• Basic Research •

Blocking VEGF signaling augments interleukin-8 secretion *via* MEK/ERK/1/2 axis in human retinal pigment epithelial cells

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Abstract

• **AIM:** To identify proangiogenic factors engaged in neovascular age-related macular degeneration (AMD) except vascular endothelial growth factor (VEGF) from human retinal pigment epithelial (hRPE) cells and investigate the underlying mechanisms.

• **METHODS:** VEGF receptor 2 (VEGFR2) in ARPE-19 cells was depleted by siRNA transfection or overexpressed through adenovirus infection. The mRNA and the protein levels of interleukin-8 (IL-8) in ARPE-19 cells were measured by quantitative real-time polymerase chain reaction and enzyme-linked immunosorbent assay respectively. The protein levels of AKT, p-AKT, MEK, p-MEK, ERK1/2, p-ERK1/2, JNK, p-JNK, p38 and p-p38 were detected by Western blotting. A selective chemical inhibitor, LY3214996, was employed to inhibit phosphorylation of ERK1/2. Cell viability was determined by MTT assay.

• **RESULTS:** Knockdown of VEGFR2 in ARPE-19 cells robustly augmented IL-8 production at both the mRNA and the protein levels. Silencing VEGFR2 substantially enhanced phosphorylation of MEK and ERK1/2 while exerted no effects on phosphorylation of AKT, JNK and p38. Inhibiting ERK1/2 phosphorylation by LY3214996 reversed changes in VEGFR2 knockdown-induced IL-8 upregulation at the mRNA and the protein levels with no effects on cell viability. VEGFR2 overexpression significantly reduced IL-8 generation at the mRNA and the protein levels.

• **CONCLUSION:** Blockade of VEGF signaling augments IL-8 secretion *via* MEK/ERK1/2 axis and overactivation of VEGF pathway decreases IL-8 production in hRPE cells. Upregulated IL-8 expression after VEGF signaling inhibition in hRPE cells may be responsible for being incompletely responsive to anti-VEGF remedy in neovascular AMD, and IL-8 may serve as an alternative therapeutic target for neovascular AMD.

• **KEYWORDS:** age-related macular degeneration; vascular endothelial growth factor signaling; anti-vascular endothelial growth factor therapy; retinal pigment epithelial cells; interleukin-8

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INTRODUCTION

Age-related macular degeneration (AMD) is the principal cause of irreversible blindness among individuals older than 55y in developed world[1]. AMD falls into two forms, a non-neovascular or "dry" form and a neovascular or "wet" form. The former results in typically gradual vision loss, while the latter gives rise to acute vision loss owing to the development of neovasculatures from choroid into subretinal space. The newly formed immature vasculatures induce hemorrhage and leakage from the neovessels within the subretinal space, leading to acute and severe visual impairment[2]. Thus far, though no efficacious treatment is available for "dry" AMD, remedy employing anti-vascular endothelial growth factor (anti-VEGF) agents by intravitreal injection has made remarkable success in preserving vision of neovascular AMD patients[3], in which anti-VEGF agents potently inhibit angiogenesis and reduce vascular permeability[4].

Retinal pigment epithelium (RPE), a monolayer of polarized pigmented epithelial cells, lies in the back of the eyeball and acts as a pivotal player in maintaining retinal homeostasis[5].

These polarized cells are viewed as the main source of VEGF in the eye, of which the basal part secrets VEGF to nurture choroid blood vessels while the apical part pigment epithelium-derived factor to inhibit neovascularization within retina[6-7]. Furthermore, there are also evidences that RPE cells are able to generate a broad spectrum of other growth factors and cytokines including members of interleukin family, fibroblast growth factor, transforming growth factor-β, ciliary neurotrophic factor, tumor necrosis factor-α (TNF-α)[5]. Besides, compelling evidences demonstrated VEGF signaling exists and plays crucial roles in RPE cells[8-9]. Dysfunction of RPE cells alters expression level and direction of its secretory cytokines, contributing to initiation and progression of ocular disorders[10]. However, it remains undetermined whether blockage of VEGF signaling in RPE cells exerts any detrimental effects in retinal homeostasis.

