Differential Endothelial Transcriptomics Identifies Semaphorin 3G as a Vascular Class 3 Semaphorin

Simone Kutschera, Holger Weber, Anja Weick, Frederik De Smet, Guillem Genove, Minoru Takemoto, Claudia Prahst, Maria Riedel, Constantinos Mikelis, Sylvain Baulande, Catherine Champseix, Petra Kummerer, Emmanuel Conseiller, Marie-Christine Multon, Melanie Heroult, Roy Bicknell, Peter Carmeliet, Christer Betsholtz, Hellmut G. Augustin

Objective—To characterize the role of a vascular-expressed class 3 semaphorin (semaphorin 3G [Sema3G]).

Methods and Results—Semaphorins have been identified as axon guidance molecules. Yet, they have more recently also been characterized as attractive and repulsive regulators of angiogenesis. Through a transcriptomic screen, we identified Sema3G as a molecule of angiogenic endothelial cells. Sema3G-deficient mice are viable and exhibit no overt vascular phenotype. Yet, LacZ expression in the Sema3G locus revealed intense arterial vascular staining in the angiogenic vasculature, starting at E9.5, which was detectable throughout adolescence and downregulated in adult vasculature. Sema3G is expressed as a full-length 100-kDa secreted molecule that is processed by furin proteases to yield 95- and a 65-kDa Sema domain–containing subunits. Full-length Sema3G binds to NP2, whereas processed Sema3G binds to NP1 and NP2. Expression profiling and cellular experiments identified autocrine effects of Sema3G on endothelial cells and paracrine effects on smooth muscle cells.

Conclusion—Although the mouse knockout phenotype suggests compensatory mechanisms, the experiments identify Sema3G as a primarily endothelial cell–expressed class 3 semaphorin that controls endothelial and smooth muscle cell functions in autocrine and paracrine manners, respectively. (Arterioscler Thromb Vasc Biol. 2011;31:151-159.)

Key Words: endothelial cell ■ smooth muscle cell ■ angiogenesis ■ semaphorin ■ neuropilin

The growth of new blood vessels (angiogenesis) is associated with a distinct transition of endothelial cells (ECs) from the quiescent growth-arrested state to an invasive, migratory, and proliferating phenotype. The early steps of the angiogenic cascade are initiated by an intricate interplay between the vascular endothelial growth factor (VEGF) and the VEGF receptor and the Notch and Delta systems to control capillary sprout formation.1 Invading capillary sprouts eventually anastomose to form a new capillary network that matures by recruiting mural cells to establish directional blood flow. Neuronal guidance molecules (ephrin-Eph, semaphorin–neuropilin (NP)–plexin, netrin-unc, and slit-robo)2–4 and vessel maturation molecules (angiopoietin/Tie and platelet-derived growth factor–platelet-derived growth factor receptor)5,6 have been identified as key regulators of these successive steps. The cross talk between ECs and periendothelial mural cells (pericytes and smooth muscle cells [SMCs]) is particularly important for controlling the activation status of the vascular endothelium. Mural cells are in intimate contact with the maturing EC monolayer to control their quiescent phenotype and their ability to respond to exogenous cytokines, including angiogenic growth factors.7 Conversely, the loosening of EC/mural cell contacts is an important early step of the angiogenic cascade involving molecules of the transforming growth factor β, platelet-derived growth factor-BB, and angiopoietin families of vasculotropic growth factors.8

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From: F. Weick, R. Bicknell, S. Kutschera, H. Weber, and H. Augustin, German Cancer Research Center Heidelberg (DKFZ-ZMBH Alliance), Heidelberg, Germany; Vascular Biology and Tumor Angiogenesis (S.K., A.W., C.P., M.H., and H.G.A.), Medical Faculty Mannheim (CBTM), Heidelberg University, Heidelberg, Germany; the Department of Vascular Biology and Angiogenesis Research (H.W., P.K., and H.G.A.), Tumor Biology Center, Freiburg, Germany; the Department for Transgene Technology and Gene Therapy (F.D.S. and P.C.), VIB, Leuven, Belgium; the Center for Transgene Technology and Gene Therapy (F.D.S. and P.C.), K. U. Leuven, Leuven, Belgium; the Division of Matrix Biology (G.G., M.T., and C.B.), the Department of Medical Biochemistry and Biophysics, Karolinska Institutet, Stockholm, Sweden; the Oncology Department (S.B., C.C., E.C., and M.-C.M.), Sanofi-Aventis, Paris Research Center, Vitry-sur-Seine, France; Cancer Research UK Angiogenesis Group (R.B.), University of Birmingham, Birmingham, England. Dr Kutschera is now with the University of Uppsala, Uppsala, Sweden; Dr Weber is now with ProQinase GmbH, Freiburg, Germany; Dr Prahst is now with Yale University, New Haven, Conn; Dr Takemoto is now with Chiba University Hospital, Chiba, Japan; Dr Mikelis is now with the National Institutes of Health, Bethesda, Md; Dr Conseiller is now with Genomic Vision, Paris, France; and Dr Heroult is now with Bayer-Schering Pharma, Wuppertal, Germany.

Dr Kutschera and Weber contributed equally to this study.

Correspondence to Hellmut G. Augustin, DVM, PhD, Joint Research Division Vascular Biology, Medical Faculty Mannheim (CBTM), Heidelberg University, and German Cancer Research Center Heidelberg (DKFZ-ZMBH Alliance), Im Neuenheimer Feld 280, D-69120 Heidelberg, Germany. E-mail augustin@angiogenes.de

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Stimulated by the prospect of therapeutically exploiting determinants associated with the angiogenic program, numerous experimental avenues have been pursued to systematically unravel the transcriptome of angiogenic ECs. In the most reductionist approach, the properties of cultured confluent quiescent ECs have been compared with those of subconfluent migrating ECs.9 Correspondingly, microarray analyses of cultured ECs stimulated by angiogenic cytokines have been pursued.10,11 Transcriptomic analyses have also been performed with ECs undergoing capillarylike tube formation in 2D and 3D cellular systems.12,13 Correspondingly, several studies have performed transcriptome analyses of angiogenic ECs in vivo.14,15

