SUPPLEMENTAL MATERIAL

Endothelial cells regulate physiological cardiomyocyte growth via VEGFR2-mediated paracrine signaling
Kivelä et al.

SUPPLEMENTAL METHODS

Mouse models
All animal experiments were approved by the committee appointed by the District of Southern Finland. The following previously published mouse lines were used in the experiments (both males and females): Pdgfb-CreERT2, Cdh5-CreERT2, Flt1\textsuperscript{lox/lox}, VEGFR2\textsuperscript{c\textsubscript{3}lox/c\textsubscript{3}lox\textsuperscript{p}}, VEGFR1\textsuperscript{TK-/-}, Rosa26-TdTomato\textsuperscript{lox/STOP/lox} (The Jackson laboratory, #007914) Myh6-CreERT2 (The Jackson Laboratory #005657), aMHC-Cre (The Jackson Laboratory #011038), and APJ KO\textsuperscript{a}. The mice were maintained in C57BL/6J or 6N background except for VEGFR1\textsuperscript{TK-/-}, which were in FVB/N. For the blocking experiments with AAVs, weight matched male wild type C57BL/6JOlaHsd mice were purchased from Envigo Harlan. The animal numbers for each group and experiment are given in the respective figure legends.

Gene deletion and Cre specificity in adult mice
To induce Cre-targeted gene deletion, Tamoxifen (Sigma) dissolved in sterile corn oil was administered by oral gavage (2 mg/day, for 3-5 consecutive days) to 7 - 8 weeks old male mice. To determine the endothelial specificity and recombination efficiency of the PdgfbCreERT2 and VECadherinCreERT2 lines, mice were mated with the Rosa26-TdTomato\textsuperscript{lox/STOP/lox} reporter line,
and tamoxifen was administered to induce Cre-mediated TdTomato expression. Endothelial specificity was validated by co-staining the heart sections with an antibody against the endothelial cell marker VEGFR2 (AF644, R&D Systems; 1:50).

**Adeno-associated viral vectors**

The recombinant adeno-associated viral vectors (AAV, serotype 9) encoding mVEGFB186, mPIGF, mVEGF164 and Scrambled control vector were constructed and generated as described previously⁷. All the vectors contained CAG-promoter. The mice were injected with $2 \times 10^{11}$ AAV9 particles intraperitoneally (i.p.) and analyzed 2-6 weeks later. In addition, we generated recombinant AAV-mErbB4ECD-mFc, which encodes the extracellular domain of mouse ErbB4 receptor fused with mouse Fc.

**Blocking VEGFR2 by monoclonal antibody DC101**

To block VEGF/VEGFR2 signaling, eight weeks old C57BL/6J wild type mice were injected with AAV-Ctrl, AAV-mVEGFB186 or AAV-mPIGF and treated with DC101 mAb (30 μg/g, BioXcell) every 3-4 days. In the prevention study, DC101 injections were started at the same time as the AAVs were injected and the mice were terminated after two weeks. In the treatment study, mice were first injected with AAVs and the two-week antibody treatment was started two weeks later, when cardiac hypertrophy was established.

**Effect of AAV9-mErbB4ECD on VEGFB-induced cardiac hypertrophy**

To study the effects of ErbB signaling inhibition on VEGFB-induced cardiac hypertrophy, recombinant AAV9-mErbB4ECD or AAV9-Ctrl was injected i.p. together with AAV9-mVEGFB186 i.p to wild type C57BL/6J mice. The AAV viral particles were injected in the following four combinations (the respective viral particle concentrations are mentioned within the
brackets): Ctrl (5 \times 10^{11}) alone, Ctrl (4 \times 10^{11}) + mVEGFB186 (1 \times 10^{11}), Ctrl (1 \times 10^{11}) + mErbB4ECD (4 \times 10^{11}), mVEGFB186 (1 \times 10^{11}) + mErbB4ECD (4 \times 10^{11}). Mice were terminated two weeks after the AAV injections.

**Blocking EGFR/ErbB signaling by Afatinib**

To inhibit EGFR, ErbB2 and ErbB4 signaling in VEGF-B -induced cardiac hypertrophy, eleven weeks old C57BL/6J wild type mice were injected i.p. with 2 \times 10^{11} AAV9 particles of Ctrl or mVEGFB186 vectors and treated with 25 mg/kg of Afatinib Dimaleate (BIBW2992, Cat# S7810, Selleckchem) by oral gavage for two weeks. To maintain the systemic levels, the inhibitor was administered every day and the mice were euthanized two weeks after the AAV-injections.

**Tissue collection**

At the end of the experiment mice were terminally anesthetised with intraperitoneal injection of ketamine and xylazin, and serum and hearts were collected. The heart weights were normalized to body weight or tibial length. A transverse mid-section of the heart was embedded in OCT and snap-frozen in liquid nitrogen-cooled 2-methylbutane containing 2% pentane, and the apex was frozen in liquid nitrogen for molecular and biochemical analyses.

**Cell culture**

RNA was isolated from human coronary microvascular endothelial cells (HCMEC), human coronary arterial endothelial cells (HCAEC), human cardiomyocytes (HCM) and human cardiac fibroblasts (HCF) (all from PromoCell) to analyze the expression of VEGFR1, VEGFR2 and Neuropilin 1 in each cell type. HCAECs, HCMECs, HDMECs and HUVECs were treated with recombinant VEGF (100 ng/ml) or PBS for 4 h and RNA was collected after stimulation. In another experiment, HCAECs were cultured with serum free medium overnight, then treated with
VEGF (50ng/ml) for 1, 3 or 6 hours. Culture media were collected and subjected to ELISA analysis for neuregulin1-β1 protein.

