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Reduction of Laser-Induced Choroidal Neovascularization in Mice With Erythropoietin RNA Interference

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Purpose: The purpose of this study was to evaluate the pathological involvement of erythropoietin (EPO) in experimental choroidal neovascularization (CNV) and its association with neovascular age-related macular degeneration (AMD) and polypoidal choroidal vasculopathy (PCV) in the Chinese population.

Methods: Treatment effect of recombinant EPO protein were assessed by human umbilical vein endothelial cell (HUVEC) proliferation, migration, and tube formation, and ex vivo choroid-sprouting ability. The effect of intravitreal injection of *Epo* siRNA against neovascularization was evaluated in the laser-induced CNV mouse model. In addition, the association of *EPO* variants with neovascular AMD and PCV was determined.

Results: Exogenous supplementation of EPO significantly enhanced the migration and tube formation of HUVECs and promoted ex vivo choroid sprouting in mouse retinal pigment epithelium (RPE)-choroid-sclera complex culture. In the experimental CNV mouse model, Epo expression was found to be significantly upregulated by 3.5-folds in RPE-choroid-sclera complex at day 10 after laser induction as compared to the baseline. Immunofluorescence analysis showed that Epo was mainly expressed around the vascular endothelial cells in the RPE-choroid-sclera complex. Intravitreal injection of siRNA targeting *Epo* reduced 40% Epo expression and 40% CNV lesion areas as compared to the scramble control. However, *EPO* variants were not associated with neovascular AMD nor PCV in the Chinese population.

Conclusions: This study revealed the promotion of human endothelial cell tube formation *in vitro* and choroid sprouting ex vivo by EPO, and the reduction of laser-induced CNV *in vivo* by *Epo* RNA interference.

Translational Relevance: Targeting EPO could be a potential additional treatment for CNV-related diseases.

Introduction

Choroidal neovascularization (CNV) refers to the ingrowth of new blood capillaries from choroid through a break at Bruch membrane into the subretinal space or the subretinal pigment epithelium (RPE) space. CNV is a common manifestation in multiple pathological vascular-related conditions, including age-related macular degeneration (AMD), pathological myopic, and idiopathic CNV, which could lead to subsequent photoreceptor damage and result in irreversible visual impairment and even blindness if not instantly treated.[1] Current clinical treatment for

CNV primarily relies on the intravitreal injection of antivascular endothelial growth factor (VEGF) agents as VEGF was found to be involved in the development of CNV.[2,3] Yet, the etiology of CNV is complex and largely remains elusive. Elucidating the disease mechanism is still warranted.

Erythropoietin (EPO) not only functions as a regulator in erythropoiesis, but also participates in angiogenesis[4] and related diseases, including diabetic retinopathy (DR),[5] retinopathy of prematurity (ROP),[6] and AMD.[7] The EPO receptor is expressed on the epiretinal membrane of patients with proliferative DR,[8] and increased EPO protein was found in the vitreous of patients with proliferative DR[9] and infants with ROP.[10] The risk of developing ROP is increased among infants who received >20 doses of recombinant human EPO as compared to those received ≤20 doses.[11] EPO treatment could promote human umbilical vein endothelial cell (HUVEC) tube formation, but not affect the proliferation and migration of HUVECs.[12] Intraperitoneal administration of Epo would increase the number of neovascular buds in the hypoxia-inducible factor-1α-like factor knockdown mice with the induction of oxygen-induced retinopathy.[13] Similarly, increased Epo signaling in gain-of-function human mutated EPO receptor knock-in mice would exacerbate the laser-induced CNV lesions with increased numbers of macrophages and greater cytokine expression.[14] In contrast, overexpression of Epo R76E mutant, a modified form of Epo attenuating erythropoietic activity, in RPE could delay retinal degeneration in RPE-specific *Sod2*-knockout mice.[7] Moreover, intravitreal injection of *Epo* small interfering RNA (siRNA) could suppress retinal neovascularization areas in a mouse model of oxygen-induced retinopathy,[15] we therefore hypothesized that EPO should be involved in the etiology of neovascularization, and reducing the *Epo* gene expression could possibly be a treatment for CNV. In this study, we aimed to evaluate the pathological involvement of EPO in experimental CNV models.

Apart from the animal studies, we previously reported significant association of the rs1617640 variant in the promoter of the *EPO* gene with proliferative DR.[16] An additional study also showed that rs1617640 and rs551238 variants are associated with increased risk of DR risk in the Chinese Han population.[17] AMD and polypoidal choroidal vasculopathy (PCV) are also related to the pathological angiogenesis. Thus, we also aimed to determine the association of these two *EPO* variants with neovascular AMD and PCV in the Chinese population.

Materials and Methods

Cell Culture

The HUVECs were purchased from American Type Culture Collection (Manassas, VA). They were cultured in basal medium (Dulbecco's Modified Eagle's Medium (DMEM; Hyclone Laboratories Inc., Logan, UT) supplemented with 10% fetal bovine serum (FBS; Biological Industries, Israel) and 1% penicillin-streptomycin (Gibco, Rockville, MD) and maintained in a 37°C incubator with 5% CO_2.

Cell Proliferation Analysis

The proliferation of HUVECs with EPO treatment was evaluated by the Cell Counting Kit-8 (CCK-8) assay (Sigma-Aldrich, St. Louis, MO), according to our previously established protocol.[18] Briefly, 1000 cells/well (3 wells for each treatment group) were seeded on the 96-well plates 1 day before the start of the treatment, and treated with 10 U/mL human recombinant EPO protein (Abcam, Cambridge, United Kingdom) for 5 days. The cells in basal medium without EPO treatment were considered as control. The CCK-8 assay was performed at days 0, 1, 3, and 5. The CCK-8 signal was measured at the wavelength of 450 nm by a plate reader (Powerwave XS; Bio-Tek Instruments, Winooski, VT).

Tube Formation Assay

Tube formation assay was carried out as previously described.[19] Briefly, HUVECs were starved for 24 hours in basal medium, harvested by trypsin detachment, seeded at a density of 5000 cells/well in 48-well plates precoated with Matrigel (Corning Inc., Corning, NY), and incubated in medium supplemented with 10 U/mL human recombinant EPO protein (Abcam). After 20-hour incubation, the images were taken at 4-fold magnification using an inverted light microscope (Nikon, Japan). The extent of tube formation was quantified by counting the number of meshes using ImageJ software (version 1.50a; National Institutes of Health, Bethesda, MD).

Cell Migration Analysis

The migration of HUVECs with EPO treatment was examined by the scratch wound assay, according to our previously established protocol.[20] Briefly, 2×10^5 cells/well HUVECs were seeded on the 12-

well plates 1 day before the start of the treatment. After cell seeding, scratch wounds were created by the 200-µL pipette tips on the attached cells, which were then washed and incubated in FBS-free medium with the supplementation of 20 U/mL human recombinant EPO protein (Abcam). The cells in basal medium without EPO treatment were considered as controls. Photographs were taken at time 0 (immediately following the scratch wound), 12, 24, and 36 hours. The wound gaps were measured by ImageJ software. The percentage migration was calculated by the average area reduction at 12, 24, or 36 hours as compared to time 0. Each well had six scratch wounds.