To functionally and molecularly mimic the properties of ECs during angiogenesis, our laboratory has developed a spheroid-based 3D cellular angiogenesis assay.16 This assay is based on the focal delivery of EC spheroids of defined cell number (between 400 and 1000 cells each) into a gel matrix. ECs sprout focally from the embedded spheroids to establish an anastomosing network of luminized capillarylike structures. This versatile assay has been adapted for several applications and was also used to establish a novel in vivo angiogenesis assay in which human ECs are grafted as spheroids to establish a complex 3D vasculature in immunodeficient mice.17 The 3D spheroid-based assay appeared to be a suitable cellular representation of the angiogenic cascade. We exploited it for this reason as a starting point for the systematic transcriptomic analysis of angiogenic ECs. The experiments revealed that the transcriptomic analysis of 3D-sprouting ECs identified a relevant transcriptomic fingerprint of angiogenic ECs, as reflected by the many expected candidate molecules that came up in this screen. The screen has yielded several novel candidate molecules. Class 3 semaphorin (semaphorin 3G [Sema3G]) was 1 of the most promising candidates; and further analyses focused on Sema3G, a novel member of the class 3 family of secreted semaphorins. Class 3 semaphorins are secreted growth factors that act through NP receptors and plexin coreceptors.18 Unlike the well-characterized class 3 semaphorins, Sema3A through Sema3F, which were identified as neuronal molecules that later exerted functions in the vascular system, Sema3G appears to be a primarily vascular-acting semaphorin.

**Methods**

Details of the cells and reagents (cell culture, antibodies and reagents), molecular techniques (RNA isolation and microarray processing, analysis of microarray chip data, siRNA selection and transfection of HUVE cells, RT-PCR), protein biochemical techniques (production of recombinant Sema3G, generation of different forms of Sema3G, immunoblot analysis, Sema3G pull-down assay, alkaline phosphatase (AP)-fusion protein binding assay on PAEC), histological techniques (immunofluorescence staining), cellular techniques (3D in vitro angiogenesis assay for transcriptome screening, 3D in vitro angiogenesis assay for target validation, EC-SMC co-culture system, SMC scattering assay, proliferation assay (WST read-out) screen), in vivo techniques (Sema3G deficient mice, knockdown and expression analysis in zebrafish) are summarized in the corresponding supplemental material (available online at http://atvb.ahajournals.org).

**Results**

**Identification of Sema3G by Transcriptomic Profiling of Angiogenic ECs**

Transcriptomic profiling of EC (human umbilical vein EC) sprouting for 24 and 48 hours in collagen and fibrin gels (supplemental Figure IA) identified a distinct gene expression profile of angiogenic ECs stimulated with either VEGF or basic fibroblast growth factor (bFGF) (compared with the gene expression profile of unstimulated human umbilical vein ECs cultured as 3D spheroids). A total of 966 distinct probe sets (Affymetrix), corresponding to 784 transcripts, were identified as differentially regulated (>2-fold change, P<0.01) compared with control. Of these sets, 92 transcripts were regulated >4-fold (63 upregulated and 29 downregulated, supplemental Figure II). Among the cohort of strongly upregulated genes, 26 (41%) were previously identified as either VEGF-induced genes or implicated in the regulation of angiogenesis (supplemental Figure II), validating the biological relevance of the experimental approach.

Next, we prioritized 29 genes from the original screening pool of 784 differentially expressed transcripts for further analysis (supplemental Table). This list of candidate genes was validated in small interfering RNA-based loss-of-function experiments (VEGF- and bFGF-induced 3D spheroid-based sprouting angiogenesis experiments and VEGF-induced proliferation assays, supplemental Table). Silencing of 4 genes (glia maturation factor γ, Semaphorin sem2 [Sema3G], SRY-box containing gene 17 (SOX17), and Down syndrome critical region-1) had an inhibitory effect in all tested parameters (supplemental Figure IB and supplemental Table). Three of these genes (ie, glia maturation factor γ, SOX17, and Down syndrome critical region-1) have recently been implicated in the regulation of EC function and angiogenesis.19–22 Therefore, further analyses focused on the characterization of Semaphorin sem2 (Sema3G) as a novel and differentially expressed EC class 3 semaphorin.

Semaphorins are grouped into 8 classes: 2 invertebrate classes (1 and 2), 5 vertebrate classes (3–7), and 1 viral class (8).23 Sema3Gs are the only vertebrate class of secreted semaphorins. Based on structural criteria, sem2 is the seventh member of the Sema3Gs and has consequently been designated as Sema3G. In contrast to the extensively characterized class 3 semaphorins, Sema3A through Sema3F, little is known about Sema3G.24–27

Human Sema3G is located on chromosome 3 (3p21.1) and is composed of 16 exons (supplemental Figure VA). It contains 782 amino acids consisting of a signal sequence, the 446–amino acid SEMA domain, and an Ig-like domain. The alignment with the other class 3 semaphorins identified Sema3G as a novel and differentially expressed EC class 3 semaphorin.
that led predominantly to full-length Sema3G production (Figure 1C). Therefore, 3 mutated forms of Sema3G were generated to study the binding properties and functions of differently processed Sema3G. First, a cleavage-resistant form of Sema3G (Sema3G-Mut) was generated in which both furin cleavage sites were mutated by point mutations (Figure 1D, lane 3; and supplemental Figure IIIA and supplemental Figure IV). Next, 2 forms of the completely processed protein (65 kDa) were generated. Sema3G-65oF was fused to a myc tag in a way that the furin cleavage site was removed, yielding a cleavage-resistant tag (Figure 1D, lane 4; and supplemental Figure IIIB and supplemental Figure IV). In contrast, the construct Sema3G-65F contained an active furin cleavage site (Figure 1D, lane 5; and supplemental Figure IIIC and supplemental Figure IV).

