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Supplemental Information

Effective Suppression of Vascular Network Formation by Combination of Antibodies Blocking VEGFR Ligand Binding and Receptor Dimerization

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Inventory

Supplemental Data:

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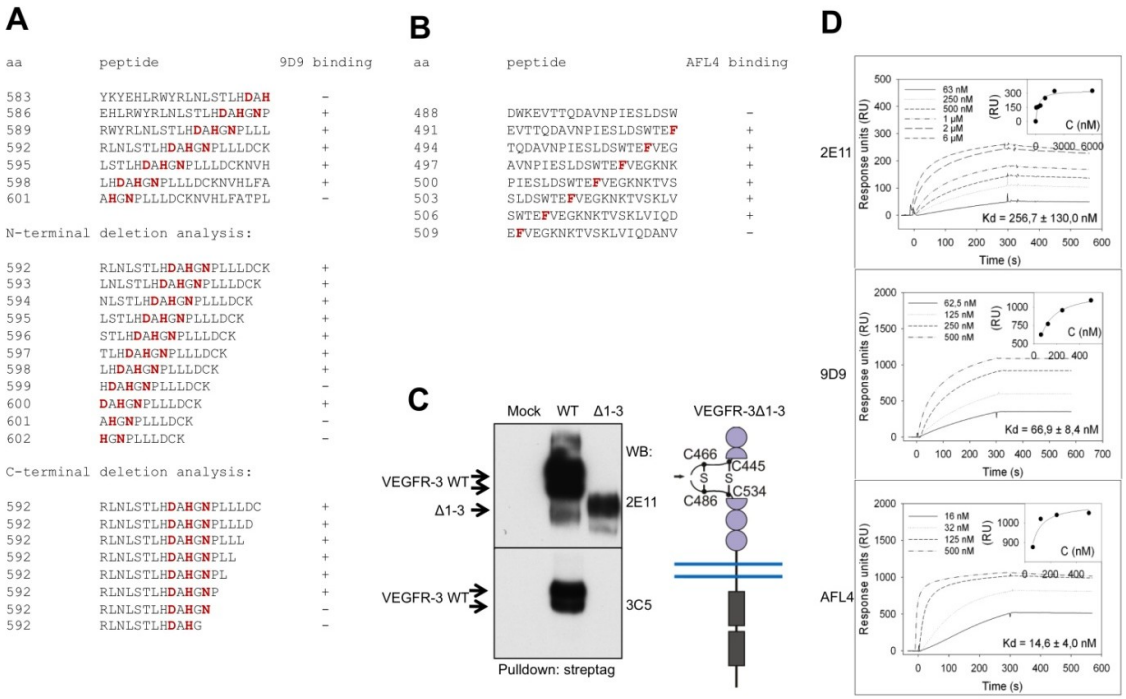
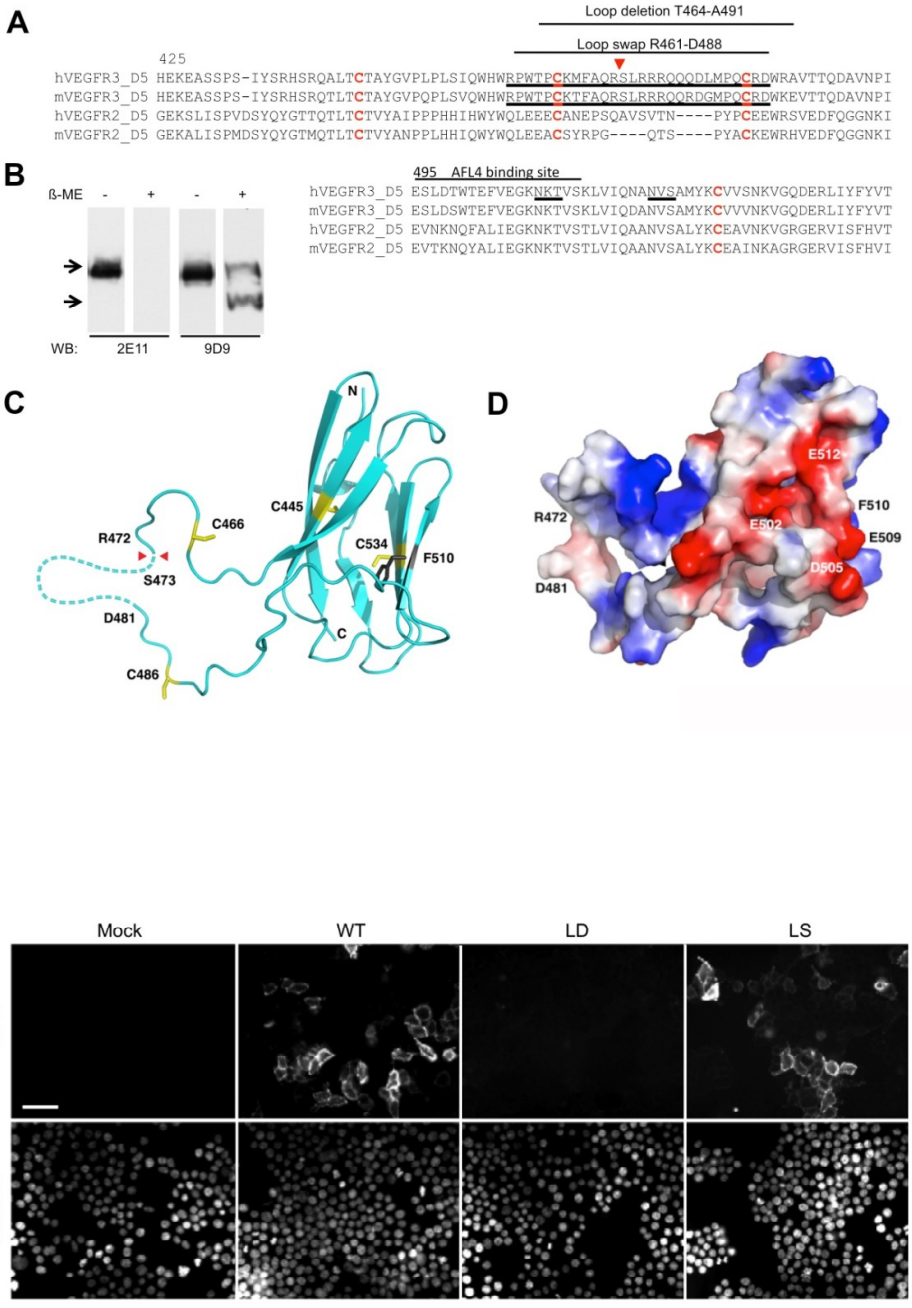
Supplemental Experimental Procedures



