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# Silencing heme oxygenase-1 gene expression in retinal pigment epithelial cells inhibits proliferation, migration and tube formation of cocultured endothelial cells

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ABSTRACT

Heme oxygenase-1 (HO-1) plays an important role in the vasculature and in the angiogenesis of tumors, wounds and other environments. Retinal pigment epithelial (RPE) cells and choroidal endothelial cells (CECs) are the main cells involved in choroidal neovascularization (CNV), a process in which hypoxia plays an important role. Our aim was to evaluate the role of human RPE-cell HO-1 in the angiogenic activities of cocultured endothelial cells under hypoxia. Small interfering RNA (siRNA) for HO-1 was transfected into human RPE cell line ARPE-19, and zinc protoporphyrin (ZnPP) was used to inhibit HO-1 activity. Knockdown of HO-1 expression and inhibition of HO-1 activity resulted in potent reduction of the expression of vascular endothelial growth factor (VEGF) under hypoxia. Furthermore, knockdown of HO-1 suppressed the proliferation, migration and tube formation of cocultured endothelial cells. These findings indicated that HO-1 might have an angiogenic effect in CNV through modulation of VEGF expression and might be a potential target for treating CNV.



## 1. Introduction

Age-related macular degeneration (AMD) is a leading cause of irreversible vision loss in the elderly throughout the world. Approximately 10–15% of the cases of AMD are the exudative type, which is characterized by choroidal neovascularization (CNV), the growth of abnormal blood vessels from the choroid via Bruch's membrane into the sub-retinal space and neurosensory retina [1,2]. These abnormal blood vessels eventually result in disciform scarring, leading to permanent loss of central vision [2]. The mechanisms involved in the development of CNV are complex and incompletely understood. Among these mechanisms, the migration and proliferation of choroidal endothelial cells (CECs), followed by their formation of capillary-like structures, are critical and final steps; the initiation of these processes has been related to hypoxia, inflammation, and imbalance of pro- and anti-angiogenic factors [3,4]. Vascular endothelial growth factor (VEGF) is the most important pathogenic factor in vaso-proliferative disorders, including CNV. Retinal pigment epithelium (RPE) under hypoxic conditions has been suggested to be the source of VEGF in these cases [5,6].

Heme oxygenases (HOs) are rate-limiting enzymes that catalyze the conversion of heme into carbon monoxide (CO), free iron and biliverdin. There are two isoforms: HO-1 (inducible) and HO-2 (constitutive). HO-1 is widely distributed and highly induced by a range of stimuli including inflammation, oxidative stress, nitric oxide (NO), and hypoxia [7]. HO-1 has a significant functional role in the vasculature, and its involvement in the angiogenesis of tumors, wounds, and various experimental models has been demonstrated [8]. HO-1 contributes to the formation of blood vessels both directly, through enhancing the angiogenic activities of endothelial cells, and indirectly, through regulating VEGF expression in vivo and in vitro [9,10]. HO-1 is a potent antioxidant enzyme and is usually considered to have beneficial effects in AMD [11,12], but this has not been confirmed directly. Whether HO-1 performs an angiogenic role in CNV is still unknown, and the aim of the present study was to investigate the role of HO-1 in the expression of VEGF in RPE cells and the impact of the knockdown of HO-1 on the proliferation, migration and tube formation of cocultured endothelial cells.

## 2. Materials and methods

### 2.1. Cell culture

The human retinal pigment epithelium cell line ARPE-19 was obtained from the American Type Culture Collection (ATCC, Mantissa, VA) and was cultured in Dulbecco's modified essential medium/Ham's F12 medium (DMEM/F12) (Gibco, Grand Island, NY), supplemented with 10% heat-inactivated fetal bovine serum (FBS,

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JRH Biosciences, Lenexa, KS), 100 U/ml penicillin, and 100 μg/ml streptomycin. The cells were cultured at 5% $CO_2$ and 37 °C and were used at passages 10–15. Human umbilical vein endothelial cells (HUVECs) were purchased from Dainippon Sumitomo Pharma Biomedical Company (DSP, Osaka, Japan) and were cultured in DMEM (Gibco, Grand Island, NY, USA), supplemented with 10% FBS, 75 μg/ml endothelial cell 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. The cells were cultured at 5% $CO_2$ and 37 °C and were used at passages 3–6.

