Supplemental Material

Expanded Materials & Methods

Online Figures I - XXI

Expanded Materials & Methods

Mice

Alk1 conditional knock-in mouse strains bearing ROSA26^{Alk1} (R26^{Alk1}) allele (Figure IA) were generated at Gachon University, in which the standard, specific pathogen-free conditions for the strains were maintained. The mice were maintained in a mixed C57BL/6 x 129 genetic background with standard chow and wood bedding. Establishment of *Alk1*^{2loxp}, *Alk1*^{GFPCre}, *Eng*^{2loxp}, and hENG-overexpression (hENG-OE) alleles was described previously.[22, 45, 66, 67] R26$^{CreER/+}$ was utilized for global gene deletion or overexpression.[68] *Scl*CreER, and *Myh11*CreER drivers were used for endothelial-specific and smooth muscle cell-specific overexpression or gene deletion, respectively.[41, 42] Genotypes were determined using genotyping PCR as previously described.[19, 24] Briefly, PCR reaction was performed following condition for 36 cycles: denaturing at 94°C for 45 seconds, priming at 60°C for 45 seconds and extending at 72°C for 1 minute. The last cycle remained at 72°C for 10 minutes. Genotyping PCR primer sequences are provided in Sup table 1. To delete DNA region flanked by *loxP* sites at the adult stage, tamoxifen (0.1 mg/g of body weight) was intraperitoneally injected five-times to *Scl*CreER or *Myh11*CreER;*Alk1*-iKO or *Eng*-iKO mice. Neonatal *Scl*CreER;*Alk1*$^{2loxp/2loxp}$, *Scl*CreER;*Alk1*$^{2loxp/2loxp}$;R26^{Alk1}, and control littermates were given an intragastric injection of 50 μg of tamoxifen at postnatal day (PN) 3, and retinas were harvested at PN5. For deletion of *Eng*-conditional alleles, 50 μg of tamoxifen were injected three-times from PN1 through PN4, and then the tissues were harvested at PN7. Adult mice were anesthetized with intraperitoneal injection of ketamine/xylazine (100 mg/15 mg/kg of body weight), and abdominal and thoracic cavities were opened for euthanasia. Postnatal pups were anesthetized using ice then tissue were collected. No animal were excluded from the analyses. Both male and female mice were utilized for experiments except for *Myh11*CreER. Since the *Myh11*CreER transgene was inserted into the Y-chromosome, all *Myh11*CreER mice were males. All pups in a litter were subjected to the experiments. Randomly chosen adult mice with prior knowledge of their genotype were used for the experiments. Data analyses for pups were performed without knowing their genotypes. For adult mice, the anonymized images were analyzed using Matlab for latex quantification. All animal procedures performed were reviewed and approved by the Institutional Animal Care and Use Committee of Gachon University (approval reference number, LCDI-2012-0074; LCDI-2014-0019), University of Florida (UF 201401417), and Barrow Neurological Institute/St. Joseph Hospital Medical Center Institutional Animal Care and Use Committee (531).

Generation of Cre-inducible Alk1 overexpression (R26^{Alk1}) mouse

Linearized pROSA-CAGGS-*Alk1*-HA-*GFP* knock-in vector (Figure 1A) was electroporated into J1 mouse embryonic stem cells (ESCs), and puromycin-resistant colonies were selected. Approximately 288 puromycin-resistant colonies were screened by Southern-blot analysis using the 5'-probe (Figure1A and 1B). Male chimeras obtained from correctly targeted ESCs were bred with C57BL/6 females to establish the R26$^{Alk1\text{-}off/+}$ mouse strain.

Skin Wound Generation and latex perfusion

Skin wounds in the mid-dorsum were generated using disposable biopsy punch (3 or 4 mm diameter) under the anesthesia as described previously.[24] Male and female mice aged between 6 and 24 weeks were utilized for skin wound generation. To delete conditional alleles of *Alk1* or *Eng*, tamoxifen (0.1 mg/ g of body weight) was administered on the same day of wounding (Day 0). To examine arterial vasculature and AVMs, the blue latex dye was injected slowly and continuously into the left ventricle with a 26-gauge syringe after sequential perfusion of 10 ml of PBS dilator [containing heparin (10 unit/ml, Sigma-Aldrich), papaverine (0.04 mg/ml, Sigma-Aldrich), and sodium nitroprusside (100 μM, Sigma-Aldrich)] and 10 ml of 10% formalin. The mice were fixed with 10% neutral buffered formalin (Fisher) overnight, and the dorsal skin was cleared with organic solvent (benzyl alcohol/benzyl benzoate, 1:1; Sigma-Aldrich) after methanol dehydration. Blood vessels containing the latex dye were imaged using a CCD camera (Leica). Vascular areas containing latex were quantified using Matlab (MathWorks Inc.).[25, 69] We excluded the latex infusion images from quantification in the following two cases. Complete filling of arterial vessels is indicated by connections of collateral arteries from the left and right subclavian arteries. When perfused latex did not make connections of dermal collateral vessels in the midline, we excluded the sample from the data. Another case is when the image could not be processed for quantification using the imaging program due to black pigmentation spots in the skin.

Hemoglobin concentration and GI hemorrhage index

Hemoglobin level in the blood was examined using a hemoglobin photometer (Hemopoint H2, STANBIO Laboratory). To categorize GI hemorrhage, the abdominal and thoracic cavities were opened, and hemorrhage levels were scored by visual inspection: 0 for none, 1.0 for weak, 2.0 for moderate, and 3.0 for severe level as described previously.[25, 43]

Physiological sign examination and complete blood count

Mice were anesthetized using isoflurane vaporizer and warmed up using a heat lamp and heating pad. Body temperature was measured with thermocouple thermometer (Amprobe). Once body temperature reached to 38°C, blood pressures and oxygen saturation/heart rate were measured using CODA Monitor (Kent Scientific) and Mouse STATR (Kent Scientific), respectively. Mouse blood was collected from submandibular vein using lancet, and a complete blood count was carried out using Element HT5 (HESKA).

