SUPPLEMENTAL MATERIAL

Supplementary Methods

Construction and preparation of the recombinant adeno-associated virus (AAV) vectors. Mouse $VEGF_{120}$, PlGF, VEGF-B_{167}, VEGF-B_{186} and human serum albumin (HSA) cDNAs were cloned into blunted MluI and NheI restriction sites of the psubCMV-WPRE recombinant AAV expression vector[1]. The AAVs (serotype 9) were produced as described previously[2]. Six to seven-week-old female FVB/NJ, ICR and C57Bl/6J mice were anesthetized with xylazine (Rompun, Bayer)-ketamine (Ketalar, Pfizer), and 5 x 10^{10} AAV particles (in 30 μl volume) were injected into *tibialis anterior* muscles. In parallel experiments, 2 x 10^{11} AAV particles (in 120 μl volume) were injected into the left ventricle.

***In vivo* ultrasound measurement of perfusion in transduced muscles.** One *tibialis anterior* muscle per mouse was injected as above and expression continued for four weeks. Values are presented as ratios between pools of treated muscles and a pool of untreated muscles (VEGF-B, *n*=6; PlGF, *n*=6; VEGF, *n*=6; HSA, *n*=3). The mice were anesthetized, and perfusion in *tibialis anterior* muscles was measured with an Acuson Sequoia 512 ultrasound apparatus (Siemens). Intensities of the ultrasound signals were analyzed with the Datapro 2.13 (Noesis) program.

Evans Blue permeability assay. Mouse *tibialis anterior* muscles were injected with AAVs encoding mouse VEGF-B (both isoforms, *n*=3), PlGF (*n*=5), VEGF (*n*=4), HSA (*n*=7), or were left untreated (*n*=7). Eight weeks later, mice were anesthetized with xylazine-ketamine, and 300 μl of 1% Evans Blue-PBS was injected into the left ventricle. The dye was allowed

to circulate for 30 min, after which the mice were euthanized and both *tibialis anterior* muscles dissected. The extravasated dye was released by incubating the muscles in formamide overnight at 55°C and quantitated by spectrophotometry at 620 nm.

Generation of αMHC-VEGF-B transgenic mice and rats. A fragment of the human VEGF-B gene (corresponding to nucleotides 745-5059 of Genbank accession number AF468110) was isolated from the K14-VEGF-B construct[3] and cloned into the alpha myosin heavy chain (αMHC) promoter expression vector (a kind gift from Dr. Jeffrey Robbins). Transgenic animals were generated by microinjection of fertilized oocytes from FVB/N mice or outbred HsdBrl:WH Wistar rats. Positive founder animals were identified using PCR analysis of ear biopsies taken at the time of weaning. The primer pair 5'-TCAGAGAGGTGGTGAAGCCT-3' and 5'-CTCCTCACTGGTTTTCCTGC-3' was used for genotyping. All animal experiments were approved by the Provincial State Office of Southern Finland and carried out in accordance with institutional guidelines.

Generation of αMHC-mVEGF-Bex1-5 mice. The mVEGF-Bex1-5 fragment (last four coding amino acids: VKPD) was isolated from a mVEGF-Bex1-5-pSubCMV-WPRE vector with MluI and blunted. The fragment was cloned into the αMHC promoter expression vector, digested with SalI and blunted with Klenow. The plasmid was further digested with BamHI, and the fragment was purified and injected into fertilized mouse oocytes of the FVB/N background. The primers 5'-CCAGAAATGACAGACAGATCC-3' and 5'-GCTTCTAGTTAGTCAGTCGACG-3' were used for genotyping.

Western blotting of cardiac extracts. Corresponding pieces of the heart were sliced into small pieces, mixed with 1 mL RIPA lysis buffer (150 mM NaCl, 1% NP-40, 0.5% DOC,

0.1% SDS, 50 mM Tris pH 8.0, 20 μg/mL leupeptin, 3.4 μg/mL aprotinin, 1 mM Na_3VO_4, 1 mM PMSF) in Lysing Matrix tubes (MP Biomedicals) and homogenized. The total protein concentrations were measured using the BCA Protein Assay Kit (Thermo Scientific). Lysates were boiled in Laemmli sample buffer (LSB) and equal amounts of total protein samples (25 μg) were subsequently separated in SDS-PAGE, transferred onto a nitrocellulose membrane and incubated with anti-VEGF-B (AF751, R&D Systems) antibodies, followed by an HRP-conjugated secondary antibody. Antibody complexes were visualized on X-ray film using chemiluminescent substrate (Thermo Scientific).

Angiogenesis antibody array analysis. Snap-frozen left ventricular samples of four-month-old rat hearts were lysed and analyzed with the R&D Proteome Profiler™ Mouse Angiogenesis Array Kit (#ARY015) according to the manufacturer's instructions. Lysates from three different transgenic and wildtype hearts, respectively, were pooled before analysis. Intensities were quantified with the ImageJ software (NIH).