### 2.2. Small interfering RNA (siRNA) transfection

Human-specific HO-1 siRNA and non-targeted negative control siRNA were purchased from Santa Cruz Biotechnology Signaling Technology (Santa Cruz, CA, USA). ARPE-19 cells were seeded onto six-well plates at the recommended density and grown to 60–80% confluence before transfection. siRNAs were transfected into ARPE-19 cells at a final concentration of 30 nM with siRNA Transfection Reagent (Santa Cruz, CA, USA) according to the manufacturer's instructions.

### 2.3. RNA extraction and semi-quantitative RT–PCR

ARPE-19 cells were grown in six-well plates in DMEM/F12 with 10% FBS until confluence. For hypoxia treatment, 200 μM $CoCl_2$ (Sigma Aldrich, St. Louis, MO) was added for 0, 2, 4, 8, 12 and 24 h. For zinc protoporphyrin (ZnPP, Sigma Aldrich, St. Louis, MO) treatment, RPE cells were pretreated by 10 μM ZnPP for 1 h and then 200 μM $CoCl_2$ was added for 8 h. Total RNA was extracted from cells using Trizol reagent (Invitrogen, Carlsbad, CA). First-strand cDNA was synthesized with 500 ng DNA-free total RNA for each sample and an oligo-(dT) primer using the TaKaRa RNA PCR Kit (AMV) Ver.3.0 (TaKaRa, Tokyo, Japan). PCR analysis was performed with the DNA Engine Opticon 2 System (Bio-Rad, Richmond, CA, USA). cDNA was amplified using the following primer pairs: HO-1: forward: 5′-ATG GAG CGT CCG CAA CCC GA-3′, reverse: 5′-GCA TAA AGC CCT ACA GCA AC-3′, 860 bp; VEGF: forward: 5′-TTG CCT TGC TGC TCT ACC TC-3′, reverse: 5′-AAA TGC TTT CTC CGC TCT GA-3′, 424 bp; GAPDH: forward: 5′-GAA GGT GAA GGT CGG AGT C-3′, reverse: 5′-GAA GAT GGT GAT GGG ATT TC-3′, 313 bp. The PCR reactions for VEGF and GAPDH consisted of an initial denaturation at 94 °C for 3 min, followed by 29 cycles of denaturation at 94 °C for 30 s, annealing at 58 °C for 30 s and extension at 72 °C for 30 s, and a final extension at 72 °C for 7 min. The PCR reaction for HO-1 consisted of an initial denaturation at 94 °C for 3 min, followed by 31 cycles of denaturation at 94 °C for 30 s, annealing at 62 °C for 30 s and extension at 72 °C for 40 s, and a final extension at 72 °C for 7 min. GAPDH was used as an internal control for sample normalization. PCR products were separated on 1.2% agarose gels and visualized by staining with ethidium bromide.

### 2.4. Western blotting

For western blotting assays, cells were treated as in the RT-PCR assay above. Cells were washed with ice-cold PBS, and the cells were lysed in CelLytic M Mammalian Cell Lysis/Extraction Reagent (Sigma, NY) with added protease inhibitor cocktail (Nacalai tesque, Kyoto, Japan). The lysates were centrifuged (15,000×*g* for 10 min at 4 °C). The protein concentration was quantified by a Pierce® BCA Protein Assay Kit (Thermo Fisher Scientific Inc, Hampton, NH). The lysates (30 μg per lane) were separated by SDS–polyacrylamide gel (Precast 5%–20% gel, Atto Corp, Tokyo, Japan) and transferred to a Clear Blot Membrane-P (Atto Corp, Tokyo, Japan) at 30 V for 30 min. The membranes were blocked in 5% non-fat milk for 1 h at room temperature and were then incubated with primary antibodies (1:500 for the antibody to HO-1, 1:200 for VEGF, 1:2000 for β-actin; Santa Cruz, CA, USA) in 5% non-fat milk overnight at 4 °C. After washing, the membranes were further incubated with horseradish peroxidase-linked secondary antibodies (1:10,000) for 1 h at room temperature. Signals were developed using the enhanced chemiluminescence system (ECL; GE Healthcare, Buckinghamshire, UK). Images were obtained and quantified using a Bio-Rad Versa Doc imaging system model 5000 (Bio-Rad, Hercules, CA) with Bio-Rad Quantity One software. The amount of protein expression was standardized against the amount of β-actin. The results were expressed relative to the untreated control.

