·Basic Research·

Small interfering RNA targeting PGC-1α inhibits VEGF expression and tube formation in human retinal vascular endothelial cells

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Abstract

• AIM: To determine whether small interfering RNA (siRNA) of PGC-1α could inhibit vascular endothelial growth factor (VEGF) expression and tube formation in human retinal vascular endothelial cells (hRVECs).

• METHODS: hRVECs transfected with peroxisome proliferator-activated receptor-γ coactivator-1α (PGC-1α) siRNA were incubated for 24h and then placed into a normoxic (20%, O_2) or hypoxic (1%, O_2) environment for another 16h. PGC-1α mRNA and protein levels were detected by real-time PCR and Western blot. VEGF mRNA and protein levels were detected by real-time PCR and ELISA. Cell proliferation was evaluated by BrdU incorporation assay. Forty-eight hours after siRNA transfection, hRVECs were planted into Matrigel-coated plates and cultured under normoxic (20%, O_2) or hypoxic (1%, O_2) conditions for another 48h. The tube formation of hRVECs was observed under an optical microscope and quantified by counting the number of branch points and calculating the total tube length.

• RESULTS: PGC-1α mRNA and protein levels were significantly reduced by PGC-1α siRNA, and VEGF mRNA and protein levels also decreased significantly. The percentage of BrdU-labeled cells in siPGC-1α groups were significantly decreased compared with control siRNA groups under normoxia and hypoxia in cell proliferation assay. In the tube formation assay, PGC-1α siRNA treated cells formed significantly fewer tubes.

• CONCLUSION: Blocking PGC-1α expression can inhibit VEGF expression in hRVECs and inhibit their ability to form tubes under both normoxic and hypoxic conditions.

• KEYWORDS: peroxisome proliferator-activated receptor-γ coactivator-1α; vascular endothelial growth factor; small interfering RNA; retinal vascular endothelial cell; tube formation

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INTRODUCTION

Neoangiogenesis is a common pathophysiological feature of many diseases. Ischemia and hypoxia induce the formation of new blood vessels by altering the balance between angiogenesis-promoting factors and angiogenesis-inhibiting factors. Nascent blood vessels grow from existing ones by sprouting. In certain diseases, new blood vessels often have abnormal vessel walls, which can lead to complications. Retinal neovascularization occurs in various ocular disorders including proliferative diabetic retinopathy, retinopathy of prematurity and secondary neovascular glaucoma which often result in blindness. Identifying novel methods of inhibiting neovascularization has long been the focus of a large body of research. Endothelial cells are important components of blood vessels and the main subject of the present study.

Peroxisome proliferator-activated receptor-γ coactivator-1α (PGC-1α) is a transcription cofactor that contains binding sites for a variety of nuclear hormone receptors. It regulates oxidation reactions and mitochondrial energy metabolism in various cells and tissues [1]. PGC-1α is expressed at high levels in many human and rodent tissues, including brown fat, skeletal muscle, the heart, kidney, liver, brain, and vascular endothelial cells [1, 2]. Under ischemic and hypoxic conditions, both expression and transcriptional regulation activity of PGC-1α are enhanced in rat cardiac myocytes[3,4] and brain cells [5,6], rabbit renal tubular cells [7], and human skeletal muscle cells [8,9]. It has recently been found that PGC-1α can induce the expression of angiogenic factors including vascular endothelial growth factor (VEGF) and promote the formation of new blood vessels[10]. PGC-1α may become a novel therapeutic target for improving the treatment of ischemic and hypoxic diseases[11].

RNA interference can inhibit gene expression with high specificity, high efficiency, and very few side effects. It has been widely used in gene function studies and gene therapies.

RNA interference involves using exogenous double-strand RNA to degrade mRNA containing homologous sequences, which silences the corresponding genes [12]. In gene therapy, RNA or DNA are often introduced into the body by liposome-mediated transfection. This method has several advantages, including low cytotoxicity, simple preparation, and considerable consistency. Liposomes are slowly metabolized *in vivo*, so they can remain in the body for a long time. They have the capacity for large DNA molecules and can protect the enclosed nucleotides from nuclease degradation. Liposomes can also transfect non-dividing cells effectively.