EC isolation from lungs and retinas

Retinal or pulmonary ECs were purified with rat anti-mouse CD31 antibody-coated Dynabeads (Invitrogen) following tissue dissociation using collagenase I (for retina; Gibco)- or collagenase/dispase (for lung; Roche). Subsequently, the retinal ECs purified from at least six PN7 retinas per group were subjected to RNA isolation using RNA easy Micro kit (Qiagen). Pulmonary ECs (pECs) isolated from PN7 pups or adult lung were cultured on 2% gelatin-coated plate in the formulated endothelial cell medium (ECM) in which Dulbecco's modified eagle medium (DMEM; GIBCO) was complemented with 20% fetal bovine serum (FBS; HyClone), 0.5% heparin (200 mg/ml; Sigma-Aldrich Co.), 1% endothelial mitogen (10 mg/ml; Biomedical Technologies, Inc.), 1% nonessential amino acids (Mediatech, Inc.), 1% sodium pyruvate (100 mM; Invitrogen), and 0.4% penicillin-streptomycin (Invitrogen) or EGM2-Bullet media

(LONZA) containing 10% FBS. To delete the floxed sequences in pECs, 1 μM of 4-hydroxyl-tamoxifen (OH-TM) was treated for three consecutive days. For complete serum-starvation, pECs were incubated in chemically defined growth factor- and serum-free ECM (Genlantis) or for 16 hours. hTERT lentivirual vectors (Applied Biological Materials Inc) were treated into pECs isolated from PN7 *Scl*CreER; $R26^{+/Alk1}$ pups in order to establish immortalized *Scl*CreER; $R26^{+/Alk1}$ pECs. Transduced pECs were selected using puromycin treatment. Primary pECs and immortalized pECs were used in experiments at passage 2 and passage 6-10, respectively.

RT-PCR

RNA was isolated from adult lungs and retinal ECs using a NucleoSpin RNA II kit (BD Biosciences) and the RNA easy Micro kit (Qiagen), respectively. cDNA was generated using a Superscript IV reverse transcriptase (Invitrogen). Real-time PCR was performed using an SYBR Premix (ABI), and the ΔΔCt method determined the relative amount of the transcripts. The relative expression represents the averages of the mRNA levels from at least three independent experiments. Primer sequences for real-time RT-PCR and semi-quantitative RT-PCR are provided in Sup table 2 and Sup table 3, respectively.

Flow stimulation

pECs were seeded on 12 well plates coated with 2% gelatin. Shear stress was given to confluent pECs, following to serum starvation with EBM-2 media containing 0.1% FBS for 16 hours. 10 ng/ml of BMP9 was treated for 90 mins and then the cells were exposed to ~11.5 dynes/cm^2 of laminar shear stress generated by an orbital shaker (210 rpm).

Western blot

Lung tissue, ESCs or pulmonary ECs were lysed with an RIPA buffer containing 50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 1% Nonidet P-40, 0.5% Deoxycholate, 0.1% SDS, 2 mM EDTA, 1X Halt™ Protease Inhibitor Cocktail (Pierce), and 1X Halt™ Phosphatase Inhibitor Cocktail (Pierce). DC™ Protein Assay kit (Bio-Rad Laboratories) determined protein concentration and the proteins were resolved by SDS-PAGE. After blotting the sample onto a nitrocellulose membrane (Bio-Rad Laboratories), the primary antibodies were probed as primary antibodies: anti-pAKT (Ser473) (4060, Cell Signaling, 1:1000), anti-pan AKT (4691, Cell Signaling, 1:1000), anti-pSMAD1,5,8 (13820, Cell Signaling, 1:1000), anti-SMAD1 (9743, Cell Signaling, 1:1000), anti-HA (MMS-101-R, Covance, 1:1000), anti-HA (ab9110, Abcam, 1:4000), anti-mouse ENG (550546, BD Biosciences, 1:500), anti-human ENG (611315, BD Biosciences, 1:300), anti-mouse ALK1 (sc-19546, Santa Cruz Biotechnology, 1:100), and anti-β-ACTIN (A1978, Sigma-Aldrich Co, 1:5000). Detection was achieved using HRP-conjugated sheep anti-mouse IgG (NA931V, GE healthcare, 1:5000), HRP-conjugated donkey anti-rabbit IgG (NA934, GE healthcare, 1:5000), HRP-conjugated donkey anti-rat IgG (712-036-153, Jackson ImmunoResearch, 1:5000) or HRP-conjugated donkey anti-goat IgG (705-036-147, Jackson ImmunoResearch, 1:5000). The band was detected using ECL reaction (Pierce) on Chemidoc MP (Biorad). Bands were quantified with the ImageJ program.

