Materials and Methods

Animals and ethics

Male C57BL/6J mice (Japan SLC, Hamamatsu, Japan), and HB-EGF conditional knockout mice, aged 8-12 weeks, were used in these experiments. The conditional HB-EGF KO mice were created with the Cre-lox-mediated conditional gene KO approach with a homeobox protein SIX3 promoter as described in detail\(^1\).\(^2\). The background strain of HB-EGF conditional KO mice was C57BL/6J. More than 60% of the standard HB-EGF KO mice die in the first postnatal week because of an enlarged heart\(^3\). We used the SIX3 promoter to create the conditional knockout mice because SIX3 plays an important role in the development of the vertebrate visual system\(^4\). This gene encodes a member of the sine oculis homeobox transcription factor family. The encoded protein plays a role in eye development. Indeed, we have already reported that HB-EGF conditional KO mice using SIX3 promoter deleted HB-EGF in the all retinal layer using β-galactosidase (LacZ) staining and RT-PCR\(^1\). The mice were kept under 12 h:12 h light:dark conditions and had access to food (CLEA rodent diet CE-2; CLEA Japan, Inc. Tokyo, Japan) and water ad libitum. The maximum number of animals/cage was 7. Experiments were also performed on 4 adult male common marmoset monkeys (Callithrix jacchus), age 2–3 years, weighing 240 g–360 g. They were kept in an air-conditioned room at 24° to 27°C with
40% to 70% humidity. All procedures were performed in accordance with the ARVO statement for the Use of Animals in Ophthalmic and Vision Research. All experiments including both mice and common marmosets studies were approved and monitored by the Institutional Animal Care and Use Committee of Gifu Pharmaceutical University, and in accordance with “2010 Guide for Care and Use of Laboratory Primates” of Kyoto University’s Primate Research Institute.

**Choroidal neovascularization (CNV) model in mice and common marmoset monkeys**

Mice were anesthetized with pentobarbital (Nakarai Tesque, Kyoto, Japan) or a mixture of ketamine (Daiichi Sankyo, Tokyo, Japan) and xylazine (Bayer Health Care, Tokyo, Japan), and the pupils were dilated with 0.5% tropicamide (Santen, Osaka, Japan). Laser photocoagulation (647 nm, 120 mW, 100 msec, 50 μm; MC500, NIDEC, Kyoto, Japan) was performed on the right eye of each animal on day 0. Six laser spots were applied around the optic nerve with a slit-lamp delivery system with a cover glass used as a contact lens as described³. The endpoint of the laser application was the appearance of a cavitation bubble which was an indication of a disruption of Bruch’s membrane.

The marmoset monkeys were anesthetized with ketamine (30 g/kg, im; Daiichi
Sankyo), medetomidine (0.15 mg/kg, im; Kyoritsu Seiyaku Corporation, Tokyo, Japan), and pentobarbital (5 mg/kg, ip; Nakarai Tesque) as developed in our laboratory. The pupils were dilated with 0.5% tropicamide (Santen), and the retinas were photocoagulated (MERILAS 532α; MERIDIAN AG, Thun, Switzerland) at 8 spots in the macular area.

**Choroidal fluorescein angiography**

Two weeks after the laser photocoagulation, mice were killed and perfused with 0.5 mL of fluorescein-conjugated dextran (MW = 2,000 kDa, Sigma) as reported. Eyes were enucleated and fixed in 4% paraformaldehyde (PFA) for 12 h. The retinal pigment epithelium (RPE)-choroid-sclera complex was flat-mounted with Fluoromount™ (Diagnostic BioSystems). The sites of the CNVs in the flat-mounted samples were examined with a confocal microscope (FV10i, Olympus, Tokyo, Japan; ×10 objective and ×3 digital zoom), and the average size of the CNV lesions was determined using the imaging software for OLYMPUS FLUOVIEW-ASW Version 02. 01 (OLYMPUS).