Histochemistry. Formalin-fixed mid-ventricular paraffin sections were stained with Resorcin Fuchsin for the internal elastic lamina, Herovici's stain for collagen, and Masson's Trichrome. The sections were viewed and imaged with a Leica DM LB research microscope with Olympus DP50 color camera.

Immunohistochemistry, microscopy and image analysis. 6-8 μm frozen transverse sections were fixed with cold acetone, washed, and blocked in TNB (PerkinElmer) or 5% donkey serum and 0.2% BSA. Thick 200 μm sections of hearts were fixed with 4% paraformaldehyde, washed and blocked with TNB. The primary antibodies used for immunostaining were: rat anti-mouse PECAM-1 (BD Pharmingen), mouse anti-human

dystrophin (Dys2, Novocastra), goat anti-human VEGF-B (AF751, R&D Systems), mouse anti-SMA (Cy3-conjugated, clone 1A4, C6189, Sigma), mouse anti-rat RECA-1 (MCA970, Serotec), rabbit anti-mouse PAI-1 (a kind gift from Peter Andreasen), mouse anti-rat ED-1 (22451D, BD Pharmingen), rat anti-mouse CD45 (BD Pharmingen), and rat anti-mouse F4/80 (AbD Serotec). Alexa Fluor 488, 594 and 647-conjugated secondary antibodies (Molecular Probes) were used for detection. Sections were post-fixed with 4% paraformaldehyde, washed, and mounted with Vectashield with DAPI (Vector Laboratories). Immunofluorescence stainings were imaged using an Axioplan2 fluorescence microscope (Zeiss) or a confocal LSM 510 Meta or LSM 5 Duo microscope (Zeiss). Alternatively, the peroxidase ABC method (Vectastain Elite ABC Kit; Vector Laboratories, Burlingame, CA) was used and the reaction revealed by 3-amino-9-ethylcarbazole (AEC; Vector Laboratories) for light microscopy.

Image analysis was carried out using the ImageJ software (NIH) from several randomly chosen photographic fields from each section. The number of arteries in rat hearts was quantified visually from whole transverse heart sections with the aid of Masson's Trichrome staining for the arterial adventitia. PECAM-1 and SMA-positive surface areas as well as CD45 and F4/80 immunostainings were quantified from transverse muscle sections. Two to three photographic fields (10x magnification) were analyzed from each section from left and right *tibialis anterior* muscles of three to four mice per group. Cardiomyocyte areas were quantified from dystrophin-stained sections with ImageJ. Four photographic fields (20x magnification) from five mice per group were analyzed.

Electron microscopy. Tissue samples from the left ventricle were fixed with 2.5% glutaraldehyde, postosmicated and embedded in epon. Semithin sections were stained with

toluidine blue, and on the basis of initial analysis in light microscopy, regions of interest were selected for thin (100 nm) sectioning and analysis using a JEOL 1400 EX Transmission Electron Microscope equipped with Morada CCD Camera (Olympus SIS).

Blood pressure measurements and echocardiography. The blood pressure of the rats was measured with the CODA Non-Invasive Blood Pressure System for Mice and Rats (Kent Scientific Corporation, Torrington, Connecticut, USA) on non-anesthetized animals restrained in a rodent holder. Measurements were performed in three sets of six cycles with five-second breaks between cycles and thirty-second breaks between sets. Transthoracic echocardiography was performed with an Acuson Sequoia 512 Ultrasound System and an Acuson Linear 15L8 14 MHz transducer (Siemens Medical Solutions, Mountain View, CA, USA). Rats were anesthetized with xylazine 10 mg/kg i.m. (Rompun 20 mg/mL Bayer) and ketamine 40 mg/kg i.m. (Ketalar 50 mg/mL Pfizer). Normal body temperature was maintained.

MicroCT imaging of the cardiac vessels. Coronary angiographies were performed using the Inveon micro-computed tomography (CT) scanner (Siemens, Knoxville, TN, USA). The rats were anesthetized with isoflurane, heparinized (0.1 mL, 60 IU) via a tail vein, and euthanized with carbon dioxide. The ascending aorta was cannulated, clamped and 0.3 mL of iodinated intravascular contrast agent eXIATM160XL (working dilution 1:5, Binitio Biomedical Inc., Ottawa Ontario, Canada) was carefully injected manually to fill the cardiac blood vessels, avoiding very high pressure. Then, both the vena cava and pulmonary artery were clamped, and the heart was excised and placed in a cylindrical container. The CT acquisition consisted of 721 projections acquired with an exposure time of 1000 ms, X-ray voltage of 80 kVp, and current of 500 μA for a full 360° rotation and a total scan time of 20 min. Images were

reconstructed using a standard filtered backprojection algorithm. The resulting matrix was 768 x 768 pixels with 512 transverse slices (pixel size 0.04×0.04×0.04 mm). The coronary arterial and venous trees were segmented using the ADW 4.4 Workstation (General Electric, Milwaukee, Wis., USA) and visualized as 3D volume rendered images.