Immunofluorescence

The following primary antibodies were used in the immunostaining: anti-CD31 (CM303, Biocare medical, 1:200), anti-cleaved CASPASE3 (9661, Cell Signaling, 1:200), anti-Ki67 (RM-9106-

S1, Thermo Scientific, 1:200) anti-EMCN (sc-65495, Santa Cruz Biotechnology, 1:200), anti-LYVE1 (NB600-1008, Novus Biologicals, 1:200), anti-ERG1/2/3-647 (sc-376293 AF647, Santa Cruz Biotechnology, 1:100) anti-mouse ALK1 (AF770, R&D systems, 1:200), anti-mouse ENG (sc-18893, Santa Cruz Biotechnology, 1:100), anti-SMA-Cy3 (C6198, Sigma-Aldrich Co.; 1:200), anti-GFP (ab290, Abcam, 1:500), anti-JAGGED1 (AF599, R&D systems, 1:100), and anti-pSMAD1,5,8 (13820, Cell Signaling, 1:200). Cell nuclei were detected with 4',6-diamidino-2-phenylindole dihydrochloride (DAPI, Invitrogen) staining. Collected tissues were fixed in 4% paraformaldehyde and subjected to paraffin section/cryosection immunofluorescence or whole-mount staining. For cryosection, samples were embedded in OCT compound (Tissue-Tek). The block was cryosectioned, followed by immunostaining procedure. For whole-mount staining on developing retinas, enucleated mouse eyes were fixed with 4% paraformaldehyde. Biotin-conjugated B. Simplicifolia isolectin B4 (L2140, Sigma-Aldrich Co.) and/or primary antibodies were applied to sections, wounded-ears, skin or retinas at 4°C overnight. Samples were incubated with secondary antibodies conjugated with Alexa Fluor goat anti-rabbit AF 488 (A11070, Thermo Fisher, 1: 500), donkey anti-rabbit AF 594 (A11058, Thermo Fisher, 1: 500), donkey anti-goat AF 594 (A11058, Thermo Fisher, 1: 500), donkey anti-rat AF 594 (A21209, Thermo Fisher, 1: 500), goat anti-rat AF 350 (A21093, Thermo Fisher, 1: 500), goat anti-rat AF 647 (A21247, Thermo Fisher, 1: 500), Streptavidin AF 594 (S11227, Thermo Fisher, 1: 500) and Streptavidin AF 405 (S 32351, Thermo Fisher, 1: 500). A secondary antibody only staining was used as negative control to confirm the specificity of immunostaining. Images were captured using a BX31 microscope (Olympus) and BZ-X710 (Keyence). The number of AVMs and the length of retinal vasculature were quantified in a whole retina using ImageJ.

Histology

Collected tissues were fixed in 4% paraformaldehyde, embedded in paraffin block and cut into 7-μm slices. Deparaffinized sections were serially rehydrated using ethanol series, followed by hematoxylin/eosin staining or immunostaining. Ki67 or ERG-positive cells were counted using ImageJ software (NIH). The Ki67-positive cell numbers were normalized by the number of ERG-positive ECs in given fields.

