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The regulatory role of hepatoma-derived growth factor as an angiogenic factor in the eye

Michelle E. LeBlanc,[1] Weiwen Wang,[1] Xiuping Chen,[1,2] Yanli Ji,[1,3] Akhalesh Shakya,[1] Chen Shen,[1] Chenming Zhang,[1,4] Vivianne Gonzalez,[1] Megan Brewer,[1] Jian-xing Ma,[5] Rong Wen,[1] Fangliang Zhang,[6] Wei Li[1,7]

(The first two authors contributed equally to this work.)

[1]Bascom Palmer Eye Institute, Department of Ophthalmology, University of Miami School of Medicine, Miami, FL; [2]Department of Ophthalmology, Zhongshan Hospital of Fudan University, Shanghai, China; [3]Department of Ophthalmology, 2nd People's Hospital of Zhengzhou, Zhengzhou, Henan, China; [4]Department of Ophthalmology, Jinan 2nd People's Hospital, Jinan, China; [5]Department of Physiology, the University of Oklahoma Health Sciences Center, Oklahoma City, OK; [6]Department of Molecular and Cellular Pharmacology, Sylvester Comprehensive Cancer Center, University of Miami, School of Medicine; [7]Vascular Biology Institute, University of Miami School of Medicine, Miami, FL

Purpose: Hepatoma-derived growth factor (HDGF) is a mitogen that promotes endothelial proliferation and neuronal survival. Using a unique technology of ligandomics, we recently identified HDGF as a retinal endothelial binding protein. The purpose of this study is to examine the role of HDGF in regulating ocular vasculature and the expression of HDGF in the retina.

Methods: HDGF expression in the retinal was analyzed with western blot and immunohistochemistry. Angiogenic activity was investigated in human retinal microvascular endothelial cells (HRMVECs) with in vitro endothelial proliferation, migration, and permeability assays. In vivo angiogenic activity was quantified with a corneal pocket assay. The Evans blue assay and western blot using anti-mouse albumin were performed to detect the capacity of HDGF to induce retinal vascular leakage.

Results: Immunohistochemistry revealed that HDGF is expressed in the retina with a distinct pattern. HDGF was detected in retinal ganglion cells and the inner nuclear layer but not in the inner plexiform layer, suggesting that HDGF is expressed in the nucleus, but not in the cytoplasm, of retinal neurons. In contrast to family member HDGF-related protein 3 (HRP-3) that has no expression in photoreceptors, HDGF is also present in the outer nuclear layer and the inner and outer segments of photoreceptors. This suggests that HDGF is expressed in the nucleus as well as the cytoplasm of photoreceptors. In vitro functional assays showed that HDGF induced the proliferation, migration, and permeability of HRMVECs. Corneal pocket assay indicated that HDGF directly stimulated angiogenesis in vivo. Intravitreal injection of HDGF significantly induced retinal vascular leakage.

Conclusions: These results suggest that HDGF is an angiogenic factor that regulates retinal vasculature in physiologic and pathological conditions. Identification of HDGF by ligandomics and its independent characterization in this study also support the validity of this new technology for systematic identification of cellular ligands, including angiogenic factors.

Angiogenic factors play an important role in the regulation of retinal vasculature. For example, morphogenesis of blood vessels during retinal development requires the coordination of different angiogenic factors [1,2]. Aberrant developmental angiogenesis may lead to retinal vascular diseases, such as retinopathy of prematurity (ROP) [3]. Angiogenic factors also contribute to the pathogenesis of neovascular age-related macular degeneration (AMD), diabetic macular edema (DME), and proliferative diabetic retinopathy (PDR) [4]. In these diseases, angiogenic factors promote not only neovascularization but also retinal vascular leakage. Most previous studies focused on vascular endothelial growth factor (VEGF), one of the well-known angiogenic factors involved in these diseases. Anti-VEGF therapies, such as ranibizumab (Lucentis) and aflibercept (Eylea), have been approved for the therapy of neovascular AMD and DME [5,6]. However, these VEGF inhibitors have limited therapeutic efficacy to treat both diseases [5,7], implicating that other angiogenic factors may also play an important role in the pathogenesis of these diseases. One example is that anti-platelet-derived growth factor (anti-PDGF) therapy is currently in a clinical trial for combination therapy with ranibizumab to treat neovascular AMD [8]. Thus, investigation of angiogenic factors other than VEGF will provide molecular insights into the regulation of retinal vasculature, pathogenic mechanisms of neovascular diseases, and therapeutic targets.

Correspondence to: Wei Li, University of Miami School of Medicine, 1638 NW 10th Avenue, Miami, FL 33136; Phone: (305) 326-6445; FAX: (305) 547-3658; email: w.li@med.miami.edu

Hepatoma-derived growth factor (HDGF) was originally purified and identified from the medium of the human hepatoma cell line Huh-7 and was capable of stimulating the proliferation of mouse 3T3 fibroblast cells [9,10]. HDGF was reported as an endothelial mitogen to stimulate DNA synthesis [11]. A large number of studies indicated that HDGF promoted cancer cell proliferation and increased tumor malignancy and their resistance to anticancer therapy [12-14]. HDGF promoted tumorigenesis through direct angiogenic activity and induction of VEGF [15]. HDGF is widely expressed in many tissues, including brain, live, lung, kidney, heart, spleen stomach, intestine, muscle, skin, and testis [16]. However, the expression and functional regulation of HDGF in the eye remain unclear.

Endothelial ligands are traditionally identified on a case-by-case basis with technical challenges. We recently developed open reading frame phage display (OPD) for unbiased identification of cellular ligands [17-19]. We further combined OPD with next-generation DNA sequencing (NGS) for high-throughput identification of cellular ligands [20,21]. OPD-NGS is the first technology of ligandomics to globally identify cell-wide ligands. We applied ligandomics to the retina and identified HRP-3 and HDGF as endothelial ligands [22]. Furthermore, HRP-3 was independently verified as a novel angiogenic factor in a recent study [22]. Here, we characterized HDGF expression in the retina and investigated its role as an angiogenic factor to regulate retinal vascular leakage.

METHODS

Materials: Human retinal microvascular endothelial cells (HRMVECs; #ACBRI 181) and a complete classic medium kit with serum and CultureBoost (Cat. #4Z0–500) were from Cell Systems (Kirkland, WA). The cells were used for experiments at passages 4–8. Recombinant HDGF was purified, as previously described [22].