PGC-1α promotes the formation of new blood vessels, suggesting that it may become a novel target for angiogenesis inhibition. In this study, PGC-1α small interfering RNA (siRNA) was transfected into human retinal vascular endothelial cells (hRVECs) using liposomes, and then the effect of PGC-1α inhibition on VEGF expression, cell proliferation and endothelial cell tube formation was determined under normoxic and hypoxic conditions.

MATERIALS AND METHODS

Cell Culture hRVECs (HUM-CELL-0112, PriCells, Wuhan, Hubei Province China) were cultured in a DMEM medium containing 10% fetal bovine serum (HyClone, Logan, UT, USA) at 37℃ in a 5% CO_2, 20% O_2 environment. Then, 24h after planting, when cells reached the logarithmic growth phase, they were given fresh medium and then divided into the following groups: normoxia without siRNA, normoxia control siRNA, normoxia siPGC-1α, hypoxia without siRNA, hypoxia control siRNA, and hypoxia siPGC-1α. Cells in the siRNA groups were transfected siRNA accordingly. Then, 24h after transfection, cells were left under normoxic conditions or placed into a hypoxic environment (1% O_2, 5% CO_2, and 94% N_2) and cultured for a further 16h.

Cell Transfection Chemically synthesized PGC-1α siRNA and negative control siRNA were used for transfection. The sequences of PGC-1α siRNA were: sense strand 5'-CCAA GACUCUAGACAACUAdTdT-3', antisense strand 5'-UAG UUGUCUAGAGUCUUGG dTdT-3'. To make the transfection mixture, solutions A and B were first prepared in 1.5 mL microcentrifuge tubes. Then 250 μL medium was mixed with 10 μL PGC-1α siRNA or negative control siRNA as solution A; and 250 μL medium was mixed with 5 μL Lipofectamine2000 (Invitrogen, Carlsbad, CA, USA) as solution B. Both solutions were allowed to sit at room temperature for 5min. Then solution A was mixed with solution B and allowed to sit at room temperature for 20min. Cells in 6-well plates were washed with PBS twice and then the transfection mixture was added to the cells. The plates were gently rocked to make sure all cells came into contact with the mixture, and then 1.5 mL medium was added to the cells.

Real-time PCR to Measure PGC-1α and VEGF mRNA Expression Whole RNA was extracted from cells using Trizol (Invitrogen, Carlsbad, CA, US). The primers used in real-time PCR were as follows: human PGC-1α (165 bp): forward 5'-AAGGATGCGCTCTCGTTCAA-3' and reverse 5'-AAGGGAGAATTTCGGTGCGT-3'; human VEGF (124 bp): forward 5'-CTGTCTAATGCCCTGGAGCC-3' and reverse 5'-ACGCGAGTCTGTGTTTTTGC-3'. Human β-actin (169 bp): forward 5'-TCTGGCACCACACCTTCTAC-3' and reverse 5'-GATAGCACAGCCTGGATAGC-3'. PCR reaction conditions were as follows: denaturation at 94℃ for 3min followed by 40 cycles of 94℃ for 30s, 59℃ for 30s, and 72℃ for 45s.

Western Blot Detection of PGC-1α Protein Expression Total protein was extracted from cells, separated using polyacrylamide gel electrophoresis, and transferred to PVDF membranes (Pierce, Rockford, IL, USA). The membranes were gently shaken in the blocking solution for 1h at room temperature and then incubated overnight with primary antibodies at 4℃ with shaking. The primary antibodies used were as follows: mouse anti-human PGC-1α (Abcam, Cambridge, MA, USA) 1:1000, mouse anti-human β-actin (Santa Cruz Biotechnology, Dallas, TX, USA) 1:800. The membranes were washed, incubated with the secondary antibodies, and washed again. Antibodies bound to the membranes were detected using ECL chemiluminescence and exposed to X-ray films in the dark. The intensity of the protein bands was analyzed using BandScan 5.0 software. β-actin served as an internal control, and the relative level of PGC-1α was calculated as PGC-1α band intensity/β-actin band intensity.

