# SUPPLEMENTAL MATERIAL

## Expanded Methods

### Construction and production of the rAAV vectors.

The rAAV encoding VEGF165 was published previously.[1] rAAVs encoding Ang1 and Ang2 were constructed in an analogous fashion. For the VA1 vectors, the fibrinogen-like domain (FLD) of human Ang1 was PCR-amplified using primers FLD-F (5'-TCGGTAGACCGAAGAAAGATAGAAGAGACTGTGC AGATGTATATC-3') and FLD-R (5'-CTCGGTAGACGAATTCTCAAAAATCT AAAGGTCGAATC-3'). To PCR-amplify FLD with a linker, FLD-linker-F primer (TCGGTAGACCGAAGAAAGATAGAATGGACACAGTCCACAACCT) and the above reverse primer were used instead. Both PCR products (containing 5' AccI site and 3' EcoRI and AccI sites) were digested with AccI and ligated into pKO-A165-AccI vector containing the VEGF homology domain (VHD) of VEGF, flanked by 5' EcoRI and 3' AccI sites. The resulting plasmid was opened with AccI and dephosphorylated. VA1 or VA2 inserts from the resulting plasmids were excised with EcoRI, blunted and ligated into the PmlI-opened psubCAG-WPRE rAAV vector (derivative of psubCMV-WPRE,[2] where the CMV promoter was replaced with the composite CAG promoter, consisting of the chicken beta-actin promoter, cytomegalovirus enhancer and beta-actin intron [3]). Production of rAAVs was carried out as previously described.[1]

**Protein expression and purification.**

Angiopoietin-1, produced in mammalian cells or overexpressed in mice and COMP-Ang1 protein, purified from CHO cells,[4] had similar biological activities and are collectively called here as Ang1. Purified VA1 protein with a C-terminal His-tag was expressed in Sf21 insect cells using the baculovirus expression system. The expression construct was obtained by PCR-amplification using the rAAV-expression construct as the template. Primers used for the amplification were: 5'-CGCGGATCCCGGTCCGAAGCGCGCGGAATTCAAAGGAGCTTTTCGCCACC ATGGAGACAGACACACTCCTGC-3' (forward) and 5'-CGCGGTACCTCAATGA TGATGATGATGATGAAAATCTAAAGGTCGAATCATCATAGT-3' (reverse). VA1 protein was expressed and purified as in [5].

For binding assays, VEGFR-2 domains 2+3 (R2D2-3; residues 118 to 326),[7] VEGFR-1 domains 1-2 (R1D1-2, residues 23-224) and Tie2 ligand-binding domains (LBD, residues 1-444) were cloned into the pFastBac baculovirus expression vector with a C-terminal Fc-tag (human IgG1; VEGFR-1 and -2) or with a C-terminal Histidine-tag (Tie2). The constructs were expressed in Sf21 insect cells and purified by Protein A Sepharose or $Ni^{2+}$-affinity step (GE Healthcare) followed by gel filtration on a Superdex 200 column in 10 mM HEPES, 0.1 M NaCl at pH 7.5. A Factor Xa cleavage site allowed the proteolytic Fc-tag removal and the preparation of the monomeric VEGFR-1 D1-2 construct.

**Affinity measurements**.

Isothermal calorimetric titrations of VA1 protein to soluble VEGFR-2 (D2-3, Fc-fusion), Tie2 (LBD, His-tagged) and VEGFR-1 (D1-2, monomeric) were carried out at 25°C using a VP-ITC calorimeter (MicroCal, GE Healthcare, Waukesha, WI). To control for heat dilution effects, all the protein buffers were adjusted to Hepes buffered sodium chloride at pH 7.5 by gel filtration. The receptor constructs were used in the calorimeter cell at a concentration of 5-8 μM, and the VA1 ligand in the syringe at a concentration of about 0.10 mM. Data were processed using the MicroCal Origin 7.0 software.

**VE-cadherin phosphorylation/internalization and Src phosphorylation assays**.

To measure VE-cadherin tyrosine phosphorylation, HUVECs were grown to confluence, serum-starved and stimulated with the indicated proteins (500 ng/ml of each) for 5 min. The cells were then fixed and permeabilized with 3% paraformaldehyde (PFA), 0.5% Triton-X100 (Tx) in PBS for 3 min followed by further 15 min fixation with 3% PFA in PBS. Affinity purified rabbit antibody to VE-cadherin P-Tyr658 peptide was produced and purified by New England Peptide. Antibody (10 μg/ml in PBS containing 1% BSA) was further purified by three absorption cycles on VE-cadherin null ECs (30 min VEGF stimulated, fixed, permeabilized and blocked with 5% BSA) for 1 h at room temperature (RT). Donkey antibodies to the appropriate species conjugated with Alexa-Fluor 555 (Molecular Probes, Invitrogen, Carlsbad, CA) were used as secondary antibodies. The primary and secondary antibodies were diluted in 5% BSA, 5% donkey serum in PBS. Epifluorescence microscope (Leica Microsystems GmbH, Wetzlar, Germany) images

were captured using a CCD camera. Confocal microscopy was performed with a Leica TCS SP2 microscope with 40X oil immersion objective (Leica Microsystems). For quantification, the intensity of fluorescence signal for VE-cadherin P-Tyr658 was evaluated using ImageJ software (The National Institutes of Health, Bethesda, MD) after segmentation with the triangle algorithm.