Tvorogov et al., Figure S1

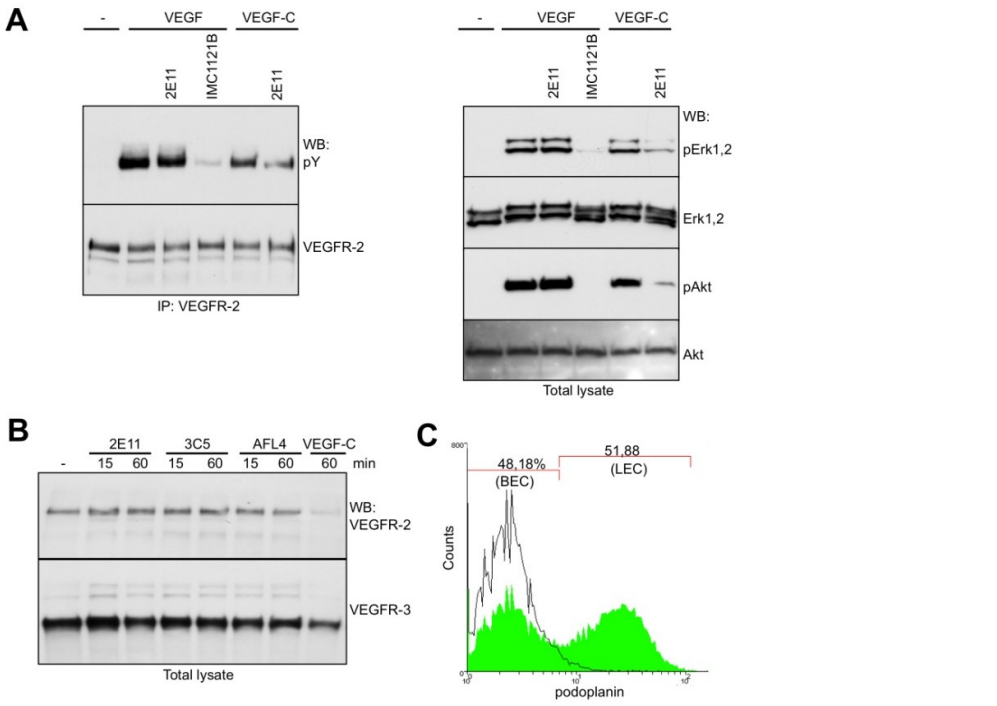
Figure S1. Epitope mapping and binding affinity of 2E11, 9D9 and AFL4 antibodies. (**A**) Peptide scan (SPOT) analysis of the whole extracellular domain of VEGFR-3 to detect the exact binding sites of antibodies 9D9, 2E11 and AFL4. Peptides of 20 aa length with a transition frame of +3 were spotted on cellulose membranes and binding of the antibodies to the membranes was assessed by immunoblotting. The 2E11 antibody did not display reactivity to a linear epitope (data not shown). For 9D9, strong binding (+) was found within a linear epitope comprising peptides covering the region from E586 to A617, which is located in D6. To find out the exact residues involved in the binding of 9D9, alanine scan and staggered end deletion analysis were employed (described in detail in the "materials and methods"). According to these analyses L598HDAHGNP605 turned out to be the minimal peptide epitope. The residues that lost 9D9 binding when mutated to alanine are marked in red. The crucial H602 corresponds to Q602 in the corresponding mouse sequence, thus providing an explanation for the species specificity of this antibody. Indeed, in our hands, 9D9 did not recognize murine VEGFR-3 in immunohistochemical stainings or in peptide scan analysis (data not shown). (**B**) Probing of a SPOTS membrane containing the mouse VEGFR-3 peptides with the AFL4 antibodies. Binding (+) was found within to the region from E491 to D525, which is located in D5. This was identical in the human VEGFR-3 sequence except for S506 was T506 in the human sequence. SPOTS analysis of the extracellular domain of human VEGFR-3 indeed showed that the AFL4 antibody binds to the corresponding human sequence (data not shown). (**C**) Mapping the 2E11 binding domain. Since it was impossible to locate the 2E11 epitope, we made VEGFR-3 receptor with deletion of first three extracellular domains (D1-D3) named VEGFR-3 Δ1-3. This construct was expressed in 293T cells along with WT VEGFR-3, both containing the StreptagIII at C-terminus. The proteins precipitated with streptactin beads, run on SDS-PAGE under non-

reducing conditions and blotted with the 2E11 and 3C5 antibodies. (**D**) Surface plasmon resonance analysis of the binding of monomeric VEGFR-3D1-7 to Mabs 9D9, 2E11 and AFL4.