Vascular Endothelial Growth Factor Protein Expression as Measured by ELISA The amount of VEGF secreted into the cell culture supernatant was measured with an ELISA kit (USCNK, Houston, TX, USA). Standard wells were filled with 100 μL of VEGF standard at various concentrations. Blank wells were filled with 100 μL of buffer. Sample wells were filled with 100 μL of sample. The microplates were then covered and incubated at 37℃ for 2h. Each well was then filled with 100 μL of the working solution of solution A, and the microplates were covered again and incubated at 37℃ for 1h. Then 100 μL working solution B was then added to each well, and the microplates were covered and incubated at 37℃ for 30min. Then 90 μL substrate solution was added to each well, and the microplates were covered and incubated at 37℃ for 20min in the dark. Finally, each well was filled with 50 μL stop solution to stop the reaction. The optical density at 450 nm was immediately measured with a plate reader.

Cell Proliferation Assay hRVECs were seeded in 96-well tissue culture plates and incubated for 24h. Cells were then starved in M199 medium (Invitrogen, Carlsbad, CA, USA)

containing 2% FBS in the absence of endothelial cell growth supplements for another 16h. After starvation, cells were transfected with siRNA for 24h, followed by culturing under normoxic conditions or hypoxic environment for another 16h. Cell proliferation was then determined using a Cell Proliferation ELISA, BrdU kit(Roche, Branchburg, NJ, USA) which based on the colorimetric detection of the incorporation of BrdU, following the manufacturer instructions.

Tube Formation Assay Forty-eight hours after cells were transfected with siRNA, the medium was replaced with endothelial cell basal medium, and the cells were planted into plates coated with Matrigel (BD Biosciences, Franklin Lakes, NJ, USA). However, 30-60min before planting the cells, 100 μL Matrigel was placed to the bottom of each well in a 24-well plate, avoiding bubbles. The plate was placed to 37℃ for 30-60min for Matrigel to solidify. Cells were dissociated and counted, and then 100-200 μL cells per well were added on top of the Matrigel. Cells were placed under normoxic (20% O_2) or hypoxic (1% O_2) conditions and cultured further for 48h. The tubes formed were observed and photographed under an optical microscope. Tube formation was quantified by counting the number of branch points and calculating the total tube length in six randomly chosen fields from each well[13].

Statistical Analysis One-way ANOVA was used for comparisons across all groups, and pair-wise comparisons between groups were performed using the LSD test. $P<0.05$ was considered statistically significant.

RESULTS

Changes in PGC-1α and VEGF mRNA Expression in hRVECs Real-time PCR was used to determine the levels of PGC-1α mRNA (Figure 1) and VEGF mRNA (Figure 2). Cells in the normoxia without siRNA and normoxia control siRNA groups expressed both PGC-1α and VEGF mRNA, and there was no significant difference between these two groups for either gene ($P>0.05$). The expression of both PGC-1α and VEGF mRNA was significantly downregulated in cells in the normoxia siPGC-1α relative to cells in the normoxia control siRNA group ($P<0.01$). The inhibition rates were 56% and 49% respectively. PGC-1α and VEGF mRNA levels were not significantly different between cells in the hypoxia without siRNA group and cells in the hypoxia control siRNA group ($P>0.05$), but were both significantly higher than cells under normoxic conditions. Cells in the hypoxia siPGC-1α group expressed significantly less PGC-1α and VEGF mRNA than cells in the hypoxia control siRNA group ($P<0.01$), and the inhibition rates were 57% and 51% respectively. In addition, cells in the hypoxia groups expressed more PGC-1α and VEGF mRNA compared with cells in their corresponding normoxia groups.