VE-cadherin internalization assay was performed as previously described.[6] Briefly, to measure internalization of endogenous VE-cadherin, HUVECs were treated with chloroquine 300 µM for 3 h. Cells were incubated with mouse monoclonal BV-6 antibody (mAb) directed against the VE-cadherin extracellular domain (Millipore, Billerica, MA) for 1 h at 4oC in MCDB131 buffer, supplemented with 1% BSA medium. Cells were then rinsed with ice-cold MCDB131 to remove unbound antibody and treated with the indicated proteins (500 ng/ml of each) for 5 or 30 min. The cells were then lysed and surface VE-cadherin was immunoprecipitated, followed by western blotting for VE-cadherin with the same mouse monoclonal BV-6 antibody.

Src (Tyr416) phosphorylation was determined by western blotting in total cell lysates of HUVECs, cultured and stimulated as described above for VE-cadherin internalization studies, using rabbit monoclonal anti-phospho-Src (Tyr416) antibody (Cell Signaling Technology Inc., Danvers, MA). Total Src was determined using rabbit monoclonal anti-Src antibody (Cell Signaling Technology Inc.). In this experiment, HRP-generated chemiluminescence was acquired and recorded using the Chemidoc system (Bio-Rad).

**MTT cell survival assay and analysis of protein expression by IP**.

For analysis of protein expression, supernatants containing the growth factors of interest were produced by rAAV vector transfection of 293T cells using the jetPEI transfection reagent (Polyplus-transfection). Proteins were metabolically labeled with [$^{35}$S]Cys/Met (Amersham Biosciences and GE Healthcare), [$^{35}$S]-labelled growth factors were precipitated with VEGFR-1-Ig, VEGFR-2-Ig or VEGFR-3-Ig [7, 8] or Tie2-Ig (R&D Systems, Minneapolis, MN) and protein A sepharose, and analyzed with SDS-PAGE and autoradiography.

hVEGFR-1/EpoR-BaF3 and mVEGFR-2/EpoR-BaF3 cells are derived from the IL-3-dependent mouse pro-B cell line, BaF3.[9,10] These cells undergo apoptosis in IL-3-deficient medium, but they can be rescued by the addition of the respective VEGFR ligands. hVEGFR-2/EpoR-BaF3 were made in a similar way (Dr Michael Jeltsch, University of Helsinki, PhD, unpublished data, 2011). For analysis of growth factor activity, 50 µl of serial dilutions of conditioned media (or purified proteins), as well as positive and negative controls, were applied to the wells of 96-well plates in triplicate. Subsequently, 20,000 VEGFR-1/EpoR-BaF3 cells in 50 µl were added to each well and the cells were incubated at 37$^{o}$C for 48 h. MTT substrate was added, and the cells were incubated at 37$^{o}$C for 2 h, lysis buffer (10% SDS, 10 mM HCl) was added, and the plates were incubated at 37$^{o}$C overnight for color development. Quantification was done by absorbance at 540 nm. When the mammalian cell supernatants were used, the small values obtained with conditioned medium from untransfected cells were subtracted.

**Immunocytochemistry.**

HUVECs (PromoCell) overexpressing Tie2 fused in-frame to green fluorescent protein (HUVEC-Tie2-GFP) were made by transfection with a Tie2-GFP retrovirus, as described.[11] In an analogous way, BECs (PromoCell) overexpressing human VEGFR-2 fused in-frame to cyan fluorescent protein CFP (BEC-VEGFR-2-CFP) were made by transfection with a VEGFR-2-GFP retrovirus. The cells were cultured in complete endothelial cell growth medium MV (PromoCell) on coverslips. Stimulation with the indicated factors was done for 45 min. The samples were analyzed with a LSM 510 Meta confocal microscope equipped with digital camera (Carl Zeiss AG, Oberkochen, Germany; objectives 40x Plan-Neofluar 1.30, DIC or 63x Plan-Neofluar 1.25, DIC, Ph3; acquisition LSM software release 3.2).

