

Semaphorin 3A blocks the formation of pathologic choroidal neovascularization induced by transforming growth factor beta

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Objective: Choroidal neovascularization (CNV) is a major cause of vision loss in retinal diseases such as age-related macular degeneration (AMD). Previously, we demonstrated that semaphorin3A (Sema3A), which is a chemorepellent guidance molecule, inhibited the formation of retina neovascularization. In the present study, we investigated the anti-angiogenic effects of Sema3A on transforming growth factor beta (TGF- β) in vitro and in vivo.

Methods: Enzyme-linked immunosorbent assays (ELISAs) were used to measure the TGF- β levels in the vitreous humor of patients with AMD and controls. Human umbilical vein endothelial cells (HUVECs) were used for the in vitro study, and a laser-induced CNV mouse model was prepared for the in vivo study. The HUVECs were incubated with TGF- β and Sema3A. The proliferation, migration, apoptosis, and tube formation of the cells were then measured using BrdU, Transwell, flow cytometry, and Matrigel assays, respectively, and the SMAD2/3 signaling pathways were analyzed using western blot analysis. The C57BL/6J mouse retina was exposed to a laser to induce choroidal neovascularization (CNV), and Sema3A was injected intravitreally. After 14 days, fundus fluorescein angiography was performed to evaluate the leakage area of the CNV. The vascular endothelial growth factor (VEGF) and TGF- β concentrations in the retina-choroid complex were measured with ELISA. Components of the p38 mitogen-activated protein kinase (MAPK), extracellular signal-regulated kinase-1/2 (ERK1/2), c-Jun NH2-terminal kinase (JNK), and SMAD2/3 signaling pathways in the Sema3A-treated groups were analyzed using western blotting.

Results: In this study, we first verified that the vitreous TGF- β level was higher in patients with neovascular AMD than in the controls. We also showed that Sema3A inhibited TGF- β -induced HUVEC proliferation, migration, and tube formation and inhibited the downstream SMAD2/3 signaling pathway. Sema3A also induced TGF- β -stimulated HUVEC apoptosis and inhibited the response of TGF- β in vitro. In vivo, the TGF- β level was increased in the CNV mouse model. Sema3A not only inhibited laser-induced CNV formation but also inhibited the uptake of VEGF and TGF- β . In the western blot analysis, Sema3A was shown to inhibit the phosphorylation of p38 MAPK, ERK1/2, and JNK and to inhibit the SMAD2/3 signaling pathway after Sema3A treatment in CNV mice.

Conclusions: Sema3A can be applied as a useful, adjunctive therapeutic strategy for preventing CNV formation.

Choroidal neovascularization (CNV) is a major cause of vision loss in retinal diseases such as age-related macular degeneration (AMD), pathological myopia, and traumatic choroidal laceration [1]. CNV refers to the growth of neovasculation derived from the choroid vessels through breaks in Bruch's membrane into the sub-retinal pigment epithelium or sub-retinal space [2]. Although the mechanisms of CNV are not well understood, the upregulation of angiogenic factors, such as vascular endothelial growth factor (VEGF), transforming growth factor beta (TGF- β), angiostatin, and hypoxia-inducible factor, play major roles in the formation

and progression of CNV [3]. Although intravitreal injection of anti-VEGF agents is the primary treatment for CNV, other mediators related to VEGF upregulation are targets for treating CNV, and TGF- β is an important molecule among these targets [4].

TGF- β is a molecule with pleiotropic effects that participates in cell proliferation and differentiation during angiogenesis and fibrotic processes, and its presence in neovascular membranes has been demonstrated [5-7]. Three isoforms of TGF- β have been discovered, of which TGF- β 1 is the most important [8]. The signaling pathways that act downstream of TGF- β 1 include canonical (Smads) and noncanonical (e.g., c-Jun NH2-terminal kinase [JNK]/p38 mitogen-activated protein kinase [MAPK], extracellular signal-regulated kinase-1/2 [ERK1/2], phosphatidylinositol 3-kinase PI3K/Akt, etc.) pathways [9]. Recently, several studies have reported that TGF- β significantly enhances VEGF secretion,

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vascular permeability, and extracellular matrix remodeling on its own or in concert with other cytokines, such as tumor necrosis factor alpha [10-13]. These findings led us to speculate that an agent that can block VEGF and TGF- β would more efficiently inhibit CNV progression.

Semaphorins (Semas), which represent one of the best-studied classes of guidance molecules, are active in axonal growth cone guidance and vessel network formation [14,15]. Semas conduct signals through multimeric receptor complexes, and neuropilins (Nrps) and plexins (Plxns) are the most important members of these complexes [16]. Among the Sema family proteins, semaphorin 3A (Sema3A) has been demonstrated to play an important role in angiogenesis [17]. Sema3A binds to Nrpl and PlexA1-4 to form the complex Nrpl/PlexA1-4. In this receptor complex, Nrpl acts as a binding element, whereas PlexA1-4 acts as a signal-transducing element [16]. Nrpl is not only a receptor for Sema3A but also a coreceptor that enhances the response to several growth factors, such as VEGF, TGF- β 1, hepatocyte growth factor, and platelet-derived growth factor [18]. Previously, we demonstrated that Sema3A could inhibit the effect of VEGF on retinal angiogenesis and on RPE proliferation, preventing the response to VEGF by competitively inhibiting Nrpl [19,20]. In the present study, we aimed to investigate the effects and possible mechanisms of Sema3A on TGF- β signaling in the formation of pathological CNV. The encouraging results of our present and previous studies provide a new strategy for treating CNV by inhibiting VEGF and TGF- β to prevent angiogenesis.

METHODS

Ethics statement: The human patient study protocol was approved by the Ethical Committee and Institutional Review Board of Peking University People's Hospital (Beijing, China) and was conducted in accordance with the Declaration of Helsinki. Written informed consent was obtained from each study subject.

All animal experiments adhered to the ARVO statements for the Use of Animals in Ophthalmology and Vision Research and to the guidelines of the Institutional Animal Care and Use Committee (IACUC) of Peking University. All of the procedures were approved by the Animal Care and Use Committee of Peking University People's Hospital (Beijing, China).

Subjects and vitreous sample collection: All subjects received a standard ophthalmic examination by two retinal specialists (Dr. Wenzhen Yu and Dr. Xiaoxin Li). The diagnosis of neovascular AMD (nAMD) was made according to the International Classification System for ARM [21]. All

patients with nAMD underwent fluorescein angiography (FA), optical coherence tomography (OCT), and indocyanine green angiography (ICGA). The controls were patients who had been diagnosed with idiopathic macular holes (IMH; stage III, Gass IMH phases). No systemic diseases, such as hypertension or diabetes, were presented by any patient included in this study.

All of the patients with nAMD included in this study required anti-VEGF treatment. Approximately 100 μ l of an undiluted vitreous sample was collected from the patients with nAMD during anti-VEGF treatment (n=14), whereas the vitreous bodies of the patients with IMH (n=12) were accessed during pars plana vitrectomy. These samples were centrifuged to remove the cells and debris and were frozen at -80 °C until analysis. All surgeries were performed by Dr. Wenzhen Yu and Dr. Xiaoxin Li at Peking University People's Hospital.

Assessment of the TGF- β 1 level in the patients' vitreous humor: The TGF- β 1 concentration in the vitreous humor of the patients with nAMD and IMH was measured with enzyme-linked immunosorbent assay (ELISA) using a human TGF- β 1 ELISA kit (Human TGF-beta 1 Quantikine ELISA kit; R&D Systems, Minneapolis, MN). Each assay was performed according to the manufacturer's instructions.

