SUPPLEMENTAL MATERIAL

Modulation of Endothelial BMPR2 Activity by VEGFR3 in Pulmonary Arterial Hypertension

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Supplemental Methods

siRNA and transfection. Human siRNA against *VEGFR3* and non-targeting control (Invitrogen) were transfected with Lipofectamine RNAiMAX (13778-150, Life Technologies) according to the manufacturer's instructions. The cells were harvested 72 hours after transfection.

RNA extraction and RT-PCR. The cells or homogenized lung tissues were resuspended in Qiazol (79306, Qiagen) and total RNA was then extracted with the miRNeasy RNA isolation kit (Qiagen). Purified total RNA was reverse transcribed with the iScript cDNA Synthesis Kit (Bio-Rad). The mRNA expression levels of human and mouse transcripts were determined by quantitative real-time PCR with TaqMan probes (Applied Biosystems) or SYBR green assays (Bio-Rad) on a CFX-96 (Bio-Rad) according to the manufacturer's instructions.

Western blot and immunoprecipitation. Western blotting and immunoprecipitation were described previously.[1] Briefly, cells were washed with PBS in 3 times and lysed in RIPA buffer (89901, Thermo Scientific) containing Halt protease and phosphatase inhibitor cocktails (78442, Thermo Scientific). Lung tissues were homogenized in RIPA buffer with Halt protease and phosphatase inhibitor cocktails, and centrifuged at 15,000 rpm for 30 min at 4°C. The protein concentrations were measured with the micro BCA protein assay kit (23235, Thermo Scientific) and samples were boiled in a 95 °C heat block for 10 min and separated by SDS-PAGE. For immunoprecipitation, equivalent protein concentrations of cell lysates were incubated with indicated antibodies for overnight at 4 °C, followed by incubation with protein A/G agarose plus beads (sc-2003, Santa Cruz) for 2 h at 4 °C. Immunoprecipitates were extensively washed, and the eluted precipitates were run on SDS-PAGE gels and the separated proteins were transferred to Immun-Blot PVDF membrane (162-0177, Bio-Rad). The membranes were blocked with 5 % BSA in PBS-T (0.1 % Tween-20 in PBS) for 1 h at room temperature and

incubated overnight at 4°C with the indicated antibodies. The membranes were washed with PBS-T and incubated with HRP tagged secondary antibodies. The signal was detected using the ECL System (34096, Thermo Scientific). Each western blot is representative of three independent experiments of triplicate samples. The antibodies used were: phospho-Smad 1/5 (Ser463/465) (Cell Signaling), Smad 1 (Santa Cruz), Smad 5 (Cell Signaling), GAPDH (Cell Signaling), Tubulin (Santa Cruz), BMPR2 (BD science), VEGFR3 (Millipore), and Id2 (Santa Cruz).

Immunohistochemistry and immunofluorescence. For human tissues, lung sections were deparaffinized with Histoclear (National Diagnostics) twice for 10 min and rehydrated through a degraded series of ethanol concentrations (100 % to 30 %). Antigen retrieval was carried out with Retrieval solution (DAKO) for 30 min at 94 ~ 96 ^{o}C, followed by cooling on the bench for 20 min at room temperature. The lung tissue sections were blocked with 5 % BSA in PBS for 1 h at room temperature and incubated with anti-CD31 antibody (M0823, Dako) and anti-VEGFR3 antibody (sc-321, Santa Cruz) overnight at 4 ^{o}C, and then incubated with Alexa Fluor 568 goat anti-rabbit IgG (A11011, Invitrogen) and Alexa Fluor 488 goat anti-mouse IgG antibodies (A11001, Invitrogen) and counter stained with DAPI and imaged using Nikon eclipse Ti confocal microscope.

For assessing YFP expression in the*Vegfr3:YFP* mice, these mice were perfused with saline through the RV and vented through the aorta. Liquid bismuth contrast agent was perfused through the RV and the mice were immediately placed on ice to solidify the contrast agent. The lungs were removed and fixed in 4% paraformaldehyde overnight at 4 ^{o}C. The lungs were then washed in PBS and equilibrated in 30% sucrose overnight at 4 ^{o}C. The lungs were embedded in OCT and sectioned at 15 μm. Sections were counterstained with DAPI and fluorescence and DIC signals were detected using Nikon Eclipse Ti confocal microscope.

Lentivirus. For VEGFR3 expression lentivirus, VEGFR3 construct was purchased from Origene Technologies and cloned into pCDH-EF1-MCS lentivirus backbone vector (System Biosciences) to generate pCDH-EF1-hVEGFR3 vector. 293T cells were cotransfected with pCDH-EF1-hVEGFR3, pVsvg and pGag-pol vectors using Lipofectamnine LTX reagent (Invitrogen). Media was replaced at 12 h after transfection. Medium with infectious lentiviruses was harvested at 72 h after transfection. HUVECs or PAECs were infected with recombinant lentivirus transducing units with 8 µg/mL Polybrene (Sigma).

