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Characterization of Semaphorin 6A-Mediated Effects on Angiogenesis Through Regulation of VEGF Signaling

Hidetaka Ohnuki[1] and **Giovanna Tosato**[2]

[1]Laboratory of Cellular Oncology, Center for Cancer Research, National Cancer Institute, Building 37, Room 4124, 37 Convent Drive, Bethesda, MD, 20892, USA. hidetaka.ohnuki@nih.gov

[2]Laboratory of Cellular Oncology, Center for Cancer Research, National Cancer Institute, Building 37, Room 4124, 37 Convent Drive, Bethesda, MD, 20892, USA.

Abstract

Angiogenesis identifies the process of endothelial cell sprouting and remodeling leading to the formation of new and functional blood vessels. Vascular expansion during development and in the adult mammal provides nutrients and oxygen to areas with increased need. Although many molecules and pathways have been identified as regulators of angiogenesis, aspects of this complex process remain unclear. Particularly undefined are the signals that orchestrate vessel survival and pruning once new blood vessels have sprouted. These poorly characterized aspects of angiogenesis need exploration. This chapter describes the experiments and methods enabling the characterization of Semaphorin 6A as a critical regulator of endothelial cell survival and vessel function.

Keywords

Semaphorin; VEGF; VEGFR; bFGF; Angiogenesis; Vasculature; Cell death; Cleaved caspase−3; Endothelial cells

1 Introduction

Recent progress in understanding the fundamental mechanisms that regulate angiogenesis has resulted in the development of drugs to modulate pathological angiogenesis, and their application to patient treatment [1]. In particular, the discovery of VEGF as a principal stimulator of angiogenesis in physiology and disease has led to the development of drugs that reduce angiogenesis by targeting VEGF and its principal receptor VEGFR2 [2, 3]. Whereas the neutralization of VEGF function has provided significant improvement in the treatment of certain intraocular diseases [4], this approach has given variable results in the context of cancer where angiogenesis contributes to tumor progression. This variability has been attributed to intrinsic differences of tumor cell dependency on angiogenesis, tumor vessel original or acquired resistance to anti-VEGF therapies and the contribution of pro −angiogenic factors other than VEGF [1, 5]. Often the reasons for poor responsiveness to VEGF neutralization are complex and poorly defined, particularly in the context of cancer −associated angiogenesis. Recent studies have highlighted a role for certain semaphorins in

the regulation of developmental and cancer−associated angiogenesis [6] raising expectations for future therapeutic applications.

The semaphorins comprise a family of proteins that play critical roles in the development and function of the neural system [7−9]. They have also been implicated in the regulation of immunity and tumor growth [7, 8]. There are eight classes of semaphorins: class 1 and 2 are present in invertebrates, and classes 3 to 7 are present in vertebrates; and the viral−encoded semaphorins comprise a separate class. Some of the proteins in this family are membrane bound whereas others are secreted, but they all share a Sema domain, a 500 residue N −terminal domain that is similar in structure to the extracellular domain of α-integrins, which mediates receptor−binding specificity [10, 11]. Plexins are semaphorin receptors mediating distinct signaling cascades [6, 12, 13]. Membrane−bound semaphorins signal directly through their cognate receptors, whereas soluble semaphorins (such as class 3 semaphorins) generally require neuropilin-1 or −2 as co−receptors, with the exception of the soluble Sema3E, which can directly activate plexin D1 [6, 11, 14]. Interestingly, neuropilins can also serve as VEGF co-receptors providing a structural basis for cross talk between semaphorin and VEGF signaling [6].

A number of class 3 semaphorins have been found to inhibit angiogenesis [8]. Semaphorin 3A (Sema3A) inhibits endothelial cell migration, growth and survival in response to VEGF in vitro. Some studies have attributed this activity to Sema3A binding to neuropilin 1 and competition with VEGF [15–19]. Other studies have suggested that Sema3A inhibits integrin function [19] or that Sema3A promotes neuropilin 1 internalization from the cell surface to the cytoplasm thus reducing neuropilin 1 availability and VEGF function [20]. However, it is not clear whether Sema3A is a critical contributor to vascular development because genetic studies in Sema3A-deficient mice have provided conflicting results [6, 19, 21]. In addition, mice lacking expression of plexin A2, A3, and A4, the known signaling receptors for Sema3A, display no overt vascular defects [21]. Similarly, mice expressing a mutant neuropilin 1, which does not support semaphorin signaling, develop a normal vascular system but display characteristic defects in the nervous system [22].