To silence Flt1 (VEGFR1), Mouse Brain endothelial cells (bEND.3, ATCC) were cultured and maintained in DMEM (Corning, #10-014-CVR) supplemented with 10% FBS, penicillin (100 U/mL) and streptomycin (100 µg/mL). The lentiviral vectors encoding two different clones of mouse Flt1 shRNA constructs (TRCN0000009606 and TRCN0000009608 obtained from FuGU core facility, University of Helsinki) were used to transfect bEND cells for 24 hours and the cells were then treated with puromycin (2 µg/mL) for 48 hours to select Flt1 silenced cells. After that the cells were harvested and used for further molecular analyses.

**ELISA**

ELISA was used to quantify protein levels of mouse VEGFR1 (DuoSet DY471, RnD Systems), mouse VEGFR2 (DuoSet DY493, RnD Systems), mouse PI GF (DY465, RnD Systems) and mouse VEGFB_{186} (in house ELISA based on RnD Systems antibodies AF590 and BAF767 and recombinant mVEGFB_{186} 767-VE). Neuregulin-1 was determined from conditioned media using Nrg-1 Elisa Kit (DuoSet DY377, RnD Systems).

**VEGFR1 recombination PCR**

To verify the recombination of VEGFR1, genomic DNA was isolated from mouse ear skin and heart. The forward and reverse primer sequences 5'-AGAAGATGCGTGGGCGTTAG-3' and 5'-CCTTCTCTGGCCTCCATCTG-3' were used to amplify the region containing the loxP sites in the construct. The PCR product was resolved in 1.5% agarose gel electrophoresis and the recombined and non-recombined amplicons were detected at ≈ 400bp and ≈ 2500bp, respectively.
**Real time quantitative PCR**

The apex portion of the adult mouse heart was homogenized in 700µl of TRIsure™ reagent (Bioline, USA) using the bead-based tissue homogenizer PowerLyzer®24 (MO BIO Laboratories, USA). Total RNA was purified and isolated using NucleoSpin RNA II Kit (Macherey-Nagel) according to the manufacturer’s protocol. RNA was reverse transcribed into cDNA with iScript cDNA synthesis Kit (Bio-Rad) and gene expression was analyzed using SYBR green gene or TaqMan gene expression assays (Applied Biosystems) using iQ™SYBR® Green Supermix and iQ™ Supermix (Bio-Rad) kit respectively. The gene expression assay was performed in BIO-RAD C1000 thermal cycler according to standardized protocol of the qPCR master mix supplier (Bio-Rad). The Cq values of the technical triplicates were averaged for each sample and normalized to the housekeeping genes HPRT-1, TBP or GAPDH. The mRNA expression level was calculated and presented as fold change (Ctrl = 1). The complete primer sequences and TaqMan probe set catalog numbers are listed in the Supplemental Table 1 and 2.

**Whole-genome microarray**

The quality of RNA was determined with Bioanalyzer (Agilent Technologies) and analysed on genome-wide Illumina Mouse WG-6 v2 Expression BeadChips (Illumina). Illumina’s GenomeStudio software was used for initial data analysis and quality control and the further data analysis was performed with the Chipster software (www.chipster.csc.fi)\textsuperscript{8}. After quantile normalization, statistically significant differences in expression of individual genes between the groups were tested using Empirical Bayes statistics and the Benjamini-Hochberg algorithm controlling false discovery rate (FDR). Adjusted FDR values of P<0.05 were considered significant. The microarray data have been submitted to the GEO database, under the series accession number GSE110532. Further, the UniProt accession numbers of the up and down regulated genes were retrieved and imported into the MetazSecKB proteome knowledgebase to characterize the
subcellular localisation, molecular function and secretome properties of the gene encoding proteins.

**Immunofluorescent staining**

10 µm thick transverse heart sections were fixed with acetone, blocked with Donkey Immuno Mix (DIM) and incubated with the respective primary antibodies (Supplemental Table 3). Primary antibodies were detected with the corresponding Alexa-conjugated 488, 594 or 647 secondary antibodies (Molecular Probes, Invitrogen). Stained sections were imaged with 40X air or oil immersion objectives using AxioImager fluorescent microscope (Carl Zeiss). The coronary vasculature was either stained with VEGFR2+ or CD31+ blood vessels or α-SMA+ arteries. The micrographs were initially adjusted for threshold and area fraction tool was used to quantify the vessels area, free hand line tool was used to measure the vessel luminal diameter and individual vessels per field were scored using multipoint selection tool to determine vessel density (Image J Software, NIH). The cardiomyocyte size were analyzed using Cell profiler pipeline in Cell Profiler™ image analysis platform (Carpenter Lab, Broad Institute, www.cellprofiler.org).

**Western blotting**

Mid-portion of the heart was homogenized in RIPA lysis buffer containing aprotinin, 1mg/ml Leupeptin, 1M PMSF, 1M NaF, 0.1M Na3VO4, 0.5% Triton X-100 (v/v) and 0.5% NP-40 (v/v) in PBS pH 7.4. The total protein concentration was determined using BCA protein assay kit (Pierce, Thermo Scientific). Equal amounts of total protein were resolved in 7.5% Mini-PROTEAN TGX Precast gels (Bio-Rad) and transferred to PVDF membrane (immobilon-P, Millipore). The blots were blocked with 5% BSA (wt/vol) in TBS containing 0.1% Tween 20 and incubated overnight at 4°C with primary antibodies (Supplemental Table 3). The secondary antibodies were labeled with suitable infrared red fluorescent dye (IR Dye) or HRP-conjugated secondary antibodies (DAKO).
The IR dye signals were directly detected with appropriate fluorescent channels in Odyssey CLx imaging system (Li-COR Biosciences), whereas the HRP signals were developed with Super-Signal West Pico Chemiluminescent substrate (#34080, Thermo Fisher Scientific) or Femto Maximum Sensitivity Substrate (#34095, Thermo Fisher Scientific) and detected by either Odyssey imager (Li-COR Biosciences) or X-ray films, wherever necessary. The Protein expression in the blots were quantified with Image Studio Lite software (Li-COR Biosciences).

