Lentivirus Vector-Mediated Knockdown of Erythropoietin-Producing Hepatocellular Carcinoma Receptors B4 Inhibits Laser-Induced Choroidal Neovascularization

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Abstract

Purpose: To evaluate the efficacy of erythropoietin-producing hepatocellular carcinoma receptors B4 (EphB4) knockdown on the development of laser-induced choroidal neovascularization (CNV) *in vivo*.

Methods: We constructed recombinant lentiviral vectors (Lv) Lv-shRNA-EphB4 to specifically knock down the expression of EphB4. The mRNA and protein expression of EphB4 was investigated by real-time reverse transcription-polymerase chain reaction (RT-PCR) and western blot. CNV was induced by laser photocoagulation in C57BL/6 mice. The mice were then randomly assigned to be intravitreally injected with phosphate-buffered saline (PBS), Lv-shRNA-EphB4 recombinant lentivirus, or an unrelated shRNA recombinant lentivirus (pFU LV-shRNA-NC). An uninjected group was used as the control. Fundus fluorescein angiography (FFA), histologic analysis, and choroidal flat mounts analysis were applied to evaluate the inhibition of CNV after an intravitreal injection.

Results: Transfection of Lv-shRNA-EphB4 led to the knockdown of EphB4, and EphB4 mRNA was down-regulated by about 80%. FFA and histologic analysis revealed that the leakage areas and the mean thickness of CNV were much smaller in the Lv-shRNA-EphB4 group than in the PBS-treated, pFU Lv-shRNA-NC group and the non-injection group. Choroidal flat mounts showed significantly less leakage and smaller leakage areas in the Lv-shRNA-EphB4 group than those in other groups.

Conclusion: Knocking down the expression of EphB4 exerts an inhibitory effect on CNV *in vivo*. It may provide a potential strategy for the treatment of CNV.

Introduction

AGE-RELATED MACULAR degeneration (AMD) is the main cause of vision loss or blindness in the elderly. Generally, AMD can be divided into 2 subtypes: dry AMD and wet AMD. Choroidal neovascularization (CNV) is the key feature of wet AMD, and is the leading reason for visual loss among adults with AMD.^{1,2} In wet AMD, the choroid begins to sprout abnormal blood vessels, and this process is known as CNV. CNV is a complicated phenomenon, including endothelial cell adhesion, migration, proliferation, and extracellular matrix production.³ CNV grows in the plane between the retinal pigment epithelium (RPE) and the Bruch membrane, or between the retina and RPE. CNV represents new blood vessel growth from the choroid that traverses Bruch's membrane and extends into the subretinal pigment epithelium.⁴ This abnormal vessel proliferation may affect vision and cause visual loss.

Recent investigations suggest that signaling through receptor tyrosine kinases (RTKs) plays an important role in vascular permeability.⁵ Vascular endothelial growth factor (VEGF) receptor, angiopoietin receptors, and the erythropoietin-producing hepatocellular carcinoma receptors (Ephs) are RTKs.⁶ Among these molecules, Ephs and their ligands, Ephrins, contain 14 receptors and 8 ligands, which comprise the largest subfamily of RTKs. It has been proposed that Ephs can regulate cell migration and angiogenesis *in vivo*. The Eph family is subdivided into 2 groups, EphA and EphB. Recent data show that the interaction between erythropoietin-producing hepatocellular carcinoma receptors B4 (EphB4) and their ligand EphrinB2 contributes to the formation of angiogenesis.⁷

EphB4 and EphrinB2 play critical roles in many biological processes, including vasculogenesis, tumor angiogenesis, and neural development.^{8,9} The overexpression of EphB4 is predicted to promote angiogenesis by stimulating reverse

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signaling through EphrinB2. Noren et al.¹⁰ reported that EphB4 could promote a more aggressive and invasive tumor phenotype using breast cancer cells transfected with EphB4 lacking the kinase domain. The authors concluded that EphB4 promoted tumor growth by stimulating angiogenesis through EphrinB2.¹⁰

