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BASIC SCIENCE

MiR-9 regulates the post-transcriptional level of VEGF165a by targeting SRPK-1 in ARPE-19 cells

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Abstract
Purpose To investigate the effect of the overexpression of miRNA-9 to the ratio of pro- and anti-angiogenic isoforms of vascular endothelial growth factor (VEGF) in human retinal pigment cells (ARPE-19).
Methods Oxidative stress was induced to ARPE-19 cells by 4-hydroxynonenal (4-HNE), *tert*-butyl hydroperoxide (t-BH), and hypoxia chamber with 1 % O_2. Expression patterns of miRNAs were validated by qPCR. Relative mRNA levels of VEGF and PEDF were measured by semi-quantitative PCR. After the transfection of miR-9 mimic and inhibitor, transcriptional levels of VEGF165a, VEGF 165b, and SRPK-1 were measured by qPCR.
Results We demonstrated that miR-9 expression is decreased in ARPE-19 human retinal pigment cells under hypoxic stress induced by 4-HNE, a lipid peroxidation end-product. We observed that miR-9 mimic transfection of ARPE-19 inhibited one of its targets, serine-arginine protein kinase-1 (SRPK-1), modulating the transcriptional level of VEGF165b. Transfection of miR-9 reduced the alternative splicing of VEGF165a mRNA in ARPE-19 cells under hypoxic conditions, suggesting that miR-mediated regulation of alternative splicing could be a potential therapeutic target in neovascular pathologies.
Conclusions Hypoxic stress decreased the miR-9 level in ARPE-19 cells, which increased the transcriptional level of SRPK-1, resulting in alternative splicing shift to pro-angiogenic isoforms of VEGF165 in human retinal pigment epithelial cells

Keywords Retinal pigment epithelial cell · VEGF · MiR-9 · SRPK-1 · Hypoxia

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Introduction

Age-related macular degeneration (AMD) is a chronic degenerative disease that causes irreversible loss of central vision function in elderly people over 65 years old [1–3]. There are two types of this disease, dry (atrophic) AMD and wet (neovascular) AMD, which can be characterized by the accumulation of drusen between Bruch's membrane and the retinal pigment epithelium (RPE) during the early stages of the disease [4, 5]. Drusen consist of deposits of extracellular debris, primarily composed of lipids and proteins that are known risk factors of this disease [6, 7]. With chronic oxidative stress, dry AMD can progress to wet AMD, forming chronic neovascularization (CNV) from choroidal blood vessels [8–11]. 4-Hydroxy-2-nonenal (4-HNE) is a peroxidation product of polyunsaturated fatty acids (PUFAs) and a component of lipofuscin in RPE [12–15]. 4-HNE has been reported to induce oxidative stress, forming 4-HNE protein adducts by covalently binding to cysteine, lysine, or histidine residues [16].

Vascular endothelial growth factor (VEGF), an endothelial cell-specific mitogen, is involved in angiogenesis, vascular permeability in hypoxic conditions of wound healing, tumor formation, and retinal neovascularization [17–19]. The VEGF family consists of VEGF-A, −B, −C, and –D as well as placenta growth factor (PIGF)-1 and −2 [20]. The VEGF receptors (VEGFRs) include VEGFR-1, −2, and −3, and

VEGF-A is highly associated with VEGFR-2 in endothelial cell recruitment, proliferation, and migration in developing new blood vessels [21]. Pigment epithelium-derived factor (PEDF), a 50-kDa glycoprotein, belongs to the serine protease inhibitor (serpin) family, which is encoded by the SERPINF1 gene on chromosome 17p13 [22, 23]. PEDF down-regulates hypoxia-inducible factor (HIF)-1α and enhances the gamma secretase-dependent cleavage of the VEGFR-1 transmembrane domain, interfering with VEGFR-2-induced angiogenesis [24, 25]. An increase in the VEGF-to-PEDF ratio induced by hypoxic stress may play a key role in CNV [26].

Of the VEGF-A isoforms, VEGF165 plays a critical role in the development of CNV [27]. VEGF165 is alternatively spliced into VEGF165a and VEGF165b by serine-arginine-rich splicing factor 1 (SRSF1) [28]. VEGF165a is generated by proximal splice-site including terminal exon 8a, while VEGF165b is generated by distal splice-site including terminal exon 8b [29]. VEGF165a stimulates angiogenesis while VEGF165b is anti-angiogenic [30]. SRSF1 is phosphorylated by serine-arginine protein kinase-1 (SRPK-1) in retinal pigment epithelial cells [31]. Selcuklu et al. reported that they identified SRPK-1 as a direct target of miR-9 from microarray profiling of MCF-7, a human breast cancer cell line [32].