**Vascular Permeability by Miles assay**

The Pdgfb-CreERT2;Flt1lox/lox mice were treated with tamoxifen and AAVs encoding VEGFB186 (2 x 10^{11} viral particles) and VEGFA164 (0.4 x 10^{11} viral particles) were injected intraperitoneally and allowed to express for two weeks and for four days, respectively. To analyse the vascular permeability by Miles assay, the mice were anesthetized with ketamine/xylazine and 100µl of 3% Evans Blue dye was dissolved in PBS and injected retro-orbitally under anesthesia. The dye was allowed to circulate for one hour and the mice were transcardially perfused with PBS. The heart, lungs, skeletal muscle (tibialis anterior) and kidney were harvested, weighed and Evans blue dye was extracted by incubating the tissues in 500µl of deionized formamide for overnight at 55°C. The optical density of the Evans blue was measured at 610 nm^{11}. The data is presented as ng of Evans Blue extravasated per mg of tissue dry weight^{12}.

**Echocardiography**

To analyse cardiac function and dimensions of the left ventricular chamber, thoracic cavity hair was removed and mice were anesthetized by inhalation with 2% isoflurane mixed with 0.5L/min 100% oxygen (Vevo Compact Dual Anesthesia System). The mice were placed on the heating stage (Vevo Imaging Station) in supine position, 1-1.5% isoflurane mixed with 0.5L/min 100% oxygen were supplied continuously via the nose cone to maintain the mice under sedation. Electrode gel
was applied to the paws connected to ECG electrodes. Prewarmed ultrasound gel was applied on the thoracic cavity, heart rate between 430 - 450 beats per minute were maintained and two-dimensional (2D) ECG images were acquired using MS550D 22–50 MHz linear array solid-state transducer (Vevo 2100 Ultrasound, FUJIFILM VisualSonics). The ECG images in B-mode were used to confirm the anatomic boundaries of the ventricular chambers and M-mode along the para-sternal short axis view using modified Simpson’s method were used to measure left ventricular internal diameter, left ventricular posterior wall thickness, interventricular septum thickness at end-systole and end-diastole. These parameters were used to calculate left ventricular mass, volume, ejection fraction and fractional shortening (Vevo Vasc Analysis software). The data were reported as mean ± SEM.

**Maximal exercise test**

The maximal endurance capacity was measured on a treadmill (LE 8710, Bioseb) by incremental maximal running test. For adaptation, mice were familiarized on the treadmill for two days for 15 min at the speed of 15 m/s. In the maximal exercise test the speed was increased from 15 m/s by 5 m/s two times in 5 min interval (for total of 15 min) and then the speed was increased by 2 m/s every 2 min until exhaustion. Total running distance (m) and time (min) were recorded.

**Blood Pressure measurement**

Tail-cuff method (CODA Blood Pressure System, Kent Scientific) was used to measure blood pressure (BP) from the tail blood volume. Prior to the actual measurements the experimental mice were trained and acclimatized to the CODA mouse holder, nose cone and tail cuff (Occlusion and Volume Pressure Recording (VPR) cuff) for 10min and repeated for 3 consequetive days. On the day of actual experiment, the mice were transferred to the mouse holder, nose cone was adjusted to prevent the mice from moving, occlusion cuff was fixed to the proximal end of the tail followed by
VPR cuff. The following parameters were set in the CODA data acquisition software: Acclimation cycles – 10, Number of sets – 1, Time between sets – 30sec, Cycles per set – 10, Time between cycles – 5sec, Maximum Occlusion Pressure – 250mmHg, Deflation time – 15 sec, Minimum volume – 15µl. The systolic and diastolic BP were recorded and represented as mean ± SEM.

Statistics

The data sets from individual experiments were statistically analyzed by One-way or Two-way ANOVA with Holm-Sidak post hoc test or two-tailed student’s t test. P<0.05 value was considered statistically significant and P values in the graphs are mentioned as *P<0.05, **P<0.01 and ***P<0.001. The data is represented as mean ± SEM and the GraphPad Prism 7 software was used for statistical analysis.
**Supplemental Table 1:** The mouse primer sequences for SYBR green gene expression assays and catalogue numbers for TaqMan probe sets.