After the SD-OCT recordings, the marmoset monkeys received a 3 mL injection of 20.0 mg/mL FITC in the femoral vein, the eyes were enucleated, and the choroid and RPE complex were isolated and flat-mounted. The areas of the CNV were photographed with a fluorescence confocal microscope (FV10i) at 30X magnification and measured using
the imaging software of OLYMPUS FLUOVIEV-ASW Version 02. 01 (Olympus). Analyses were conducted in a masked way by Mr. Shinsuke Takata and evaluation was done by Mr. Yuki Inoue.

**Oxygen-induced retinopathy (OIR) model**

The OIR mouse model was produced as described in detail\(^7\). In brief, 7-day-old pups and their mothers were placed in a custom-built chamber and exposed to an atmosphere of 75% ± 1% oxygen for 5 days at a temperature of 24 ± 2°C. The oxygen level was continuously monitored with an oxygen controller (PRO-OX 110; Reming Bioinstruments Co., Redfield, SD, USA). On P12, the animals were returned to room air (21% O\(_2\)) until P17.

**Retinal angiography**

Mice were deeply anesthetized intraperitoneally with 30 mg/kg of sodium pentobarbital (Nembutal; Dainippon-Sumitomo Pharmaceutical Co. Ltd., Osaka, Japan). The mice were perfused through the left ventricle with high molecular weight (MW = 2,000 kDa) fluorescein-conjugated dextran (Sigma, St. Louis, MO, USA) dissolved in PBS. The eyes were then enucleated and placed in 4% PFA. The cornea and lens were
removed under a dissecting microscope, and the retinas were isolated, flat-mounted, and covered with a coverslip with a few drops of Fluoromount™ aqueous mounting medium for fluorescent staining (Diagnostic BioSystems, Pleasanton, CA, USA). The size of the retinal neovascularization was measured with an imaging software as described. To quantify the degree of pathological neovascularization, the retinal vasculature was analyzed with the Angiogenesis Tube Formation Module in Metamorph (Universal Imaging Corp., Downingtown, PA, USA). We evaluated the number of nodes and node areas, which are parameters that are obtained from these analyzed images. The node is the region of the connected ‘blobs’, which are shown as green labels in the analyzed images and corresponded well with the areas of pathological neovascularization area including the tortuous and dilated blood vessels and abnormal vascular structures.

**Immunoblotting**

For the *in vivo* studies, mice were euthanized by cervical dislocation on 1, 3, 5, and 7 days after laser irradiation or by decapitation at P12, P14, P17. The eyes were enucleated, and the corneas and lenses were removed. Then, the eye cups with the RPE-choroid-sclera complexes or retinas were quickly frozen in dry ice. The eyecups were homogenized in cell-lysis buffer with a homogenizer (Phycotron; Microtec Co. Ltd., Chiba, Japan). The
lysate was centrifuged at 12,000 × g for 20 min, and the supernatant was used for the following studies. The protein concentration was measured by comparing it to known concentrations of bovine serum albumin using a BCA Protein Assay Kit (Pierce Biotechnology, Rockford, IL, USA). A mixture of equal parts of an aliquot of protein and sample buffer with 10% 2-mercaptoethanol was subjected to 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The separated protein was then transferred onto a polyvinylidene difluoride membrane (Immobilon-P; Millipore Corporation, Bedford, MA, USA). For immunoblotting, the following primary antibodies were used: HB-EGF (kind gift from Dr. Higashiyama of Ehime University) (0.2 μg/mL), VEGF (Merck Millipore, Billerica, MA, USA, Cat #PC315) (5 μg/mL), ADAM17 (Abcam, Cambridge, UK, Cat #ab2051), (1 μg/mL), ADAM12 (Abcam, Cat #ab39155) (1 μg/mL), and β-actin mouse monoclonal antibody (Sigma-Aldrich, Tokyo, Japan, Cat #A2228) (0.2 μg/mL). The secondary antibodies were goat anti-rat HRP-conjugated (50 ng/mL; Funakoshi, Tokyo, Japan), goat anti-rabbit HRP-conjugated (50 ng/mL; Thermo Fisher Scientific), and goat anti-mouse HRP-conjugated (50 ng/mL; Thermo Fisher Scientific).