Immunohistochemistry: C57BL/6 mice (6–8 weeks old, Jackson Laboratory, Bar Harbor, ME) were anesthetized with intraperitoneal injection of ketamine (90 µg/g) and xylazine (8 µg/g). Mice were intracardially perfused with 10% formalin. Eyes were enucleated and fixed overnight at 4 °C. After the cornea and lens were removed, the eye cups were incubated with sucrose gradient solutions (10% and 20% for 3 h each; 30% for overnight) at 4 °C, followed by three rounds of freeze–thaw and embedding in optimal cutting temperature compound (OCT). Frozen tissue sections in 7-µm thickness were incubated with monoclonal mouse anti-HDGF antibody (Proteintech, Chicago, IL), followed by Alexa Fluor 594-labeled goat anti-mouse immunoglobulin G (IgG) antibodies. Nuclei were visualized with 4',6-diamidino-2-phenylindole (DAPI). Signals were analyzed with fluorescence microscopy.

All animal procedures in this study were approved by the Institutional Animal Care and Use Committee at the University of Miami and complied with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

RT–PCR: Mice (C57BL/6, 6–8 weeks old) were euthanized by CO_2 inhalation, followed by cervical dislocation. Eyes were enucleated, and total RNA was extracted from isolated retinas using TRIzol reagent (Life Technologies, Grand Island, NY), as described [23]. Reverse transcription-PCR (RT-PCR) with the following program: 95 °C for 2 min, (95 °C for 30 s, 58 °C for 40 s, 72 °C for 1 min) x 45 cycles, 72 °C for 10 min. HDGF primers: 5′-TCG AAT TCG GAT CCA TGA TTG ATG AGA TGC CTG AGG-3′ and 5′-AGT CTA GAC TAC AGG CTC TCA TGA TCT CTG-3′ (underlined for HDGF sequence; GenBank Accession # NM_008231). GAPDH primers: 5′-CTT CAC CAC CAT GGA GAA GGC-3′ and 5′-ATG AGG TCC ACC ACC CTG TTG-3′ (GenBank Accession # NM_008084).

Western blot: Retinas were isolated from euthanized C57BL/6 mice as above, homogenized in RIPA buffer (Thermo Fisher Scientific, Waltham, MA) and analyzed with western blot using anti-HDGF antibody or anti-β-actin antibody (Cell Signaling, Danvers, MA) [22]. The total brains were collected from the same mice, homogenized, and used as a positive control.

We quantified HDGF expression in the retina and vitreous fluid of mice with oxygen-induced retinopathy (OIR) or laser-induced choroidal neovascularization (CNV) as follows. We induced OIR in mice, as previously described [24]. Briefly, C57BL/6 mice at postnatal day 7 (P7) with nursing mothers were exposed to 75% oxygen in a regulated chamber for 5 days. On P12, mice were returned to room air. On P17, mice were euthanized by CO_2 inhalation, followed by cervical dislocation. After the vitreous fluid was collected with a Hamilton syringe using a 33-gauge needle, the retinas were isolated and homogenized in RIPA buffer. Both samples were analyzed with western blot as above. HDGF signal intensity was quantified [25], normalized against the signal intensity of β-actin, and compared between healthy and OIR retinas. We verified OIR by staining flatmount retinas with Alexa Fluor 488-isolectin B4 (Life Technologies), as described [24].

We induced CNV by laser photocoagulation, as previously described [26]. Briefly, C57BL/6 mice (6–8 weeks old, male) were subjected to laser photocoagulation (Argon laser,

532 nm, 100 mW, 100 ms, 100 µm, four spots/retina) [26,27]. Laser-induced CNV was verified using spectral domain-optical coherence tomography (SD-OCT) and fluorescein angiography 7 days post laser photocoagulation, as previously described [26,28]. Retinas and vitreous fluid were collected for western blot analysis as above.

Cytoplasmic and nuclear fractionations were prepared from pig retinas, as previously described with minor modifications [29]. Briefly, fresh eyes were collected as residual tissues from euthanized normal pigs at the University of Miami. Retinas were isolated, homogenized in STM buffer (250 mM sucrose, 50 mM Tris-HCl, pH 7.4, 5 mM $MgCl_2$, and a cocktail of protease inhibitors (Sigma, St. Louis, MO)), and centrifuged at 800 ×*g* for 15 min. To prepare cytoplasmic fractionation, the supernatant was recentrifuged at 11,000 ×*g* for 10 min to remove additional cellular organelles. The supernatant was precipitated with cold 100% acetone (final 80% acetone) at −20 °C and centrifuged at 12,000 ×*g* for 5 min. The pellet (cytoplasmic fractionation) was resuspended and analyzed with western blot. To prepare nuclear fractionation, the pellet of pig retina from the initial centrifugation was washed and resuspended in NET buffer (20 mM HEPES, pH 7.9, 1.5 mM $MgCl_2$, 0.5 NaCl, 0.2 mM EDTA, 20% glycerol, 1% Triton X-100, and a cocktail of protease inhibitors). After incubation on ice for 30 min, the solution was passed through an 18-gauge needle and centrifuged at 9,000 ×*g* for 30 min. The supernatant (nuclear fractionation) was analyzed with western blot.

Proliferation assay: HRMVECs were seeded in 96-well plates in basal medium at 1×10^3 cells/well with HDGF, VEGF165 (R&D Systems, Minneapolis, MN), or control medium at the indicated concentrations. Fresh medium and growth factors were added every 24 h. Cells in each well were collected by trypsin digestion at 48 h and quantified in PBS (1X; 155 mM NaCl, 1.5 mM KH_2PO_4, 2.7 mM Na_2HPO_4, pH7.4) with 1 mM trypan blue using a hemocytometer [22].

Wound healing assay: HRMVEC migration was analyzed with in vitro wound healing assay, as described [22,30]. Briefly, HRMVECs were cultured in 12-well plates until approximately 90–100% confluence. Cells were starved for 3 h in 293 SFM II medium (Life Technologies) supplemented with 2% fetal bovine serum (FBS). A sterile 200 µl tip was used to create a defined and clear scratch approximate 1 mm in width in each well. The dislodged cells were immediately removed by rinsing, and the remaining cells were supplemented with fresh 293 SFM II medium containing 2% FBS in the presence of HDGF, VEGF, or PBS. The migration of cells was monitored at 0 and 20 h as follows. At 0 h, at least six images per well were acquired with phase-contrast microscopy, and the average width of the original scratch (AW) was calculated. At 20 h, cells were incubated with 2 µM Calcein AM (Life Technologies) for 30 min at 37 °C and analyzed with fluorescence microscopy. At least six images were acquired for each well. The area percentage of the migrated cells was calculated using ImageJ software (NIH). Briefly, each image was cropped with the width of AW at 0 h. The area percentage of the migrated cells within the cropped image was calculated for each image with the threshold function of ImageJ software (NIH). The data of all images in the same well were averaged. We analyzed three wells for each group.

