Endocan Blockade Suppresses Experimental Ocular Neovascularization in Mice

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PURPOSE. Ocular neovascularization (NV) is a pathologic process characterized by the proliferation and infiltration of various types of cells such as RPE, glial, and endothelial cells, which interact with proangiogenic factors and inflammatory cytokines. Endocan is known to be enriched in retinal endothelial tip cells under hypoxia, but the effect of endocan on ocular NV progression is largely unknown. In this study, we investigated the role of endocan in the ocular NV pathologic process and the possible mechanisms involved.

METHODS. In the eyes of mice with oxygen-induced retinopathy (OIR); choroidal NV (CNV); and rhodopsin promoter (rho)/VEGF transgenic mice, endocan expression was assessed by quantitative real-time PCR (RT-PCR) and Western blot. In vivo, a specific functional antibody was used to neutralize endocan and ocular NV levels were evaluated by RT-PCR, Western blot and immunostaining of flat-mounts. In vitro, the effect of endocan on human retinal microvascular endothelial cell (HREC) tube formation was observed using a routine method.

RESULTS. Endocan was significantly elevated in these three experimental mice models. Endocan blockade with the neutralizer intravitreal injection not only suppressed the area of retinal, choroidal and subretinal NV, but also resulted in a decrease in several angiogenesis-associated molecules. Recombinant endocan protein (rhEndocan) was found to induce tube formation on HRECs directly.

CONCLUSIONS. The current data suggest that endocan is a potential therapeutic or an additional target for retinal and subretinal NV diseases.

Keywords: endocan, ocular neovascularization, vascular endothelial growth factor, endothelial cell

Ocular neovascularization (NV) is the critical pathologic process and the leading cause of blindness in a variety of eye diseases such as retinopathy of prematurity (ROP); diabetic retinopathy (DR); and AMD.[1] These diseases can be divided into retinal vascular diseases, in which there is leakage and/or NV from retinal vessels, and subretinal or choroidal NV, in which new vessels grow into the normally avascular outer retina and subretinal space.[2] These fragile new vessels can easily hemorrhage and cause irreversible vision loss. It has been well elucidated that VEGF plays an important role in this pathologic process.[2] Although treatments involving VEGF antagonists have provided major benefits for patients with ROP and neovascular AMD,[3,4] frequent injections are required to sustain efficacy and treatment resistance frequently occurs. In addition, the procedure poses a heavy economic burden on patients due to multiple injections. In 2012, aflibercept, a fusion protein that blocks VEGFA, VEGFB, and placental growth factor (PIGF), was approved for the treatment of macular degeneration.[5] Intravitreal injection of aflibercept achieved an equivalent effect in improving the best-corrected visual acuity (BCVA) and preventing BCVA loss in AMD while requiring fewer injections when compared to ranibizumab monotherapy,[6] thus indicating the need to develop additional antagonists to other ocular NV targets that could be used as potential combination therapy.

One way to address this unmet need is to target other relevant angiogenic biomarkers like endocan. In our previous study, our microarray analysis suggested that endocan was one of the most significantly upregulated genes with a more than 15-fold increase in mRNA expression levels in the OIR model compared to control. Endocan (also known as endothelial cell specific molecule-1), a soluble dermatan sulfate proteoglycan, has been found to be expressed in endothelial tip cells in several retinal angiogenesis models.[7] For example, animal experiments demonstrated that following hypoxic exposure, the expression of endocan in immature retinas, especially in endothelial tip cells of 1-day old Wistar rats, was increased from 3 hours to 14 days.[7] Clinical research demonstrated that endocan levels were significantly higher in the vitreous fluid from patients with proliferative DR (PDR) compared to nondiabetic control patients. Furthermore, the expression level of endocan in active PDR patients was also higher than that in inactive PDR.[8] Endocan is also strongly overexpressed in various carcinomas such as renal cancer, non-small lung cancer, hepatocarcinoma, glioblastoma, and bladder cancer. High

endocan expressions can be identified as part of the molecular signatures defining a poor prognosis and prediction of recurrence in some malignant tumors.[9] Interestingly, in mice models of tumor xenografts, overexpression of human endocan induced tumor growth of nontumorigenic human embryonic kidney cells and accelerated tumor growth of the tumorigenic cells. Blockade of endocan with a monoclonal antibody in these models resulted in a notable reduction of tumor growth.[10] In addition, multiple studies have demonstrated that endocan silencing decreased tumor cells survival, migration, and invasion.[11] By using endocan knockout mice, Rocha et al.[12] reported that endocan was required for an optimal response to VEGF stimulation and played a role in retinal angiogenesis. In vitro, in the absence of endocan, human umbilical vein endothelial cells disturbed VEGF-induced or fibroblast growth factor 2–induced sprouting angiogenesis.[13] Given the existing literature regarding endocan, we hypothesized that endocan may be involved in the regulation of ocular NV. The aim of this study was to investigate the role of endocan in ocular NV regulation and its potential interactions with VEGF in several experimental NV mouse models and cell assays.

MATERIALS AND METHODS

Cell Culture

In in vitro studies, HRECs were cultured with endothelial cell medium (ECM) containing 5% fetal bovine serum (FBS) and 100 U/mL penicillin-streptomycin at 37°C under a humidified 95%: 5% (vol/vol) mixture of air and CO_2. Before the tube formation assay, 100 μL of chilled membrane matrix (Matrigel; Corning Life Sciences, Tewksbury, MA, USA) was added into 96-well culture plates on ice and incubated for 30 minutes at 37°C, followed by preparing HRECs suspensions in complete ECM. Approximately 2.5×10^4 cells in 200 μL medium with or without rhEndocan were plated onto a layer of the matrix (Corning Life Sciences) in each well. Cell tube formation images were recorded 6 hours later under an inverted microscope (×100), and the number of tubes was counted as described previously.[14] In other experiments, cells were seeded in triplicate in 60×15 mm petri dishes. Until cell attachment, the medium was replaced with fresh ECM containing 50 ng/mL and 100 ng/mL of rhEndocan or recombinant human VEGFA (rhVEGFA; Cell Signaling Technology, MA, USA) for the following investigation. Cell lysates were collected and prepared for total RNA and protein isolation.

Animals

We used C57BL/6 mice for the purpose of this study and in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and the guidelines of the Animal Care and Use Committee at Shanghai Jiaotong University Medical School. Mice were housed in a specific pathogen-free environment and rho/VEGF transgenic mice were provided by Professor Peter A. Campochiaro of Johns Hopkins Hospital (Baltimore, MD, USA).