| Gene symbol | Forward Primer (5’-3’) | Reverse Primer (5’-3’) |
|-------------|------------------------|------------------------|
| APJ         | CGCCAGGCCATTCTCAAAG    | AGCTGCCCTCTGCTGCTATCTGTC |
| Apln        | CAGGCCTATTCCCAGGCTCA   | CAAGATCAAGGGCGCAGTCA    |
| STC1        | ATTTGACACTCAGGGAAAAG   | AAACGTGAAGCTTGCTGTAAG  |
| SPARC       | GAACCCACATGGGAAGTCTTA  | AAAGCCCAATTGCGAGGATG    |
| VEGFB       | GATCCTCCTAGATCCAGTACC  | TTTGGTCTGCTACCATGTTG    |
| GDF10       | TGAGAAGTACAACCCGAAAGAG| GAGGATCATATTCTGAGTCTTG  |
| ESM-1       | CTGGAGCGCCAAATATCGC    | TGAGACTGTACGGTAGGCTG    |
| NRP-1       | GGAGCTACTGCGCTGAAAG    | CCTCCTGTGAGCTGGAAGTC    |
| VEGFR2      | GCGTGATTCTGAGGAAAGGAT  | CCGTGGAAATGAGTGTGTG    |
| CD31        | GAACTGCACCCATCCTTAC    | ATGCTCTGCTGAGGCTTATC    |
| ANP         | GAAAAGCAAAACTGAGGGGCTG| CCTACCCCGAGAGCTG       |
| BNP         | AGGCGAGACAAAGGGGAACA   | GGAGATCCATGCGCGAGA     |
| COL3        | TGGTCCTCAGGGTGAAAGG    | GTCCAGCATCACCTTTTGT     |
| Skeletal α-actin | TCCTCCGCCGTTGGCT    | AATCTATGTACACGTACAAAAA |
| PI3K-p110β  | AACCTTCCTCCTCCCTTACC  | GGTCCGCTGAAAAAGCCCAG   |
| Akt         | ATCCCCCTAAACTCTTCAGT   | CTTCCGCTCCTCTCTCTTC    |
| C/EBPβ      | ACGACTTTCCCTCCGCACCTCT | ACGCTACGTAACCGGTACG    |
| GSK3β       | CACTCTTCAACTTCTACC    | ATTAGTACCTGAGGCTG      |
| TBX3        | TGTCTGGGAGGGAGCCTAAA  | CAACAGCAGCCTGGTTACACA  |
| ANKRD1/CARP | AGAGTAGAGGAGCGTGGTAACA| TTGGCCGGAAGTGCTCTCAGG  |
| Gene      | Primer Sequence 1          | Primer Sequence 2          |
|-----------|---------------------------|----------------------------|
| SRF       | CATCATGAAGAAGGCTATG       | TCTCAGTGGTGATCATGG         |
| MEK1      | TGCCACAGAGATCTACATG       | TGGCATCAGAGAGGAATG         |
| MYH6      | GCAGCTGTGCAATCATTCAAT    | CACTCAATGCCCTCCTTCTTG      |
| cTnnT2    | AGAGATTACAAACGGAGMA       | GAGTTTGGAGACTTTCTGG        |
| GAPDH     | ACAACTTTGGCATTGTGGAAG    | GATGCAAGGATGATGTTCTG       |
| TBP       | GGAAGCTGGCTACAATCCAG     | CCCCTTGTACCCCTCACCAGAAT   |
| HPRT-1    | TTGCTCGAGATGTCATGAGGAA  | AGCAGGTCAGCAAAGAACTTATAG  |
| VEGFRI    | TTCATCAGTGTGAAACATCG     | CGAGCCATCTTTAACCATACTAG   |
|           | (Exon 8-9)                |                             |
| VEGFRI    | TTGAGGAGCTTTCCACGAAC     | GGAGGAGTACAACACCACCGG     |
|           | (Exon 29-30)              |                             |
| VEGFRI    | Mm00438980_m1            |                             |
|           | (Ex15-16)                |                             |
| VEGFR2    | Mm01222419_m1            |                             |
| VEGFA     | Mm00437304_m1            |                             |
| NRP-1     | Mm00435372_m1            |                             |
| Pecam-1   | Mm01246167_m1            |                             |
| NOTCH1    | Mm00435245_m1            |                             |
| NOTCH4    | Mm00440525_m1            |                             |
| JAG1      | Mm00496902_m1            |                             |
| Dll4      | Mm00444619_m1            |                             |
Supplemental Table 2: The human primer sequences for SYBR green gene expression assays.

| Gene symbol | Forward Primer (5'-3') | Reverse Primer (5'-3') |
|-------------|------------------------|------------------------|
| NRG1        | GTGGAATCAACGCTACATC    | AAAGGTCTTTCAACCATGAAG  |
| HB-EGF      | GCTTATATACCTATGACCACAC| GTACCTAAACATGAGAAAGCC  |
| ADAM12L     | CACCATTGAAAAAATAGGTTGT| GAGCCTGACAGGGTTGGAAG   |
|             | GT                     |                        |
| ADAM12S     | CTGGGCACCTCCCTTCTG     | TGCTCTGTGCTGCGGGA      |
| HPRT-1      | TGAGGATTTGGAAGGGGTGT   | TCCCCTGTGACTGTCATT     |
**Supplemental Table 3:** List of primary and secondary antibodies used in immunohistochemistry (IHC) and Western blotting (WB).