Mitogen activated protein kinases (MAPKs), consisting of p38, c-Jun N-terminal kinase (JNK) and extracellular signal-regulated kinase (ERK1/2), are a group of ubiquitous and highly conserved serine/threonine protein kinases. These kinases, serving as important mediators in signal transduction systems *in vivo*, participate in a variety of the physiological and pathological processes, such as cell proliferation, differentiation, apoptosis and so forth. Moreover, the MAPKs signaling pathways are also involved in the activation and production of proinflammatory mediators and cytokines, such as nitric oxide, cyclooxygenase-2, TNF-α, interleukin-8 (IL-8), and interleukin-6[11]. In RPE cells, MAPKs signaling plays crucial parts in modulating secretion of proinflammatory factors and cytokines under stress conditions[12].

Though the current prevalent anti-VEGF therapy for neovascular AMD has made striking breakthrough, incomplete response to anti-VEGF agents has been reported in numerous studies[13]. Therefore, apart from VEGF which is mainly from RPE cells in the eye, other angiogenic factors and pathways may be implicated in neovascular AMD. In this research, we sought to identify from RPE cells other vascular mediators engaged in angiogenesis of AMD, with a view to investigate potential regulatory mechanisms and to develop novel therapeutic strategies.

MATERIALS AND METHODS

Cell Culture and Treatment Human RPE (hRPE) cell line ARPE-19 cells were purchased from American Type Culture Collection (ATCC) and cultured in Dulbecco's modified Eagle's medium/Nutrient Mixture F-12 Ham (DMEM/F12) supplemented with 10% fetus bovine serum (FBS), 100 U/mL streptomycin and penicillin in a humidified incubator at 37℃ with 5% CO_2. For ERK1/2 phosphorylation inhibition, 100 nmol/L of selective ERK1/2 inhibitor reagent (MedChemExpress, #LY3214996) was added to the culture medium with FBS and lasted for 24h, followed by collection of the cell lysates in RIPA or Trizol.

siRNA Transfection and Adenovirus Infection The human VEGFR2 siRNA or control siRNA were transfected into ARPE-19 cells using Lipofectamine RNAiMAX reagent (Invitrogen, #13778-150) by following the manufacturer's instructions. Forty-eight hours after transfection, the cells were harvested for quantitative real-time polymerase chain reaction (qRT-PCR) or Western blotting. For VEGFR2 overexpression, Ad-VEGFR2 virus was employed to infect ARPE-19 cells while Ad-GFP group was used as a control. Forty-eight hours after infection, the cells were harvested for qRT-PCR or Western blot analysis.

Quantitative Real-time Polymerase Chain Reaction Total RNA was extracted using Trizol reagent and reverse transcribed into cDNA *via* a PrimeScript RT reagent Kit (TAKARA Biotechnology, Kusatsu, Shiga, Japan) according to the manufacturer's instructions. qRT-PCR was performed using qPCR SYBR® Green Master Mix (Roche, Basel, Switzerland) and a Roche 480 Light Cycler. The Light Cycler 480 software (Roche, Basel, Switzerland) was utilized to analyze the results. For each gene, the threshold cycle value was normalized to 18sRNA. Sequences of all the primers used in qRT-PCR assays were as followed: *VEGFR2*, F: 5'-CGTGTCTTTGTGGTGCACTG-3', R: 5'-GGTTTCCTGTGATCGTGGGT-3', *IL-8*, F: 5'-AAGGTGCAGTTTTGCCAAGG-3', R: 5'-CAACCCTCTGCACCCAGTTT-3', *18sRNA*, F: 5'-CGGCTACCACATCCAAGGAA-3', R: 5'-GCTGGAATTACCGCGGCT-3'.

Western Blot For total protein extraction, cells were lysed in RIPA buffer (Sobiobiomart, Beijing, China) with protease inhibitor and phosphatase inhibitor cocktails (Thermo Fisher Scientific, Waltham, MA, USA), kept on ice for 15min, and centrifuged for 10min. Protein concentration was determined by BCA Protein Assay Kit (Beyotime, #P0012). Equal amounts of proteins were separated by 8% or 10% SDS-polyacrylamide gel and transferred onto PVDF membranes. After transfer, the membranes were then blocked in 5% non-fat milk for 1h at room temperature and probed with the following primary antibodies overnight at 4℃: VEGFR-2 (CST, #9698S, 1:1000); MEK (CST, #4694, 1:1000); p-MEK (Ser217/221; CST, #9154, 1:1000); ERK1/2 (CST, #4696S, 1:1000); p-ERK1/2 (Thr202/Tyr204; CST, #4370, 1:1000); p38 MAPK (CST, #8690, 1:1000); p-p38 MAPK (Thr180/Tyr182; CST, #9216, 1:1000); p-JNK (Thr183/Tyr185; CST, #9255, 1:1000); JNK (CST, #9252, 1:1000); AKT (CST, #2920, 1:1000); p-AKT (Ser473; CST, #4060S, 1:1000); α-tubulin (Sigma, #T6074, 1:2000). The membranes were washed with TBST for 3 times (10min each time) to remove unbound antibodies, labeled