Mouse Model of Oxygen-Induced Ischemic Retinopathy

C57BL/6 mice were exposed to 75% oxygen from postnatal day 7 (P7) with their nursing mother and returned to room air at P12. These mice were subsequently randomly divided into five groups. One eye received an intravitreal injection of 1 μL mouse endocan neutralizing antibody (NAb, R&D Systems, Minneapolis, MN, USA) at a concentration of either 0.01, 0.1, 0.5, or 1 μg/μL; the contralateral eye was treated with PBS. At P17, mice were euthanized and eyes were prepared for immunofluorescent flat-mounts with FITC-labeled isolectin B4 (1:50; Vector Laboratories, Inc., Burlingame, CA, USA) for 40 minutes, retinas were washed three times in PBS and flat-mounted with fluorescence mounting medium (DAKO, Agilent Technologies, CA, USA) and sealed with a cover slip. Digital photographs were obtained with a fluorescence microscope (Carl Zeiss Microscopy, Thornwood, NY, USA) at ×5 magnification, and images were merged into a single image to show the entire retina using the photomerge option of a raster graphics editor (Photoshop CS 6.0; Adobe Systems, San Jose, CA, USA). Imaging software (Image Pro Plus; Media Cybernetics, Inc., Rockville, MD, USA) was used to measure the area of retinal NV per retina by a blinded investigator.

Laser-Induced Choroidal NV Model

Choroidal NV was generated by laser photocoagulation with rupture of Bruch's membrane as previously described.[15,16] In one group, 3 days after laser photocoagulation, mice were euthanized for posterior segment separation (retina/choroid complex) and total RNA or protein exaction. In the second group, mice received a 1 μL intraocular injection of 0.5 μg mouse endocan NAb in one eye and PBS in the contralateral eye on day 0 and day 7 after rupture of Bruch's membrane. Mice were euthanized on day 14 to perform immunostaining and flat-mount analysis.

Mouse Model of Rho/VEGF Transgenic Mice and Flat-Mounts Analysis

Rho/VEGF transgenic mice, in which the rhodopsin promoter drives the expression of VEGF in photoreceptors so that new vessels sprout from the deep capillary bed of the retina,[17] received an intraocular injection of 0.5 μg/μL endocan NAb in one eye and PBS in the contralateral eye at P15. Retinas were dissected from one group of these mice for total RNA and protein exaction 3 days later. In the second group, rho/VEGF transgenic mice were anesthetized and perfused with fluorescein-labeled dextrans at P21. Retinal flat-mounts were examined by fluorescence microscopy at a ×200 magnification providing a narrow depth of field, so that when focusing on NV on the outer edge of the retina, the retinal vessels were out of focus, allowing easy observation of the NV. The outer edge of the retina, which corresponded to the subretinal space in vivo, was easily identified, ensuring standardization of the focal plane from slide to slide. Imaging software (Media Cybernetics, Inc.) was used to measure the number of subretinal NV lesions per retina.

Immunofluorescence Staining of Endocan and Isolectin

Mice with OIR and CNV or age-matched controls were euthanized. Their eyes were enucleated and rapidly frozen in embedding medium (Sakura Finetek, Torrance, CA, USA) prior to sectioning. Sections (10 μm) were thawed, air-dried and fixed in 4% paraformaldehyde at room temperature for 10 minutes. These sections were incubated with blocking buffer (PBS containing 10% FBS) for 1 hour, and were then subsequently with goat anti-mouse endocan antibody (R&D Systems) for 2 hours. They were then incubated with AlexaFluor 555 donkey anti-goat secondary antibody (1:500; Invitrogen, Carlsbad, CA, USA) and isolectin for 1 hour at room temperature in the dark. The nuclei were stained with DAPI (Beyotime Biotechnology, Shanghai, China) for 5 minutes. The sections were washed with PBS between these incubations. Finally, the sections were
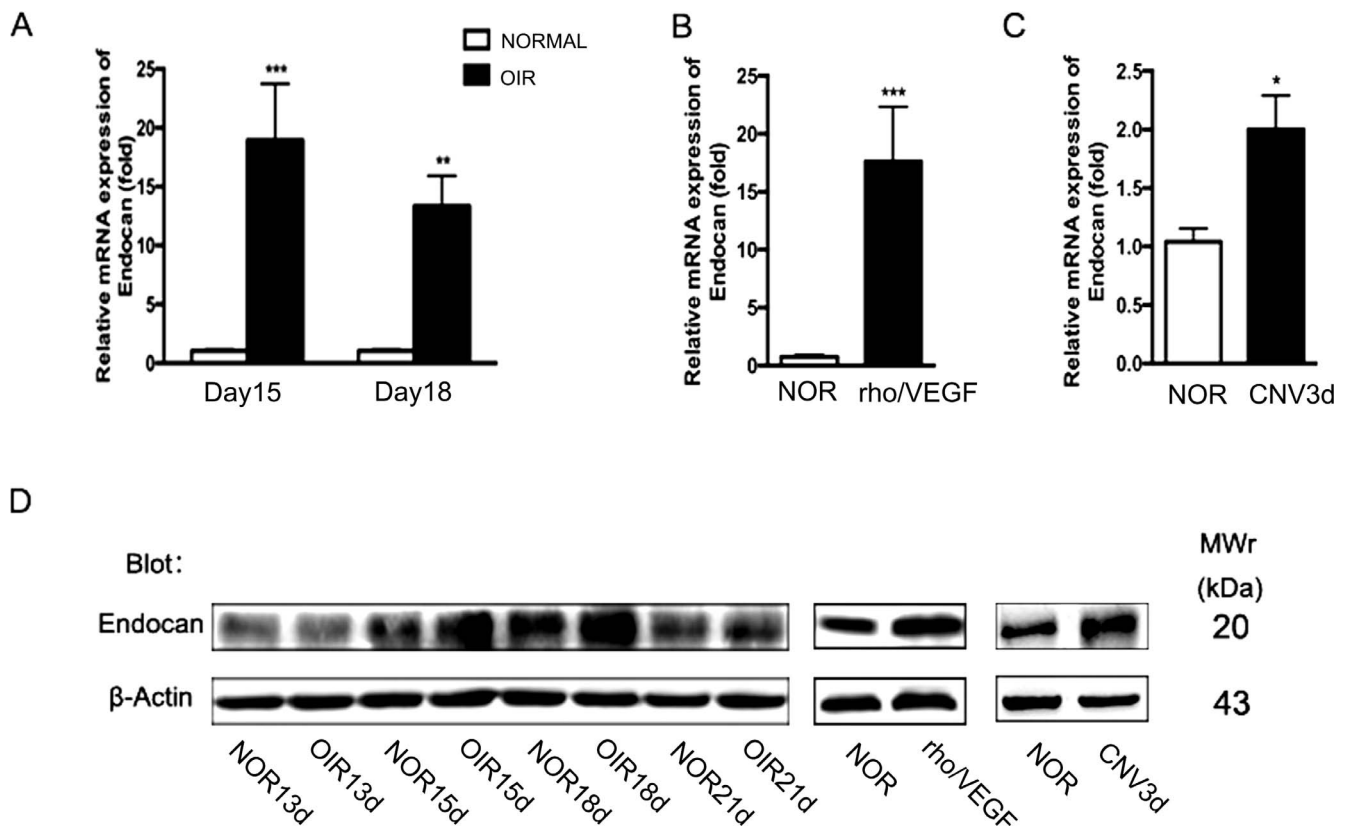
FIGURE 1. Endocan expression levels increased in eyes of OIR, CNV, and rho/VEGF transgenic mice models. (**A**) RT-PCR analysis for endocan mRNA in the retinas of OIR mice at P15 ($n = 8$) and P18 ($n = 8$). (**B**, **C**) Representative RT-PCR assay results of endocan mRNA expression in retinas from rho/VEGF transgenic ($n = 8$) at P15 and retina/choroidal complex from CNV mice ($n = 8$) at day 3 after photocoagulation, respectively. (**D**) Western blot demonstrates protein expression levels of endocan in OIR models ($n = 3$) at P13, P15, P18, and P21, rho/VEGF transgenic mice ($n = 3$) and mice with CNV ($n = 3$). In these assays above, the eyes from age-matched mice served as control. RT-PCR was used to determine mouse endocan mRNA levels, which were endogenously normalized to cyclophilin A. The protein levels of endocan in these mice models were measured by Western blot assays and β-actin was used for equal protein loading. These data were shown as the mean ± SEM (*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$).