| Antibody                      | Clone      | Application | Company/Cat.no            |
|-------------------------------|------------|-------------|--------------------------|
| rat anti-mouse CD31          | MEC 13.3   | IHC         | BD Pharmingen/553370     |
| goat anti-mouse VEGFR2       | Polyclonal | IHC, WB     | R&D Systems /AF644       |
| mouse αSMA-Cy3                | Polyclonal | IHC         | Sigma/ C6198             |
| rabbit Laminin-1             | Polyclonal | IHC         | Thermo scientific/ Rb-082|
| mouse Dystrophin             | Polyclonal | IHC         | Novocastra/ NCL-DYS2     |
| goat anti-mouse VEGFR1       | Polyclonal | WB          | R&D Systems / AF471      |
| rabbit pVEGFR2               | Polyclonal | WB          | Cell signaling/#2478     |
| Peroxidase conjugated rabbit | Polyclonal | WB          | DAKO/ P0161              |
| anti-mouse IgG               |            |             |                          |
| Peroxidase conjugated swine  | Polyclonal | WB          | DAKO/ P0217              |
| anti-rabbit IgG              |            |             |                          |
| mouse anti-Hsc70             | Polyclonal | WB          | Santa Cruz/SC-7298      |
| rabbit pEGF Receptor (Y1086) | Polyclonal | WB          | Cell signaling/#2220S    |
| rabbit pHER3/ErbB3 (Y1289)   | 21D3       | WB          | Cell signaling/#4791S    |
| rabbit pHER4/ErbB4 (Y1284)   | 21A9       | WB          | Cell signaling/#4757S    |
| rabbit PI3K-p85              | Polyclonal | WB          | Cell signaling/#4292S    |
| rabbit pAkt (Ser473)         | Polyclonal | WB          | Cell signaling/#9271L    |
| Antibody                        | Type       | Format | Source                      |
|--------------------------------|------------|--------|-----------------------------|
| rabbit Akt                     | Polyclonal | WB     | Cell signaling/#9272S      |
| rabbit pErk1/2 (T202/Y204)     | E10        | WB     | Cell signaling/#9106S      |
| rabbit Erk1/2                   | Polyclonal | WB     | Cell signaling/#9102L      |
| rabbit pS6K (Ser235/236)       | Polyclonal | WB     | Cell signaling/#2211L      |
| mouse S6K                       | 54D2       | WB     | Cell signaling/#2317S      |
| mouse Vinculin                 | hVIN-1     | WB     | Sigma/#V9131                |
| rabbit EGFR (1005)             | Polyclonal | WB     | Santa Cruz/SC-03            |
| rabbit ErbB3 (C-17)            | Polyclonal | WB     | Santa Cruz/SC-285           |
| rabbit anti-ErbB4              | E200       | WB     | Abcam/ab32375               |
| human NRG1- β1                 | 147705     | WB     | R&D Systems/MAB3771         |
| rabbit anti-HB EGF             | Polyclonal | WB, PD | Abcam/ab192545              |
| rabbit NRG4                    | Polyclonal | WB     | Biorbyt/b229181             |
| rabbit Nrg-1 α/β/1/2 (C-20)    | Polyclonal | WB     | Santa Cruz/SC-348           |
| goat-Actin (I-19)              | Polyclonal | WB     | Santa Cruz/SC-1616          |
| IRDye® 680RD Donkey anti-Goat  |           | WB     | Li-COR Biosciences/926-68074|
| IRDye® 800CW Donkey anti-Rabbit|           | WB     | Li-COR Biosciences/926-32213|
| IRDye® 680RD Donkey anti-Rabbit|           | WB     | Li-COR Biosciences/926-68073|
| IRDye® 800CW Donkey anti-Mouse |           | WB     | Li-COR Biosciences/926-32212|
| IRDye® 680RD Donkey anti-Mouse |           | WB     | Li-COR Biosciences/926-68072|
| HRP-Goat anti-Rabbit IgG (H+L) | WB | Invitrogen/A16104 |
### Supplemental Table 4: Cardiac function and ventricular chamber dimension analysis by echocardiography

| Treatment groups | HR (bpm) | IVS,d (mm) | IVS,s (mm) | LVID,d (mm) | LVID,s (mm) | LVPW,d (mm) | LVPW,s (mm) | LV Vol,d (μl) | LV Vol,s (μl) | LV mass (mg) | EF (%) | FS (%) |
|------------------|----------|------------|------------|-------------|-------------|-------------|-------------|---------------|---------------|--------------|--------|--------|
| Cre (-); R1ECΔΔ + Ctrl n=6; 3.5 mo old | 413±8.7 | 0.72±0.05 | 1.18±0.09 | 3.63±0.08 | 2.36±0.12 | 0.80±0.07 | 1.06±0.07 | 55.6±2.9 | 19.8±2.3 | 75.7±5.3 | 64.7±3.3 | 34.9±2.4 |
| Cre (+); R1ECΔΔ + Ctrl n=4; 3.5 mo old | 433±2.0 | 0.77±0.04 | 1.17±0.07 | 3.76±0.12 | 2.45±0.09 | 0.96±0.11 | 1.29±0.11 | 60.5±4.6 | 21.5±1.9 | 93.8±4.8 | 64.6±1.5 | 34.6±1.1 |
| Cre (+); R1ECΔΔ + mB186 n=5; 3.5 mo old | 458±6.2 | 0.83±0.04 | 1.15±0.09 | 4.08±0.04 | 2.72±0.08 | 1.07±0.06 | 1.37±0.06 | 73.8±4.3 | 27.8±4.7 | 123.3±5.3 | 62.5±4.1 | 33.4±2.9 |

#### 3 weeks post tamoxifen and AAV9-Ctrl/mB186 administration

| Treatment groups | HR (bpm) | IVS,d (mm) | IVS,s (mm) | LVID,d (mm) | LVID,s (mm) | LVPW,d (mm) | LVPW,s (mm) | LV Vol,d (μl) | LV Vol,s (μl) | LV mass (mg) | EF (%) | FS (%) |
|------------------|----------|------------|------------|-------------|-------------|-------------|-------------|---------------|---------------|--------------|--------|--------|
| Cre (-); R1ECΔΔ + Ctrl n=6; 5.5 mo old | 396±19.7 | 0.87±0.04 | 1.15±0.08 | 3.78±0.05 | 2.52±0.08 | 0.79±0.04 | 1.15±0.08 | 60.9±1.9 | 22.9±1.8 | 90.4±6.8 | 62.3±2.8 | 33.2±2.0 |
| Cre (+); R1ECΔΔ + Ctrl n=4; 5.5 mo old | 443±7.6 | 0.86±0.07 | 1.14±0.06 | 3.88±0.06 | 2.62±0.05 | 0.98±0.10 | 1.38±0.11 | 65.2±2.3 | 25.0±1.2 | 109.3±9.1 | 61.3±3.1 | 32.5±2.2 |
| Cre (+); R1ECΔΔ + mB186 n=5; 5.5 mo old | 438±18.3 | 1.02±0.09 | 1.33±0.10 | 4.35±0.10 | 2.98±0.13 | 1.06±0.07 | 1.47±0.06 | 85.5±4.7 | 34.9±3.8 | 154.7±7.8 | 59.5±2.7 | 31.6±1.8 |