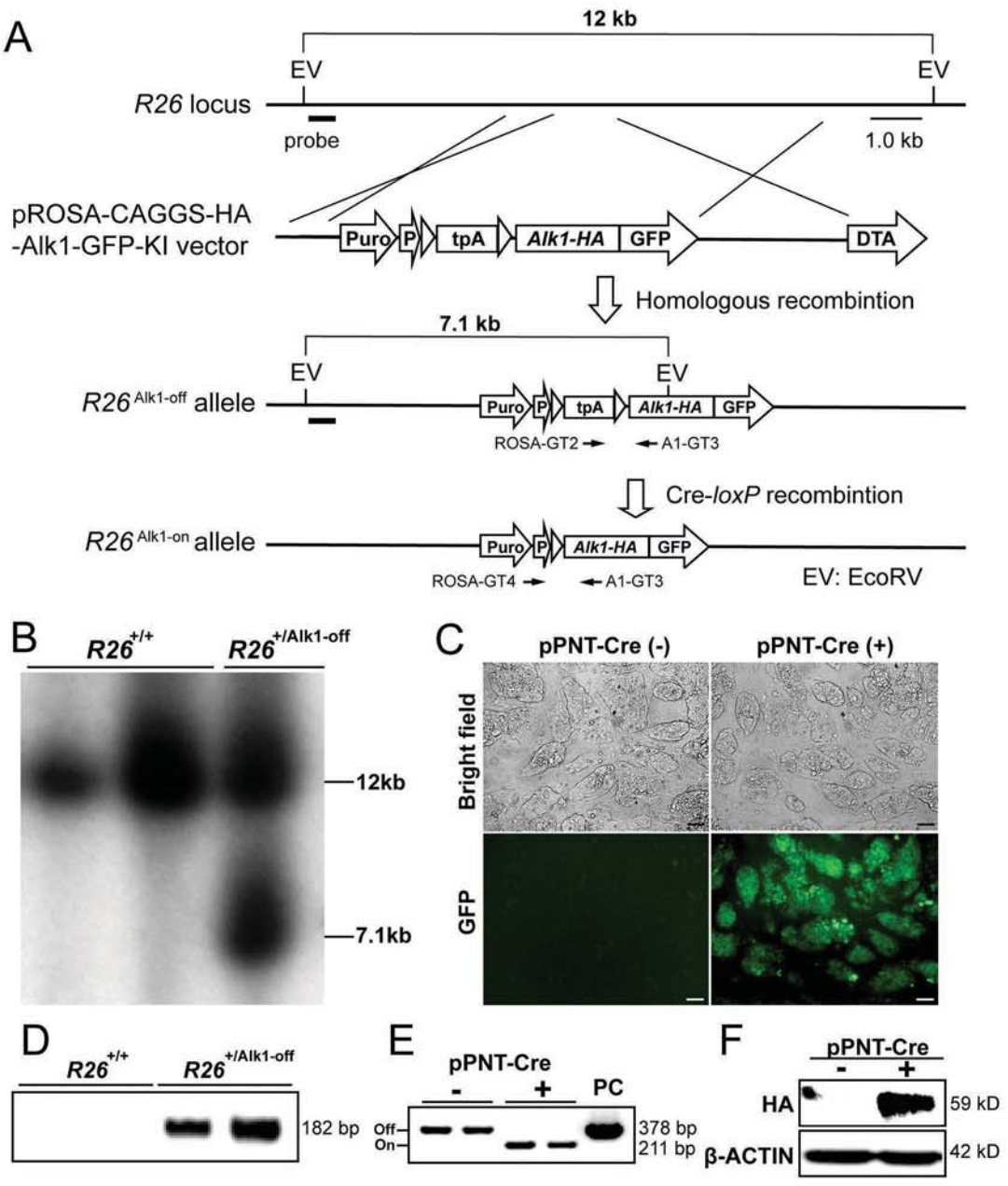
X-Gal staining

Wounded-skin was fixed with in a fixative solution (1% formaldehyde, 0.2% glutaraldehyde, 2 mM $MgCl_2$, 5 mM EGTA, and 0.02% NP-40 in PBS) at room temperature, and it was replaced with X-Gal staining solution [5 mM $K_3Fe(CN)_6$, 5 mM $K_4Fe(CN)_6$, 2 mM $MgCl_2$, 0.01% NaDeoxycholate, 0.02% NP-40, 100 mM phosphate buffer (pH 7.3), 0.5 mg/ml X-Gal], and samples were incubated overnight at 37°C.

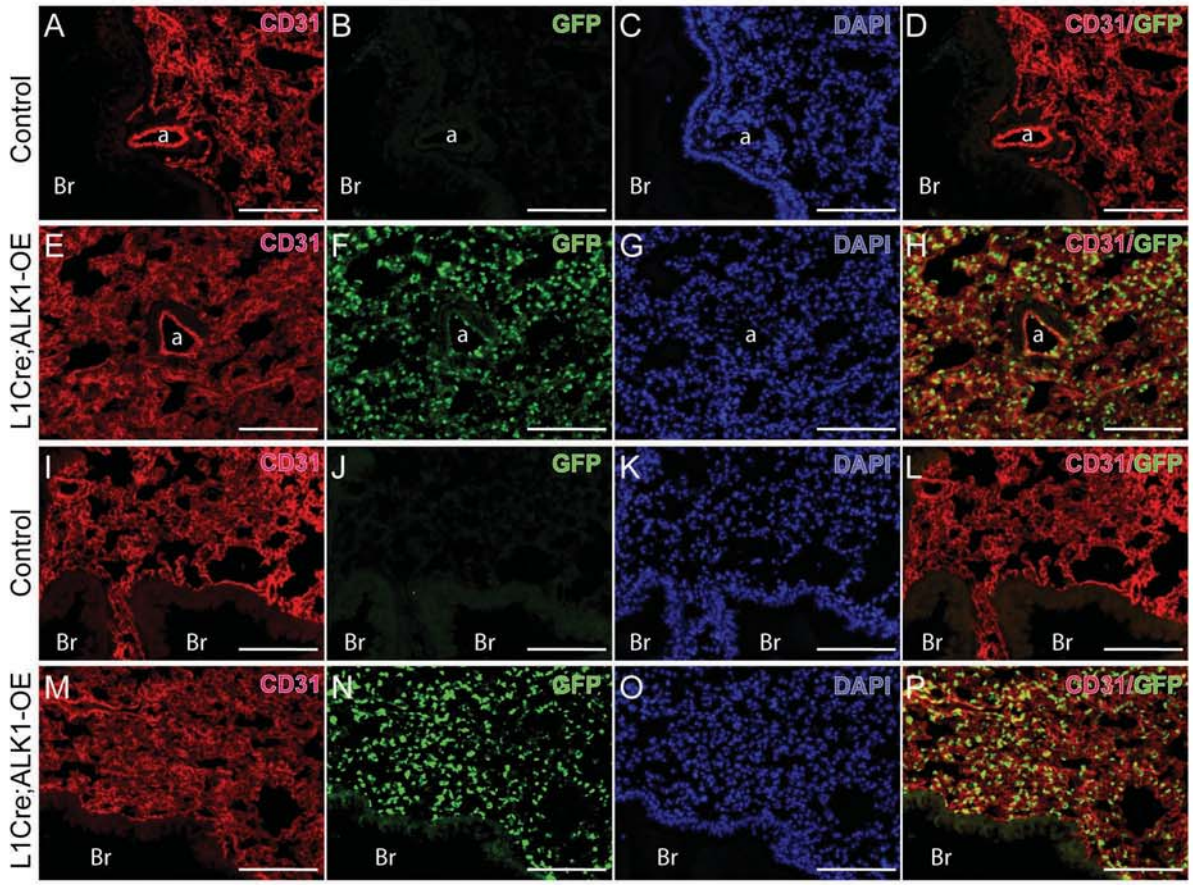
Statistics

Sample sizes were chosen on the basis of previous studies in which we examined AVM formation with *Alk1*- and *Eng*-iKO mice [19, 69], and power calculations were not conducted. Representative images were selected based on the proximity to the mean of the full range of data. Statistical analyses were carried out using GraphPad Prism version 8. Data were represented as mean ± standard errors (SDs). Statistical significance were determined with at least 3 pairs per group. We first tested the results for equal variance and normality (Shapiro-Wilk normality test) to analyze the significance between two groups. Data that passed both tests were analyzed by