Choroid Sprouting Assay

The choroid sprouting assay was carried out as previously described.[21] Briefly, RPE-choroid-sclera complex from postnatal day 8 C57BL/6J mice were isolated and cut into pieces (approximately 1 × 1 mm^2). The pieces were immediately embedded in 30 µL Matrigel in the 24-well plates on ice and incubated at 37°C for 30 minutes. After polymerizing, EBM-2 medium (Lonza Bioscience, Basel, Switzerland) supplemented with mouse recombinant Epo protein (Abcam) was added into the wells, and the medium was changed every 2 days. The cells in EBM-2 medium without Epo treatment were considered as controls. The RPE-choroid-sclera complex were imaged by an inverted microscope at time 0 (immediately after addition of the medium), and at 2, 4, and 6 days. The choroid sprouting area was quantified using ImageJ software.

Laser-Induced Choroidal Neovascularization Model

The animal experiments were conducted according to the Association for Research in Vision and Ophthalmology (ARVO) Statement on the Use of Animals in Ophthalmic and Vision research, and approved by the Animal Experimentation Ethics Committee of Joint Shantou International Eye Center of Shantou University and the Chinese University of Hong Kong (approval number: EC20121123(5)-P21). Two-month-old C57BL/6 mice were purchased from Beijing Vital River Laboratory Animal Technology Co. Ltd., China. All mice were housed under standard conditions at 22 ± 1°C, 40 ± 10% humidity, and with 12:12 hour dark-light cycle. Standard rodent chow and water were provided ad libitum. The mice were anesthetized with 100 mg/kg ketamine and 10 mg/kg xylazine, and the pupils were dilated with 0.5% tropicamide eyedrops. Bruch's membrane rupture was created using a 532-nm laser photocoagulator (NOVUS Spectra; Lumenis, San Jose, CA) with a slit lamp delivery system that was used to create 3 burns, which were located at 3 disc-diameters from the optic disc at 3, 6, and 9 o'clock with the power of 120 mW, spot size of 75 µm and duration of 0.1 seconds. Only burns with production of a bubble at the time of the laser were included, as it indicates the rupture of Bruch's membrane.

Intravitreal Injection of Erythropoietin siRNA

The *Epo* siRNA was designed according to the mouse *Epo* mRNA sequence (NM_007942.2)[15]: sense: 5′-GACCCUUCAGCUUCAUAUATT and anti-sense: 5′-UAUAUGAAGCUGAAGGGUCTT. The sequences of scramble control siRNA were designed as: sense, 5′-FUUCUCCGAACGUGUCGUTT; and anti-sense, 5′-ACGUGACACGUUCGGAGAATT. The potential off-target effect of the siRNA was evaluated by BLAST (less than 78% query coverage with other genes and ≤15 nucleotides out of 19 matching with the respective siRNA considered to be tolerable).[22] The *Epo* siRNA and scramble control siRNA were diluted in saline at a concentration of 2 µg/µL. *Epo* siRNA or scramble control siRNA (1 µL) was intravitreally injected at the limbus with a 30-gauge needle, immediately after the laser burns. At day 7 after intravitreal injection, the mice were euthanized for subsequent analyses.

Immunoblotting Analysis

At days 0, 3, 7, 10, 14, and 17 after laser application, the mice were euthanized. The eyeballs were enucleated, and the RPE-choroid-sclera complex was dissected. The RPE-choroid-sclera complex was homogenized in chilled radioimmunoprecipitation assay lysis buffer (Millipore, Billerica, MA) supplemented with protease inhibitor. Equal amounts of total protein were resolved in SDS-polyacrylamide gel and electro-transferred onto a PVDF membrane (Millipore). The membrane was blocked with 5% non-fat milk for 1 hour at room temperature and incubated overnight with primary rabbit anti-Epo antibody (Santa Cruz Biotechnology, Santa Cruz, CA) at 4°C and subsequently with secondary anti-rabbit antibody conjugated with horseradish peroxidase (Santa Cruz Biotechnology) for 2 hours at room temperature. Gapdh was used as the housekeeping protein for normalization. Signals were detected by enhanced chemiluminescence (Amersham Pharmacia, Cleveland, OH) with the ChemiDocTM XRS^+ system (Bio-Rad Laboratories, Hercules, CA). The densitometry was analyzed by the ImageJ software.

Table 1. Demographics of the Study Subjects in the Genetic Association Study

	AMD ($n = 428$)	PCV ($n = 397$)	Control ($n = 988$)	P_{AMD}	P_{PCV}
Male, *n* (%)	299 (69.9)	276 (69.5)	512 (51.8)	2.95×10^{-10}	1.80×10^{-9}
Female, *n* (%)	129 (30.1)	121 (30.5)	476 (48.2)		
Age range, years	40–90	36–81	43–97		
Age years (Mean ± SD)	66.99 ± 9.70	63.20 ± 9.36	69.50 ± 8.85	4×10^{-6}	5.05×10^{-29}

AMD, age-related macular degeneration; PCV, polypoidal choroidal vasculopathy; SD, standard deviation.

Immunofluorescence Analysis

At day 7 after laser application, the mice were euthanized, and the eyeballs were enucleated and fixed in 4% paraformaldehyde for 2 hours at room temperature. The fixed eyeballs were cryoprotected with 10% to 30% sucrose gradient in phosphate-buffered saline (PBS), and sectioned using a cryostat (MZ9.5, Leica, Germany). The eyeball sections (10 µm) were blocked with 1.5% normal goat serum for 10 minutes at room temperature, incubated with anti-Epo antibody (Santa Cruz Biotechnology) for overnight at 4°C, and subsequently with FITC-conjugated secondary anti-rabbit antibody (Molecular Probes, Grand Island, NY) and Alexa Flour 594-conjugated GS-IB4 isolectin (Sigma-Aldrich) for 1 hour at room temperature. The slides were imaged using a confocal microscope (Leica TCS SP5 II; Leica Microsystems, Wetzlar, Germany). For each sample, 5 retinal sections were used, and 10 fields were imaged.

Quantification of Neovascularization Areas

At day 7 after laser application, the mice were euthanized, and the eyeballs were enucleated and fixed in 4% paraformaldehyde for 2 hours at room temperature. The retina and vitreous were removed. The RPE-choroid-sclera complex flat mount was dissected with 4 radial cuts and incubated with Alexa Flour 488-conjugated isolectin GS-IB4 (Sigma-Aldrich) overnight at 4°C. The slides were imaged using a confocal microscope (Leica TCS SP5 II). The neovascularization areas were quantified by the ImageJ software.