In the in vitro studies, human retinal microvascular endothelial cells (HRMECs, DS Pharma Biomedical, Osaka, Japan) were seeded in 12-well plates at a density of 4 × 10^5 cells/well and incubated for 24 h. The medium was then replaced with fresh medium
containing 1% FBS. HB-EGF (R&D systems, Minneapolis, MN, USA), VEGF (R&D systems), or HB-EGF+VEGF was added to the cell cultures at 10 ng/mL. After 5, 10, 30, 60, 180, and 360 min, the cells were washed with PBS and then lysed with a cell-lysis buffer including protease inhibitor (Sigma-Aldrich) and phosphatase inhibitor cocktails (Sigma-Aldrich). Diphtheria Toxin CRM mutant, an HB-EGF inhibitor (CRM-197; Merck CALBIOCHEM) was used at a concentration of 10 μM. The BCA Protein Assay Kit (Pierce Biotechnology, Rockford, IL, USA) was used to measure the protein concentration. About 2 μg of total protein/sample was diluted with sample buffer with 20% 2-mercaptoethanol (Wako). After heating for 5 min at 95°C, the samples were electrophoresed with 5-20% sodium dodecyl sulfate-polyacrylamide electrophoresis gel (Wako). The separated proteins were electroblotted onto polyvinylidene difluoride membrane (Immobilon-P; Millipore Corporation, Bedford, MA, USA). For immunoblotting, the following primary antibodies were used: phosphorylated extracellular signal-regulated kinase (p-ERK) 1/2 (CST, Cat #4377) (97 ng/mL), t-ERK1/2 (CST, Cat #9102) (41 ng/mL), phosphorylated EGFR (p-EGFR) (Abcam, Cat #ab5644) (1 μg/mL), t-EGFR (CST, Cat #2232) (130 ng/mL), phosphorylated VEGFR (p-VEGFR) (CST, Cat #2478) (153 ng/mL), t-VEGFR (CST, Cat #2479) (26 ng/mL), and β-actin mouse monoclonal antibody (0.2 μg/mL; Sigma-Aldrich, Tokyo, Japan, Cat
We used the following secondary antibodies; goat anti-rabbit HRP-conjugated (50 ng/mL; Thermo Fisher Scientific), or goat anti-mouse HRP-conjugated (50 ng/mL; Thermo Fisher Scientific).

The immunoreactive bands were made visible with SuperSignal West Femto Maximum Sensitivity Substrate (Thermo Fisher Scientific Inc.). The intensities of the bands were measured with a Lumino Imaging Analyzer (Fujifilm, Osaka, Japan).

**Immunohistochemistry**

For immunohistochemistry in CNV model, mice were perfused with 0.5 mL fluorescein-conjugated dextran (MW = 2,000 kDa, Sigma) 3 days after the laser irradiation. The mice were euthanized by cervical dislocation, and their eyes quickly removed and fixed in 4% PFA overnight at 4°C. For immunohistochemistry in OIR model, mice were euthanized by decapitation at P17. The eyes were enucleated fixed in 4% PFA for 3 h at 4°C. For 5-Bromo-2′-deoxyuridine (BrdU) staining, mice were injected 50 mg/kg BrdU (Sigma-Aldrich) by i.p. Three hours after BrdU treatment, the eyes were enucleated and used for immunohistochemistry. Next, their cornea and lens were removed and the RPE-choroid-sclera complexes or retinas isolated and flat-mounted.