**Biotinylation experiments.**

BECs were starved o/n in serum-free medium, stimulated with growth factors as indicated and treated with Sulfo-NHS-LC-Biotin (Thermo Fisher Scientific, Rockford, IL), as recommended by the manufacturer. After the treatment, the cells were lysed (1% Triton X-100 in PBS, supplemented with protease inhibitors), biotinylated proteins were precipitated by using the streptavidin-agarose resin (Thermo Fisher Scientific) and analyzed by SDS-PAGE and western-blotting with goat anti-human Tie1, Tie2 or VEGFR-2 antibodies (R&D Systems).

**Ubiquitinylation experiments.**

BECs were starved o/n in serum-free medium, stimulated as indicated and lysed (1% NP-40 in 40 mM Tris-HCl, pH7.4, 150 mM NaCl, supplemented with protease

inhibitors). VEGFR-2 or Tie2 were immunoprecipitated with polyclonal antibodies (R&D Systems). Precipitated proteins were run in SDS-PAGE and analyzed by Western blotting using mouse monoclonal antibodies against polyubiquitin-conjugated proteins (Enzo Life Sciences).

**Cross-linking experiments.**

BECs were starved for 3 h, treated with the indicated proteins for 20 min, and then with DTSSP (3,3'-Dithiobis(sulfosuccinimidyl propionate)) for 30 min. The cells were lysed, solubilized proteins were immunoprecipitated with anti-VEGFR-2 antibodies and Western-blotted with anti-Tie2.

**Tissue transduction with the rAAV vectors.**

For transduction of the ear skin, six to seven week-old female C57BL/6 mice were anesthetized with xylazine and ketamine and 5 x $10^9$ rAAV particles (in 10 µl volume) were injected into each ear. One week after the injections the mice were perfusion-fixed, and the ears were harvested, PFA-fixed and whole-mount stained.

**Quantification of protein expression in transduced skeletal muscle.**

rAAV-transduced muscles were weighed and homogenized in 0.2% Triton X-100 in PBS supplemented with protease inhibitors, using PowerLyzer™ 24 Bench Top Bead-Based Homogenizer (MO BIO Laboratories Inc., Carlsbad, CA). A ninety-six well maxisorp plate (Nunc, Thermo Fisher Scientific) was coated with 10 µg/ml hVEGFR-1-Ig[6] for VEGF or Tie2-Ig (R&D Systems) for VA1 in PBS, blocked with 5% BSA in TBST, after which the samples were applied and incubated for 2 h at RT. After extensive washing with TBST, the bound proteins (VEGF and VA1) were

detected with goat anti-human VEGF antibody AF293 (R&D Systems), diluted in 2.5% BSA in TBST, followed by washing and incubation with rabbit anti-goat HRP conjugate (Dako, Glostrup, Denmark). Excess conjugate was washed away with TBST and the substrate SureBlue (KPL Inc, Gaithersburg, MD) was added for color development. The reaction was stopped by addition of an equal volume of 1M HCl, and OD at 450 nm was determined. The calibration curve was made with pure VA1 protein, made in S2 cells (see above), or VEGF (R&D Systems).

**Whole-mount staining of mouse ears.**

Separate ear leaflets, pinned to Sylgard plates were fixed with 4% PFA, washed with 0.3% Triton-X in PBS and blocked with donkey immunomix. Primary antibodies and secondary antibody-Alexa fluor conjugates (Moecular Probes, Invitrogen) were also diluted in donkey immunomix.

**Acute skin permeability test.**

FvB/N mice were anesthetized and injected i.v. with 300 µl of 1% EvansBlue. Back skin on the back was shaved and 20 µl of VEGF or VA1 in PBS (500 ng/µl) was injected into the dermis. After 1 hour, the mice were perfused with 10 ml of PBS via cardiac perfusion, skin pieces were excised, photographed and weighed. The dye was extracted overnight with deionized formamide (1 ml per a muscle) and measured at OD620 nm.

**Doppler ultrasound measurement of blood perfusion.**

Doppler ultrasound (VEVO 770 Micro-Ultrasound System, VisualSonics Inc., Toronto, Canada) was used to analyze blood perfusion in the transduced healthy *t.a.* muscles of anesthetized mice two weeks post-transduction. Image stacks were

generated by three-dimensional scanning, and percent vascularity in the muscle mid region was analyzed using the VEVO 700 analysis software (VisualSonics).