Genetic Association Analysis

This study was approved by the Ethics Committee for Human Medical Research of the Joint Shantou International Eye Center of Shantou University and the Chinese University of Hong Kong (approval number: EC20121123(5)-P21), which is in accordance to the tenets of the Declaration of Helsinki. Written informed consent was obtained from all study subjects after explanation of the nature and possible consequences of the study. A total of 988 control subjects, 428 patients with neovascular AMD, and 397 patients with PCV were recruited at the Joint Shantou International Eye Center of Shantou University and the Chinese University of Hong Kong (Table 1). All study subjects underwent complete ophthalmic examinations. AMD was graded according to an international classification and grading system.[23] Patients with neovascular AMD had non-drusenoid RPE detachment, serous or hemorrhagic retinal detachments, CNV, subretinal or sub-RPE hemorrhage, and/or fibrosis. Patients with PCV had subretinal red or orange nodules and hemorrhagic pigment epithelial detachment and characteristic sacculated vascular abnormalities in the inner choroid as visualized on indocyanine green angiography (ICGA). The diagnosis of PCV was distinguished from neovascular AMD by fluorescein angiography (FA) and ICGA staining.[24] Patients with PCV were excluded if they had geographic atrophy or early signs of AMD, or were at the late stage of disease that possess fibrosis and disciform scar, or at an advanced stage with intensive hemorrhage. The control subjects were individuals older than 60 years old, without any signs of AMD, PCV, or other major eye diseases, except for mild myopia or senile cataract.

Peripheral whole blood was collected from all study subjects. Genomic DNA from peripheral whole blood was extracted using Qiagen QIAamp DNA Blood Mini kit (Qiagen, Hilden, Germany), according to the manufacturer's protocol. Two *EPO* variants (rs1617640 and rs551238) were selected from our previous study[16,17] for genotyping by TaqMan assay (Applied Biosystems, Foster City, CA).

Statistical Analysis

For cellular and animal experiments, the data were presented as mean ± standard error of mean. Mann-Whitney *U* test or 1-way analysis of variance (ANOVA) with post hoc Tukey test was used to compare the means in different treatment groups. All the statistical analysis was performed using commercially available software (SPSS, version 21.0; SPSS Inc., Chicago, IL). Any $P < 0.05$ was considered as statistically significant.

For genetic association analysis, the categorical demographic data was compared by the χ^2 test, whereas the continuous variables were compared by independent *t*-test. Hardy-Weinberg equilibrium (HWE) of the two *EPO* variants were assessed in the control subjects by χ^2 test using Haploview (version 4.2; http://www.broadinstitute.org/). Association of the two *EPO* variants with AMD and PCV was calculated using the χ^2 or Fisher's exact test in six genetic models (genotypic, allelic, dominant, recessive, heterozygous and homozygous). The odds ratio (OR) and corresponding 95% confidence interval (CI) were estimated with the reference allele. Statistical significance was defined as $P < 0.05/2$ variants $= 0.025$ after Bonferroni's correction.

Results

Erythropoietin Promoted Human Endothelial Cell Migration and Tube Formation *In Vitro*

Neovascularization is determined by different properties of endothelial cells, including cell proliferation, migration, and tube formation capability. Scratch wound assay showed that supplementation of EPO protein could significantly increase HUVEC migration at 24 (42.04 ± 0.46%, $P = 0.034$) and 36 hours (54.19 ± 0.67%, $P = 0.034$) as compared to the control (24 hours: 40.29 ± 0.27%, 36 hours: 52.28 ± 0.65%; Fig. 1A). Consistently, tube formation assay demonstrated that HUVECs treated with EPO (36.33 ± 15.54) formed higher number of meshes than the control group (6.67 ± 4.93, $P = 0.034$; Fig. 1B). However, EPO treatment did not alter the proliferation of HUVECs (2.06 ± 0.12) as compared to the control (1.80 ± 0.15, $P = 0.083$; Fig. 1C). Our results indicated that EPO could promote endothelial cell migration and tube formation ability, but would not influence its proliferation.

Erythropoietin Promoted Choroid Sprouting in *Ex Vivo* Mouse Choroid Explant Culture

To delineate the effect of EPO on CNV, we first evaluated the growth of choroid capillaries in ex vivo RPE-choroid-sclera explant culture. Choroid sprouting assay demonstrated that RPE-choroid-sclera