mounted with fluorescence mounting medium, and examined and captured with a fluorescence microscope.

Quantitative Real-Time PCR

Total RNA in eye tissues and cells was extracted using a commercial reagent (TRIzol; Thermo Fisher Scientific, Waltham, MA, USA) following the manufacture's guidelines. Total RNA was quantified by spectrophotometer (Thermo NanoDrop 8000; Thermo Fisher Scientific); 1 μg total RNA of each sample was reversely transcribed into complimentary DNA (cDNA) using M-MLV reverse transcriptase system according to the manufacture's protocol. RT-PCR detection was performed in a 10 μL volume with SYBR Green Mix on an RT-PCR instrument (Light-Cycler 480; Roche Applied Science, Mannheim, Germany). Relative gene expression levels were normalized to cyclophilin A and calculated using the $2^{-\Delta\Delta Ct}$ method. Primer sequences could be obtained as follows: Endocan(mouse), 5′-GGAGGATGATTTTGGTGACG-3′ (sense), 5′-CTGTCACA TATGCCCGACTG-3′ (antisense); Endocan(human), 5′-CTTCGGGATGGATTGCAGAG-3′ (sense), 5′-CAGATGCCATGT CATGCTCC-3′ (antisense); MMP-2(mouse), 5′-GCTCTGTCCTCCTCTGTAGTTA-3′ (sense), 5′-GGTACAGT CAGCACCTTTCTT-3′ (antisense); MMP-9(mouse), 5′-TGCACTGGGCTTAGATCATTC-3′ (sense), 5′-TGCCGTCTATGTCGTCTTTATTC-3′ (antisense); PIGF (mouse), 5′-CTTCTGTGACCACTGTCAATCT-3′ (sense), 5′-TCAAAGCTCGCGTTACCTTAT-3′ (antisense); INOS(mouse), 5′-CCGGCATGAAGTACCTGATATT-3′ (sense), 5′-GGTGA

ACAGGTTTCCTCCTATG-3′ (antisense); VEGFA(human), 5′-CTTCAAGCCATCCTGTGTGTGC-3′ (sense), 5′-CGCTCCAG GACTTATACCG-3′ (antisense); VEGF164(mouse), 5′-CAGGCTGCTGTAACGATGAA-3′ (sense), 5′-AATGCTTTC TCCGCTCAGAA-3′ (antisense); VEGFR1(mouse), 5′-GAGC TAAAAATCTTGACCCACATTG-3′ (sense), 5′-CAGTATTCAA CAATCACCATCAGAG-3′ (antisense); VEGFR2(mouse), 5′-CACCTGCCAGGCCTGCAA-3′ (antisense), 5′-GCTTGGTGCAGGCGCCTA-3′ (antisense).

