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BASIC SCIENCE

Inhibition of proliferation, migration and tube formation of choroidal microvascular endothelial cells by targeting HIF-1α with short hairpin RNA-expressing plasmid DNA in human RPE cells in a coculture system

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Abstract
Background Retinal pigment epithelial (RPE) cells and choroidal microvascular endothelial cells (CECs) are the main cells involved in choroidal neovascularization (CNV), and hypoxia plays an important role in CNV formation via hypoxia inducible factor 1 (HIF-1). Our aim was to evaluate the role of HIF-1 in human RPE cells with regard to proliferation, migration and tube formation of CECs under hypoxia.
Methods RPE cells were cultured under chemical hypoxia induced by 200 μM $CoCl_2$, and RNA interference (RNAi) technique was used to knock down HIF-1α gene in RPE cells. mRNA and protein expression of HIF-1α and VEGF in RPE cells were investigated by real-time RT-PCR and Western blot. Three kinds of coculture models were used to observe the effects of RPE cells transfected by short hairpin RNA (shRNA)-expressing plasmid DNA (pDNA) (pshHIF-1α) on the proliferation, migration and tube formation of CECs respectively.
Results Transfection of shRNA-expressing pDNA targeting HIF-1α to RPE cells resulted in the knock down of HIF-1α gene and reduction of the corresponding mRNA and protein of HIF-1α and VEGF under hypoxia. Consequently, the proliferation, migration and tube formation of CECs were significantly inhibited by the knocked-down RPE cells compared with the control in the coculture system. The proliferation rates of CECs decreased by 40.2%, 36.6% and 36.8% on days 3, 4 and 5 respectively. Migration reduced by 49.6% at 5 h, and tube formation decreased by 40.4% at 48 h.
Conclusion RNAi of HIF-1α in RPE cells can inhibit angiogenesis in vitro and provide a possible strategy for treatment of choroidal neovascularization diseases by targeting HIF-1α.

Keywords Retinal pigment epithelium · Choroidal microvascular endothelial cell · Choroidal neovascularization · Growth factors

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Introduction

Age-related macular degeneration (AMD) is the leading cause of visual loss in persons more than 50 years of age. The exudative form of the disease is characterized by choroidal neovascularization (CNV). Although the morphology of angiogenesis in CNV secondary to AMD has been described in detail, the pathogenesis is still poorly understood [1].

Vascular endothelial growth factor (VEGF) secreted by retinal pigment epithelium (RPE) cells is a major pathogenic factor in the development of CNV, and hypoxia has been considered to play an important role [2, 3]. Some studies have showed that the relative hypoxia caused by the disturbed balance between the limited blood supply in the macula and the high oxygen demand by the photoreceptors may contribute to the formation of CNV by up-regulating VEGF [4]. Consequently, several novel therapies for CNV have emerged based on antagonism of VEGF or the VEGF receptor [5, 6], such as intravitreal administration of pegaptanib (Macugen), ranibizumab (Lucentis), bevacizumab (avastin) and a systemically delivered, modified VEGF

receptor (VEGF-Trap) [7]. However, a potential drawback of these therapies is that only one of multiple potentially important angiogenic factors is targeted. Other therapies, such as thermal laser, photodynamic therapy (PDT) or intravitreal triamcinolone, are believed to exert their effects partly through the down-regulation of multiple angiogenic factors [8, 9]. These therapeutic methods, however, all have significant side effects.

Recently, hypoxia-inducible factor 1 (HIF-1), which is a transcription factor that regulates genes such as VEGF and erythropoietin (EPO) involved in the response to hypoxia, has been proposed as a novel therapeutic target [3]. HIF-1 is a heterodimer composed of HIF-1α and HIF-1β subunits. HIF-1β is constitutively expressed, while HIF-1α is induced by hypoxia. HIF-1 transactivates a repertoire of genes, including VEGF, which mediate angiogenesis, cell proliferation/survival, and glucose/iron metabolism to hypoxia [10].

Recently, ribonucleic acid (RNA) interference (RNAi) emerged as a potentially exciting therapeutic method for various disease states. Clinical trials involving RNAi targeting VEGF or its receptor through intravitreal injection delivery are currently underway [11]. RNAi in the form of short interfering RNA (siRNA) targeting VEGF has previously been shown to inhibit VEGF production of human RPE cells [12].

Although VEGF is an attractive target for RNAi experiments on CNV, the effects of HIF-1α inhibition on CNV are not yet known, especially in coculture systems composed of RPE cells and choroidal microvascular endothelial cells (CECs). Our study group recently demonstrated that VEGF expression could be inhibited by targeting HIF-1α with siRNA in human RPE cells [3]. It is unclear if silencing HIF-1α in RPE cells could inhibit angiogenesis. We therefore used coculture models to investigate the effect of RPE cells knocked down HIF-1α on the proliferation, migration and tube formation of CECs.

Materials and methods

shRNA-expressing pDNA

shRNA-expressing pDNAs driven by human H1 promoter were constructed from pGCsi.H1.neo.GFP vector (Genechem, Shanghai, China) according to the manufacturer's instructions. Target site in human genes encoding HIF-1α was as follows: HIF-1α, 5′-GGA AGA ACT ATG AAC ATA A-3′. The pDNA transcribes a stem-loop-type RNA with loop sequences of TTCAAGAGA. pGCsi.H1.neo.GFP vector, which transcribes a non-related sequence of RNA with partial duplex formation, was used as a control pDNA throughout the study. Each pDNA was amplified in the DH5α strain of Escherichia coli and purified using a Biospin Plasmid DNA Extraction Kit (BioFlux, Hangzhou, China). They were pshHIF-1α (shRNA-expressing pDNA targeting HIF-1α) and control pDNA respectively.

Cell culture

Human RPE were harvested from keratoplasty donor eyes within 24 h post mortem; their use was approved by the Ethics Committee of the Fourth Military Medical University, and followed the tenets of the Declaration of Helsinki. The isolation and cultivation of RPE were performed as described previously [13]. Briefly, the anterior segment, vitreous and neurosensory retina were removed and an eye cup was made. The RPE were immersed in a trypsin (0.05%)-EDTA (0.02%) solution at 37°C for 1 h. Culture medium with 20% FBS was added, and the RPE were isolated and collected with a pipette, using a dissecting microscope. Isolated cells were centrifuged, resuspended and seeded to a culture flask. The cells were cultured in DMEM supplemented with 10% FBS, 100 U/ml penicillin and 100 μg/ml streptomycin at 37°C in a 5% CO_2 humidified incubator. Medium was changed twice a week. Once cells reached confluence, they were passaged using the trypsin-EDTA solution. All experiments were performed on cells from passage 3 to 6.

