previously been suggested that RPE cells secrete VEGF and VEGF-associated polypeptides in response to hypoxic insult [14], and may therefore be important in the progression.
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of CNV. PEDF, a glycoprotein (50 kDa), is an endogenous inhibitor of angiogenesis and is present in the vitreous at high levels [15]. It has previously been demonstrated that the decrease of PEDF in the eye is correlated with CNV formation [16-18]. In addition, hypoxia may downregulate PEDF via proteolytic degradation [19]. Consistent with these previous studies, the present study observed that hypoxia significantly upregulated VEGF expression and downregulated PEDF expression. However, knockdown of EGFL6 restored the imbalance of pro- and anti-angiogenic factors in hypoxia-stimulated ARPE-19 cells. This demonstrated the angiogenic effect of EGFL6 in RPE cells under hypoxic conditions, which may be important in the development of CNV.

Inflammation is associated with the development of CNV [20-22]. A study demonstrated that the administration of an anti-inflammatory drug, bromfenac, decreased the formation of laser-induced CNV [23]. SDF-1 is important in ocular neovascularization diseases [24-26], and hypoxic insult effectively augmented the expression of SDF-1α in RPE cells [27]. Furthermore, IL-1β is the primary inflammatory cytokine produced by a variety of cells, and is fundamental in inflammatory responses [28]. Various studies have previously revealed the protein secretion and mRNA expression of VEGF in ARPE-19 cells was increased by IL-1β [29, 30]. In accordance with the previous data, the present study observed that knockdown of EGFL6 reduced the expression of IL-1β in RPE under hypoxic culture conditions. These results suggested that EGFL6 may regulate the expression of hypoxia-induced angiogenic factors in RPE cells via regulation of inflammatory factors.

In conclusion, the results of the present study demonstrated that EGFL6 exhibited an angiogenic role in CNV, via regulation of angiogenic factor expression in RPE cells under hypoxic conditions. However, the exact underlying molecular mechanism remains to be elucidated in further investigations.

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Disclosure of conflict of interest

None.

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References

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