Sema3E, which can bind and activate the cognate Plexin D1 receptor, without a requirement for neuropilin 1, plays an important role in developmental angiogenesis [23]. Mice that are deficient for Sema3E or Plexin D1 have similar vascular patterning defects in the intersomitic space characterized by exuberant ectopic extension of the intersomitic vessels, which are no longer restricted to the somite [24, 25]. As a consequence, the normal segmented somite patterning is obfuscated. This defect was attributed to the absence of Sema3E-derived repulsive signals restricting vessel growth to the intersomitic space, and was linked biochemically to altered regulation of VEGF signaling [26]. Sema3E also contributes to proper development of the dorsal aorta, which is abnormally narrow in Sema3E knockout mice [27], and to the postnatal extension of retinal vessels, which display reduced branching and an uneven growing front in Sema3E or Plexin D1−null mice [26, 28]. Recent studies in vitro and tumor-bearing mice have shown that Sema3E−Plexin D1 signaling reduces integrin−mediated endothelial cell adhesion, migration and tube formation and inhibits tumor angiogenesis [29−31].

Class−6 semaphorins are also emerging as regulators of angiogenesis. Sema6D, through activation of its receptor plexin A1, magnifies VEGFR2 phosphorylation and function. Biochemically, this effect was linked to physical association between plexin A1 and VEGFR2 [32]. A similar pro−angiogenic effect was described for Sema6B [33]. The silencing of Sema6B or its receptor Plexin A4 impairs endothelial cell proliferation in response to FGF2 and VEGF, suggesting that Sema6B is a stimulator of endothelial cell proliferation. This function was attributed to Plexin A4 forming stable complexes with the FGFR1 and VEGFR2 receptors resulting in amplification of VEGF and FGF functions [33].

Consistent with the pro−angiogenic functions of the other two Class−6 semaphorins, Sema6B and Sema6D, we have recently discovered that Sema6A critically contributes to endothelial cell survival and angiogenesis [34]. The silencing of Sema6A in primary endothelial cells results in cell death, which is not rescued by exogenous VEGF or FGF2. Mechanistic studies revealed that Sema6A promotes the expression and function of VEGFR2. When Sema6A is silenced, VEGFR2 mRNA and protein levels are reduced in endothelial cells. Consistent with this reduction of VEGFR2, exogenous VEGFA fails to properly activate VEGFR2 phosphorylation and downstream signaling, and to prevent cell death induced by the silencing of Sema6A. However, FGFR1, the principal FGF2 receptor in endothelial cells, is functionally normal in Sema6A−silenced endothelial cells, raising the question why exogenous FGF2 fails to rescue endothelial cells from death. The explanation lies on the failure of Sema6A−silenced endothelial cells to utilize endogenous VEGF, which is essential for cell survival [34]. This conclusion is consistent with the results of genetic experiments in VEGFA−deficient mice showing that endogenous VEGF is required for autocrine endothelial cell survival, and that this requirement is not overcome by exogenous VEGF [35]. We found that the silencing of Sema6A does not alter VEGFA expression, but that VEGFR2 deficiency associated with the silencing of Sema6A is responsible for the failure of endogenous VEGF to support endothelial cell survival, which is mediated by VEGFR2 [34].

These experiments in vitro demonstrated that Sema6A is an essential stimulator of endothelial cell survival by sustaining VEGFR2 expression, raising the possibility of a similar function in vivo. Sema6A-deficient mice had previously been derived [36−38]. Phenotypically, these mice display developmental defects in the central nervous system, which are compensated in the adult mouse [36–43]. However, Sema6A-null mice were not reported to have overt defects in the vascular system. We examined the hyaloid and retinal vessels in Sema6A-deficient mice and compared to wild-type (WT) mice. We found that both these ocular vascular systems express Sema6A in the WT endothelium [34]. Interestingly, we uncovered a role for Sema6A in the involution of hyaloid vessels, which occurs physiologically during the first 7−10 days after birth in the mouse. Specifically, we found an acceleration of cell death in Sema6A−deficient hyaloid vessels compared to WT vessels. Additionally, we uncovered a delay in retinal angiogenesis, which develops as the hyaloid vasculature regresses. Thus, during the first week after birth, Sema6A−deficient retinal vessels do not cover the retinal surface to the same degree as the WT retinal vessels. Despite this delayed development, however, Sema6A-deficient retinal vessels eventually form a normal-appearing retinal vascular bed.

We further examined whether adult angiogenesis is compromised in Sema6A−deficient mice. Our results found a defect in Matrigel−assisted subcutaneous angiogenesis, and in choroidal and tumor angiogenesis [34]. In these experimental situations, Sema6A−deficient vessels displayed reduced angiogenesis. This reduction resulted in reduced tumor progression. Collectively, this investigation uncovered a novel role for Sema6A as a critical regulator of endothelial cell survival and angiogenesis, and identified Sema6A as a potential drug target for reducing pathological angiogenesis [34]. Herein, we describe the experiments and methods enabling the characterization of Semaphorin6A as a critical regulator of endothelial cell survival and vessel function.