Assessment of myocardial perfusion, oxygen consumption and efficiency of work. Eight rats were given a slow bolus of 30±24 MBq of [^{11}C]acetate (0.4–1.0 ml) and imaged for ten minutes using the Inveon microPET/CT scanner (Siemens, Knoxville, TN, USA). Myocardial blood flow was estimated using the single compartment model and expressed as rate constant K_1 (1/min). Myocardial oxygen consumption was assessed by applying monoexponential fitting to calculate the [^{11}C]acetate clearance rate (Kmono). Myocardial efficiency of forward work was estimated as forward LV work power per gram/LV Kmono[4]. LV mass was calculated from LV dimensions in the long axis M-mode image and cardiac output from LV outflow tract pulsed Doppler measurements using the Visualsonics Vevo 770 ultrasound apparatus.

Statistical Analysis. Values are indicated as mean±SD unless otherwise indicated. Statistical analysis was performed with one-way ANOVA (post-hoc with Tukey's test), or with the two-tailed unpaired Student's *t*-test unless otherwise specified in the Results. Differences were considered statistically significant at $P<0.05$.

Supplementary References

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Supplementary Figure Legends

Supplementary Figure 1. Perfusion and permeability of muscles after transduction of AAVs encoding VEGF-B (both isoforms), PlGF, VEGF or HSA. **A.** Blood perfusion in *tibialis anterior* muscles as quantified by ultrasound. **B.** Evans Blue dye was injected into the left ventricle of mice and dye leakage from muscles was quantified as the ratio between the absorbance at 620 nm and muscle weight. Significance values were determined between the test groups and the HSA control group. **, $P<0.005$; ***, $P<0.0005$.

Supplementary Figure 2. Comparison of the vascular effects of VEGF-B and PlGF overexpressed for two weeks in cardiac muscle via AAV vector delivery. Representative PECAM-1 and SMA-stained sections of hearts transduced with AAVs encoding VEGF-B_{167}, PlGF or HSA. Scale bar = 100 μm.

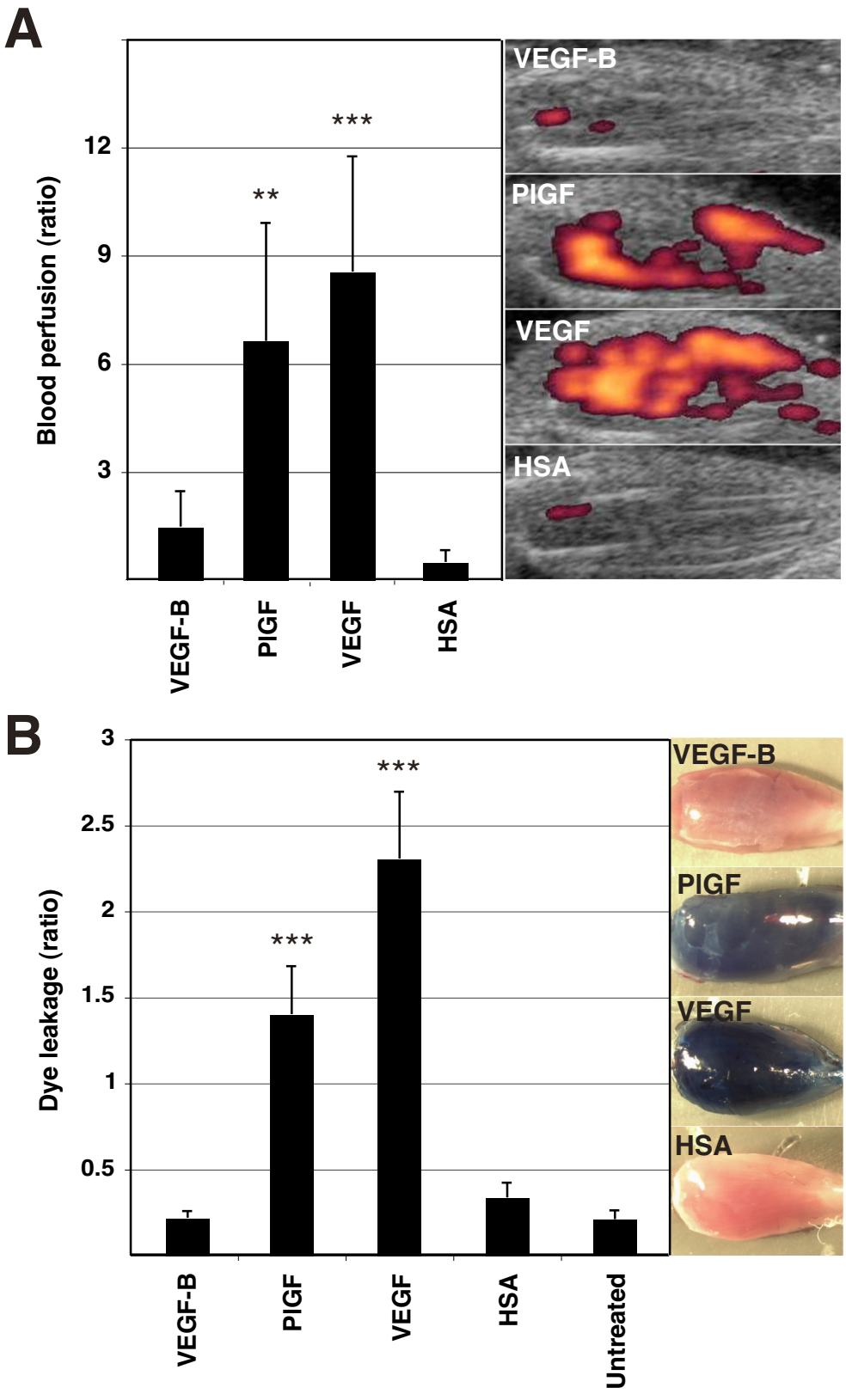
Supplementary Figure 3. Inflammatory cell infiltration in mouse skeletal and cardiac muscles transduced with AAVs. **A.** CD45 and F4/80-staining of skeletal muscles injected with AAVs encoding VEGF-B_{167}, VEGF-B_{186}, PlGF, VEGF or HSA. **B.** Quantification of **A. C.** CD45-staining of hearts injected with AAVs encoding VEGF-B (1:1 mixture of both isoforms), PlGF or HSA. Scale bars = 100 μm. **, $P<0.01$; *, $P<0.05$.