MicroRNAs, single-stranded noncoding small (~22 nucleotides) RNA molecules, post-transcriptionally regulate expression of target genes at the 3′-untranslated regions (UTRs) of mRNAs [33, 34]. Although miR-9 has been reported to exert diverse effects in neuronal development and tumor formation related to the cellular oxidative state, it is difficult to classify a clear role of miR-9 in human retinal pigment cells [35, 36]. In the present study, we screened several miRNA patterns in human retinal pigment epithelial (RPE) cells under oxidative stress. We demonstrated that miR-9 could increase the transcriptional level of VEGF165b by down-regulating SRPK-1 in RPE cells.

Materials and methods

Cell culture

ARPE-19 cells were purchased from American Type Culture Collection (Manassas, VA, USA). The cells were maintained in Dulbecco's modified Eagle's medium/F12 (DME/F12 1:1, Invitrogen, Carlsbad, CA, USA) supplemented with 10 % fetal bovine serum and 1 % penicillin-streptomycin. Cells were incubated at 37 °C in a humidified atmosphere of 5 % CO_2 and 95 % air and the culture medium was changed every 3 days.

Cell viability

Cell viability was measured with the sulfonated tetrazolium salt WST-1 (Takara, Japan) according to the manufacturer's instructions. Cells were seeded in a 96-well microplate at a density of 2×10^4 cells/well and incubated at 37 °C for 24 h before assay. The cells were then treated with various concentrations of 4-HNE and t-BH. After the addition of WST-1 solution (10 μl/well), cell viability was determined at 450-nm absorbance.

Hypoxia induction

For hypoxia induction, cells were placed in a MIC-101 modular incubator chamber (Billups-Rothenberg, Inc., CA, USA) with a mixture of 1 % O_2, 5 % CO_2, and 94 % N_2 at 37 °C.

Determination of mRNA and miRNA Expression

After RNA was isolated from cultured cells using TRIZOL reagent (Invitrogen, Carlsbad, CA, USA), cDNA was synthesized using AccuPower PreMix (Bioneer, Daejeon, Republic of Korea) in the presence of oligo (dT) primer. PCR was performed using a gradient thermal cycler (Takara, Japan). The reaction cycle consisted of incubation at 94 °C for 5 min, then 30 cycles of denaturation at 94 °C for 30 s, annealing at 55–62 °C for 30 s, and extension at 72 °C for 30 s. The primer sequences were as follows: VEGF-A: 5′-CCA TGA ACT TTC TGC TGT CTT-3′ and 5′-TCG ATC GTT CTG TAT CAG TCT-3′, PEDF-1: 5′-CAG AAG AAC CTC AAG AGT GCC-3′ and 5′-CTT CAT CCA AGT AGA AAT CC-3′, HIF-1α: 5′-AAG AAA CCG CCT ATG ACG TG-3′ and 5′-CCA CCT CTT TTT GCA AGC AT-3′, SRPK1: 5′-CAC GGC ATG CAT GGC CTT TGA-3′and 5′-CGG CGG CAG TGG CTC TCT TC-3′, VEGF165a: 5′-TGT TTG TAC AAG ATC CGC AGA CGT G-3′ and 5′-TCA CCG CCT CGG CTT GTC ACA TCT GCA AGT ACG TT-3′, VEGF165b: 5′-TGT TTG TAC AAG ATC CGC AGA CGT G-3′ and 5′-GTT CTG TAT CAG TCT TTC CTG GTG AGA GAT CTG CA-3′, Ang-2: 5′-ATC AGC CAA CCA GGA AAT GA-3′ and 5′-AGG ACC ACA TGC ATC AAA CC-3′, FGF-2: 5′-TCA AGC AGA AGA GAG AGG AGT TGT-3′ and 5′-AAA GAA ACA CTC ATC CGT AAC ACA-3′, β-actin: 5′-ATC CAC GAA ACT ACC TTC AA-3′ and 5′-ATC CAC ACG GAG TAC TTG-3′. Experiments were performed in triplicate in three independent experiments.