2. Materials

2.1 Components for Derivation and Culture of HUVEC

1. Primary human umbilical cord endothelial cells (HUVEC) are derived as described under methods (Subheading 3.1). The cells are used for experiments in vitro through passage 6 (*see* Note 1).
2. HUVEC culture medium: 370 ml Medium 199 (Thermo Fisher Scientific) supplemented with 0.5 ml 50 mg/ml l−ascor−bic acid (Sigma−Aldrich), 0.5 ml 25 mg/ml heparin sodium salt (Sigma−Aldrich), 4 ml 200 mM l−glutamine (Sigma−Aldrich), 5 ml 110× stock Pen/Strep, 5 ml endothelial cell growth supplement (ECGS; Sigma−Aldrich), 25 ml human serum (Thermo Fisher Scientific), and 100 ml newborn calf bovine serum (Thermo Fisher Scientific) (*see* Note 2).
3. Gelatin solution: Prepare 0.5 % gelatin solution (Sigma−Aldrich) in PBS.
4. Luer adaptors (male and female adaptors; Custom Automatic): Place in sterilizing bags and sterilize by autoclaving.
5. Plastic ties (PGC Scientifics Co.): Place in sterilizing bags and sterilize by autoclaving.
6. Collagenase: Prepare 2 mg/ml Collagenase (Type II; Worthington Biochemical) in sterile PBS.
7. Trypsin: 0.05 % Sterile solution (Thermo Fisher Scientific).
8. Freezing solution: 12 % DMSO Hybri−Max in fetal bovine serum (FBS). Filter prior to using.
9. Freezing vials (Corning).
10. Human CD31 antibody: Fluorescein isothiocyanate (FITC)−conjugated mouse monoclonal antibody (BD Pharmingen; clone WM59).

2.2 Components for Gene Silencing

1. pMDLg/pRRE: plasmid DNA for third−generation packaging vector containing Gag and Pol under the control of the CMV promoter (Addgene).

2. pRSV−Rev: Third plasmid DNA for generation packaging vector containing Rev (Addgene).
3. pCMV−VSV−G: Plasmid DNA for envelope protein for producing lentiviral particles (Addgene).
4. pLKO.1−SEMA6A shRNA: Plasmid DNA for human SEMA6A Mission shRNA (Sigma−Aldrich, cat. No. TRCN0000061111 or Sigma−Aldrich, cat. No. TRCN0000061112) effectively silences SEMA6A in HUVECs.
5. pLKO.1-VEGFA shRNA: Plasmid DNA for human VEGFA Mission shRNA (Sigma-Aldrich, cat. No. TRCN000003343) effectively silences VEGFA in HUVECs.
6. pLKO.1−Puro: Plasmid DNA for control vector of shRNA vectors containing the puromycin resistance gene cassette under human PGK promoter and ampicillin resistance gene for bacterial selection (Sigma−Aldrich, SHC001).
7. One Shot TOP10 Chemically Competent *E. coli* (Thermo Fisher Scientific).
8. S.O.C. medium (Thermo Fisher Scientific).
9. L.B. broth (KD Medical) containing 100 μg/ml ampicillin.
10. L.B. agar plate containing 100 μg/ml ampicillin.
11. PureLink HiPure Plasmid Maxiprep Kit (Thermo Fisher Scientific).
12. Human embryonic kidney (HEK) 293T cells; these are HEK 293 cells that have been infected with the large T antigen of SV40 that inactivates pRb (*see* Note 3). HEK 293T cells are transfected easily and support high−level expression of lentivirus packaging systems.
13. HEK 293T culture medium: DMEM supplemented with 10 % FBS.
14. Lipofectamine 2000 Transfection Reagent (Thermo Fisher Scientific).
15. Opti−MEM I Reduced Serum Medium (Thermo Fisher Scientific).
16. Supplemented Medium 199 growth medium: Contains Medium 199 (Thermo Fisher Scientific), 25 μg/ml heparin sodium salt from porcine intestinal mucosa (Sigma−Aldrich), 50 μg/ml ascorbic acid (Sigma−Aldrich), 2 mM 1−glutamine (Thermo Fisher Scientific), 10 U penicillin/100 μg/ml streptomycin (Thermo Fisher Scientific), 75 μg/ml endothelial cell growth supplement (Sigma −Aldrich), 20 % FBS and 10 % human serum (Thermo Fisher Scientific).
17. Polybrene solution: Dissolve 8 mg/ml polybrene (hexadime−thrine bromide, Sigma−Aldrich) in PBS and store at −20 °C until use.
18. Puromycin: ~0.5 μg/ml Diluted in Medium 199.