### 2.5. Cell proliferation assay

The coculture system of ARPE-19 cells and HUVECs was designed based on previously described methods [6]. All Transwell systems used in this study were purchased from Corning (NY, USA). HUVECs were seeded at a density of $5 \times 10^4$/ml in 24-well plates in complete medium and allowed to adhere overnight. Medium was then exchanged with DMEM with 1% FBS. ARPE-19 cells transfected with either HO-1 siRNA or control siRNA and non-transfected cells were seeded at a density of $1 \times 10^5$/ml on Transwell inserts with 0.4-μm pore size membrane and allowed to adhere overnight. The inserts were then put into the wells, and 200 μM $CoCl_2$ was added into the inserts. Experiments were performed on three groups: HUVECs cocultured with non-transfected ARPE-19 cells, HUVECs cocultured with ARPE-19 cells transfected with HO-1 siRNA or HUVECs cocultured with ARPE-19 cells transfected with control siRNA. All groups were under hypoxic conditions. Cell proliferation was analyzed by a 3-(4, 5-dimethylthiazol-2-yl)-2, 5 diphenyl tetrazolium bromide (MTT, Sigma, St. Louis, MO) assay on days 1, 2 and 3. Briefly, 100 μl MTT solution was added per well after the inserts were taken out, and they were incubated for 4 h. The formazan crystals formed were dissolved by dimethylsulfoxide after media were aspirated. The solution was transferred to a 96-well plate, and the optical density value was measured at 570 nm using a microplate reader (MTP-800; CORONA, Tokyo, Japan).

### 2.6. Cell migration assay

APRE-19 cells were seeded at a density of $5 \times 10^4$/ml in 24-well plates. The three groups were separated as described above in the proliferation assay. RPE cells were transfected with HO-1 siRNA or control siRNA as described above. 200 μM $CoCl_2$ was added into the wells after transfection. HUVECs ($5 \times 10^4$) were seeded on Transwell inserts with 8-μm pore size membrane and were incubated with DMEM with 1% FBS. After 1 h attachment, the inserts were put into the 24-well plates containing RPE cells. After incubation for 8 h, the inserts were fixed with methanol for 10 min and stained with 0.5% crystal violet solution for 30 min. The number of migrated cells was counted using phase-contrast microscopy (200×). Five randomly chosen fields were counted per insert.

### 2.7. Tube formation assay

In the tube formation assay, 24-well plates were coated with 200 μl growth factor-reduced (GFR) Matrigel (BD Biosciences, CA, USA) and incubated at 37 °C for 1 h to form gels. HUVECs ($1 \times 10^5$/ml) were seeded on the gel of each well and incubated with 1% FBS DMEM. Then, the inserts (described above in the section about the proliferation assay) were placed into the wells. Five different fields were chosen randomly in each well, and

photographs were taken with a phase-contrast microscope after 48 h. The length of the tubes was measured using Image J software.

### 2.8. Statistical analysis

All assays were performed using at least three separate experiments in triplicate, and data were expressed as the mean ± standard error (SE). Statistical analysis of data was performed by one-way ANOVA and a Student–Newman-Keuls test, and statistical significance was set at $P < 0.05$.

## 3. Results

### 3.1. Expression of HO-1 and VEGF in RPE cells under hypoxia

To determine whether hypoxia affects the expression of HO-1 and VEGF in human retinal pigment epithelial cells, ARPE-19 cells were treated with 200 μM $CoCl_2$, and then RT-PCR and western blotting were performed. HO-1 and VEGF mRNA were present under normal conditions, and their levels were increased 2 h after $CoCl_2$ treatment at the same rate. The maximum level was reached at 8 h (Fig. 1A and B). Levels of VEGF and HO-1 protein were up-regulated in a similar manner as the mRNA. The highest level of HO-1 protein was observed at 12 h under hypoxia, whereas the VEGF protein reached its maximum level at 8 h (Fig. 1C and D).

