Supplemental information

VEGF receptor 2/-3 heterodimers detected *in situ* by proximity ligation on angiogenic sprouts

Ingrid Nilsson[1)¶§], Fuad Bahram[1)¶], Xiujuan Li[1)], Laura Gualandi[1)], Sina Koch[1)], Malin Jarvius[1)], Ola Söderberg[1)], Andrey Anisimov[2)], Ivana Kholová[3)], Bronislav Pytowski[4)], Megan Baldwin[5)], Seppo Ylä-Herttuala[3)], Kari Alitalo[2)], Johan Kreuger[6)], and Lena Claesson-Welsh[1)†]

1) Uppsala University, Dept. Genetics and Pathology, Rudbeck Laboratory,
Dag Hammarskjöldsv. 20, 751 85 Uppsala, Sweden
2) University of Helsinki, Molecular and Cancer Biology Research Program
00014 Helsinki, Finland
3) University of Kuopio, A.I. Virtanen Institute for Molecular Sciences, Department of Biotechnology and Molecular Medicine, 70211 Kuopio, Finland
4) Imclone Systems Incorporated, 180 Varick Street, New York, NY 10014 USA
5) Vegenics Limited, Level 1, 10 Wallace Avenue, Toorak, 3142 Victoria, Australia
6) Uppsala University, Dept. Medical Biochemistry and Microbiology, Biomedical Center, 751 23 Uppsala, Sweden

¶ Equal contribution

Materials and Methods

Pericyte–embedded angiogenic sprouts in 3D embryoid bodies (EBs)
EBs in 3D collagen were washed twice in phosphate-buffered saline (PBS) and fixed in 4% *para*-formaldehyde (PFA) in PBS for 30 minutes at room temperature. After permeabilization in 0.1% Triton X-100 and block with TNB (PerkinElmer Life Science), antibodies against CD31 or NG2 (Chemicon) were applied. All samples were treated with Hoechst 33342 to visualize nuclei. Stained EBs were mounted on glass slides in Fluoromount-G (Southern Biotechnology) and analyzed by a Nikon Eclipse E1000 microscope with a Nikon Eclipse DXM 1200 camera (Nikon, Tokyo, Japan) or an LSM 510 META confocal microscope (Carl Zeiss, Oberkochen, Germany).

Recombinant AAV vector preparation
The recombinant adeno-associated viruses (rAAVs) were produced as described (Anisimov et al., 2009).

Muscle transduction by the rAAV vectors
Six to seven week-old female C57BL/6J mice (three to four per group) were anesthetized with xylazine (Rompun, Bayer)-ketamine (Ketalar, Pfizer), and 5E+10 rAAV particles (in 30 µl volume) were injected into each tibialis anterior (t.a.) muscle. All mouse experiments were approved by the Provincial State Office of Southern Finland and carried out in accordance with institutional guidelines and as described in detail previously (Anisimov et al., 2009).

Immunohistochemistry
Muscles were isolated and frozen in O.C.T (TissueTek, Sakura Finetek). Cryosections (8 µm) were cut, acetone-fixed, and immunostained using the following antibodies: rat anti-PECAM-1 (Pharmingen), rat anti-MECA32 (Pharmingen), and DAPI for DNA staining. Secondary

antibodies were Alexa Fluor-conjugated (Molecular Probes, Eugene, OR). Microvessel area density was quantified using ImageJ software (NIH).

Embryoid body culture, treatment and analysis by immunostaining and proximity ligation. Procedures for embryoid body (EB) culture and processing for immunostaining as well as proximity ligation (PLA) are described in the accompanying main paper and in Li et al., 2008. Quantification of branching in the co-treated EB cultures was performed on x20 micrographs. The number of manually counted branch points was related to the CD31-positive vascular area of that field and expressed as branch points/CD31 area (arbitrary units). Six EBs/condition were analyzed.

References

Anisimov A, Alitalo A, Korpisalo P, Soronen J, Kaijalainen S, Leppänen VM, Jeltsch M, Ylä-Herttuala S, Alitalo K. Activated forms of VEGF-C and VEGF-D provide improved vascular function in skeletal muscle. Circ Res. 2009 Jun 5;104(11):1302-12. Epub 2009 May 14.