2.3 Components for VEGFR2 Activation and Modulation

1. EBM−2: Endothelial basal medium, EBM−2 (Lonza).

2. Supplemented EBM−2 medium: EBM−2 (Lonza) supplemented with FGF2, EGF, IGF−1 (provided as EGM−2 Bullet kit, Lonza). Add 0.1 % human serum (Thermo Fisher Scientific).
3. EBM−2/orthovanadate: EBM−2 medium (Lonza) plus 100 μM orthovanadate (Na_3VO_4, Sigma−Aldrich).
4. EBM−2/orthovanadate/VEGF neutralizing antibody: EBM−2 medium (Lonza) plus 100 μM orthovanadate plus 10 μg/ml Avastin (Genentech).
5. EMB−2/orthovanadate/tyrosine kinase inhibitor: EBM−2 medium (Lonza) plus 100 μM orthovanadate plus 2 sorafenib (Selleck Chemicals). Sorafenib is a small −molecule inhibitor of the tyrosine kinase VEGFR2 and other kinases, including PDGFR, cKit, and Raf kinases.
6. EMB−2/VEGF: EBM−2 medium (Lonza) plus 100 ng/ml human VEGFA (R&D Systems).

2.4 Immunoblotting Components

1. Supplemented NuPAGE LDS sample buffer: NuPAGE LDS sample buffer (Thermo Fisher Scientific) supplemented with 1 mM Na_3VO_4, 20 mM NaF, 10 mM $Na_4P_2O_7$, and complete protease inhibitor cocktail (Roche).
2. Micro BCA Protein Assay Kit (Thermo Fisher Scientific).
3. NuPAGE 4−12 % Bis−Tris gels (Thermo Fisher Scientific).
4. 1×MOPS SDS running buffer containing NuPAGE AntiOxidant: dilute 20×MOPS SDS Running Buffer (Thermo Fisher Scientific) in water and add NuPAGE AntiOxidant (Thermo Fisher Scientific).
5. 1×MES SDS running buffer containing NuPAGE AntiOxidant: dilute 20×MES SDS Running Buffer (Thermo Fisher Scientific) in water and add NuPAGE AntiOxidant (Thermo Fisher Scientific).
6. Nitrocellulose membrane (Whatman).
7. Mini Trans−Blot Electrophoretic Transfer Cell (Bio−Rad).
8. 1×Tris glycine transfer buffer with methanol: Dilute 10× Tris glycine transfer buffer (Quality Biological) in water and add 20 % methanol.
9. Blocking buffer (for nitrocellulose membrane): 5 % BSA, 0.1 % Tween 20, 50 mM Tris−HCl, pH 7.4, and 150 mM NaCl.
10. TBS−T: Add 1 ml Tween 20 per liter of TBS (Quality Biologicals).
11. 1×1st Antibody binding enhancer solution: Dilute 10× 1st antibody binding enhancer (Crystalgen) in water and add 5 % BSA.
12. Primary antibodies for Western blotting: Rabbit mAb to phos−phorylated (p) −VEGFR2 (Tyr 1175) (Cell Signaling Technology), rabbit mAb to p−VEGFR2 (Tyr 951) (Cell Signaling Technology), rabbit IgG to human VEGFR2 (Cell

Signaling Technology), rabbit mAb to cleaved Caspase 3 (Cell Signaling Technology), and goat anti−actin (Santa Cruz Biotechnology).

13. 1×2nd Antibody binding enhancer solution: Dilute 10× 2nd antibody binding enhancer (Crystalgen) in water and add 5 % BSA.
14. Secondary antibodies for Western blotting: ECL Anti−Rabbit IgG, horseradish peroxidase (HRP)−linked whole antibody (GE Healthcare) and donkey anti−goat IgG−HRP (Santa Cruz Biotechnology).
15. SuperSignal West Femto Maximum Sensitivity Substrate (Thermo Fisher Scientific).

2.5 Components for Evaluation of Cell Death

1. Chamber slides are culture chambers designed for growing adherent cells such as HUVEC, which can be reduced to slides for microscopy. They consist of a plastic or glass slide with chambered superstructure (1−8 chambers) sealed over the slide with a biologically inert silicone gasket (Thermo Fisher Scientific, Nunc Lab Tek II).
2. 4 % Paraformaldehyde (PFA): Freshly prepared prior to use as a fixative 4 % (weight/volume) PFA (Sigma−Aldrich) solution in PBS.
3. 1.0 % Triton X−100 in PBS
4. Blocking solution for immunostaining: PBS containing 0.5 % Triton X−100 and 10 % goat serum.
5. Rabbit mAb to cleaved Caspase 3 (Cell Signaling Technology).
6. Alexa Fluor−conjugated goat anti−rabbit IgG (H + L) (Thermo Fisher Scientific).
7. DAPI−containing mounting fluid (VectaShield mounting medium with DAPI, Vector Laboratories, cat. No. H-1200).
8. HRP−conjugated donkey anti−rabbit IgG (GE Healthcare Life Sciences). PBS−T containing 3 % BSA.
9. PBS−T containing 2 % BSA.
10. Chemiluminescent HRP substrate (Millipore).

