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Research Paper

# Sema3f Protects Against Subretinal Neovascularization In Vivo

Ye Sun [a], Raffael Liegl [a], Yan Gong [a], Anima Bühler [b], Bertan Cakir [b], Steven S. Meng [a], Samuel B. Burnim [a], Chi-Hsiu Liu [a], Tristan Reuer [b], Peipei Zhang [b], Johanna M. Walz [b], Franziska Ludwig [b], Clemens Lange [b], Hansjürgen Agostini [b], Daniel Böhringer [b], Günther Schlunck [b], Lois E.H. Smith [a], Andreas Stahl [b,*]

[a] Department of Ophthalmology, Harvard Medical School, Boston Children's Hospital, 300 Longwood Ave, Boston, MA 02115, USA
[b] Eye Center, Medical Center, Faculty of Medicine, University of Freiburg, Killianstrasse 5, 79106 Freiburg, Germany

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ABSTRACT

Pathological neovascularization of the outer retina is the hallmark of neovascular age-related macular degeneration (nAMD). Building on our previous observations that semaphorin 3F (Sema3f) is expressed in the outer retina and demonstrates anti-angiogenic potential, we have investigated whether Sema3f can be used to protect against subretinal neovascularization in two mouse models. Both in the very low-density lipid-receptor knockout (*Vldlr*$^{-/-}$) model of spontaneous subretinal neovascularization as well as in the mouse model of laser-induced choroidal neovascularization (CNV), we found protective effects of Sema3f against the formation of pathologic neovascularization. In the *Vldlr*$^{-/-}$ model, AAV-induced overexpression of Sema3f reduced the size of pathologic neovascularization by 56%. In the laser-induced CNV model, intravitreally injected Sema3f reduced pathologic neovascularization by 30%. Combined, these results provide the first evidence from two distinct in vivo models for a use of Sema3f in protecting the outer retina against subretinal neovascularization.


## 1. Introduction

Age-related macular degeneration (AMD) is one of the leading causes of visual impairment and legal blindness in industrialized countries (Bressler, 2004; Finger et al., 2011). Especially the neovascular form of AMD (nAMD) can lead to substantial vision loss within months after onset if left untreated. Anti-vascular endothelial growth factor (VEGF) therapy introduced in 2006 can slow down disease progression and restore vision. However, long term clinical data demonstrate that despite continuous anti-VEGF therapy up to one third of patients will lose more than three lines of visual acuity over 5–7 years despite continuous anti-VEGF therapy (Wecker et al., 2016; Rofagha et al., 2013). In addition, the CATT study provided evidence for a continuous growth of the subretinal neovascular lesion area in AMD patients despite anti-VEGF therapy (Comparison of Age-related Macular Degeneration Treatments Trials Research et al., 2016). These data from clinical long-term studies clearly demonstrate the need for novel treatment approaches that are additive or supplementary to anti-VEGF treatment in nAMD.

Variation in patient responses to anti-VEGF therapy has been seen in clinical trials. One of the reasons why some patients do not respond to anti-VEGF therapy may be that nAMD subtypes differ in their response to anti-VEGF treatment (Gulat-Marnay et al., 1989; Sulzbacher et al., 2017; Ying et al., 2013). Retinal angiomatous proliferations (RAP) for example are lesions that originate from the deep retinal vasculature and progress towards the retinal pigment epithelium (RPE) to form subretinal neovascular membranes. This is different from the classic choroidal neovascularization (CNV) phenotype in nAMD with subretinal neovascularization originating from the choroid, not the retinal vasculature. In both instances, however, the end result is similar: the normally avascular outer retinal space becomes compromised by invading pathological blood vessels.