HUVEC cell culture and cell proliferation assay: Human umbilical vein endothelial cells (HUVECs, CRL-1730 American Type Culture Collection [ATCC], Manassas, VA) were used in this study as previously described [22]; all HUVECs used were between passage 3 and passage 5. Sema3A (50,631-M01H; Sino Biologic, Inc., Beijing, China) was dissolved in PBS (Ca²⁺ and Mg²⁺ free) to 1 mg/ml as a stock solution and then diluted in Dulbecco's Modified Eagle Media (DMEM, Hyclone, Grand Island, NY) to the indicated concentrations. Sema3A at concentrations of 250 ng/ml and 500 ng/ml was incubated with HUVECs in a general culture medium or medium containing TGF- β 1 (10 ng/ml, 240-B-010, R&D Systems, Inc., Minneapolis, MN) in 96-well plates for 24, 48, and 72 h.

A BrdU cell proliferation assay (#6813, Cell Signaling Technology, Danvers, MA) was used according to the manufacturer's instructions and read with an ELISA microplate reader (Finstruments Multiskan Models 347; MTX Lab Systems, Vienna, VA). Each experiment was performed in five wells and was repeated at least three times.

HUVEC migration assay: A migration study was performed using a Transwell system (Cat#3422; Corning, Tewksbury, MA) as described previously [22]. Briefly, DMEM (containing 1% fetal bovine serum [FBS]) with 250 ng/ml

or 500 ng/ml Sema3A or Sema3A and TGF- β 1 (10 ng/ml) was placed in the bottom chamber. All migration assays were conducted at 37 °C for 4 h. After incubation, the cells were fixed and stained with 4,6-diamidino-2-phenylindole (DAPI; Roche Diagnostics, Indianapolis, IN). The cells that had not migrated were removed, and the membrane was imaged with fluorescence microscopy (Zeiss Axiophot, Thornwood, NY).

Flow cytometry analysis of HUVEC apoptosis: An apoptosis study (FITC-Annexin V Apoptosis Detection kit; BD Science) was performed as previously reported [20]. Briefly, HUVECs were incubated for 24, 48, and 72 h with Sema3A, TGF- β 1 and Sema3A, or controls. Then, the cells were detached with EDTA and stained with Annexin-V-FITC and propidium iodide (PI). Flow cytometry (FACSCalibur; BD Biosciences, Franklin Lakes, NJ) analysis was immediately performed (ex/em=488/530 nm). The collected cells were divided into four groups: dead cells (Annexin V-/PI+, UL), late apoptotic cells (Annexin V+/PI+, UR), viable cells (Annexin V-/PI-, LL), and early apoptotic cells (Annexin V+/PI-, LR). The apoptotic rate was calculated as the percentage of early apoptotic (LR) and late apoptotic cells (UR).

HUVEC tube formation study: Aliquots of 150 μ l of Matrigel (Cat#354234, BD Sciences) were added to each well of the 48-well plates, followed by incubation at 37 °C for 30 min [22]. The HUVECs (5×10^4) were treated with Sema3A or Sema3A and TGF- β 1, and the cells were then seeded on the Matrigel and cultured for 8–10 h at 37 °C. The networks from five randomly chosen fields of the Matrigel were counted and photographed, and the lengths of the tubes were measured using ImageJ software (National Institutes of Health, Bethesda, MD). The experiments were repeated three times.

TGF- β 1 measurement with ELISA using the HUVEC cell culture supernatant: Using the same treatment process as for the proliferation assay, the cell culture supernatant was harvested after incubation for 24, 48, and 72 h and centrifuged. The free TGF- β 1 in the culture medium was measured using a TGF- β 1 ELISA kit as described for the vitreous humor study. Each experiment was performed in five wells and was repeated at least three times.

Murine CNV models: Forty-five C57BL/6J mice (6–8 weeks old, 25–30 g) were used for the laser-induced CNV animal model. The mice were anesthetized using intraperitoneal injection of ketamine, xylazine, and acepromazine (50/5/1 mg/kg). The pupil of the experimental eye was dilated. CNV was induced with laser photocoagulation (Coherent 130SL, Coherent, Santa Clara, CA) with the following settings: 532 nm, 150 mW, 100 ms, and 100 mm. Then, four laser lesions were applied in a homodispersed distribution in a standardized manner around the optic disc in the right eye.

The contralateral eye was used as a control. The morphologic endpoint of the laser injury was the appearance of a subretinal bubble at the time of laser photocoagulation due to the disruption of Bruch's membrane. In total, 1.5 μ l of Sema3A (10 ng/ml, 100 ng/ml) was intravitreally injected immediately after the CNV model was established. Each group contained 15 mice.

Assessment of VEGF₁₆₅ and TGF- β 1 levels in the mouse retina: At the end of the Sema3A-treatment experiments, the retina-choroid complexes of the CNV mice were collected. The concentrations of retina VEGF₁₆₅ and TGF- β 1 after Sema3A treatment were measured using mouse VEGF₁₆₅ (Mouse VEGF Quantikine ELISA kit, cat#MMV00, R&D Systems) and TGF- β 1 ELISA kits (Mouse/Rat/Porcine/Canine TGF-beta 1 Quantikine ELISA kit, cat#MB100B, R&D Systems). Each assay was performed according to the manufacturer's instructions. Each group contained five mice.

Measurement of CNV with fluorescein angiography: FA was performed on day 14 after laser photocoagulation using a digital imaging system (Phoenix Micron IV Retinal Imaging Microscope, Pleasanton, CA). In total, 0.03 ml of 5% fluorescein was administered by intraperitoneal injection after each mouse was anesthetized using the method described, and FA was then performed with pupil dilation. The late-phase (5 min after injection) fundus angiograms were analyzed, and the fluorescein leakage area for each lesion was measured using the ImageJ software.

RNA extraction and real-time PCR for TGF- β 1: The mice were euthanized 14 days after the laser induction, and the retina-choroid complexes were isolated and lysed in TRIzol reagent. Reverse transcriptase reactions were performed using a RevertAid First Strand cDNA Synthesis kit with oligo-dT primers (Fermentas, Pittsburgh, PA). Real-time PCR reactions were performed with the SYBR Green PCR mix (Thermo, Pittsburgh, PA) using an ABI7300 real-time PCR system (Applied Biosystems, Life Technologies, Foster City, CA). The following real-time PCR primers were used: TGF- β 1, forward 5'-AAC AAT TCC TGG CGT TAC CTT-3', reverse 5'-GAA TCG AAA GCC CTG TAT TCC-3'; and glyceraldehyde 3-phosphate dehydrogenase (GAPDH), forward 5'-GAG TCC ACT GGC GTC TTC AC-3', reverse 5'-GTT CAC ACC CAT GAC GAA CA-3'. Each experiment was repeated three times.

Western blot analysis of HUVECs and retina-choroid complexes: The HUVECs and the retina-choroid complexes were prepared using protein extraction and protease inhibitor kits (Pierce, Rockford, IL) according to the manufacturer's instructions. Equal amounts of protein were separated with 10% sodium dodecyl sulfate–polyacrylamide gel

electrophoresis (SDS–PAGE) and transferred electrophoretically to polyvinylidene difluoride membranes (PVDF, Amersham, Little Chalfont, UK). The proteins were visualized using enhanced chemiluminescence western blot detection reagents (Pierce).

After the HUVECs were incubated with Sema3A for 48 h *in vitro*, the cells were harvested for SMAD2/3 and phospho-Smad2 (Ser465/467)/Smad3 (Ser423/425) western blot analysis. For the *in vivo* study, the retina-choroid complexes of the CNV mouse model were isolated after 14 days of Sema3A treatment. The TGF-β1, SMAD2/3, phospho-Smad2 (Ser465/467)/Smad3 (Ser423/425), phospho-p38 MAPK (Thr180/Tyr182), phospho-p44/42 MAPK (Erk1/2), and phospho-SAPK/JNK (Thr183/Tyr185) levels were evaluated. All antibodies were purchased from Cell Signaling Technology and were diluted 1:1,000. The loading was controlled using antibodies against β-actin (1:5,000, CST). For digital quantification, the membranes were analyzed using the ImageJ software. The western blot analyses were repeated three times, and qualitatively similar results were obtained.