Western Blot

Fresh isolated eye tissues and cells were lysed, quantified, blotted and developed as previously described.[18] The following primary antibodies were used for the purpose of this experiment: goat anti-mouse endocan antibody (0.1 μg/mL; R&D Systems); rabbit anti-matrix metalloproteinase (MMP)-2 antibody (1:1000; Cell Signaling Technology); MMP-9 antibody (1:1000; Cell Signaling Technology); rabbit anti-PIGF antibody (1:1000; Abcam, Cambridge, UK); goat anti-VEGF antibody (1:1000; Abcam); rabbit anti-VEGFR1 antibody (1:1000; Abcam); rabbit anti-VEGFR2 antibody (Cell Signaling Technology); rabbit anti-P38 antibody (1:1000; Cell Signaling Technology); rabbit anti–phospho (p)-P38 antibody (1:1000; Cell Signaling Technology); rabbit anti-extracellular signal-regulated kinase (ERK) antibody (1:1000; Cell Signaling Technology); rabbit anti–p-ERK (1:1000; Cell Signaling Technology); rabbit anti–c-Jun N-terminal kinases (JNK) antibody (1:1000; Cell Signaling Technology) and rabbit anti–p-JNK antibody (1:1000; Cell Signaling
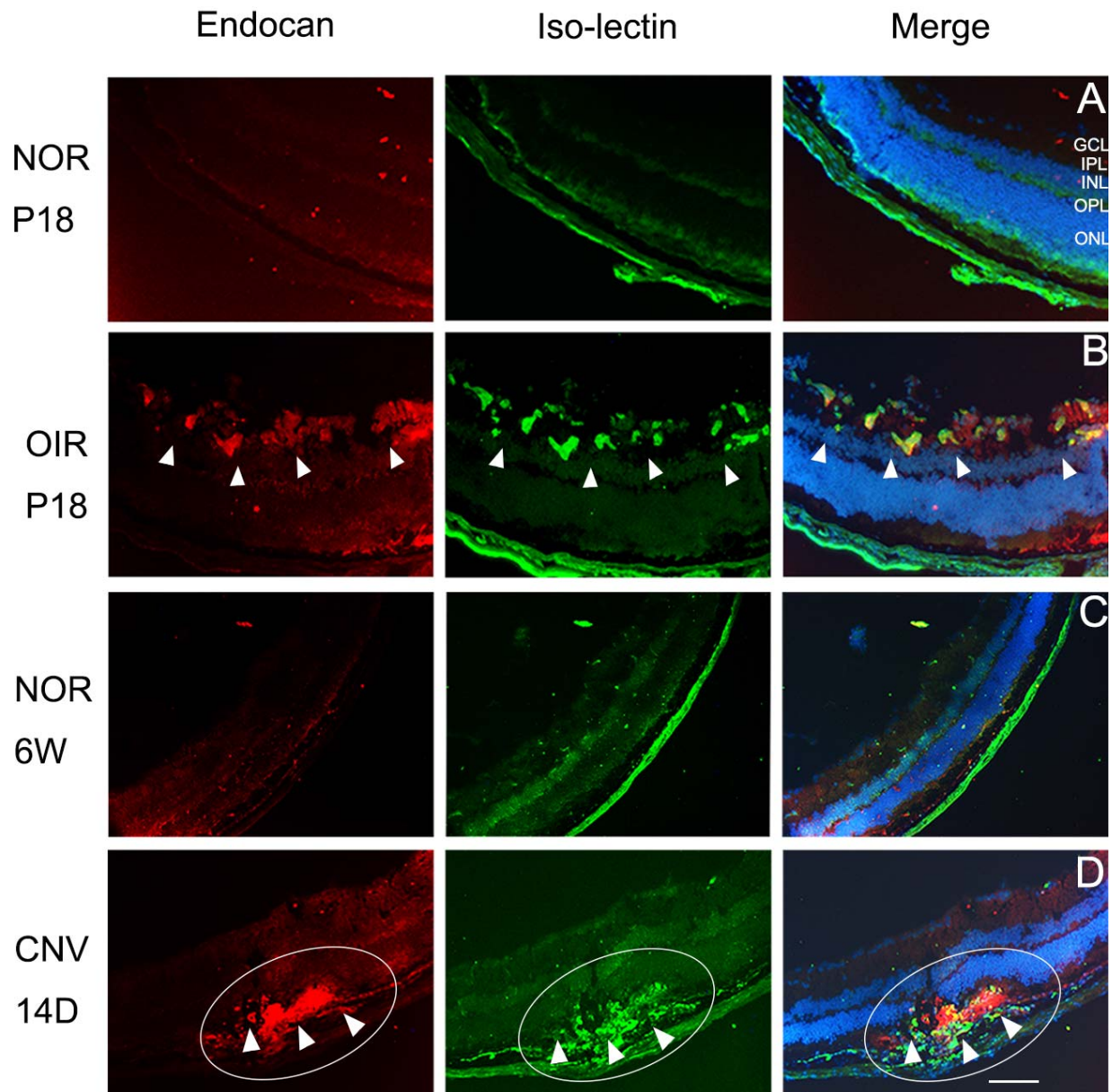
FIGURE 2. Immunofluorescent stained ocular frozen sections from the OIR and CNV mice model with anti-endocan antibody and isolectin B4. OIR mice were euthanized at P18 and CNV mice were euthanized at day 14 after photocoagulation. Eyes were removed and frozen, and cryosectioning was performed with subsequent immunofluorescent staining of endocan (*red*) and isolectin B4 (*green*). (**A, C**) Normal age-related mice were used as control groups. (**B**) *Arrowheads* in the merged images indicate the colocation between lectin and endocan in OIR mice. (**D**) The merged photographs also showed the double labeling of lectin and endocan in the CNV photocoagulated lesion site (*white circles*). The retinal layers shown are: ganglion cell layer (GCL); inner plexiform layer (IPL); inner nuclear layer (INL); outer plexiform layer (OPL); outer nuclear layer (ONL).

Technology) and β-actin (1:1000; Cell Signaling Technology) was used as an internal control to normalize band intensity.

RESULTS

Endocan Expression Was Significantly Upregulated in Animal Models of Retinal, Subretinal, and Choroidal NV

We examined endocan mRNA and protein expression levels in OIR mice from P13 to P21. RT-PCR analysis revealed that there was a more than 15-fold induction of endocan mRNA expression in the OIR model at P15 and P18 (Fig. 1A), which is in agreement with our microarray results. In addition, immuno-

blots also showed high expression in retinas of mice with OIR at P15 and P18 (Fig. 1D). While investigating the role of endocan in the subretinal or choroidal NV mice models, we detected that endocan mRNA and protein expression levels were both elevated in CNV (Fig. 1C) and rho/VEGF transgenic mice (Fig. 1B). We therefore concluded that endocan expression was upregulated in our murine ocular NV mouse models and may play an important role in ocular NV development.