EphB4 and EphrinB2 are essential for vascular development. Previous studies have indicated that cultured human retinal endothelial cells express EphB4 and EphrinB2, and the expression has been localized to the arteries and veins, respectively. Davies et al.¹¹ evaluated the expression of EphB4 and EphrinB2 in a model of oxygen-induced retinopathy (OIR), and found that the expression of EphB4 was reduced after hyperoxia. They demonstrated that EphB4 and EphrinB2 expression was associated with a subset of capillaries in the developing deep retinal vascular plexus.¹¹ Recent studies have investigated that the soluble monomeric derivative of the extracellular domain of EphB4 (sEphB4) could block activation of EphB4 and EphrinB2.¹² Ehlken et al. have demonstrated that an injection of sEphB4 inhibits retinal NV in the mouse model of OIR.¹³ The role of a soluble monomeric form of EphB4 (sEphB4) in CNV was first reported by He et al., in which they showed that sEphB4 can inhibit endothelial tube formation and choroidal endothelial cell (CEC) migration.¹⁴

The effects of EphB4 knockdown on CNV are not yet known; however, it is intriguing to speculate that EphB4 silencing may inhibit the formation of CNV. RNAi has been widely used to knock down gene expression, and lentiviral vectors have been shown to be useful vectors for the long-term regulation of gene expression.^{15–17} We, therefore, designed a recombinant lentiviral vector to knock down the expression of EphB4. An intravitreal injection of the recombinant lentivirus was used to evaluate the therapeutic effect in mice with laser-induced CNV.

Methods

Reagents and antibodies

Monoclonal antibodies against EphB4 and goat anti-rabbit immunoglobulin G (IgG) antibodies were purchased from Santa Cruz Biotechnology. pFU-GW-RNAi vector was constructed by Genechem. BCA protein assay kit was from Pierce. Sodium pentobarbital was provided by Sigma. 4', 6-diamidino-2-phenylindole (DAPI) and fetal bovine serum (FBS) were provided by Invitrogen. Fluorescein sodium was purchased from WuZhou Pharmaceutical. Bouin's fixative was obtained from ZhongShan Biotechnology. C57BL/6 mice were obtained from the Fourth Military Medical University. All mouse experiments were approved by the Animal Care and Use Committee, and all mice were treated in accordance with the Association for Research in Vision and Ophthalmology Statement.

Cell culture

All cells were purchased from American Type Culture Collection. Lewis, Hepa1-6, and NIH-3T3 cells were maintained in RPMI-1640 medium containing 10% FBS. Two hundred ninety-three T-cells were maintained in Dulbecco's modified Eagle medium containing 10% FBS at 37°C with 5% CO₂ to evaluate the efficiency of lentivirus transfection.

Construction of lentiviral vectors

A recombinant lentiviral vector expressing EphB4-shRNA was constructed by Shanghai Genechem. EphB4-shRNA was introduced into pFU-GW-RNAi vector that carried the green fluorescent protein (GFP) reporter gene driven by the U6 promoter. Three vectors were designed: Lv-shRNA1-EphB4 (KD1), Lv-shRNA2-EphB4 (KD2), and Lv-shRNA3-EphB4 (KD3). The suppression of mRNA expression was analyzed by real-time reverse transcription-polymerase chain reaction (RT-PCR), and EphB4 protein levels were detected by western blot. The most efficient recombinant vector was used in later experiments. pFU-GW-RNAi vector, which produces a non-targeting sequence TTCTCCGAACGTGTCACGT, was used as the negative control (pFU LV-shRNA-NC) throughout the study. The recombinant vectors and the shRNA-LVs were co-transfected into 293 T-cells, and the titer of recombinant lentivirus was 2×10^9 infectious units per mL.

Western blot

Western blot was performed to detect the protein levels of EphB4 in Lewis cells, Hepa1-6 cells, and NIH-3T3 cells, as previously described.^{18,19} Cells were lysed in RIPA buffer (Sigma). The cell lysates were quantified using a BCA protein assay kit (Pierce), and an equal amount of protein (10 µg) was resolved by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred onto nitrocellulose membranes (Bio-Rad). The membranes were blocked in 1% blocking reagent containing 0.05% tween-20 for 1 h and incubated with monoclonal antibodies against EphB4 overnight at 4°C. An anti-rabbit horseradish peroxidase-conjugated antibody (Santa Cruz Biotechnology) was used as the secondary antibody. The target bands were detected by a Gel Imaging System (Bio-Rad).