Statistical analysis: The data analysis was performed using Prism 5 statistical software (GraphPad Software Inc., San Diego, CA). The data are presented as the frequencies or the means±standard error of the mean (SEM). Differences in the TGF-β1 concentrations among the groups were estimated using the Student *t* test and the nonparametric Mann–Whitney U test. For other data, the differences were evaluated using an ANOVA followed by a Student–Newman–Keuls test

for multiple comparisons or the Student *t* test for pair-wise comparisons. Two-tailed probabilities of less than 0.05 were considered to indicate statistical significance.

RESULTS

Vitreous TGF-β: The vitreous humor TGF-β levels in the patients with nAMD and IMH were measured, and the differences between the two groups were compared. The mean age of the patients was 67.1±4.3 years in the nAMD group and 71.4±5.6 years in the IMH group. Women constituted 42.9% of the nAMD group and 50% of the IMH group. No significant differences in number, mean age, or sex were found between the two groups. The TGF-β1 level was 943.7±78.78 pg/ml (n=14) in the patients with nAMD and 277.6±54.94 pg/ml (n=12) in the patients with IMH (Figure 1). A significant difference was observed in the TGF-β1 levels in the vitreous humor (p=0.003) of the groups.

Sema3A inhibited TGF-β-induced HUVEC proliferation and migration: The capacity of cells to proliferate and migrate is important for the formation and extension of neovasculature. In this study, we used BrdU and Transwell assays to evaluate the inhibitory effects of Sema3A on HUVECs. Sema3A inhibited HUVEC proliferation under normal culture conditions (1% FBS) at different time points compared with the 1% FBS treatment group and inhibited HUVEC proliferation under TGF-β stimulation at both studied concentrations (250 ng/ml and 500 ng/ml) compared with the TGF-β-treated group (Figure 2A–C, *p<0.05, **p<0.01).

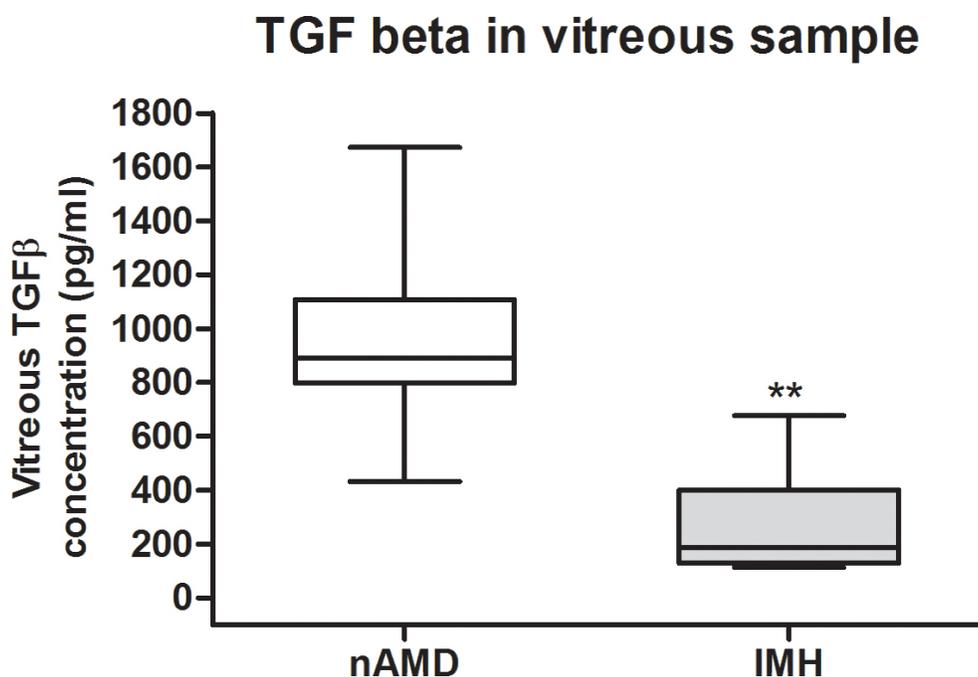


Figure 1. The vitreous concentrations of TGF-β in patients with nAMD. The boxplots show the vitreous concentrations of TGF-β in nAMD eyes and in the controls with IMH. The TGF-β levels were significantly different between the two groups (nAMD compared with IMH, p=0.0003) nAMD (n=14) vs IMH (n=12), **p<0.01, error bars are SEM.

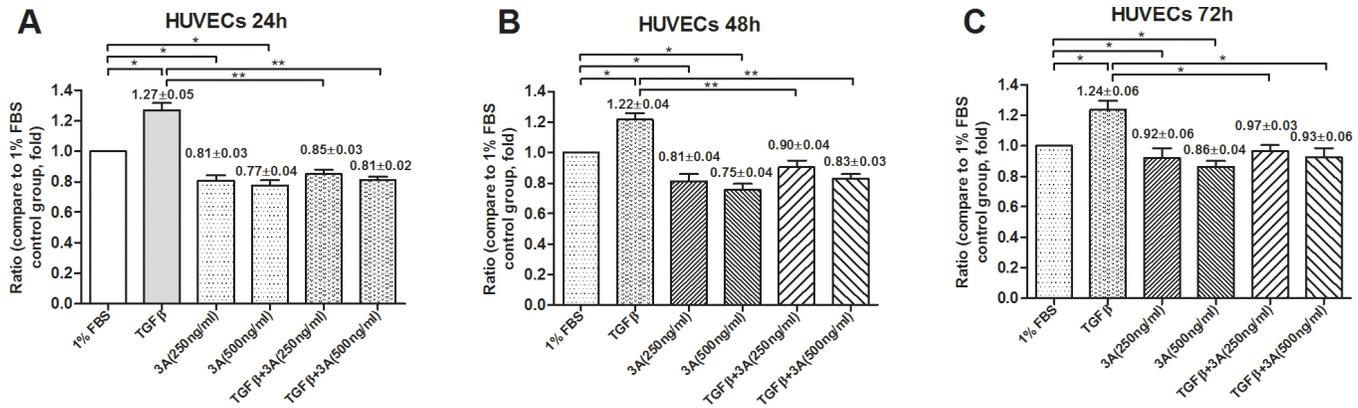


Figure 2. Effect of *Sema3A* on HUVEC proliferation. **A**, **B**, and **C** show the results and statistical analysis of HUVEC proliferation at different time points (24 h (**A**), 48 h (**B**), and 72 h (**C**)). *Sema3A* inhibits HUVEC proliferation in general culture medium and under TGF-β-stimulated conditions. The experiment was performed in six wells and was repeated at least three times. The data are presented as the means±SEM. *p<0.05; **p<0.01.

The numbers of cells that passed through the membrane in the *Sema3A*-treated (250 ng/ml and 500 ng/ml) groups were significantly lower than that in the control group (1% FBS; Figure 3A,B,D,E, ** p<0.01, *** p<0.0001). In addition, for the exogenous TGF-β-stimulated groups, *Sema3A* also inhibited the crossing of HUVECs, as revealed by counting the DAPI-staining HUVEC cells in a Transwell study (Figure 3A,C,F,G, *** p<0.0001).