Supplementary Figure 4. Expression and structure of the αMHC-VEGF-B transgenes. **A.** Shown are exons and introns with the alternative splice acceptor (SA) sites that produce the VEGF-B_{167} and VEGF-B_{186} isoforms. Arrowhead indicates the site of proteolytic processing of VEGF-B_{186}. hGH pA, human growth hormone polyadenylation signal. Red, sequence encoding the VEGF homology domain. **B.** Schematic structure of the αMHC-VEGF-B_{Ex1-5}

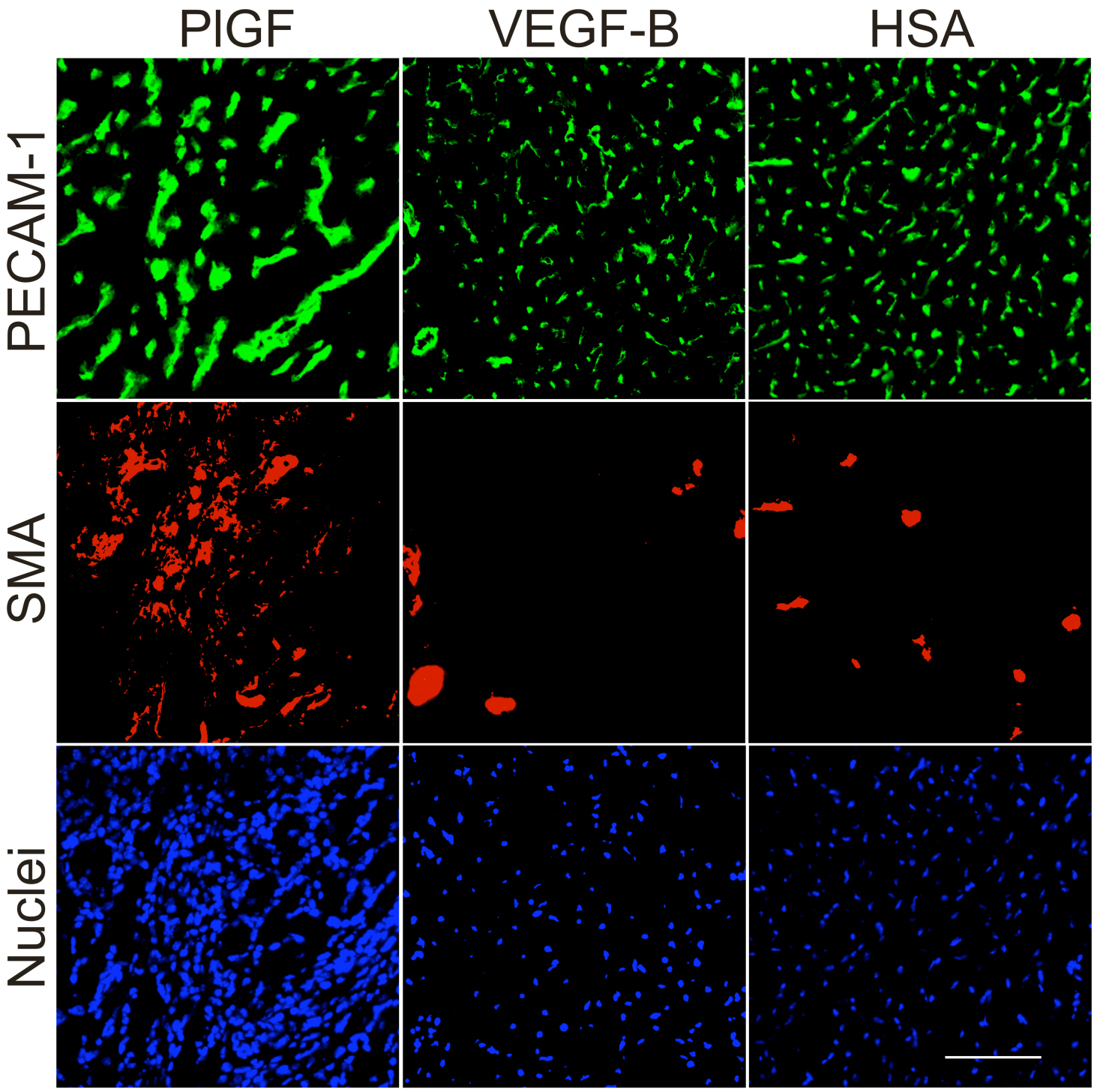
cDNA transgene. **C.** Western blot analysis of VEGF-B in heart lysates from five transgenic rat founder lines (TG1-5) and a wildtype control (WT). Founder lines 2-4 were used for subsequent analyses. The polypeptides generated from the 167 and 186 transcripts are indicated. **D.** Western blot analysis of VEGF-B in heart lysates from αMHC-VEGF-B_{Ex1-5} (TG) and wildtype mice. **E.** Immunofluorescence staining of heart sections with antibodies against VEGF-B. Blue, DAPI staining of the nuclei. Scale bar = 50 μm.

Supplementary Figure 5. Degenerative changes in αMHC-VEGF-B mouse but not rat hearts. **A.** Representative hematoxylin-eosin stained heart sections from one-year-old αMHC-VEGF-B mice and rats. Note the vacuolar lesions indicated by arrows in the mouse hearts. Scale bar = 50 μm. **B.** Transmission electron micrographs from four-month-old αMHC-VEGF-B rat hearts. Scale bar = 800 nm.

Supplementary Figure 6. Angiogenesis antibody array analysis of VEGF-B transgenic and wildtype rat heart lysates. In the TG rat, the expression of Cyr61, Dll4, Osteopontin and PAI-1 was upregulated more than 2.5-fold when compared to WT rat. VEGF-B expression was 6-fold higher in TG rat.