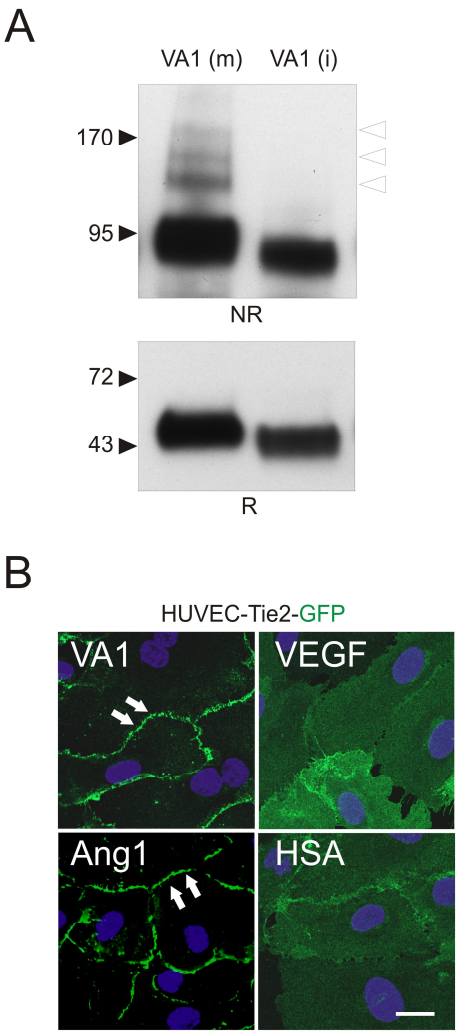
**Mouse hindlimb ischemia model with rAAV delivery.**

CAnN.Cg-Foxn1 nu/CrljOri nude male mice were obtained from Orient Bio, Korea. All mice were 6-12 weeks (16-24 g) of age at the time of study. Hind limb ischemia was induced by ligation and excision of the right femoral artery and vein under ketamine-xylazine anesthesia. For studies of the angiogenic potential, the mice were divided into 3 groups one day before the induction of ischemia. Mice (n=5 in each group) received intramuscular injection of rAAV-HSA (control), rAAV-VEGF or rAAV-VA1 (30 μl of 4 x $10^8$ vp/μl of each virus dose). For NIR fluorescence imaging, the previously described customized optical systems was used.[13] Briefly, this system employs a CCD digital camera (PIXIS 1024; Princeton Instruments, Trenton, NJ) with a custom-made 830-nm band-pass filter (Asahi Spectra USA Inc., Torrance, CA) and 760-nm light-emitting diode arrays (SMC760; Marubeni America, New York, NY). For time-series ICG imaging, mice under ketamine-xylazine anesthesia were injected with an i.v. bolus of ICG (0.16 ml of 400 mM/l; Sigma-Aldrich) into the tail vein. ICG fluorescence images were obtained in a dark room for 6 minutes at 1-second intervals immediately after the injection. After acquiring the serial imaging, customized computer programs were used to obtain perfusion maps and necrosis ratio.

**Mouse hindlimb ischemia model with protein delivery.**

BALB/cAnNCrljOri male mice were obtained from Orient Bio. The mice were 6–8 weeks (19–25 g) of age at the time of study. Murine hind limb ischemia was induced by ligation and excision of the right femoral artery and vein under ketamine-xylazine

anesthesia. For therapeutic angiogenesis studies, the mice were divided into 3 groups after induction of ischemia. Mice (n=5 in each group) received intramuscular injection of BSA (20 µl of 0.1% BSA) as a control, VEGF (20 µl of 130 ng/µl), or VA1 (20 µl of 260 ng/µl). A higher VA1 dose, compared to VEGF was used to equalize molar amounts of the proteins. Serial ICG perfusion imaging was performed immediately after surgery, and on post-operative days (POD) 3 and 7. NIR fluorescence imaging was performed as described above.

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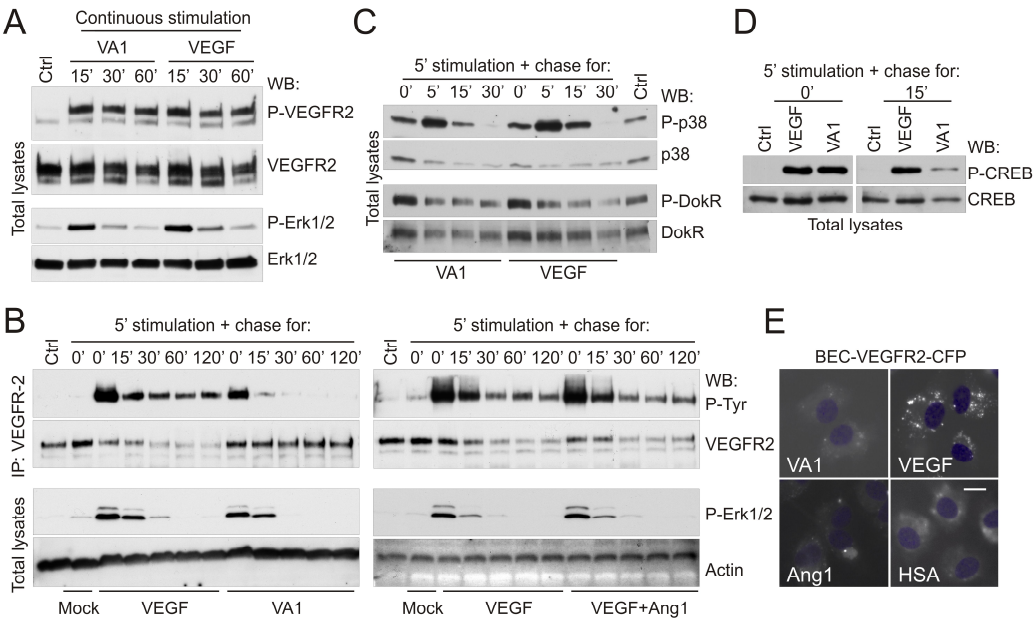