**Binding Properties of Sema3G**

Class 3 semaphorins bind NPs expressed by neuronal and vascular cells. Consequently, we probed Sema3G binding to NPs by performing immunoprecipitation experiments with Sema3G-containing supernatants and NP1-fragment crystallizable region (Fc) or NP2-Fc to pull down bound Sema3G. Western blot analysis using either the S1 antibody (identifying full-length Sema3G [F1] and the processed Sema3G fragments F2 and F3) or myc staining (detecting only full-length unprocessed Sema3G [F1]) confirmed the previously reported binding of Sema3G to NP2 (Figure 2A, S3G-wt).24–27 Surprisingly, these experiments revealed that furin proprotein convertase processed Sema domain–Containing F2 and F3 Sema3G fragments but not full-length Sema3G (F1) also bound to NP1 (Figure 2A, S3G-wt). In addition, the binding assay was performed for the cloned forms of Sema3G, Sema3G-Mut, Sema3G-65oF, and Sema3G-65F (Figure 1D and supplemental Figure III and supplemental Figure IV). Interestingly, the cleavage-resistant form of Sema3G (Sema3G-Mut) strongly bound to NP2 but not to NP1 (Figure 2A, S3G-Mut), whereas the cleaved 65-kDa forms (Sema3G-65oF and Sema3G-65F) did not bind to NP2 or NP1, independently of the exposure of the natural C-terminus (Figure 2A, S3G-65oF and S3G-65F).

To further validate Sema3G binding in a cellular context, we performed a binding assay on porcine aortic ECs overexpressing NP1 or NP2. Sema3G and the control Sema3F were C-terminally fused to alkaline phosphatase (AP). The amount of bound AP was visualized using nitroblue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate substrate (Figure 2B) and quantified with the soluble substrate p-nitrophenyl phosphate (PNPP) (Figure 2C). The assay demonstrated strong
binding of full-length wild-type Sema3G to NP2 and weaker binding to NP1. Similarly, Sema3F bound to NP2 and weaker to NP1, as expected, whereas the control-AP yielded no signal.

Expression of Semaphorin3G in Mammalian Tissues

To further explore vascular Sema3G functions, we analyzed the expression of Sema3G in detail by tracing LacZ marker gene expression, which was knocked into the Sema3G locus of mice during the generation of Sema3G-deficient mice (supplemental Methods and supplemental Figure VA and VB). Compatible with the high degree of redundancy of class 3 semaphorin family members,18 Sema3G-deficient mice were viable and fertile and exhibited no overt vascular morphogenic defects (supplemental Figure VC and VD). Corresponding data were obtained in zebrafish gene-silencing experiments, which similarly yielded no overt vascular phenotype. Both a transcriptional start site morpholino and a splice site morpholino did not cause overt defects during zebrafish development (supplemental Figure VI).

Specific vascular expression of Sema3G was first observed in the fetal aorta around E9.5, spreading to major trunk and brain arteries at E10.5 to E14.5. Sema3G displayed pronounced preferential arterial expression in all organs, which was most evident in the prenatal and postnatal heart, revealing prominent vascular expression in major arteries and branching small arteries (Figure 3F and supplemental Figure VII). To further trace the arterial expression of Sema3G, retinas of 12-day-old Sema3G-null mice were costained for LacZ and the EC and SMC markers Bandeiraea simplicifolia (BS)-1 lectin and α-SMA (red) showed that Sema3G is mainly expressed in SMC-covered vessels. E, Only background LacZ expression was detectable in α-SMA-positive retinal vessels in adult Sema3G-null mice. F, LacZ staining of the embryonic heart at E18.5 confirmed the vascular expression of Sema3G.

Gene expression of Sema3G was first observed in the fetal aorta around E9.5, spreading to major trunk and brain arteries at E10.5 to E14.5. Sema3G displayed pronounced preferential arterial expression in all organs, which was most evident in the prenatal and postnatal heart, revealing prominent vascular expression in major arteries and branching small arteries (Figure 3F and supplemental Figure VII). To further trace the arterial expression of Sema3G, retinas of 12-day-old Sema3G-null mice were costained for LacZ and the EC and SMC markers Bandeiraea simplicifolia (BS)-1 lectin and α-SMA (red) showed that Sema3G is mainly expressed in SMC-covered vessels. E, Only background LacZ expression was detectable in α-SMA-positive retinal vessels in adult Sema3G-null mice. F, LacZ staining of the embryonic heart at E18.5 confirmed the vascular expression of Sema3G.
cells in the islets of Langerhans of the pancreas (supplemental Figure VIII.A) and glomerular podocytes in the kidney. Neuronal expression was restricted to dorsal and trigeminal ganglions and the granular layer in the cerebellum (data not shown), confirming the previously observed limited expression of Sema3G in the brain.

Cellular RT-PCR analysis of Sema3G indicated expression by several EC populations, including human umbilical vein ECs, human umbilical artery ECs, and human saphenous vein ECs, but not by SMCs (Figure 4A). To validate the EC-specific expression of Sema3G in the vessel wall, we performed high-resolution double staining of LacZ and α-SMA in mouse tissues. These experiments unambiguously confirmed that Sema3G was expressed by ECs and not by SMCs (Figure 4B).

Next, we performed a binding assay of Sema3G to SMCs because SMCs in cell culture express the Sema3G receptor NP2 (Figure 4E) and might, therefore, be a target cell of secreted Sema3G. Thus, Sema3G, coupled to AP and control AP, was added to SMCs. Quantification of bound AP revealed significant binding of Sema3G to SMCs (Figure 4C). The images in Figure 4D also confirmed binding of Sema3G to SMCs. The cellular and in situ expression profiling experiments and the double staining of LacZ and α-SMA in mouse tissue clearly showed that Sema3G was not expressed by SMCs. This suggested that Sema3G, which is a secreted protein, bound to and acted on SMCs.

**Paracrine Effects of Semaphorin3G on SMCs**

The nature of the transcriptomic screening and validation procedure had identified Sema3G as a novel autocrine regulator of EC function (supplemental Figure IB). Yet, the binding of Sema3G to SMCs in vitro suggested that Sema3G...
may also exert paracrine functions. Therefore, we pursued cellular experiments to study the effects of Sema3G on EC-SMC interactions and on SMC functions.