They were then blocked with 10% normal goat serum and 0.3% Triton X-100 (Nacalai
Tesque, Kyoto, Japan) for 1 h at room temperature. The flat-mounted RPE-choroid sclera complexes or retinal flat-mounts were incubated with primary antibodies against rat anti-HB-EGF (kind gift from Dr. Higashiyama of Ehime University) (2 μg/mL), rabbit anti-VEGF (Merck Millipore, Cat #PC315) (5 μg/mL), Merck Millipore), or rat anti-BrdU (Abcam, Cat #ab6326) (5 μg/mL) overnight at 4°C. After washes with PBS, the immunoreactivity was made visible by incubation with goat anti-rat (secondary antibody) conjugated with Alexa-546 (Life Technologies, Carlsbad, MD, USA) (2 μg/mL) and goat anti-rabbit (secondary antibody) conjugated with Alexa-633 (Life Technologies) (2 μg/mL) for 1 h at room temperature. For isoelectin B4 (IB4) staining, retinal flat-mounts were incubated Fluorescein labeled Griffonia Simplicifolia Lectin I (GSL I) isoelectin B4 (Vector laboratories, Burlingame, CA, USA) overnight at 4°C. After washing with PBS, the samples were mounted with Fluoromount™.

For the BrdU staining, the retinal or RPE-choroidal flat-mounts were pre-treated for 30 min with 2M hydrochloric acid (HCl) 2M for 30 min. They were incubated with 0.3% Triton X-100 (Bio-Rad Labs, Hercules, CA, USA) for 30 min. They were then treated with 0.1% trypsin (Wako Pure Chemical Industries, Ltd.) at 37 °C for 10 min.

The fluorescent images were photographed through a confocal microscope (FV10i).
Cell Culture

Human retinal microvascular endothelial cells (HRMECs) were cultured in a growth medium (CSC complete defined medium, DS Pharma Biomedical) at 37 °C in a humidified atmosphere of 5% CO₂ in air. The medium was supplemented with a defined cell boost. Subconfluent monolayers of HRMECs from passages 3 to 8 were used in the experiments.

Cell proliferation assay

HRMECs were seeded into 96 well plate at a density of 2 × 10³ cells/well at 37 °C for 24 h in a humidified atmosphere of 5% CO₂, and preincubated in CSC medium containing 10% fetal bovine serum (FBS) without cell boost at 37 °C for 24 h. The media of the HRMECs were supplemented with different concentrations of HB-EGF (0.1-10 ng/mL), VEGF (10 ng/mL), HB-EGF+VEGF, AG1478, CRM-197 (10 μg/mL), or anti-human HB-EGF antibody (0.1, 1, 10μg/mL) (Angio-proteomie, Boston, MA, USA). Then, the HRMECs were incubated for 24, 48 or 72 h. After incubation, the viable cell numbers were determined with the WST-8 assay. Briefly, 10 μL of CCK-8 (Dojindo, Kumamoto, Japan) was added to each well, incubated at 37 °C for 3 h, and the absorbance was measured at 492 nm.
**Endothelial scratch assay**

A modified endothelial scratch assay was used to measure unidirectional migration as described in detail in our previous reports. HRMECs were seeded into 12 well plates at a density of $4 \times 10^4$ cells/well at 37 °C for 24 h in a humidified atmosphere of 5% CO$_2$. The preincubation CSC medium contained 1% FBS without cell boost at 37° C for 6 h. After 24 h of incubation, the monolayers of the HRMEC were scratched to a 1 mm depth in a straight line using a 1000 µL micro-tip. After the HRMECs were exposed to HB-EGF, VEGF, HB-EGF+VEGF, or CRM-197, the cells were incubated for an additional 24 h. Images of the HRMECs in each well were taken at the time of the wounding and at 24-h intervals thereafter using a phase-contrast microscope (Olympus, Tokyo, Japan). The amount of migration was estimated by counting the cell numbers within the wounded region. The invading cells were counted by a single observer and taken as the number of migrating cells. For each monolayer sample, four measurements were taken from four fields from each well.