For miRNA detection, reverse transcription of 100 ng small RNA using the EasyScript cDNA Synthesis Kit (Applied Biological Materials, Richmond, BC, Canada) and qPCR using TOPreal SYBR qPCR System (Enzynomics, Republic of Korea) were performed according to the manufacturer's instructions. The primer sequences were as follows; miRNA-9: 5′-TCT TTG GTT ATC TAG CTG TAT GA-3′, miRNA-210: 5′-CTG TGC GTG TGA CAG CGG CTG A-3′, miRNA-21: 5′-TAG CTT ATC AGA CTG ATG TTG A-3′, miRNA-126: 5′-TCG TAC CGT GAG TAA TAA TGC G-3′, miRNA-146a: 5′-TGA GAA CTG AAT TCC ATG GGT

T-3′, U6: 5′-CTC GCT TCG GCA GCA CA-3′ and 5′- AAC GCT TCA CGA ATT TGC GT- 3′. Thermal cycle conditions were as follows: 95 °C for 30 s; 40 cycles of 95 °C for 5 s and 60 °C for 32 s. The expression levels of target genes were determined by using the $2^{-\Delta\Delta Ct}$ method. U6 was used as an endogenous control.

Transfection of miRNA mimic and inhibitor

ARPE-19 cells were seeded at 2×10^5 cells/well in a six-well plate (BD Biosciences). The next day, using 6.25 μl Lipofectamine 2000 reagent (Invitrogen) per well according to the manufacturer's instructions, the cells were transfected with one of the following RNA oligonucleotides at a 100nM final concentration: miRNA-9 duplex (Bioneer, Republic of Korea) or miRNA-9 antisense (Bioneer). After 4 h, the transfection medium was replaced with fresh growth medium with 10 % FBS.

Immuno blotting

Whole cell lysate from ARPE-19 cells was prepared in extraction buffer (Invitrogen) containing protease inhibitor (Sigma-Aldrich, St. Louis, MO, USA). The lysate (10 μg) was denatured by boiling in SDS sample buffer (Bio-Rad, Hercules, CA, USA), resolved by SDS–PAGE and then transferred to PVDF membrane by electroblotting. Blots were then incubated with a mouse anti-human VEGF165a antibody (Abcam, Cambridge, UK), a mouse anti-human VEGF-165b (Abcam), antibody or a mouse anti-human β-actin antibody (Thermo, Rockford, IL, USA) plus a horseradish peroxidase-linked secondary antibody, and detected by chemiluminescence using a Fujifilm LAS-3000 system (Fujifilm, Tokyo, Japan). The bands were quantified by densitometric analysis using ImageJ software(version 1.48, NIH, Bethesda, MD, USA).

Statistical analysis

Statistical analysis was performed using SPSS version 12.0 (SPSS, Chicago, IL, USA). Data are expressed as means± SEM. Student's *t* test was used to examine the correlation analysis. *p* values less than 0.05 were considered significant.

Results

MiR-9 is down-regulated by hypoxic stress in ARPE-19 cells

We found that miR-9 was down-regulated in a dose-dependent manner by the addition of 4-HNE or t-BH to ARPE-19 cells (Fig. 1a and b). MiR-9 expression was significantly decreased after hypoxic stress in a time-dependent manner compared with normoxia controls (Fig. 1c). With 5-aza-2'-deoxycytidine, a DNA methylation inhibitor, miR-9 expression increased up to about 16-fold in ARPE-19 cells. We then confirmed that miR-9 was significantly decreased following the addition of 4-HNE (Fig. 1d).

4-Hydroxynonenal increased the VEGF-A/PEDF ratio in ARPE-19 cells

Human retinal pigment epithelial cells (ARPE-19) were treated with various concentrations (20, 40, 60, and 80 μM) of 4-HNE for 1 h, and cell viability was determined by WST-1 assay. 4-HNE at 20–30 μM yielded over 50 % cell viability (data not shown). Treatment of ARPE-19 cells with 30 μM 4-HNE for 1 h significantly increased the mRNA levels of VEGF-A isoforms and also increased the mRNA level of NLRP-3 compared with normoxia- and LPS-(10 μg/ml) induced hypoxia controls (Fig. 2a). 4-HNE stimulation of ARPE-19 cells had no effect on the mRNA level of PEDF. Prolonged stimulation of ARPE-19 cells with 4-HNE for 4 days dose-dependently increased the mRNA level of VEGF-A isoforms (Fig. 2c). However, no significant differences in the mRNA levels of VEGFR-1, 2, and PEDF were observed (Fig. 2b). With various concentrations of 4-HNE (10–50 μM), ARPE-19 cells showed increased VEGF-A/PEDF mRNA ratios (threefold) compared with the normoxia control (Fig. 2d).