Sema3A induced HUVEC apoptosis: Flow cytometry was used to evaluate the effects of *Sema3A* on apoptosis (early apoptosis and late apoptosis). FITC-Annexin V staining was measured at 24, 48, and 72 h after *Sema3A* (250 ng/ml, 500

ng/ml) treatment. As shown in Figure 4, HUVEC apoptosis was induced in both *Sema3A*-treated groups as well as in the TGF-β-stimulated groups compared with the control groups (Figure 4, *p<0.05, ** p<0.01).

Sema3A inhibited TGF-β-induced HUVEC tube formation: A tube formation study is a convenient and quantifiable assay for testing the angiogenic properties of compounds on vascular endothelial cells, and the Matrigel assay is one of the most widely used methods for evaluating the angiogenic ability of endothelial cells in vitro. HUVECs had an impaired capacity to form a regular network when exposed to *Sema3A* concentrations of 250 ng/ml and 500 ng/ml (Figure 5) in

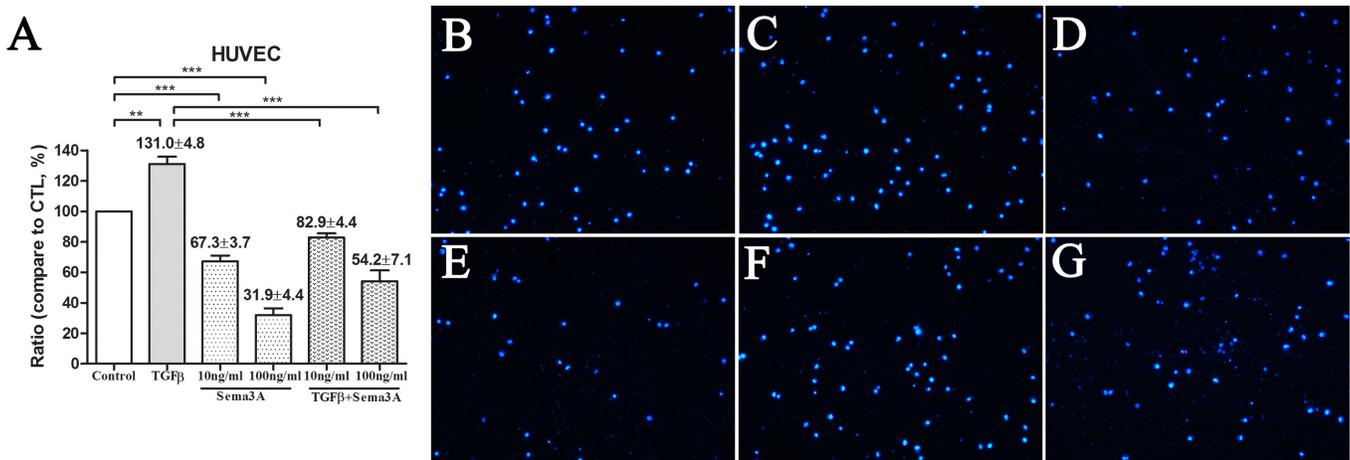


Figure 3. The effect of *Sema3A* on HUVEC migration. The cell nuclei, which were stained with DAPI, are shown as blue dots. The cells from five random fields were counted, and the average was used for the statistical analysis. **A**: Statistical analysis of the HUVEC migration study. *Sema3A* inhibits HUVEC migration in the general culture medium and in VEGF-stimulated conditions. **B**: 1% FBS-treated group. **C**: TGF-β-treated group. **D**: *Sema3A* (250 ng/ml)-treated group; **E**: *Sema3A* (500 ng/ml)-treated group. **F**: *Sema3A* (250 ng/ml)- and TGF-β-treated groups. **G**: *Sema3A* (500 ng/ml)- and TGF-β-treated groups. CTL represents the control group. *p<0.05; **p<0.01; ***p<0.0001.

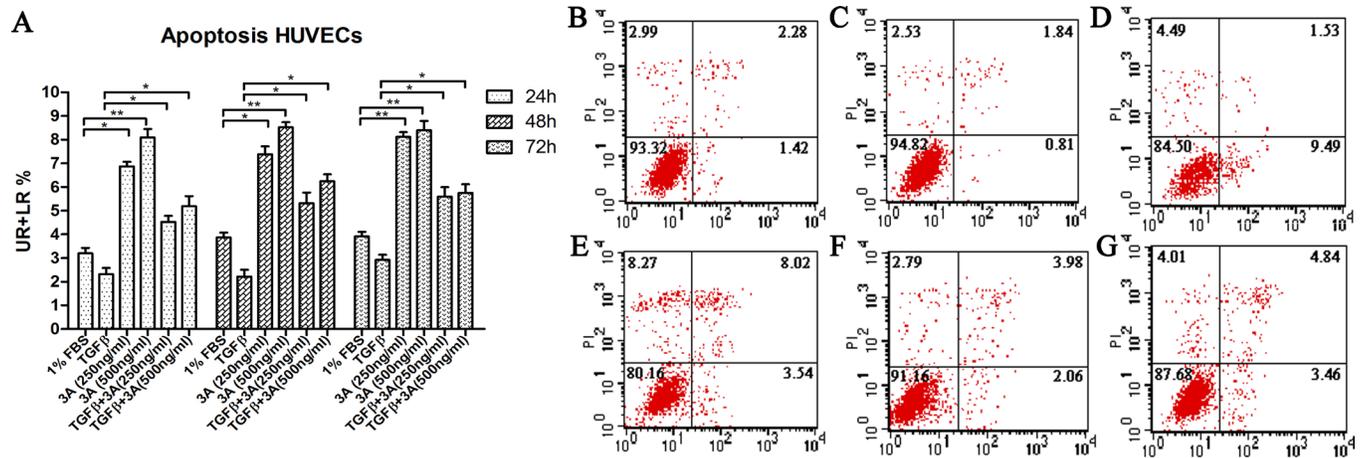


Figure 4. Effect of Sema3A on HUVEC apoptosis. Sema3A induced HUVEC apoptosis in the general culture medium and in the TGF-β-stimulated conditions. FITC-Annexin V staining was measured at the treatment time points of 24, 48, and 72 h. **A**: Statistical analysis of the HUVEC apoptosis study. **B**: Representative image of the 1% FBS-treated group. **C**: Representative image of the TGF-β-treated group. **D**: Representative image of the Sema3A (250 ng/ml)-treated group. **E**: Representative image of the Sema3A (500 ng/ml)-treated group. **F**: Representative image of the Sema3A (250 ng/ml)- and TGF-β-treated groups. **G**: Representative image of the Sema3A (500 ng/ml)- and TGF-β-treated groups. UR=early apoptotic cells; LR=late apoptotic cells; UR+LR=total apoptotic cells. The experiment was repeated at least three times. The data are presented as the means±SEM. *p<0.05; **p<0.01.

either the general culture medium or the TGF-β-containing medium, and the lengths of the angiogenesis network differed significantly from those of the controls (Figure 5A, *p<0.05, ** p<0.01).

Sema3A inhibited the response of HUVECs to TGF-β: The concentration of free TGF-β1 was evaluated with an ELISA assay. No increase in secreted TGF-β was observed for the Sema3A-treated HUVEC groups (Figure 6, Figure

3D (250 ng/ml) and 3E (500 ng/ml) groups) under general culture conditions; however, when the Sema3A-treated HUVEC groups were also stimulated by TGF-β (10 ng/ml), the detected TGF-β level increased significantly (Figure 6, TGF-β+3A (250 ng/ml) and TGF-β+3A (500 ng/ml)). The results indicated that Sema3A did not affect TGF-β secretion; instead, Sema3A inhibited the response to exogenous TGF-β and impeded the function of TGF-β in HUVECs.