3 Methods

3.1 Isolation and Derivation of HUVEC

1. Primary HUVEC are derived from umbilical cords that have been removed within 12 h and are kept at 4 °C at all times prior to processing.
2. Wipe the outside of the umbilical cord with sterile gauze to remove blood, and cut off both ends of the cord with a sterile razor to produce a clean cut.

3. Cannulate the umbilical vein with sterile luer adaptors (male and female adaptors) and tie them to each end of the vein with sterile plastic ties. Fill the vein with 5−10 ml sterile Collagenase at 37 °C and clamp both ends with sterile forceps.

4. Place the umbilical cord in a sterile 500 ml beaker containing sterile PBS at 37 °C, and place the beaker containing the umbilical cord in a 37 °C incubator for 8−10 min.

5. After bringing to room temperature and massaging the cord with sterile gloves, flush the vein with 20−30 ml sterile PBS into a 50 ml sterile tube to collect the endothelial cells that have sloughed off the vein, and centrifuge at 1200 × *g* for 10 min to pellet the cells.

6. Prepare gelatin-coated plates by adding gelatin solution to cover the bottom of a 60 mm dish. Incubate for 30 min at 37 °C and aspirate liquid. Suspend the pellet of cells from **step 5** in HUVEC culture medium and plate all cells onto a gelatin coated 60 mm dish. Incubate at 37 °C.

7. After 18−24 h, remove non−adherent cells from the plate, wash the plate with HUVEC culture medium and continue culture in HUVEC culture medium until the plate is confluent. This generally occurs within 24 h. Cells that have formed a confluent monolayer at this time−point are called “passage 0.”

8. The cell monolayer is trypsin-treated to detach the cells from culture plate, and the cells are re-plated to 25 % confluency (1:4 dilution) onto 100 mm plate until a confluent monolayer is obtained (passage 1).

9. For subsequent expansion, cells are split at a ratio of 1:4. Endothelial cell purity is assessed by immunostaining with a human CD31 antibody and flow cytometry [20]. Using this method >95 % of the cells are usually CD31 positive.

10. Passage 1 HUVEC are frozen (0.5×10^6cells/vial) in liquid nitrogen after pelleting and suspension in sterile 1 ml freezing solution. Recovery from frozen state is excellent.

3.2 Silencing Sema6A in HUVEC : Preparation of Packaging and shRNA Vectors

Gene silencing in endothelial cells is achieved using a third−generation HIV−derived lentiviral vectors [44].

1. Prepare 100 pg to 100 ng (1−2.5 μl) of pMDLg/pRRE, pRSV−Rev, pCMV−VSV−G, pLKO. 1−SEMA6A shRNA, and pLKO.1−VEGFA−shRNA in 10 μl of nuclease−free water.

2. For each plasmid DNA, mix gently with 25 μl of One Shot T0P10 Chemically Competent *E. coli.*

3. Incubate on ice for 30 min.

4. Heat shock at 42 °C for 45 s.

5. Incubate on ice for 1 min.

6. Add 250 μl of S.O.C. medium; place at 37 °C for 1 h in a shaking incubator set at 225 rpm.
7. Place 5 μl suspension of the transformed *E. coli* on L.B. agar plate containing ampicillin.
8. Invert the plate, and incubate at 37 °C overnight.
9. Select one colony, and transfer into 250 ml of L.B. broth containing Ampicillin.
10. Culture overnight.
11. Purify each of the plasmid DNAs using PureLink HiPure Plasmid Maxiprep Kit according to the manufacturer's protocol (http://tools.lifetechnologies.com/content/sfs/manumailto:http://tools.lifetechnologies.com/content/sfs/manuals/purelink_hipure_plasmid_dna_purification_man.pdf