4-HNE up-regulated pro-angiogenic VEGF165a and SRPK-1

Hypoxic stimulation by 4-HNE increased the mRNA levels of HIF-1α and SRPK-1 in ARPE-19 cells (Fig. 3a). The relative mRNA ratio of VEGF165a (a pro-angiogenic isoform of VEGF165) and VEGF 165b (an anti-angiogenic isoform of VEGF165) increased as higher doses of 4-HNE (>25 μM) were used to stimulate ARPE-19 cells (Fig. 3b).

Effect of miR-9 on the expression of SRPK-1, VEGF165a, and VEGF165b

To elucidate the role of miR-9 in regulating the mRNA levels of SRPK-1, VEGF165a, and VEGF165b in hypoxia, ARPE-19 cells were transfected with miR-9 mimic and inhibitor to affect the endogenous miR-9 level. The transfection with miR-negative control had no specific effect on the SRPK-1 level in cells stimulated by 4-HNE (Fig. 4a). Under 4-HNE-induced hypoxia, ARPE-19 cells transfected with miR-9 mimic did not show hypoxic elevation of SRPK-1 and VEGF165a compared with the hypoxic control and exhibited a similar sensitivity to hypoxia compared with the hypoxic control. Treatment with miR-9 mimic decreased the mRNA level of VEGF165a in hypoxia (Fig. 4b, c). The transfection of miR-9 inhibitor led to an approximately two-fold increase in the