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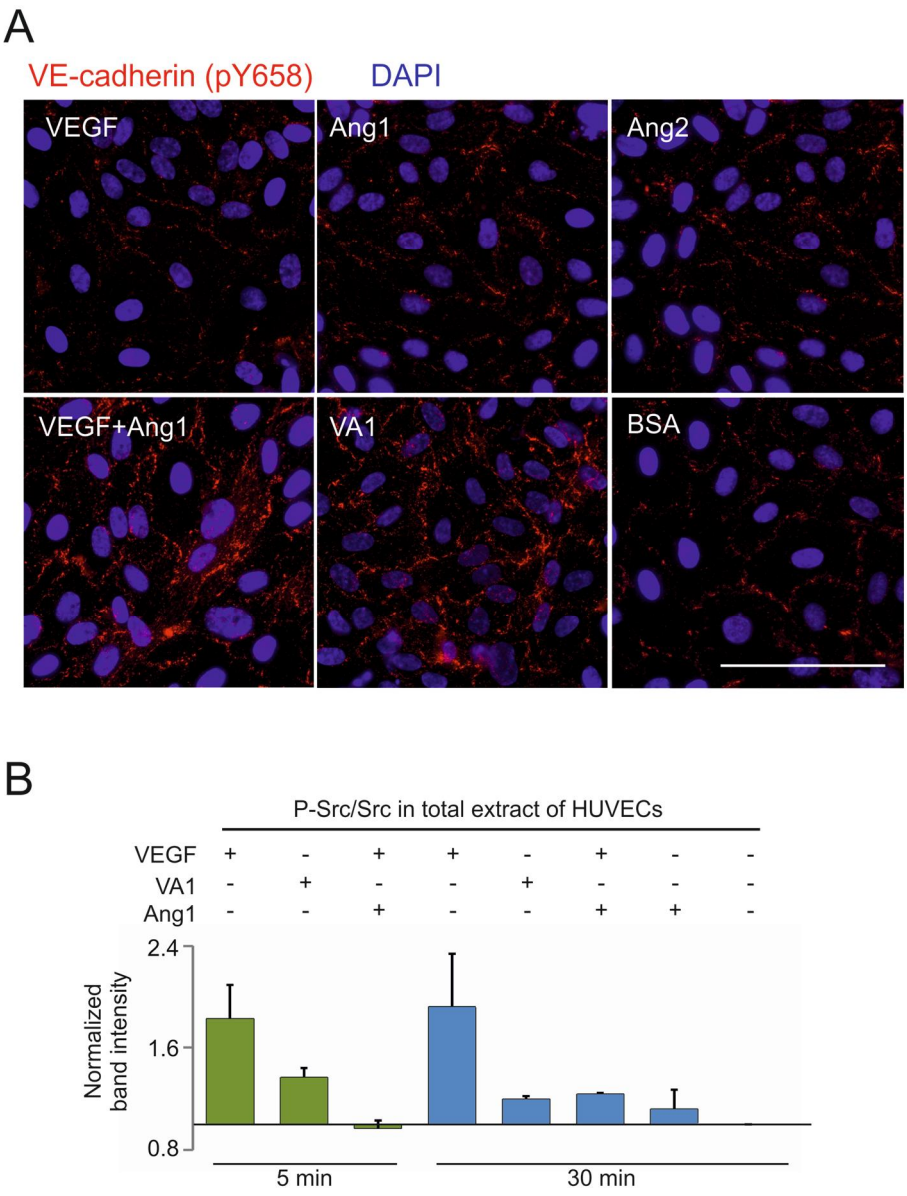


