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Characterization of the Effects of Semaphorin 4D Signaling on Angiogenesis

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Summary

The semaphorins and plexins comprise a family of cysteine-rich cell surface and secreted proteins originally shown to control nerve growth and the immune response, but that have recently been implicated in a wide variety of developmental and pathological processes that are influenced by cell adhesion and migration. Along those lines, our group and others have found that Semaphorin 4D (SEMA4D) plays an important role in angiogenesis by promoting chemotaxis of endothelial cells, which express its receptor, Plexin-B1. Indeed, some neoplasms produce SEMA4D along with other pro-angiogenic proteins for the purpose of enhancing blood vessel growth into a developing neoplasm. Here we describe the application of *in vitro* migration and tubulogenesis assays and the Directed *In Vivo* Angiogenesis Assay (DIVAA) in the measurement of the angiogenic potential of cell-derived and soluble SEMA4D.

Keywords

Semaphorin 4D; Plexin-B1; Angiogenesis; Endothelial cells; Chemotaxis; Tubulogenesis; Basement membrane extract

1. Introduction

The semaphorins and plexins are a group of proteins characterized by a large, cysteine-rich semaphorin domain that are known to control cell migration, adhesion, and hence the development and function of many different tissues, including the nervous system, the immune system, and the vasculature. Our group and others have demonstrated that Semaphorin 4D (SEMA4D) is chemotactic for endothelial cells, thereby promoting angiogenesis, the process by which new blood vessels arise from a pre-existing vasculature [1,2]. While angiogenesis can actively occur during normal growth and development, it is also an integral part of much pathology. Indeed, we have shown that SEMA4D is overexpressed by many different aggressive carcinomas, and that its activity on endothelial

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cells, which express its receptor Plexin-B1, promotes enhanced growth and vascularity of tumors [3].

The importance of angiogenesis for many pathological processes has meant a great deal of research effort is devoted to the signaling mechanisms that control it and more specifically towards its inhibition. However, a consistent challenge in all studies attempting to determine the pro- or anti-angiogenic potential of any compound is the quantification of these effects, both for *in vitro* assays that permit easy manipulation of experimental conditions and *in vivo* models for testing potential therapeutics. While no one angiogenesis assay can fully recapitulate this rather complex process, here we describe three assays used successfully to measure the pro-angiogenic potential of SEMA4D: *in vitro* migration and tubulogenesis assays using cultured human umbilical vein endothelial cells (HUVECs), and the Directed *In Vivo* Angiogenesis Assay (DIVAA), in immunosuppressed athymic (nude) mice.

2. Materials

2.1 *In vitro* trans-well migration assay

1. In vitro trans-well migration assay (*see* Note 1) is carried out in an acrylic NeuroProbe 48-Well Micro Chemotaxis Chamber (AP48, NeuroProbe).
2. Membranes used for the In vitro migration assay are polycarbonate track-etch (PCTE) membranes pretreated with polyvinylpyrrolidone (PVP) (PRB8, NeuroProbe), 8μ pore size.
3. HUVEC Cells (PCS-100–010, ATCC).
4. Endothelial Cell Growth Kit-BBE (PCS-100–040, ATCC): includes Vascular Cell Basal Medium (ATCC), 0.2% bovine brain extract, 5 ng/mL recombinant epidermal growth factor, 10 mM L-glutamine, 0.75 Units/mL heparin sulfate, 1 μg/mL hydrocortisone hemisuccinate, 50 μg/mL ascorbic acid, and 2% fetal bovine serum. Reserve a small amount of this medium with 10% FBS for neutralization of trypsin (see 3.1, step 9, below).
5. Serum-free endothelial growth media: the same media listed as contained within the Endothelial Cell Growth Kit-BBE (PCS-100–040, ATCC) but without the 2% fetal bovine serum.
6. Fibronectin Solution: Create a solution of 0.1% acetic acid by adding 1mL of acetic acid to 1 L of Phosphate Buffer Saline (PBS from a commercial supplier). Dissolve fibronectin (Gibco) to a concentration of 10μg/ml in this solution.
7. BSA Solution: dissolve 0.1g of bovine serum albumin (BSA) into 100mL of serum-free endothelial growth media to generate 0.1% BSA.
8. FGF (basic fibroblast growth factor, bFGF) positive control: dissolve FGF (R&D Systems) to a concentration of 100 to 400ng/ml in serum-free endothelial growth media.
9. HGF (hepatocyte growth factor) positive control: dissolve HGF (R&D Systems) to a concentration of 100 to 400ng/ml in serum-free endothelial growth media.