To study the effect of Sema3G on EC-SMC interactions, we used a spheroidal EC-SMC coculture system that mimics the 3D assembly of the normal vessel wall. This assay was previously used to assess the vessel destabilizing effects of angiopoietin-2. Recombinant Sema3G, produced in S9 cells, was used to stimulate coculture spheroids of ECs and SMCs (Figure 5A). Exogenous Sema3G, but not Sema3A used as control, led to rapid and dramatic denudation of the EC monolayer growing on top of a core of SMCs (Figure 5A), suggesting a vascular destabilizing effect of Sema3G. To study the direct effects of Sema3G on SMCs, SMC spheroids were embedded in a gel matrix and outgrowth of migrating SMCs into the gel was quantified. Sema3G led to significant SMC outgrowth, similar in efficacy to the effect of bFGF (Figure 5B). The effects of Sema3G and bFGF on SMC outgrowth were additive (Figure 5B). Sema3A, used as a control, had no effect in this assay.

Next, the SMC migration assay was used to test the effect of the different Sema3G forms on SMC migration. Stimulation with wild-type Sema3G activated outgrowth of SMCs to a similar extent as bFGF, whereas the cleavage-resistant form (Sema3G-Mut) and the completely processed forms of Sema3G (Sema3G-65oF and Sema3G-65F) were not able to induce SMC migration (Figure 5C).

**Discussion**

Several families of neuronal guidance molecules have been identified in recent years to exert guidance and assembly functions in the vascular system. These include ephrins, semaphorins, netrins, and slit molecules. Among the semaphorins, class 3 semaphorins are secreted proteins that are capable of exerting propulsive and repulsive functions by activating their cognate NP and plexin receptors. We report the identification of Sema3G in a differential angiogenic EC transcriptomic screen as a preferentially vascular semaphorin expressed by arterial ECs during vascular development.

Semaphorins 3A through 3F have been extensively studied as propulsive and repulsive path finding molecules controlling neuronal and vascular cells. In contrast, little is known about Sema3G. The first Sema3G publication appeared a few years ago, established the limited expression of Sema3G in about neuronal and vascular cells. In contrast, little is known as propulsive and repulsive path finding molecules control arterial ECs during vascular development.

The present study has identified Sema3G as a primarily vascular expressed class 3 semaphorin. Class 3 semaphorins appear to be capable of exerting diverse functions in the vascular system. Secretion by peri-ECs transduces paracrine signaling with repulsive and migration-inhibiting functions on ECs. Repulsive EC functions, translating into a net antiangiogenic outcome, have been best characterized for Sema3A and Sema3F. Sema3A has also been characterized as an autocrine-acting negative regulator of EC integrin activation, thereby exerting antiangiogenic functions. However, class 3 semaphorins have also been reported to elicit attractive cues and antiangiogenic functions of semaphorins have also been described. The membrane-bound Sema4D is capable of exerting angiogenic effects after proteolytic cleavage. Intriguingly, class 3 semaphorins appear to be able to act context dependently as negative and positive regulators of EC function. For example, full-length Sema3E acts repulsive, whereas proteolytically cleaved Sema3E acts as an attractive guidance cue.

Compared with the predominantly repulsive functions of the unraveled class 3 semaphorin functions, Sema3G acts differently in that it positively controls ECs in an autocrine manner and SMCs in a paracrine manner. The mutated uncleaved form of Sema3G was not able to stimulate sprouting, and full processing abolished the biological activity of Sema3G. Thus, only the wild-type Sema3G protein, yielding all cleavage products, acts as the biologically active form of Sema3G.

Mechanistically, the vascular functions of class 3 semaphorins are poorly understood. The original concept that semaphorins act negatively by competing with VEGF for NP binding is not sufficient to explain vascular semaphorin functions. Genetic experiments have provided compelling evidence that NPs act in concert with VEGF to control vascular functions, whereas semaphorins act through NPs and plexins to control neuronal guidance. In turn, plexin-D1-deficient mice have an overt vascular phenotype, suggesting direct and rate-limiting semaphorin-mediated effects on vascular patterning. There is also significant phenotypic variation of class 3 semaphorin knockout phenotypes, depending on the genetic background. For example, the vascular phenotype of cluster of differentiation molecule (CD)-1 Sema3A null mice was not observed in a 129/SV or C57/Bl6 background. Similarly, the mortality of Sema3C homozygous mice is <50% in 129/SV and C57/Bl6 and almost 100% in the CD-1 background. The mild phenotypes of class 3 semaphorin-deficient mice and the phenotypic variation based on genetic background also point toward redundancy mechanisms between different class 3 semaphorins, which are also suggested by their overlapping expression patterns and the observed functional cooperativity. The identification of Sema3G with preferential vascular expression in mammalian tissues adds an additional level of complexity to the analysis of class 3 semaphorin functions. The deletion of Sema3G in mice or zebra fish did not cause obvious morphological or functional defects. Yet, the future generation of mice with targeted deletion of Sema3G and another class 3 semaphorin will likely yield important novel insights into redundancy mechanisms of class 3 semaphorins.

In summary, the present study has led to the identification and initial functional characterization of Sema3G as a preferentially vascular-acting class 3 semaphorin. Sema3G is expressed by angiogenic and developing arterial ECs. It controls ECs in an autocrine manner and SMCs in a paracrine manner. Unlike the previously characterized primarily repulsive and antiangiogenic functions of class 3 semaphorins in the vascular system, Sema3G acts as a positive regulator of angiogenic functions by stimulating ECs and activating...
Figure 5. Effect of Sema3G on EC-SMC interactions and on SMC migration. A, Surface CD31 whole mount staining of EC-SMC coculture spheroids treated for 3 hours with Sema3G, Sema3A, or control supernatants (left). Sema3G stimulation, but not Sema3A, led to massive denudation of the surface EC layer, as evidenced by quantification image analysis of the percentage of denuded surface area (right). B, Effect of Sema3G on the migration of SMCs embedded as 3D spheroids into a collagen matrix. Sema3G, but not Sema3A, induced in gel migration of SMCs with similar efficacy as bFGF, which was used as positive control (left). The effect of Sema3G and bFGF was additive (right). *P<0.005 vs unstimulated, and **P<0.0001 vs unstimulated. C, Only Sema3G-wt induced SMC migration, whereas the other forms of Sema3G (Sema3G-Mut, Sema3G–65oF, and Sema3G–65F) and the control-transfected supernatants were not able to do so. The quantification is on the left. Results are expressed as mean±SD of 10 randomly selected spheroids per experiment. *P<0.01 vs basal medium (BM) and mock. Representative images are shown on the right. The bar indicates 500 μm.
SMCs. Further analysis of this vascular class 3 semaphorin will shed novel light into the biological features of a novel secreted regulator of vascular functions during angiogenesis and may also contribute to the better understanding of the other class 3 semaphorins by shedding light into unrecognized redundancy mechanisms.