In vitro permeability assay: The assay was performed, as previously described with modifications [31]. Briefly, we precoated Transwell inserts of 24-well plates (Corning Life Sciences, Tewksbury, MA) with 1% gelatin. HRMVECs were plated on the Transwell inserts at 1×10^5 cells/well and cultured overnight to confluence. The medium was replaced with additional HDGF, VEGF, or PBS in the bottom chamber. Fluorescein isothiocyanate (FITC)–dextran (3–5 kDa, 0.5 mg/well, Sigma) was added to the bottom chamber. The medium in the upper chamber was collected 6 h later and quantified for FITC with a fluorescence plate reader using FITC-dextran as a standard curve.

Corneal pocket assay: The assay was performed as described [22]. Briefly, sterilized Whatman filter paper (Grade 3; GE Healthcare Bio-Sciences, Piscataway, NJ) was cut into pieces (0.125 mm^2/piece). The papers were soaked in the solution of HDGF (1 µg/µl), VEGF (100 ng/µl), or PBS for 2 h at 4 °C and implanted into corneal pockets in anesthetized C57BL/6 mice (6–8 weeks old; one paper/cornea; two pockets/mouse), as described [32]. After 6 days, angiogenesis in each eye was evaluated using a slit-lamp microscope and photographed. The number of new sprouting vessels into the cornea and their branching points were quantified. In addition, we semiquantitatively analyzed the number, density, and length of the visible corneal blood vessels with a comprehensive scoring system, as previously described [22]. Mice were then euthanized by CO_2 inhalation and immediately perfused intracardially with lipophilic fluorescent DiI dye [33]. The eyes were removed and fixed in 10% formalin for 24 h at 4 °C. Corneas were dissected at the limbus, flatmounted in 50% glycerol/PBS, and imaged with confocal microscopy to detect DiI-labeled blood vessels.

Retinal vascular leakage: Retinal vascular leakage was quantified with the Evans blue assay, as described [34,35]. Briefly, HDGF (0.25 µg/µl, 2 µl/eye), VEGF (0.1 µg/µl, 2 µl/eye), or BSA (BSA, 0.25 µg/µl, 2 µl/eye) was intravitreally injected into one eye of anesthetized C57BL/6 mice (6–8 weeks old).

The contralateral eye always received PBS (2 µl) as a negative control. Evans blue (0.15 mg/g bodyweight, 150 µl) was intravenously injected 1.5 h post intravitreal injection. Mice under anesthesia were intracardially perfused with sodium citrate buffer (pH 4.5) 2.5 h post the Evans blue injection. Retinas were isolated and incubated with formamide (50 µl/retina) at 70 °C overnight to extract the dye. The solutions were centrifuged at 180,000 ×*g* at 4 °C for 1 h. Evans blue in the supernatant was quantified at 620 nm and 740 nm (background) and compared to a standard curve. The blood samples were collected before the intracardial perfusion, directly centrifuged at 3,550 ×*g* for 15 min at 25 °C, diluted, and quantified at the same wavelengths. Evans blue leakage was calculated with the following formula: [leaked Evan blue concentration (mg/ml)/retinal weight (mg)]/[blood Evans blue concentration (mg/ml) × circulation time (h)]. The amount of leaked Evans blue was always normalized against the contralateral eye treated with PBS.

Alternatively, we analyzed retinal vascular leakage with western blot to detect leaked mouse albumin, as previously described [36]. Briefly, mice were directly perfused with PBS 4 h post the injection of angiogenic factors without Evans blue. Retinas were isolated, homogenized, and analyzed with western blot using affinity-purified anti-mouse albumin polyclonal antibody (Thermo Fisher Scientific) or anti-β-actin antibody.

Data analysis: Data were expressed as mean ± standard error of the mean (SEM) and analyzed with one-way ANOVA test. Data were considered significant when the p value was less than 0.05.

RESULTS

HDGF expression in the retina: HDGF expression in the mouse retina was detected with RT–PCR in Figure 1A. This was verified with western blot using anti-HDGF antibody in Figure 1B. Mouse total brain homogenate and purified HDGF were used as positive controls. The results indicated that HDGF is expressed in the retina and the brain at a similar level. Immunohistochemistry detected HDGF expression in multiple layers of the retina (Figure 1C). The HDGF signal is predominantly expressed in the inner nuclear layer, outer nuclear layer, photoreceptor outer segments, and choroid. Additionally, HDGF is present in retinal ganglion cells (RGCs) and photoreceptor inner segments at relatively low levels. However, fewer signals were detected in the inner and outer plexiform layers. These data suggest that HDGF is expressed in the nucleus and the cytoplasm of the photoreceptor cells but only in the nucleus of other retinal neurons.

Of note, a much weaker signal was also detected in the photoreceptor outer segments with no primary antibody control than with anti-HDGF antibody (top row, Figure 1C). In all other retinal layers, no signal was found for the control without the primary antibody, suggesting that the HDGF signals in all these retinal layers were primary antibody-dependent.

HDGF without a classical signal peptide was previously reported to be expressed in the cytoplasm, nucleus, and extracellular space [11,16,37,38]. Similarly, we confirmed the presence of HDGF in the cytoplasmic and nuclear fractionations of the pig retina (Figure 1D). Furthermore, western blot analysis detected HDGF in the vitreous fluid of mice (Appendix 1 and Appendix 2), suggesting that HDGF can extrinsically regulate vascular function.

HDGF promotes proliferation of retinal endothelial cells: We characterized HDGF activity to stimulate the proliferation of HRMVECs. Purified HDGF significantly induced the growth of HRMVECs at 100 ng/ml ($p<0.05$) and 500 ng/ml ($p<0.0001$) at a level comparable to that of the positive control VEGF (100 ng/ml; Figure 2). The results suggest that HDGF is capable of promoting the proliferation of retinal microvascular endothelial cells.

HDGF induces migration of retinal endothelial cells: The migration of endothelial cells represents an important process of angiogenesis. Therefore, we investigated the capability of HDGF to induce the migration of HRMVECs by performing an in vitro wound healing assay. The results showed that HDGF at 100 or 500 ng/ml significantly facilitated endothelial migration ($p<0.05$), similar to VEGF (100 ng/ml) as a positive control (Figure 3). These results suggest that HDGF is likely an angiogenic factor to stimulate the migration of retinal endothelial cells.