10. Fetal Bovine Serum (FBS) positive control: add 1mL of FBS to 10mL of serum-free endothelial growth media to generate 10% FBS.
11. SEMA4D solution: dissolve soluble SEMA4D (sSEMA4D; [1]) in serum-free endothelial growth media to a final concentration of 400ng/ml. Note that SEMA4D is not obtained commercially. We generate it by transfecting a his tagged construct designed for secretion (pSecTag2 (Invitrogen)) into 293T cells and then purify conditioned media with TALON affinity resin or similar nickel or cobalt-based exchange columns. Secretion is compared to a PSA control and quantification performed by comparing to a BSA standard curve on a silver stained gel [1].
12. Trypsin Solution: 0.05% Trypsin, 0.53mM EDTA in Hank's balanced salt solution (HBSS; Corning cellgro).
13. Diff-Quick Stain kit (Diff-Quick, Dade Behing)
14. Permount (Fisher Scientific)
15. Hemocytometer
16. Methanol
17. Image J (NIH)

2.2 Tubulogenesis assay

Some of the reagents used in this assay are also used (and listed) in Materials 2.1.

1. The cells used to carry out the tubulogenesis assay (*see* Note 2) are HUVEC (PCS-100–010, ATCC).
2. Growth factor reduced basement membrane extract (BME), either Matrigel (BD Biosciences) or Cultrex (Trevigen), without phenol red
3. 35mm, 6-well plates
4. Vascular endothelial growth factor (VEGF) solution: dissolve VEGF (R&D Systems) to 100ng/ml in serum-free endothelial growth media.
5. Glutaraldehyde fixative: dilute glutaraldehyde solution to 0.05% in PBS.

2.3. Directed *In Vivo* Angiogenesis Assay

1. The Directed In Vivo Angiogenesis assay (DIVAA) (*see* Note 3) is performed using athymic (nude) or other immunocompromised strains of mice, although this may work in immunocompetent animals such as those in the C57 background, as the angioreactors fail to elicit a strong inflammatory response.
2. Sterile surgical tools (forceps, scalpel, blunt ended dissection scissors, sutures or skin stapler for wound closure).
3. Ketamine 75–100 mg/kg intraperitoneal (IP), or other anesthetic.

4. The DIVAA Kit (Trevigen) contains 10mm long sterile, non-resorbable silicon tubes termed “angioreactors” (*see* Note 4). These should be kept clean and sterile for eventual placement in the experimental animals. The kit also contains dispersal buffer.
5. Reconstituted basement membrane material including growth factor reduced BME, either Matrigel (BD Biosciences) or Cultrex (Trevigen).
6. VEGF with reconstituted basement membrane substrate: create a solution of 5.56mg/ml VEGF (equal to 100ng VEGF in the 18μl angioreactors) in BME for a positive control, maintaining sterile conditions (keeping BME on ice to avoid early polymerization).
7. PBS with reconstituted basement membrane substrate: add an equal volume of PBS (as used in the positive control) to BME as a negative control, under sterile conditions and kept on ice.
8. sSEMA4D with reconstituted basement membrane substrate: create a solution of 400ng/ml sSEMA4D in BME, under sterile conditions and kept on ice.
9. FITC-labeled Griffonia lectin (FITC-lectin)
10. 96-well assay plate and fluorescent plate reader.

3. Methods

3.1. *In vitro* trans-well migration assay (*see* Note 1)

1. Prepare the PCTE membrane by coating a membrane (or several for storage and later use, see below) in the fibronectin solution by filling a small container or tissue culture plate with the solution and adding membranes, incubating overnight at 4°C on a rocker platform.
2. The following day, remove membranes, rinse in PBS and allow to air dry (this is best accomplished by pinning and hanging the membrane off of the edge of a Styrofoam container or other object where both sides are exposed to air, facilitating drying and preventing the membrane from sticking to any surfaces). Dried membranes can be stored at least a year at room temperature in a clean container for later use (*see* Note 5).
3. To prepare the acrylic NeuroProbe 48-Well Micro Chemotaxis chamber (Fig. 1A) for the migration assay wash in de-ionized water (*see* Note 6).
4. The migration assay is carried out over a short period of time and therefore does not need to be done under sterile conditions.
5. Wash HUVEC cells the day before the assay and incubate overnight in serum-free endothelial growth media. The cells should be subconfluent the day of the assay.
6. The wells of the lower member of the Chemotaxis chamber will contain the chemoattractant (Fig. 1A). Pipette a volume of 28μl of serum-free endothelial