Bry et al., Suppl. Fig. 1



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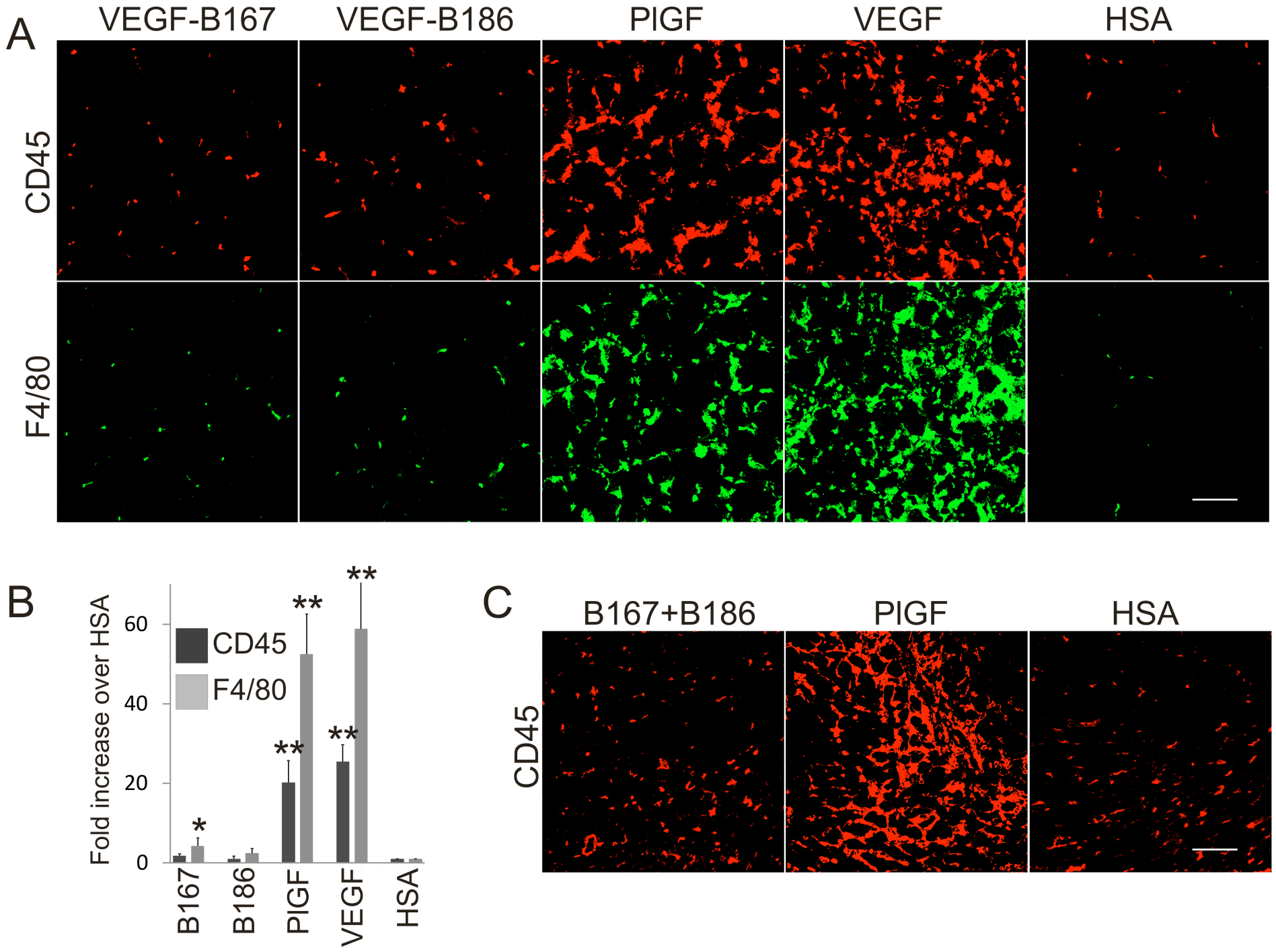