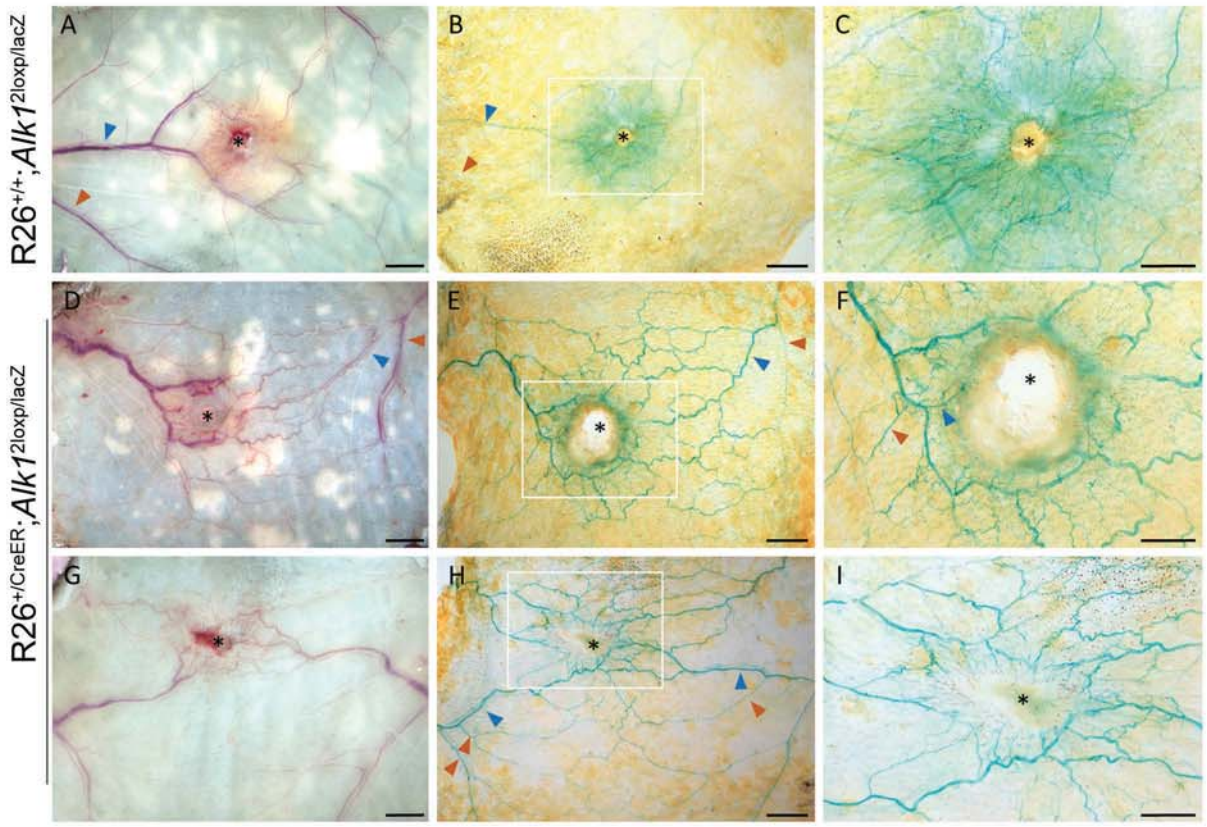
unpaired Student's *t*-test. Mann-Whitney test was applied to analyze data that passed the test for equal variance but not for normality. The unequal variance *t*-test (Welch *t*-test) was used if data failed the equal variance test. For multiple comparison, data which passed normality test (Shapiro-Wilk normality test) were analyzed with ANOVA (one-way or two-way) and Tukey's multiple comparisons test were applied as multiple-comparison post-hoc correction. Kruskal–Wallis test was used to analyze the data which did not pass the normality test, and Dunn's test was used to adjust for multiple comparisons. An across experiment multiple test correction was not performed. A value of $P<0.05$ was considered statistically significant.



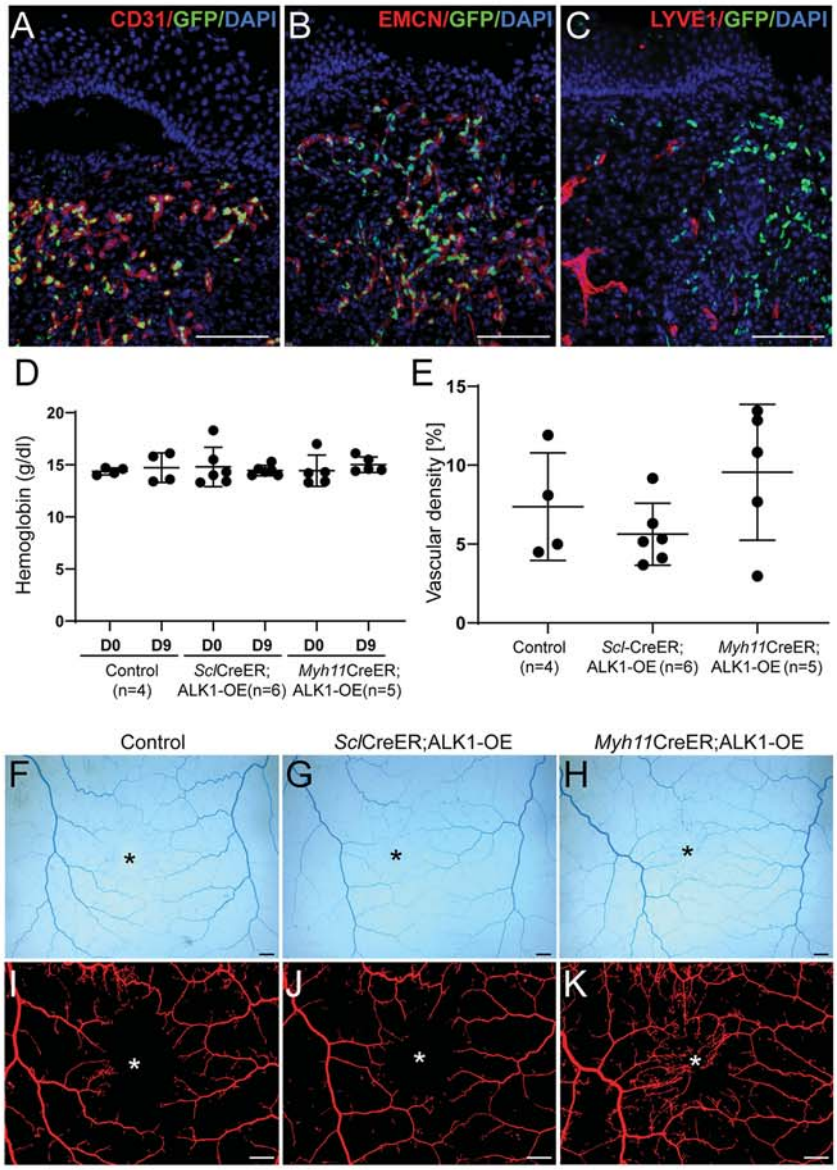
Online Figure I. Generation of Cre-inducible ALK1 overexpression alleles. **A**, Schematic diagram of the R26 wild-type and R26$^{Alk1\text{-}off}$ and R26$^{Alk1\text{-}on}$ alleles. Mouse ALK1 carrying an HA tag at the C-terminus is bicistronically transcribed with eGFP by the CAGGS promoter from the R26$^{Alk1\text{-}on}$ allele due to the excision of tpA in the presence of Cre recombinase. LoxP sequences are indicated by arrowheads. Genotyping primers and Southern blot probe are also noted. Puro, puromycin resistance gene; P, a CAGGS promoter; tpA, transcription stop sequences, DTA, diphtheria toxin A. EV, EcoRV. **B**, Southern blot analysis with EcoRV-digested genomic DNA isolated from embryonic stem cells (ESCs). **C**, GFP expression in pPNT-Cre transfected ESCs. Scale bars: 50 μm. **D** and **E**, PCR detection of the R26$^{Alk1\text{-}off}$ allele (D) and conversion of genotypes from R26$^{Alk1\text{-}off}$ to R26$^{Alk1\text{-}on}$ upon introduction of Cre recombinase (E). **F**, Western blot analyses against HA-tag to detect induction of HA-ALK1 proteins by Cre recombinase introduction into ESCs.