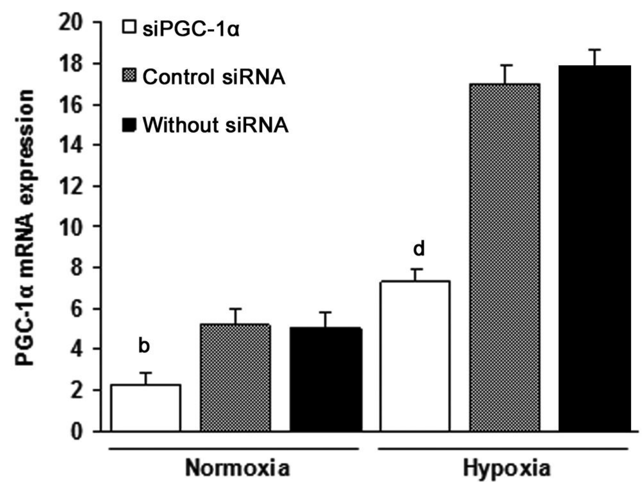

Figure 1 Changes in PGC-1α mRNA expression PGC-1α siRNA-mediated downregulation of PGC-1α mRNA expression in hRVECs at 16h of normoxia and hypoxia after transfection were examined by real-time PCR. The expression of PGC-1α mRNA were significantly downregulated in cells in the siPGC-1α groups relative to cells in the control siRNA groups under normoxia and hypoxia. $^bP<0.01$ *vs* normoxia control siRNA group, $^dP<0.01$ *vs* hypoxia control siRNA group.

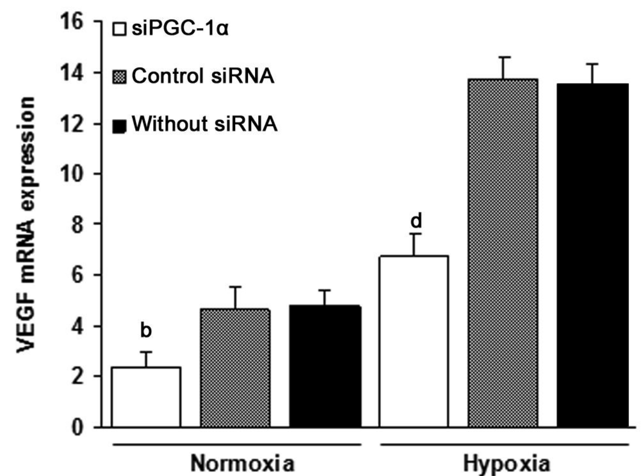

Figure 2 Changes in VEGF mRNA expression Effect of PGC-1α siRNA on VEGF mRNA expression in hRVECs at 16h of normoxia and hypoxia after transfection were examined by real-time PCR. The VEGF mRNA levels of siPGC-1α groups were significantly downregulated when compared with control siRNA groups during normoxia and hypoxia. $^bP<0.01$ *vs* normoxia control siRNA group, $^dP<0.01$ *vs* hypoxia control siRNA group.

Changes in PGC-1α and Vascular Endothelial Growth Factor Protein Levels in hRVECs Western blot assays were used to detect PGC-1α protein levels (Figure 3), and ELISA was used to detect VEGF protein levels (Figure 4). PGC-1α and VEGF proteins were expressed in cells in the normoxia without siRNA and normoxia control siRNA groups, and there was no significant difference between these two groups for either protein ($P>0.05$). The levels of both PGC-1α and VEGF proteins were significantly lower in cells in the normoxia siPGC-1α group compared with cells in the normoxia control siRNA group ($P<0.01$), and the inhibition rates were 53% and 43% respectively. PGC-1α and VEGF protein levels were not significantly different between cells in the hypoxia without siRNA group and cells in the hypoxia