### 3.2. Inhibition of HO-1 activity reduced the expression of VEGF in RPE cells under hypoxia

To determine the effect of HO-1 activity on VEGF expression, ARPE-19 cells were incubated with 10 μM ZnPP for 9 h under normoxia and hypoxia, and then RT-PCR and western blotting were performed. VEGF mRNA and protein expression under normoxia were not affected by ZnPP administration compared with the control group. Hypoxia enhanced VEGF expression, whereas concomitant administration of ZnPP abolished hypoxia-induced VEGF mRNA and protein expression in ARPE-19 cells (Fig. 2).

### 3.3. Knockdown of HO-1 down-regulated the expression of VEGF in RPE cells

To further explore the involvement of HO-1 in regulation of VEGF expression, HO-1 siRNA was transfected into ARPE-19 cells

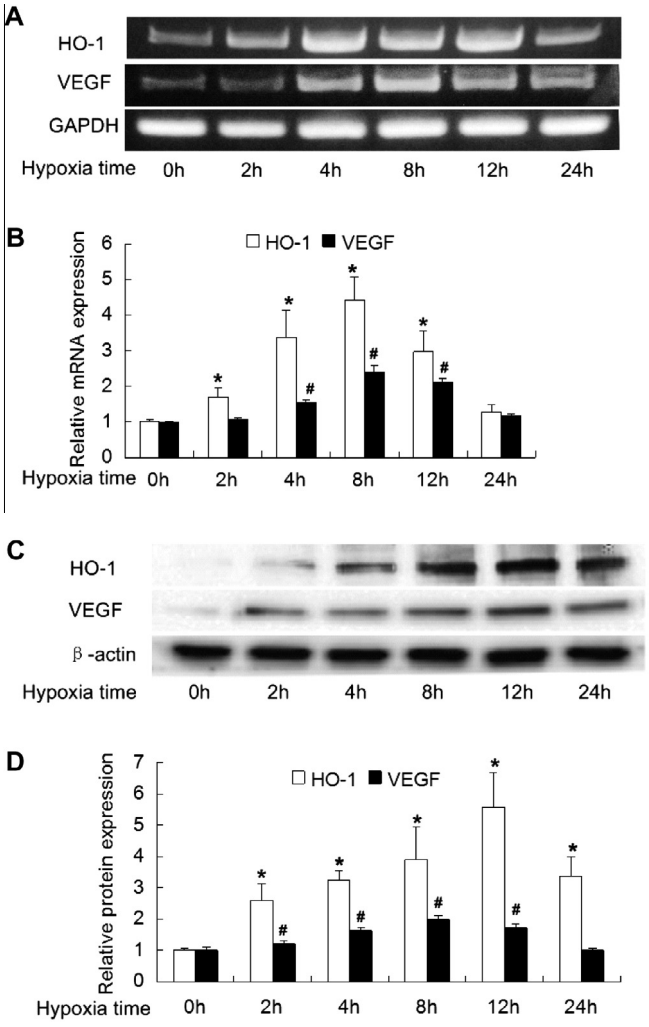


**Fig. 1.** Effects of hypoxia on the expression of HO-1 and VEGF in ARPE-19 cells. RT-PCR and western blotting analysis were used to measure the mRNA (A) and protein (C) expression, respectively, of HO-1 and VEGF. ARPE-19 cells were exposed to 200 μM $CoCl_2$ for 0, 2, 4, 8, 12 and 24 h. Compared with 0 h, the expression of HO-1 and VEGF mRNA (B) and protein (D) was up-regulated in response to hypoxia ($^{*}P < 0.05$ vs 0 h of HO-1, $^{\#}P < 0.05$ vs 0 h of VEGF, $n = 3$).