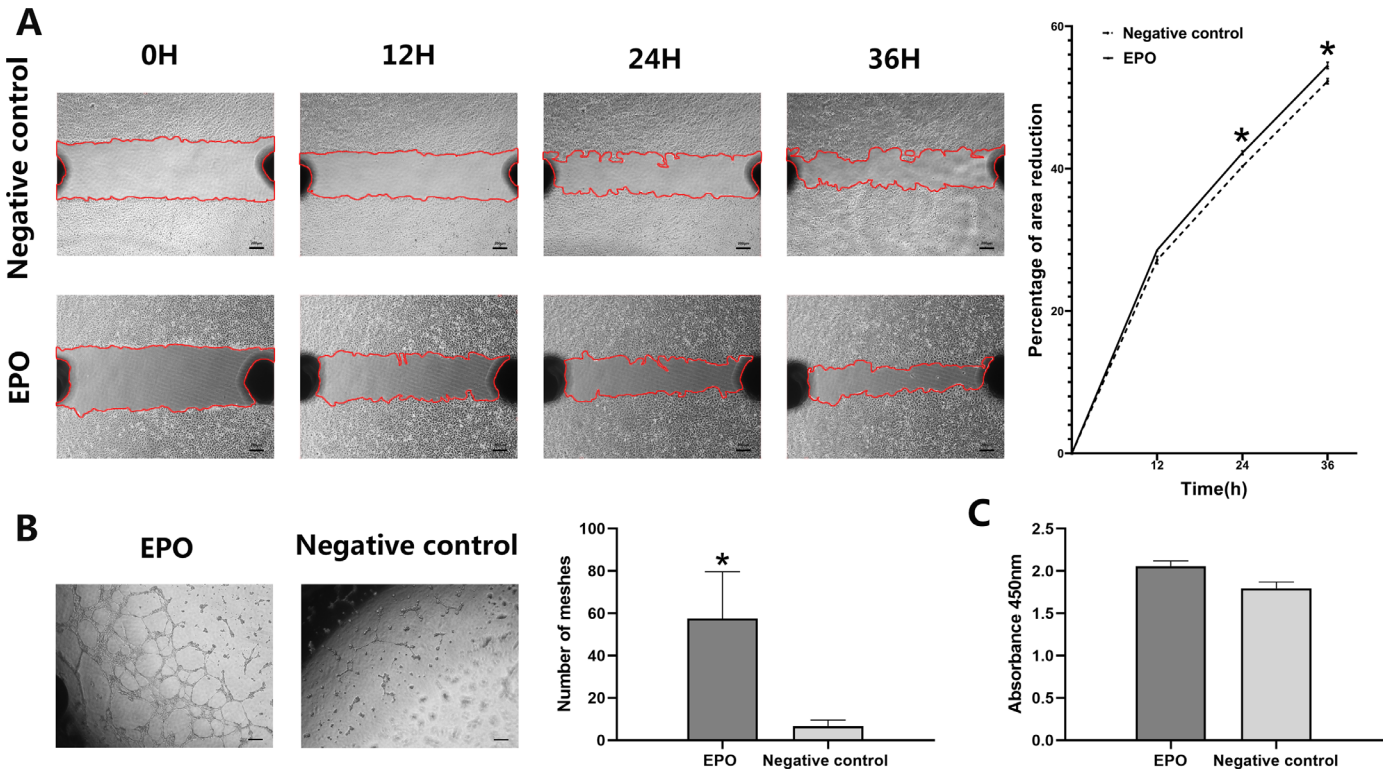

Figure 1. **Erythropoietin treatment on human umbilical vein endothelial cell proliferation, migration and tube formation.** (**A**) The migration of human umbilical vein endothelial cells (HUVECs) with human erythropoietin (EPO) was evaluated by scratch wound assay at 0, 12, 24, and 36 hours. Scale bar = 200 µm. *Triangle* = EPO; circle = control. (**B**) Tube formation assay on EPO-treated HUVECs. (**C**) Cell Counting Kit-8 proliferation assay of HUVECs with EPO treatment. The data were presented as mean ± standard error of mean. $^*P < 0.05$ (Mann-Whitney *U* test); $n = 4$ for each group.

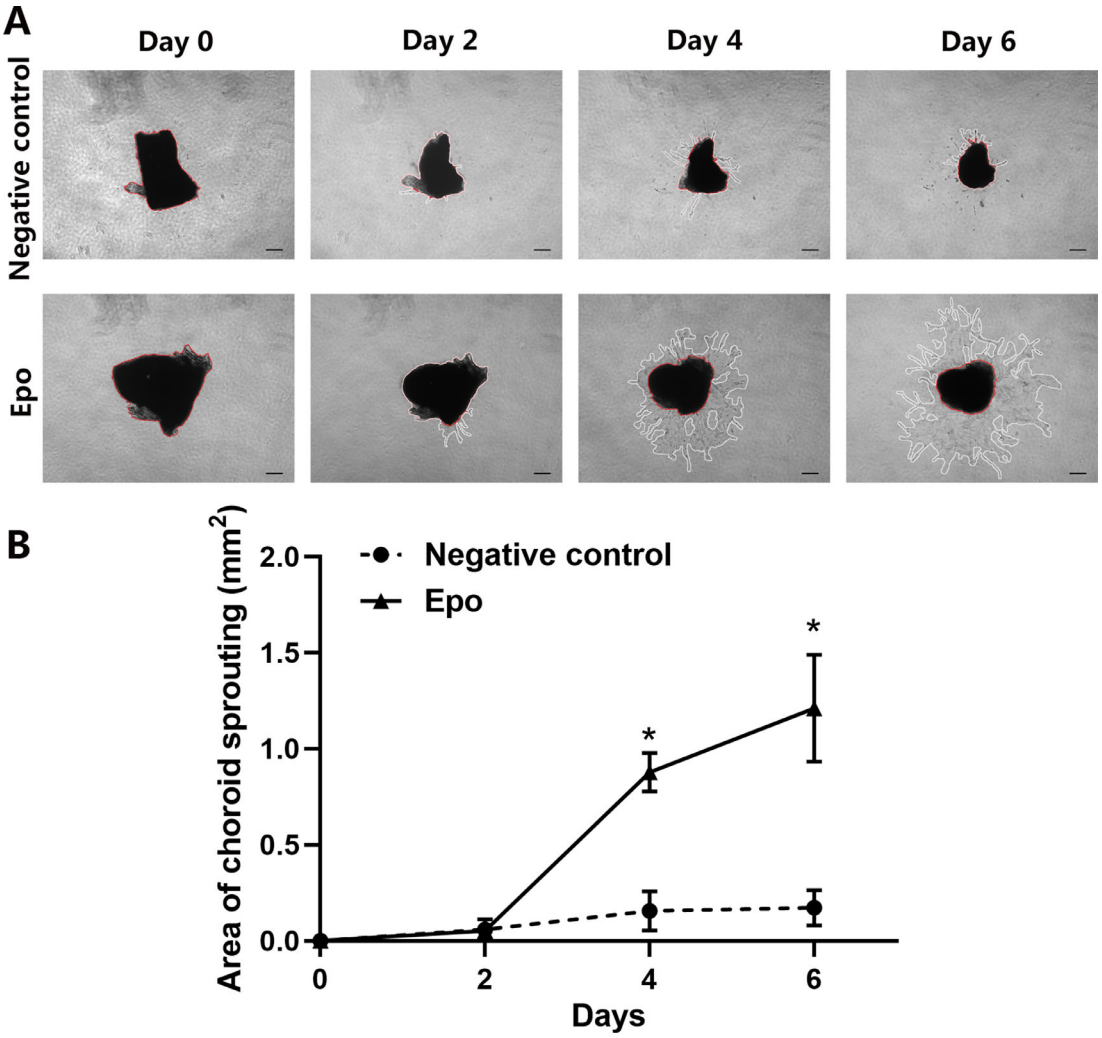

Figure 2. Erythropoietin treatment on choroid sprouting in mouse choroid explant culture. (**A**) Representative images of mouse choroid explant culture with mouse erythropoietin (Epo) treatment at days 0, 2, 4, and 6. Scale bar = 200 µm. (**B**) Quantification of choroidal sprouting areas. *Triangle* = Epo; *circle* = control. The data were presented as mean ± standard error of mean. $^{*}P < 0.05$ (Mann-Whitney *U* test); $n = 5$ for each group.

explant treated with Epo protein showed larger choroid sprouting area at day 4 (0.88 ± 0.23 mm^2, $P = 0.025$) and six (1.21 ± 0.62 mm^2, $P = 0.025$) as compared to the control (day 4: 0.16 ± 0.18 mm^2 and day 6: 0.17 ± 0.16 mm^2; Fig. 2). Our results suggested that Epo could promote choroid capillary growth in ex vivo explant culture.

Elevated Expression of Erythropoietin in Vascular Endothelial Cells in Laser-Induced Choroidal Neovascularization Mouse Model

To confirm the involvement of EPO in CNV, we determined the expression of Epo in the laser-induced CNV mouse model. Immunoblotting analysis demonstrated that the expression of Epo was gradually increased in the RPE-choroid-sclera complex from 3 to 10 days after laser injury as compared to the baseline (Fig. 3A, Supplementary Fig. S1). The expression of Epo at day 10 after laser injury was 3.5-fold higher than the baseline ($P = 0.037$). The Epo levels returned to the baseline level after 14 days of laser injury. Immunofluorescence analysis confirmed that Epo protein was expressed around the vascular endothelial cells in the RPE-choroid-sclera complex (Fig. 3B). Our results indicated that the elevated Epo expression could be involved in the CNV development after laser injury in mice.