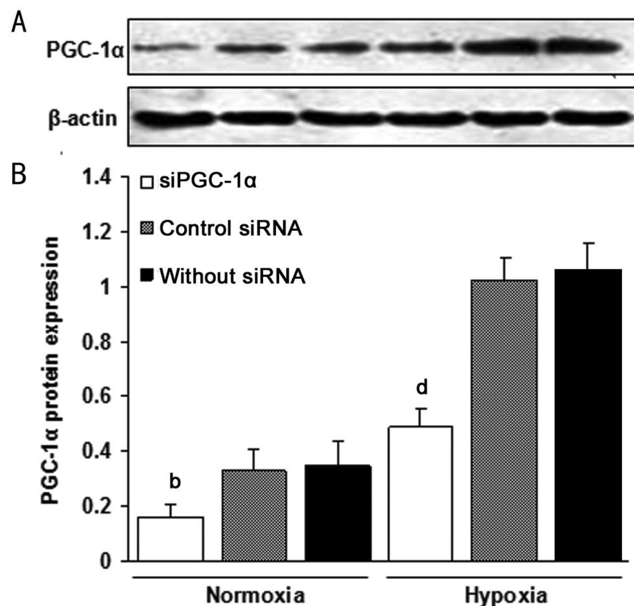

Figure 3 Changes in PGC-1α protein levels A: PGC-1α siRNA-mediated downregulation of PGC-1α protein production in hRVECs at 16h of normoxia and hypoxia after transfection were detected by Western blot; B: Quantitative results of the Western blot analysis in A. The levels of PGC-1α protein were significantly lower in cells in the siPGC-1α groups compared with cells in the control siRNA groups under normoxia and hypoxia. $^{b}P<0.01$ *vs* normoxia control siRNA group, $^{d}P<0.01$ *vs* hypoxia control siRNA group.

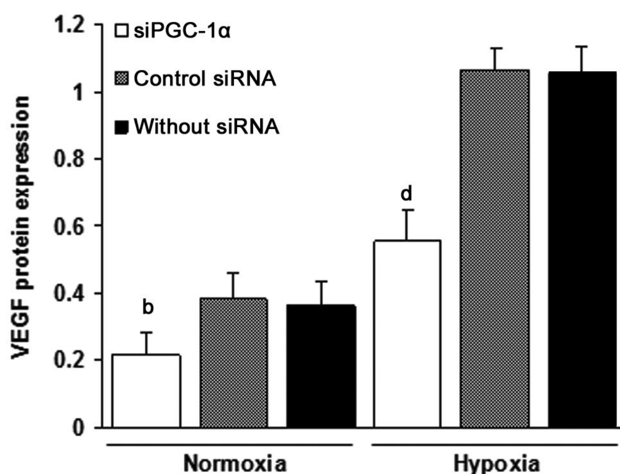

Figure 4 Changes in VEGF protein levels Effect of PGC-1α siRNA on VEGF protein production in hRVECs at 16h of normoxia and hypoxia after transfection were detected by ELISA. The VEGF protein production in siPGC-1α groups were significantly downregulated when compared with control siRNA groups under normoxia and hypoxia. $^{b}P<0.01$ *vs* normoxia control siRNA group, $^{d}P<0.01$ *vs* hypoxia control siRNA group.

control siRNA group ($P>0.05$), but were both significantly higher than in cells under normoxic conditions. Cells in the hypoxia siPGC-1α group expressed significantly lower levels of PGC-1α and VEGF proteins than cells in the hypoxia control siRNA group ($P<0.01$), and the inhibition rates were 52% and 48% respectively. In addition, cells in the hypoxia groups expressed more PGC-1α and VEGF proteins than cells in their corresponding normoxia groups.

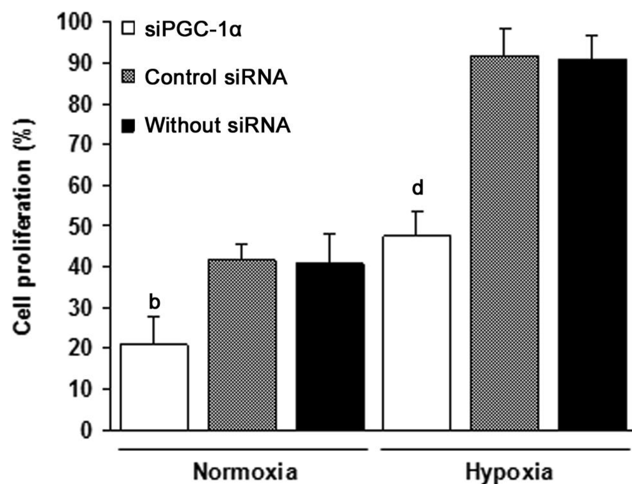

Figure 5 PGC-1α siRNA inhibits cell proliferation in hRVECs Effect of PGC-1α siRNA on cell proliferation in hRVECs at 16h of normoxia and hypoxia after transfection. The percentage of BrdU-labeled cells in siPGC-1α groups significantly decreased compared with control siRNA groups under normoxia and hypoxia. $^{b}P<0.01$ *vs* normoxia control siRNA group, $^{d}P<0.01$ *vs* hypoxia control siRNA group.