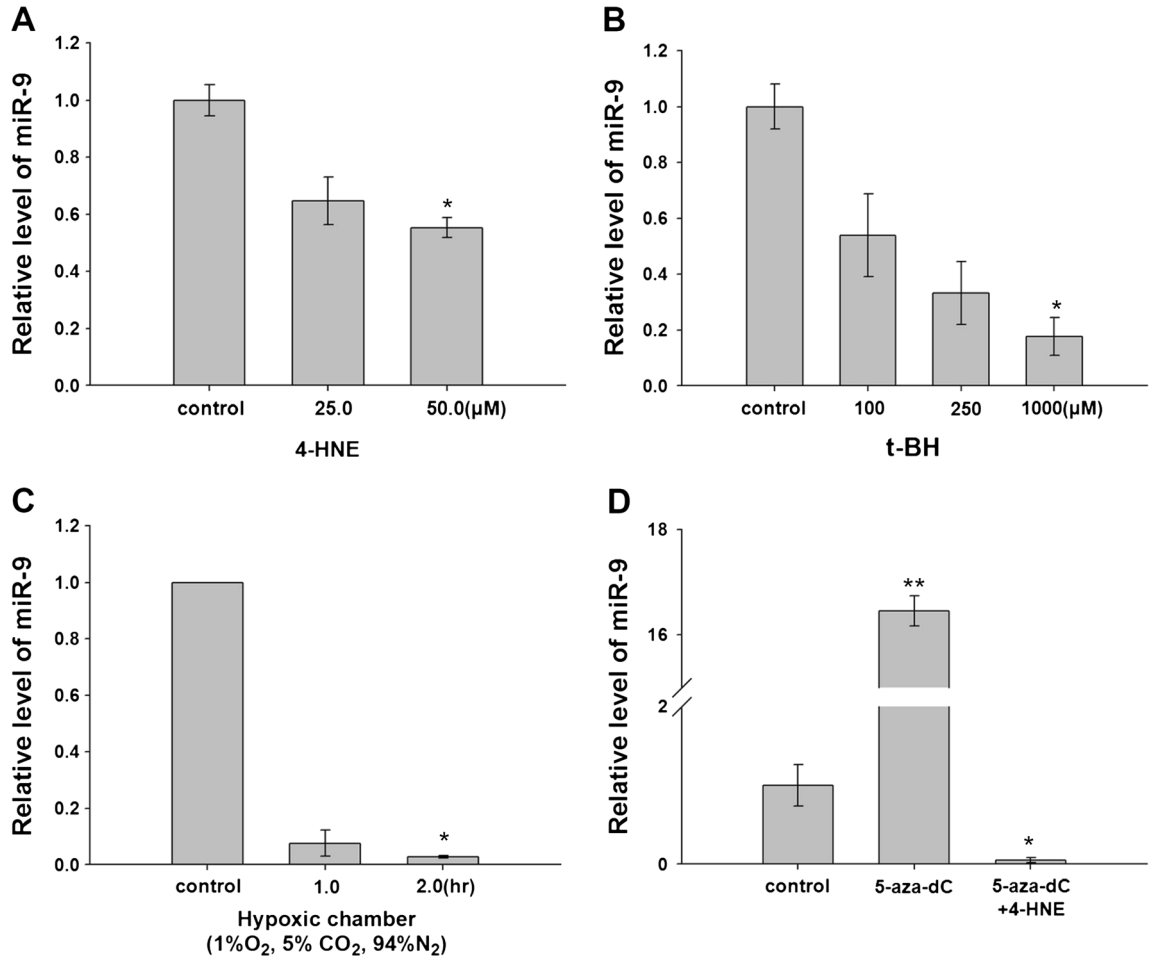

Fig. 1 Oxidative stress induces miR-9 decrease in ARPE-19 cells. The relative levels of miRNA-9 expression were measured by quantitative real-time PCR after ARPE-19 cells were subjected to 4-HNE (**a**), t-BH (**b**), and hypoxic chamber (**c**) with 1 % O_2. **d** Expression levels of miR-9 in cells treated with 5-Aza-dC (3 days) were analyzed using U6 snRNA as an internal control. Values are presented as mean±SD from triplicate wells. *, $p<0.05$, **, $p<0.01$ compared to control groups

SRPK-1 mRNA level in normoxia. Both in normoxia and hypoxia, miR-9 mimic transfection increased the mRNA level of VEGF165b in ARPE-19 cells, while the miR-9 inhibitor decreased the VEGF165b mRNA level (Fig. 4d). The protein expression of VEGF-165 isoform (VEGF-165a and VEGF-165b) was mildly increased in the presence of 4-HNE (10 μM) for 24 h. The expression rate of VEGF165a to VEGF165b was inversed by miR-9 overexpression in the presence of 4-HNE (Fig. 4e).

Discussion

The purpose of this study was to identify differentially expressed miRNAs that affect the expression of pro-angiogenic VEGF165a and to investigate the potential of retinal miRNA as a biomarker for predicting chronic neovascularization in the RPE. Increases of miR-9 in ARPE-19 cells can be measured by using oxidative stress molecules such as LPS, N-(4-hydroxyphenyl) retinamide (4-HPR), hydrogen peroxide, and molecular hydrogen [37–39]. We observed a decreased level of intracellular miR-9 in ARPE-19 cells under hypoxic conditions by sub-lethal concentrations (25–30 μM) of 4-HNE. The cellular concentrations of 4-HNE vary from 0.1–0.3 μM under normoxic conditions, while it accumulates up to concentrations of 10 μM to 5 mM under oxidative stress [40].

The transcriptional activity of miR-9 seems to be related to gene silencing by DNA methylation. Previous studies have demonstrated that the high frequency of hypermethylated CpG islands at miR-9 genes resulted in down-regulation of miR-9 in human cancer [41]. Epigenetic inactivation of miR-9 level may be associated with DNA hypermethylation in human RPE. We demonstrated that the expression level of miR-9 could be restored by hypomethylation of genomic DNA in ARPE-19 cells.

The VEGF gene is comprised of eight exons and seven introns approximately 14 kilobases in length. Members of the VEGF family are alternatively spliced to form pro-angiogenic