Endocan Staining Increased in OIR and CNV Mice, and Colocalized With Endothelial Cells

From immunofluorescent staining images of ocular frozen sections of OIR and CNV mice, high expression levels of
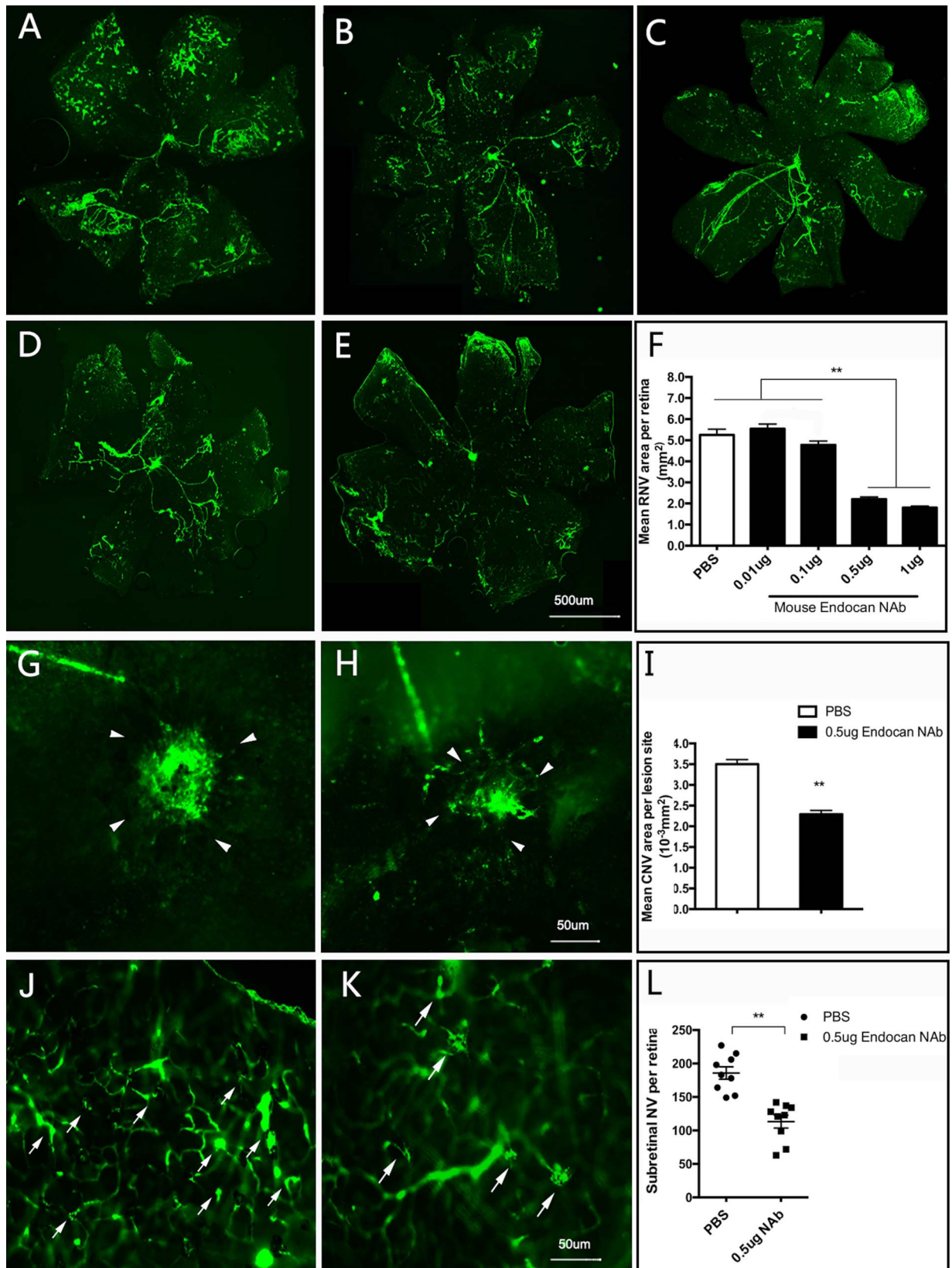
FIGURE 3. Immunofluorescence staining of retinal and choroidal flat-mounts of mice treated with mouse endocan NAb in the OIR, CNV, and rho/VEGF transgenic mouse models. Mice with OIR were randomly divided into several groups and one eye was injected with different concentrations of mouse endocan Nab; the contralateral eye treated with PBS at P12 served as control. (A–E) Retinas were flat-mounted and stained with FITC-lectin at P17 ($n = 6$ mice/group). (G–H) Choroidal flat-mounts from CNV mice were performed at day 14 ($n = 10$ mice/group) after laser photocoagulation with ocular treatments at days 0 and 7, as described. (J–K) Rho/VEGF transgenic mice received an intravitreal treatment at P12 and retinas were dissected and stained with FITC-lectin and flat-mounted at P21 ($n = 8$ mice/group). (F, I, L) Imaging software (Media Cybernetics, Inc.) was used to quantify the area of ocular NV. Statistics were analyzed using 1-way ANOVA with the least-significant difference (LSD) method. Data were shown as mean $\pm$ SEM (*$P < 0.05$; **$P < 0.01$).
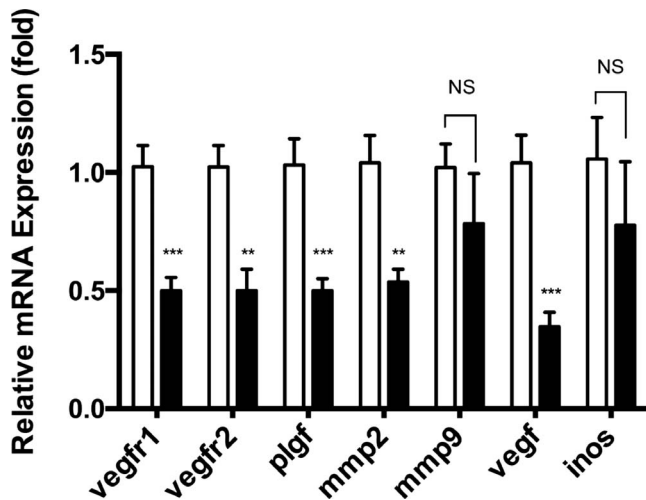
FIGURE 4. Angiogenic genes mRNA levels in endocan-blockade OIR mice. Mice with OIR received an intravitreal injection of 1 μL of 0.5 μg/μL endocan NAb in one eye and PBS in the contralateral eye at P12. Retinas were separated 5 days later for total RNA isolation. RT-PCR was conducted to quantify the mRNA expression levels of VEGFR1 ($n = 7$); VEGFR2 ($n = 6$); PIGF ($n = 7$); MMP-2 ($n = 7$); MMP-9 ($n = 6$); VEGF ($n = 7$); and INOS ($n = 5$). Data were analyzed from 5 to 7 independent retinas and represented as the mean ± SEM. The statistical analysis was performed using an unpaired *t*-test (*$P < 0.05$; **$P < 0.01$; NS, no significance).

endocan could be observed in the inner layer of the retina of OIR mice (Fig. 2B) and the photocoagulated lesion site with CNV (Fig. 2D). However, lectin-stained vasculature and endocan in the control sections showed faint staining (Figs. 2A, 2C). Merged images did show colocalization of endocan and lectin, which stained for endothelial cells. These findings indicated that endothelial cells might be the main source of

endocan secretion participating in retinal and choroidal NV progression.