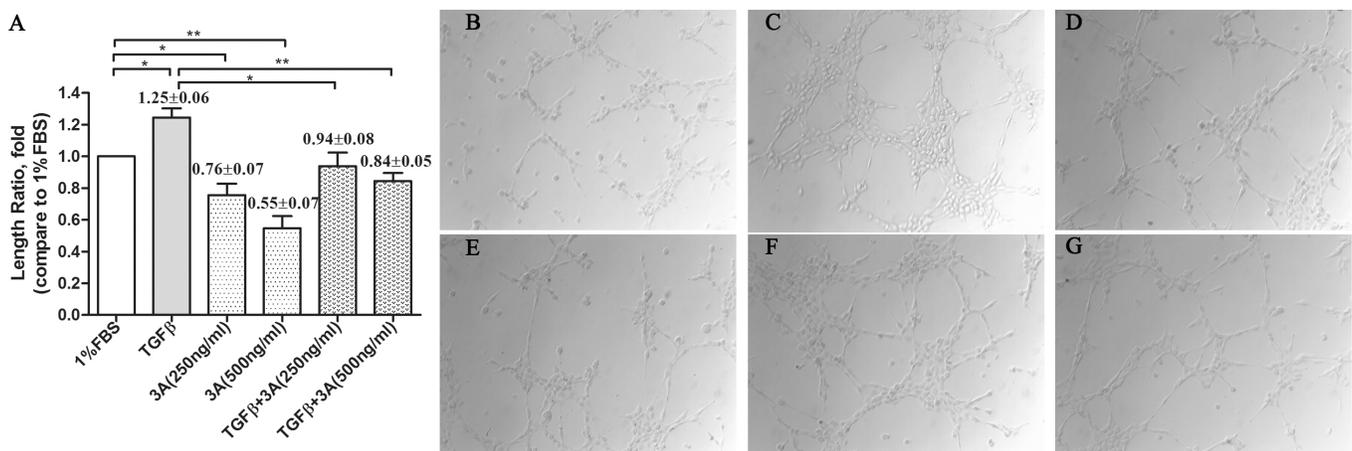


Figure 5. Effect of Sema3A on HUVEC tube formation. The Matrigel assay was used to evaluate the angiogenic effect in Sema3A-treated HUVECs. This assay measures the ability of endothelial cells to form capillary-like structures. **A**: Statistical analysis of the HUVEC tube formation study. **B**: 1% fetal bovine serum (FBS)-treated group. **C**: TGF-β culture group. **D**: Sema3A (250 ng/ml)-treated group. **E**: Sema3A (500 ng/ml)-treated group. **F**: Sema3A (250 ng/ml)- and TGF-β-treated groups. **G**: Sema3A (500 ng/ml)- and TGF-β-treated groups. All of the pictures (panel **B** to **G**) were taken at 10× magnification under a Zeiss light microscope. All of the data were measured at least three times and are presented as the means±SEM. *p<0.05; **p<0.01.

TGFβ in HUVECs

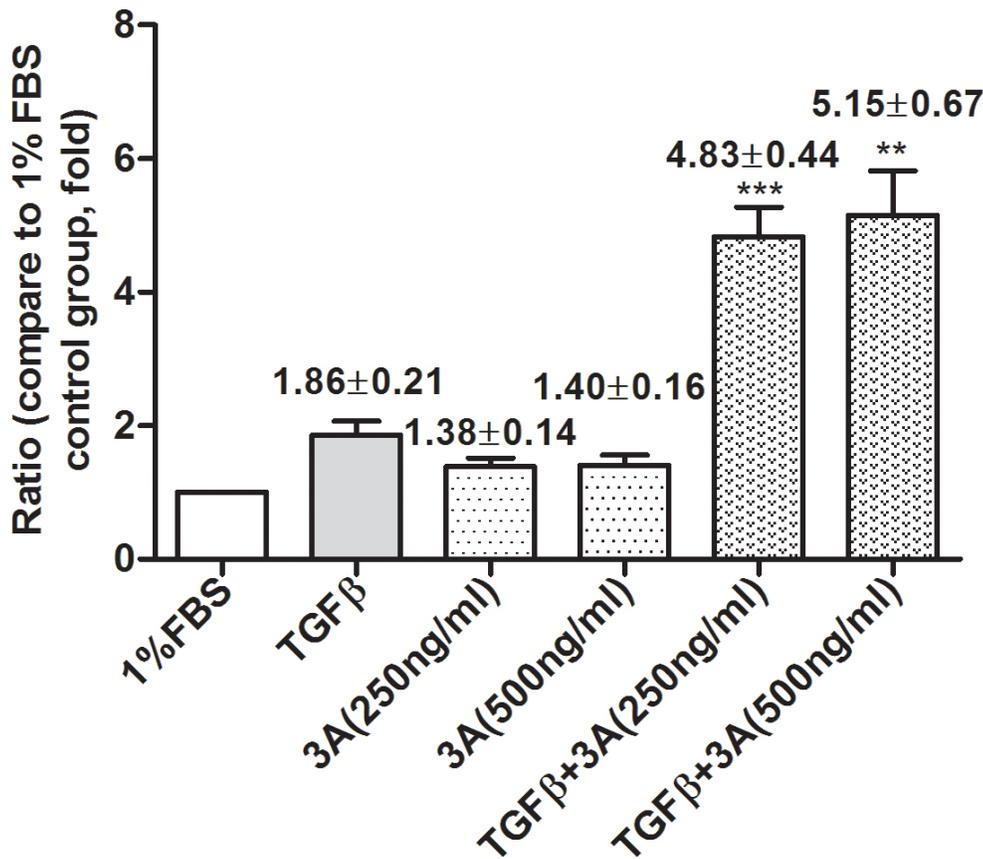


Figure 6. The TGF-β concentration in *Sema3A*-treated HUVECs. The concentration of free TGF-β1 in the supernatant was measured using an ELISA kit. The statistical analysis of the free TGF-β measured with ELISA at 48 h is presented. *Sema3A* inhibited the response of TGF-β. Each experiment was performed in six wells and was repeated at least three times. The data are presented as the means ± SEM. **p < 0.01; ***p < 0.0001.

TGF-β was upregulated in the laser-induced CNV mouse model: The TGF-β concentration in the retina-choroid complexes was measured using ELISA. Fourteen days after laser-induced CNV lesions, real-time PCR (Figure 7A, **p < 0.01) and western blot (Figure 7B,C, *p < 0.05) studies indicated that TGF-β was upregulated. These results are

consistent with our finding that the amount of TGF-β is increased in the vitreous bodies of patients with nAMD.

Sema3A inhibited CNV formation in mice: To evaluate the effects of *Sema3A* in the CNV mouse model, CNV leakage in the retina after *Sema3A* treatment was assessed with FA. On

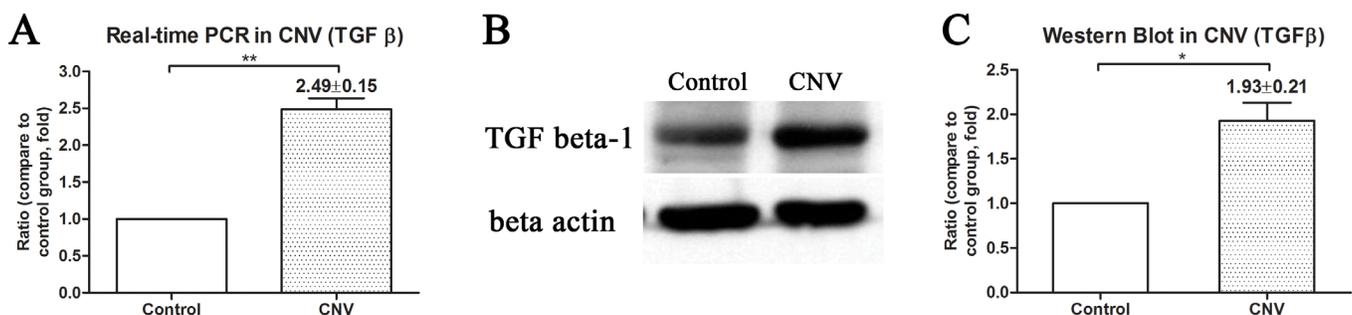


Figure 7. TGF-β expression in the CNV mouse model. **A:** The expression of TGF-β in a real-time PCR array. TGF-β expression is upregulated significantly 14 days after the CNV lesion. **B:** The immunoblot image shows that TGF-β is overexpressed. **C:** The statistical analysis of the western blot results. The real-time PCR and western blot analyses were repeated three times, and qualitatively similar results were obtained. The data are presented as the means ± SEM. *p < 0.05; **p < 0.01.