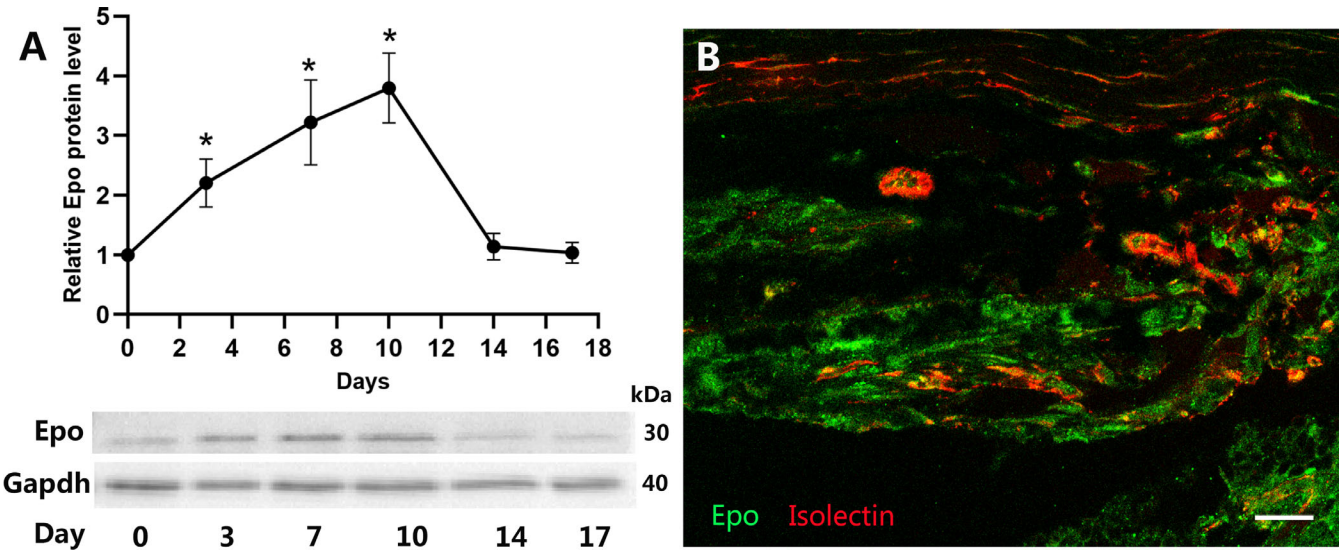

Figure 3. Expression of erythropoietin protein in laser-induced choroidal neovascularization mouse model. (**A**) Immunoblotting analysis on erythropoietin (Epo) protein expression in the retinal pigment epithelium (RPE)-choroid-sclera complex from day 0 to day 17. Gapdh was used as housekeeping control. The relative expression was compared to the level at day 0. The data were presented as mean ± standard error of mean. $^{*}P < 0.05$ (Mann-Whitney *U* test). (**B**) Immunofluorescence analysis of Epo protein expression in the RPE-choroid-sclera complex. *Green* =: Epo; *red* = isolectin for vascular endothelial cells. Scale bar = 20 µm, $n = 3$ for each group.

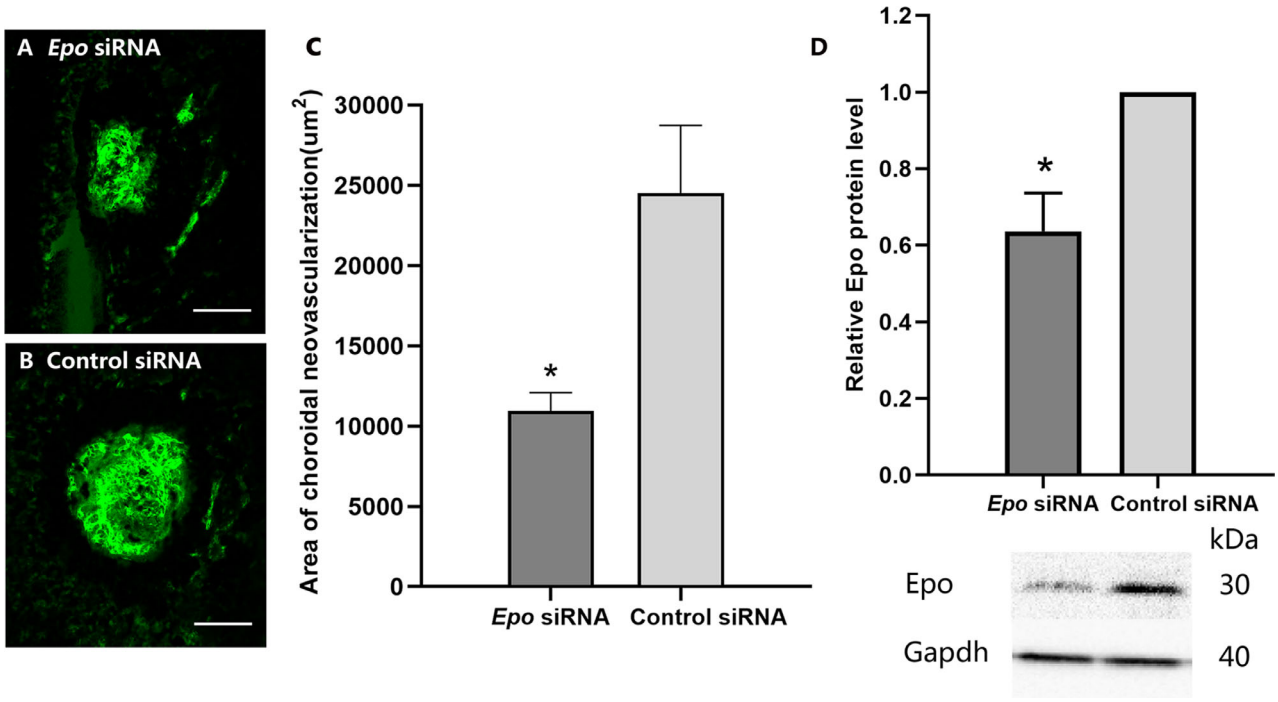

Figure 4. Erythropoietin small interfering RNA treatment on laser-induced choroidal neovascularization lesions. Laser-induced choroidal neovascularization (CNV) lesions in mouse eyes with (**A**) treatment of small interfering RNA (siRNA) against erythropoietin (*Epo*) gene or (**B**) scramble control siRNA at day 7. Scale bar = 100 µm. (**C**) Quantification of the CNV lesion areas. The data were presented as mean ± standard error of mean; $n = 14$ for each group. (**D**) Immunoblotting analysis on erythropoietin (Epo) protein expression in the retinal pigment epithelium (RPE)-choroid-sclera complex of mouse eyes with treatment of small interfering RNA (siRNA) against *Epo* gene or scramble control siRNA at day 7. The data were presented as mean ± standard error of mean; $n = 4$ for each group. $^{*}P < 0.05$ (Mann-Whitney *U* test).