Reduction of Retinal, Subretinal, and Choroidal NV by Endocan Neutralization in Ocular NV Animal Models

Mice with OIR received 1 μL intravitreal injections of endocan NAb in one eye and PBS in the contralateral eye at P12. To determine the lowest effective concentration, dose-effect experiments were designed to treat OIR mice. Analysis of the retinal flat-mounts revealed that endocan blockade significantly suppressed retinal NV at concentrations of 0.5 μg/μL and 1 μg/μL (Figs. 3A–F). We therefore chose 0.5 μg/μL to carry out the subsequent experiments. We also found that the areas of CNV decreased in 0.5 μg/μL endocan NAb neutralized mice (Figs. 3G–I). To further investigate whether endocan NAb treatment was as effective in the VEGF-overexpressing murine model, retinal flat-mounts were examined under fluorescence microscopy, revealing that 0.5 μg/μL endocan NAb significantly suppressed subretinal NV of rho/VEGF mice (Figs. 3J–L). These results demonstrate that endocan may play a supportive role in ocular NV progression related to VEGF.

Neutralizing Endocan Modulated the Expression of Several Proangiogenic Molecules

OIR and CNV mice received a 1 μL intravitreal injection of 0.5 μg/μL endocan NAb in one eye and PBS in the contralateral eye at either P12 (OIR) or day 0 (CNV) respectively. RT-PCR was performed to evaluate the mRNA expression of angiogenesis-associated factors in OIR retinas at P15. Angiogenic factors such as VEGF, VEGFR1, VEGFR2, PIGF, and MMP-2 were significantly downregulated (with the exception of MMP-9 and INOS; Fig. 4). Downregulation was further assessed by measuring expression at the protein level during the same period. Intraocular treatment with endocan
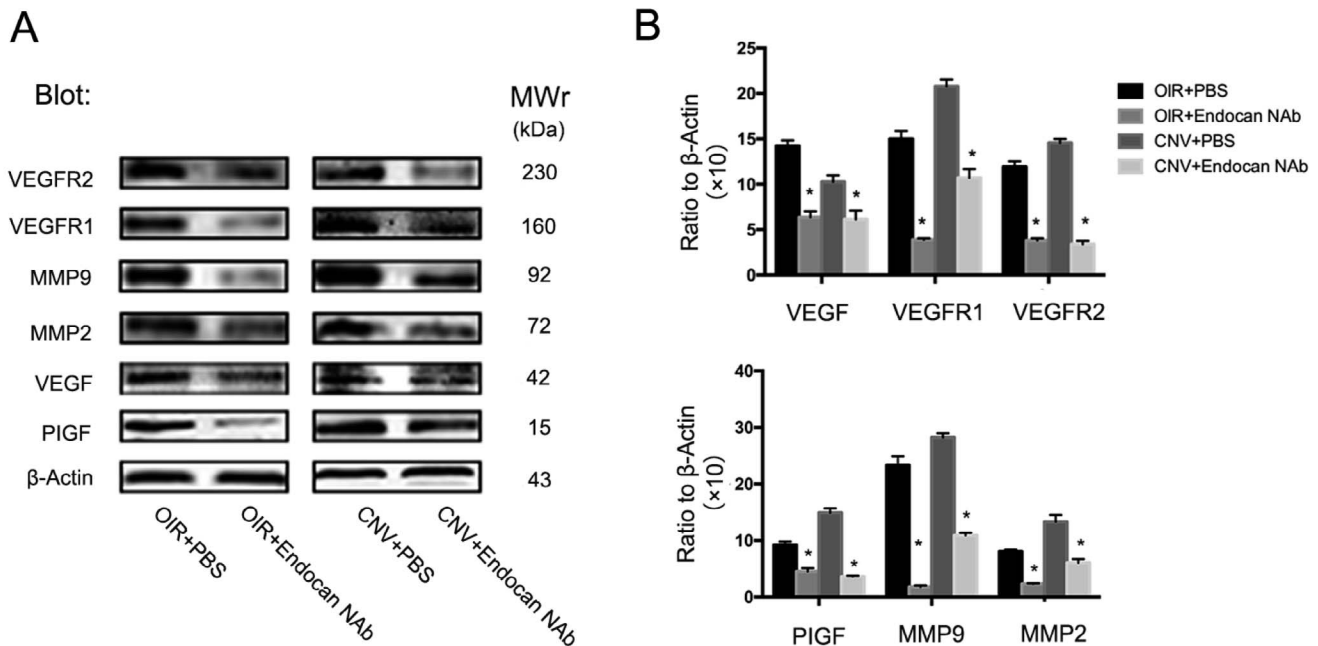

FIGURE 5. Protein levels of angiogenic factors in endocan-neutralized OIR and CNV mice. Mice received an intraocular injection of endocan NAb in one eye and PBS in the contralateral eye at P12 (OIR mice) or day 0 after photocoagulation (CNV mice). Immunoblot was performed to detect the protein expression levels of VEGF, PIGF, VEGFR1, VEGFR2, MMP-2, and MMP-9. β-actin was used as control. Data were analyzed and expressed as mean ± SEM from three independent experiments (*$P < 0.05$; **$P < 0.01$).
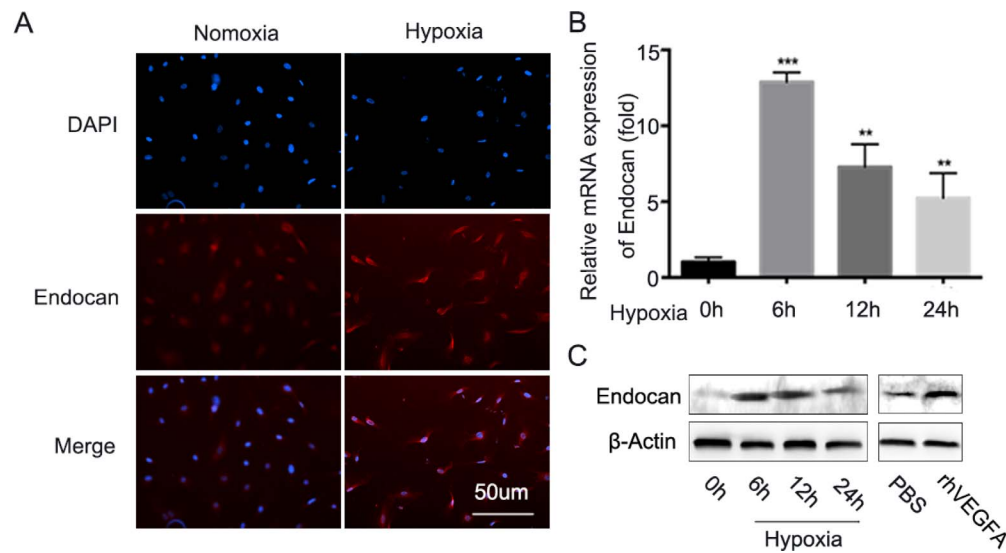
FIGURE 6. Hypoxia and VEGF-induced endocan synthesis in HRECs. (**A**) Immunofluorescence images of endocan expression on HRECs under hypoxia. (**B**) Quantification of endocan expression in HRECs with or without hypoxia treatment. (**C**) Western blot analysis of endocan expression in lysates of HRECs induced by hypoxia or 50 ng/mL recombinant VEGFA.