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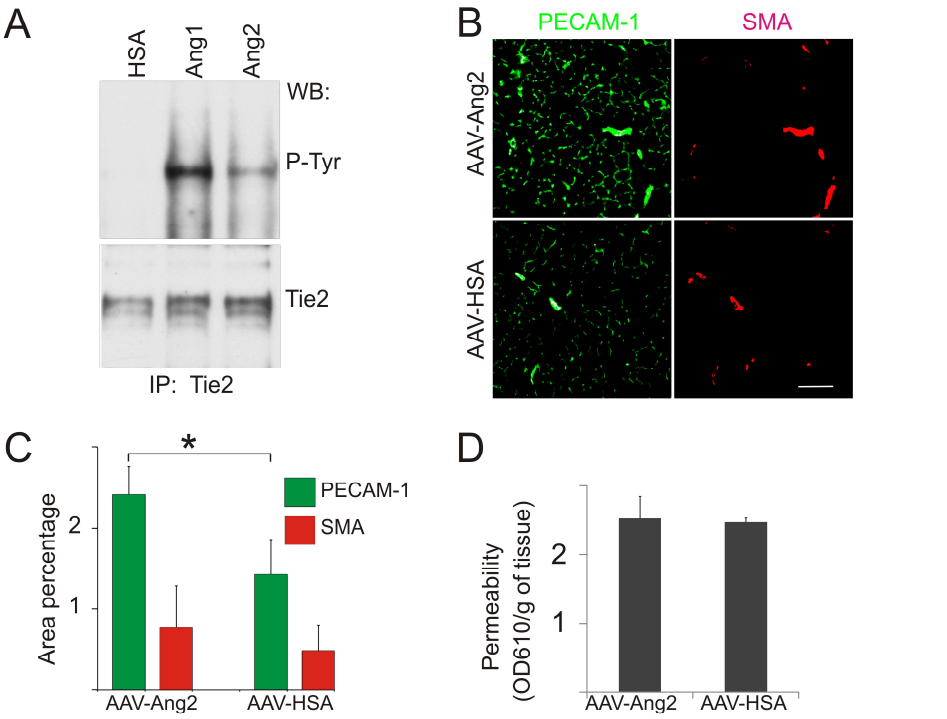


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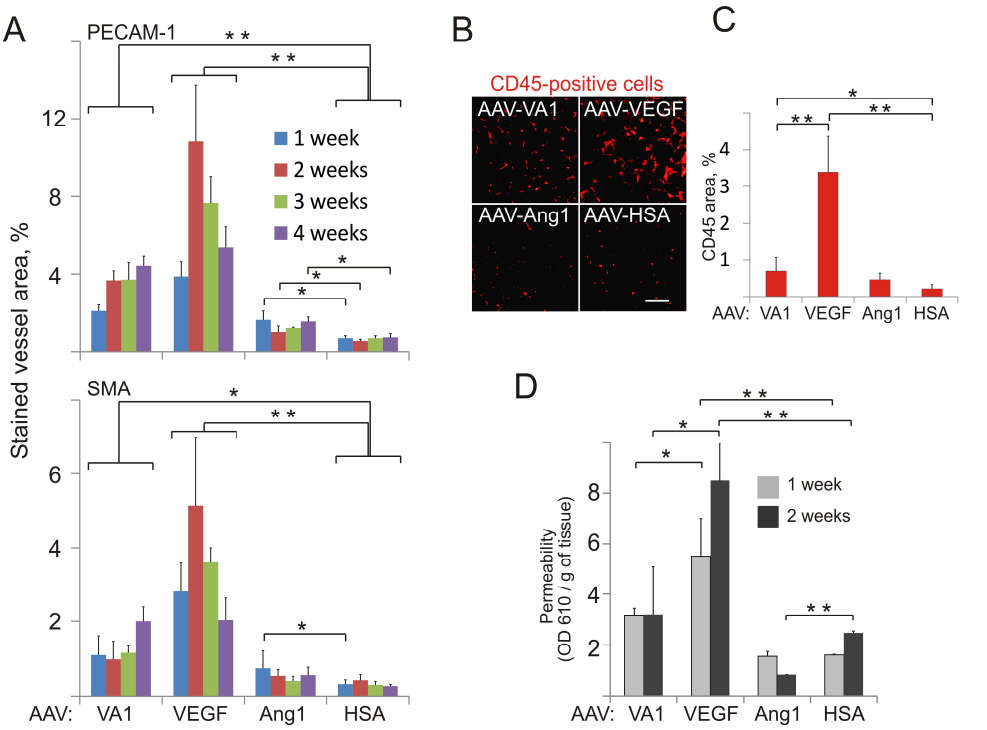