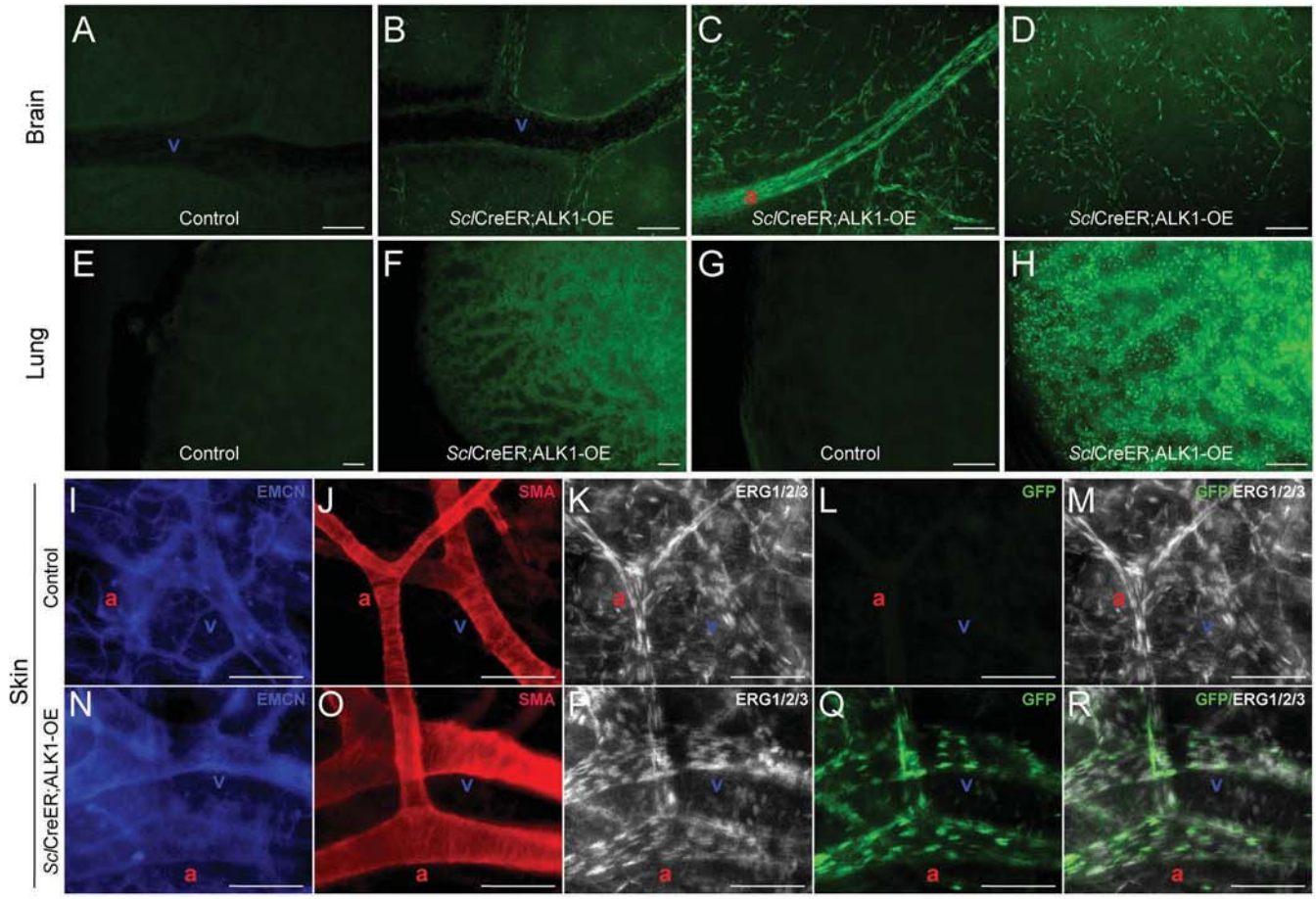
Online Figure II. L1Cre-mediated ALK1-OE are predominantly detected in endothelial cells. Immunofluorescence staining of CD31 (red) and GFP (green) in lung sections of adult wild-type control (L1Cre-negative;ALK1-OE, **A-D** and **I-L**) and L1Cre;ALK1-OE (**E-H** and **M-P**) mice. Nuclei are stained with DAPI (blue). a, artery; br, bronchus. Scale bars: 100 μm.