NAb lowered protein levels in both OIR and CNV models (Fig. 5B) suggesting endocan is involved in the ocular NV process by interacting with angiogenesis-associated molecules.

Hypoxia and VEGF Induced Endocan Synthesis in HRECs

We next sought to detect whether endocan could directly modulate endothelial cells in vitro. To mimic the hypoxia-induced OIR model, we cultured HRECs in 1% oxygen. The time curve of mRNA expression showed that endocan was elevated within 24 hours and reached its peak at 6 hours after hypoxia (Fig. 6B). Similar results were obtained from immunoblot (Fig. 6C) and immunofluorescent assays (Fig. 6A). As VEGF was considered to play a central role in ocular NV, we quantified endocan expression after recombinant VEGF stimulation. Notably, endocan expression was induced by VEGF stimulation.

Endocan Promoted HRECs Tube Formation by Modulating VEGF Signaling

Compared to controls, incubating HRECs with recombinant endocan could promote endothelial tube formation at 50 ng/mL and 100 ng/mL (Fig. 7A). To further elucidate the underlying mechanism of endocan's effect on HRECs in vitro, we examined molecules associated with the VEGF pathway (Fig. 7C). We found that VEGF receptor levels were not increased by rhEndocan stimulation. However, phosphorylation levels of both ERK (p-ERK) and p-P38 were increased (p-JNK was not included). These data suggest that endocan may mediate endothelial function by regulating the VEGF signaling pathway via p-ERK and p-P38, the well-established downstream effectors of the VEGF pathway.

DISCUSSION

Endocan is known to be essential in the process of pathologic angiogenesis and vascular permeability.[12] Expression levels of endocan are significantly elevated in multiple malignant tumors, and endocan is used as a biomarker for monitoring cancer progression and as a potential therapeutic target.[19] Previous reports established that endocan was implicated in the regulation of inflammation and angiogenesis.[20] However, it is still unclear whether endocan plays a functional role in ocular NV diseases. This study is the first to confirm that endocan expression is significantly elevated at critical time points in established ophthalmic neovascular disease models including OIR, CNV, and rho/VEGF transgenic mice models. We also demonstrated a consistent increase of endocan in HRECs following hypoxia exposure in vitro. Immunofluorescent double-labeling staining showed that endocan was enriched in the surface of the retina of OIR mice and the photocoagulated lesion site of CNV mice, colocalizing with isolectin, an endothelial cell marker,[21] suggesting that endothelial cells might be the main source of endocan secretion. Recently, endocan was described to be enriched in "tip cells" which are the motile endothelial cells that mediate sprouting of developing vessels during the process of angiogenesis.[22] Therefore, we hypothesized that endocan might be integral to retinal and subretinal NV progression by regulating retinal endothelial tip cells behavior.

In our prior research, we demonstrated that disruption of the balance between antistatic and angiogenic factors resulted in the formation of pathologic new blood vessels, in which several angiogenic factors were elevated at P15 of the OIR model and at day 3 of CNV mice.[23] Interestingly, these angiogenic molecules, such as VEGFR1, VEGFR2, MMP-2, MMP-9, and PIGF were downregulated after blocking endocan. Consistent with our findings, our new results indicate that the area of retinal, subretinal, and choroidal NV was significantly decreased following the neutralizing effect of an intraocular injection of endocan neutralizing antibody providing additional evidence that endocan plays a proangiogenic role in the ocular NV pathologic process. Further studies are needed to evaluate endocan neutralizing antibody's potential effects on ocular vascular permeability.