Bovine CECs were primarily isolated and cultured as previously described [14]. CECs were cultured in complete medium, which was DMEM supplemented with 10% FBS, 75 μg/ml Endothelial cells growth supplement (ECGS, Sigma-Aldrich, St.Louis, MO, USA), 100 μg/ml heparin sulfate (Gibco BRL, Grand Island, NY, USA), 100 U/ml penicillin and 100 μg/ml streptomycin. Positive immunostaining for von Willebrand factor (Santa Cruz Biotech, Santa Cruz, CA, USA) and uptake of di-acetylated low density lipoprotein (Dil-Ac-LDL, Biomedical Technologies, Stoughton, MA, USA) confirmed endothelial cell specificity. Experimentation was carried out using subconfluent CECs at cell passage 3 to 6.

In vitro transfection

1×10^5 RPE cells/cm^2 were plated in 0.40 μm transwell inserts (Millecell CM, Millipore, Bedford, MA, USA) or 6-well plates. After an overnight incubation in DMEM containing 10% FBS without antibiotics, transfection of pDNA was performed using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. In brief, for 24-well plates, 0.8 μg pDNA was mixed with 2 μl Lipofectamine 2000 at a final concentration of 1.3 μg pDNA/ml dissolved in DMEM, and the resulting complex was added to the cells and the cells were incubated with the complex for 6~8 h. Cells were washed with DMEM and further incubated with DMEM with 1% FBS

for specified time periods up to 5 d, and transfection was confirmed by viewing the cells under a fluorescent microscope. For 6-well plates, the pDNA and Lipofectamin 2000 were 3 μg and 6 μl respectively. The cells were used after transfection for 24 h. The amount of siRNA and transfection agent used was optimised, and under the conditions used we observed 100% transfection under fluorescence microscopy and little to no toxicity under light microscopy.

Real-time RT-PCR

RNA was isolated using Trizol (Invitrogen, Life Technologies, USA) and reverse-transcribed to cDNA using the ExScript RT Reagent kit (Takara, Japan). Real-time PCR was performed with Bio-Rad iQ™5 Real-Time PCR Detection System (Bio-Rad, USA) using SYBR Premix Ex Taq Kit (Takara, Japan) according to the manufacturer's instructions. The amplification program included an initial denaturation step at 95°C for 10 min, followed by denaturation at 95°C for 10 s, and annealing and extension at 60°C for 30 s, for 40 cycles. SYBR Green fluorescence was measured after each extension step, and the specificity of amplification was evaluated by melting curve analysis. Primers used to amplify specific gene products from RPE cells cDNA were GAPDH sense, 5′-GCG CTG AGT ACG TCG TGG AG-3′; GAPDH antisense, 5′-CAG TTG GTG GTG CAG GAG G-3′; VEGF sense, 5′- CGC CTC TCC AAA AAG CTA CAC-3′; VEGF antisense, 5′-CTC ACA GGA AAC CGG ACA TC-3′; HIF-1α sense, 5′-TCG GCG AAG TAA AGA ATC TGA A-3′; HIF-1α antisense, 5′-CAA ATC ACC AGC ATC CAG AAG-3′. The relative gene expression levels were calculated using the comparative Ct (ΔΔCt) method (according to Applied Biosystems), where the relative expression is calculated as $2^{-\Delta\Delta Ct}$, and Ct represents the threshold cycle.

Western blot analysis

RPE cells were grown in six-well plates in DMEM with 10% FBS until confluence. For hypoxia treatment, 200 μM $CoCl_2$ was added at 1, 3, 6, 12 and 24 h. For the transfection treatment, 200 μM $CoCl_2$ was added to wells for 3 h. Then cells were lysed and proteins were separated using a Tris-HCl 8% or 12% polyacrylamide gel at 120 V. The proteins were transferred to a polyvinylidene difluoride (PVDF) blotting membrane (Millipore, Bedford, MA, USA). The membranes were blocked in 5% milk and probed with mouse monoclonal anti-HIF-1α (Chemicon, Temecula, CA, USA) or mouse monoclonal VEGF (Santa Cruz Biotech, Santa Cruz, CA, USA) antibody overnight at 4°C. Membranes were washed and incubated with a horseradish peroxidase (HRP)–conjugated secondary antibody (Santa Cruz Biotech, Santa Cruz, CA, USA) for 1 h at 37°C. Images were developed by adding chemiluminescent HRP substrate solution (Millipore, USA).

Cell ELISA assay

Secreted VEGF protein in the media from proliferation assay (described below) all grown for 24 h was measured using Quantikine human VEGF ELISA kit (Jingmei, Shanghai, China). The respective conditioned media (including transwell insert) from each well, were removed, spun down to remove debris, and placed immediately into -80 °C until ELISA assay. The results were expressed relative to VEGF measured in the CEC monoculture cultured media.

Coculture Assay

Three kinds of coculture model, which maintained the natural anatomical relationship between basal RPE cells and CECs, were used for proliferation, migration and tube formation assay respectively. Briefly, for proliferation and tube formation assay models, RPE cells were plated in 0.40 μm transwell inserts which were put in wells where CECs were plated. However, the wells for tube formation assay had been coated by 200 μl extracellular matrix (ECM, Sigma-Aldrich, St. Louis, MO, USA). For migration assay, CECs were plated in 8-μm transwell inserts which

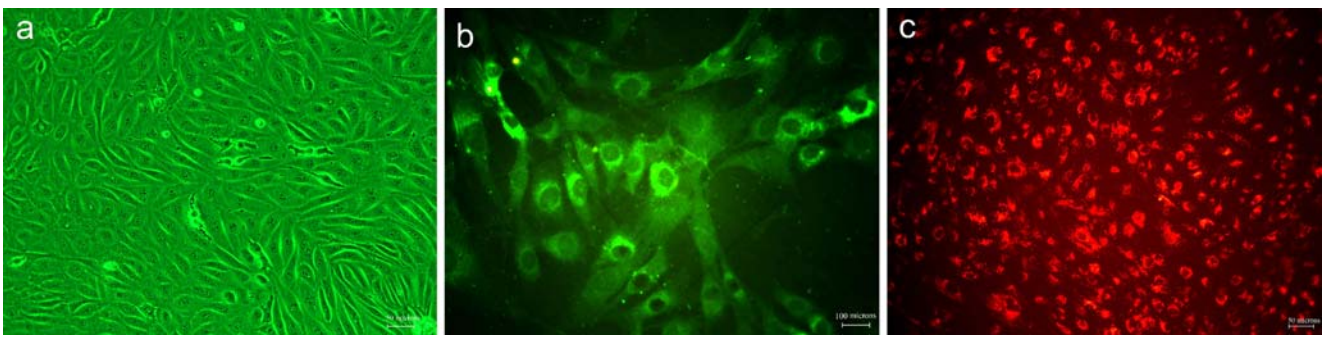

Fig. 1 Cultured CECs. **a** Confluent bovine CECs. **b** Immunofluorescent staining for von Willebrand factor. **c** Incorporation of Dil-Ac-LDL (*Bar*=50 μm)