PGC-1α siRNA Inhibits Cell Proliferation in hRVECs Endothelial cell proliferation is an essential step in the progress of angiogenesis. To assess the anti-angiogenic activity of PGC-1α siRNA, we first evaluated its inhibitory effects on cell proliferation in hRVECs. As shown in Figure 5, there was no significant difference in the percentage of BrdU-labeled cells between normoxia without siRNA and normoxia control siRNA groups ($P>0.05$). The percentage of BrdU-labeled cells significantly decreased in the normoxia siPGC-1α group compared with normoxia control siRNA group ($P<0.01$). There was no significant difference in the percentage of BrdU-labeled cells between hypoxia without siRNA and hypoxia control siRNA groups ($P>0.05$), but the percentage of BrdU-labeled cells of hypoxia groups was significantly higher than cells in their corresponding normoxia groups. The percentage of BrdU-labeled cells significantly decreased in the hypoxia siPGC-1α group compared with hypoxia control siRNA group ($P<0.01$). PGC-1α siRNA significantly inhibited cell proliferation in hRVECs both under normoxia and hypoxia conditions.

PGC-1α siRNA Inhibits Cell Tube Formation in hRVECs When hRVECs were cultured in Matrigel-coated plates, they started to form tubes after 6h. The tubes appeared stabilized by 48h. Cells in both the normoxia without siRNA and normoxia control siRNA groups formed tubes. They showed no significant difference between these two groups in branch point numbers and total tube length ($P>0.05$). Cells in the normoxia siPGC-1α group formed significantly fewer tubes than cells in the normoxia control siRNA group ($P<0.01$). Cells in the hypoxia without siRNA group and the hypoxia control siRNA group markedly enhanced tube