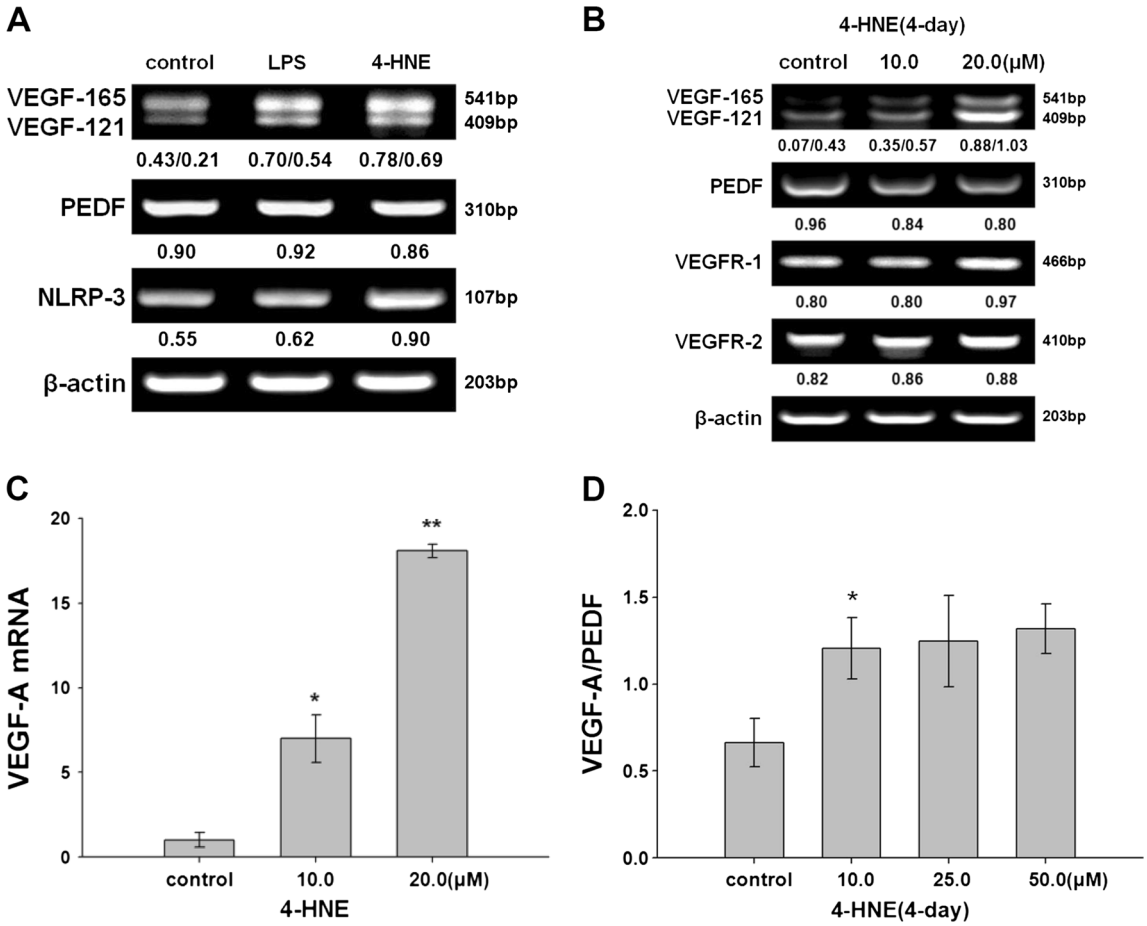

Fig. 2 Effects of 4-HNE on the VEGF/PEDF mRNA ratio in ARPE-19 cells. Reverse transcriptase-polymerase chain reaction (RT-PCR) analyses of VEGF-A isoforms. The resulting PCR products were analyzed on a 1.2 % agarose gel. **a** Treatment of ARPE-19 cells with 30 μM 4-HNE for 1 h significantly increased the mRNA levels of VEGF-165 and 121 isoforms and also increased the mRNA level of NLRP-3 compared with normoxia- and LPS-(10 μg/ml) induced hypoxia controls. Total RNA was extracted after incubation with 4-HNE for 4 days (**b-d**). **b** The relative mRNA expressions of VEGF-165, VEGF-121, PEDF, NLRP-3, VEGFR-1, and VEGFR-2 were analyzed using RT-PCR. **c** Quantitative analysis for VEGF-A mRNA was performed using real-time PCR. **d** 4-HNE increased VEGF/PEDF mRNA ratio. The results were normalized to β-actin and expressed as fold increase over control (*, $p<0.05$ and **, $p<0.01$)

VEGFxxx and anti-angiogenic VEGFxxxb, where xxx indicates the number of amino acids on the polypeptide chain. Several studies demonstrated that VEGFxxxb expression is lower than VEGFxxx in tumor cells. The selection of a distal splicing site of VEGF exon 8 (exon 8a and exon 8b) results in expression of VEGFxxxb, which differs from VEGFxxx at the C-terminal region of the pre-mRNA. The receptor-binding C-terminal domain of VEGF165b acts as a competitive inhibitor of VEGF165a and allows the inhibition of endothelial proliferation, migration, and vasodilatation [29].