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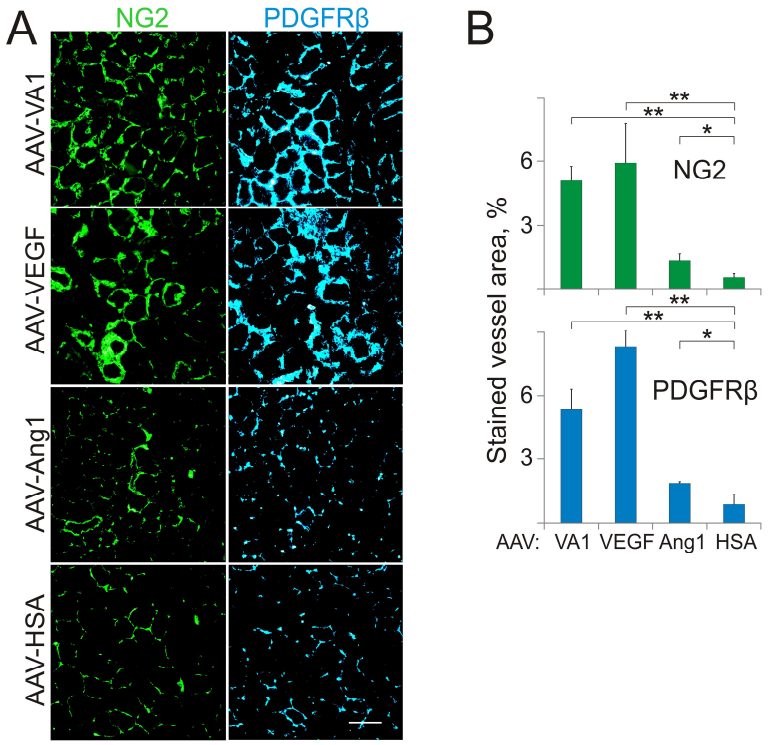


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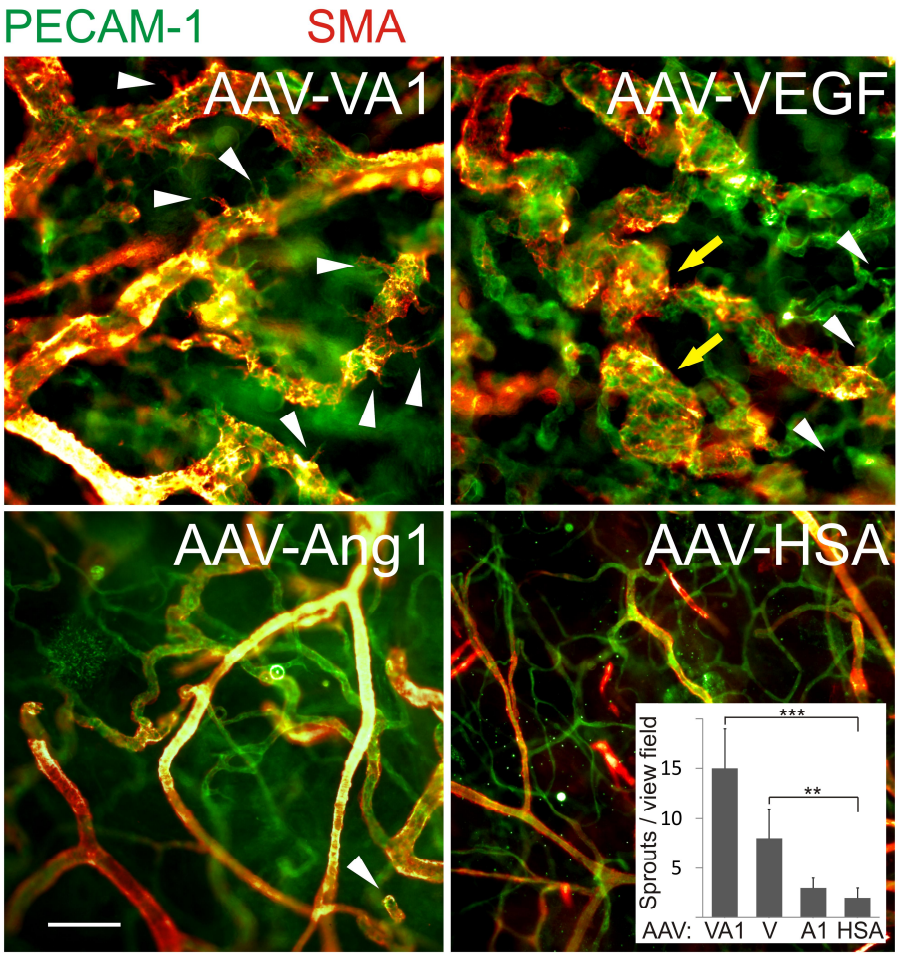


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**Supplemental figure legends**

**Figure S1. VA1 promotes Tie2 accumulation in cell-cell junctions.** (A) VA1 proteins produced in 293T cells and Sf21 insect cells were analyzed by gel electrophoresis in non-reducing (NR) vs. reducing (R) conditions and western blotting with polyclonal antibody directed against VEGF. Note that VA1 produced in mammalian 293T cells reveals a small fraction of high-order multimers. (B) Fluorescent images of junctional Tie2 localization in HUVEC-Tie2-GFP cells treated with the mammalian cell-derived factors for 45 min. Representative images from three independent experiments are presented. Scale bar: 20 µm.