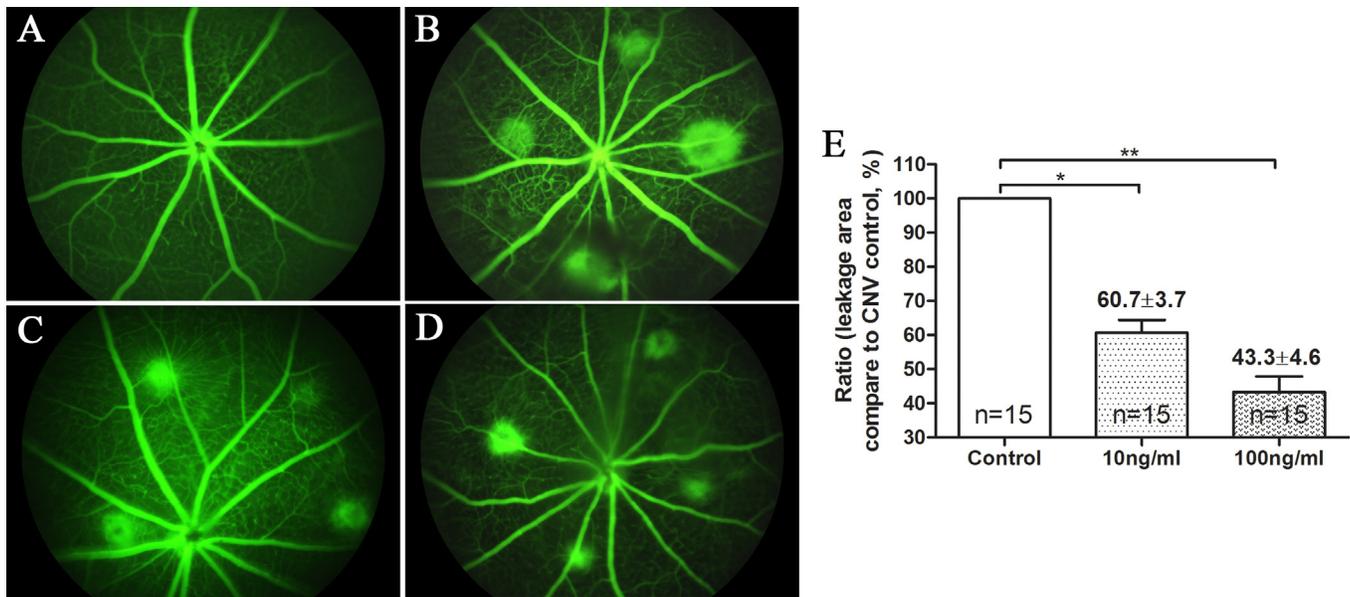


Figure 8. CNV leakage with FA assessment of the CNV mouse retina. Angiographic analysis of CNV leakage 14 days after laser photocoagulation in the different treatment groups and in the control group. **A:** The normal control. **B:** The CNV control. **C:** The Sema3A (10 ng/ml)-treated group. **D:** The Sema3A (100 ng/ml)-treated group. **E:** The results and the statistical analysis of the CNV leakage by FA. The data are presented as the means±SEM. * $p<0.05$; ** $p<0.01$.

day 14 after laser-induced CNV, hyperfluorescence leakage was observed at the lesion sites in the mice. In the Sema3A treatment group, the leakage was significantly reduced compared with that in the CNV control group (Figure 8, * $p<0.05$, ** $p<0.01$), which indicated that Sema3A could inhibit the formation of laser-induced CNV in mice.

Sema3A inhibited the response of retina-choroid complexes to VEGF₁₆₅ and TGF- β : VEGF₁₆₅ and TGF- β are important growth signals in CNV, and the Sema3A receptor (Nrp-1) is known to be the coreceptor of VEGF₁₆₅ and TGF- β . As shown in Figure 9, the VEGF₁₆₅ and TGF- β levels are upregulated after intravitreal injection of Sema3A, indicating that Sema3A inhibited the uptake of these two important growth factors (Figure 9, * $p<0.05$, ** $p<0.01$).

Sema3A reduced SMAD2/3 and p-SMAD2/3 expression in HUVECs and in mouse CNV retina-choroid complexes: SMAD2/3 and p-SMAD2/3 expression in HUVECs and in mouse CNV retina-choroid complexes was assessed with western blot assays. Exposure to TGF- β significantly increased SMAD2/3 and p-SMAD2/3 expression, whereas exposure to Sema3A partially inhibited the effects of TGF- β on SMAD2/3 and p-SMAD2/3 upregulation in HUVECs (Figure 10A–C, * $p<0.05$, ** $p<0.01$). In the CNV mouse model, SMAD2/3 and p-SMAD2/3 were upregulated in the retinas, whereas Sema3A partially inhibited their expression (Figure 10D–F, ** $p<0.01$).

Sema3A reduced the expression of p-p38 MAPK, p-ERK1/2, and p-JNK in the mouse retina-choroid complexes: In addition to the evaluation of SMAD2/3 and p-SMAD2/3 expression in mouse retina-choroid complexes, p-p38 MAPK, p-ERK1/2, and p-JNK expression was measured. After Sema3A treatment in the CNV model, all signaling pathways were partially inhibited (Figure 11, * $p<0.05$, ** $p<0.01$).

DISCUSSION

CNV is a complex process that develops in several retinal diseases, leading to significant vision loss [3,23]. Although the molecular details underlying the formation and progression of pathogenetic CNV remain to be fully elucidated, recent studies suggested the dynamic balance between positive and negative regulators is a key step [24,25]. Positive regulators of CNV include VEGF, angiopoietins, TGF- β , stromal-derived factor-1 (SDF-1), basic fibroblast growth factor (b-FGF), and other inflammation factors, such as interleukins and chemokines, whereas the negative regulators of CNV include pigment epithelium-derived factor (PEDF), tissue inhibitors of matrix metalloproteinases (TIMPs), and endostatin [23,26,27]. Considerable evidence indicates that VEGF, which stimulates endothelial cell survival, proliferation, migration, and tube formation, may be the single most important causal agent of angiogenesis in CNV [28,29]. Currently, VEGF antagonists, such as ranibizumab and