Li X, Claesson-Welsh L, Shibuya M. VEGF receptor signal transduction. Methods Enzymol. 2008;443:261-84-

Figure legends

Supplemental figure S1. Formation of pericyte-embedded angiogenic sprouts in 3D EBs

EB cultures in 3D collagen treated with VEGFA (30 ng/ml) or VEGFC (300 ng/ml) from day 4 to day18 were whole-mount fixed and immunostained to detect CD31 (red) and the pericyte marker Nerve-glia2 (NG2; green). Staining with Hoechst 33342 marks nuclei (blue). Merged panel to the right shows that both VEGFA and –C induced pericyte-embedded angiogenic sprouts. Scale bar=100 μm.

Supplemental figure S2. Vessel formation by VEGFA and VEGFC in peripheral muscle

A. Skeletal muscle tissue from mouse hind limb after intramuscular administration of adeno-associated virus (AAV) encoding human serum albumine (HSA), mouse VEGFA or VEGFC followed by perfusion fixation and immunohistochemical staining for CD31 or MECA32. Nuclei were counter-stained using DAPI.

B. Quantification of mean vessel area in the sections (n=10).

Supplemental figure S3. Increased heterodimerization and branching morphogenesis in EBs treated with combination of VEGFA+VEGFC

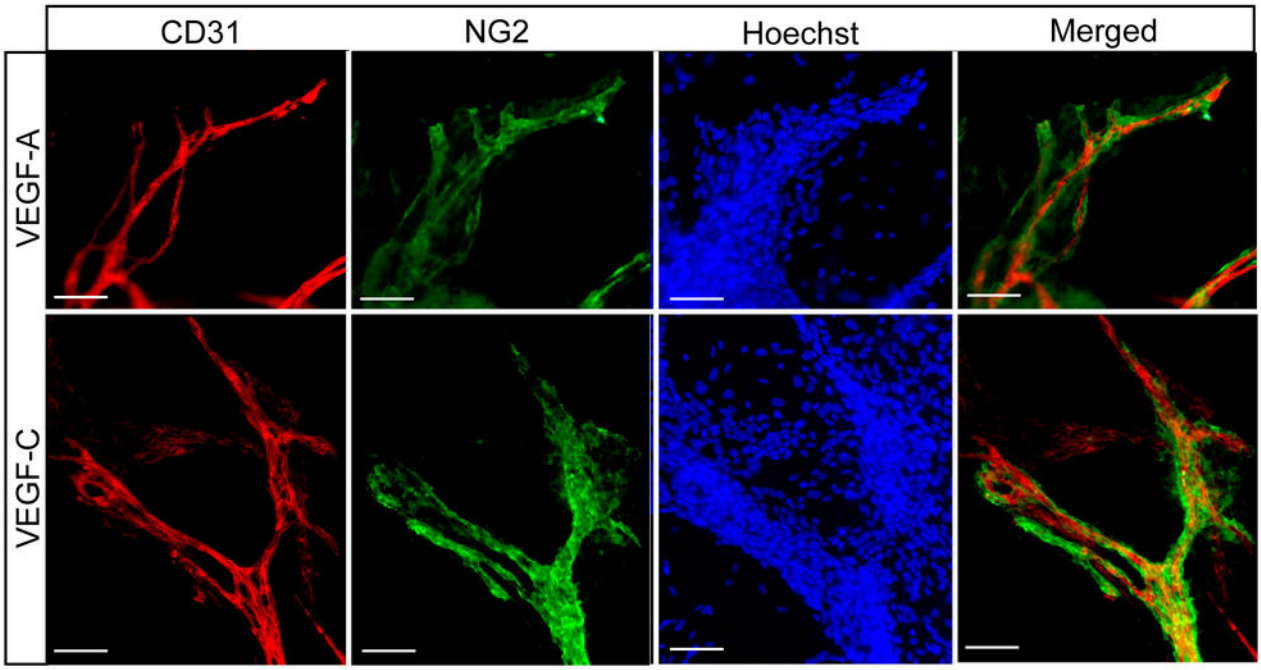
A. EBs in 3D collagen were treated from day 4 to day 18 with vehicle (-), VEGFA (30 ng/ml), VEGFC (30 ng/ml) or combined VEGFA+VEGFC (30 ng/ml of each). Growth factors were added fresh every 4 days. Cultures were processed for whole-mount fixation and immunostaining to detect CD31-positive angiogenic sprouts. Arrows indicate sprout length. Scale bar=300 μm.

B. Quantification of branch points in A expressed as branch points/CD31-positive vessel area. The number of branch points from 6 separate cultures/condition were analyzed and set in relation to their CD31-positive vessel area (acquired as arbitrary units). Mean ±S.D. are shown.