It is well documented that VEGF is an incredibly important stimulator that initiates the sprouting of new blood vessels in retinal and subretinal neovascularization, maintenance of endothelial cell division sustainment and cell survival.[24] PIGF, VEGF, VEGFR1, and VEGFR2 are crucial
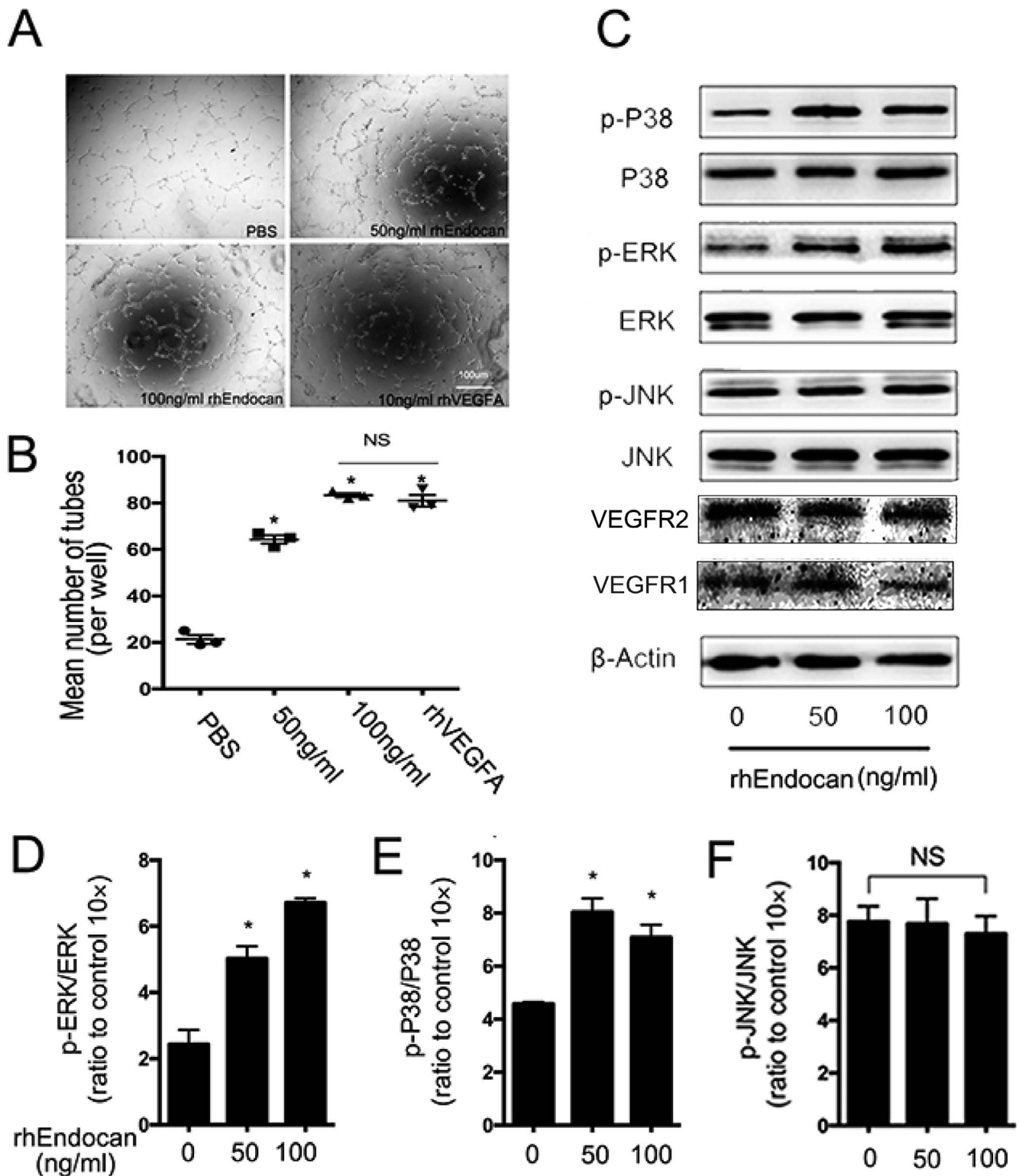
FIGURE 7. Endocan promotes HRECs tube formation by regulating VEGF signaling. (A) Tube formation assays were taken in HRECs in the presence of 50 ng/mL, 100 ng/mL rhEndocan, PBS, or 10 ng/mL rhVEGFA, which served as controls. (B) Data presented represent the mean number of tubes in each treatment group. (C) Western blot to detect protein levels of VEGFR1, VEGFR2, p-P38, and total P38, p-ERK, and total ERK, p-JNK, and total JNK of HRECs lysates after rhEndocan stimulation. Data are expressed as mean ± SEM from three independent experiments. Statistical analysis was performed by 1-way ANOVA with the LSD method (*$P < 0.05$).

members of the VEGF family. Similar to VEGF, PlGF can directly bind with VEGFR1 and mobilize bone marrow–derived cells and stimulate retinal NV.[1] Clinical investigations have shown that the expressions of PlGF, VEGF, and endocan are upregulated in vitreous fluid from patients with proliferative diabetic retinopathy.[25,26] Based on our results, inhibition of endocan caused a decrease in VEGF, PlGF and their receptors in the OIR and CNV models, suggesting that the blockade of endocan can modulate angiogenic activities in pathologic NV progression by interacting with VEGF family members. Our results were consistent with previous reports showing that VEGF signaling was reduced in the absence of endocan in the early postnatal mouse retina.[12] Endocan secretion by endothelial cells may act in a paracrine manner in nearby cells during angiogenesis[27] and blockade of endocan may interrupt this network. It has been shown that both MMP-2 and MMP-9 are involved in ocular NV progression. MMPs digest extracellular matrix, attributing to early endothelial cell migration in the angiogenic process.[28,29] Kang et al.[11] demonstrated that endocan contributed to cellular invasiveness through the modulation of MMPs resulting in epithelial tumors migration and metastatic process, indicating that endocan might affect MMP activity during NV development. However, the underlying mechanism remains to be investigated. There is limited evidence to verify whether endocan is an upstream or downstream factor of VEGF signaling in ocular NV development. In our experiments, we chose the VEGF overexpressing murine model to investigate the interactions between endocan and VEGF. We observed increased endocan expression levels in VEGF-overexpressing mice and found that inhibition of endocan could suppress VEGF-induced NV in rho/VEGF transgenic mice. In vitro, endocan secretion by HRECs was significantly increased upon recombinant VEGF stimulation. We identified a prominent increase in tube formation of HRECs in the presence of endocan. However, recombinant human endocan alone did not promote HRECs proliferation (data not shown). These results seem reasonable given that new blood vessels formation from preexisting vessels is dependent not only on endothelial proliferation, but also on the appropriate sprouting activity of endothelial cells by interacting with angiogenic factors.[30] Our findings are consistent with prior studies involving bladder cancer research demonstrating that endocan was secreted by microvasculature-derived endothelial cells upon VEGF stimulation and bound VEGF on the cell surface, facilitating its interaction with VEGFR2 and increasing the intensity of VEGF signaling.[31] To uncover the underlying mechanism of endocan mediating HRECs, molecules associated with the VEGF pathway were examined.[32] We demonstrated that endocan stimulation alone did not increase VEGF receptor levels in HRECs. This result is consistent with findings of Rocha et al.[12] that endocan interacts with fibronectin and heparan sulfate proteoglycan 2 instead of directly leading to VEGFR2 phosphorylation. Moreover, we found that endocan modulated the VEGF signaling pathway by regulating downstream effectors p-ERK1 and p-P38. Further studies are needed to determine if the blockade of endocan can provide an additional advantage over current anti-VEGF agents.

In conclusion, our findings in this study indicate that endocan promotes angiogenesis progress in ocular NV by modulating endothelial cell function via upregulation of the VEGF signaling pathway. In addition, the experimental ocular NV in mice and VEGF signaling can be suppressed by endocan blockade. These findings may support targeting endocan as a potential therapy to prevent ocular NV diseases.

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