with horseradish peroxidase-conjugated secondary antibodies (1:2000, GE, USA) for 1h at room temperature and visualized by enhanced chemiluminescence reagents (Roche, Mannheim, Germany).

Enzyme-linked Immunosorbent Assay The cells were cultured or treated with or without 100 nmol/L LY3214996 (DMSO as vehicle) in serum-free DMEM/F12 medium for 24h after treatments. The medium was collected and centrifuged to discard any scraps 24h later. The supernatant was gathered to determine IL-8 level using an enzyme-linked immunosorbent assay (ELISA) kit (R&D, #D8000C) according to the manufacturer's instructions.

MTT Assay MTT [3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2-H-tetrazolium bromide, Sigma] assay was utilized to determine cell viability. Briefly, 24h after siRNA transfection, 8×10^3 cells/well were seeded with 100 μL complete medium in 96-well plates and incubated at 37℃ for 12h. Then the cells were treated with complete medium containing DMSO or 100 nmol/L ERK1/2 inhibitor LY3214996 for 24h, followed by adding 10 μL MTT (5 mg/mL) per well and incubation at 37℃ for 4h. The reduced MTT crystals were dissolved in 100 μL DMSO after discarding the MTT solution and incubated at 37℃ for 15min. The absorbance at 570 nm was measured by FLx800 Fluorescence Reader (BIO-TEK, Winooski, VT, USA).

Statistical Analysis and Figure Layout Data were presented as mean±standard error of the mean (SEM). Differences between two groups were analyzed by two-tailed unpaired Student's *t*-test in GraphPad Prism software. For multiple comparisons, *P* value was corrected by utilizing the Bonferroni's method. Statistical significance was considered when $P<0.05$. When values of $P<0.05$, $P<0.01$, and $P<0.001$, ^aP, ^bP and ^cP were designated, respectively. Figures were laid out using Adobe Illustrator CS6 software.

RESULTS

Robustly Increased Secretion of IL-8 and Activation of MEK/ERK1/2 Cascade by Silencing VEGFR2 in hRPE Cells Previously, our colleagues have reported VEGF signaling exists and plays important roles in RPE cells[8-9]. Hence, to investigate whether blockage of VEGF signaling exerts any impacts on IL-8 expression in RPE cells, we first evaluated abundance of IL-8 expression in ARPE-19 cells, a hRPE cell line. As reported in other studies, ARPE-19 cells robustly secreted IL-8 (Figure 1A and 1D)[14-15], indicating this cell line is suitable for our present study. Then we utilized siRNA-mediated silencing of VEGFR2 to mimic blockage of VEGF signaling pathway in ARPE-19 cells. Upon VEGF signaling was highly suppressed, indicated by efficient depletion of VEGFR2 (Figure 1A and 1B), a prominent increase in IL-8 expression was observed at both the RNA and the protein level (Figure 1A and 1D).

MAPKs cascade, inclusive of ERK1/2, JNK and p38, is widely involved in cytokines and chemokines modulation[12,14,16-19]. Hence, we first tested whether MAPKs activity was altered upon inhibition of VEGF signaling in ARPE-19 cells. Western blotting showed that phosphorylated ERK1/2 but not JNK or p38 was substantially enhanced (Figure 1B and 1C). MEK has been demonstrated to act as an upstream factor of ERK1/2[20-21]. We then tested MEK phosphorylation level. Significantly elevated phosphorylation of MEK was observed as expected (Figure 1B and 1C). Apart from MEK/ERK1/2 axis, PI3K/AKT signaling was also shown to regulate cytokines expression in RPE cells[22-23], while no visible changes were detected in phosphorylated or total form of AKT (Figure 1B and 1C), indicating upregulated IL-8 expression is independent of PI3K/AKT signaling pathway. Phosphorylated ERK1/2 has been demonstrated to be able to translocate into nucleus and activate transcriptional promoters of IL-8, thereby driving IL-8 expression[24]. Collectively, these data suggested that suppressed VEGF signaling activates MEK/ERK1/2 cascade and augments production of IL-8 in hRPE cells.