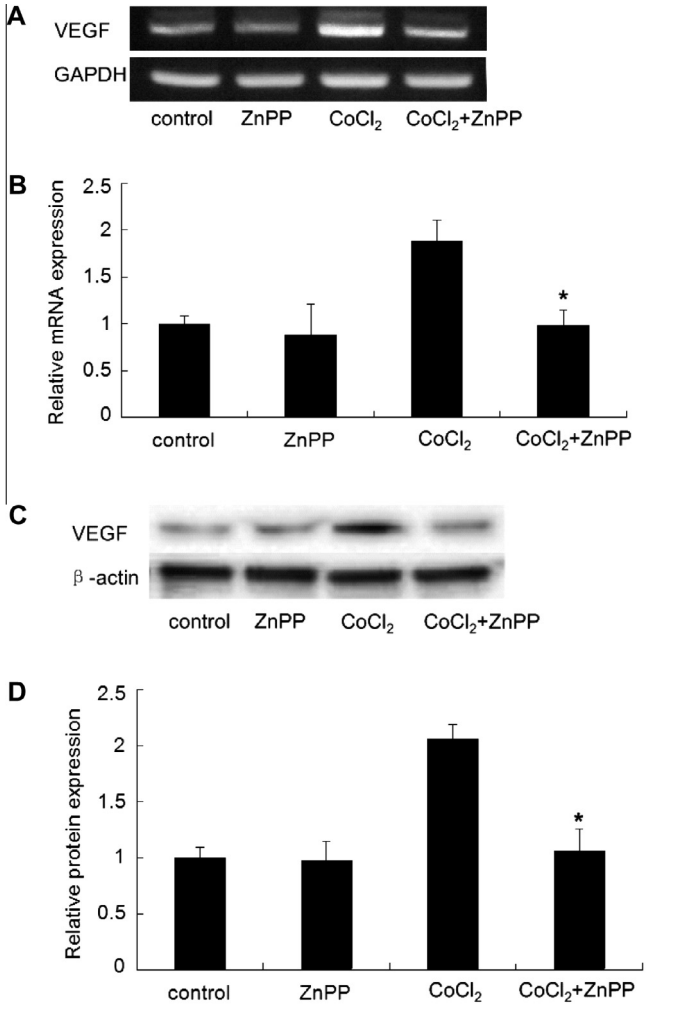


**Fig. 2.** Effects of ZnPP administration on VEGF expression in ARPE-19 cells RT-PCR and western blotting analysis were used to measure VEGF mRNA (A) and protein (C) expression, respectively. ARPE-19 cells were pretreated with 10 μM ZnPP for 1 h, then exposed to 200 μM $CoCl_2$ for 8 h. ZnPP administration significantly diminished the up-regulation of VEGF mRNA (B) and protein (D) expression induced by hypoxia ($^{*}P < 0.05$ vs $CoCl_2$, $n = 3$).

to knock down the expression of HO-1. HO-1 siRNA transfection significantly inhibited the expression of HO-1 mRNA under both normoxia and hypoxia. The expression of VEGF mRNA was not affected by HO-1 siRNA transfection under normoxia; however, it was enhanced by hypoxia treatment, and the up-regulation was clearly abrogated by HO-1 siRNA (Fig. 3A and B). The expression of HO-1 and VEGF protein gave similar results to the expression of mRNA, except that under normoxia HO-1 protein expression was not affected by HO-1 siRNA; this may be due to its extremely low basal expression detected by western blotting (Fig. 3C and D).