**Boyden Chamber Assays**

Boyden Chamber Assays were conducted using Transwell® (Coring Life Sciences,
Tewksbury, MA, USA) following the manufacturer’s protocol. Briefly, the preincubation CSC medium contained 1% FBS without cell boost at 37° C for 6 h. After 6 h starvation, HRMECs were seeded at a density of 5×10^4 cells/well to the insert wells. To receiver well, we added medium with 10% FBS or 10% FBS plus HB-EGF, VEGF, HB-EGF+VEGF, or CRM-197 for 24 h at 37° C in 5% CO₂. After aspirate the medium, we washed the well with PBS and stained the cells. Then, the inserts were washed with water until the water run clear and dry completely. Images of the HRMECs in each well were taken using a phase-contrast microscope (Olympus, Tokyo, Japan). The amount of migration was estimated by counting the cell numbers. The invading cells were counted by a single observer and taken as the number of migrating cells. For each Transwell® insert, the number of stained cells were taken in five random fields. Finally, we calculated and determined the migration cell number per inserts.

**Transfection**

HRMECs were seeded at a density of 1×10^4 cells/well in 12-well plates and incubated for 24 h at 37° C in 5% CO₂. They were then transfected using Lipofectamine RNAiMax (Thermo Fisher Scientific) according to the manufacturer’s instructions. The HRMECs were transfected with 50 nM of Stealth RNAiTM siRNA Duplex Oligo ribonucleotides
targeting HB-EGF, ADAM17, or ADAM12 (Thermo Fisher Scientific) for 48 h. The HRMECs transfected with a non-targeting siRNA (50 nM; Thermo Fisher Scientific) served as a negative control.

**Cell treatment and RNA isolation.**

To examine the expression of some of the members of the EGF family such as HB-EGF and EGF, HRMECs were exposed to VEGF and sampled after 1, 3, or 6 h. Aflibercept, anti-VEGF antibody, was treated 1 h before VEGF exposure. The RNAs were isolated from the cells with the NucleoSpin® RNA kit (Takara, Shiga, Japan) following the manufacturer’s protocol. The concentrations of the RNAs were measured with the NanoVue Plus spectrophotometer (GE Healthcare Japan, Tokyo, Japan) at 260 nm. We synthesized 10-μL reaction volume of the first-strand cDNA using a PrimeScript™ RT reagent kit (Perfect Real Time; Takara) according to the manufacturer’s protocol.

**Real-time quantitative RT-PCR**

The expressions of the HB-EGF, EGF, ADAM17, and ADAM12 genes were measured by real-time quantitative RT-PCR. The expression of the β-actin gene was used as the
housekeeping gene. We used SYBR Premix Ex Taq™ II (Takara) and a TP 8000 Thermal Cycler Dice Real Time system (Takara). The PCR primer sequences used were: HB-EGF (forward, 5’-TGCCTGTAGCTTTTGGTCCC-3’; reverse, 5’-CCCCACCTCAAACCTCTCGG-3’), EGF (forward, 5’-TGTCTGCTGGTGCTTG-3’; reverse, 5’-CTGCGACTCCTCACATCTCTGC-3’), ADAM17 (forward, 5’-CCTGGGCACCTCCCTGT-3’; reverse, 5’-AATCGCCTCTGGGACTTCTTCTTG-3’), ADAM12 (forward, 5’-CTGGGCACCTCCCTGT-3’; reverse, 5’-TGCTTCTGCTTGCCGGAG-3’), and β-actin (forward, 5’-TCAAGATCATTGCTCCTG-3’; reverse, 5’-CTGCTTGCTGATCCACATCTG-3’).

**Intravitreal administration of CRM-197 in common marmoset monkeys**

Diphtheria Toxin CRM mutant (CRM-197; Merck CALBIOCHEM) at a concentration of 12.5 mg/mL was injected into the vitreous cavity by a 33-gauge needle inserted at the corneal-scleral junction. This was done immediately after the laser irradiation under anesthesia. For buffer control experiments, the marmoset monkeys were injected with the same volume of PBS. The injection volume was 10 μL in all cases.
**Fundus fluorescein angiography for common marmoset monkeys**

Fundus fluorescein angiography was performed to compare the degree of extravasation at the photocoagulated spots in the marmoset monkeys. Fluorescein angiography was performed 3 weeks after the photocoagulation. The fluorescein angiograms were graded using standard angiograms as follows: Grade 1, no hyperfluorescence; grade 2, hyperfluorescence without leakage; grade 3, early hyperfluorescence or mid transit and late leakage; and grade 4, transit bright hyperfluorescence with leakage at the borders of the treated areas\(^9,10\).