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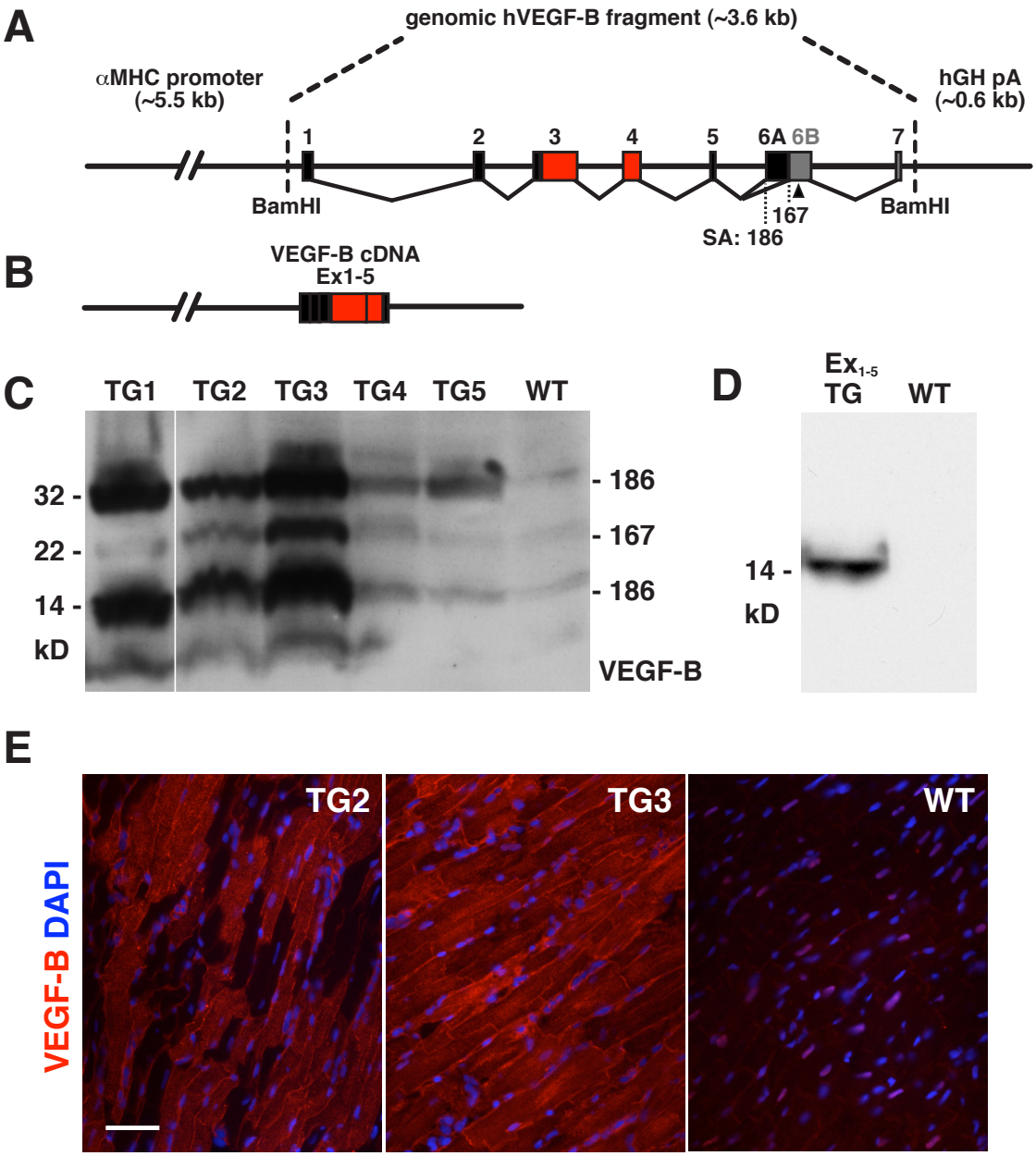
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αMHC promoter
(~5.5 kb)
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3