Critical Role of MEK/ERK1/2 Axis in VEGFR2 Depletion-induced IL-8 Upregulation in hRPE Cells To further dissect the role of MEK/ERK1/2 in VEGF signaling inhibition-induced IL-8 upregulation in hRPE cells, we exploited a chemical inhibitor LY3214996, which is able to selectively inhibit ERK1/2 phosphorylation so as to repress ERK1/2 activity. Treatment with 100 nmol/L LY3214996 for 24h was capable of efficiently inhibiting ERK1/2 phosphorylation (Figure 2A and 2B) without toxic effects on cell viability indicated by MTT assay in ARPE-19 cells (Figure 2C). It turned out that transcription of IL-8 in VEGFR2 knockdown group treated with 100 nmol/L LY3214996 was significantly downregulated in comparison with its counterpart treated with vehicle (Figure 2D). Similar trend was also observed in ELISA results (Figure 2E). Taken together, these data indicated that MEK/ERK1/2 is required for VEGFR2 knockdown-induced IL-8 upregulation in hRPE cells.

Downregulated IL-8 Generation through VEGFR2 Overexpression in hRPE Cells To further confirm the role of VEGF signaling inhibition in IL-8 upregulation in hRPE cells, we performed gain-of-function assay by overexpressing VEGFR2 in ARPE-19 cells. As shown by quantitative RT-PCR and Western blotting, VEGFR2 was robustly expressed in ARPE-19 cells through adenovirus infection (Figure 3A and 3B). To our surprise, IL-8 transcription and secretion levels were markedly diminished in VEGFR2 overexpressed group compared with GFP group (Figure 3B and 3C). Together, these results suggested VEGF signaling negatively modulates IL-8 production in hRPE cells.