#### 2.5 months post tamoxifen and AAV9-Ctrl/mB186 administration

| Treatment groups | HR (bpm) | IVS,d (mm) | IVS,s (mm) | LVID,d (mm) | LVID,s (mm) | LVPW,d (mm) | LVPW,s (mm) | LV Vol,d (μl) | LV Vol,s (μl) | LV mass (mg) | EF (%) | FS (%) |
|------------------|----------|------------|------------|-------------|-------------|-------------|-------------|---------------|---------------|--------------|--------|--------|
| Cre (-); R1ECΔΔ + Ctrl n=6; 8 mo old | 358±20.3 | 0.69±0.03 | 0.81±0.05 | 3.89±0.06 | 2.76±0.06 | 0.81±0.06 | 1.11±0.10 | 65.7±2.7 | 28.9±1.5 | 82.5±5.6 | 55.8±2.6 | 28.7±1.8 |
| Cre (+); R1ECΔΔ + Ctrl n=4; 8 mo old | 390±27.3 | 0.63±0.06 | 0.90±0.04 | 4.22±0.18 | 3.18±0.26 | 0.84±0.13 | 1.00±0.17 | 80.1±7.8 | 41.7±7.5 | 91.5±12.4 | 48.9±7.0 | 24.9±4.3 |
| Cre (+); R1ECΔΔ + mB186 n=5; 8 mo old | 437±18.0 | 0.87±0.02 | 1.11±0.06 | 4.41±0.14 | 3.15±0.21 | 1.16±0.13 | 1.69±0.12 | 88.9±6.7 | 40.7±6.3 | 155±15.9 | 55.2±4.0 | 28.8±2.6 |

mo: months; HR: heart rate, beats per minute; IVS,d: Interventricular septum thickness at end-diastole; IVS,s: Interventricular septum thickness at end-systole; LVID,d: Left ventricular internal dimension at end-diastole; LVID,s: Left ventricular internal dimension at end-systole; LVPW,d: Left ventricular posterior wall thickness at end-diastole; LVPW,s: Left ventricular posterior wall thickness at end-systole; LV Vol,d: Left ventricular volume at end-diastole; LV Vol,s: Left ventricular volume at end-systole; LV mass: Left ventricular mass; EF: Ejection fraction; FS: Fractional shortening.

Data is presented as mean ± SEM and analyzed by One-Way ANOVA (Holm-Sidak test).

* p<0.05, ** p<0.01: R1nAEC cre (+) + AAV9-Ctrl/mB186 vs WT (Cre-) + AAV9-Ctrl, † p<0.05: cre (+) + AAV9-Ctrl vs cre (+) + AAV9-mB186 within each time point
SUPPLEMENTAL FIGURES

Supplemental Figure 1. AAV-VEGF-B increase cardiac vasculature and heart weight in both WT and VEGFR1 TK KO mice. (A-B) HW/BW and CMC size in WT and VEGFR1 tyrosine kinase deficient (TK KO) mice treated with AAV-VEGF-B or a control vector. (C-D) Cardiac vessel area and arterial area. (E) VEGFR2 protein levels in the heart measured with ELISA, (F) VEGFR1 and VEGFR2 mRNA expression in the heart and (G) cardiac VEGFR2 protein levels by WB. (H) Silencing of VEGFR1(Flt1) in mouse brain endothelial cells using lentiviral (LV) vectors encoding two independent shRNA constructs and a scrambled control (Scr). Note increased VEGFR2 mRNA expression. (A-F) Two-way ANOVA (Holm-Sidak’s multiple comparison test), *p<0.05, **p<0.01, ***p<0.001 (N = 4/group). (G-H) Student’s t-test, ***p<0.001.

Supplemental Figure 2. Analysis of Pdgfb-CreERT2 mediated deletion of VEGFR1 in the heart. (A) Fluorescence images of the heart showing tdTomato/RFP (red fluorescent protein) signal exclusively in endothelial cells (co-staining with VEGFR2) after tamoxifen-treatment of the Pdgfb-CreERT2; Rosa26-tdTomatoSTOPfl/STOPflo/STOP mice. (B) Schematic of the Vegfr1 (Flt1) loxP sites in the 5’ region of the targeted Vegfr1 allele. (C) Analysis of Cre-mediated DNA recombination in skin and heart of Pdgfb-CreERT2;Vegfr1fl/fl mice (R1ECΔA), analysed by PCR. Red and blue arrows point to the recombined (400 bp) and non-recombined (≈ 2400 bp) allele, respectively. (D) Body weight of the mice. (E-F) Quantification of VEGF and VEGFR1 mRNA expression in the heart. (G) sVEGFR1 protein level in the serum by ELISA and (H) VEGFR1 protein in the heart by Western Blot. Two-way ANOVA (Holm-Sidak test), *p<0.05, **p<0.01, ***p<0.001 (N = 4/group). Scale bar 50µm.
Supplemental Figure 3. AAV-PIGF induced cardiac hypertrophy in wild-type littermates and R1ECΔΔ mice. Heart weight normalized to body weight (mg/g). Two-way ANOVA (Holm-Sidak test), *p<0.05, (N = 4/group).

Supplemental Figure 4. Miles assay for vascular leakage analysis. Vascular leakage in the heart, skeletal muscle, lung or kidney was analyzed in VEGFR1 EC-deleted, VEGF-B overexpressing or double-treated mice after injecting Evans Blue. AAV-VEGF164 was used as a positive control. Note that a five-fold lower dose and shorter expression period (4 days vs. 2 weeks) was used for VEGF. Data are represented as mean ± SEM. Two-way ANOVA (Holm-Sidak test), *p<0.05, **p<0.01, ***p<0.001 (N= 4-5/group).