HDGF stimulates corneal angiogenesis: To directly test the angiogenic potential of HDGF in vivo, we used the well-established corneal pocket assay to examine the effect of HDGF on angiogenesis. We found that HDGF and VEGF induced corneal angiogenesis, as detected with slit-lamp microscopy (Figure 4A). These results were independently verified with DiI staining of the corneal blood vessels (Figure 4B). Quantification of the total number of new corneal vessels and their branching points indicated that HDGF significantly induced corneal angiogenesis ($p<0.01$, Figure 4C,D). In addition, quantification with a comprehensive scoring system for the number, density, and length of visible corneal blood vessels [22] also confirmed that HDGF significantly promoted corneal angiogenesis ($p<0.01$, Figure 4E). VEGF as a positive control showed similar activity to significantly

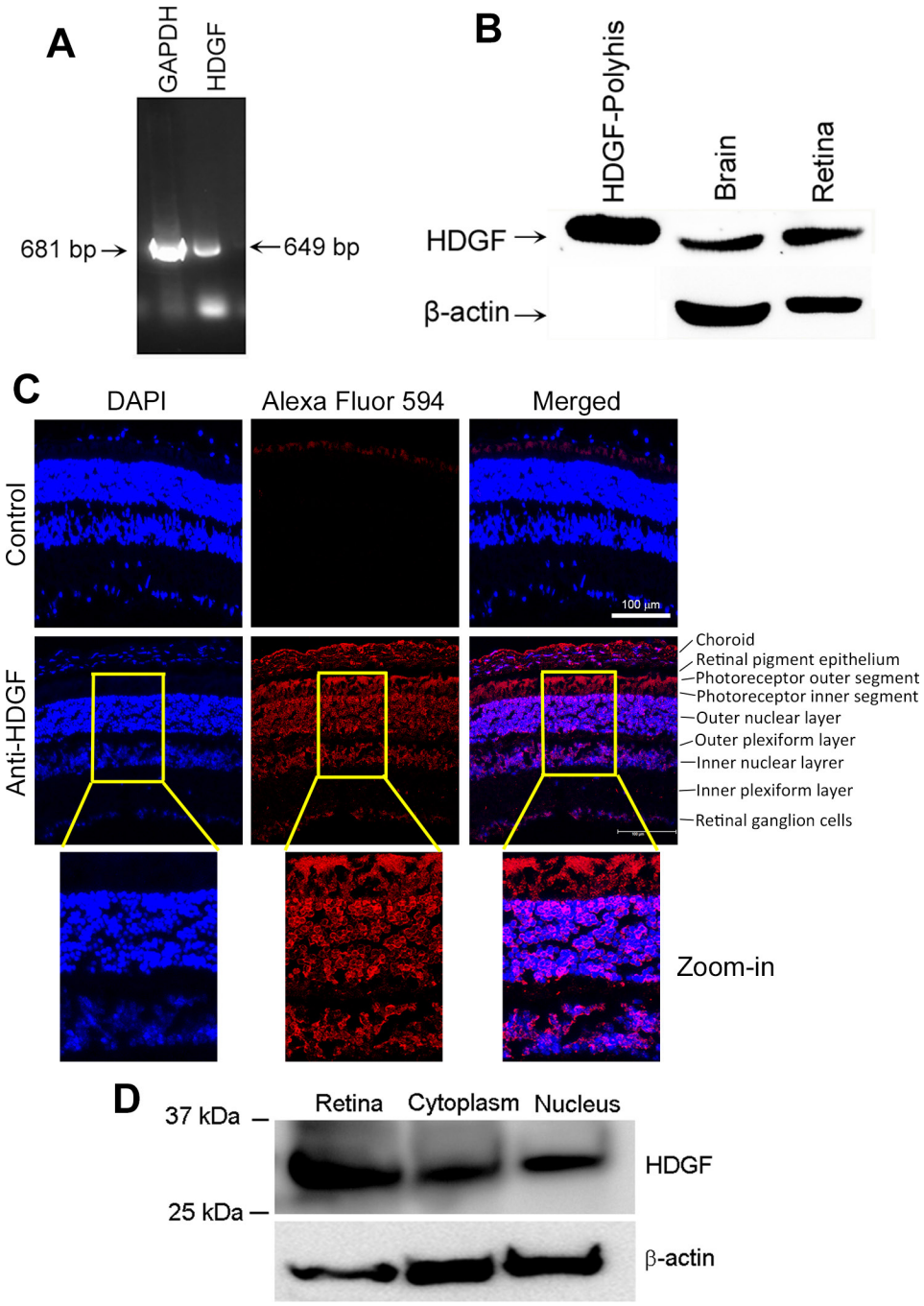

Figure 1. HDGF expression in the retina. **A**: Hepatoma-derived growth factor (HDGF) expression in the mouse retina was detected with reverse transcription PCR (RT–PCR). GAPDH was included as a positive control. **B**: Western blot analysis of HDGF expression in the mouse retina. Mouse total brain homogenate (middle lane) and purified recombinant HDGF (left lane) with extra C-terminal polyhistidine were included as positive controls. Five hundred μg protein/lane for retinal or brain homogenate. One μg protein/lane for purified HDGF. The predicted molecular weight (MW) for HDGF is 25.5 kDa. **C**: Immunohistochemical analysis of HDGF expression in the mouse retina. HDGF is predominantly expressed in the inner and outer nuclear layers, photoreceptor outer segments, and choroid. HDGF was also detected in the retinal ganglion cell layer and the photoreceptor inner segments at relatively low levels. Few HDGF signals were found in the inner and outer plexiform layers. Bar = 100 μm. **D**: HDGF is present in the cytoplasmic and nuclear fractionations by western blot. These results were validated three times with similar outcomes.

induce corneal angiogenesis (Figure 4). These in vivo results suggest that HDGF is an angiogenic factor.

HDGF induces retinal vascular leakage: VEGF is an angiogenic factor well known for its activity to stimulate retinal vascular leakage [5,35]. To determine whether HDGF is able to trigger the leakage, we analyzed the effect of HDGF on the permeability of HRMVECs in vitro (Figure 5A) The results indicated that HDGF and VEGF at 100 ng/ml increased the permeability of 4 kDa FITC-dextran through the confluent layer of HRMVECs on Transwell inserts ($p<0.01$, Figure 5B). These data suggest that HDGF induced retinal vascular leakage. To independently verify this finding in vivo, we intravitreally injected purified HDGF in the mouse eyes. To detect HDGF-induced leakage, we performed intracardial perfusion 4 h post injection and detected leaked albumin in the retina with western blot using an anti-mouse albumin antibody (Figure 5C). The results showed that HDGF (0.5 µg/eye) and VEGF (0.2 µg/eye) induced albumin leakage. As a negative control, BSA induced no leakage. To reliably quantify retinal vascular leakage, Evans blue with a high binding affinity to albumin was intravenously injected 1.5 h post HDGF injection and allowed to circulate for 2.5 h. The leaked dye was quantified 4 h post HDGF injection. The results showed that HDGF significantly induced retinal vascular leakage ($p<0.01$, Figure 5D). VEGF as a positive control elicited similar retinal leakage. The negative control BSA failed to stimulate the leakage.