Real-time reverse transcription-polymerase chain reaction

Total RNA was isolated using Trizol (Invitrogen). Primers were designed by Beacon designer 2 and were synthesized by Shanghai Genechem. GAPDH was used as an internal control. Primers were as follows: EphB4 forward primer: 5'-GCCATTGAACAGGACTACCG-3'; reverse: 5'-CGCCCA CAGAACCGAAAG-3'; GAPDH forward primer: 5'-TGGT GAAGG TCGGTGTGAAC-3'; reverse: 5'-GCTCCTGGAAG ATGGTGATGG-3'. Real-time PCR was performed in a real-time PCR detection system (Takara Biotechnology). The cycling conditions were as follows: pre-treatment at 95°C for 15 s, then 40 cycles of denaturation at 95°C for 5 s, and extension at 60°C for 30 s. Relative quantitation of gene expression was calculated using the comparative ΔC_T (C_T : cycle threshold) method.

Laser-induced experimental CNV model in C57BL/6J mice

Forty-eight male C57BL/6J mice weighing 18–20 g each were used. The mice were anesthetized with intraperitoneal injections of 45 mg/kg 0.5% sodium pentobarbital, and the pupils were dilated with 0.5% tropicamide. The induction of CNV was induced by Nd:YAG laser irradiation (532 nm; Quantel medical) in one randomly selected eye of each animal. In each eye, 4–6 focal laser photocoagulation lesions (100 mW, 100 ms, and 75 µm) were placed around the optic

discs. The mice were then randomly assigned to be injected intravitreally with 2 μ l phosphate-buffered saline (PBS), pFU LV-shRNA-NC, and LV-shRNA-EphB4, respectively, and the non-injection group was set as the control.

Immunofluorescence

We observed the distribution of the lentivirus within the eyes by immunofluorescence as previously described.²⁰ Cryosections of 10 μ m thickness were obtained from a frozen mouse 3 days after laser treatment and stored at -20°C . Slides were fixed in 4% paraformaldehyde (PFA) and blocked with 5% bovine serum albumin and 1% Triton for 3 h at room temperature. The slides were incubated for 12 h with rabbit anti-mouse antibodies against EphB4, then incubated with rhodamine-labeled goat anti-rabbit IgG antibodies, after washing in PBS, and labeled by DAPI before being examined with a confocal laser-scanning microscope (FluoView FV-1000; Olympus).

Fundus fluorescein angiography

The CNV lesions were studied 7, 14, and 28 days after laser photocoagulation by fundus fluorescein angiography (FFA) with a digital fundus camera (Heidelberg Retina Angiography) as previously described.^{21,22} Six laser spots were made (75 μ m spot size, 75 ms, and 90-mW power) in the area surrounding the optic disc in the eye. Early-phase angiograms were taken within 1–2 min of the tail vein injection. The mean area of CNV was derived from the measurement of all the fluorescein leakage. In each eye, the area of fluorescein leakage on FFA was measured and calculated as the number of pixels with Image-Pro Plus software, and 10 lesions of each group were chosen in this study.

Histopathology analysis

After laser induction of CNV for 14 days, the eyes were carefully enucleated, and fixed in Bouin's fixative for standard hematoxylin-eosin (HE) staining. Eye cups were made as just described. After paraffin embedding, 4 mm-thick serial sections were cut and stained with HE. These sections were mounted on glass slides to determine the center of each lesion. HE-stained sections were digitized by light micros-

copy. The distance from the top of the lesion to the bottom of the RPE layer was measured as the thickness of CNV using image analysis software Image-Pro Plus 6.0. Ten lesions of each group were selected for a statistical analysis.