growth media containing the chemoattractant of choice (or controls) into each well, which will fill the entire well and create a small convex bead of solution that will help reduce the risk of air bubbles entering the system when the membrane is placed over the lower half of the chamber. If any bubbles form at this stage the well should be suctioned out completely and re-filled until a bubble free bead is formed. Wells should be reserved for BSA solution as a negative control, and FGF or HGF [4,5] or 10% FBS as a positive control solution, in addition to wells containing the chemoattractant compound to be tested (such as SEMA4D solution). In evaluating sSEMA4D, we find that 400ng/ml concentration yields a good migration response for HUVECs (*see* Note 7).

7. Cut a small notch or other identifying mark into the membrane at one far corner, to help in orientation when the assay is completed.

8. Lay a dry, coated membrane on top of the chemoattractant wells, avoiding the creation of bubbles that might block diffusion of the chemoattractant or migration of cells. Place the rubber gasket (included with the chamber) over the membrane and the upper member of the chamber on top of this, sealing the two together by tightening the finger screws over the screw posts (Fig. 1A). The screws should be tightened symmetrically (in other words, screws opposite to each other) to place even pressure over the gasket, which will reduce the risk of bubble formation.

9. Use trypsin solution to trypsinize HUVEC cells from Step 5 and neutralize trypsin with endothelial cell growth media containing 10% serum. Centrifuge HUVECs at 100-140 × g for 4–5 min. and remove the media, adding back serum-free endothelial growth media for a wash. Re-spin at 100-140 × g for 4–5 min, remove the media and re-suspend the pellet in BSA solution, using a Coulter counter or hemocytometer to create a solution of about 50,000 HUVECs per 50μl. This is the volume to be added to the top chamber (which is a stock solution of 1×10^6 cells per ml). There are 48 wells, so while only 2,400μl of cells are needed, there should be extra volume available for any possible errors.

10. Add 50μl of the cell solution to the top chamber, being careful to pipette directly into the bottom of the well at an angle so that the end of the pipette tip rests against the wall of the well just above the membrane. Eject the solution rapidly while drawing up on the pipette tip in order to reduce the risk of air bubble formation. Be careful not to puncture a hole in the migration membrane with the pipette tip (if the membrane is broken that well cannot be reliably used). If air bubbles are suspected to have formed at the base of the upper wells against the membrane, lightly tap the chamber. Air bubbles rising to the top of the wells will not affect migration. The chamber is set up so the same conditions can be done in triplicate (the wells are arranged in groups of three) but any number of replications can be done.

11. Incubate at 37°C in a container kept humid with a damp paper towel for 6 to 8 hours. This should be adequate to elicit a migratory response in HUVECs to the

positive controls and sSEMA4D. Longer incubation times may require that the assay be set up under sterile conditions.

12. After incubation, disassemble the chamber and remove the membrane with forceps, being careful not to disrupt the cells on the portion of the membrane that had been facing down toward the chemoattractant wells (these are the cells migrating through the pores to the underside of the membrane in response to chemoattractant) (*see* Note 8). Immerse the membrane in 100% methanol for 2 minutes. At this stage, the chamber should be immersed in distilled, de-ionized water for storage and re-use (see above).

13. Stain the membrane with the Diff-Quick Stain kit according to the manufacturer's instructions. Briefly, this is done by dipping the membrane for 2 minutes in eosin, then 3 minutes in hematoxylin, followed by two washes in distilled water, being careful to not touch the spots containing migrating cells.

14. Place the membrane on a glass slide with the surface that had been facing the chemoattractant face down. Wipe the top surface gently with a wet Kim-wipe, rubber policeman or cotton swab to remove non-migrated cells on top of the membrane. Cover with another glass slide or coverslips secured with Permount. The stained cells that remain will be visible as spots on the membrane (Fig. 1B).

15. The readout for this assay is the migration of cells though the pores in the membrane and adherence of those cells onto the opposite side. Cells that migrate to the lower surface of the filter or that remain within the pores can be counted using light microscopy. We have found that scanning the membrane in a desktop scanner and then quantifying staining density of each spot using Image J or other appropriate quantitation software yields a more rapid and objective result (Fig. 1C).