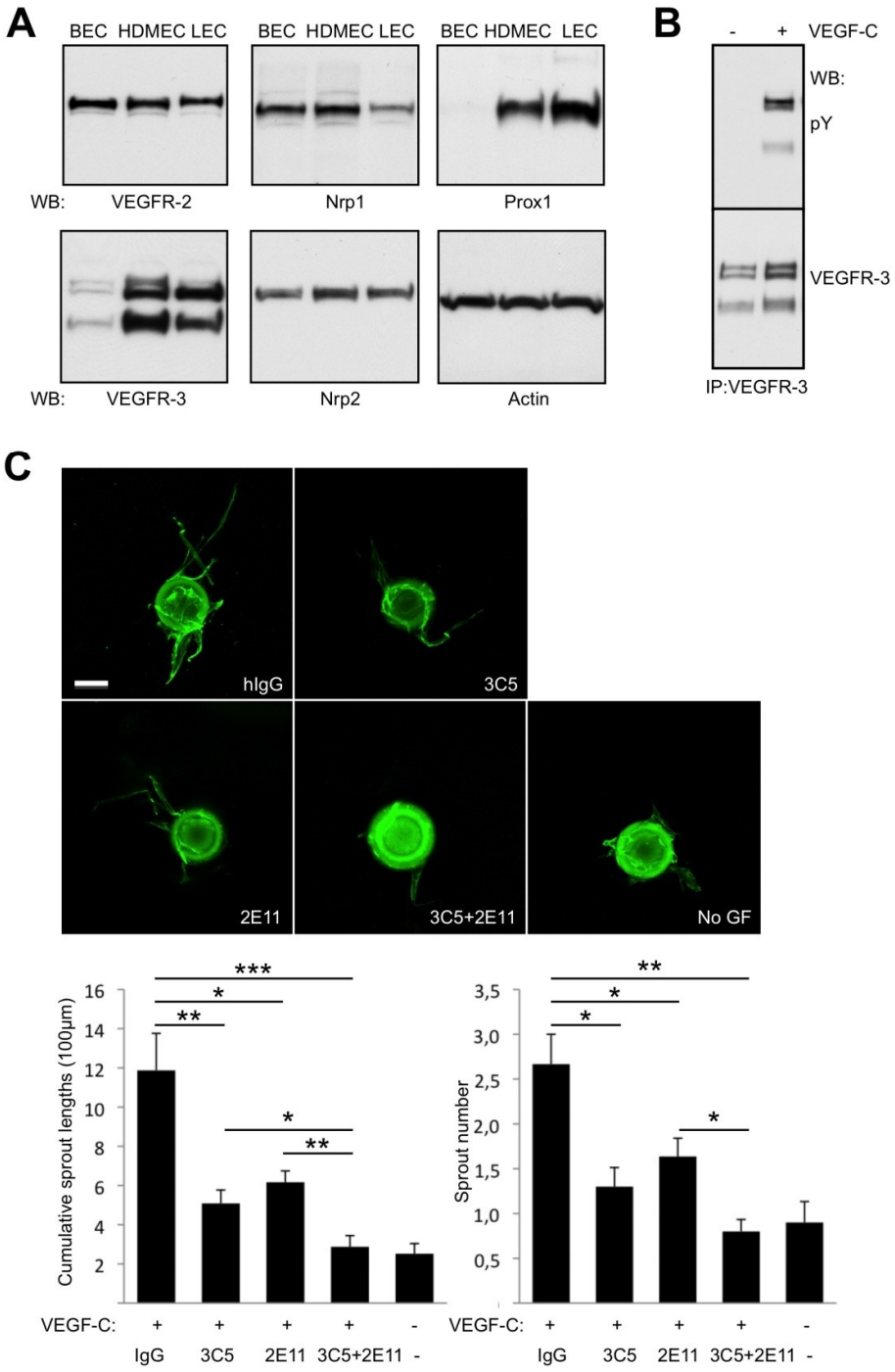
Tvorogov et al., Figure S2

Figure S2. Analysis of VEGFR-3 D5 epitopes. (**A**) Sequence alignment of D5 of human and mouse VEGFR-3 and VEGFR-2. The predicted extra loop and proteolytic processing site have been marked, as are the deleted and swapped sequences, plus the AFL4 binding peptide. The cysteine residues are marked in red and the two N-linked glycosylation sites have been underlined. (**B**) Sensitivity of the antibody epitopes to reduction of disulfide bonds. VEGFR-3-streptag III was stably expressed in 293T cells, precipitated with streptactin sepharose and analyzed by blotting with 2E11 and 9D9 antibodies under reducing and non-reducing conditions. (**C**) A three-dimensional VEGFR-3 D5 model (Phyre), based on the MyBP-C structure (PDB code 1GXE), with the cysteine residues highlighted in yellow. Note that in VEGFR-3 D5, C445 and C534 make a disulfide bridge typical for immunoglobulin (Ig) homology domains. C466 and C486 are far apart in the model but probably interact in VEGFR-3 D5. In MyBP-C, there are no counterparts for residues S473-Q480 (dotted line). R472-S473 is the identified protease cleavage site. In (**D**), the surface is colored according to the electrostatic potential (red = negative, blue = positive charge). Note that the acidic residues in the AFL4 antibody-binding site center around F510 (see also Figure S1). Notably, the loop area, including the residues missing from MyBP-C (SLRRRQQQ, dotted line) is positively charged. (**E**) Immunofluorescent staining of 293T cells transfected with WT, LD and LS VEGFR-3. 293T cells were transfected with different VEGFR-3 constructs and stained for VEGFR-3 with 2E11 antibodies as in Fig. 2C. Scale bar 20 µm.