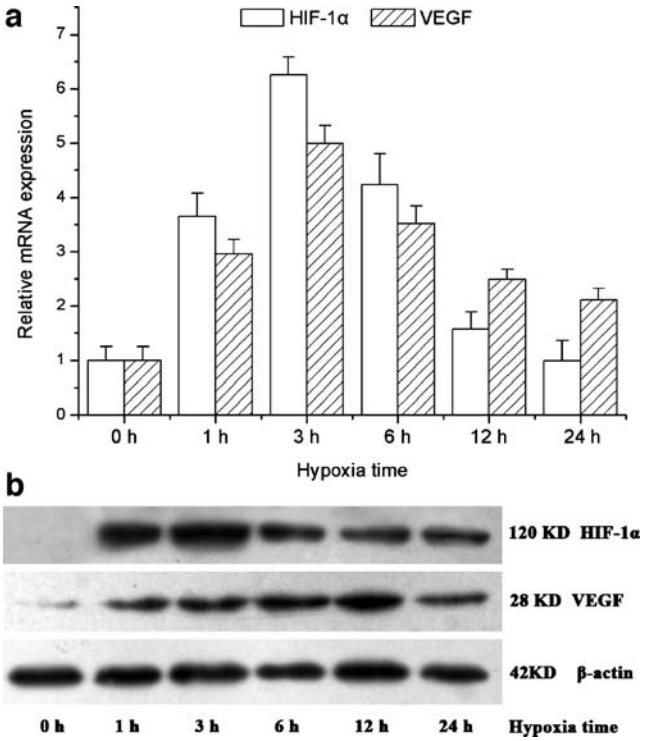

Fig. 2 mRNA (**a**) and protein (**b**) expressions of HIF-1α and VEGF in RPE cells under hypoxia induced by 200 μM $CoCl_2$ at 0, 1, 3, 6, 12 and 24 h. Basal reference levels in control (0 h) are defined as 1 (mean±SD, $n=3$)

were put in wells where RPE cells was plated. Specific procedures are described thoroughly in respective assays below.

Proliferation assay

To test the effect of RPE cells on CEC proliferation under hypoxia, a proliferation assay model was used (Fig. 5a). CECs were plated at density of 4×10^4 cells/cm^2 in 24-well plates in complete medium, and allowed to adhere overnight. Media were changed by DMEM with 1% FBS. The pshHIF-1α transfection insert and control pDNA transfection inserts were then put into the wells, and 200 μM $CoCl_2$ was added into the inserts. Three controls were used; one insert without transfection, one without transfection and $CoCl_2$, and the third without RPE cells. Media were changed on day 3. Cell proliferation was measured by a modified MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) assay on days 1, 2, 3, 4 and 5. Briefly, 100 μl MTT was added per well after the inserts were taken out, and CECs were incubated for 4 h. The formazan crystals formed were dissolved in 750 μl dimethyl sulfoxide after media were aspirated. The solution was transferred to 96-well plate and the optical density value was recorded at 570 nm on Microplate Reader (Model 680, Bio-Rad, USA). The results were expressed relative to OD value in the CEC monoculture on the first day.

Migration assay

To test the effect of RPE cells on CEC migration under hypoxia, a migration assay model was used (Fig. 6a). RPE cells were plated at density of 1.0×10^5/ cm^2 in the 24-well plates. RPE cells of two wells were transfected by pshHIF-1α, control pDNA as described above for transfection. 200 μM $CoCl_2$ was added into the wells after transfection for 24 h. There were three control wells: one without transfection, one without transfection and $CoCl_2$, and the last without RPE cells plated. The CEC migration assay was performed using fibronectin-coated, transwell inserts with 8-μm pore size. Briefly, 50,000 cells were seeded into inserts incubated with DMEM with 1% FBS. After 1 h attachment, the inserts were put into the 24-well plates described above. After incubation for 5 h, the inserts were fixed with methanol and stained with hematoxylin. The number of migrated cells was counted using phasecontrast microscopy (200×). Five randomly chosen fields were counted per insert.

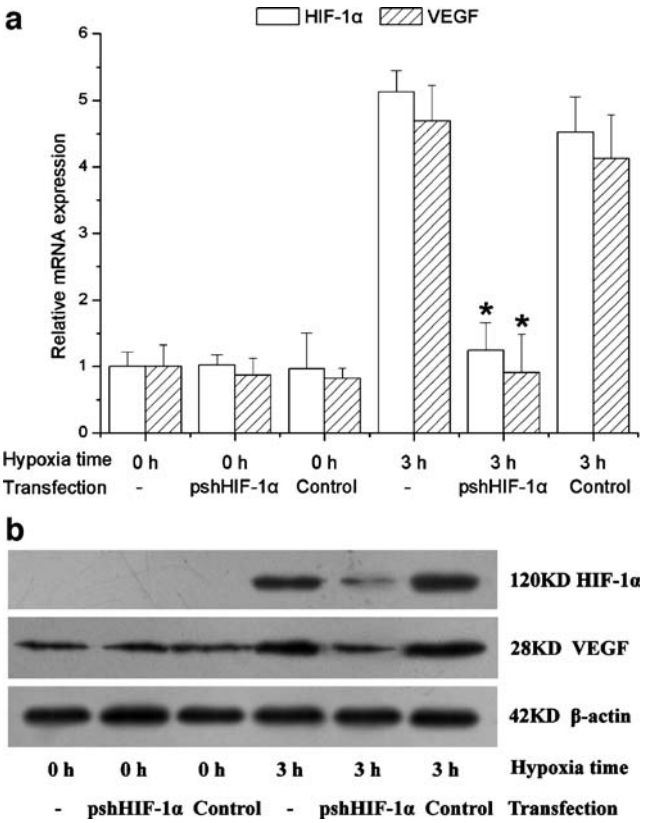

Fig. 3 mRNA (**a**) and protein (**b**) expressions of HIF-1α and VEGF in RPE cells with or without transfection with pshHIF-1α and control pDNA. RPE cells were exposed to 200 μM $CoCl_2$ for 3 h to achieve chemical hypoxia. Basal reference levels in normoxic cells without transfection are defined as 1 (mean±SD, $n=3$). (* $P<0.01$ vs control at 3 h)