Treatment of Erythropoietin Small Interfering RNA Against Laser-Induced Choroidal Neovascularization in Mice

To further illustrate the contribution of EPO to CNV, we evaluated the potential of targeting *Epo* as the treatment for CNV. Bioinformatics off-target analysis confirmed the specificity of our siRNA only to *Epo* gene (Supplementary Table S1). Immunofluorescence analysis showed that the isolectin-stained CNV lesions were significantly smaller in the laser-injured mice with intravitreal injection of *Epo* siRNA (10,943 ± 4116 μm^2) than in those injected with scramble control siRNA (24,156 ± 15,778 μm^2, $P = 0.020$) at day 7 (Fig. 4A–C). Meanwhile, the Epo protein level in the RPE-choroid-sclera complex of the *Epo* siRNA-injected eyes reduced to 60% of controls (the eyes injected with scramble control siRNA) in the laser-induced CNV mice ($P = 0.014$; Fig. 4D). Our results suggested that reducing the elevated Epo levels could attenuate the CNV development in mice with laser injury.

Association of Erythropoietin Gene Variants With Age-Related Macular Degeneration and Polypoidal Choroidal Vasculopathy

To extend the analysis of EPO from experimental CNV to the CNV-related ocular diseases in humans, we determined the genetic association of two *EPO* variants (rs1617640 and rs551238) with AMD and PCV. Both *EPO* variants followed HWE in the control subjects (Supplementary Table S2). Yet, both *EPO* variants showed no significant association with neovascular AMD (rs1617640: $P_{Dominant} = 0.737$, OR = 0.89, 95% CI = 0.45–1.75; rs551238: $P_{Dominant} = 0.656$, OR = 1.16, 95% CI = 0.60–2.23) or PCV (rs1617640: $P_{Dominant} = 0.407$, OR = 1.30, 95% CI = 0.70–2.40; rs551238: $P_{Dominant} = 0.169$, OR = 1.53, 95% CI = 0.83–2.84) in the southern Han Chinese population

Table 2. Genetic Association Analysis of *EPO* Variants With Age-Related Macular Degeneration and Polypoidal Choroidal Vasculopathy

	Ref/Hetero/Homo				AMD Versus Control		PCV Versus Control		AMD + PCV Versus Control	
EPO Variants	AMD ($n = 428$)	PCV ($n = 397$)	Control ($n = 988$)	Model	*P* Value	OR (95% CI)	*P* Value	OR (95% CI)	*P* Value	OR (95% CI)
rs1617640	12/127/289	16/100/281	31/278/679	Genotype	0.811	–	0.419	–	0.922	–
C > A				Allele	0.779	1.03 (0.84–1.27)	0.713	0.96 (0.77–1.20)	0.993	1.00 (0.78–1.28)
				Dominant	0.737	0.89 (0.45–1.75)	0.407	1.30 (0.70–2.40)	0.759	0.92 (0.55–1.55)
				Recessive	0.655	0.94 (0.74–1.21)	0.445	1.10 (0.86–1.42)	0.867	1.02 (0.83–1.24)
				Homozygous	0.785	0.91 (0.46–1.80)	0.484	1.25 (0.67–2.32)	0.784	0.93 (0.55–1.57)
				Heterozygous	0.642	0.85 (0.42–1.70)	0.271	1.44 (0.75–2.74)	0.714	0.90 (0.53–1.55)
rs551238	14/147/267	17/110/270	28/312/648	Genotype	0.504	–	0.176	–	0.543	–
G > T				Allele	0.258	1.12 (0.92–1.37)	0.765	0.97 (0.78–1.20)	0.686	0.95 (0.75–1.21)
				Dominant	0.656	1.16 (0.60–2.23)	0.169	1.53 (0.83–2.84)	0.270	0.75 (0.44–1.26)
				Recessive	0.247	0.87 (0.69–1.10)	0.388	1.12 (0.87–1.43)	0.825	0.98 (0.81–1.19)
				Homozygous	0.563	1.21 (0.63–2.34)	0.231	1.46 (0.79–2.71)	0.277	0.75 (0.44–1.26)
				Heterozygous	0.862	1.06 (0.54–2.08)	0.093	1.72 (0.91–3.27)	0.279	0.74 (0.44–1.27)

AMD, age-related macular degeneration; Hetero, heterozygous genotype; Homo, homozygous genotype; PCV, polypoidal choroidal vasculopathy; Ref, reference genotype.

Table 3. Genetic Association Analysis of *EPO* Variants With Age-Related Macular Degeneration and Polypoidal Choroidal Vasculopathy in Female Subjects

	Ref/Hetero/Homo				AMD Versus Control		PCV Versus Control		AMD + PCV Versus Control	
EPO Variants	AMD ($n = 219$)	PCV ($n = 121$)	Control ($n = 476$)	Model	*P* Value	OR (95% CI)	*P* Value	OR (95% CI)	*P* Value	OR (95% CI)
rs1617640	2/39/88	7/33/81	13/137/326	Genotype	0.724	–	0.247	–	0.808	–
C > A				Allele	0.863	0.97 (0.67–1.40)	0.401	1.17 (0.81–1.67)	0.785	0.95 (0.63–1.41)
				Dominant	0.749	0.56 (0.13–2.52)	0.151	2.19 (0.85–5.61)	0.516	0.75 (0.32–1.78)
				Recessive	0.953	0.99 (0.65–1.50)	0.745	0.93 (0.61–1.43)	0.807	0.96 (0.69–1.33)
				Homozygous	0.747	0.57 (0.13–2.57)	0.151	2.17 (0.84–5.61)	0.513	0.75 (0.31–1.79)
				Heterozygous	0.532	0.54 (0.12–2.50)	0.143	2.24 (0.83–6.04)	0.536	0.76 (0.31–1.86)
rs551238	3/44/82	7/34/80	12/152/312	Genotype	0.894	–	0.158	–	0.542	–
G > T				Allele	0.744	1.06 (0.75–1.50)	0.632	1.09 (0.76–1.56)	0.716	0.93 (0.63–1.37)
				Dominant	> 0.999	0.92 (0.26–3.31)	0.081	2.37 (0.91–6.17)	0.269	0.62 (0.26–1.46)
				Recessive	0.675	0.92 (0.61–1.38)	0.906	1.03 (0.67–1.56)	0.841	0.97 (0.70–1.33)
				Homozygous	> 0.999	0.95 (0.26–3.45)	0.145	2.20 (0.87–5.97)	0.277	0.62 (0.26–1.47)
				Heterozygous	> 0.999	0.86 (0.23–3.20)	0.070	2.61 (0.96–7.11)	0.278	0.62 (0.26–1.49)

AMD, age-related macular degeneration; Hetero, heterozygous genotype; Homo, homozygous genotype; PCV, polypoidal choroidal vasculopathy; Ref, reference genotype.