Preparation of choroidal flat mounts

The eyes were harvested on day 14 after intravitreal injections for flat mount preparations. The size of CNV lesions was measured on choroidal flat mounts according to Shen's method.²³ The mice were anesthetized, and the eyes were enucleated and fixed in 4% PFA for 30 min at 4°C , followed by PBS for 2 h. The anterior segment and the lens of the eyes were removed, and the vitreous and the entire retina were

FIG. 1. Western blot analysis of EphB4 protein expression in Lewis cells, Hepa1-6 cells, and NIH-3T3 cells. EphB4, erythropoietin-producing hepatocellular carcinoma receptors B4.

FIG. 2. Expression of EphB4 in Lewis cells transfected with either Lv-shRNA-EphB4 vectors or pFU LV-shRNA-NC vectors was detected by real-time polymerase chain reaction and western blot. (A) EphB4 mRNA levels were down-regulated by 61.9% (KD1, Lv-shRNA1-EphB4), 12.7% (KD2, Lv-shRNA2-EphB4), and 61.2% (KD3, Lv-shRNA3-EphB4), respectively; MOI = 20. (B) EphB4 mRNA levels were down-regulated by 82.4% (KD1), 48.9% (KD2), and 80.4% (KD3), respectively; MOI = 40. (C) The expression of EphB4 protein, assayed by western blot. MOI, multiplicity of infection.

peeled away from the eyecups. Four or five radial places were cut and mounted in PermaFluor™ Aqueous Mounting Medium (Thermo Fisher Scientific). The CNV area was analyzed using ricinus communis agglutinin staining. Image-Pro Plus software was used to measure the area of CNV lesions.

Statistical analysis

Statistical analysis was performed using one-way analysis of variance (ANOVA) by SPSS11.0 software (SPSS, Inc.). All data are expressed as mean $\pm$ standard error of the mean. A P value <0.05 was considered statistically significant.

Results

Establishment of the lentivirus-infected cell system

The protein levels of EphB4 were verified using Western blot (Fig. 1). EphB4 protein was clearly detected in Lewis cells. However, only small amounts of protein were detected in Hepa1-6 cells and NIH-3T3 cells. Therefore, we chose Lewis cells to conduct EphB4-targeting experiments.

Knockdown of EphB4 expression in Lewis cells

We used a lentiviral vector system to knock down the expression of EphB4. Lewis cells were transfected with either Lv-shRNA-EphB4 vectors or pFU LV-shRNA-NC vectors, and the EphB4 mRNA and protein levels were determined by real-time PCR and western blot. When the multiplicity of infection (MOI) was 20, the mRNA levels of EphB4 were down-regulated by 61.9% (KD1), 12.7% (KD2), and 61.2% (KD3), respectively (Fig. 2A). When the MOI was 40, the down-regulation of EphB4 mRNA was 82.4% (KD1), 48.9% (KD2), and 80.4% (KD3), respectively. There were discrepancies in mRNA and protein levels for KD2, but the reason was not clear. We speculated that it was due to

translational efficiency or posttranslational regulation. The EphB4-silencing induced by Lv-shRNA1-EphB4 and Lv-shRNA3-EphB4 ($>80\%$) was greater than that of the Lv-shRNA2-EphB4. In addition, western blotting demonstrated that Lv-shRNA3-EphB4 generated a loss of EphB4 protein expression (Fig. 2C). We, therefore, concluded that Lv-shRNA3-EphB4 transfection was functional for silencing EphB4 expression.

Immunofluorescence

Immunofluorescence staining was carried out on the third day after intravitreal injections. There was no fluorescence of GFP in the PBS group (Fig. 3A) or the non-injection group (Fig. 3B). In Fig. 3C (pFU LV-shRNA-NC), a diffuse and faint GFP fluorescence was present in the neural retina, RPE, CNV lesions, and underlying choroid, indicating the possibility that the pFU LV-shRNA-NC lentiviral vector is being taken and expressed in many of the retinal layers. However, the fluorescence of GFP in the Lv-shRNA-EphB4 group (Fig. 3D) could only be detected in CNV lesions. It indicated that the pFU LV-shRNA-NC lentiviral vector was non-specific to EphB4. The expression of EphB4 (Fig. 3E) observed in the CNV specimens of the Lv-shRNA-EphB4 group was diminished compared with the PBS group, and non-injection group, suggesting that the shRNA was functional for silencing EphB4.