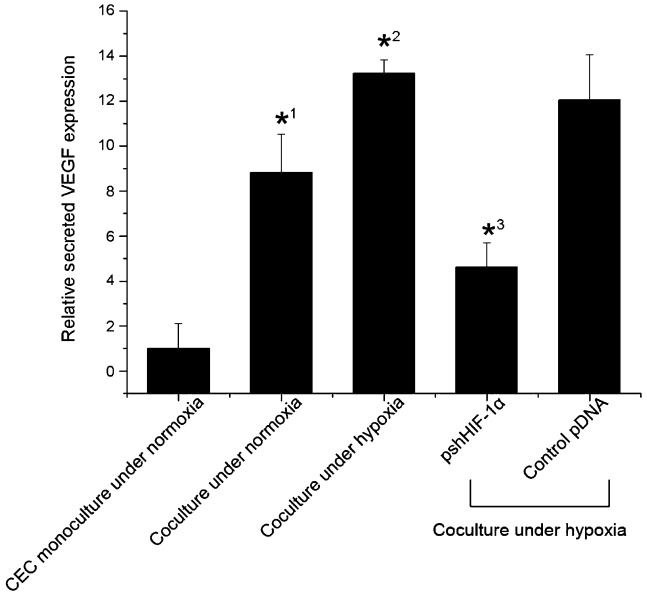

Fig. 4 Secreted VEGF expression in proliferation assay at 24 h. Soluble VEGF protein in conditioned media was expressed relative to CEC monoculture. Media from the coculture under normoxia had significantly greater VEGF expression than that of CEC monoculture under normoxia, and media from coculture under hypoxia had significantly greater VEGF than that of coculture under normoxia (*[1] $P<0.01$ vs CEC solo. *[2] $P<0.01$ vs coculture under normoxia). After RPE cells were transfected with pshHIF-1α, soluble VEGF level was significantly decreased compared to the control with pDNA transfection (*[3] $P<0.01$ vs control pDNA)

Tube formation assay

A coculture model for tube formation assay was used (Fig. 8a), in which a two-dimensional tube formation was measured in a collagen gel. 200 μl ECM was placed in 24-well plates and incubated at 37°C for 1 h to form gels. After polymerization of the gels, 1.0×10^5 CECs were seeded on each well and incubated with 1% FBS DMEM (1.0 ml). Then, the inserts described above in proliferation assay were put into the wells. Five different fields were chosen randomly in each well, and photographs were taken with a phase-contrast microscope in 48 h. The length of the tubes was measured using Image-Pro Plus software (Media Cybernetics, L.P., Silver Spring, MD, USA) and expressed as a total length (mm) per microscopic field for each single well.

Statistical analysis

Experiments were performed three times, and each experiment was performed in triplicate. All data from quantitative assays were expressed as the mean±standard deviation and statistically analyzed using one-way ANOVA analysis and independent-samples *t*-test. $P<0.01$ was considered to be statistically significant.

Results

Cell culture

CECs formed confluent monolayers which showed a cobblestone shape when observed with a phase-contrast microscope (Fig. 1a). They were confirmed to be vascular endothelial cells by their positive immunostaining for von Willebrand factor and by their incorporation of Dil-Ac-LDL (Fig. 1b,c).

Time-course of HIF-1α expression and subsequent VEGF induction in RPE cells

Under normoxia, even though HIF-1α protein was undetectable, the mRNA of HIF-1α was expressed. The mRNA levels of HIF-1α were enhanced 1 h after hypoxic exposure. they reached maximum level at 3 h (6.1-fold compared with normoxia) and decreased back to normal

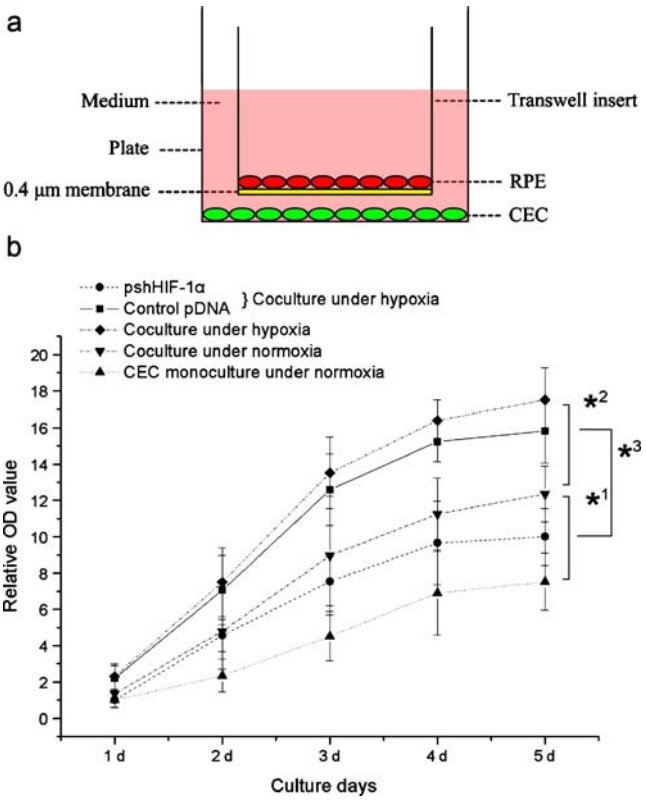

Fig. 5 Effect of pshHIF-1α on CEC proliferation in proliferation assay. **a** Proliferation assay model RPE, retinal pigment epithelial cell; CEC, choroidal microvascular endothelial cell. **b** CEC growth curves from five groups were depicted, and results showed that the proliferation of coculture group increased compared with CEC monoculture (*[1] $P<0.01$ vs CEC monoculture) and hypoxia significantly increased CEC proliferation in the coculture system (*[2] $P<0.01$ vs coculture under normoxia) on days 3, 4 and 5. After RPE cells were transfected by pshHIF-1α, the proliferation of CECs was significantly inhibited compared with the group transfected by control pDNA (*[3] $P<0.01$ vs control pDNA)

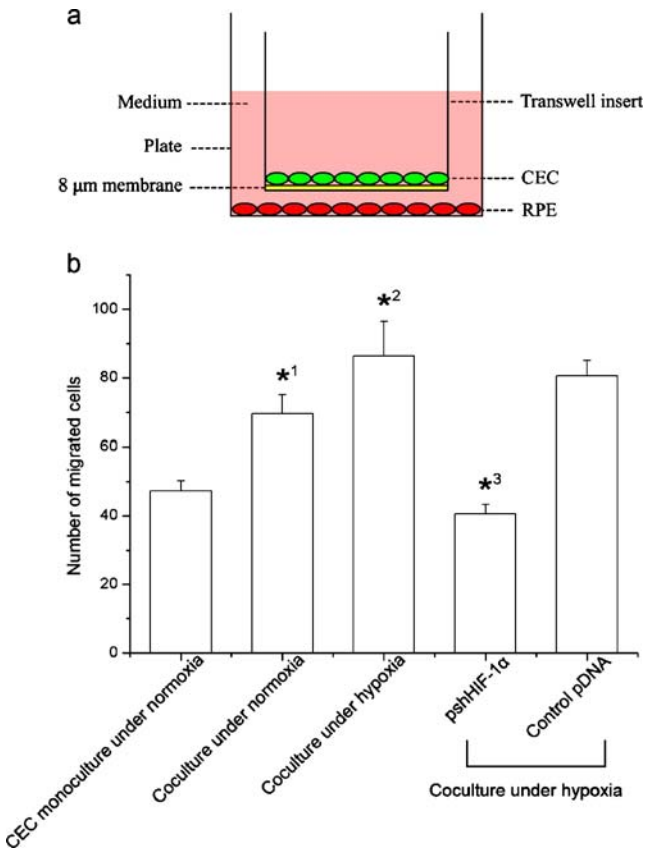

Fig. 6 Effect of pshHIF-1α on CEC migration in migration assay. **a** Migration assay model. **b** The coculture system increased the number of migrated CECs by 47.5% for 5 h. (*[1] $P<0.01$ vs CEC monoculture). The number of migrated cells increased by 24.3% in response to hypoxia (*[2] $P<0.01$ vs coculture under normoxia). The number of migrated CECs was significantly reduced by 49.6% compared with control pDNA group after RPE cells tranfected by pshHIF-1α (*[3] $P<0.01$ vs control pDNA)

level at 12 h (Fig. 2a). HIF-1α protein was stable under hypoxia, increased to its maximum 3 h after hypoxic exposure, and thereafter slowly declined (Fig. 2b).