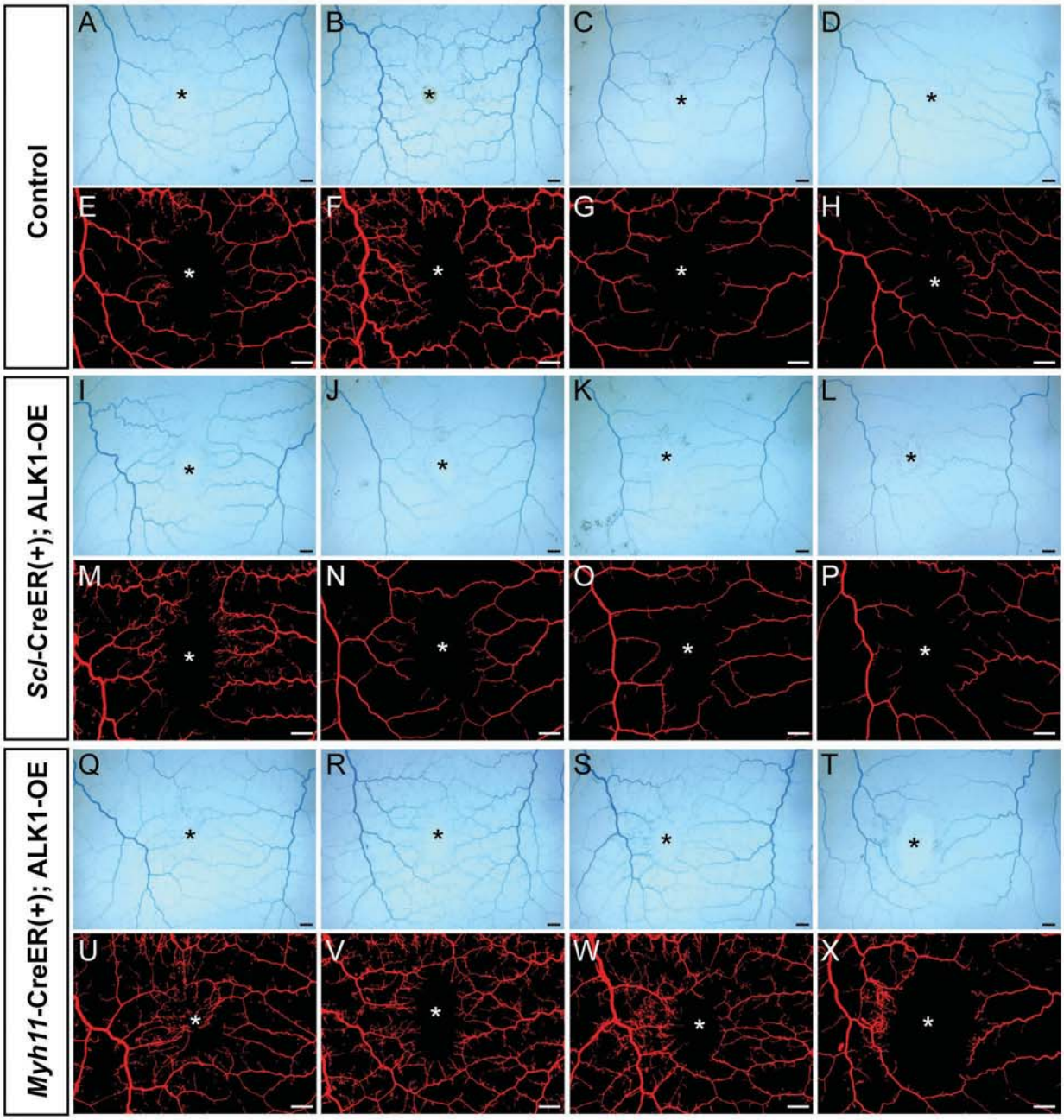
Online Figure III. Arterial EC-specific expression of Alk1 in AVM development.
A, D, and **G**, Vascular morphologies of R26$^{+/+}$;*Alk1*$^{2loxp/lacZ}$ (**A**) and R26$^{+/CreER}$;*Alk1*$^{2loxp/lacZ}$ (**D** and **G**) mice on the day 9 after wounding and the tamoxifen treatment. Note that dilated and tortuous AVM vessels are observed in the wounded skin of R26$^{+/CreER}$;*Alk1*$^{2loxp/lacZ}$ (**D** and **G**), but not that of R26$^{+/+}$;*Alk1*$^{2loxp/lacZ}$ (**A**) control mice. **B, C, E, F, H,** and **I,** Representative images of X-Gal staining in wounded-skin of R26$^{+/+}$;*Alk1*$^{2loxp/lacZ}$ (**B** and **C**) and R26$^{+/CreER}$;*Alk1*$^{2loxp/lacZ}$ (**E, F, H,** and **I**) mice. Magnified views of the white-boxed wounded skin areas in the panels **B, E,** and **H** are shown on the right panels (**C, F,** and **I**). X-Gal positive vessels are predominantly arterial vessels supplying to the wound area. Note that the arterial branches toward the wound have more intense X-Gal staining (indicated by blue arrowheads) than arteries away from the wound (red arrowheads) . The wound sites are marked by asterisks. Scale bars: A, B, D, E, G, and H, 2 mm; C, F and I, 1 mm.