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6A
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BamHI
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SA: 186
BamHI
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VEGF-B cDNA
Ex1-5
C
TG1
TG2
TG3
TG4
TG5
WT
32 -
22 -
14 -
kD
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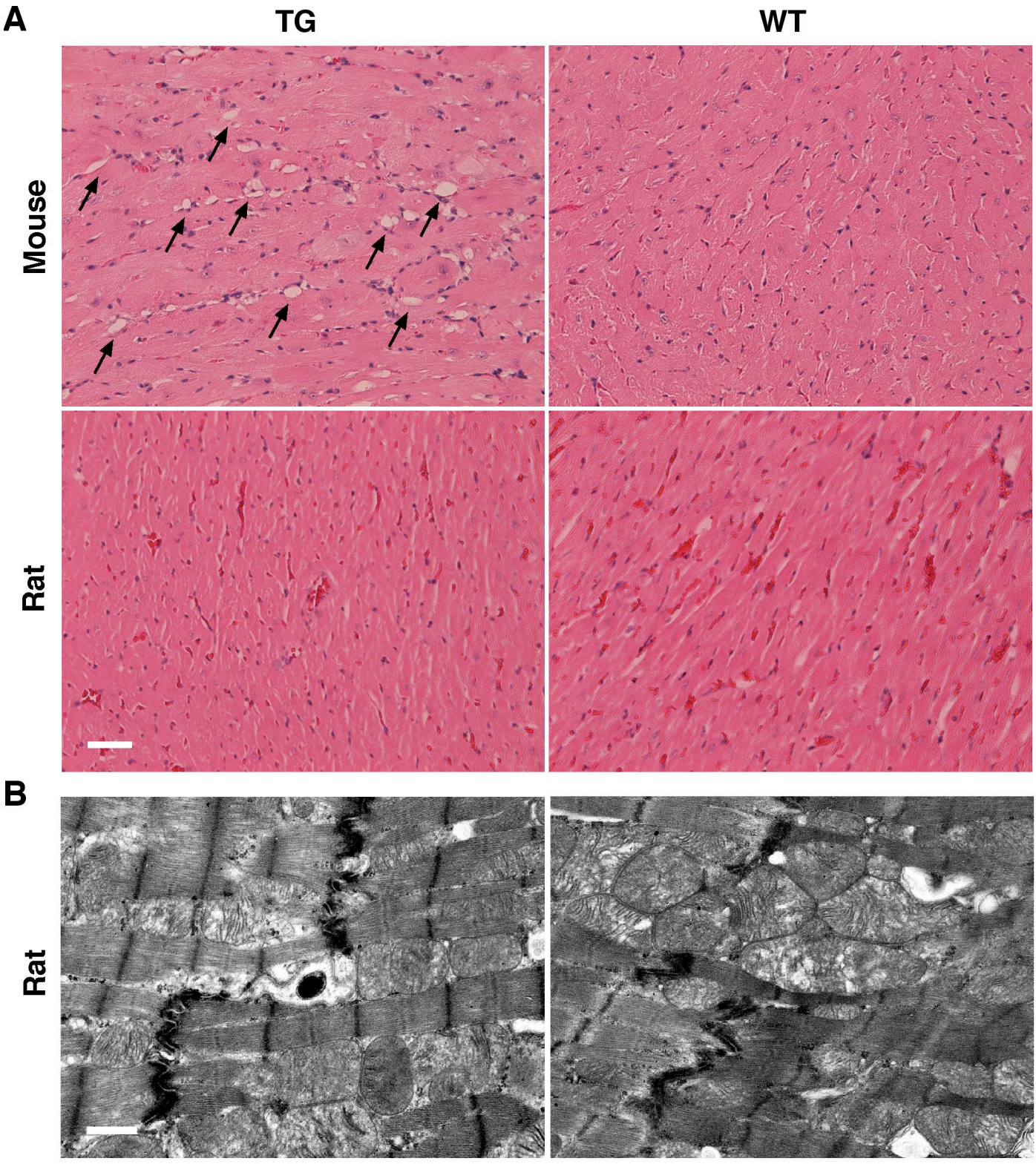
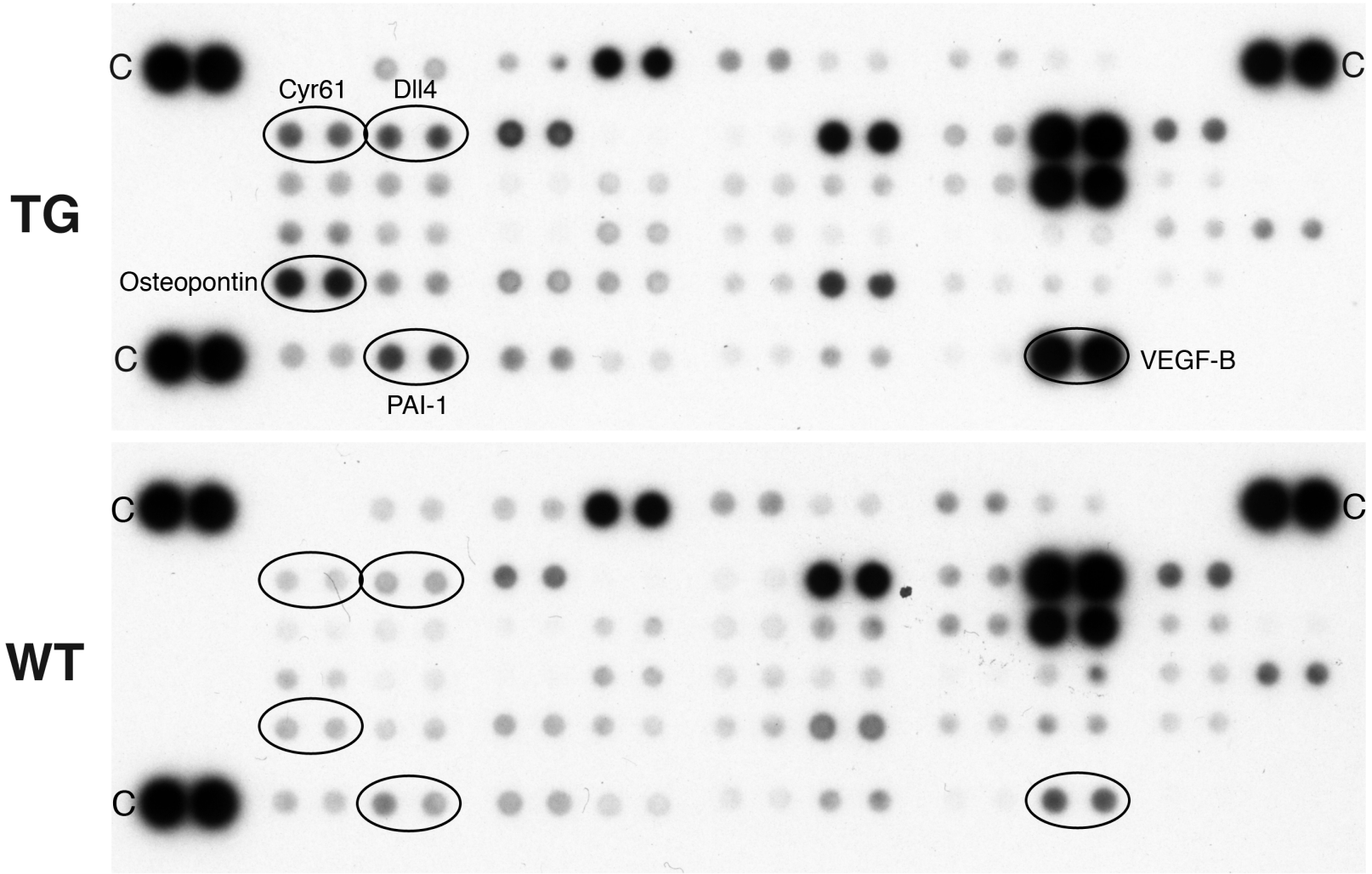


Bry et al., Suppl. Fig. 5



Bry et al., Suppl. Fig. 6