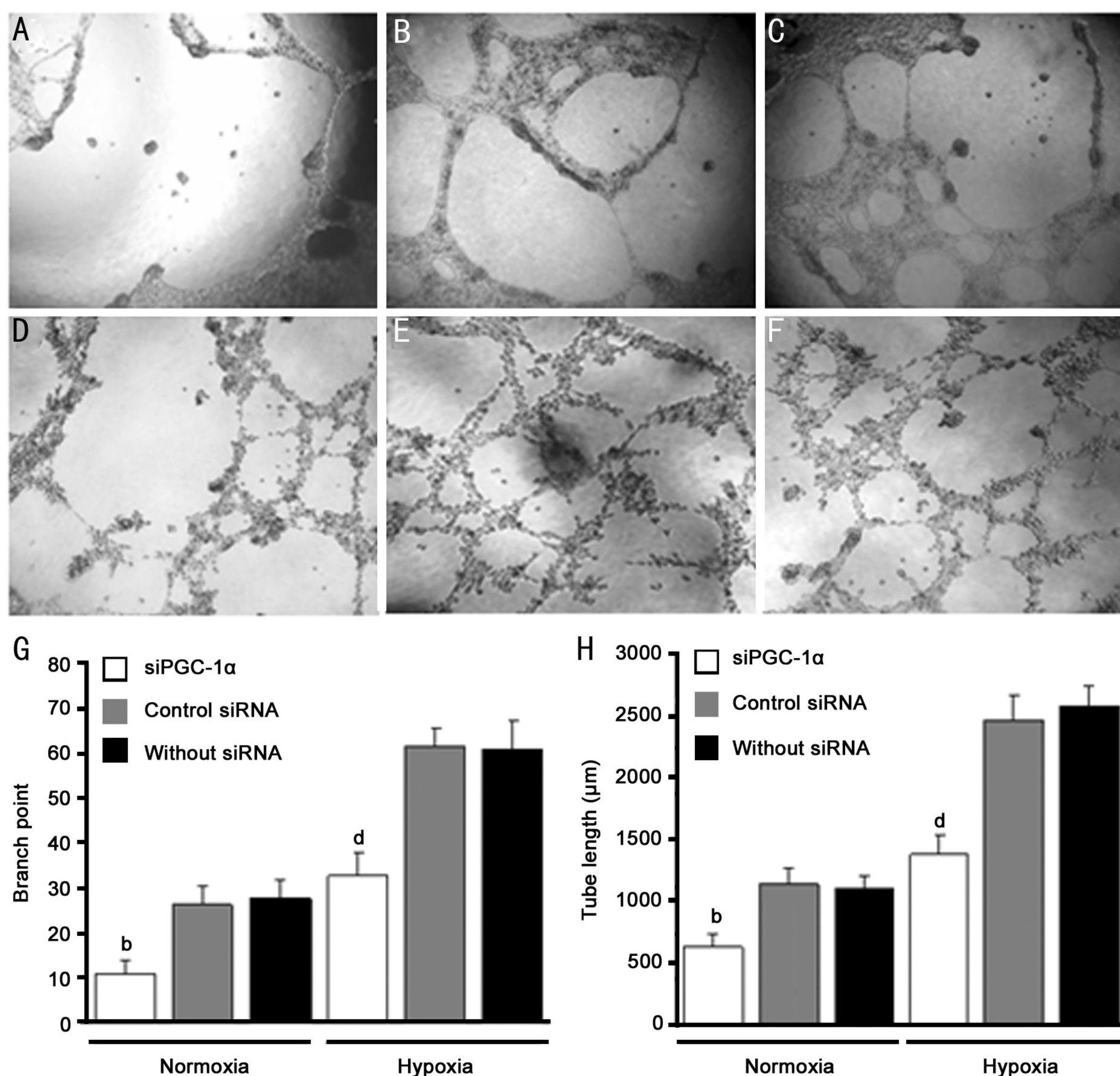

Figure 6 PGC-1α siRNA inhibits cell tube formation in hRVECs Tubular structures were photographed (A-F) and then quantified by counting the number of branch points (G) and calculating total tube length (H) in cultured hRVECs. A: Normoxia siPGC-1α group; B: Normoxia control siRNA group; C: Normoxia without siRNA group; D: Hypoxia siPGC-1α group; E: Hypoxia control siRNA group; F: Hypoxia without siRNA group. PGC-1α siRNA significantly reduced tubular-like formation (A and D), branch points (G), and total tube length (H) in cells compared with control siRNA group under normoxia (B) and hypoxia (E). Cells in the hypoxia without siRNA group and the hypoxia control siRNA group formed significantly more tubes than cells in their corresponding normoxia groups. $^{b}P<0.01$ *vs* normoxia control siRNA group, $^{d}P<0.01$ *vs* hypoxia control siRNA group. Magnification: ×20.

formation as quantitatively evaluated by branch point numbers and total tube length than cells in their corresponding normoxia groups. Cells in the hypoxia siPGC-1α group significantly attenuated tube formation than cells in the hypoxia control siRNA group ($P<0.01$) (Figure 6). Taken together, these results suggest that PGC-1α siRNA can directly repress angiogenic activity in hRVECs.

DISCUSSION

Neoangiogenesis is the formation of new blood vessels from pre-existing vessels, usually small veins, by sprouting. Neoangiogenesis occurs in wound healing and other pathological conditions such as proliferative diabetic retinopathy and retinopathy of prematurity. During angiogenesis, vascular endothelial cells secrete a number of factors to stimulate the proliferation of mesenchymal cells, promoting the maturation of the vascular wall[14].

Vascular endothelial cells form a barrier between the vascular wall and the blood, and injuries to endothelial cells can lead to many vascular diseases. In wound healing, inflammation, and cancer formation, endothelial cells can proliferate and form new blood vessels. The abnormal growth of new vessels are very fragile, leaking, and susceptible to hemorrhage. The resultant hemorrhage and accumulation of blood in ocular cavities, such as vitreous, leads to the further blockade of light transportation and decrease of visual acuity. For these reasons, retinal vascular endothelial cells have become an important research subject in the study of angiogenesis-related ocular diseases.