3.2 Tubulogenesis assay (*see* Note 2)

1. To assay tube formation, coat 35mm plates with Cultrex BME by thawing out the stock solution (stored at −80 °C) on ice. Once thawed, pipette out 150 μl and coat the plates evenly, cover and allow the material to set in a tissue culture hood at room temperature. Count out and plate early passage HUVEC at a density of about 45,000 cells/cm^2 and incubate overnight in BSA solution (negative control), FBS positive control, or with a pro- or anti- angiogenic compound of choice (such as VEGF solution and SEMA4D solution).

2. 16 to 20 hours later cells are fixed in 0.5% glutaraldehyde and photographed (Fig. 2).

3. Quantification of results is determined using Image J, measuring and summing the length of all tubular structures observed in 10 random fields in a phase contrast microscope, for three independent experiments (*see* Note 9).

3.3 Directed *In Vivo* Angiogenesis Assay (*see* Note 3)

1. To prepare the angioreactors, make enough solution of reconstituted basement membrane substrate with PBS (negative control), VEGF (positive control), and sSEMA4D for the number of angioreactors that will be used, calculating the volume of each at 18μl and fill as described in Fig. 3A. Make a small amount of extra volume and prepare all solutions under sterile conditions and on ice to prevent the BME from setting/polymerizing.

2. Implant the angioreactors in ketamine anesthetized mice. Elevate the skin of the nude mouse, incise (but not deeply down into the underlying connective tissue or muscle) and bluntly dissect a small pocket horizontally along the plane of the skin with scissors or forceps creating a pocket large enough for insertion of two angioreactors side by side (Fig. 3B). This is done on both flanks, for four angioreactors per mouse. The incision is sealed with a suture, surgical staples, or surgical adhesive, being careful to maintain a clean and sterile surgical environment.

3. Over the next several days, blood vessels and supporting structures grow into the open ends of the reactors in a response that is modified by their content (Fig. 3B, **inset**) (*see* Note 10). After nine days the reactors are removed. Invasion of blood vessels into the reactors can be measured and recorded directly (Fig. 3C, **left panel**) and the basement membrane material and its cellular contents pushed through the open end of the reactor into an Eppendorf tube for processing and measurement in a plate reader.

4. The basement membrane material is digested in dispersal buffer (included in the DIVAA kit) so the cellular contents can be recovered, washed and incubated with FITC-lectin, a compound that binds to endothelial glycoproteins [6,7]. The endothelial cells that were in the reactor therefore will be bound with FITC-Lectin while unbound FITC-Lectin is removed following centrifugation and washing, following the manufacturer's instructions.

5. The relative cell number is then quantified by fluorescence in a plate reader as a representation of invasion of endothelial cells into the angioreactors [8]. Fig. 3C demonstrates the feasibility of this system and shows robust blood vessel growth into angioreactors in response to two different concentrations of the pro-angiogenic factors FGF and VEGF, relative to the negative control. Fluorescence measurements carried out on the contents of the reactor (in triplicate) graphically demonstrate the strong pro-angiogenic response (Fig. 3C, **right panel**). We have successfully used this system to measure the *in vivo* angiogenic potential of SEMA4D under different conditions and continue to use it in our recent publications [1,9–11].

Notes

1. Many eukaryotic cells exhibit chemotaxis, or cell movement towards a gradient of increasing chemical concentration [12], which serves as the basis for such

diverse processes as growth and development, healing, homing of immune cells towards sites of infection, trauma or inflammation, and angiogenesis. An *in vitro* assay used to test if a compound is chemotactic for a particular cell type is the Boyden chamber or trans-well migration assay [13]. The assay described here is a trans-well migration assay modified to test the pro- or anti-angiogenic potential of a compound by using HUVEC or other endothelial lines as the cells of interest [14]. Briefly, a solution of cells suspended in serum-free endothelial growth media are placed in a small well that is separated from a reservoir of chemoattractant below by a porous filter coated with the extracellular matrix protein fibronectin. A gradient forms by the chemoattractant compound diffusing out from the lower chamber. The difference in concentrations of the chemoattractant combined with its intrinsic qualities and the cell type and cell signaling pathways activated will determine the strength of the chemotactic response.