DISCUSSION

Heparin-binding HGDF is the founding member of the HDGF family, which includes HDGF, HDGF-related proteins (HRP) 1–4, and lens epithelium-derived growth factor [39]. All family members share a conserved N-terminal PWWP/HATH domain. Similar to fibroblast growth factor (FGF) for the well-known unconventional secretion [40], HDGF without a classical signal peptide was also reported to be secreted [9,11].

HDGF and HRP-3 were reported to be expressed in the nucleus as well as the cytoplasm of neurons with neurotrophic activity [16,37,41]. We recently characterized HRP-3 expression in the retina with immunohistochemistry [22]. HDGF and HRP-3 are expressed in the retina but with distinct expression patterns. HRP-3 is predominantly expressed in retinal ganglion cells and the inner nuclear layer [22]. Minimal HRP-3 was detected in the inner and outer plexiform layers, suggesting that the protein is expressed in the

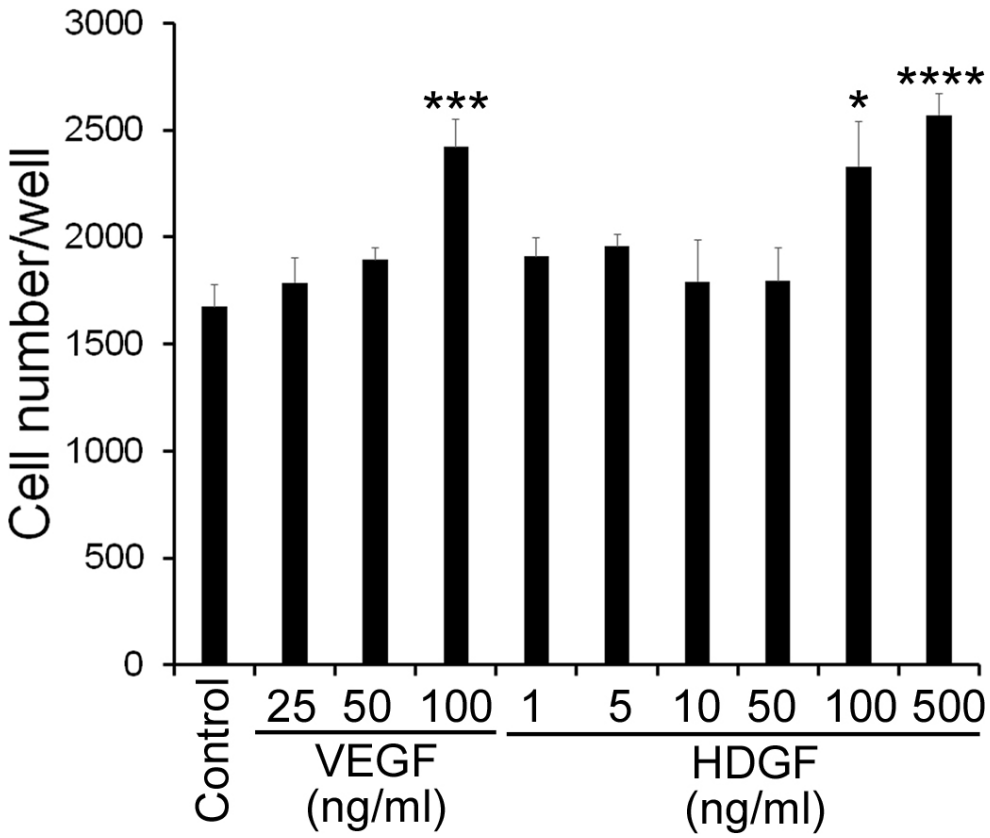

Figure 2. HDGF induces endothelial cell growth. The proliferation activity of hepatoma-derived growth factor (HDGF) and vascular endothelial growth factor (VEGF) was analyzed after 48-h incubation with human retinal microvascular endothelial cells (HRMVECs). The experiment was repeated three times with similar results. One representative experiment is shown. Mean ± standard error of the mean (SEM), n = 7 wells/group, *$p<0.05$, ***$p<0.001$, ****$p<0.0001$, versus control, one-way ANOVA *t* test.