3.3 Silencing Sema6A in HUVEC : Preparation of Lentivirus for Gene Silencing

1. Prepare 80–90 % confluent HEK 293T cells (see Note 4) in a 10 cm dish.
2. Change medium to fresh 5 ml of HEK 293T culture medium/dish.
3. Prepare a plasmid DNA mixture containing 2.5 μg of pRSV−REV, 3.5 μg of pCMV−VSV−G, 5 μg of pMDLg/pRRE, and 12 μg of the desired shRNA vector in 0.5 ml Opti−MEM.
4. Dilute 20 μl of Lipofectamine 2000 with 0.5 ml Opti−MEM, and then incubate for 5 min at room temperature.
5. Mix the plasmid DNA mixture and the diluted Lipofectamine 2000 solution, and then incubate for 20 min at room temperature.
6. Add the plasmid DNA−Lipofectamine complex solution to the HEK 293T cells in the 10 cm dish.
7. After 5 h, add 5 ml of HEK 293 T culture medium to the dish.
8. Culture for 18 h.
9. Change medium to fresh Medium 199 supplemented growth medium and culture for 72 h post−transfection.
10. Collect HEK 293T culture supernatant and centrifuge the supernatant at 15,000 × *g* for 10 min at 4 °C.
11. The virus−containing supernatant is stored at −80 °C (*see* Note 5).

3.4 Silencing Sema6A in HUVEC : Lentivirus Infection and Gene Silencing

1. Prepare HUVEC to a 75 % confluent monolayer on gelatin−coated 10 cm dishes (similar to Subheading 3.1).
2. Remove the culture medium and substitute with 1.5 ml/dish Opti-MEM.

3. Add 0.5 ml of the culture supernatants From Subheading 3.3, **step 11** containing shRNA lentiviral particles (*see* Note 6).
4. Add 2 μl/dish of polybrene solution.
5. Incubate the cells for 1 h in a CO_2 incubator (5 % CO_2, 37 °C). During the incubation, periodically gently mix to avoid drying the cells.
6. Add 4 ml/dish of Medium 199.
7. Culture the cells for 18 h.
8. Remove the medium and replace with 10 ml/dish of fresh Medium 199.
9. After 2 days (approximately 72 h after lentiviral infection), the medium is replaced with 10 ml/dish of fresh Medium 199.
10. Add puromycin for selection of cells expressing the puromycin resistance gene (puromycin N−acetyl−transferase) as a marker of shRNA vector transduced cells (*see* Note 7).
11. After 5 days, gene silencing effectiveness is evaluated by measuring mRNA and protein levels of the targeted gene (*see* Note 8).

3.5 Evaluating the Effect of Sema6A Silencing on Intracellular VEGF Signaling

An important role has been identified for intracellular VEGF in the maintenance of endothelial cell survival [35]. These studies showed that most mice with a genetic deletion of VEGF targeted to the endothelium display a progressive loss of endothelial cells and suddenly die by 6 months of age. Endothelial cells are a poor cell source of VEGF, which is mostly contributed by other cell types through paracrine mechanisms. Consistent with this, the authors found that tissue levels of VEGF mRNA and protein were normal in the mutant mice lacking VEGF expression in the endothelium. The authors also found that VEGFR2 phosphorylation is present in VEGF−deficient endothelium. This provides evidence that exogenous VEGF does not compensate for the absence of endothelial−intrinsic VEGF, and uncovers an essential role for autocrine VEGF signaling in endothelial cell survival [35].

In Sema6A−silenced endothelial cells, VEGFR2 levels and signaling are reduced but FGFR1 levels and signaling are intact. Nonetheless, the addition of FGF2 (a potent inducer of endothelial cell growth and survival in vitro) failed to rescue Sema6A−deficient cells from death, despite normal signaling [34]. This finding raised the possibility that paracrine FGF2 could not compensate for reduced VEGFR2 function. To test for the possibility that endogenous VEGF required a functional VEGFR2 to exert its pro−survival effects, we selectively silenced VEGFA expression in endothelial cells [34]. Confirming the results in mice [35], we found that endothelial cells die and death is not prevented by the addition of exogenous VEGFA. In addition, by using neutralizing VEGFA antibodies, which do not penetrate cells but block exogenous VEGF, and sorafenib, a small−molecule inhibitor of VEGFR2 (and other receptor tyrosine kinases), which is cell permeable, we could dissect the roles of exogenous versus endogenous VEGF/VEGFR2 signaling as mediators of cell survival in Sema6A−silenced endothelial cells.