Tvorogov et al., Figure S3

Figure S3. The 2E11 antibody does not inhibit VEGF-A induced VEGFR-2 phosphorylation or induce VEGFR-2 or VEGFR-3 downregulation. (**A**) Inhibition of VEGF-A and VEGF-C induced VEGFR-2 phosphorylation and intracellular signaling in HDME cells with 2E11 and IMC1121B (positive control) antibodies. HDME cells were pre-incubated with different antibodies for 15 min and then stimulated with VEGF-A or VEGF-C for 5 min for VEGFR-2 phosphorylation or 30 min for Erk1,2 and Akt phosphorylation. Lysates were either precipitated with polyclonal VEGFR-2 antibodies and blotted with pY antibodies or analysed for Erk1,2 and Akt phosphorylation. (**B**) Western blotting analysis of cellular VEGFR-2 and VEGFR-3 after ligand and antibody treatment. HDME cells were icubated with indicated antibodies for different time periods and the cell lysates were analyzed by blotting with VEGFR-2 and VEGFR-3 antibodies. Note downregulation by VEGF-C, used as a positive control. (**C**) Analysis of the HDME cells by anti-podoplanin immunofluorescence staining followed by flow cytometry. The percentages of positive (LEC) and negative (BEC) cells are indicated above the graph.



Tvorogov et al., Figure S4

Figure S4. Expression, phosphorylation and inhibition of VEGFRs (**A**) Expression of VEGFRs and neuropilins in the endothelial cells. VEGFR-2, VEGFR-3, Nrp1, Nrp2 and Prox1 levels were determined by western blot analysis in BECs, HDMECs and LECs. (**B**) VEGFR-3 phosphorylation in BECs stimulated with 25 ng/ml of VEGF-C. The cells were immunoprecipitated with VEGFR-3 antibodies followed by western blot with pTyr and VEGFR-3 antibodies. (**C**) Antibody inhibition of LEC sprouting. Shown are PECAM-1 stained immunofluorescent images of LECs sprouting from microbeads in the presence of 3C5, 2E11 or the combination, and statistical evaluation of sprout length and number. Note that the antibody combination decreases sprout length significantly more than either antibody alone, and a similar trend is seen for the number of sprouts. *$p<0.05$, **$p<0.01$, ***$p<0.001$ compared to hIgG. Error bars represent +/- SEM.

Supplemental experimental procedures

Epitope mapping. The extracellular domains of mouse and human VEGFR-3 were synthesized on a membrane PVDF filter as peptide spots of 20 amino acid residues in length, with a frame shift of three. Alanine scanning analysis was done for the following peptides (comprising the binding region of 9D9): EHLRWYRLNLSTLHDAHGNP, RLNLSTLHDAHGNPLLLDCK, LDHAHGNPLLLDCKNVHLFA. For deletion analysis, these peptides were reduced by one amino acid residues from the N- or C-terminus down to the length of six residues. All peptides were immobilized as spots on cellulose membranes and stored at -20° C. After thawing, the membranes were equilibrated in methanol for 10 min at room temperature. The membrane was washed three times in Tris-buffered saline (TBS, pH 8.0) for 10 min and then incubated overnight in blocking buffer (TBS containing 0.05 % Tween-20, 5 % non-fat dry milk and 5 % sucrose). On the next day the membrane was washed three times for 10 min in TBST (TBS with 0.05 % Tween-20), followed by incubation with the primary antibody (1 µg/ml in blocking buffer) for 1 h. The membrane was then washed three times with TBST and then incubated with the second antibody (anti-human IgG-HRP; Dako; 1:10,000) in blocking buffer. Then the membrane was washed two times with TBST and then again two times with PBS. The spots were visualized by chemiluminescence (Pierce). Before incubation with another antibody, the membrane was regenerated as follows: incubations (three times for 10 min) were done with: TBST, regeneration buffer A (48 % urea, 1 % SDS, 0.1 % □-mercaptoethanol), regeneration buffer B (50 % ethanol, 10 % acetic acid), DMF (N'N'-dimethylformamide) and methanol.

FACS analysis. Cells were detached with the narrow-spectrum proteinase Accutase (PAA, Laboratories) to avoid degradation of VEGFR-3 and incubated at + 4 ^{o}C with the 2E11 or

9D9 antibodies (5 µg/ml) for 30 min. After washing three times with cold phosphate buffered saline (PBS), the cells were incubated with anti-mouse Alexa488 antibody (Molecular Probes, 1:400) at + 4 ^{o}C, washed again three times in cold PBS, fixed in paraformaldehyde for 30 min at room temperature and subjected to FACS analysis in a Becton Dickinson LSR flow cytometer.