VEGF in RPE cells was up-regulated in a similar fashion. The highest level of VEGF mRNA was observed at 3 h under hypoxia (5.2-fold compared with normoxia) (Fig. 2a), while VEGF protein reached its peak at 12 h (Fig. 2b).

Reduction in mRNA and protein by transfection of pshHIF-1α to RPE cells

Under normoxic conditions (0 h), transfection of RPE cells with pshHIF-1α had no effect on basal mRNA expression of HIF-1α or VEGF compared with control and non-transfected cells. The HIF-1α mRNA enhanced 5.1-fold 3 h after $CoCl_2$ treatment. The VEGF mRNA was up-regulated in a similar fashion 3 h after chemical hypoxia. The $CoCl_2$-induced up-regulation of both HIF-1α and VEGF at mRNA level was clearly abrogated by shRNA against HIF-1α, while control pDNA had no effect (Fig. 3a).

The protein of HIF-1α and VEGF had similar results to those for mRNA expressions, except that under normoxic conditions there was no HIF-1α expression detected (Fig. 3b).

Secreted VEGF protein expression in RPE-CEC coculture systems under hypoxia and transfection with pshHIF-1α

The supernatant from five groups of the proliferation assay model at 24 h was collected, and secreted VEGF expression was determined by ELISA. Results showed that coculture of RPE cells and CECs significantly increased the VEGF secretion compared with CEC monoculture ($P<0.01$ vs CEC monoculture). For the coculture situation, hypoxia also significantly increased VEGF secretion ($P<0.01$ vs coculture under normoxia). After RPE cells were transfected by pshHIF-1α, soluble VEGF was significantly decreased by 61.5% compared with control pDNA transfection ($P<0.01$ vs control pDNA) (Fig. 4).

Inhibition of CEC proliferation in the coculture system

The proliferation assay showed that hypoxia and coculture with RPE cells increased the proliferation of CECs respectively. Moreover, hypoxia and coculture had a synergistic effect. When the RPE cells were exposed to hypoxia and cocultured with CECs, CEC proliferation reflected by OD value was the highest. However, after RPE cells were transfected in the coculture system by pshHIF-1α, the proliferation of CECs was significantly inhibited compared with the group transfected by control pDNA on days 3, 4 and 5, and the inhibition rate was 40.2%, 36.6% and 36.8% respectively. ANOVA analysis revealed that each of the groups (pshHIF-1α vs control pDNA, coculture under hypoxia vs coculture under normoxia, and coculture under normoxia vs CEC monoculture under normoxia) showed statistically significant differences on day 3, 4 and 5 ($P<0.01$) (Fig. 5b).

Inhibition of CEC migration in the coculture system

CECs migrated more effectively when cocultured with RPE cells under normoxia. The coculture system increased the number of migrated CECs by 47.5% at 5 h. In the coculture condition, the number of migrated cells increased by 24.3% in response to hypoxia. Furthermore, after RPE cells tranfected by pshHIF-1α, the number of migrated CECs was significantly reduced by 49.6% compared with control pDNA group. ANOVA analysis revealed that each of the groups (pshHIF-1α vs control pDNA, coculture under hypoxia vs coculture under normoxia, and coculture under

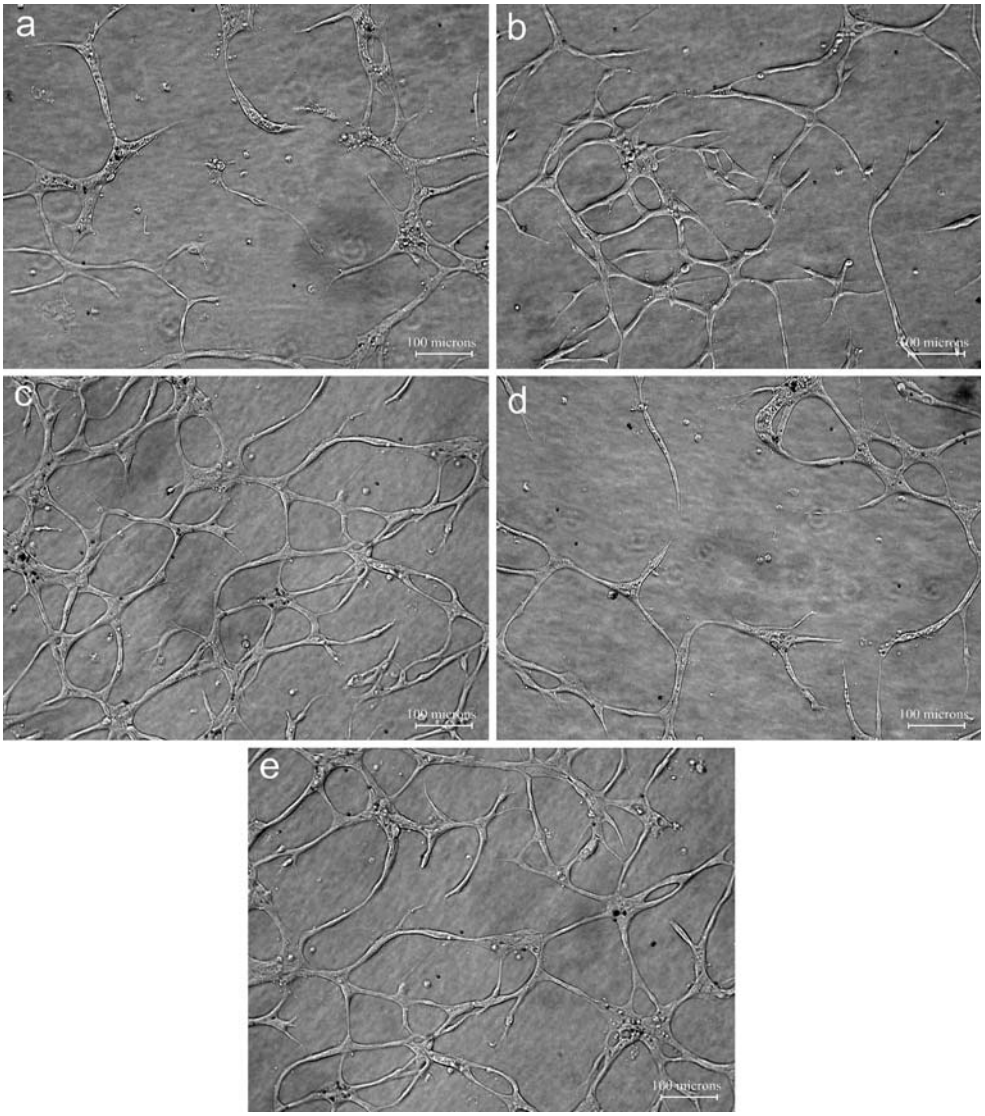

Fig. 7 Effect of pshHIF-1α on CEC tube formation in tube formation assay. Representative photographs of each group are shown. **a** CEC monoculture under normoxia. **b** Coculture under normoxia. **c** Coculture under hypoxia. **d** pshHIF-1α. **e** Control pDNA (*Bar*=100 μm)

normoxia vs CEC monoculture under normoxia) showed statistically significant differences ($P<0.01$) (Fig. 6b).