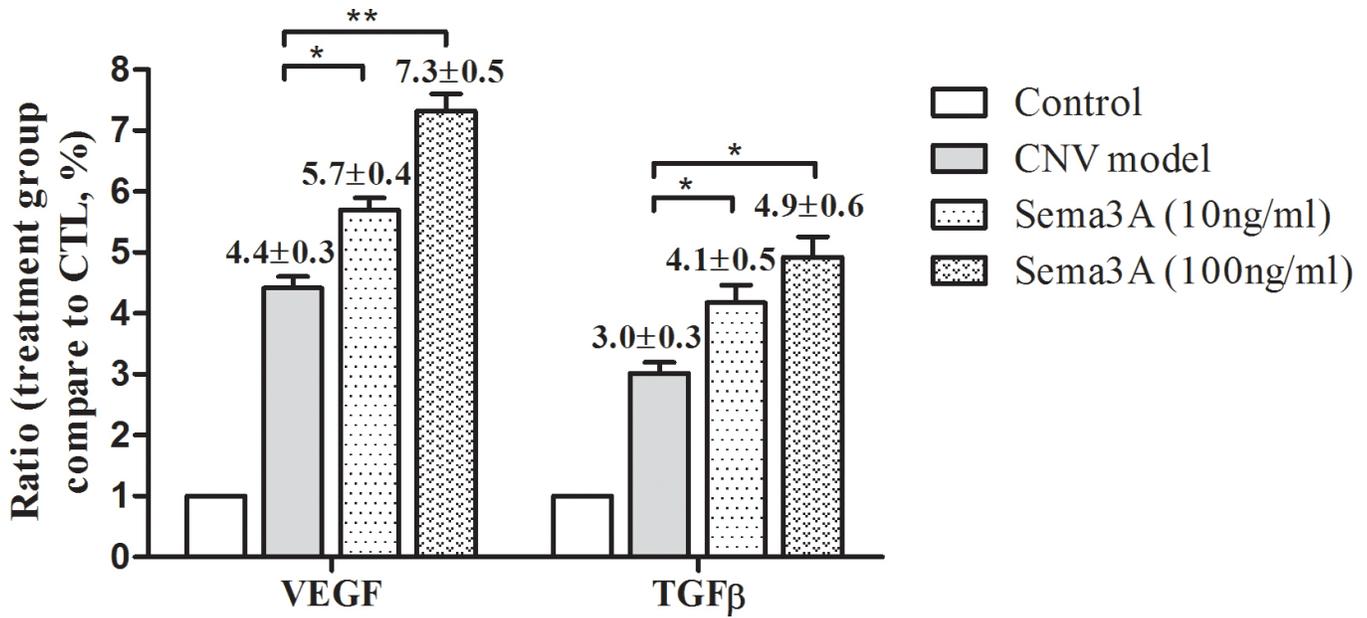


Figure 9. The VEGF and TGF-β concentrations in Sema3A-treated retina-choroid complexes. The VEGF and TGF-β1 concentrations in the retina-choroid complexes were measured using an ELISA kit. The statistical analysis of the free VEGF and TGF-β measured at 14 days after Sema3A treatment is presented. Each experiment was repeated at least three times. The data are presented as the means±SEM. *p<0.05; **p<0.01.

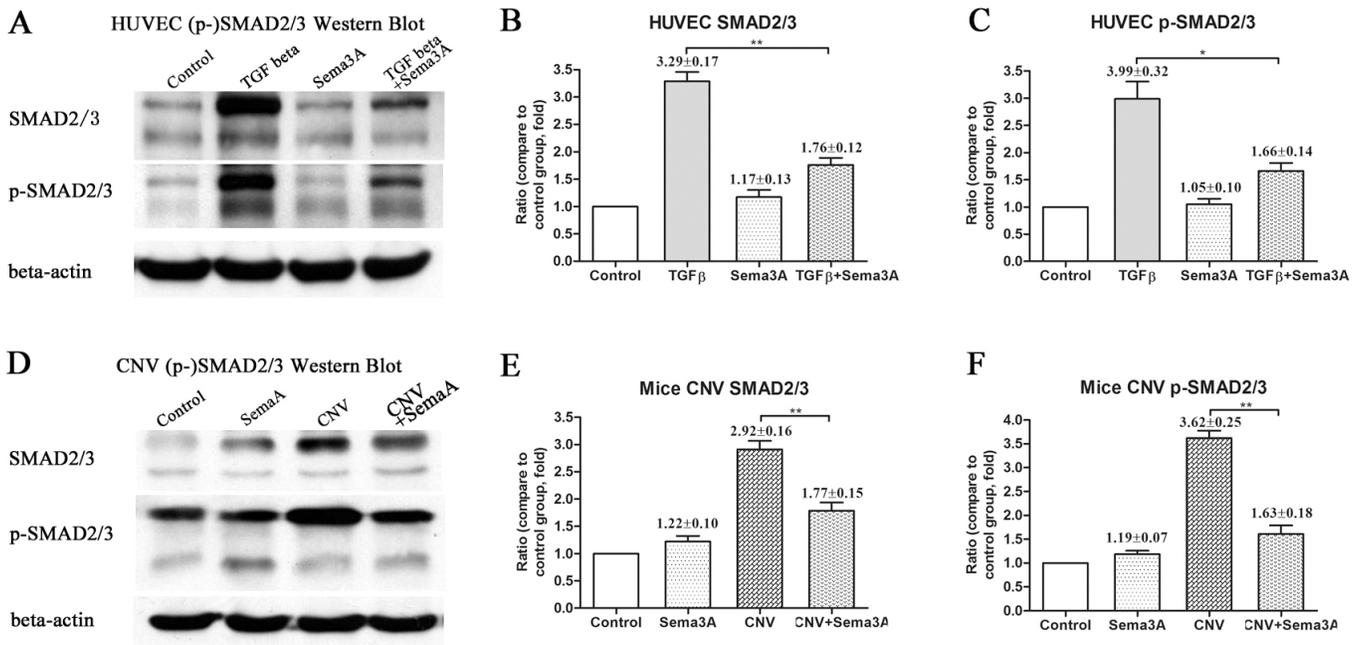


Figure 10. Effect of Sema3A on SMAD2/3 and phosphorylated SMAD2/3 in HUVECs and in the CNV mouse model. Immunoblot images (A, D) and statistical analyses (B, C, E, and F) for the SMAD2/3 and p-SMAD2/3 signaling pathways. The results show that Sema3A inhibits SMAD2/3 and p-SMAD2/3 in HUVECs and in the CNV mouse model. The western blot analyses were repeated three times, and qualitatively similar results were obtained. The data are presented as the means±SEM. *p<0.05; **p<0.01.