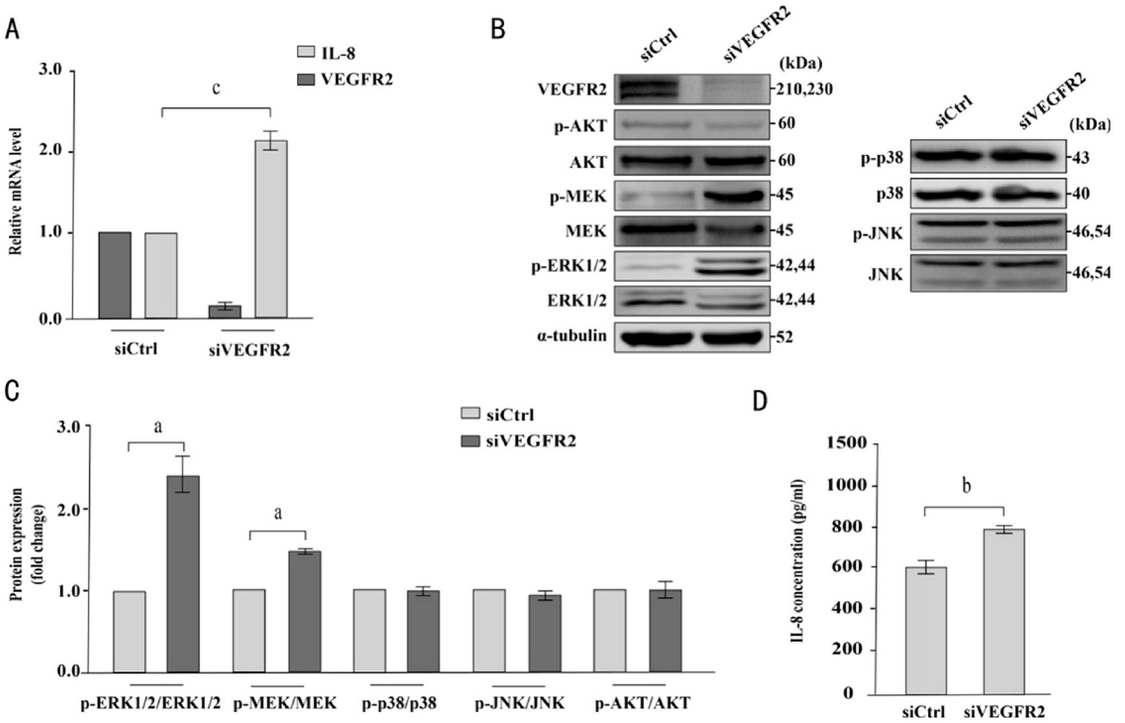

Figure 1 Silencing VEGFR2 robustly increased IL-8 secretion and activated MEK/ERK1/2 cascade in hRPE cells A: VEGFR2 knockdown efficiency and IL-8 expression at the RNA level were assessed by qRT-PCR; B: Western blotting analysis of VEGFR2, p-AKT, AKT, p-MEK, MEK, p-ERK1/2, ERK1/2, p-p38, p38, p-JNK, JNK in ARPE-19 cells after VEGFR2 knockdown *via* siRNA; C: The ratio of phosphorylated forms to unphosphorylated forms of AKT (p-AKT/AKT), MEK (p-MEK/MEK), ERK1/2 (p-ERK1/2/ERK1/2), p38 (p-p38/p38), JNK (p-JNK/JNK) were analyzed in ARPE-19 cells after VEGFR2 knockdown *via* siRNA; D: IL-8 protein level after VEGFR2 depletion was determined *via* ELISA assay. α-tubulin was used as loading control. Data are mean±SEM. $^{a}P<0.05$; $^{b}P<0.01$; $^{c}P<0.001$.