Fundus fluorescein angiography

FFA examinations were performed to observe the development of CNV. On day 3, 7, 14, and 28 after the laser induction of CNV, the mice were treated with Lv-shRNA-EphB4 recombinant lentivirus. The laser lesions were evaluated by FFA in the treated group (Fig. 4A–D). FFA showed white lesions in the retina around the optic disc 3 days (Fig. 4A) after laser photocoagulation. The fluorescence signal could clearly be seen 7 days after laser treatment (Fig. 4B).

FIG. 3. Fluorescence microphotographs of CNV lesions. Fluorescence microphotographs of CNV lesions in (A) PBS, (B) non-injection group 3 days after laser photocoagulation. (C) Immunofluorescence staining showed a diffuse and faint GFP fluorescence (green) present in the neural retina, RPE, CNV lesions, and underlying choroid in the pFU LV-shRNA-NC group. (D) The GFP fluorescence could be observed in and around CNV lesions 3 in the Lv-shRNA-EphB4 group. (E) The expression of EphB4 (red) observed in the CNV of the Lv-shRNA-EphB4 group was diminished compared with the PBS group and non-injection group; (F) was the merged image of (D) and (E). (Bar: 100 μ m). CNV, choroidal neovascularization; PBS, phosphate-buffered saline; GFP, green fluorescent protein; RPE, retinal pigment epithelium.

FIG. 4. Treatment with Lv-shRNA-EphB4 recombinant lentivirus decreased the area of CNV leakage by FFA. FFA of CNV lesions in Lv-shRNA-EphB4 group on 3 days (A), 7 days (B), 14 days (C), and 28 days (D) revealed a reduction in fluorescein leakage on days 14 and 28 compared with days 3 and 7. (E–H) FFA in the non-injection group showed bigger fluorescein leakage than the Lv-shRNA-EphB4 group. At 14 days after the intravitreal injection, the Lv-shRNA-EphB4 group showed decreased fluorescein leakage compared with the PBS-treated group (I), pFU LV-shRNA-NC group (J), and non-injection group (G). (K) In each group, 10 lesions from 5 mice were included (** $P < 0.01$: represents statistically significant). FFA, fundus fluorescein angiography.

However, there was a significant decrease in fluorescein leakage of CNV lesions on the 14th day (Fig. 4C), and a fluorescein signal could not be detected 28 days after the injection (Fig. 4D). The data suggest that an intravitreal injection of Lv-shRNA-EphB4 recombinant lentivirus was able to diminish the area of CNV leakage. Images on day 3, 7, 14, and 28 for the non-injection group showed bigger fluorescein leakage of CNV lesions (Fig. 4E–H).

Fourteen days after an intravitreal injection of PBS, Lv-shRNA-EphB4, or pFU LV-shRNA-NC, each group of mice showed moderate-to-severe fluorescein leakage in CNV membranes. Treatment with Lv-shRNA-EphB4 led to a reduced fluorescein leakage (Fig. 4C; $2.40 \pm 0.32 \times 10^3$ pixels; $n=10$) compared with the PBS-treated group (Fig. 4I; $4.01 \pm 0.86 \times 10^3$ pixels; $n=10$), pFU LV-shRNA-NC group

(Fig. 4J; $3.78 \pm 0.83 \times 10^3$ pixels; $n=10$), and non-injection group (Fig. 4G; $3.35 \pm 0.42 \times 10^3$ pixels; $n=10$). ANOVA showed significant differences ($P < 0.01$) between the Lv-shRNA-EphB4 group and the PBS group, pFU LV-shRNA-NC group, or non-injection group.