2. Endothelial cells form capillary-like structures *in vitro* when plated on a protein mixture secreted by Engelbreth-Holm-Swarm mouse sarcoma cells. This material is rich in collagen type IV and laminins, among other extracellular matrix proteins [15], and therefore is used as a surrogate for the basement membrane (and is sometimes referred to as reconstituted basement membrane extract (BME) in experimental applications). The formation of a vascular network by HUVECs and other endothelial cell lines on this substrate suggests the activation of a program of differentiation involving cell adhesion, migration and protease secretion, partially recapitulating many of the steps of vasculogenesis or angiogenesis, without actually eliciting any proliferation [16]. This assay therefore has the advantage of measuring multiple steps of angiogenesis, with results that can be quantified [15].

3. It has always been a challenge getting quantifiable results of angiogenesis *in vivo*. The standard technique has been the Matrigel plug assay, where a bolus of Matrigel BME containing a pro- or anti-angiogenic compound of choice is injected subcutaneously into a host animal, allowed to set, and then removed after a prescribed period of time to look for blood vessel in-growth as a measure of angiogenesis. This method has several drawbacks, the most serious of which is spread, leakage and loss of material when placed and resorption of the BME over time, making locating the plug at the conclusion of the experiment particularly difficult. Additionally, quantification, based upon hemoglobin content or histological examination of the plug for vessels is inexact, labor intensive, and highly subjective. A more recent advance for *in vivo* angiogenesis that solves the problem of material loss and resorption, while allowing for precise quantification of results, is the DIVAA.

4. In this assay, silicon tubes termed “angioreactors,” which have an opening at one end, are filled with BME containing the pro- (in this case, SEMA4D) or anti-angiogenic compound of choice, and implanted subcutaneously into host mice [8]. After a designated time period (usually 9 days) the rectors are excised and the basement membrane material and its contents processed with FITC-labeled

Griffonia lectin (FITC-lectin), a compound that binds to glycoproteins located on the surface of endothelial cells [6,7]. If endothelial cells are attracted to the contents of the reactor, newly formed blood vessels will grow into the open end and the endothelial content, and hence vascularity, can be quantified by fluorescence in a plate reader [8].

5. If using pre-coated, dried and stored membranes, rinse in PBS again and air dry before use in the assay.

6. Proteins and other contaminants accumulating on the walls of the wells of the chamber (Fig. 1A) can affect cell migration. It is best to soak the chamber in de-ionized water immediately after use. To better clean the chamber, soak in a solution of chlorine bleach and distilled de-ionized water for 30 to 60 minutes, followed by washing with distilled de-ionized water until all traces of bleach are removed. The chamber should not be autoclaved or the wells aggressively scrubbed, and should only be used when clean and dry. The rubber gasket (Fig. 1A) can be autoclaved or cleaned in de-ionized water. However, avoid immersing the gasket in chlorine bleach.

7. Migration toward a chemoattractant can be affected by using too high a concentration of a compound, which either desensitizes the receptors to further responses from the ligand, or becomes so high as to prevent the establishment of a proper gradient [17]. Use of too many cells may also give false positives, just as use of too few cells may yield a negative result, regardless of the chemoattractant. Careful use of positive and negative controls will help alleviate this problem, but results might have to be optimized under different experimental conditions.

8. It is important that no air bubbles are in contact with the membrane, which would block diffusion of chemoattractant and migration of cells. In addition, while it is possible that some cells may migrate completely though the membrane and fall into the lower chamber, (depending upon the cell type used [18]) this is not likely to be a significant factor for adherent cells such as HUVECs.

9. Visual assessment of tubes can be an accurate, qualitative way to determine the results of this assay, but for more accurate quantification, several methods can be used. We use Image J to measure tube lengths, but other labs use tube area, number of tubes, or the number of ‘nodes’ from which emanate the capillary structures. Endothelial cells can also be treated with antibodies after fixation and viewed under fluorescence microscopy [19].

10. The angioreactors are tubes of sterile silicone. They do not elicit an inflammatory response (which could otherwise alter an angiogenic response) and are non-resorbable, so they maintain their integrity in the subcutaneous compartment for the duration of the experiment (*see* Method 3.3, **Step 3**).