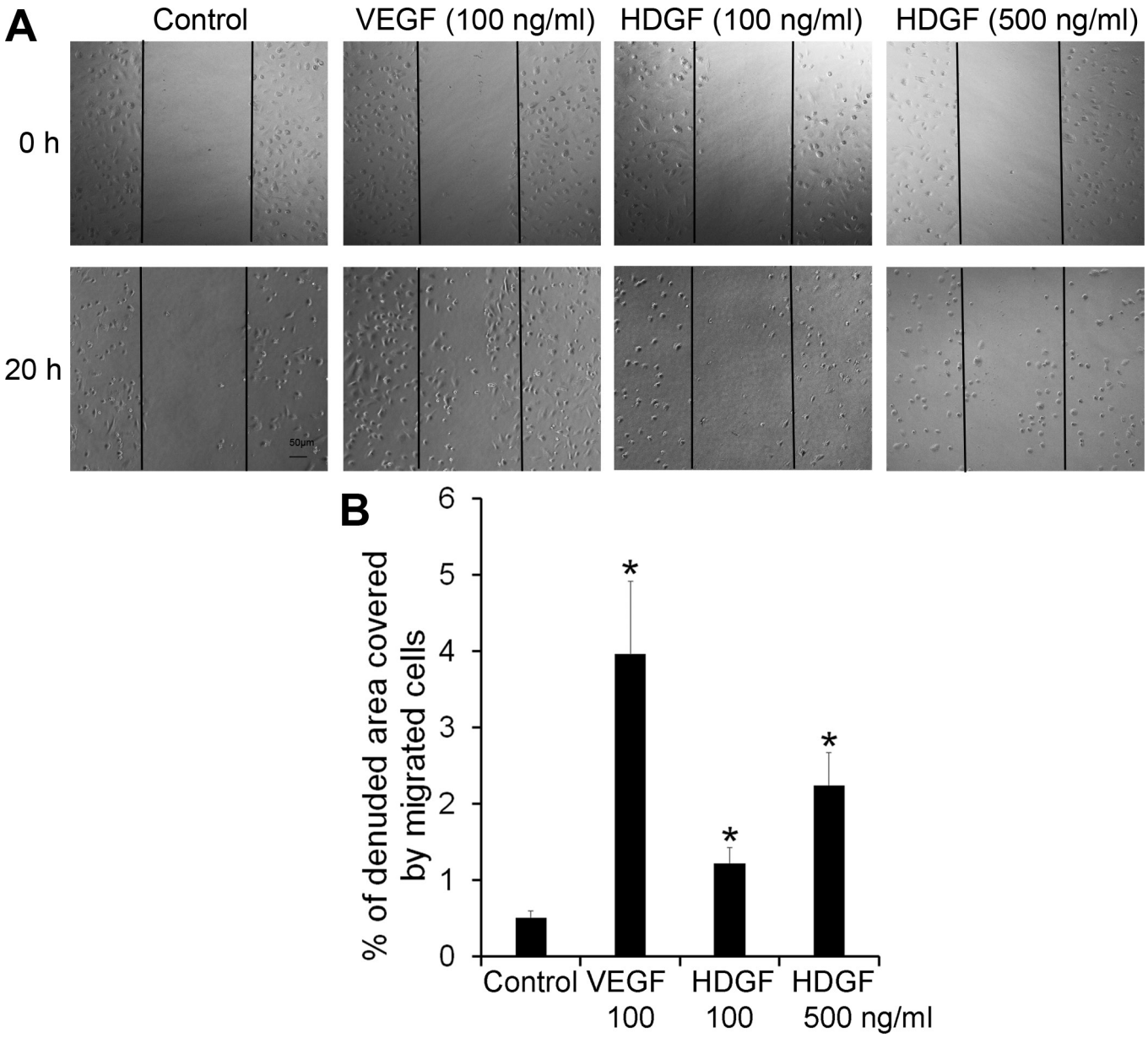

Figure 3. HDGF stimulates endothelial migration. **A**: Representative images of endothelial migration by wound healing assay. Human retinal microvascular endothelial cells (HRMVECs) were cultured in 12-well plates to confluence. A scratch was created in each well. Hepatoma-derived growth factor (HDGF; 500 ng/ml), vascular endothelial growth factor (VEGF; 100 ng/ml), or PBS was incubated with the cells for 20 h. Bar = 100 µm. **B**: The percentage of the denuded area covered by migrated cells within the original scratch was quantified. This assay was independently performed three times with similar outcomes. Mean ± standard error of the mean (SME), n = 3 wells/group, *p<0.05, versus control, one-way ANOVA test.

nucleus instead of the cytoplasm. No HRP-3 was detected in the photoreceptors, including the outer nuclear layer, and the photoreceptor inner and outer segments. In contrast, HDGF is expressed in inner and outer nuclear layers as well as the photoreceptor inner and outer segments (Figure 1C). The most predominant expression of HDGF was found in the outer segments. HDGF is expressed in retinal ganglion cells but at a much lower level. Minimal HDGF expression was detected in the inner and outer plexiform layers. This expression pattern suggests that HDGF is localized to the nucleus of non-photoreceptor neurons in the retina but is distributed in the nucleus and the cytoplasm of the photoreceptors.

The primary antibody used in immunohistochemistry (Figure 1C) was mouse monoclonal antibody. Accordingly,