1. Control, Sema6A-, and VEGFA−silenced HUVEC are washed twice with PBS.
2. Culture the cells in supplemented EBM−2 medium for 6 h.
3. Culture the cells for 24 h in each of the following: EBM−2, EBM−2/ orthovanadate (*see* Note 9), EBM−2/orthovanadate/ VEGF neutralizing antibody (*see* Note 10), and EBM−2/ orthovanadate/tyrosine kinase inhibitor (*see* Note 11). As a positive control for phosphorylation of VEGFR2, also culture cells in EBM−2/VEGF for 5 min.
4. Collect the cells in supplemented NuPAGE LDS sample buffer.
5. Freeze cell lysates at −80 °C prior to use for SDS−PAGE and Western blotting.
6. Defrost samples and measure protein concentration of the lysate using a Micro BCA Protein Assay Kit. Adjust protein concentration to 2 mg/ml with NuPage LDS sample buffer (*see* Note 12).
7. Add β−mercaptoethanol is added to the lysate at 10 % (v/v) and boil at 70 °C for 10 min.
8. Load 40 μg protein of the lysate/lane into a NuPage 4−12 % gradient Bis−Tris gel.
9. Perform SDS−PAGE in 1× MOPS or 1× MES SDS running buffer containing NuPAGE AntiOxidant (*see* Note 13).
10. Transfer proteins from the gel to a nitrocellulose membrane using a Mini Trans-Blot Electrophoretic Transfer Cell with 1× Tris glycine transfer buffer with methanol.
11. Block the nitrocellulose membrane is blocked with blocking buffer for 30 min with orbital shaking.
12. Rinse in TBS−T and incubate the membrane for 18 h at 4 °C with orbital shaking in a primary antibody diluted in 1× 1st antibody binding enhancer solution. Each of the primary antibodies (anti−phospho−VEGFR2 (Tyr1175), anti−phospho −VEGFR2 (Tyr951), and anti−VEGFR2) is used in sequence after stripping the membrane (*see* Note 14).
13. Wash the membrane at room temperature three times for 5 min in TBS-T on an orbital shaker. Dilute the secondary HRP−conjugated donkey anti−rabbit IgG antibody 1:25,000 in 1× 2nd antibody binding enhancer solution (*see* Note 15). Incubate in the secondary antibody for 30 min at room temperature on an orbital shaker.
14. Wash the membrane in TBS−T with orbital shaking for 15 min, three times. Then, drain the membrane and expose it to HRP detection reagents (SuperSignal West Femto Maximum Sensitivity Substrate) (*see* Note 16). Capture the chemiluminescent signals from the membrane via imaging technology or film.

15. To ensure equal protein loading in different lanes, strip blots and re−probe with primary antibodies against actin (goat antiactin) followed by an HRP−linked secondary anti−goat antibody (*see* Note 14).

16. The presence of phosphorylated VEGFR2 and VEGFR2 is reflected by the visualization of appropriate bands at the expected relative size of about 150 kDa. The presence of actin is reflected by the visualization of appropriate bands at the expected relative size of about 42 kDa.

3.6 Assessing Endothelial Cell Death by Immune Detection of Cleaved Caspase−3 in Sema6A−Silenced HUVEC

Caspase-3 is a member of the cysteine-aspartic acid proteinase (caspase) family of enzymes that play an important role as mediators of cell death. These enzymes are inactive proenzymes that undergo proteolytic cleavage to generate two subunits (a large and a small subunit), which subsequently generate the active enzyme [45]. When activated, caspase−3 is cleaved to generate fragments (17/19 kDa) of activated caspase−3, which is specifically recognized by a variety of antibodies that do not detect the full-length caspase−3, which is inactive, or other cleaved caspases. Detection of cleaved caspase−3 is an indication of cell death.

3.6.1 Detection of Cleaved Caspase−3 by Immunofl uorescent Staining

1. Grow HUVEC transduced with vector only or with Sema6A silencing lentivirus on glass chamber slides coated with gelatin solution (2 % gelatin solution not 0.5 %).

2. Grow cells until 50−60 % confluency and then remove the chamber slides chambered superstructure and silicone gasket.

3. Fix the HUVEC monolayer−containing slide with freshly prepared 4 % PFA for 10 min at room temperature.

4. Remove the PFA solution and wash the slides twice in PBS for 10 min at room temperature.

5. Permeabilize the cells for 20 min with 1.0 % Triton X−100. Then, wash the slides twice in PBS for 10 min at room temperature.

6. Block the slides with blocking solution for immunostaining for 1 h at room temperature and then remove the blocking buffer.

7. Dilute the primary antibody (rabbit mAb to cleaved caspase−3) in PBS−T containing 3 % BSA and add to the slide for 1 h at room temperature (or overnight at 4 °C) in a humidified chamber. The dilution of the primary antibody is predetermined by selection of optimal specificity/background ratios (start at 1:50 dilution).

8. Wash in PBS-T for three times at 15 min each at room temperature and then add the secondary antibody (Alexa Fluor 488−conjugated goat anti−rabbit IgG) in PBS−T containing 2 % BSA and incubate at room temperature for 1 h. The

dilution of the secondary antibody should be determined experimentally for optimal specificity/background ratios (start at 1:1000 dilution).

9. Wash in PBS−T for three times at 15 min each at room temperature and then mount the glass slides in DAPI−containing mounting fluid (for identification of nuclei).