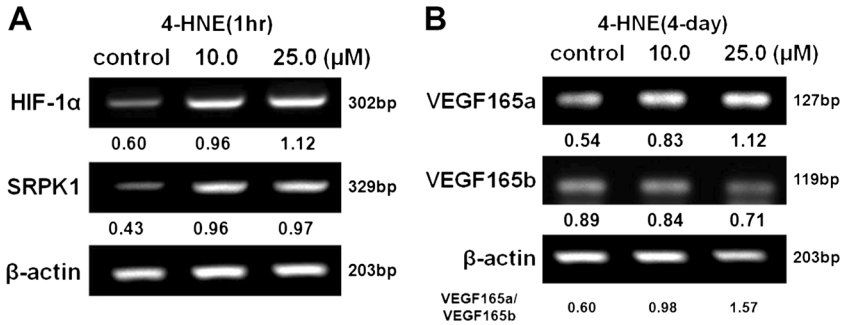

Fig. 3 mRNA expression of HIF-1α, SRPK-1 (**a**), and VEGF165 isoforms (**b**) in ARPE-19 cells. Total RNA from ARPE-19 cells was analyzed for mRNA expression of HIF-1α, VEGF165a, VEGF165b, SRPK-1, and β-actin. Semi-quantitative RT-PCR products were separated on a 1.2 % agarose gels and stained with ethidium bromide. Values represent densitometry analysis (ImageJ software) in the expression of the selected genes