Supplemental Figure 5. VEGFR2 blocking antibody DC101 reverses the effects of VEGF-B in the heart. (A-B) VEGF-B concentration in serum (ng/ml) and heart lysates (ng/mg) measured by ELISA. (C-D) Vascular area and cardiomyocyte size in the rescue experiment at 4-week time point. In this experiment, DC101 treatment was started two weeks after AAV-injection, when cardiac hypertrophy was already present. (E-F) mRNA expression of Apj, Stc1, Esm1 and pathological gene markers (Anp, Bnp, Col3a and a-skeletal actin) in the heart. Data are represented as mean ± SEM. Two-way ANOVA (Holm-Sidak test), **p<0.01, ***p<0.001 (N= 5/group).

Supplemental Figure 6. AAV-PIGF induced vasculature growth and cardiac hypertrophy are blocked by inhibition of VEGFR2 signaling using DC101 antibody. (A-C) Heart weight normalized to body weight (mg/g) and quantification of cardiomyocyte size and blood vessel area (%). (D-E) mPlGF2 levels in serum and heart quantified by ELISA. Data are represented as mean ± SEM. Two-way ANOVA (Holm-Sidak test), *p<0.05, **p<0.01, ***p<0.001 (N= 5/group).
**Supplemental Figure 7.** Constitutive or conditional deletion of VEGFR1 in cardiomyocytes does not affect heart growth. (A) Heart weight normalized to body weight (mg/g) in Myh6CreER<sup>T2</sup>; Vegfr1<sup>f/f</sup> mice, in which VEGFR1 was deleted from cardiomyocytes at the age of 8 weeks by tamoxifen treatment. The hearts were analyzed two months later. (B) Heart weight normalized to body weight (mg/g) in αMHC-Cre; Vegfr1<sup>f/f</sup> mice, in which VEGFR1 had been deleted using constitutive deleter already during development. Two-way ANOVA (Holm-Sidak test), N= 5-8/group. R1<sup>+/+</sup> = mice with two wt alleles for VEGFR1, R1<sup>Δ/+</sup> = mice with one VEGFR1 floxed allele and one wt allele, R1<sup>ΔΔ</sup> = mice with two VEGFR1 floxed alleles.

**Supplemental Figure 8.** APJ deficiency does not affect AAV-VEGF-B induced vascular changes or cardiac hypertrophy. (A) Heart weight normalized to body weight (mg/g) and (B) quantification of CMC size (µm²) and (C) blood vessel area (%). (D) Representative images of immunofluorescent staining of cardiomyocyte basal lamina (Laminin-1) and blood vasculature (CD31). One-way ANOVA (Holm-Sidak test), (N=5-6/group), *p<0.05, **p<0.01, ***p<0.001. Scale bars 100µm.

**Supplemental Figure 9.** VEGF stimulates Nrg1 release from ECs. (A) Detection of Nrg1 in the cardiac microvascular endothelial cells (CMEC) and cardiomyocytes (CMC). (B) Shedding of Nrg-1 into conditioned media of CMECs upon VEGF stimulation at 10, 20 and 50 ng/ml concentration. (C) Total and phosphorylated Akt levels in adult rat ventricular myocytes (ARVM) treated with conditioned media of CMECs stimulated with VEGF. Recombinant Nrg (50 ng/ml) treated ARVM as a positive control. (D) Schematic illustration of the proposed mechanism of how increased VEGF production by CMCs promotes an angiogenic responses in ECs and induces Nrg1 shedding from ECs. Nrg1, in turn, signals back to CMCs and promotes Akt phosphorylation and cardiomyocyte growth.
Supplemental Figure 10. Blocking ErbB signaling with AAV9-mErbB4ECD or Afatinib. (A-B) Vascular area (%) in adult mouse heart expressing VEGF-B and/or mErbB4ECD. Heart weight and HW/BW ratio in AAV-VEGF-B and AAV-mErbB4ECD treated mice (C-D) and in Afatinib and AAV-VEGF-B treated mice (F-G). (E and H) Protein levels of VEGF-B in serum. Two-way ANOVA (Holm-Sidak test), *p<0.05, **p<0.01, ***p<0.001. Number of mice animals N=5/per group in A and shown in brackets in B and C. Scale bars 100µm.

Supplemental Figure 11. Angiogenesis-induced cardiac hypertrophy does not progress into heart failure. (A) Ejection fraction 3 weeks, 2.5 months and 5 months after VEGFR1 deletion and/or AAV-VEGF-B treatment. (B) Total running distance (m) and time (min) in an incremental maximal exercise test on a treadmill 5 months after the treatments. (C) mRNA expression of pathological gene markers (Anp, Bnp, Col3a, α-skeletal actin) in the heart. (D) Systolic and diastolic blood pressure (mmHg) in AAV-VEGF-B treated and VEGFR1 deleted mice and their combination. Two-Way ANOVA (Holm-Sidak test), In A and B (N= 4-6/group), in C (N=4/group), in D (N=6-10/group) *p<0.05, **p<0.01.
Supplemental Figure 1

A. HW/BW (mg/g) 
B. CMC Size (μm²) 
C. CD31+ vessel area (%) 
D. α-SMA+ vessel area (%) 
E. VEGFR2 (ng/mg) 
F. Fold change (Ctrl = 1) of VEGFR2
G. Western Blot of VEGFR2 and HSC70 
H. Fold change (Ctrl = 1) of VEGFR1 and VEGFR2
Supplemental Figure 2

A. Pdgfb-Cre\textsuperscript{ERT2}\textsubscript{ER}\textsubscript{2} negative control mice (Rosa26-tgTomato\textsuperscript{R/STOP}} were treated with tamoxifen to induce expression of tdTomato. 