Binding affinity measurements by surface plasmon resonance. The binding of monomeric forms of VEGFR-3 D1-7 to Mabs 9D9, 2E11 and Afl4 were analyzed with surface plasmon resonance in the Biacore 2000™ biosensor (GE Helthcare). CM5 biosensor chip flow cells were covalently coated either with the VEGFR-3 variants in studying the Mabs as mobile phase analytes, or vice versa to obtain binding affinities of VEGFR-3 analytes to the immobilized Mabs. The coatings were done via standard amine coupling chemistry to 2,000 resonance units (RU) of the receptors or 1,000 RU of Mabs. The bindings were analyzed in HBS running buffer (10 mM Hepes, pH 7.4, 150 mM sodium chloride, 1 mM EDTA, 0.005 % surfactant P-20) by varying the analyte concentrations (16–500 nM of VEGFR-3 proteins and 63-6000 nM of Mabs). The contact time of analytes to ligands was 5 min and the flow rate 20 µL/min. The flow cells were regenerated after every injection with 10 mM glycine, pH 1.7. The data were evaluated by first subtracting the sensorgram obtained from the empty flow cell from the sensorgrams of the flow cells containing the ligands. The steady-state binding levels (RU) over analyte concentrations were plotted and fitted (SigmaPlot 8.0 software package) assuming 1:1 binding, for which the dissociation constant, Kd, and standard variations were derived.

Spheroid assay with LEC infected with KSHV. Wild type (wt) Kaposi sarcoma herpesvirus (KSHV) was produced from the naturally KSHV-infected primary effusion cell line BCBL-1

(NIH AIDS Research and Reference Reagent Program (Cat# 3233 from McGrath and Ganem) induced with 20 ng/ml PMA. The supernatant was collected after three days by ultracentrifugation (Beckman SW28.1 rotor, 21,000 rpm at 4^{o}C for 2 h), and resuspended in TNE buffer (150 mM NaCl, 10 mM Tris pH 8, 2mM EDTA, pH 8). For infection, the LECs grown in EC culture medium plus supplement pack (PromoCell) with additional 5% human serum (Sigma) were split at a density of $2.5x10^5$ cells per a 6-well, and spin-infected with KSHV at MOI of ~3 in serum free EC medium supplied with 8μg/ml polybrene.

Confluent monolayers of LECs infected with KSHV for 6-8 days (K-LECs), and grown in basal EC culture medium plus supplement pack (PromoCell) for 12 to 24 hours were seeded into 0.5% agarose pre-coated, non-adherent round-bottom 96-well plates at 4000 cells per well with or without VEGF-C (100 ng/ml). During the formation of spheroids the medium was also supplemented with either 10 □g/ml control IgG, 10 □g/ml 2E11 or 10 □g/ml 3C5 antibodies or with the mixture of 2E11 and 3C5 (5 □g/ml+5 □g/ml). After 16 to 24 h incubation at 37°C, the formed spheroids were harvested and embedded into fibrin gel consisting of plasminogen-free human fibrinogen (final concentration 3 mg/ml; Calbiochem) and human thrombin (final concentration 2 U/ml; Sigma) in 50 μl Hank's Balanced Salt Solution supplemented with 400 μg/ml aprotinin (Sigma). The gels were cast onto the bottom of 24-well plates and incubated for 1-2 h at 37°C to allow complete gelling followed by addition of the EC culture medium supplemented with the same combinations and concentrations of antibodies as described above. The sprouting was followed by phase-contrast microscopy for 3 days. To quantify the sprouting of the spheroids in duplicate wells the number of sprouts per spheroid was determined from phase contrast images acquired with a Zeiss Axiovert 200 epifluorescence microscope (eight to 16 spheroids were quantified per condition). Next the average length of the sprouts was determined using Zeiss AxioVision 3.1

software from the same phase contrast images. Relative sprouting was obtained by multiplying the number of sprouts with the average length of the sprout.