Inhibition of CEC tube formation in the coculture system

CEC grown in collagen matrix gel alone showed minimal tube formation (Fig. 7a). In the CEC gel cultures with RPE inserts, tubes formed well (Fig. 7b) and when 200 μM $CoCl_2$ was added into the RPE inserts, more tubes were formed (Fig. 7c). After the RPE inserts transfected by pshHIF-1α, tubes were reduced by 40.4% compared with the control pDNA group (Fig. 7d,e). The total length of the tubes was measured and analyzed by ANOVA. Results revealed that each of the groups (pshHIF-1α vs control pDNA, coculture under hypoxia vs coculture under normoxia, and coculture under normoxia vs CEC monoculture under normoxia) showed statistically significant differences ($P<0.01$) (Fig. 8b).

Discussion

In our study, the expression of HIF-1α protein and mRNA in RPE cells could be increased under chemical hypoxia, followed by increasing expression of VEGF. Consequently, hypoxia significantly increased the proliferation, migration and tube formation of CECs in the coculture systems. It was interesting that the coculture systems also enhanced the proliferation, migration and tube formation of CECs under normoxia. It can be understood that RPE cells can basally secrete VEGF and other growth factors. As shown in the study, the VEGF mRNA and protein were expressed in RPE cells under normoxia. In addition, the VEGF expression of the coculture system was higher than that of the noncoculture system at 24 h. Other studies have confirmed that, and showed that the CEC-RPE coculture increased production of soluble VEGF of RPE cells, which in turn facilitated CEC transmigration [15, 16].

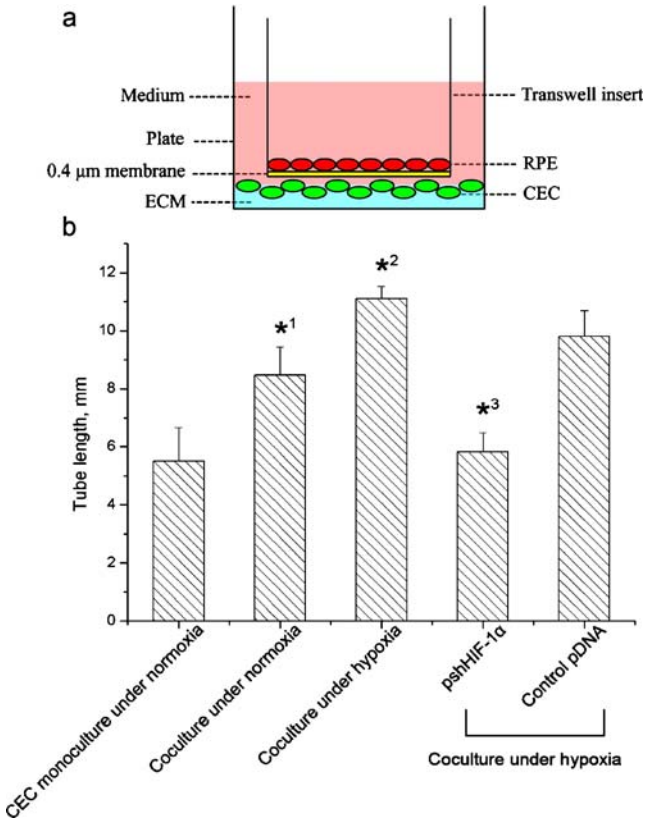

Fig. 8 The total length of tube formed in tube formation assay. **a** Tube formation assay. ECM, extracellular matrix. **b** There were more tubes formed when CECs cocultured with RPE cells under normoxia (*[1] $P<0.01$ vs CEC monoculture). Hypoxia enhanced the tube formation in the coculture system (*[2] $P<0.01$ vs coculture under normoxia). Less tubes formed when RPE cells were knocked down the HIF-1α (*[3] $P<0.01$ vs control pDNA)

Using the RNAi technique targeting HIF-1α of RPE, we observed that HIF-1α mRNA and protein expression was abrogated in cultured RPE cells at 3 h under hypoxia; consequently, the VEGF mRNA and protein expression also diminished, compared with the control pDNA transfection. There were similar results in RPE cells at 6 h under hypoxia (data not shown). We therefore concluded that pshHIF-1α transfection was functional to silence HIF-1α expression. As we predicted, silencing HIF-1α of RPE cells further significantly attenuated the proliferation, migration and tube formation of CECs under hypoxia. Accordingly, the soluble VEGF in the supernatant of coculture systems in which RPE cells were transfected by pshHIF-1α was diminished significantly at 24 h under hypoxia. The results indicated that by utilizing the novel therapeutic target, HIF-1α, partly through attenuating the VEGF transcription, angiogenesis could be inhibited.

Our study utilized the RPE-CEC coculture systems and chemical hypoxia induced by $CoCl_2$ to observe the effect of RPE cells on CECs. Even though the chemical hypoxia method was not identical to physical hypoxia, it was easy and manipulable to mimic the hypoxic condition of solo RPE cells, and could induce the expression of HIF-1α and the downstream transcript, VEGF [17, 18]. However, physical hypoxia seemed chronic compared with hypoxia induced by $CoCl_2$. Under physical hypoxia (3% O_2), HIF-1α protein levels showed an increasing trend during the first 24 h, and at 36 h were decreased to basal line in RPE cells [19]. But $CoCl_2$ just induced an acute function to increase the expression of HIF-1α in RPE cells and other cells [3, 18, 20, 21]. Several studies have used the RPE-CEC coculture systems to observe the interaction between the two kinds of cells [15, 16, 22]. However, these coculture systems did not use the intervention of hypoxia. Furthermore, the physical hypoxia will not only affect RPE cells but also CECs in the coculture systems composed by RPE cells and CECs. Therefore, it cannot simulate the relative hypoxia of RPE layer in vivo. So our models have some predominance to investigate effects of hypoxia in such conditions.