**Spectral-domain Optical coherence tomography (SD-OCT)**

SD-OCT was performed on day 21 to obtain in situ images of the CNV as described\(^6\). Under anesthesia, the pupils of the marmoset monkeys were dilated with 0.5% tropicamide, and the corneas were covered with a contact lens (zero power; Unicon, Tokyo, Japan) to prevent eye dehydration. Two and three dimensional images of the CNVs were recorded with a SD-OCT system (RS-3000, NIDEC) using a 15 diopter lens.

**Statistical analysis**

Statistical analyses of the data of the in all experiments were performed with the
Statistical Package for the Social Sciences 15.0J for Windows software (SPSS Japan Inc, Tokyo, Japan). Data are presented as the means ± standard error of the means (SEMs). Groups were compared using the Student’s *t*-test or one way ANOVA for parametric data, assuming equal variance and normality of the data. Statistical comparisons of the in this study were made *via* one-tailed Student’s *t*-tests (Fig. 6D), two-tailed Student’s *t*-tests (Fig. 1B, 1D, 1F, 1G, 1I, 2F, 2G, 4C, and 4D), Dunnett’s multiple comparison tests (Fig. 2D, 2E, 2J, 2K, Supp. Fig. IIA, and IIC), or Tukey’s tests (Fig. 3A, 3C, 3D, 3E, 3F, 3H, 4B, 4E, 4G, 4I, 4K, 4M, 5A, 5B, 5C, 5D, 5E, and Supp. Fig. III, and IV), and Mann-Whitney *U*-test (Fig. 6B). A *P* value of <0.05 was taken to be statistically significant.
Supplemental Figure I. Localization of HB-EGF and VEGF in the normal retina.

(A) The localization of HB-EGF and VEGF in the normal RPE-choroidal complexes. Scale bar; 100 μm. (B) The localization of HB-EGF and VEGF in P17 control mice retinal flat-mount. Scale bar; 100 μm.

Supplemental Figure II. HB-EGF- and VEGF-induced cell proliferation and migration of HRMECs. (A) HB-EGF induced cell proliferation in a concentration-dependent manner. Addition of HB-EGF+VEGF induced more proliferation of the HRMECs. Data are the means ± SEMs. (n = 6). *: P <0.05, **: P <0.01 vs. Normal. #: P <0.05, ##: P <0.01 vs. VEGF-treated group (Dunnett’s test). (B) Representative images of HRMECs showing the effects of exposure to HB-EGF (10 ng/mL), VEGF (10 ng/mL), or HB-EGF+VEGF. (C) HB-EGF induces cell migration in a concentration-dependent manner. Exposure to HB-EGF+VEGF leads to greater migration than exposure to VEGF alone. Data are the means ± SEMs. (n = 4). **: P <0.01 vs. Normal. ##: P <0.01 vs. VEGF treated group (Dunnett’s test).

Supplemental Fig. III. Silencing HB-EGF did not decrease HB-EGF-induced cell proliferation in HRMECs. HB-EGF increases endothelial cell proliferation. However,
silencing $HB-EGF$ does not decrease cell proliferation induced by HB-EGF. Data are the means ± SEMs. (n = 6). **: P <0.01 vs. none siRNA. #: P <0.05 vs. negative control siRNA group (Tukey’s test).

Supplemental Fig. IV. Anti-human HB-EGF antibody decreases the VEGF-induced cell proliferation in HRMECs. Data are the means ± SEMs. (n = 6 or 8). **: P <0.01, vs. Control, ##: P <0.01, vs. VEGF (Tukey’s test).

Supplemental Fig. V. Representative fluorescein angiographic (FA) grades and typical OCT images in laser-induced CNV model using common marmoset monkeys. (A) Representative images of FA grades of common marmosets. (B) OCT images of CNV area of vehicle or CRM-197-treated common marmosets. 2-Dimensional and 3-dimensional images of CNV are shown.
References