In vivo Matrigel plug assay. 50 000 of BECs or LECs transfected with lentiviral Cherry-fluorescence vector were diluted in growth factor reduced Matrigel (BD Biosciences) containing 200 ng/ml of VEGF-C and 5 µg/ml of blocking antibody as single treatment or 2.5 µg/ml of each blocking antibody as combination, and injected intradermally to the mouse ear of NOD SCID gamma mice (Jackson Laboratories) in a volume of 30 µl. Mice were injected intravenously with 1 mg/kg of blocking antibodies every day. The mice were sacrificed 10 days following plug implantation by intracardiac perfusion with 1% paraformaldehyde (PFA). The plugs were dissected and processed for frozen sectioning. Samples were mounted with Vectashield (VectorLabs) and analyzed with a confocal microscope (Zeiss LSM 510 DUO, 10x objective with a numerical aperture of 0.4) by using multichannel scanning in frame mode. Three-dimensional projections were digitally constructed from confocal z-stacks. The color images were converted to 8-bit grayscale using Adobe PhotoShop software (San Jose, CA). The images were then exported to ImageJ software for quantification of the area covered by BECs or LECs (mCherry positive area), which was divided by the number of individual BEC or LECs clusters to yield the median cluster size in pixels. Statistical analysis was carried out using one-way ANOVA; a *P*-value of less than 0.05 was considered to be statistically significant.

MTT cell survival assay. Serial dilutions of the antibodies were mixed with predetermined amounts of human full-length VEGF-C (250 ng/ml) in 50 µl volumes in 96-well plates. Twenty thousand BaF3-VEGFR-3 cells in 50 µl were then added and the plates were incubated at 37° C for 2 days. All assays were in triplicates. At the end of the incubation

period, 10 µl of MTT substrate (Sigma; 5 mg/ml in PBS) was added and incubated for additional 2 h. One hundred microliters of lysis solution (10 % SDS, 10 mM HCl) was then added to every well, and the plates were incubated overnight at 37° C, followed by determination of the optical density at 540 nm in a Bichromatic plate reader (Labsystems). All experiments were repeated at least three times and gave similar results.

3D bead sprouting assay. Cytodex 3 microcarrier beads (GE Healthcare) were coated with endothelial cells (400 cells per bead) in endothelial growth medium -2 MV (EGM-2 MV, Lonza), and embedded in 2 mg/ml fibrin gels in 48-well plates by mixing 2 mg/ml fibrinogen (dissolved in Hank's Balanced Salt Solution), 1.25 U/ml thrombin, and 150 ng/ml aprotinin. Endothelial growth medium (EGM-2, Lonza) containing lung fibroblasts (WI-38, 11 000 cells per well) was added to each well in the presence of human VEGF-C (hVEGF-C, 150 ng/ml), HSA conditional medium, anti-VEGFR-3 (7 µg/ml), anti-VEGFR-2 (7 µg/ml), or their indicated combinations. The cultures were maintained for 6-9 days by changing the medium every other day before fixation with 4% paraformaldehyde (PFA) for 1 h at room temperature (RT). Bright field images were captured with Axiovert 200 (Zeiss) and sprout lengths were measured with Image J.

Migration assays. Polycarbonate transwells (6.5 mm diameter, 8 µm pore diameter) were coated on the underside with 10 µg/ml gelatin overnight at 4°C. Nonspecific binding sites were blocked with heat-denatured 1% BSA in PBS for 1 hour at 37°C. Cells were then trypsinized and washed with DMEM containing 0.5 mg/ml trypsin inhibitor. The cells were counted and $10x10^4$ cells were added to each transwell and allowed to attach and migrate for 4h at 37°C. Afterwards, the top of each chamber was cleaned with a cotton swab to remove

all cells. The cells remaining on the underside were fixed and stained with crystal violet and four randomly chosen fields from triplicate wells were counted at 200x magnification.

Western blotting and immunoprecipitations. For immunoprecipitation and Western blotting, the cells were lysed in 1 ml PLCLB lysis buffer (150 mM NaCl, 5 % glycerol, 1 % Triton X-100, 1.5 M $MgCl_2$, 50 mM HEPES, pH 7.5) supplemented with 1 mM sodium orthovanadate, 2 mM phenylmethylsulphonyl fluoride (PMSF), 2 µg/ml leupeptin and 0.07 U/ml aprotinin. Cleared lysates were incubated with 2 µg primary antibody for 2 h. Subsequently, the immunocomplexes were captured using protein G-sepharose. After three washing steps in PLCLB buffer, the proteins were separated in 7.5 % polyacrylamide gels under reducing or non-reducing conditions. After blotting of the proteins to nitrocellulose membranes and blocking of the membranes in 5 % BSA, the filters were probed with the monoclonal antibodies (0.5 µg/ml). After incubation with the second-step HRP-coupled antibodies (Dako) the signal was visualized by chemiluminescence (Pierce).