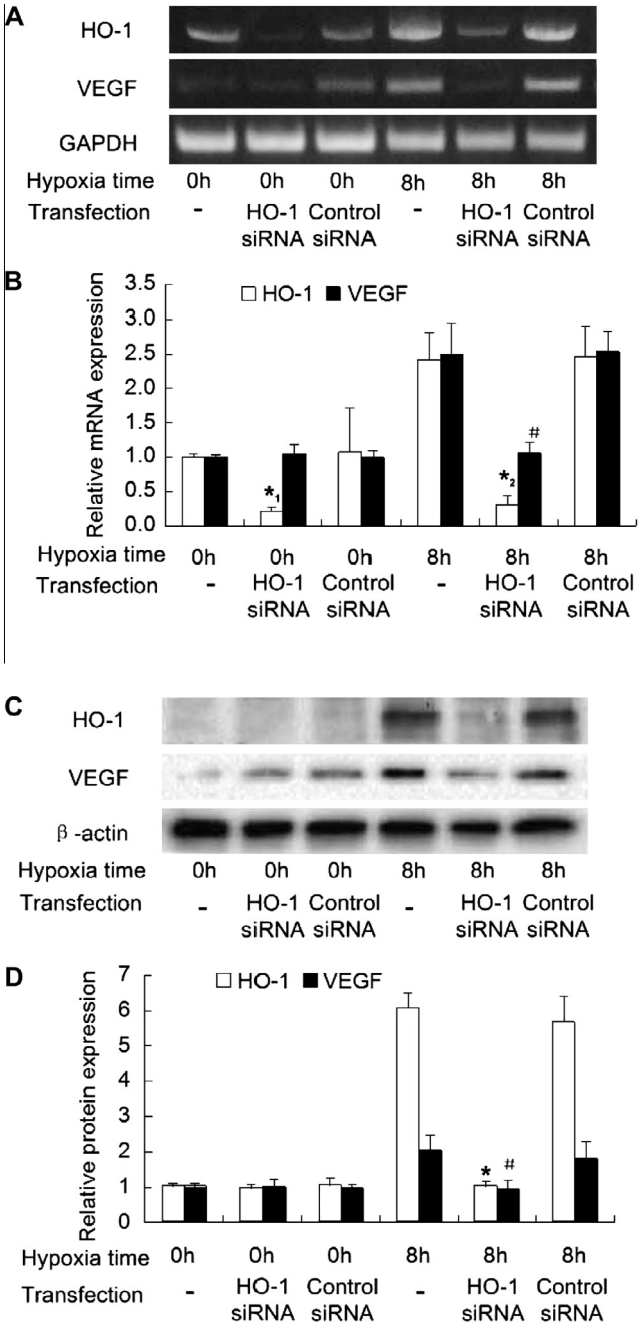


**Fig. 3.** Effects of HO-1 siRNA transfection on HO-1 and VEGF expression in ARPE-19 cells. RT-PCR and western blotting analysis were used to measure the mRNA (A) and protein (C) expression, respectively, of HO-1 and VEGF. ARPE-19 cells were transfected with HO-1 siRNA or control siRNA. For hypoxia treatment, 200 μM $CoCl_2$ was added for 8 h. HO-1 mRNA expression was significantly inhibited by HO-1 siRNA under normoxia ($^{*}{}_{1}P < 0.05$ vs non-transfected and control siRNA at 0 h). Under hypoxia, the expression of HO-1 and VEGF mRNA (B) and protein (D) was significantly inhibited by HO-1 siRNA but not control siRNA ($^{*}{}_{2}$, $^{*}P < 0.05$ vs non-transfected and control siRNA at 8 h of HO-1, $^{\#}P < 0.05$ vs non-transfected and control siRNA at 8 h of VEGF, $n = 3$).

### 3.4. Knockdown of HO-1 in ARPE-19 cells inhibited the proliferation of cocultured HUVECs

To determine the effect of knockdown of HO-1 in RPE cells on the proliferation of endothelial cells under hypoxic conditions, ARPE-19 cells and HUVECs were cocultured in a Transwell system and analyzed by the MTT assay. On day 1, there was no difference between the three groups. On days 2 and 3, for the HUVECs cultured with RPE cells that were transfected with HO-1 siRNA, the proliferation was significantly inhibited compared with the group transfected with control siRNA or the non-transfected group (Fig. 4A).

### 3.5. Knockdown of HO-1 in ARPE-19 cells inhibited the migration of cocultured HUVECs

To determine the effect of knockdown of HO-1 in RPE cells on the migration of endothelial cells, ARPE-19 cells and HUVECs were cocultured in a Transwell system and analyzed by counting the number of migrated HUVECs. When HUVECs were cultured with RPE cells in which HO-1 had been knocked down, the number of migrated cells was significantly reduced compared with the control siRNA-transfected group or the non-transfected group (Fig. 4B and C).

### 3.6. Knockdown of HO-1 in ARPE-19 cells inhibited the tube formation of cocultured HUVECs

To determine whether knockdown of HO-1 in RPE cells affects the tube formation of endothelial cells, ARPE-19 cells and HUVECs were cocultured in a Transwell system, and the formation of tubes by HUVECs was monitored. Tube formation was significantly inhibited when HUVECs were cultured with RPE cells transfected with HO-1 siRNA, compared with the group transfected with control siRNA or the non-transfected group (Fig. 4D and E).

## 4. Discussion

There is an increasing number of studies investigating the role of HO-1 in angiogenesis. Our findings indicate that HO-1 plays an important role in the expression of VEGF in RPE cells under hypoxic conditions and consequently promotes the proliferation, migration and tube formation of cocultured endothelial cells.