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Disclosures

None.

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Supplement Figure I: (A) Schematic protocol for the transcriptomic analysis of angiogenic EC employing a spheroid-based three dimensional sprouting angiogenesis assay. Spheroids of 400 HUVEC [1] were mixed with a collagen or fibrin matrix [2] and allowed to solidify [3]. Embedded cells [4] were stimulated with the angiogenic growth factors VEGF and bFGF [5] and allowed to form capillary-like tubes for 24 h and 48 h [6] after which total RNA was harvested [7] and processed for hybridization on Affymetrix GeneChips HG-U133A/B [8]. (B) SiRNA-based loss-of-function validation of one of the prioritized candidate genes measuring the cumulative length of capillary-like sprouts growing upon angiogenic stimulation from gel embedded HUVEC spheroids. Sema3G siRNA silencing was validated by RT-PCR (inset). Both, VEGF and bFGF induced a robust sprouting angiogenesis response. VEGFR2 siRNA was used as positive control. Silencing of EC Sema3G expression inhibited VEGF- as well as bFGF-induced sprouting angiogenesis. The data show the results for one out of two tested siRNAs with similar efficacy.
**Supplement Figure II:** Summary of highly regulated genes (4 fold upregulation or downregulation; \( P < 0.01 \)) of angiogenically sprouting EC embedded as 3D spheroids into a collagen or fibrin matrix and stimulated with either VEGF-A (V) or bFGF (F). Data are expressed as heat maps (log2 ratios). Genes previously associated to angiogenesis are represented in italics.
**Supplement Figure III:** (A) Schematic representation of Sema3G-Mut. Indicated are the two furin cleavage sites (1,2) and their amino acid sequences as well as the mutated sequences. (B) Schematic representation of the 65kDa form of Sema3G (Sema3G-65oF). The furin cleavage site 1 is mutated in the same way as in A so that the myc-tag is not cleaved off. (C) Schematic representation of the 65kDa form of Sema3G (Sema3G-65F). The furin cleavage site 1 is not mutated. The myc tag can be cleaved to generate a physiological C-terminal end. Both forms, with and without myc-tag are detected in the supernatant when the construct is expressed in mammalian cells. Red marks the antibody binding sites, SS = signal sequence.
**Supplement Figure IV:** Western blot analysis of Hek293 cells overexpressing the different forms of Sema3G. **(A)** Analysis of conditioned medium of stably transfected cells with the myc-antibody. **(B)** Analysis of cell lysates of stably transfected cells with the S1 antiserum (left) or the myc-antibody (right). Lane 1: mock transfected Hek; lane 2: Sema3G-wt form; lane 3: cleavage resistant form (Sema3G-Mut); lane 4: 65 kDa form of Sema3G with myc-tag and inactive furin cleavage site (Sema3G-65oF); lane 5: 65 kDa form of Sema3G with myc-tag and intact furin cleavage site (Sema3G-65F).
Supplement Figure V: (A) Schematic representation of Sema3G gene structure and Sema3G gene targeting. The sequence between exon 2 and exon 16 was replaced by a lacZ/Neo cassette by gene targeting. (B) Genotyping with the primers marked in A (red arrow head) shows the wildtype band (1) and the knockout band (2). RT-PCR with the primers marked in A (black arrowheads) shows a band for exon 2-7 (3) and for exon 13-15 (4) in the wildtype mice but not in knockout mice. Band 5 shows the GAPDH control. (C) Sema3G KO mice are viable; breeding of heterozygous mice led to normal Mendelian ratio offspring. (D) Sema3G KO mice are fertile. No significant difference in the number of offspring was observed between WT or KO breedings.
Supplement Figure VI: Silencing and expression of Sema3G in zebrafish embryos (A) Efficacy of the AUG-start codon targeting morpholino as assessed by an in vitro luciferase reporter assay. The AUG-start codon within its endogenous surrounding sequence (±25bp), which also encompassed the entire morpholino recognition site, was cloned in frame with a luciferase reporter gene. After in vitro transcription and translation in the presence of 0 mM (control), 0.1mM, 1mM, 10mM or 100mM zSema3G-ATG-MO, the luciferase activity was measured. Addition of increasing amounts of morpholino resulted in reduced luciferase activity. (B-D) Developmental expression of zSema3G assessed by in situ hybridization. (B) Expression of zSema3G in WT zebrafish embryos at 20 hpf revealed expression in several brain structures including diencephalon and telencephalon, otic vesicle, hypochord and tail bud. (C) At 30hpf, the expression of zSema3G was extended to the branchial arches and the fin bud. (D) By 48 hpf, zSema3G expression was mostly limited to the head region, the branchial arches and the fin bud.
Supplement Figure VII: LacZ staining identified Sema3G expression in the aorta starting at E9.5 (A, B) spreading to major trunk and brain arteries from E10.5 (C) to E14.5 (D). Analysis of late embryonic and early postnatal Sema3G expression revealed pronounced arterial expression in major arteries and branching smaller arteries throughout the body, including the kidney (E) and the heart (F).
Supplement Figure VIII: (A) Double staining for insulin and LacZ in the pancreas of P6 mice revealed the expression of Sema3G in the islets of Langerhans. (B) RT-PCR analysis of whole tissue homogenates of adult mouse organs revealed intense Sema3G expression in vascular rich tissues, including the kidneys, the spleen, the lungs, and the heart.
Supplement Figure IX: (A) Peptide sequence used for generation of the Sema3G antibody S1. Comparison with the other class 3 semaphorins shows partial homology. (B) Comparison of the human and murine Sema3G sequences shows complete homology.
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Supplement Table I