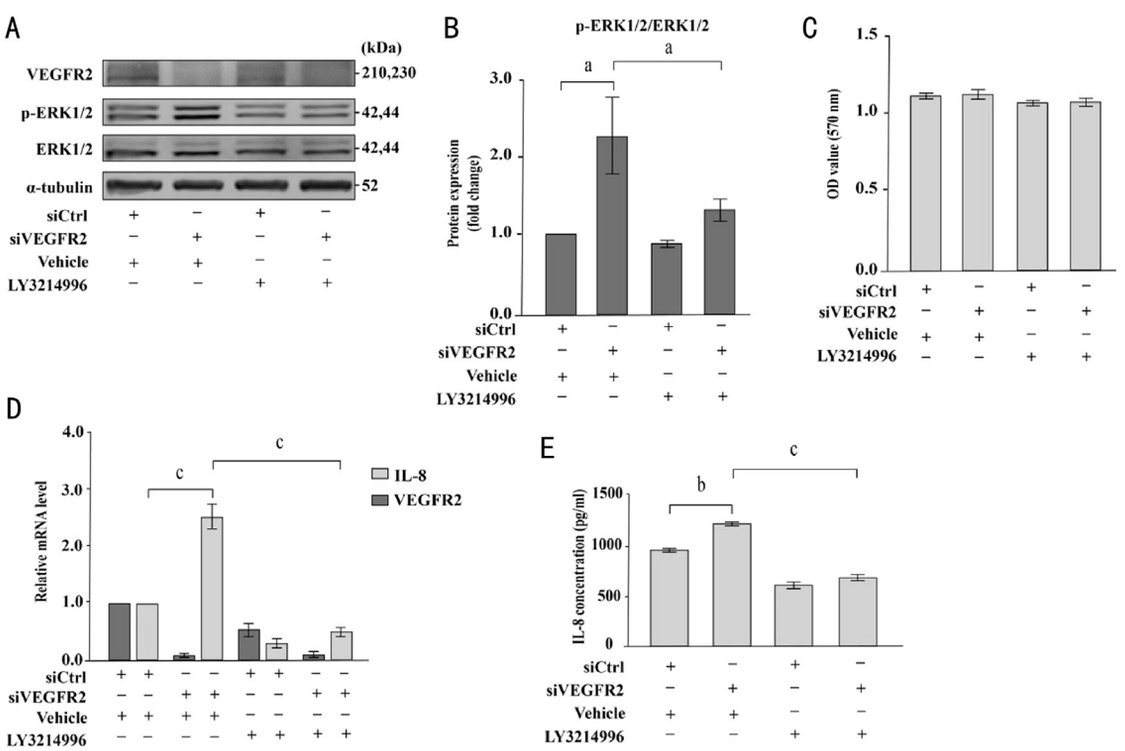

Figure 2 MEK/ERK1/2 is critical for VEGFR2 depletion-induced IL-8 upregulation in hRPE cells A: VEGFR2 knockdown efficiency by siRNA and phosphorylation of ERK1/2 after 100 nmol/L LY3214996 treatment for 24h in ARPE-19 cells were assessed by Western blotting; B: The ratio of phosphorylated forms to unphosphorylated forms of ERK1/2 (p-ERK1/2/ERK1/2) was assessed in ARPE-19 cells after 100 nmol/L LY3214996 treatment for 24h; C: Cell viability was detected by MTT assay after VEGFR2 knockdown and treatment with 100 nmol/L LY3214996 for 24h; D-E: LY3214996 100 nmol/L treatment reversed the changes in expression of IL-8 as determined by qRT-PCR and ELISA assay. α-tubulin was used as loading control. Data are mean±SEM. $^{a}P<0.05$; $^{b}P<0.01$; $^{c}P<0.001$.

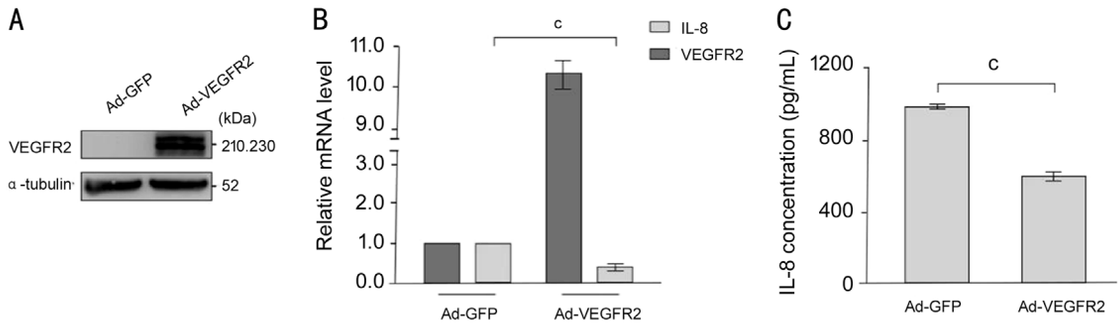

Figure 3 VEGFR2 overexpression downregulated IL-8 generation in hRPE cells A: Western blot of VEGFR2 in ARPE-19 cells after VEGFR2 overexpression through adenal virus infection; B-C: IL-8 expression level was assessed by qRT-PCR and ELISA assay. α-tubulin was used as loading control. Data are mean±SEM. $^{c}P<0.001$.

DISCUSSION

Incomplete response to anti-VEGF agents in neovascular AMD patients has recently been reported[13], implying alternative angiogenic factors may participate in neovascularization of AMD. In the present study, we sought to identify other implicated angiogenic factors except VEGF and clarify potential regulatory mechanisms. We demonstrated that suppression of VEGF signaling gave rise to increased production of IL-8, a potent proangiogenic factor, through MEK/ERK1/2 axis in hRPE cells. These findings suggest that VEGF signaling inhibition-induced IL-8 upregulation in hRPE cells may be responsible for incomplete response to anti-VEGF treatment in neovascular AMD, and IL-8 may serve as an alternative therapeutic target for neovascular AMD.

VEGF signaling robustly exists in neovascular endothelium so that anti-VEGF agents have been widely applied in treating angiogenic diseases, particularly in neovascular AMD and diabetic retinopathy[25]. Nonetheless, VEGF pathway also exists in RPE cells[8-9]. RPE cells are considered multifunctional epithelial cells capable of secretion, phagocytosis and potent anti-oxidation[5]. Most studies investigated prevention and treatment of choroidal neovascularization (CNV) in neovascular AMD by focusing on vascular endothelial cells[26-27]. In the present study, we started out centering on RPE cells instead of vascular endothelial cells to study the impacts of blocking VEGF signaling on IL-8 expression. We found that blockage of VEGF signaling *via* silencing VEGFR2 robustly augmented IL-8 production (Figure 1A and 1D) while overexpression of VEGFR2 notably decreased IL-8 generation (Figure 3B and 3C), suggesting VEGF signaling plays an important role in regulating IL-8 expression in hRPE cells. Hitherto, researchers have identified VEGF signaling is also present in other cell types of retina, such as Müller cells[28], pericytes[29] and astrocytes[30]. Moreover, Müller cells have been demonstrated to produce IL-8 under stress condition[31]. Therefore, whether blocking of VEGF signaling exerts similar impacts on Müller cells, pericytes and astrocytes as on RPE cells requires further investigation.