B. Schematic representation of the VEGFR1 locus showing the 5'UTR and ORF regions.

C. PCR analysis of Skin DNA (2500bp and 500bp) and Heart DNA with Cre+/− knock in mice treated with tamoxifen.

D. Body weight (BW) analysis of R1\textsuperscript{EC}\textsubscript{AS} mB186−/+−/++ mice treated with tamoxifen.

E. Relative expression of VEGF transcript levels in different genotypes.

F. Relative expression of VEGFR1 transcript levels in different genotypes.

G. Quantification of sVEGFR1 protein levels in different genotypes.

H. Western Blot analysis of VEGFR1 protein levels in Heart tissue.
Supplemental Figure 3

![Graph showing HW/BW (mg/g) for different conditions]

- HW/BW (mg/g) for different conditions:
  - R1 ECΔΔ-
  - mPIGF2-
  - R1 ECΔΔ+
  - mPIGF2+

* Significant differences indicated by asterisks.
Heart Skeletal Lung Kidney

Evans blue extravasated (pg)/Dry tissue mass (mg)

Supplemental Figure 4
Supplemental Figure 5

A. Serum VEGFB (ng/ml)

B. Heart VEGFB (ng/mg)

C. CD31+ Vessel area (%)

D. CMC Size (μm²)

E. Fold Change (Ctrl = 1)

F. Fold Change (Ctrl = 1)
Supplemental Figure 7

A

Myh6-CreERT2

\( R^{1^+/+} \)

\( R^{1^{Δ/Δ}} \)

\( + \)  

\( + \)

\( + \)

\( n.s \)

\( 6 \)

\( 4 \)

\( 2 \)

\( 0 \)

HW/BW (mg/g)

B

\( αMHC-Cre \)

\( R^{1^{++/+}} \)

\( R^{1^{Δ/Δ}} \)

\( + \)  

\( + \)

\( + \)

\( + \)

\( n.s \)

\( 6 \)

\( 4 \)

\( 2 \)

\( 0 \)

HW/BW (mg/g)
Supplemental Figure 8

A

B

C

D

APJ KO

mB186

APJ WT

AAV9-Ctrl

AAV9-mB186

AAV9-Ctrl

AAV9-mB186
Supplemental Figure 9

**A**

|        | CMEC | Myocyte |
|--------|------|---------|
| Nrg1   | [image] | [image] |
| GAPDH  | [image] | [image] |

**B**

Anti-Nrg-1β (53KDa)  

| VEGF (ng/ml) | CMEC conditioned medium |
|--------------|--------------------------|
| 0            | [image]                   |
| 10           | [image]                   |
| 20           | [image]                   |
| 50           | [image]                   |

**C**

Conditioned medium (CMEC treated with VEGF)  

| Nrg (50ng/ml) | pAKT (60KDa) |
|---------------|--------------|
| Positive ctrl | [image]      |
| 1h            | [image]      |
| 3h            | [image]      |
| 6h            | [image]      |

**D**

Cardiomyocytes  

| Akt1 ↑ | Myocyte Growth ↑ | Neuregulin |
|--------|-------------------|------------|

Pressure overload or Myocyte Akt1 activation  

Coronary Angiogenesis  

Endothelial cells
### Serum VEGFB (ng/ml)

| Condition                        | AAV9-Ctrl (n=23) | AAV9-mB186 (n=22) | AAV9-Ctrl + mErbB4 + ECD (n=19) | AAV9-mB186 + mErbB4 + ECD (n=23) |
|----------------------------------|------------------|-------------------|----------------------------------|----------------------------------|
| **0.05**                         |                  |                   | **0.05**                          |                                  |
| **0.10**                         |                  |                   | **0.10**                          |                                  |
| **0.15**                         |                  |                   | **0.15**                          |                                  |
| **0.20**                         |                  |                   | **0.20**                          |                                  |

### HW/BW (mg/g)

| Condition   | AAV9-Ctrl (n=5) | AAV9-mB186 (n=4) | AAV9-Ctrl + Afatinib (n=5) | AAV9-mB186 + Afatinib (n=5) |
|-------------|-----------------|------------------|---------------------------|------------------------------|
| **2**       |                 |                  |                           |                              |
| **4**       |                 |                  |                           |                              |
| **6**       |                 |                  |                           |                              |

### CD31+ vessel area (%)

| Condition   | AAV9-Ctrl (n=5) | AAV9-mB186 (n=4) | AAV9-Ctrl + Afatinib (n=5) | AAV9-mB186 + Afatinib (n=5) |
|-------------|-----------------|------------------|---------------------------|------------------------------|
| **20**      |                 |                  |                           |                              |
| **40**      |                 |                  |                           |                              |
| **60**      |                 |                  |                           |                              |
Supplemental Figure 11

A

|        | 3wk | 2.5mo | 5mo |
|--------|-----|-------|-----|
| EF (%) |     |       |     |
| R1ECΔΔ | -   | +     | -   |
| mB186  | -   | -     | +   |

B

|        | 3wk | 2.5mo | 5mo |
|--------|-----|-------|-----|
| Running Distance (m) |     |       |     |
| R1ECΔΔ | -   | +     | -   |
| mB186  | -   | -     | +   |

C

Fold Change (Ctrl = 1)

|        | Anp | Bnp | Col3a | α-skeletal actin |
|--------|-----|-----|-------|------------------|
| WT+AAV9-Ctrl |   1 |     |       |                  |
| WT+AAV9-mB186 |   2 |     |       |                  |
| R1ECΔΔ +AAV9-Ctrl |   3 |     |       |                  |
| R1ECΔΔ +AAV9-mB186 |   4 |     |       |                  |

D

Systolic

|        | 3wk | 2.5mo | 5mo |
|--------|-----|-------|-----|
| BP(mmHg) |     |       |     |
| R1ECΔΔ | -   | +     | -   |
| mB186  | -   | -     | +   |

Diastolic

|        | 3wk | 2.5mo | 5mo |
|--------|-----|-------|-----|
| BP(mmHg) |     |       |     |
| R1ECΔΔ | -   | +     | -   |
| mB186  | -   | -     | +   |
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