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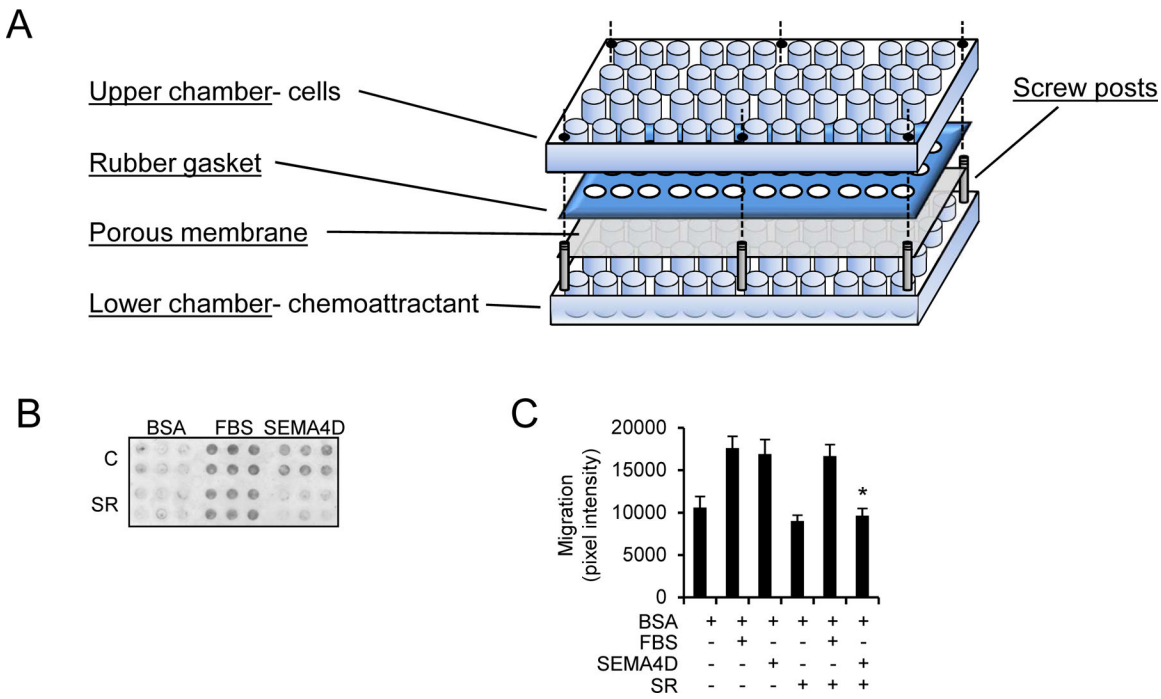

Figure 1:

A) Schematic showing the proper assembly of a 48-well migration assay chamber. The top member contains cylindrical wells traversing the entire thickness of the lid, with the base resting upon the migration assay membrane containing pores of 8μm diameter. The lower chamber contains wells open at one end, which hold the chemoattractant compound. Fasteners are attached to the screw posts between the upper portion and the migration membrane. A rubber gasket ensures a leak-proof seal. **B)** Stained migration assay membrane on HUVEC, control infected (C), or infected with the I-κB super-repressor (SR) towards BSA, FBS or SEMA4D. **C)** Quantification of staining, measured by scanning the membrane and determining pixel intensity with Image J *(18)*.

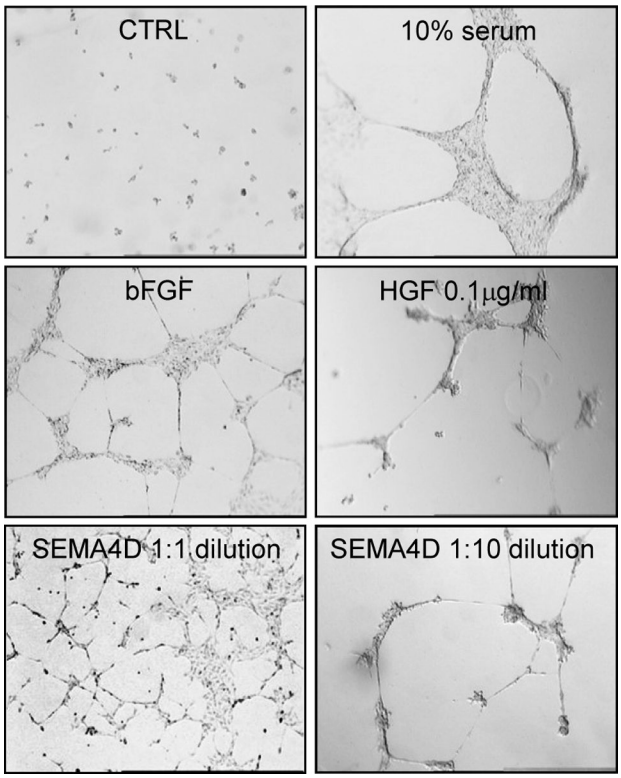

Figure 2:

Endothelial cells were grown in serum-free endothelial growth media with the indicated factors on a substrate of reconstituted basement membrane extract, then fixed and analyzed for tube formation. Media containing 0.1% BSA was used as the negative control (CTRL). Media with BSA and 150 ng/ml bFGF or 10% FBS were the positive controls. Media containing SEMA4D is shown at the indicated dilutions *(1).*

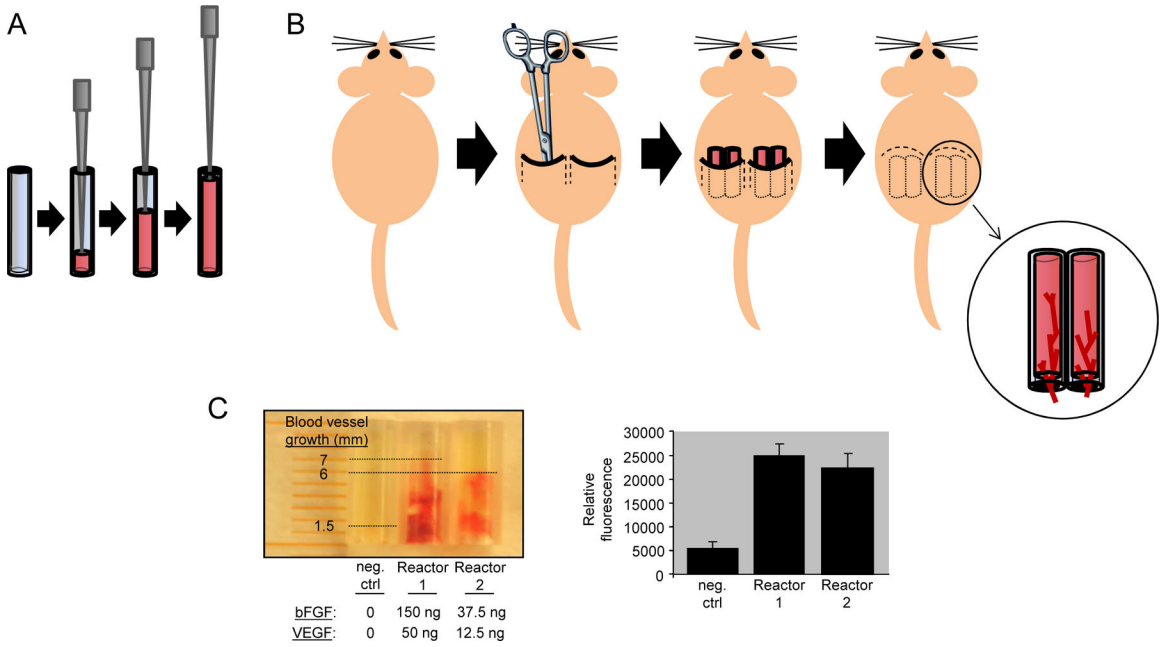

Figure 3:

A) Angioreactors are filled with reconstituted basement membrane material, with pipette tips initially placed at the bottom and drawn upwards as material is ejected, in order to avoid creating air bubbles. The reactors are kept on ice and under sterile conditions until ready to be surgically implanted. **B)** Surgical implantation of 4 reactors per mouse (two per flank) by creation of a subcutaneous pocket through blunt dissection. Over time, if attracted to the contents of the reactors, blood vessels will grow into the open ends (inset), and angiogenesis can be measured in a plate reader. **C)** In this sample experiment, reactors were removed from nude nice after 9 days. The reactors in the photograph (left panel) contain reconstituted basement membrane material with PBS (neg. ctrl), 150ng bFGF/50ng VEGF (Reactor 1) and 37.5ng bFGF/12.5ng VEGF (Reactor 2). Blood vessels have grown into the angioreactors up to 1.5 mm, 7 mm, and 6mm, respectively. The contents of the reactors were removed and processed for FITC-lectin fluorescence, measured in a plate reader (right panel).