Table 4. Genetic Association Analysis of *EPO* Variants With Age-Related Macular Degeneration and Polypoidal Choroidal Vasculopathy in Male Subjects

	Ref/Hetero/Homo				AMD Versus Control		PCV Versus Control		AMD + PCV Versus Control	
EPO Variants	AMD ($n = 299$)	PCV ($n = 276$)	Control ($n = 512$)	Model	*P* Value	OR (95% CI)	*P* value	OR (95% CI)	*P* Value	OR (95% CI)
rs1617640	10/88/201	9/67/200	18/141/353	Genotype	0.845	–	0.585	–	0.954	–
C > A				Allele	0.692	1.06 (0.81–1.37)	0.337	0.87 (0.66–1.16)	0.824	1.04 (0.76–1.42)
				Dominant	0.898	0.95 (0.43–2.09)	0.851	0.93 (0.41–2.09)	0.848	1.07 (0.55–2.06)
				Recessive	0.611	0.92 (0.68–1.25)	0.303	1.19 (0.86–1.64)	0.777	1.04 (0.80–1.34)
				Homozygous	0.951	0.98 (0.44–2.16)	0.765	0.88 (0.39–2.00)	0.827	1.08 (0.56–2.08)
				Heterozygous	0.780	0.89 (0.39–2.02)	0.907	1.05 (0.45–2.47)	0.907	1.04 (0.53–2.06)
rs551238	11/103/185	10/76/190	16/160/336	Genotype	0.553	–	0.538	–	0.892	–
G > T				Allele	0.291	1.15 (0.89–1.47)	0.506	0.91 (0.70–1.20)	0.827	0.97 (0.71–1.31)
				Dominant	0.671	1.18 (0.54–2.59)	0.709	1.17 (0.52–2.60)	0.632	0.85 (0.44–1.65)
				Recessive	0.282	0.85 (0.63–1.14)	0.361	1.16 (0.85–1.58)	0.888	0.98 (0.77–1.26)
				Homozygous	0.580	1.25 (0.57–2.75)	0.809	1.11 (0.49–2.48)	0.633	0.85 (0.44–1.66)
				Heterozygous	0.873	1.07 (0.48–2.39)	0.519	1.32 (0.57–3.04)	0.647	0.85 (0.43–1.69)

AMD, age-related macular degeneration; Hetero, heterozygous genotype; Homo, homozygous genotype; PCV, polypoidal choroidal vasculopathy; Ref, reference genotype.

under the six genetic models (Table 2), even adjusted for sex (Tables 3, 4).

Discussion

Results from this study showed that (1) EPO could promote the migration and tube formation of human endothelial cells; (2) it could also promote ex vivo choroid sprouting in mouse explant culture; (3) the expression of Epo protein was upregulated in laser-induced CNV mouse model; (4) *Epo* siRNA reduced Epo protein expression and CNV lesions in mice after laser injury; (5) *EPO* rs1617640 and rs551238 variants were not associated with AMD nor PCV in the Chinese population. Collectively, our results demonstrated the involvement of EPO in CNV development and the potential of *EPO* silencing as CNV treatment.

Neovascularization is a common pathology in multiple vascular-related ocular diseases, including AMD, PCV, myopic CNV, idiopathic CNV, and ocular histoplasmosis.[1] EPO has been reported to participate in various angiogenic-related processes, including wound healing,[25] tumor,[26] embryonic development,[27] and retinal angiogenesis.[8] Increased EPO protein has been found in the vitreous of patients with proliferative diabetic retinopathy[9] and infants with ROP.[10] Similarly, we previously showed that *Epo* gene expression is elevated in the mouse retina with oxygen-induced retinal neovascularization.[16] Coherent to the previous reports, this study demonstrated that the expression of Epo protein gradually increased from day 3 and reached a peak at day 10 in the laser-induced CNV mouse model (see Fig. 3A). The expression of Epo was found at the choroidal layer of the RPE-choroid-sclera complex (see Fig. 3B), indicating its association with the vasculature. The Epo expression is co-localized with the CNV lesion after laser injury,[28] suggesting that Epo should be involved in the development of CNV.

The mechanism of EPO promoting CNV remains elusive. Addition of EPO could stimulate brain capillary endothelial cell proliferation and migration.[29] In contrast, the proliferation and migration of HUVECs were reported not to be affected by EPO treatment, but did promote HUVEC tube formation.[12] In this study, we observed that supplementation of EPO could promote endothelial cell migration and their tube formation ability (see Figs. 1A, 1B), but not influence the proliferation of HUVECs (see Fig. 1C). Importantly, the ex vivo choroid-sprouting assay demonstrated that Epo strongly promoted choroid sprouting in mouse RPE-choroid-sclera explant culture (see Fig. 2). Collectively, EPO could promote CNV through stimulate endothelial cell migration and tube formation.

EPO has been tested as a treatment strategy in various experimental ocular disease models. Constitutive overexpression of human EPO could protect photoreceptors against light-induced degeneration in mice.[30] Moreover, addition of EPO could increase the viability of hydrogen peroxide-treated RPE cells and recover RPE integrity *in vitro*.[31] On the contrary to the retinal protective effect of EPO overexpression, increased Epo signaling in gain-of-function human mutated EPO receptor knock-in mice would exacerbate the laser-induced CNV lesions with increased numbers of macrophages and greater cytokine expression.[14] Furthermore, intravitreal injection of *Epo* siRNA could suppress retinal neovascularization areas in a mouse model of oxygen-induced retinopathy.[15] Consistently, this study showed that intravitreal injection of *Epo* siRNA could reduce 40% of Epo protein in the RPE-choroid-sclera complex (see Fig. 4D) and

attenuate the CNV lesions in mice with laser injury as compared to scramble control (see Figs. 4A, 4B, 4C). We speculated that the protective effect of EPO could be different in different cell types through different mechanisms. Additionally, although the bioinformatics analysis indicated low potential of off-target by our siRNA (see Supplementary Table S1), the siRNA could still possibly exert the microRNA-like effects that require only short regions of sequence complementarity[32]; therefore, there is a possibility that our siRNA might also suppress the expression of other genes in addition to *Epo*.

We previously reported that the T allele of rs1617640 variant in the promoter of the *EPO* gene is significantly associated with proliferative diabetic retinopathy and end stage renal disease in Caucasian populations, which enhances the expression of EPO protein as compared to the G allele.[16] Additional study confirmed that rs1617640 and rs551238 variants are associated with increased risk of DR risk in the Chinese Han population.[17] However, this study did not find significant association of *EPO* variants (rs1617640 and rs551238) with AMD and PCV (see Table 2). Notably, *EPO* rs551238 variant showed marginal significant association with PCV in female subjects ($P_{Dominant}$ = 0.081, OR = 2.37, 95% CI = 0.91–6.17; see Table 3), which might imply that EPO could be involved in the development of PCV. Nevertheless, replications in a lager cohort and in different ethnicities are needed to confirm the genetic association of *EPO* variants with AMD and PCV.

In summary, this study revealed the pathological involvement of EPO in the experimental CNV *in vitro* and *in vivo*. Despite further confirmation needed for the association of *EPO* variants with neovascular AMD and PCV, targeting EPO could still be a potential treatment against CNV.