Cell surface biotinylation and Internalization assay. Biotinylation assay was performed as previously described.[2] Briefly, ECs were grown to confluence and washed twice with ice cold PBS. 5ml/10cm dish 0.25mg/ml Biotin in ice cold PBS was added to the culture dish. After ECs were incubated with Biotin for 60 min at 4 ^{o}C on orbital shaker, the remaining Biotin was quenched by washing with ice cold 50 mM glycine in PBS. Cells were washed three times with ice cold PBS. Subsequently, cells were harvested and lysed with NP-40 lysis buffer. Immunoprecipitation with NeutrAvidin beads was performed at 4 ^{o}C to pull down biotinylated proteins. Beads were subsequently rinsed four times with 500 μL of lysis buffer. Beads were resuspended in 60 μL of 2X sample buffer, and used to detect BMPR2 or VEGFR3 by western blot.

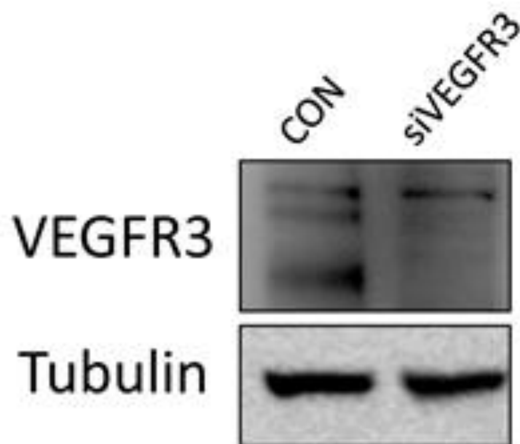
Supplemental Table

Supplemental Table 1. Genes targeted by morpholino injection in zebrafish.

Gene	Full Name	Function	BMP Signaling Related Phenotype
aplnra	apelin receptor a	G protein-coupled receptor	N
ap2m1a	adaptor-related protein complex 2, mu 1 subunit, a	endocytosis, internalization and trafficking	N
arrb2b	arrestin, beta 2b	endocytosis, internalization and trafficking	N
arrdc2	arrestin domain containing 2	N/A	N
bmpr2a	bone morphogenetic protein receptor, type II a	signaling receptor	**Y**
bmpr2b	bone morphogenetic protein receptor, type II b	signaling receptor	**Y**
cltca	clathrin, heavy polypeptide a (Hc)	endocytosis, internalization and trafficking	**Y**
dab2	disabled homolog 2 (Drosophila)	endocytosis, internalization and trafficking	**Y**
erg	v-ets erythroblastosis virus E26 oncogene like (avian)	transcription factor	N
f2r	coagulation factor II (thrombin) receptor	G protein-coupled receptor	N
flt4 (vegfr3)	fms-related tyrosine kinase 4	signaling receptor	**Y**
gpr182	G protein-coupled receptor 182	G protein-coupled receptor	N
krit1	KRIT1, ankyrin repeat containing	cytoskeleton-related	N
lamp2	lysosomal membrane glycoprotein 2	lysosome-related	N
lyve1	lymphatic vessel endothelial hyaluronic acid receptor 1	endocytosis, internalization and trafficking	N
mrc1a	mannose receptor, C type 1a	scavenger receptor	N
npl	RAB14, member RAS oncogene family	endocytosis, internalization and trafficking	N
slc17a5	sorting nexin 1	endocytosis, internalization and trafficking	N