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Supplement Table I: List of functionally validated genes: Selection of these genes was based on (1) novelty in the context of angiogenesis, (2) temporal concordance between the 24 h and 48 h values, (3) upregulation by both VEGF and bFGF, and (4) upregulation in collagen gels and in fibrin gels. This list of candidate genes was validated in siRNA-based loss-of-function experiments. A total of 75 siRNA (2 to 3 per candidate gene) was transfected into HUVEC and the cells were functionally studied in VEGF- and bFGF-induced 3D spheroid-based sprouting angiogenesis experiments as well in VEGF-induced proliferation assays. Column 3-6 depict the fold change in microarray after the indicated stimulation compared to unstimulated collagen gels. Column 7-8 depict the induction of sprouting after siRNA based silencing of the gene. Column 9 depicts the results of the proliferation assay, ++: very strong effect, +: strong effect, -: no effect, n.d.: not determined.
Supplement Methods

Cells and reagents

Cell Culture

Human umbilical vein ECs (HUVEC), human umbilical artery smooth muscle cells (HUASMC), EC growth medium (ECGM), smooth muscle cell growth medium (SMCGM), EC basal medium (EBM) and corresponding supplements were purchased from Promocell (Heidelberg, Germany). Cells were cultured at 37°C, 5% CO2 and 100% humidity in the appropriate medium containing 10% FCS (Invitrogen, Carlsbad, CA) and 1% Penicillin/Streptomycin (PAA, Pasching, Austria). HUVEC were used between passages 2 and 5. HUASMC were used between passages 2 and 8.

Antibodies and reagents

The rabbit-anti-Sema3G antiserum S1 was produced by Eurogentec, the antiserum was generated against 15aa of the Sema domain of Sema3G and recognized mouse and human Sema3G. Sequence comparison of the Sema3G peptide used to generate the antibody showed partial homology with the other class 3 semaphorins (Supplement Fig. IX A) and was identical to mouse Sema3G (Supplement Fig. IX B). The mouse-anti-myc was purchased from Santa Cruz (Santa Cruz, CA); the biotinylated goat-anti-mouse antibody was from Zymed (San Francisco, CA); the goat-anti-mouse-Alexa488, goat-anti-rabbit-Alexa546 and streptavidin-Alexa488 were purchased from Molecular Probes (Carlsbad, CA); the rabbit-anti-NP2 was from Santa Cruz (Santa Cruz, CA). VEGF-A, bFGF, NP1-Fc, NP2-Fc and Sema3A were from R&D (Minneapolis, MN). Soluble Tie2-Fc (control-FC) was purified from supernatant of sTie2-Fc-expressing Sf9 cells as previously described. The Furin inhibitor Dec-RVKR-CMK was purchased from Biomol (Hamburg, Germany) and used at a final concentration of 10M. The Tri Reagent LS was from Sigma-Aldrich (Deisenhofen, Germany).
Molecular techniques

RNA isolation and microarray processing

Gels containing EC spheroids were directly homogenized in TRI reagent LS and total RNA was isolated according to manufacturer’s instructions. The isolated RNA was additionally purified using RNeasy Total RNA Mini Kit (Qiagen, Hilden, Germany). Purified RNA of approximately 100 gels was pooled to obtain 15 µg total RNA. An additional step of DNase digestion was used to avoid contamination with small amounts of genomic DNA. The quality of RNA samples was assessed by using RNA 6000 Nano chips (Agilent, Santa Clara, CA) and quantitated by measuring the absorbance at 260 nm. Preparation of cRNA was performed according to the protocols recommended by Affymetrix. Generally, 30 to 80 µg of purified cRNA were obtained when using 8 µg of total RNA. Then, cRNA samples were fragmented at 94°C for 35 min (in 200 mM Tris-Acetate, pH 8.1, 500 mM potassium acetate, 150 mM magnesium acetate). Three biological samples (2 x collagen, 1 x fibrin) were collected. The RNA of each biological sample was split and hybridized in duplicate. Aliquots of 10 µg of each sample were hybridized to Affymetrix HG-U133A and B arrays and processed according to the Affymetrix protocol. Arrays were scanned on an Affymetrix GeneChip scanner.

Analysis of microarray chip data

Array data were analysed with the GECKO software. Replicate hybridizations were normalized and merged for further ratio calculation. Element by element, ratios were computed from two experimental datasets creating a new dataset containing ratios and p-values. Genes were considered differentially expressed if ratios for qualifiers were regulated by a factor two with a p-value lower than 0.01. Qualifiers found to be not significantly different from background were manually removed. Hierarchical clustering was performed using Spotfire software based on the UPGMA (unweighted average) clustering method.
**SiRNA selection and transfection of HUVE cells**

SiRNA was purchased from Qiagen (Hilden, Germany) and Ambion (Austin, TX). On average, two of three siRNA degraded the specific mRNA by more than 70% (http://www.ambion.com/catalog/supp/pd_guarantee.html). Therefore, at least 2 siRNAs were used in the screen per gene. Given the screening character of the experiments, gene silencing was not specifically validated for each tested siRNA.

Oligofectamine (Invitrogen, Carlsbad, CA) was used for siRNA transfection of HUVEC as recommended by the manufacturer. Briefly, cells at 30%-50% confluence were transfected with 200 nM siRNAs for 4h in OptiMEM medium, recovered in ECGM overnight and used for RNA extraction, in the 3D angiogenesis assay or for the proliferation assay (WST1 read-out).

**RT-PCR**

RNA was isolated from whole mouse organs or cultured human cell lines according to the protocol of the manufacturer’s instructions (Qiagen) and transcribed into cDNA. PCR was performed using either human Sema3G primers (forward 5’-GAGATCCTTTGACAGAGTGCG-3’, reverse 5’-CTGACAGTGACATGGTTCGAG-3’) or mouse Sema3G primers (forward 5’-G ACTGACCAGGTGAAGAC-3’, reverse 5’-TCCTCAGTCATCTTCTATGTG-3’) and the control primers (human actin forward 5’-ACCCTGAAGTACCCCAT-3’, reverse 5’-TAGAAGCATTTGCGGTG-3’; mouse GAPDH forward 5’-CTCCTGGAAGATGGTGATGG-3’, reverse 5’-AGGTCAAGTGTGACACGGATTT-3’).