10. Cleaved caspase−3−positive cells are visualized by fluorescent microscopy as the green cells and are quantified as the ratio of green cells/DAPI−positive blue nuclei.

3.6.2 Detection of Cleaved Caspase−3 by Immunoblotting

1. Cell lysates of Sema6A−silenced and control HUVEC are prepared, resolved, and transferred to nitrocellulose membrane, and blocked as described in Subheading 3.5, **steps 5−13**.

2. After washing membrane with TBS−T, add rabbit mAb to cleaved caspase 3 (at 1:1000 dilution).

3. After 18−h incubation and subsequent washing in TBS−T, add the HRP −conjugated donkey anti−rabbit IgG secondary antibody for 1−h incubation at room temperature (1:10,000 dilution).

4. Wash the membrane in TBS-T with orbital shaking for 15 min and visualize the bound antibody with a chemiluminescent HRP substrate as described in Subheading 3.5, **step 14**.

5. The presence of cleaved caspase−3 is reflected by the visualization of appropriate bands at the expected relative size of 17/19 kDa.

4 Notes

1. Primary endothelial cells are currently available commercially. The protocols for production of commercial HUVEC are proprietary and cannot be compared to the current protocol.

2. Culture media for primary endothelial cells are commercially available. In general, these media are provided in the form of kits that include a basal growth medium to which components are added, including supplements, growth factors, and cytokines.

3. HEK 293T cells are available commercially.

4. HEK 293T cells should not be confluent for optimal viral production.

5. Lentivirus titer gradually decreases during storage.

6. The volume of lentivirus containing culture supernatants to achieve >80 % cell infection is determined empirically.

7. Primary endothelial cells from different donors display variable puromycin resistance. We strongly recommend optimization of puromycin concentration

and duration of selection resulting in 100 % cell death of non−transduced primary endothelial cells but 0.5 μg/ml dilution of puromycin is a good starting concentration to us.

8. The lentiviral plasmids for silencing Sema6A, plexin A2, plexin A4, VEGFA, and VEGFR2 are available commercially. Protocols for construction of similar plasmids have been published [34].

9. Sodium orthovanadate is not toxic to endothelial cells at 100 μM, and increases cell density and yield of total protein. Endogenous intracellular VEGF−VEGFR2 signaling is undetectable by Western blotting without sodium orthovanadate.

10. The VEGF−neutralizing antibody (Avastin) is used herein to neu−tralize extracellular VEGF based on the following results: Avastin does not decrease phosphorylation of VEGFR2 (which is visualized by sodium orthovanadate); instead, a decrease of VEGFR2 phosphorylation is induced by VEGFA silencing or by incubation with an intracellular tyrosine kinase inhibitor Sorafenib.

11. Sorafenib is used herein to inhibit intracellular VEGF−VEGFR2 signaling based on the following results: Sorafenib can inhibit the phosphorylation of VEGFR2 (which is revealed by addition of sodium orthovanadate); VEGFR2 phosphorylation is also inhibited by VEGFA silencing.

12. The cell lysate in 1×LDS sample buffer is diluted (1:250) in PBS before performing MicroBCA Protein Assay. The BSA standard is also diluted in 0.004× LDS sample buffer.

13. For the details of SDS−PAGE, *see* the manufacture's web page for the NuPAGE Technical Guide (http://tools.lifetechnologies.com/content/sfs/manuals/nupage_tech_man.pdf).

14. The ability to probe a nitrocellulose membrane multiple times is based on the ability to completely remove bound primary and secondary antibodies, without compromising levels of proteins blotted onto the nitrocellulose membrane. To achieve this, the membrane is (a) soaked in PBS/0.1 % Tween (twice for 15 min each with fresh buffer on an orbital shaker at room temperature), (b) incubated in stripping buffer (Thermo Fisher Scientific) (twice with fresh solution for 5 min each on orbital shaker), and (c) washed in PBS/Tween (five times for 5 min each with fresh buffer on orbital shaker); and then blocked with blocking buffer (for nitrocellulose membranes).

15. Different lots of HRP−conjugated polyclonal second antibody differ somewhat in potency. We recommend that the fold dilution of your second antibody be optimized.

16. Chemiluminescence (emission of light from dissipation of energy from a chemical reaction) is used for detection of an HRP−labeled antibody (bound to a specific protein on a nitrocellulose membrane). HRP, in the presence of hydrogen peroxide, catalyzes the oxidation of diacylhydrazides such as luminol in alkaline conditions. Immediately after oxidation, luminol assumes an excited state

followed by decay to a ground state via emission of light. The amount of light and the length of light emission are variable depending on the addition of chemical enhancers, such as phenols. Many commercially available preparations are available for detection of HRP−based chemiluminescence.

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