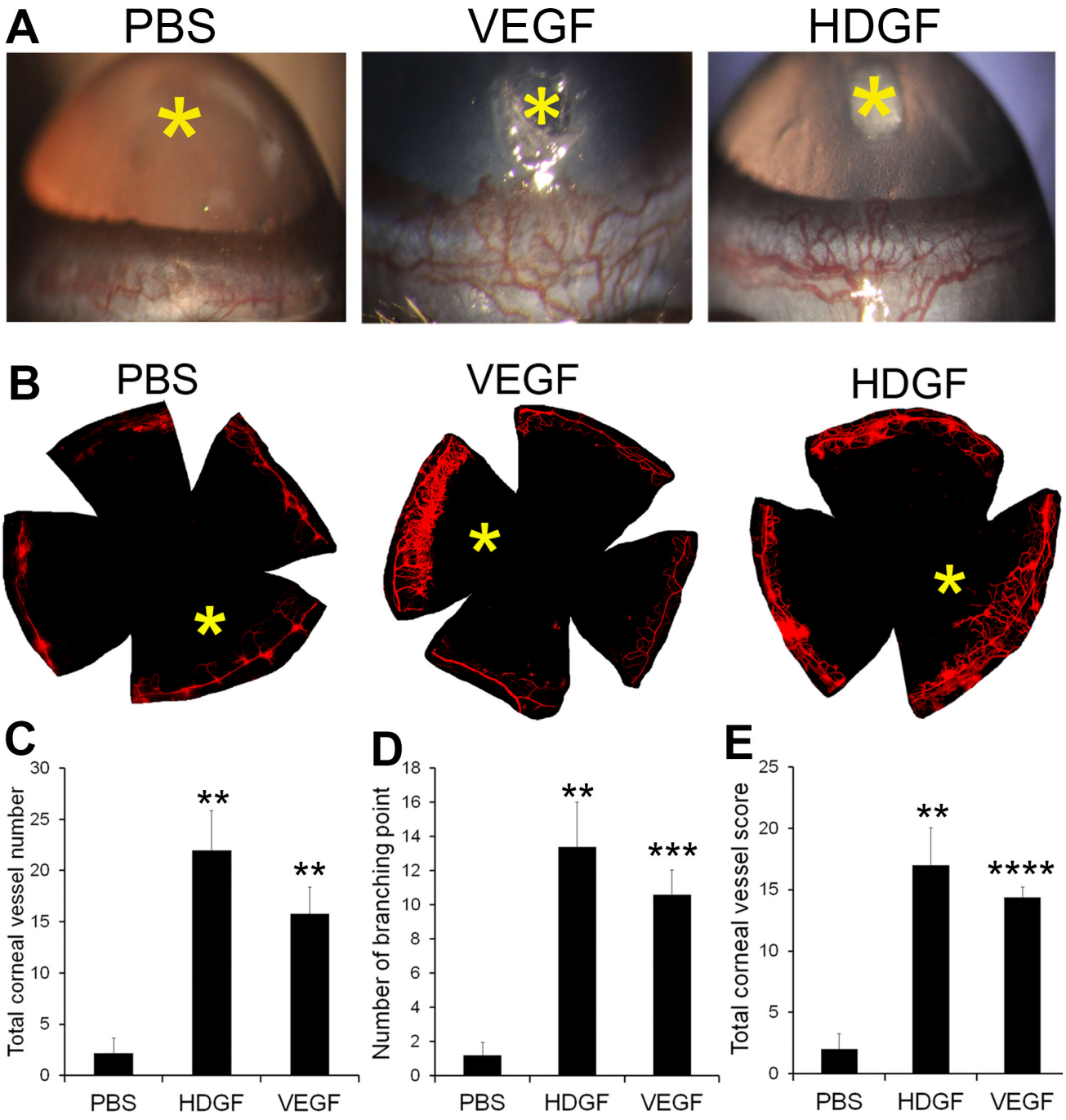

Figure 4. HDGF induces corneal angiogenesis. **A**: Representative images of corneal angiogenesis assay. Small pieces of filter papers presoaked in hepatoma-derived growth factor (HDGF; 1 µg/µl), vascular endothelial growth factor (VEGF; 100 ng/µl), or PBS were implanted in corneal pockets to induce vascular sprouting for 6 days. * indicates filter paper position. **B**: Representative images of corneal blood vessels labeled with fluorescent DiI dye. **C**–**E**: Quantification of corneal angiogenesis assay. **C**: The number of blood vessels growing into the cornea. **D**: The number of branching points for corneal vessels. **E**: Comprehensive score for new vessels. Mean ± standard error of the mean (SEM), n=5 eyes/group, **p<0.01, ***p<0.001, ****p<0.0001, versus PBS, one-way ANOVA test.

an anti-mouse secondary antibody was used to detect the bound primary antibody. This secondary antibody may also detect endogenous mouse IgG. Thus, a control without the primary antibody is necessary to eliminate non-specific signals. The minor signal detected in the photoreceptor outer segments with no primary antibody control (top row, Figure 1C) may be related to this background signal. All signals in other retinal layers were detected only with the anti-HDGF primary antibody.

A related question is why HDGF is predominantly expressed in the photoreceptor outer segments. Owing to its neurotrophic activity [37], HDGF may be secreted to promote photoreceptor survival. VEGF plays a critical role in the survival and maintenance of many other cells, including retinal pigment epithelial (RPE) cells, neurons, and endothelial cells [42-44]. Inhibition of VEGF induces RPE and neuronal cytotoxicity [42,45]. VEGF and HDGF are growth factors with angiogenic and neurotrophic activities. Thus, we speculate that HDGF may be secreted from the photoreceptor outer segments through unconventional secretion to promote