HO-1 is a ubiquitous stress-inducible enzyme; nitric oxide, hypoxia, as well as cytokines and growth factors also enhance HO-1 expression in a number of cell types. Although it has been reported that HO-1 expression was reduced by hypoxia in human umbilical vein endothelial cells [13], it was not reduced in RPE cells [14]. In this study, we showed that HO-1 mRNA and protein levels were elevated when RPE cells were treated with chemical hypoxia achieved by $CoCl_2$, followed by increased expression of VEGF, which implicated involvement of HO-1 in the response of RPE cells to hypoxia.

VEGF is the most important mediator for CNV and other neovascular disorders. Numerous studies have revealed the involvement of HO-1 in VEGF synthesis [8]. Inhibition of HO-1 activity resulted in the reduction of the synthesis of VEGF induced by hypoxia and prostaglandin $J_2$ in vascular smooth muscle cells [15] and endothelial cells [16]. Furthermore, genetic overexpression of HO-1 potently enhanced the synthesis of VEGF both in vivo [17] and in vitro [18]. Using ZnPP to inhibit HO activity, we observed that

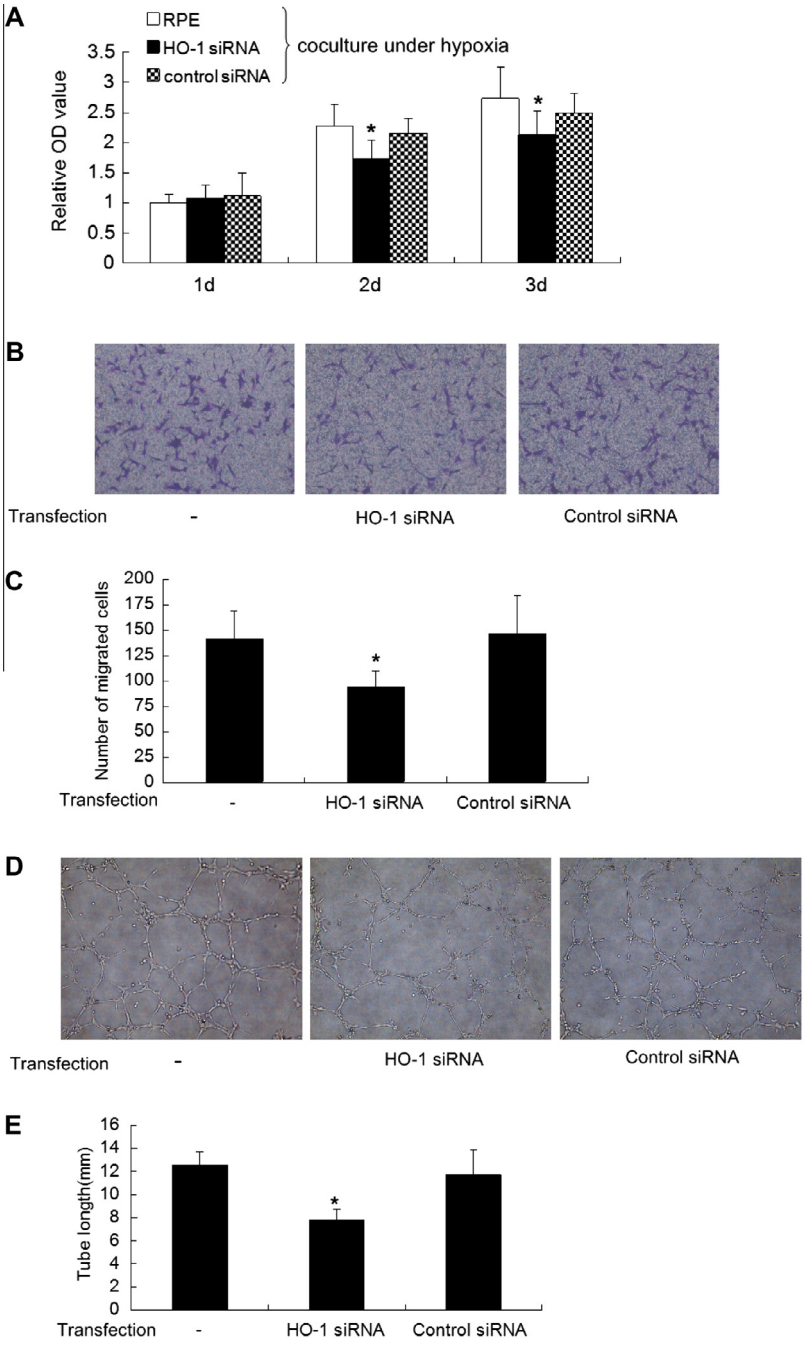


**Fig. 4.** Effects of HO-1 siRNA transfection in RPE cells on the proliferation, migration and tube formation of cocultured HUVECs under hypoxia ARPE-19 cells transfected with HO-1 siRNA or control siRNA and non-transfected cells were cocultured with HUVECs in a Transwell coculture system. 200 μM $CoCl_2$ was added to induce hypoxia. (A) Proliferation assay. HO-1 siRNA transfection in RPE cells significantly inhibited the proliferation of cocultured HUVECs on days 2 and 3 ($^*P < 0.05$ vs non-transfected and control siRNA, $n = 5$). (B) Migration assay. Cells were photographed under a phase-contrast microscope at 200× magnification. (C) The number of migrated cells was significantly decreased by transfection with HO-1 siRNA in RPE cells ($^*P < 0.05$ vs non-transfected and control siRNA, $n = 5$). (D) Tube formation assay. Tubes were photographed under a phase-contrast microscope at 200× magnification. (E) Tube formation was significantly inhibited by transfection with HO-1siRNA in RPE cells ($^*P < 0.05$ vs non-transfected and control siRNA, $n = 5$).

the VEGF mRNA and protein expression of RPE cells induced by hypoxia was diminished, and the expression of VEGF under normoxia was not affected, indicating the involvement of HO-1 in hypoxia-induced VEGF expression. Because ZnPP can inhibit the activities of all types of HOs, but not specifically HO-1, we then utilized siRNA targeting HO-1 and observed that HO-1 mRNA and protein expression was eliminated in RPE cells at 8 h under hypoxia, followed by a decrease of VEGF expression compared to cells transfected with the control siRNA. This genetic evidence further confirmed that hypoxia enhanced VEGF expression partly in an HO-1 dependent manner.