Histopathological analysis

HE staining was carried out to measure the thickness of CNV on the 14th day after laser induction. The rupture of RPE and Bruch's membrane in the area of each laser burn could be seen in all mice. There were multilayered fusiform proliferative membranes in the central area underlying the RPE to the choroid in the CNV lesions. Figure 5D shows an image of treated choroidal neovascular membrane 14 days

FIG. 5. Histopathology analysis of treated and control mice. Mice eye sections were cut and stained with hematoxylin-eosin 14 days after an intravitreal injection. The distance from the disrupted RPE layer to the top of the lesions was measured (white arrows). Histopathology analysis confirmed that CNV lesions in the Lv-shRNA-EphB4 group (D) were of a thinner thickness than the PBS group (A), pFU LV-shRNA-NC group (B), and non-injection group (C). (E) In each group, 10 lesions from 5 mice were included (** $P < 0.01$: represents statistically significant; Bar: 50 μm).

after laser induction. Compared with the PBS-treated group (Fig. 5A), the pFU LV-shRNA-NC (Fig. 5B), and non-injection group (Fig. 5C), CNV lesions had a significantly decreased thickness in Lv-shRNA-EphB4 mice. The average thickness of each group was $64.69 \pm 6.89 \mu\text{m}$ (Lv-shRNA-EphB4 group), $100.08 \pm 9.49 \mu\text{m}$ (PBS), $87.54 \pm 6.92 \mu\text{m}$ (pFU LV-shRNA-NC), and $85.87 \pm 8.87 \mu\text{m}$ (non-injection group), respectively. ANOVA revealed significant differences ($P < 0.01$) between the Lv-shRNA-EphB4 group and PBS group, pFU LV-shRNA-NC group or non-injection group.

Choroidal flat mounts analysis

The size of CNV was assessed by postmortem measurements on choroidal flat mounts. Images of CNV on choroidal

flat mounts on the 14th day after the intravitreal injection are shown in Fig. 6. The mean area of CNV lesions was measured using Image-Pro Plus software. The average area was significantly decreased in the Lv-shRNA-EphB4 group (Fig. 6D; $9,756.86 \pm 2,315.24 \mu\text{m}^2$) compared with the PBS-treated group (Fig. 6A; $16,123.05 \pm 3,227.75 \mu\text{m}^2$; $n = 20$, $P < 0.01$), pFU LV-shRNA-NC (Fig. 6B; $17,666.34 \pm 4,137.61 \mu\text{m}^2$; $n = 20$, $P < 0.01$), and non-injection group (Fig. 6C; $16,985.30 \pm 3,047.12 \mu\text{m}^2$; $n = 10$, $P < 0.01$).

Discussion

Treatments currently available for CNV include laser photocoagulation, verteporfin photodynamic therapy, and intravitreal injections of bevacizumab or ranibizumab.²⁴

FIG. 6. Images of choroidal flat mounts revealed decreased size of CNV leakage in Lv-shRNA-EphB4 mice. CNV in the PBS group (A), pFU LV-shRNA-NC group (B), and non-injection group (C) were much larger than Lv-shRNA-EphB4 group. (D) CNV in Lv-shRNA-EphB4 mice reached the minimum size within 14 days. (E) In each group, 20 lesions from 10 mice were included. (** $P < 0.01$: represents statistically significant; Bar: 50 μm).

Recently, anti-angiogenic drugs have offered improvements in visual acuity for a large number of patients. VEGF is important in the formation of CNV.²⁵ Several therapies for CNV are based on antagonism of VEGF, such as pegaptanib, bevacizumab, and ranibizumab, which are widely used to treat CNV and other eye diseases.^{26,27}

In the current experiments, we have found that the Eph family is involved in vascular development, and the interplay between VEGF and Ephs plays an important role in angiogenesis. Studies demonstrate that VEGF induces the expression of EphrinA1, thus increasing the activation of EphA2. Blocking the expression of EphA2 receptor inhibits