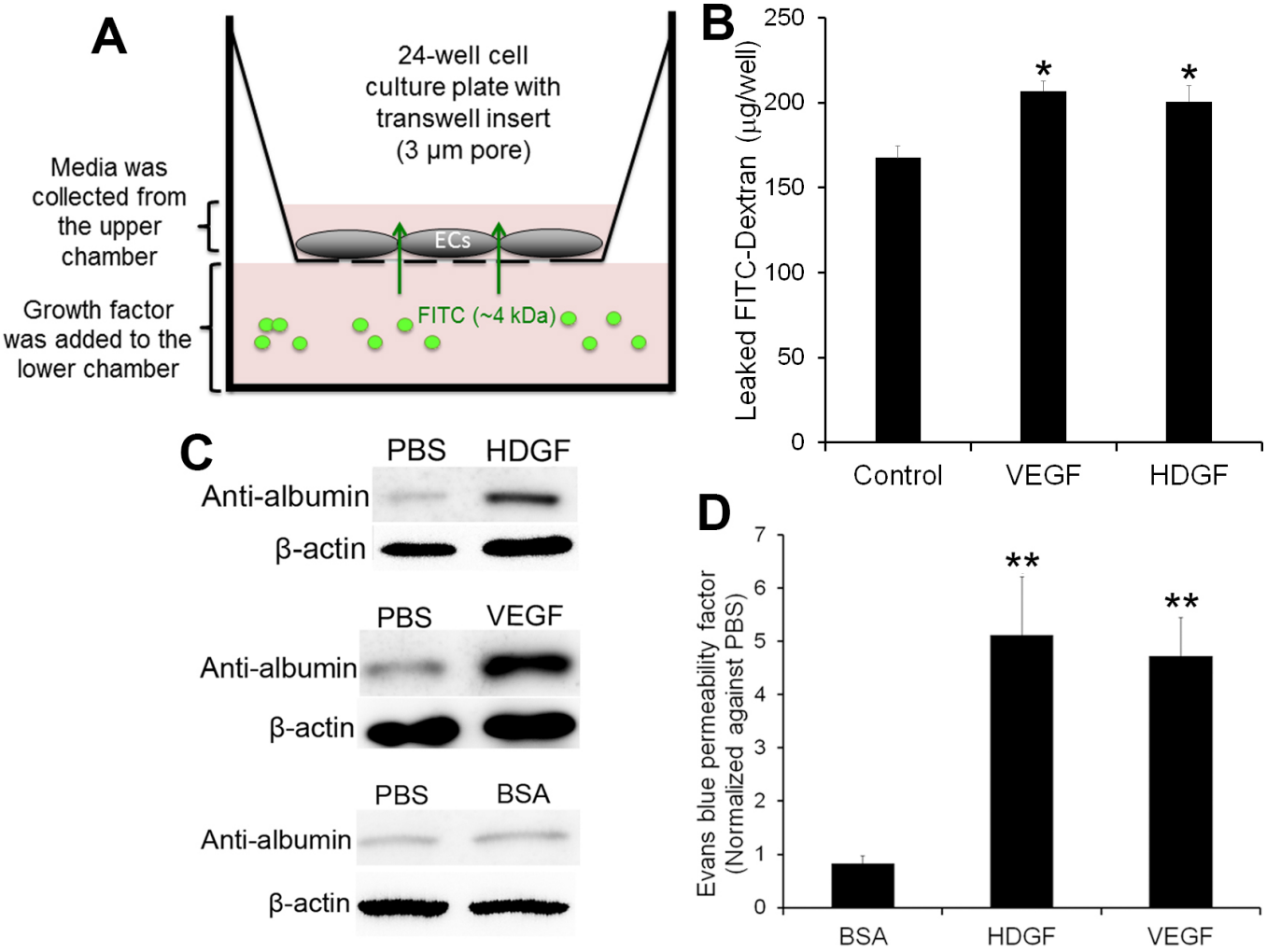

Figure 5. HDGF stimulates retinal vascular leakage. **A**, **B**: Hepatoma-derived growth factor (HDGF) increases the permeability of human retinal microvascular endothelial cells (HRMVECs). **A**: Schematic of in vitro permeability assay. HRMVECs were plated on the Transwell inserts and cultured until confluence. HDGF (100 ng/ml), vascular endothelial growth factor (VEGF; 100 ng/ml), or PBS along with fluorescein isothiocyanate (FITC)–dextran was added to the bottom chamber. **B**: Leaked FITC-dextran was quantified in the upper chamber at 6 h. **C**, **D**: Mice were intravitreally injected with HDGF (0.5 µg/eye), VEGF (0.2 µg/eye), or bovine serum albumin (BSA; 0.5 µg/eye) in one eye with the contralateral eye for PBS. After 4 h, retinal vascular leakage was analyzed. **C**: Representative images of leaked mouse albumin detected with western blot using anti-mouse albumin. **D**: Retinal vascular leakage was quantified with the Evans blue assay. Leaked dye is normalized against the PBS-treated contralateral eye. Mean ± standard error of the mean (SEM), n = 4 eyes/group, *p<0.05, **p<0.01, versus control or BSA, one-way ANOVA test.

the survival of the photoreceptors, RPE, and other retinal neurons or to maintain angiogenic homeostasis. However, this speculation has yet to be tested.

HDGF has been extensively characterized for its angiogenic activity with various in vitro functional assays using isolated endothelial cells in several studies. For example, HDGF was reported as an endothelial mitogen based on its activity to stimulate DNA synthesis of endothelial cells in culture [11]. Other studies also demonstrated HDGF angiogenic activity using human umbilical vein endothelial cells (HUVECs) in culture [15,46]. Another study analyzed HDGF's angiogenic activity with the chick chorioallantoic membrane (CAM) assay in chicken eggs [47]. To our knowledge, all in vivo characterizations of HDGF as an angiogenic factor were performed in cancer models.

HDGF is upregulated in different tumors, and its upregulation is correlated to cancer prognosis [48,49]. HDGF was reported with mitogenic activity to promote cancer cell growth, malignancy, and resistance to anticancer therapy [12-14]. HDGF can stimulate cancer angiogenesis in vitro and in vivo [15,46]. However, it is difficult to dissect whether in vivo angiogenic activity is due to a direct function of HDGF or an indirect action of cancer-derived angiogenic factors, such as VEGF [15]. The indirect action could be facilitated by mitogenic activity of HDGF to stimulate cancer growth, which in turn upregulates other angiogenic factors to support cancer growth. The direct angiogenic activity of HDGF was demonstrated only with in vitro assay with isolated endothelial cells, but not with in vivo analysis [15].

The corneal pocket assay in this study unequivocally validated the direct angiogenic activity of HDGF in mice (Figure 4). One of the advantages of this assay is that the cornea is an avascular tissue for reliable and sensitive detection of angiogenic activity. A major technical challenge for this assay is the generation of slow-releasing pellets containing angiogenic factors. Our experience indicated that slow-releasing pellets can be successfully generated for VEGF or FGF by following several published protocols [32,50,51]. However, such pellets cannot be formed for HDGF or HRP-3, probably due to their different chemical properties. We recently adapted and modified filter-based corneal angiogenesis assay from a previous study to demonstrate the angiogenic activity of HRP-3 [22,52]. Together with this study, these results unambiguously support HDGF and HRP-3 as direct angiogenic factors in vivo.

The results of this study implicate that these two ligands may contribute to retinal vascular diseases. For instance, cancer-associated retinopathy (CAR) is often coupled with vascular remodeling, and VEGF plays an important role in pathogenesis of CAR [53]. Upregulation of HDGF and HRP-3 in cancer [12,13,49,54] implies that they may be involved in CAR-related vascular remodeling. This notion must be tested in future work.

VEGF and other angiogenic factors have been well documented for their roles in neovascular AMD, DME, and PDR [4]. HDGF and HRP-3 expressed in the retina may also exert similar angiogenic activity in these diseases. However, not all angiogenic factors may play a role in the pathogenesis of retinal vascular diseases. Anti-VEGF-resistant AMD and DME [55,56] implicate that even VEGF may not always contribute to the pathogenesis of these two well-investigated diseases. Therefore, the role of HDGF and HRP-3 in these retinal vascular diseases should be individually investigated. VEGF and its receptor have been reported to be upregulated in various disease conditions [57-59]. Our initial analysis indicated that HDGF expression levels in the retina and vitreous fluid of mice with OIR or laser-induced CNV were not altered (Appendix 1 and Appendix 2). Given the unknown receptor of receptor, it is difficult to fully characterize its pathological relevance at this time.

HDGF and HRP-3 were identified by ligandomics as retinal endothelial ligands [22]. Independent characterization of these two ligands as angiogenic factors in this and previous studies [22] supports the validity of ligandomics. In unpublished studies, we used this new technology to systematically identify disease-specific angiogenic factors with their receptors upregulated in disease conditions. These findings may lead to rational design of novel antiangiogenesis therapies of diabetic retinopathy and neovascular AMD. We envisage that this new technology will accelerate systematic identification of disease-specific angiogenic factors and facilitate the development of novel antiangiogenesis therapies for retinal vascular diseases.

APPENDIX 1. HDGF EXPRESSION LEVEL WAS MINIMALLY CHANGED IN THE RETINA AND VITREOUS FLUID OF OIR MICE.

To access the data, click or select the words "Appendix 1." HDGF expression was detected by Western blot. Its signal was digitalized, normalized against β-actin. (A) Representative images of Wester blot. (B) Quantification of HDGF in the retina. (C) Quantification of HDGF in the vitreous fluid. (D) Detection of OIR in flat-mount retina stained with Alexa Fluor 488-labeled isolectin B4. OIR was characterized by central avascular region, pathological neovascularization in the peripheral region (arrow), neovascular tufts and tortuous vessels (arrowhead). Mean ± s.e.m., n=6 mice/group. No

significance was found for control vs. OIR mice. One-way ANOVA test.

APPENDIX 2. HDGF EXPRESSION LEVEL WAS MINIMALLY CHANGED IN THE RETINA AND VITREOUS FLUID OF LASER-INDUCED CNV MICE.

To access the data, click or select the words "Appendix 2." HDGF expression was detected by Western blot. Its signal was digitalized, normalized against β-actin. (A) Representative image of Wester blot. (B) Quantification of HDGF in the retina. (C) Quantification of HDGF in the vitreous fluid. (D) Detection of CNV by SD-OCT. Arrows indicate laser-induced damage on the retina. (E) Detection of CNV by fluorescein angiography. Mean ± SEM, n=6 mice/group. No significance was found for control vs. CNV mice. One-way ANOVA test.

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