Semaphorins, also known as collapsins, were first identified as a family of genes encoding guidance molecules for the embryologic development of the nervous system (Gaur et al., 2009; Luo et al., 1993). The Semaphorin class 3 consists of seven soluble proteins of ~100 kDa (designated by the letters a–g), which are secreted by cells of multiple lineages, including epithelial cells, neurons, and specific tumor cells (Gaur et al., 2009). We have previously demonstrated that Sema3f is physiologically expressed in the outer retina while Sema3a is predominantly induced in the inner retina under hypoxic conditions (Buehler et al., 2013). We and others have also demonstrated an angiomodulatory role for semaphorins and their neuropilin (Nrp) and plexin receptors

* Corresponding author at: Eye Center, Medical Center, Faculty of Medicine, University of Freiburg, Killianstrasse 5, 79106 Freiburg, Germany.
*E-mail address:* andreas.stahl@uniklinik-freiburg.de (A. Stahl).

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(Fukushima et al., 2011; Joyal et al., 2011; Soker et al., 1998). In this study, we investigated whether modulation of Sema3f in the outer retina alters the formation of pathological subretinal neovascularization in two different in vivo models.

The very low-density lipoprotein receptor knockout mouse (*Vldlr*$^{-/-}$) is an established model to study the development of spontaneous subretinal neovascular lesions originating from the deep retinal vascular plexus. These lesions are comparable to RAP in human patients (Grossniklaus et al., 2010; Heckenlively et al., 2003). Interestingly, we found in this study that *Vldlr*$^{-/-}$ retinas have reduced expression levels of Sema3f. Our results show that restoring Sema3f in these mice using an AAV approach significantly reduces both the number and size of subretinal neovascular lesions. In our second in vivo model, laser-induced CNV membranes originate from the choroidal circulation and thus resemble classic CNVs from human patients with nAMD. Our results show that in this model, the intravitreal injection of recombinant Sema3f protein significantly reduces the size of subretinal neovascular membranes following laser photocoagulation.

Combined, these data provide evidence that Sema3f levels can be modulated to protect the physiologically avascular outer retina from invasion by retinal as well as choroidal pathological neovascularization.

## 2. Materials and Methods

### 2.1. Animals

All animal studies were performed according to protocols approved by the Institutional Animal Care and Use Committee at the Boston Children's Hospital and the University of Freiburg Medical Center. *Vldlr*$^{+/-}$ (heterozygous) mice from Jackson Laboratory (Stock #002529) were bred to generate homozygous and wild type littermates. Wild type C57BL/6J mice from Charles River were used for laser CNV experiments.

### 2.2. Preparation of AAV2 Virus

AAV2 vector expressing mouse *Sema3f* cDNA (accession number: BC010976), which was cloned into a pAAV2-CMV-MCS vector driven by CMV promoter, was provided by SIRION Biotech (Cat. SB-S-AA-102-03). pAAV2-CMV-MCS vector without *Sema3f* cDNA was used as control. Recombinant AAV2 vectors were produced as previously described (Grieger et al., 2006; Vandenberghe et al., 2010). Briefly, AAV vector, rep/cap packaging plasmid, and adenoviral helper plasmid were mixed with polyethylenimine (Sigma) and transfected into HEK293T cells (catalog HCL4517; Thermo Scientific). Sixty hours after transfection, cells were harvested and the cell pellet was resuspended in virus buffer, followed by three cycles of freeze-thaw, and homogenization (Dounce). Cell debris was pelleted at 5000*g* for 20 min, and the supernatant was run on an iodixanol gradient. Recovered AAV vectors were washed three times with PBS using Amicon 100 K columns (EMD Millipore). Real-time PCR was used to determine genome titers of the recombinant AAV. This protocol also was used to prepare a control (AAV2-shControl). Viruses were diluted to various concentrations to test infection, and a concentration of approximately $2 \times 10^{12}$ gc/ml was used for the experiments.