VEGF-induced angiogenesis.²⁸ Moreover, VEGF induces the expression of EphrinB2 and the subsequent activation of EphB4, and EphB4/EphrinB2 stimulation leads to VEGF secretion. EphB4 also plays a role in pathologic neovascularization in the mouse model of OIR. Targeted disruption of EphB4 using the EphB4 extracellular domain (sEphB4) could reduce retinal neovascularization in a model of proliferative retinopathy.²⁹ He et al.³⁰ reported that EphB4 and EphrinB2 are expressed in cultured RPE, and sEphB4 can inhibit EphB4 and EphrinB2 signaling in RPE. They found that RPE proliferation, attachment, and migration are inhibited by sEphB4.³⁰

In the present experiments, we speculate that EphB4 plays a critical role in the formation of CNV, and knockdown of the expression of EphB4 may inhibit CNV. We designed an experiment to investigate the suppression of CNV by targeting the expression of EphB4. We knocked down the EphB4 gene by lentiviral-mediated RNAi, which has been widely used to knock down gene expression in mammalian cells. RNAi has been a useful tool that is used for studying gene function and therapeutic potentials for various diseases.³¹ Noren used siRNA interference to down-regulate EphB4 in MCF7 and MDA-MB-435 cancer cells. They found that 4 different EphB4 siRNAs effectively knocked down EphB4 expression in the MDA-MB-435c clonal cell line, and did not find possible off-target effects.³² Numerous vectors have been utilized to infect cells and express shRNAs for the treatment of angiogenic diseases. Among these vectors, lentivirus-based vectors could deliver a large number of genetic messages efficiently. Furthermore, lentiviruses are able to infect both dividing and non-dividing cells, and have achieved long-term regulation of gene expression.³³ These advantages made lentiviral-based vectors the best to study the effect of the treatment of EphB4 silencing on CNV. We constructed the pFU-GW-RNAi lentivirus vector, which could efficiently knock down the expression of EphB4 mRNA.

The major finding of our study was that EphB4 knockdown suppressed the formation of CNV. FFA showed that 14 days after an intravitreal injection, an EphB4-targeting lentivirus could attenuate vascular leakage. A histopathological analysis revealed that the CNV thickness of Lv-shRNA-EphB4 mice was thinner than the other groups. We, therefore, conclude that targeting EphB4 could inhibit CNV growth in laser-induced mice. Choroidal flatmounts analysis reinforces this claim. In addition, compared with the PBS group and non-injection group in the results of the FFA and HE studies, the non-injection group displayed a decreased CNV neovascular area. It appears to be an injection effect, while many studies have demonstrated that intravitreal bevacizumab or ranibizumab in patients was generally well tolerated and improved visual acuity in eyes with CNV.^{34,35}

The function of EphB4 has been described in great detail for tumor angiogenesis and the nervous system. However, it has not been well characterized in CNV.³⁶ The interaction of EphB4 and EphrinB2 only functions at the arterial-venous interface, indicating that the interaction is important in arterial and venous cells differentiation. EphB4 reverse signaling is significant for proper morphogenesis and formation of the vascular system. The signal might lead to the switch in the initial vascularization system, and individual endothelial cells may begin to leave the existing vascular tree and interplay with the contiguous sprouts to form the vascular network.³⁷ Besides, bio-directional signaling between EphB4 and its ligand might then promote endothelial cell migration and capillary tube formation. Based on these findings, we could hypothesize that EphB4 reverse signaling promoted CEC migration and tube formation, thereby leading to the occurrence and development of CNV. It is possible that EphB4 gene silencing blocked the transmission of EphB4 signaling and inhibited vessel maturation, migration of CEC, and reduced the leakage of CNV.

In summary, we provide evidence that using the RNAi technique, targeting EphB4 could effectively decrease the expression of EphB4 and inhibit the formation of CNV. In

addition, studies have suggested that no single treatment offers a perfect cure for CNV, but combined therapies provided more sustained treatments for human CNV. Therefore, EphB4 knockdown might achieve a synergistic effect and offer potential treatments for CNV. Although we found that EphB4 knockdown inhibited CNV, the role of EphB4 in CNV development is still poorly understood, and further studies are needed to investigate the EphB4 signaling mechanisms.

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Author Disclosure Statement

None of the authors has any financial association that might raise a conflict of interest in relation to this article.

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