RNA interference mediated by siRNA is a powerful technique, allowing the silencing of mammalian genes with great specificity and potency. Previous studies have demonstrated that HIF-1α siRNA could be used to effectively and specifically inhibit the expression of VEGF levels in human cell lines, such as glioma cells, pancreatic and hepatobiliary cancer cells [23, 24]. It has also been shown that subretinal delivery of siRNA directed against murine VEGF significantly inhibited CNV after laser photocoagulation [25]. And intravitreal injection of VEGF siRNA was capable of inhibiting the growth and vascular permeability of laser-induced CNV in a nonhuman primate in a dose-dependent manner [26]. Recent studies using siRNA directed against VEGF or VEGF receptors in CNV animal models have showed promising results [27].

Although siRNA targeting VEGF allows potent attenuation of VEGF, thus preventing CNV and provides promise for cure of AMD, we believe that siRNA targeting HIF-1α is more powerful than siRNA targeting VEGF for the treatment of CNV. Recently, some studies have showed that siRNA targeting HIF-1α significantly attenuated VEGF expression of RPE cells under hypoxia, and human umbilical vein endothelial cells (HUVEC) tube formation was diminished after being cultured with the conditioned media of RPE cells [3, 28]. There has been some evidence demonstrating that increased expression of VEGF in RPE alone is not sufficient to cause CNV [29]. Several possible factors have been shown to play an important role in the pathogenesis of retinal and choroidal neovascularization independent of VEGF, such as EPO, insulin-like growth factor (IGF-1), angiopoietin and stroma-derived factor-1 (SDF-1). In addition, endogenous angiogenic inhibitors, such as PEDF, play an important role in development of CNV. Many clinical studies more consistently suggest that the decrease of PEDF in the eye is correlated with CNV formation [30, 31]. These findings

strongly suggest that the decrease of PEDF in the RPE layer and the choroid is the major event of the disturbed balance between angiogenic-stimulating and angiogenic-inhibiting systems, and contributes to consequent CNV formation [32]. Previous studies have suggested that hypoxia and VEGF can downregulate PEDF through proteolytic degradation [33]. Therefore, HIF-1 is a remarkable example of a single transcription factor that can be regarded as a "master switch" regulating the oxygen-dependent genes, such as VEGF, EPO and IGF-1. Furthermore, more and more studies have showed that for the treatment of CNV, a single treatment was not very effective, and combined therapies were promising to enhance the therapeutic efficacy [34]. So, siRNA targeting HIF-1α perhaps not only downregulates the VEGF expression, but also adjusts some other uncertain factors to achieve a synergistic effect.

Although it has been demonstrated that using the RNAi technique targeting HIF-1α of RPE can effectively decrease the VEGF expression and inhibit the angiogenesis of CECs in vitro, there have been many problems in its application in vivo, such as administration route and efficiency of siRNA. The conventional administration route of gene therapy to the retina and choroid includes intravitreal and subretinal injection, both of which are invasive and have potential side effects including intraocular infection, hemorrhage and retinal detachment. Recent progress in periocular delivery of siRNA or virus vector in the treatment of retinal and choroidal neovascularization has raised a great promise to overcome these disadvantages [27, 35]. So, further studies are needed to find more effective methods such as virus vector and investigate the treatment effect of HIF-1α silencing on CNV in animals or humans.

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References

1. Lambooij AC, van Wely KH, Lindenbergh-Kortleve DJ, Kuijpers RW, Kliffen M, Mooy CM (2003) Insulin-like growth factor-I and its receptor in neovascular age-related macular degeneration. Invest Ophthalmol Vis Sci 44:2192–2198, Medline. DOI 10.1167/iovs.02–0410
2. Schlingemann RO (2004) Role of growth factors and the wound healing response in age-related macular degeneration. Graefe Arch Clin Exp Ophthalmol 242:91–101, Medline. DOI 10.1007/s00417–003–0828–0
3. Zhang P, Wang Y, Hui Y, Hu D, Wang H, Zhou J, Du H (2007) Inhibition of VEGF expression by targeting HIF-1 alpha with small interference RNA in human RPE cells. Ophthalmologica 221:411–417, Medline. DOI 10.1159/000107502
4. Penfold PL, Madigan MC, Gillies MC, Provis JM (2001) Immunological and aetiological aspects of macular degeneration. Prog Retin Eye Res 20:385–414, Medline. DOI 10.1016/S1350–9462(00)00025–2
5. Ferrara N, Damico L, Shams N, Lowman H, Kim R (2006) Development of ranibizumab, an anti-vascular endothelial growth factor antigen binding fragment, as therapy for neovascular age-related macular degeneration. Retina 26:859–870, Medline. DOI 10.1097/01.iae.0000242842.14624.e7
6. Kinose F, Roscilli G, Lamartina S, Anderson KD, Bonelli F, Spence SG, Ciliberto G, Vogt TF, Holder DJ, Toniatti C, Thut CJ (2005) Inhibition of retinal and choroidal neovascularization by a novel KDR kinase inhibitor. Mol Vis 11:366–373
7. Aiello LP (2005) Angiogenic pathways in diabetic retinopathy. N Engl J Med 353:839–841, Medline. DOI 10.1056/NEJMe058142
8. Ruiz-Moreno JM, Montero JA, Zarbin MA (2007) Photodynamic therapy and high-dose intravitreal triamcinolone to treat exudative age-related macular degeneration: 2-year outcome. Retina 27:458–461, Medline. DOI 10.1097/IAE.0b013e318030c77c
9. Iriyama A, Obata R, Inoue Y, Takahashi H, Tamaki Y, Yanagi Y (2008) Effect of posterior juxtascleral triamcinolone acetonide on the efficacy and choriocapillaris hypoperfusion of photodynamic therapy. Graefe Arch Clin Exp Ophthalmol 246:339–344, Medline. DOI 10.1007/s00417–007–0667–5
10. Bracken CP, Fedele AO, Linke S, Balrak W, Lisy K, Whitelaw ML, Peet DJ (2006) Cell-specific regulation of hypoxia-inducible factor (HIF)-1alpha and HIF-2alpha stabilization and transactivation in a graded oxygen environment. J Biol Chem 281:22575–22585, Medline. DOI 10.1074/jbc.M600288200
11. Check E (2005) A crucial test. Nat Med 11:243–244, Medline. DOI 10.1038/nm0305–243
12. Murata M, Takanami T, Shimizu S, Kubota Y, Horiuchi S, Habano W, Ma JX, Sato S (2006) Inhibition of ocular angiogenesis by diced small interfering RNAs (siRNAs) specific to vascular endothelial growth factor (VEGF). Curr Eye Res 31:171–180, Medline. DOI 10.1080/02713680500514636
13. Wang YS, Hui YN, Wiedemann P (2002) Role of apoptosis in the cytotoxic effect mediated by daunorubicin in cultured human retinal pigment epithelial cells. J Ocul Pharmacol Ther 18:377–387, Medline. DOI 10.1089/10807680260218542
14. Wang YS, Eichler W, Friedrichs U, Yafai Y, Hoffmann S, Yasukawa T, Hui YN, Wiedemann P (2005) Impact of endostatin on bFGF-induced proliferation, migration, and matrix metalloproteinase-2 expression/secretion of bovine choroidal endothelial cells. Curr Eye Res 30:479–489, Medline. DOI 10.1080/02713680590959358
15. Geisen P, McColm JR, Hartnett ME (2006) Choroidal endothelial cells transmigrate across the retinal pigment epithelium but do not proliferate in response to soluble vascular endothelial growth factor. Exp Eye Res 82:608–619, Medline. DOI 10.1016/j.exer.2005.08.021
16. Peterson LJ, Wittchen ES, Geisen P, Burridge K, Hartnett ME (2007) Heterotypic RPE-choroidal endothelial cell contact increases choroidal endothelial cell transmigration via PI 3-kinase and Rac1. Exp Eye Res 84:737–744, Medline. DOI 10.1016/j.exer.2006.12.012
17. Slomiany MG, Rosenzweig SA (2004) Autocrine effects of IGF-I-induced VEGF and IGFBP-3 secretion in retinal pigment epithelial cell line ARPE-19. Am J Physiol Cell Physiol 287:C746–753, Medline. DOI 10.1152/ajpcell.00568.2003
18. Slomiany MG, Rosenzweig SA (2006) Hypoxia-inducible factor-1-dependent and -independent regulation of insulin-like growth factor-1-stimulated vascular endothelial growth factor secretion. J Pharmacol Exp Ther 318:666–675, Medline. DOI 10.1124/jpet.106.104158