### 2.3. Subretinal Injection

Subretinal injection into *Vldlr*$^{-/-}$ P0 neonate eyes was performed as previously described (Matsuda and Cepko, 2004; Wang et al., 2014; Xiong et al., 2015) under a dissection microscope. P0 *Vldlr*$^{-/-}$ or wild type pups were anesthetized on ice for several minutes. The eyelid was prepped with Betadine, followed by water, then 70% ethanol using cotton swabs. A blade was used to gently cut open the eyelid. The pulled angled glass pipette was inserted into the subretinal space. Approximately 0.5 μl solution containing AAV2-control or AAV2-*Sema3f* ($2 \times 10^{12}$ gc/ml) was introduced into the subretinal space and the injection volume was controlled through a micro-injector (FemtoJet, Eppendorf). After injection, curved forceps were used to slowly close the eyelid. Mice were placed on a circulating water blanket for warmth. The retinas were collected at P12 for PCR assay and P16 for whole mount analysis.

### 2.4. Laser-Induced CNV, Intravitreal Injection and Quantification

Argon laser treatment was performed as previously described (Ogata et al., 1997). In brief, animals were anesthetized and the pupils were dilated. Care was taken to use mice of the same age (6–8 weeks). The animals were positioned in front of an argon laser (VISULAS 532s, Zeiss). Settings for laser coagulation were spot size 100 μm, emission time 100 ms and laser energy 150 mW at emission wavelength 532 nm. Three laser burns were applied per eye. The development of a small white bubble as a sign for Bruch's membrane rupture was required for a sufficient laser burn (Tobe et al., 1998). Intravitreal injection was performed using a Hamilton syringe with 33G canula. Directly following laser, eyes were injected with 1 μl recombinant Sema3f (100 ng) or PBS control under microscopic visual control. On day 14 after laser photocoagulation, eyes were enucleated, fixed in 4% PFA for 30 min and then transferred to PBS. Choroid was dissected and the choroidal whole mount was mounted onto slides (Thermo Fisher Scientific) with the scleral side down in SlowFade anti-fade mounting medium (Life Technologies). After staining with isolectin IB4, CNV lesion size was quantified in a blinded fashion using the ImageJ software.

### 2.5. Quantification of Subretinal Neovascularization in the *Vldlr*$^{-/-}$ Model

Neovascularization analysis in the *Vldlr*$^{-/-}$ mice was performed as described (Stahl et al., 2009; Sun et al., 2015). The whole mounts of retinas from *Vldlr*$^{-/-}$ and wild type were stained with isolectin IB4 and imaged using Zeiss AxioObserver.Z1 microscope with a monochrome digital Zeiss camera AxioCam MRm focusing on the terminal end of lesions on the RPE layer (usually at P16), and individual images were merged to create one whole retinal image using automated merge function (mosaiX; Zeiss) in the software AxioVision 4.6.3.0 (Zeiss). ImageJ (National Institutes of Health, http://imagej.nih.gov/ij/) was used for quantification of subretinal neovascularization lesion number and area in *Vldlr*$^{-/-}$ retinas with designed plugins adapted from the method used to measure retinal neovascularization (SWIFT_NV) in the OIR model (Stahl et al., 2009) which use a user-designated threshold to mark lesion structures that clearly stand out from background fluorescence of normal vessels, and can automatically remove small artifacts by selecting objects with a minimum size of 100 pixels. Other larger artifacts such as occasional cellular debris or retinal periphery with hyperfluorescence can be manually excluded from quantification. Lesion numbers and areas were quantified with researchers masked to the identity of samples.

### 2.6. Confocal Imaging and 3D Reconstruction

Eyes were enucleated from *Vldlr*$^{-/-}$ mice at P16 and fixed in 4% paraformaldehyde followed by dissection and staining of the retinas with fluoresceinated isolectin IB4 (Invitrogen) to visualize subretinal neovascularization in whole mounted retinas. 3D reconstructed images were taken with confocal microscopy (Leica TCS SP2 AOBS) and z-stacks were 3D reconstructed using Volocity software (Perkin Elmer) as described (Sun et al., 2015).

### 2.7. Hematoxylin and Eosin Staining

Slides were washed in xylene and dehydrated in absolute ethanol, 95% and 70% alcohol, PBS respectively. Harris hematoxylin solution

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