**Figure S2. Comparison of VA1 and VEGF-induced signal transduction and VEGFR-2 trafficking.** (A) VEGFR-2 (Y1175) phosphorylation and total VEGFR-2 protein in BECs subjected to continuous stimulation with purified VA1 and VEGF for the indicated periods. Ctrl: control. Note that VA1 induces somewhat weaker Erk1/2 phosphorylation than VEGF. (B) 5 min stimulation of BECs was followed by incubation without ligands (chase) for the indicated time periods. Cells were lysed, VEGFR-2 was immunoprecipitated and P-Tyr was determined by western-blotting. In parallel, P-Erk1/2 was determined in the total lysates; (C) Comparison of VA1 and VEGF induced phosphorylation of MAP kinase p38 and DokR in BECs. (D) CREB phosphorylation at Ser133 in BECs stimulated with the indicated ligands. Note that the phosphorylation decays faster after with VA1 than with VEGF. (E) VA1 promotes less internalization of fluorescent VEGFR-2 than VEGF. Serum-starved BEC-VEGFR-2-CFP cells were stimulated with the indicated factors for 45 min, fixed and subjected to fluorescence microscopy. The nuclei were counterstained with DAPI. Scale bar: 20 µm

**Figure S3. VE-cadherin and Src phosphorylation in HUVEC cells after treatment with the indicated proteins.** (A) VE-cadherin phosphorylation in HUVECs. The details of the experiment are described in the legend of **Fig. 3A** and in the Materials and methods. The panels are representative of two independent experiments. Scale bar: 100 µm. (B) Cumulative quantification data of Src phosphorylation western blots, obtained from two independent experiments. The details are explained in Fig. 3C legend and in the materials and methods.

**Figure S4. Analysis of Ang2 activity.** (A) Phosphorylation of Tie2 was analyzed in HUVECs stimulated for 10 min with Ang1 or Ang2 (both at 250 ng/ml); (B) Comparison of PECAM-1 and SMA staining in *t.a.* muscles two weeks after transduction with rAAVs encoding Ang2 or HSA; (C) Quantification data from (B); (D) Blood vessel permeability measured by Miles assay in skeletal muscle treated in the same conditions as in (B). Data in A and D are representative of two independent experiments. Data in B are representative of three independent experiments. In (C) Students' unpaired *t*-test was used. Error bars: mean ± SD. (*) – $p \leq 0.05$. Scale bar in B: 100 µm.

**Figure S5. Time-dependent vascular changes and inflammatory cell recruitment in *t.a.* mouse muscles transduced with the indicated rAAVs.** (A) Quantification of total PECAM-1 and SMA positive vessel areas. Experimental conditions and asterixes are described in Figure 4 (A, B) legend. ANOVA, followed by Dunnett *post-hoc* test was used. (B) Representative images of anti-CD45 staining in sections from *t.a.* muscles of FVB/N female mice treated for two weeks with the indicated rAAVs.

Scale bar: 100 µm.; (C) Quantification of (B); (D) Miles permeability assay one and two weeks after transduction with rAAV at 1.34E+10 vp in 30 µl per muscle. In (C) and (D) ANOVA, followed by Tukey *post-hoc* test was used.

**Figure S6. VA1 and VEGF induce similar levels of pericyte coverage.** *T.a.* muscles were injected with rAAVs encoding VA1, VEGF, Ang1 and HSA. After two weeks the muscles were dissected, sectioned and stained for the indicated antigens. Data in (A) are representative of three independent experiments. Representative images are shown. Scale bar: 100 µm. (B) Quantification data from (A). Error bars, mean ± SD. ANOVA, followed by Dunnett *post-hoc* test was used. (*): $p \leq 0.05$, , (**): $p \leq 0.01$.

**Figure S7. VA1 leads to vessel dilation and induces more sprouting than VEGF in the ear skin, but does not lead to development of glomeruloid angiomas.** Ears of FVB/N female mice were injected with the indicated rAAVs (8.0E+10 vp/ear) and analysed by whole-mount staining for PECAM-1 and SMA one week thereafter. Representative images are shown. White arrowheads show sprouts, yellow arrows point to angiomas. The inset shows sprout quantification. Scale bar: 100 µm.

## Supplemental references

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