IL-8, alternatively known as CXCL8, is one of the most intensively studied chemokines and plays important parts in modulating inflammation[24]. As a chemoattractant, it functions to recruit neutrophils and granulocytes to the sites of inflammation *via* binding to its receptors CXCR2 and CXCR1 in numerous diseases[32]. In recent years, it has been well established that IL-8 boasts strong angiogenic potentials[33-35]. Furthermore, one recent research pointed out IL-8 exerts cytotoxic effects on retinal ganglion cells[36]. In our study, a robust elevation of IL-8 level was detected ensuing from suppression of VEGF signaling in hRPE cells (Figure 1A and 1D). Given IL-8 possesses great potentials in proinflammation and proangiogenesis as well as cellular toxicity, it is preferable to eliminate any side effects stemming from anti-VEGF therapy by targeting both VEGF and IL-8. Additionally, regarding RPE cells are capable of secreting a wide variety of growth factors and cytokines except IL-8, it is also of critical importance to unveil their possible involvement.

MAPKs, consisting of p38, JNK and ERK1/2, are involved in modulating IL-8 expression in epithelial cells[12,37-38]. Interestingly, our data showed knockdown of VEGFR2 strongly enhanced phosphorylation of MEK/ERK1/2 rather than that of p38 or JNK (Figure 1B and 1C). To confirm whether MEK/ERK1/2 axis is required for VEGFR2 silencing-induced IL-8 upregulation, an ERK1/2 phosphorylation inhibitor LY3214996 was employed. Indeed, inhibition of ERK1/2 phosphorylation reversed VEGFR2 knockdown-induced IL-8 upregulation in hRPE cells (Figure 2A-2B and 2D-2E), demonstrating it is specifically dependent on MEK/ERK1/2 signaling. Aside from IL-8, proangiogenic factors like angiopoietin 2, endothelin 1, follistatin and so forth[39] may be upregulated *via* MAPK cascades in RPE cells and serve as alternative potential therapeutic targets for treating neovascular AMD.

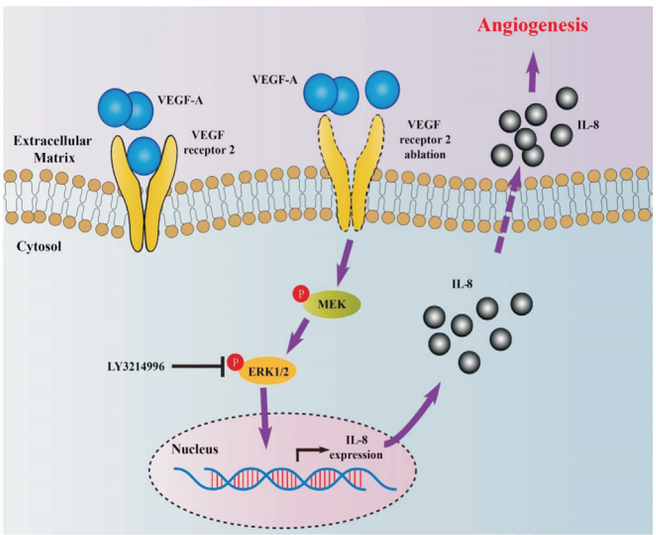

Figure 4 Overview of VEGFR2 knockdown-induced IL-8 upregulation in hRPE cells Depletion of VEGFR2, a putative receptor for VEGF signaling, enhanced phosphorylation of both MEK and its downstream factor ERK1/2. The phosphorylated ERK1/2 translocated into the nucleus and activated transcriptional promoters of IL-8, further driving IL-8 production in hRPE cells and thereby promoting angiogenesis. Employing LY3214996, a chemical inhibitor for ERK1/2 phosphorylation, reversed such changes in IL-8 generation.

To conclude, our research identified IL-8 as an alternative proangiogenic factor in neovascular AMD and unveiled that MEK/ERK1/2 axis linked VEGF signaling suppression to increased IL-8 production in hRPE cell (Figure 4). These results provide significant evidences for a better understanding of incomplete response to anti-VEGF agents in neovascular AMD, and identify an alternative therapeutic target to treat neovascularization in AMD.

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