The PGC-1α is a transcriptional coactivator identified as an upstream regulator of lipid catabolism, mitochondrial number and function[15]. Consistent with its emerging role as a central regulator of energy metabolism, PGC-1α is abundantly expressed in tissues with high metabolic rates. PGC-1α is a potent modulator of oxidative metabolism in numerous settings [16]. In particular, PGC-1α powerfully regulates oxidative phosphorylation, mitochondrial biogenesis, and respiration [17]. Several lines of evidence have suggested that ischemia and hypoxia significantly induced the expression of PGC-1α [3-9,18,19]. Arany *et al* [10] found that PGC-1α could upregulate the expression of angiogenic factors such as VEGF, promoting the formation of new blood vessels. In this study, cells were cultured in a hypoxic environment and simulate hypoxia *in vivo*. Cells cultured in the hypoxic environment grew well and exhibited no significant cell death, suggesting that hypoxia did not affect the growth or survival of endothelial cells. Under hypoxic conditions, the expression of both PGC-1α and VEGF was significantly upregulated at both the mRNA and protein levels, confirming that hypoxia can upregulate the expression of PGC-1α and VEGF.

Here, a liposome-mediated method was used to transfect PGC-1α siRNA into cells. Under both normoxic and hypoxic conditions, PGC-1α mRNA and protein levels were significantly lower in cells transfected with PGC-1α siRNA than in cells transfected with the control siRNA, indicating that PGC-1α siRNA can inhibit gene expression under both normoxic and hypoxic conditions. When PGC-1α expression was blocked by siRNA, VEGF mRNA and protein expression was also significantly reduced under both normoxic and hypoxic conditions, confirming that PGC-1α regulates VEGF expression. VEGF is a specific mitogen for endothelial cells and is one of the most important inducers of angiogenesis. It induces the migration and proliferation of endothelial cells and increases the permeability of the endothelium. VEGF acts directly on vascular endothelial cells to increase vascular permeability, which leads to extravasation of fibrin, which forms a fibrin gel with fibronectin and serves as a temporary matrix for the migration and invasion of fibroblasts, endothelial cells, and other cells. These cells are then incorporated into the vasculature. In cell proliferation assay, hypoxia increased cell proliferation in hRVECs by stimulation of VEGF expression. After treated with PGC-1α siRNA, VEGF expression in hRVECs was downregulated, followed by declination of cell proliferation. Therefore, PGC-1α siRNA could inhibit cell proliferation in hRVECs.

To further define the molecular mechanisms of the PGC-1α siRNA responsible for attenuation of angiogenic activity in hRVECs, we analyzed the potential effect of PGC-1α siRNA on angiogenic activity *in vitro* by tube formation assays, which mimic multiple key steps of the angiogenic process, including endothelial cell adhesion, migration, differentiation, and growth [20]. In this assay, Matrigel coating provided the necessary extracellular matrix for human endothelial cells to form into a network of tubes. The endothelial cells started to form tubes 6h after they were placed in Matrigel-coated plates, and the tubes became stable after 48h. The endothelial cells formed tubes under both normoxic and hypoxic environments, but more tubes formed in hypoxic environments, suggesting that hypoxia can promote tube formation. Because PGC-1α can promote blood vessel formation through VEGF [10], it was here determined whether PGC-1α siRNA could inhibit tube formation. Cells transfected with PGC-1α siRNA under normoxic and hypoxic conditions formed fewer tubes than their corresponding control siRNA transfected cells, suggesting that PGC-1α siRNA downregulates VEGF by inhibiting PGC-1α, thereby reducing tube formation. In this way, experiments *in vitro* suggest that PGC-1α siRNA can inhibit angiogenesis. In addition, cells grown in hypoxia formed more tubes, but the tubes were less organized. Despite the increase in tube formation, the tubes formed under hypoxia were not well-organized, like newly formed blood vessels *in vivo*. In summary, PGC-1α siRNA can inhibit the expression of VEGF under normoxic and hypoxic conditions, thereby reducing endothelial cell tube formation, suggesting that PGC-1α may be a novel target for inhibition of angiogenesis.

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Jian Jiang and Xiao-Bo Xia designed the experiments; Jian Jiang and Lu Zhang performed the experiments; Jian Jiang analyzed the data and drafted the manuscript; Xiao-Bo Xia revised the manuscript and participated in revisions; Xiao-Bo Xia revised the manuscript for English writing; and all authors participated in critical revision of the manuscript and approval of the final manuscript.

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