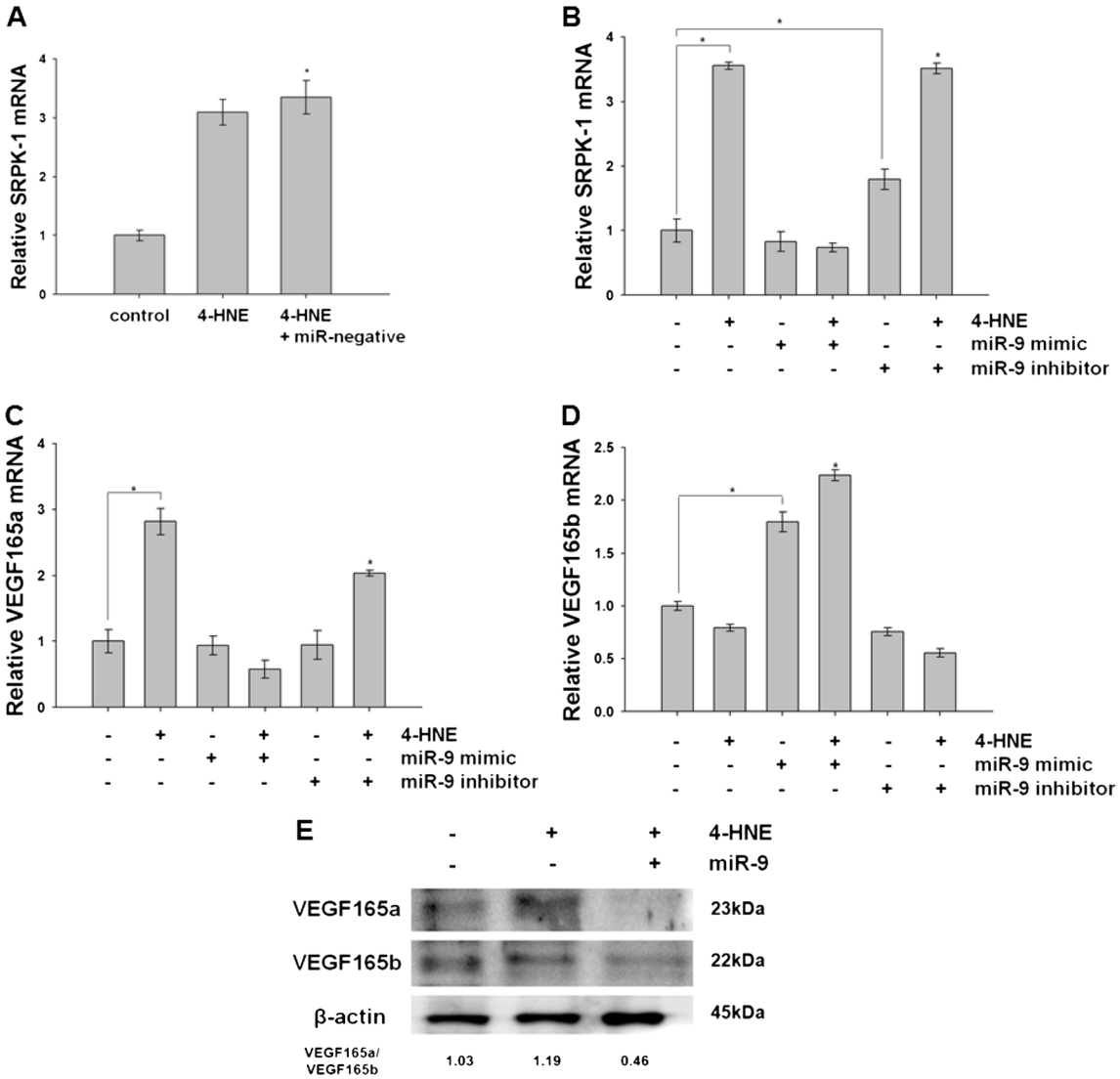

Fig. 4 miR-9 as a negative regulator of SRPK-1 in ARPE-19 cells under oxidative stress. ARPE-19 cells were transfected with 50 nM miR-negative control or mimics. At 48 h after transfection, cells were incubated with 30 μM 4-HNE for 1 h. The levels of SRPK-1, VEGF165a, and VEGF165b were determined by real-time quantitative PCR (**a**–**d**). Values are presented as mean±SD from triplicate wells. *, $p<0.05$ compared with the control. **e** Western blot analysis of VEGF165a and VEGF165b. ARPE-19 cells were treated with 4-HNE (10 μM) for 24 h with or without miR-9 transfection. Relative ratio of VEGF-165a and VEGF-165b expression was determined by densitometry (ImageJ 1.48 software, NIH image)

Alternative splicing of VEGF165 at exons 8a/8b depends on the balance of the serine/arginine rich (SR) proteins that recognize splicing enhancers or silencers at serine-arginine rich domains of the spliceosome. SR proteins can be directly regulated by SRPK-1-mediated phosphorylation, which causes SRs to translocate to the nucleus [42]. Gammons et al. reported that SRIN340, a selective SRPK inhibitor, reduced pro-angiogenic VEGF165a expression in the 50/10 oxygen-induced retinopathy (50/10 OIR) model which exposes newborn rats to repeated cycles of 24 h of 50 % and 10 % oxygen [43]. Selcuklu et al. tested *SRPK1* for a direct target of miR-9 using luciferase reporter system containing full-length 3'-UTR of SRPK-1 in a human breast cancer cell line (MCF-7). We hypothesized that the overexpression of

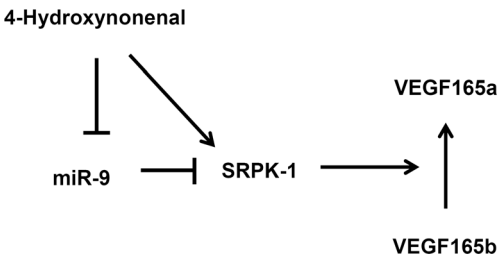

Fig. 5 4-Hydroxynonenal reduces the intracellular miR-9 level in ARPE-19 cells and increases the transcriptional level of SRPK-1. Under 4-HNE-induced hypoxic conditions, ARPE-19 showed increased production of pro-angiogenic VEGF165a via SRPK-1-mediated alternative splicing

miR-9 may influence the ratio of pro- and anti-VEGF isoforms in human retinal pigment epithelial cells under oxidative stress by transcriptional regulation of SRPK-1 mRNA. Western-blot analysis showed that miR-9 could selectively reduce the expression of pro-angiogenic VEGF165a isoform in oxidative milieu caused by 4-HNE, while anti-angiogenic VEGF165b remained unaffected.

In summary, we demonstrated that miR-9 is down-regulated in ARPE-19 cells under 4-HNE-induced oxidative stress. Increased miR-9 expression enhanced the transcriptional level of anti-angiogenic VEGF165b in ARPE-19 cells (Fig. 5). Reduced miR-9 expression in human retinal pigment cells under hypoxia might serve as a biomarker for chronic neovascularization.

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Declaration of interest The authors report no conflicts of interest.

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