The precise mechanisms involved in the development of CNV are not entirely understood, though it is certain that both RPE cells and ECs participate in this process. To investigate the role of HO-1 in angiogenesis during the development of CNV, our studies utilized RPE-HUVEC coculture systems and chemical hypoxia induced by $CoCl_2$ to observe the effect of silencing HO-1 in RPE cells on the angiogenic activities of endothelial cells. We found that the

proliferation, migration and tube formation of HUVECs cultured with RPE cells were significantly inhibited by knockdown of HO-1 in RPE cells with siRNA. Combining the results above, we concluded that under hypoxic conditions, HO-1 expression was induced in RPE cells and that siRNA targeting HO-1 in RPE cells could effectively decrease VEGF expression and inhibit the angiogenesis of HUVECs in vitro. This demonstrated the angiogenic effect of HO-1 in RPE cells under hypoxic conditions, which may play a role in the development of CNV.

There has been increasing evidence for a causal relationship between inflammation and CNV, and HO-1 is commonly regarded as a potent anti-inflammatory enzyme. Yoshinaga [19] reported that the administration of the anti-inflammatory drug bromfenac decreased the formation of laser-induced CNV through an HO-1 dependent pathway, which inhibited the infiltration of macrophages. However, this needs to be confirmed by directly using genetic techniques targeting HO-1 to observe its impact on the formation of CNV. Because the laser-induced CNV model is an acute wound-healing process rather than a chronic and long-lasting tissue repairing process as in wet AMD, it is still possible that HO-1 inhibits inflammation while promoting tissue repair by facilitating angiogenesis [20,21].

In summary, our studies showed that HO-1 played an angiogenic role in CNV, partly through regulation of VEGF expression in RPE cells under hypoxic conditions. Although the contributions of HO-1 to CNV are complicated and have yet to be fully understood, our findings may facilitate a better understanding about the mechanisms of CNV, and further studies with in vivo models investigating the exact role of HO-1 in CNV are needed.

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