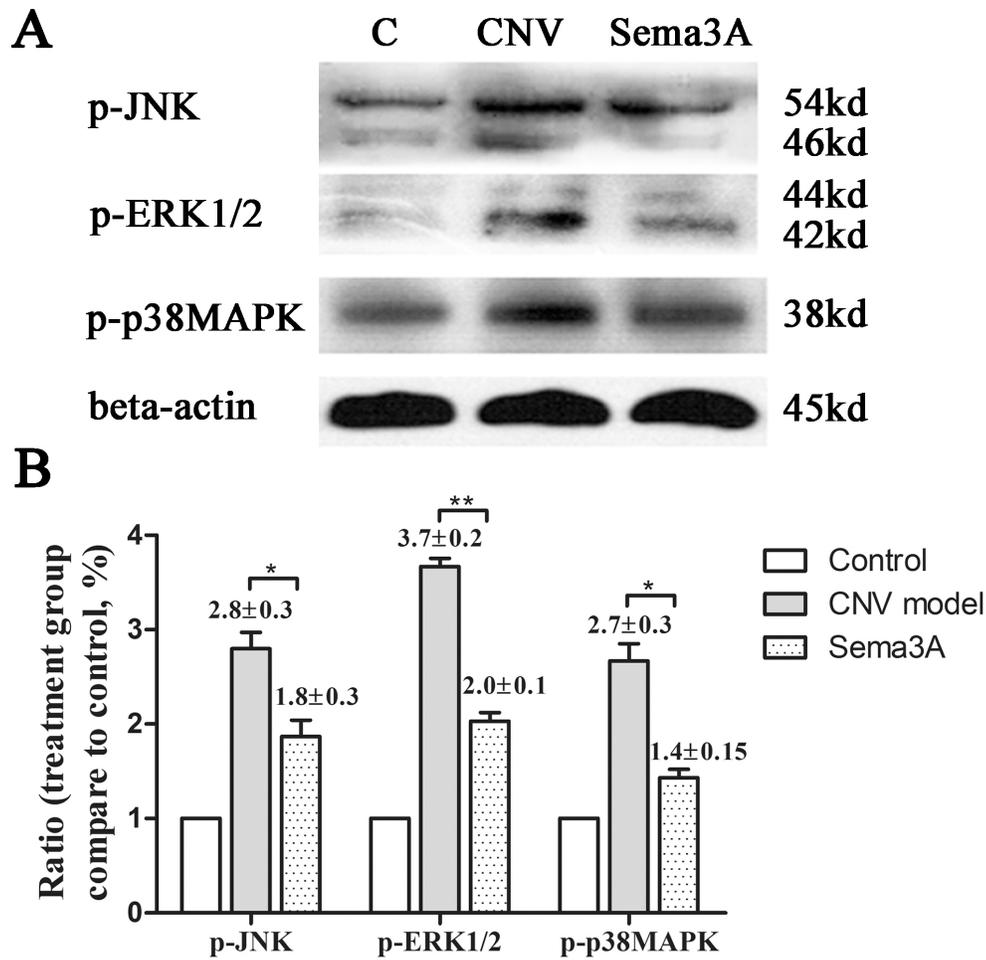


Figure 11. Effect of Sema3A on phosphorylated p38 MAPK, ERK1/2, and JNK in the CNV mouse model. Immunoblot (A) and statistical (B) analyses of the p-p38 MAPK, p-ERK1/2, and p-JNK signaling pathways. The results show that Sema3A inhibits p-p38 MAPK, p-ERK1/2, and p-JNK in the CNV mouse model. The western blot analyses were repeated three times, and qualitatively similar results were obtained. The data are presented as the means±SEM. *p<0.05; **p<0.01.

Aflibercept, are used as the standard treatment for nAMD and have been found to be a valuable adjunctive treatment strategy for several other vascular retinal diseases [28]. However, deeper investigations of the mechanisms underlying CNV have suggested the importance of treatment strategies that target other angiogenic factors in addition to VEGF and inflammatory cytokines [30,31]. Among these factors, TGF-β is a potential target for treating CNV [32].

TGF-β has been identified as a powerful pleiotropic growth factor that regulates cell growth, differentiation, apoptosis, cell motility, extracellular matrix production, and cellular immune responses during angiogenesis, wound healing, joint inflammation, tumor growth and metastasis, and immunoregulation [5,9,33]. Downstream TGF-β signaling occurs through canonical and noncanonical pathways. The canonical pathway, which is also called the SMAD pathway, involves three signaling components (type I (RI), type II (RII), and type III (RIII)). After RIII binds to TGF-β, RII is recruited and then phosphorylates RI. Subsequently, RI phosphorylates Smad2 and Smad3, which form a

heteromeric complex with Smad4. This complex translocates to the nucleus, binds to DNA, and regulates the transcription of many genes [34]. TGF-β receptors also signal through multiple noncanonical pathways, including the JNK/p38 MAPK, ERK1/2, and PI3K/Akt pathways [35].

Accumulating evidence indicates that TGF-β is essential for angiogenesis [5,36]. Knockout studies of factors in TGF-β signaling have shown that the TGF-β pathway is indispensable for angiogenesis [37,38]. A neutralizing antibody against TGF-β strongly inhibits angiogenesis in vitro and in vivo [36]. Additionally, TGF-β plays an important role during CNV formation and progression [16]. Recently, studies have reported that TGF-β is present in the retinal membranes of patients with CNV [10]. Other studies have demonstrated that TGF-β significantly enhances VEGF upregulation on its own or in concert with other cytokines in RPE cells and exerts a strong effect on extracellular matrix remodeling [23,35,37]. In addition to stimulating angiogenesis, TGF-β recruits inflammatory cells, which, in turn, initiate other proangiogenic cytokine-release cascades [39]. In the later phase of CNV,

fibroblasts become more proliferative, leading to scar tissue formation, in which collagen remodeling and scar contraction are partially mediated by TGF- β [25]. According to these findings, the multiple roles played by TGF- β in the different stages of CNV development and progression led us to hypothesize that TGF- β inhibition could be an adjunctive therapeutic strategy for anti-VEGF treatment.

In the present study, we first verified that TGF- β is highly expressed in patients with nAMD (Figure 1) and in a CNV mouse model (Figure 7); this expression further confirmed the importance of TGF- β in CNV. Then, we showed that *Sema3A* not only inhibited TGF- β -induced HUVEC proliferation (Figure 2), migration (Figure 3), and tube formation (Figure 5) but also induced TGF- β -stimulated HUVEC apoptosis (Figure 4). In vivo, *Sema3A* inhibited laser-induced CNV formation (Figure 8) and impeded the response to VEGF and TGF- β (Figure 9). In the in vitro and in vivo studies, *Sema3A* inhibited the downstream SMAD2/3 signaling pathway (Figure 10) and inhibited p38 MAPK, JNK, and ERK1/2 phosphorylation (Figure 11). The mechanism underlying these effects could be that *Sema3A* impedes the VEGF and TGF- β responses by competitively inhibiting Nrp-1 (Figure 7, Figure 9) and by inhibiting their signaling pathways (Figure 10, Figure 11).

Nrps are multifunctional proteins involved in the development and progression of angiogenesis [40]. Nrp1, which is primarily a receptor for class 3 semaphorins (*Sema3A*), is involved in axonal guidance and angiogenesis [16,40]. Additionally, Nrp1 is a coreceptor that enhances the responses to integrins and several growth factors as well as their receptors, such as TGF- β and its receptors and hepatocyte growth factor and its receptor [18]. These ligands and their signaling pathways are all relevant to angiogenesis. Nrp1 might affect angiogenesis through mechanisms including capturing ligands; regulating receptor expression, endocytosis, and recycling; or possibly even independent signaling [21]. Nrp-1 has been reported to modulate the response to TGF- β 1 by binding active and inactive TGF- β [18]. Prud'homme reported that Nrp1 interacts with the TGF- β signaling receptors (RI and RII) and enhances canonical Smad2/3 signaling in response to TGF- β [16,22]. We know that receptor activation is a key factor in regulating the response to TGF- β . Thus, competitive inhibition of the activating factor will significantly inhibit its downstream signaling.

Previously, we verified that *Sema3A* could be a useful therapeutic strategy for preventing hypoxia/ischemic-induced retinal neovascularization by inhibiting the response to VEGF [20], and our results are comparable to those reported by Joyal [41]. We also demonstrated that *Sema3A* could inhibit

VEGF-induced RPE proliferation and proliferative vitreoretinopathy in a rabbit model [19]. In the present study, we showed that *Sema3A* could inhibit laser-induced CNV at least partially by inhibiting TGF- β . However, *Sema3A* could not inhibit VEGF or TGF- β that was already bound to their receptors. This inability to inhibit bound VEGF or TGF- β will be a limitation for the clinical use of *Sema3A* in already formed CNV. In addition, the mechanisms that underlie the inhibitory effects of *Sema3A* in angiogenesis must be explored in more detail. As Joyal et al. reported, *Sema3A* contributes to vascular decay. Conversely, deleting *Sema3A* enhances normal vascular regeneration, thus diminishing aberrant neovascularization [41]. Although distinct mechanisms have been proposed in different studies, according to the present study, we have good reason to believe that *Sema3A* will be a useful therapeutic strategy or an adjunctive treatment strategy for treating pathological CNV.

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