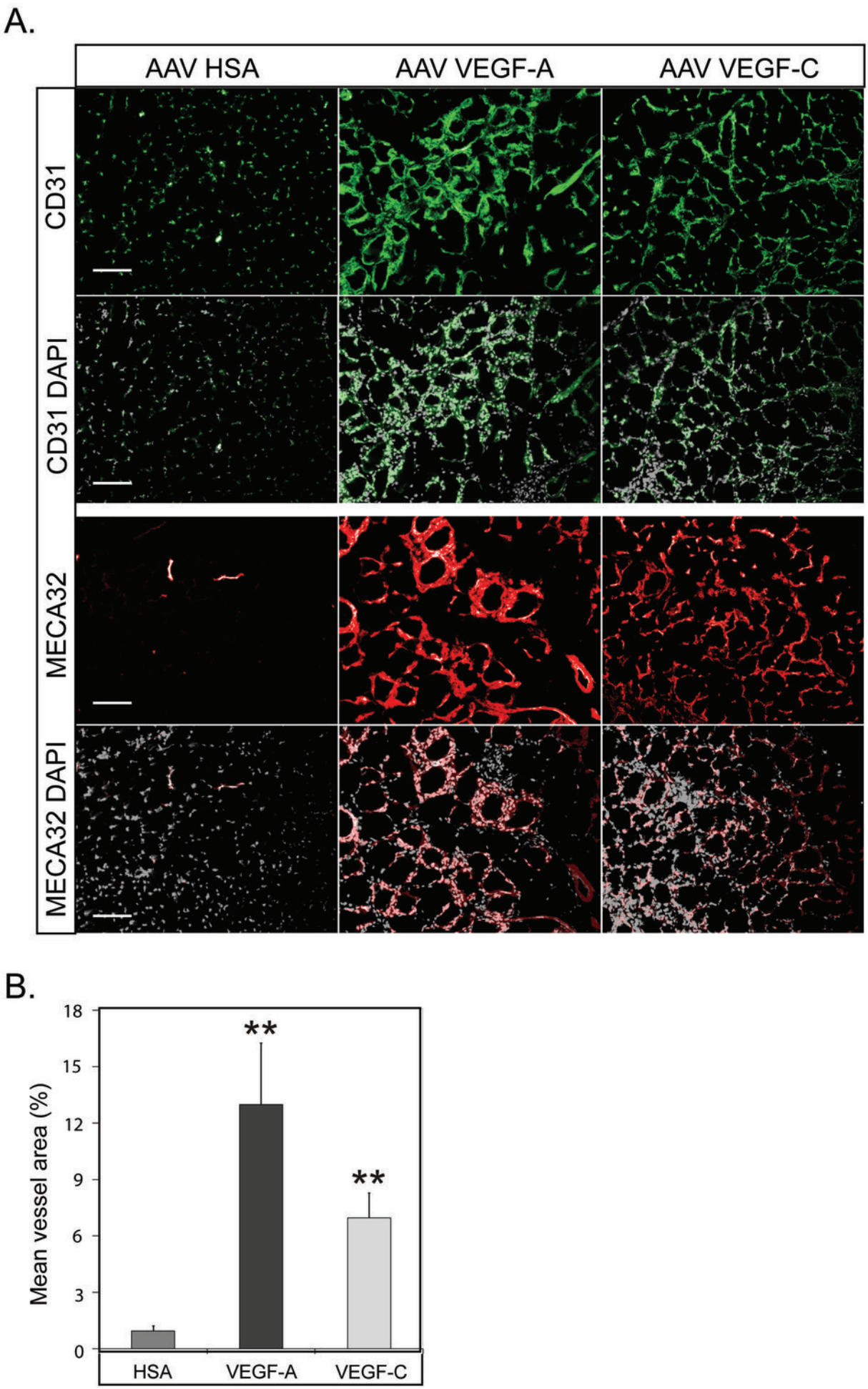
C. Proximity ligation analyses showed increased VEGFR2/-3 heterodimerization in response to combined treatment with VEGFA+VEGFC. Vessel structures were

detected by fluorescent immunostaining for CD31. PLA signals indicating heterodimers is shown in red.

Supplemental Figure 1.
Nilsson and Bahram et al.



Supplemental Figure 2.
Nilsson and Bahram et al.



Supplemental Figure 3.
Nilsson and Bahram et al.