19. Forooghian F, Razavi R, Timms L (2007) Hypoxia-inducible factor expression in human RPE cells. Br J Ophthalmol 91:1406–1410, Medline. DOI 10.1136/bjo.2007.123125
20. Mojsilovic-Petrovic J, Callaghan D, Cui H, Dean C, Stanimirovic DB, Zhang W (2007) Hypoxia-inducible factor-1 (HIF-1) is involved in the regulation of hypoxia-stimulated expression of monocyte chemoattractant protein-1 (MCP-1/CCL2) and MCP-5 (Ccl12) in astrocytes. J Neuroinflammation 4:12, Medline. DOI 10.1186/1742–2094–4–12
21. Newcomb EW, Lukyanov Y, Schnee T, Ali MA, Lan L, Zagzag D (2006) Noscapine inhibits hypoxia-mediated HIF-1alpha expression andangiogenesis in vitro: a novel function for an old drug. Int J Oncol 28:1121–1130
22. Fan W, Zheng JJ, McLaughlin BJ (2002) An in vitro model of the back of the eye for studying retinal pigment epithelial-choroidal endothelial interactions. In Vitro Cell Dev Biol Anim 38:228–234, Medline. DOI 10.1290/1071–2690(2002)038
23. Mizuno T, Nagao M, Yamada Y, Narikiyo M, Ueno M, Miyagishi M, Taira K, Nakajima Y (2006) Small interfering RNA expression vector targeting hypoxia-inducible factor 1 alpha inhibits tumor growth in hepatobiliary and pancreatic cancers. Cancer Gene Ther 13:131–140, Medline. DOI 10.1038/sj.cgt.7700871
24. Jensen RL, Ragel BT, Whang K, Gillespie D (2006) Inhibition of hypoxia inducible factor-1alpha (HIF-1alpha) decreases vascular endothelial growth factor (VEGF) secretion and tumor growth in malignant gliomas. J Neurooncol 78:233–247, Medline. DOI 10.1007/s11060–005–9103-z
25. Reich SJ, Fosnot J, Kuroki A, Tang W, Yang X, Maguire AM, Bennett J, Tolentino MJ (2003) Small interfering RNA (siRNA) targeting VEGF effectively inhibits ocular neovascularization in a mouse model. Mol Vis 9:210–216
26. Tolentino MJ, Brucker AJ, Fosnot J, Ying GS, Wu IH, Malik G, Wan S, Reich SJ (2004) Intravitreal injection of vascular endothelial growth factor small interfering RNA inhibits growth and leakage in a nonhuman primate, laser-induced model of choroidal neovascularization. Retina 24:132–138, Medline. DOI 10.1097/00006982–200402000–00018
27. Campochiaro PA (2006) Potential applications for RNAi to probe pathogenesis and develop new treatments for ocular disorders. Gene Ther 13:559–562, Medline. DOI 10.1038/sj.gt.3302653
28. Forooghian F, Das B (2007) Anti-angiogenic effects of ribonucleic acid interference targeting vascular endothelial growth factor and hypoxia-inducible factor-1alpha. Am J Ophthalmol 144:761–768, Medline. DOI 10.1016/j.ajo.2007.07.022
29. Schwesinger C, Yee C, Rohan RM, Joussen AM, Fernandez A, Meyer TN, Poulaki V, Ma JJ, Redmond TM, Liu S, Adamis AP, D'Amato RJ (2001) Intrachoroidal neovascularization in transgenic mice overexpressing vascular endothelial growth factor in the retinal pigment epithelium. Am J Pathol 158:1161–1172
30. Holekamp NM, Bouck N, Volpert O (2002) Pigment epithelium-derived factor is deficient in the vitreous of patients with choroidal neovascularization due to age-related macular degeneration. Am J Ophthalmol 134:220–227, Medline. DOI 10.1016/S0002–9394(02)01549–0
31. Bhutto IA, McLeod DS, Hasegawa T, Kim SY, Merges C, Tong P, Lutty GA (2006) Pigment epithelium-derived factor (PEDF) and vascular endothelial growth factor (VEGF) in aged human choroid and eyes with age-related macular degeneration. Exp Eye Res 82:99–110, Medline. DOI 10.1016/j.exer.2005.05.007
32. Zhang SX, Ma JX (2007) Ocular neovascularization: Implication of endogenous angiogenic inhibitors and potential therapy. Prog Retin Eye Res 26:1–37, Medline. DOI 10.1016/j.preteyeres.2006.09.002
33. Notari L, Miller A, Martinez A, Amaral J, Ju M, Robinson G, Smith LE, Becerra SP (2005) Pigment epithelium-derived factor is a substrate for matrix metalloproteinase type 2 and type 9: implications for downregulation in hypoxia. Invest Ophthalmol Vis Sci 46:2736–2747, Medline. DOI 10.1167/iovs.04–1489
34. Spaide RF (2006) Rationale for combination therapies for choroidal neovascularization. Am J Ophthalmol 141:149–156, Medline. DOI 10.1016/j.ajo.2005.07.025
35. Saishin Y, Silva RL, Saishin Y, Kachi S, Aslam S, Gong YY, Lai H, Carrion M, Harris B, Hamilton M, Wei L, Campochiaro PA (2005) Periocular gene transfer of pigment epithelium-derived factor inhibits choroidal neovascularization in a human-sized eye. Hum Gene Ther 16:473–478, Medline. DOI 10.1089/hum.2005.16.473