**Protein biochemical techniques**

*Production of recombinant Sema3G*

Recombinant Sema3G was produced in Sf9 cells using the BaculoGold™ transfection kit following standard protocols (Pharmingen, San Diego, CA). For protein production, Sf9 cells grown in serum-free medium at a density of 2 x 10^6 cells/ml were infected with a multiplicity of infection of 10. Sema3G was purified from Sf9 supernatants 72 h after infection. After addition of protease inhibitors (Protease Inhibitor Mix G, Serva, Heidelberg, Germany),
supernatants were bound to heparin sepharose (Amersham Biosciences, Uppsala, Sweden) (0.5 ml of Sepharose/100 ml Sf9 supernatants) for 2 h at room temperature. The PD-10 column was washed with 10 column volumes of 50 mM Tris-HCl, pH6.5, 100 mM NaCl, 5 mM MgCl₂, 5 mM CaCl₂. Sema3G was eluted with 5 column volumes of 50 mM Tris-Hcl, pH6.5, 600 mM NaCl, 5 mM MgCl₂, 5 mM CaCl₂. Protein was precipitated with 60% ammonium sulphate, resuspended in PBS, dialyzed against PBS overnight at 4°C and quantitated by measuring the OD at 280 nm.

Generation of the different forms of Sema3G

In order to generate the Furin resistance form of Sema3G (Sema3G-Mut), both recognition sites for Furin cleavage were mutated by point mutations with the QuickChange II Site-Directed Mutagenesis Kit from Stratagene according to the manufacturer's instructions. After confirmation of positive clones, the plasmids were transfected in Hek293 cells.

The 65kDa form of Sema3G is the N-terminal part of the molecule that occurs after Furin processing at cleavage site one. Two constructs of this form were made. The construct named Sema3G-65F still contains an active Furin cleavage site. The construct named Sema3G-65oF contains a Furin resistant site. Both constructs were cloned in frame into the pcDNA3.1 myc/His from Invitrogen (Carlsbad, CA) between the Kpn1 and EcoR1 sites so that the proteins became fused to a myc-tag. For generation of Sema3G-65F, cDNA was taken from Sema3G-65 Topo. For generation of Sema3G-65oF cDNA was taken from pcDNA3.1 Sema3G-Mut. Positive tested clones were cultured and plasmids were isolated with the Plasmid MaxiPrep Kit for further experiments.

Immunoblot analysis

For immunoblot analysis, cell lysates or purified protein was boiled with Laemmli buffer and loaded on an 8% SDS-PAGE. Western blots were analyzed with the corresponding antibodies.
Sema3G pull-down assay

Supernatant of Sema3G producing Hek-cells or of mock transfected Hek-cells was incubated with 1 µg of NP1-Fc, 1 µg of NP2-Fc, or 1 µg of control-FC (Tie2-Fc) and 30 µl of protein-G-Sepharose in 1 ml of TBS, 2% BSA for 1 h at room temperature. Sepharose beads were spun down and washed three times with TBS, 0.2% Nonidet P-40. The beads were boiled in Laemmli buffer and loaded on an 8% SDS-PAGE. Western Blots were analyzed with the anti-Sema3G antiserum S1 or an anti-myc antibody.

Alkaline phosphatase (AP)-fusion protein binding assay on PAEC

PAEC expressing NP1 and -2 and control PAEC were seeded in a 24well tissue culture dish (200,000 cells/well) and in a 96well tissue culture dish (50,000 cells/well). The next day, the medium was aspirated and conditioned medium containing Sema3G-AP, Sema3F-AP or control AP was added to the cells. After incubation for 5min at 37°C, the cells were washed and fixed with 4% formaldehyde. Incubation at 65°C for 2h destroyed the endogenous AP activity (the AP of the fusion protein is heat stable). Then the activity of cell bound AP was developed with two different substrates. In the 24well plate the cells were equilibrated with 100mM Tris pH9.5 and then the NBT/BCIP substrate was added. After 1h the enzymatic reaction was stopped by washing with water and the bound AP-fusion proteins were visible as a purple cell staining under the Olympus iX71 microscope. In the 96well plate the substrate PNPP was added. After 30min of development a soluble yellow reaction product had formed and was quantified with the ELISA reader at OD405. Each condition was carried out in triplicate.

Histological techniques

Immunofluorescence staining

For staining of Sema3G-deficient mouse retinas, the retinas were stained with β-galactosidase as previously reported and postfixed in 4% PFA in PBS. Retinas were incubated in 1% BSA-0.5% Tween 20 in PBS overnight, washed twice in PBlec (1% Tween
20, 0.1 mM CaCl$_2$, 0.1 mM MgCl$_2$, 0.1 mM MnCl$_2$ in PBS [pH 6.8]), and incubated in biotinylated isolectin from *Bandeiraea simplicifolia* (1:10; Sigma) and α-smooth muscle actin (αSMA) Cy3-conjugated (Sigma) in PBlec at 4°C overnight. After washing in PBS, isolectin was detected using 10 µg/ml of a fluorescent streptavidin conjugate (1:200 Alexa Fluor 488; Molecular Probes). The retinas were then washed again several times in PBS, post-fixed in 4% PFA, flat mounted with Mowiol 4-88 (Hoechst), and analyzed by confocal microscopy (Zeiss 510 Meta). For immunostaining of LacZ and SMA in Sema3G-deficient mice, embryos were fixed and whole mount stained using XGal according to standard protocols. Sections taken from XGal-stained embryos were incubated using FITC-conjugated (αSMA) antibodies (1:100 Abcam, Cambridge, UK).

**Cellular techniques**

**3D in vitro angiogenesis assay for transcriptome screening**

HUVEC spheroids of defined cell number (400 cells) were generated as described.$^4$ The following day, 400 spheroids were embedded into 1 ml of collagen or fibrin gel using 0.4 U of thrombin for polymerization of fibrin gels. After polymerization, embedded spheroids were cultured unstimulated for 24 h or they were stimulated with 25 ng/ml VEGF-A or bFGF for 24 or 48 h at 37°C, 5% CO$_2$, and 100% humidity.