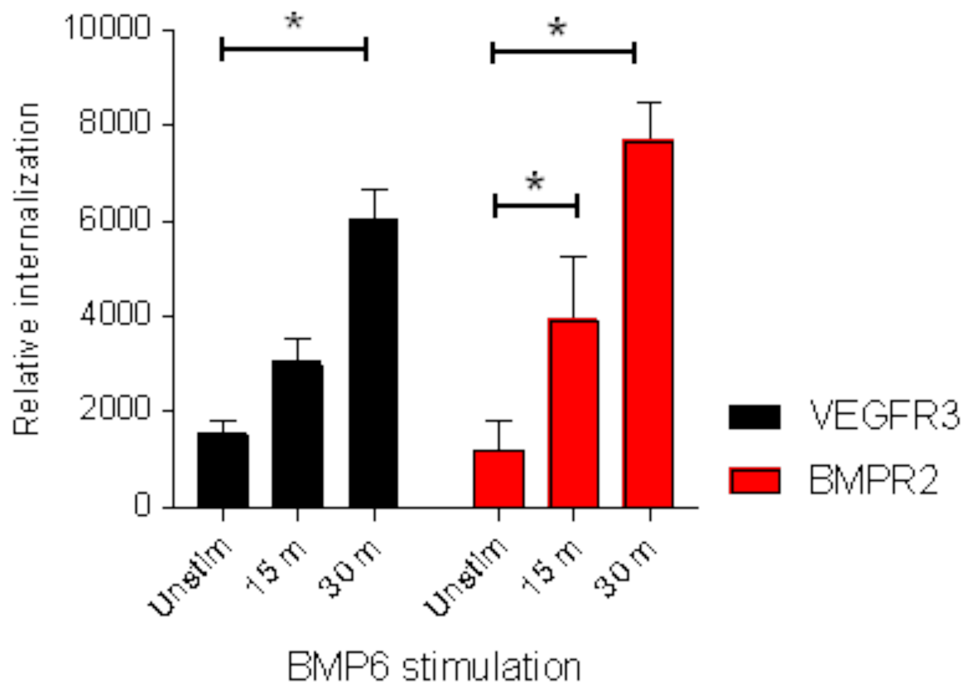
slc4a2b	sorting nexin 5	endocytosis, internalization and trafficking	N
snx1	sorting nexin 8	endocytosis, internalization and trafficking	N
snx5	stabilin 2	scavenger receptor	N
snx8	transcription factor binding to IGHM enhancer 3a	transcription factor	N
stab2	vessel-specific 1	endocytosis, internalization and trafficking	N

Supplemental Figures



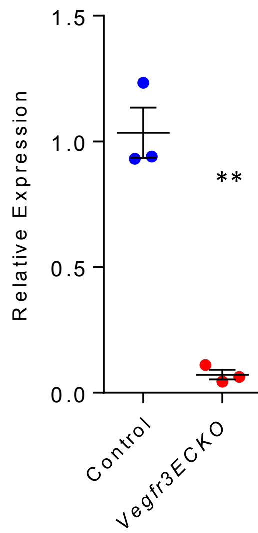
Supplemental Figure 1. Knockdown efficacy of *VEGFR3*

Efficacy of VEGFR3 overexpression via lentiviral construct infection in PAECs shown by western blot. Treatment of siVEGFR3 drastically reduced the amount of VEGFR3 protein.

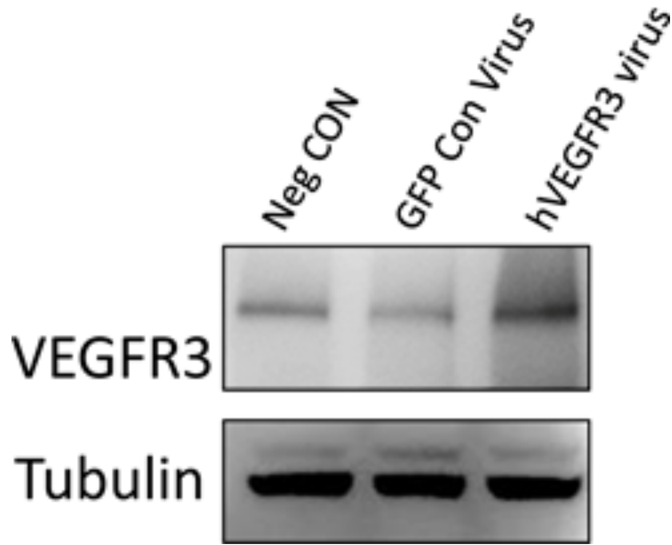


Supplemental Figure 2. Quantification of BMPR2 and VEGFR3 internalization

Quantification of BMPR2 and VEGFR3 internalization by BMP6 stimulation of HUVECs (from Figure 2C). * $P < 0.05$ Both BMPR2 and VEGFR3 appear to undergo endocytosis upon BMP6 treatment.



Supplemental Figure 3. Deletion efficiency of *Vegfr3.* The lungs from the different mouse strains were used to perform RT-PCR to determine the expression levels of VEGFR3. ** $P < 0.01$.



Supplemental Figure 4. Efficacy of VEGFR3 overexpression in PAECs

Efficacy of VEGFR3 overexpression via lentiviral construct infection in PAECs shown by western blot.

Supplemental References

1. Kim J, Hwangbo C, Hu X, Kang Y, Papangeli I, Mehrotra D, Park H, Ju H, McLean DL, Comhair SA, Erzurum SC and Chun HJ. Restoration of impaired endothelial myocyte enhancer factor 2 function rescues pulmonary arterial hypertension. *Circulation*. 2015;131:190-199.

2. Lanahan AA, Hermans K, Claes F, Kerley-Hamilton JS, Zhuang ZW, Giordano FJ, Carmeliet P and Simons M. VEGF receptor 2 endocytic trafficking regulates arterial morphogenesis. *Dev Cell*. 2010;18:713-724.