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References

1. Yeo NJY, Chan EJJ, Cheung C. Choroidal Neovascularization: Mechanisms of Endothelial Dysfunction. *Front Pharmacol.* 2019;10:1363.
2. Liao X, Jin C, Chen W, et al. Quantification of anterior chamber reaction after intravitreal injections of conbercept and ranibizumab: a pilot study. *Eye (Lond)*. 2020;34:595–596.
3. Campbell M, Doyle SL. Current perspectives on established and novel therapies for pathological neovascularization in retinal disease. *Biochem Pharmacol.* 2019;164:321–325.
4. Kimáková P, Solár P, Solárová Z, Komel R, Debeljak N. Erythropoietin and Its Angiogenic Activity. *Int J Mol Sci*. 2017;18:1519.
5. Reid G, Lois N. Erythropoietin in diabetic retinopathy. *Vision Res*. 2017;139:237–242..
6. Fahim NM, Georgieff MK, Zhang L, Naisbitt S, Rao RB, Inder TE. Endogenous erythropoietin concentrations and association with retinopathy of prematurity and brain injury in preterm infants. *PLoS One*. 2021;16:e0252655.
7. Biswal MR, Wang Z, Paulson RJ, et al. Erythropoietin Gene Therapy Delays Retinal Degeneration Resulting from Oxidative Stress in the Retinal Pigment Epithelium. *Antioxidants (Basel)*. 2021;10:842.
8. Kase S, Saito W, Ohgami K, et al. Expression of erythropoietin receptor in human epiretinal membrane of proliferative diabetic retinopathy. *Br J Ophthalmol.* 2007;91:1376–1378.
9. Watanabe D, Suzuma K, Matsui S, et al. Erythropoietin as a retinal angiogenic factor in proliferative diabetic retinopathy. *N Engl J Med.* 2005;353:782–792.
10. Sato T, Kusaka S, Shimojo H, Fujikado T. Vitreous levels of erythropoietin and vascular

endothelial growth factor in eyes with retinopathy of prematurity. *Ophthalmology.* 2009;116:1599–1603.

11. Suk KK, Dunbar JA, Liu A, et al. Human recombinant erythropoietin and the incidence of retinopathy of prematurity: a multiple regression model. *J AAPOS.* 2008;12:233–238.
12. Liu X, Tufman A, Behr J, Kiefl R, Goldmann T, Huber R. Role of the erythropoietin receptor in Lung Cancer cells: erythropoietin exhibits angiogenic potential. *J Cancer.* 2020;11:6090–6100.
13. Morita M, Ohneda O, Yamashita T, et al. HLF/HIF-2 alpha is a key factor in retinopathy of prematurity in association with erythropoietin. *EMBO J.* 2003;22:1134–1146.
14. Bretz CA, Divoky V, Prchal J, et al. Erythropoietin Signaling Increases Choroidal Macrophages and Cytokine Expression, and Exacerbates Choroidal Neovascularization. *Sci Rep.* 2018;8:2161.
15. Chen J, Connor KM, Aderman CM, Willett KL, Aspegren OP, Smith LEH. Suppression of Retinal Neovascularization by Erythropoietin siRNA in a Mouse Model of Proliferative Retinopathy. *Invest Ophthalmol Vis Sci.* 2009;50:1329–1335.
16. Tong Z, Yang Z, Patel S, et al. Promoter polymorphism of the erythropoietin gene in severe diabetic eye and kidney complications. *Proc Natl Acad Sci USA.* 2008;105:6998–7003.
17. Fan Y, Fu YY, Chen Z, Hu YY, Shen J. Gene-gene interaction of erythropoietin gene polymorphisms and diabetic retinopathy in Chinese Han. *Exp Biol Med (Maywood).* 2016;241:1524–1530.
18. Yao Y, Ma D, Xu Y, et al. Hydroxychloroquine treatment on SARS-CoV-2 receptor ACE2, TMPRSS2 and NRP1 expression in human primary pterygium and conjunctival cells. *Exp Eye Res.* 2022;214:108864.
19. Huang Z, Ng TK, Chen W, et al. Nattokinase Attenuates Retinal Neovascularization Via Modulation of Nrf2/HO-1 and Glial Activation. *Invest Ophthalmol Vis Sci.* 2021;62:25.
20. Yang Y, Chen SL, Xu Y, et al. Green Tea Catechins Attenuate Human Primary Pterygium Cell Survival and Migration Via Modulation of ERK p42/p44 and p38 Pathways. *J Agric Food Chem.* 2021;69:12209–12218.
21. Shao Z, Friedlander M, Hurst CG, et al. Choroid Sprouting Assay: An Ex Vivo Model of Microvascular Angiogenesis. *PLoS One.* 2013;8:e69552.
22. Kim YJ. Computational siRNA design considering alternative splicing. *Methods Mol Biol.* 2010;623:81–92.
23. Bird A, Bressler N, Bressler S, et al. An international classification and grading system for age-related maculopathy and age-related macular degeneration. The International ARM Epidemiological Study Group. *Surv Ophthalmol.* 1995;39:367–374.
24. Cackett P, Wong D, Yeo I. A classification system for polypoidal choroidal vasculopathy. *Retina.* 2009;29:187–191.
25. Siebert N, Xu W, Grambow E, Zechner D, Vollmar B. Erythropoietin improves skin wound healing and activates the TGF-β signaling pathway. *Lab Invest.* 2011;91:1753–1765.
26. Yasuda Y, Fujita Y, Masuda S, et al. Erythropoietin is involved in growth and angiogenesis in malignant tumours of female reproductive organs. *Carcinogenesis.* 2002;23:1797–1805.
27. Crivellato E, Nico B, Vacca A, Djonov V, Presta M, Ribatti D. Recombinant human erythropoietin induces intussusceptive microvascular growth *in vivo*. *Leukemia.* 2004;18:331–336.
28. Edelman J, Castro M. Quantitative image analysis of laser-induced choroidal neovascularization in rat. *Exp Eye Res.* 2000;71:523–533.
29. Yang X, Wang Y, Zhang J, et al. Role of PI3K/Akt and MEK/ERK in mediating hypoxia-induced expression of HIF-1alpha and VEGF in laser-induced rat choroidal neovascularization. *Invest Ophthalmol Vis Sci.* 2009;50:1873–1879.
30. Grimm C, Wenzel A, Stanescu D, et al. Constitutive overexpression of human erythropoietin protects the mouse retina against induced but not inherited retinal degeneration. *J Neurosci.* 2004;24:5651–5658.
31. Wang Z, Shen L, Tu L, et al. Erythropoietin protects retinal pigment epithelial cells from oxidative damage. *Free Radic Biol Med.* 2009;46:1032–1041.
32. Jackson AL, Linsley PS. Recognizing and avoiding siRNA off-target effects for target identification and therapeutic application. *Nat Rev Drug Discov.* 2010;9:57–67.