**3D in vitro angiogenesis assay for target validation**

The 3D angiogenesis assay was performed with siRNA transfected cells using 50 spheroids per collagen gel. Cells were stimulated with VEGF-A or bFGF (25 ng/ml each) for 24 h. Thereafter, sprouting angiogenesis was digitally quantitated. Experimental controls included a validated negative control siRNA (Ambion), a positive control siRNA (siRNA against VEGFR-2), as well as untreated ECs that had not been exposed to transfection reagent or siRNA.
**EC-SMC co-culture assay**

EC-SMC co-culture spheroids were generated as described\(^5\). Spheroids were stimulated with Sema3A, Sema3G, or control supernatants and the effect on EC-SMC co-culture assembly was quantitated by assessing the surface area of denuded ECs after 4 h.

**SMC scattering assay**

SMC spheroids (450 cells each) were formed in SMCGM containing 0.25% (w/v) methylcellulose (Sigma-Aldrich) in 25 µl hanging drops. The spheroids were harvested and embedded in collagen (50 spheroids per gel). The gels were stimulated with bFGF (25 ng/ml), Sema3A, the different forms of Sema3G, or control supernatant. After 24 h, SMC sprouting was digitally quantitated by measuring the length of the sprouts that had grown out of each spheroid (ocular grid at 40x magnification, CSL: cumulative sprout length) using the digital imaging software analySIS (Soft imaging system, Muenster, Germany). The mean and standard deviation of the CSL from 10 randomly selected spheroids per gel is shown.

**Proliferation assay (WST1 read-out) screen**

Proliferation of HUVECs was assessed using the WST1 assay (Roche, Mannheim, Germany) according to the manufacturer’s instructions. The test is a formazan-based colorimetric assay, performed in 96 microwell plates. HUVECs were transfected with siRNA and stimulated in 1% FCS EBM medium with VEGF-A (50ng/ml) and bFGF (10 ng/ml). After 48h, 20 µl of WST1 reagent was added per well and reading (photometric) was performed after 2 h incubation at 37°C. Each data point corresponded to six replicates.

**In vivo techniques**

**Sema3G-deficient mice**

A bacterial artificial chromosome (BAC) containing the 12.5 Kb Sema3G gene and flanking sequences was modified to generate a BAC-based targeting vector, which was then linearized and used to replace the Sema3G gene sequence in F1H4 (C57BL/6:129 hybrid) mouse embryonic stem (ES) cells. Correctly targeted ES cells were identified by using the
loss of native allele (LONA) assay as previously described. Two independent correctly targeted ES lines were used to generate chimeric male mice which were then bred to C57BL/6 females to generate F1 mice. Heterozygous F1 mice (backcrossed to C57BL/6) were bred to homozygosity, and correct targeting was reconfirmed by reverse transcription and quantitative real-time PCR. Analyses were done on this generation of homozygous knockouts and littermate heterozygous as well as wildtype controls. Animals were housed under standard conditions in the Scheele Animal Facility at MBB, Karolinska Institute. All procedures were carried out in accordance with institutional policies following approval from the animal ethical board of Northern Stockholm. Genotyping of Sema3G mutant mice was done by PCR amplification of genomic DNA extracted from ear biopsies. Briefly, ear samples were incubated in DirectPCR Lysis Reagent (Viagen Biotech Inc., Los Angeles, CA) containing 0.3 mg/ml Proteinase K overnight at 56°C. PCR was performed using 3 specific oligonucleotide primers amplifying the wild-type and knock-in alleles simultaneously (wildtype forward: 5’-ATGACGCAGGAAACTACACT-3’; common reverse: 5’-AGTTCTGGACTCCTTTCC-3’; mutant forward; 5’-TTGCCAAGTTCTAATTCCAT-3’) producing a 490 bp wildtype product and a 203 bp mutant product (Supplement Fig. VI B). The PCR reactions contained 1xPCR buffer with 1.5 mM MgCl2, 0.2 mM dNTP, 0.2 uM of each primer, 1x PCR enhancer (Boule ME81210), 2.5 units HotStarTaq DNA polymerase, 200-500 ng DNA template and dH2O in a final volume of 12.5 μl. Amplification was performed using a Perkin-Elmer 9700 PCR machine with the following program: 95°C 5 min, 94°C 30sec, 57.5°C 30sec, and 72°C 2.5 min for 35 cycles, and finally 72°C 10 min.

Knock down and expression analysis in zebrafish

Tg(fli1:EGFP)y1 zebrafish were maintained under standard laboratory conditions. The following morpholino oligonucleotides were purchased from Gene Tools (LLC, Corvallis, OR): 5’-CCGGTAATTCCATGCTGAACAGACC -3’ (Sema3G-MO-ATG) and 5’-CATTTAGAAACTCACCTCGGATCT - 3’ (Sema3G-MO-splice) based on NCBI sequence XM_001340771. Different doses of morpholino were injected into single- to four-cell stage
zebrafish embryos, using procedures as previously described. Fifteen ng of morpholino per embryo was considered as the maximum tolerated dose, as higher amounts induced non-specific, toxic defects. Between 30 and 60 injected embryos were analyzed per experiment to identify alterations in axial vessel assembly and sprouting of intersegmental vessels of the trunk region, and each experiment was repeated at least three times. The efficacy of the MO-ATG morpholino was determined by a luciferase reporter assay and by RT-PCR for MO-splice morpholino using primers flanking the splice-site (ACTTGTCGGCTGTGTTTTTG and CCAGTAGATGGGTGCGGTTA). Embryos were analyzed by whole body fluorescence microscopy at 28 and 48 hpf. At the desired developmental stage, dechorionated embryos were fixed overnight in 4% paraformaldehyde at 4°C. Whole-mount in situ hybridization was performed as described, using an antisense probe for zSema3G (primers for cloning: AGGAGAACGAGACGTCAGGA and CAGGGCTTTGACTTCTTTTGC). Pictures were taken on the Zeiss Lumar® microscope using Axiovision v4.5.

Supplement References