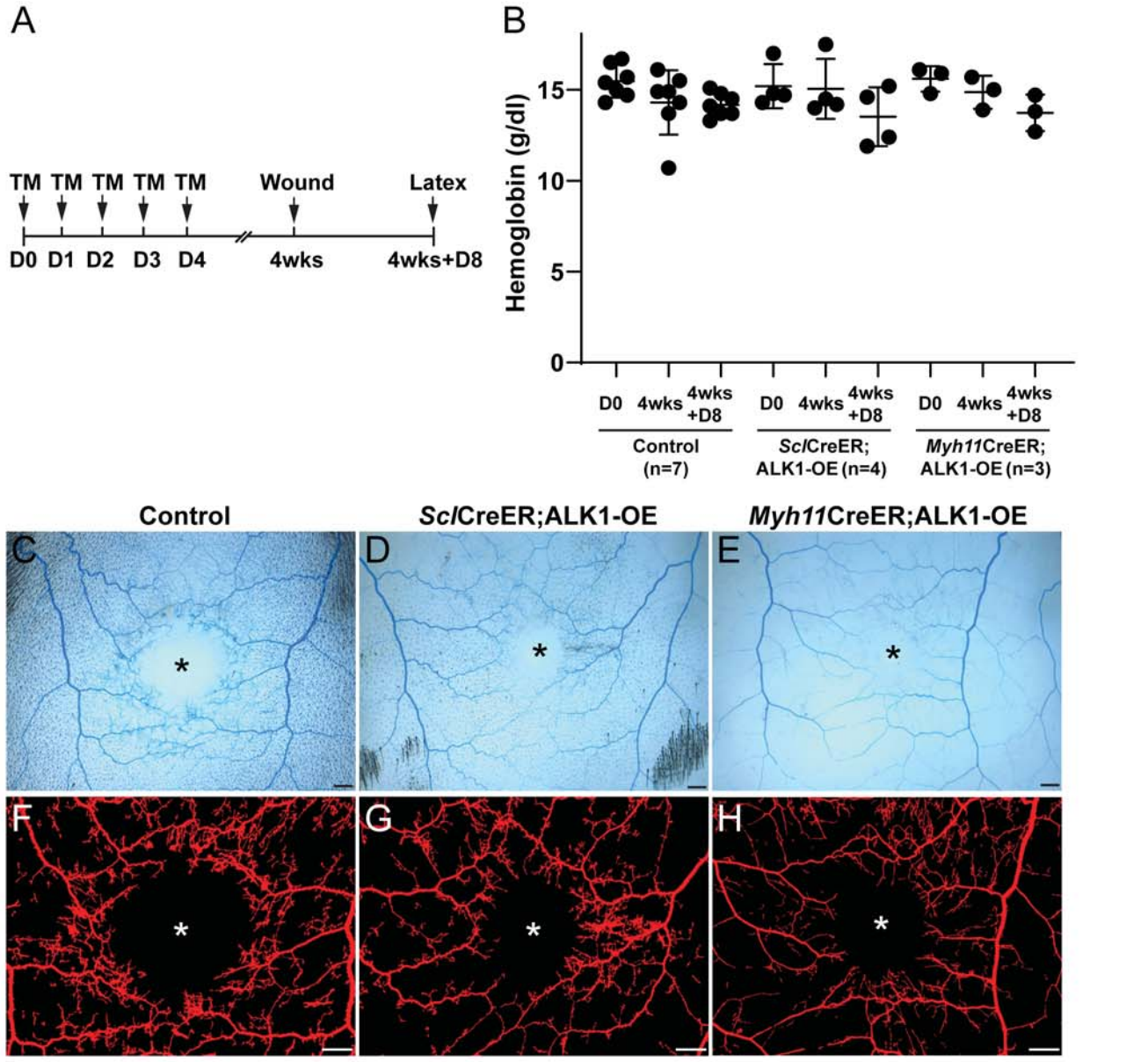
Online Figure IV. ALK1-OE in pan-endothelial cells or smooth muscle cells does not result in AVMs. A-C, Immunofluorescence staining of CD31 (**A**, red), EMCN (**B**, red), or LYVE1 (**C**, red) on skin sections from the wounded area of *Scl*CreER;ALK1-OE mice. GFP indicates endothelial cells expressing HA-ALK1 driven by *Scl*CreER. Nuclei are stained with DAPI (blue). **D**, Hemoglobin levels of control (CreER-negative ALK1-OE, n=4), *Scl*CreER;ALK1-OE (n=6) or *Myh11*CreER;ALK1-OE (n=5) mice on the first day (D0) and 9 days (D9). Tamoxifen was administrated five-times from D0 through D4. Two-way ANOVA with Tukey's correction were used for statistical analyses. **E,** Quantification of the vascular area containing latex in wounded skin areas. Control (n=4), *Scl*CreER;ALK1-OE (n=6) and *Myh11*CreER;ALK1-OE (n=5). One-way ANOVA with Tukey's correction for staistical anslysis. **F-K.** Representative latex dye-perfused images of vasculature in wounded skins on 9 days after wounding. Control (**F** and **I**), *Scl*CreER;ALK1-OE (**G** and **J**), and *Myh11*CreER;ALK1-OE (**H** and **K**). The wound sites are marked by asterisks. All data are means ±SDs. Scale bars: **A-C**, 100 μm; **F-K**, 1 mm.



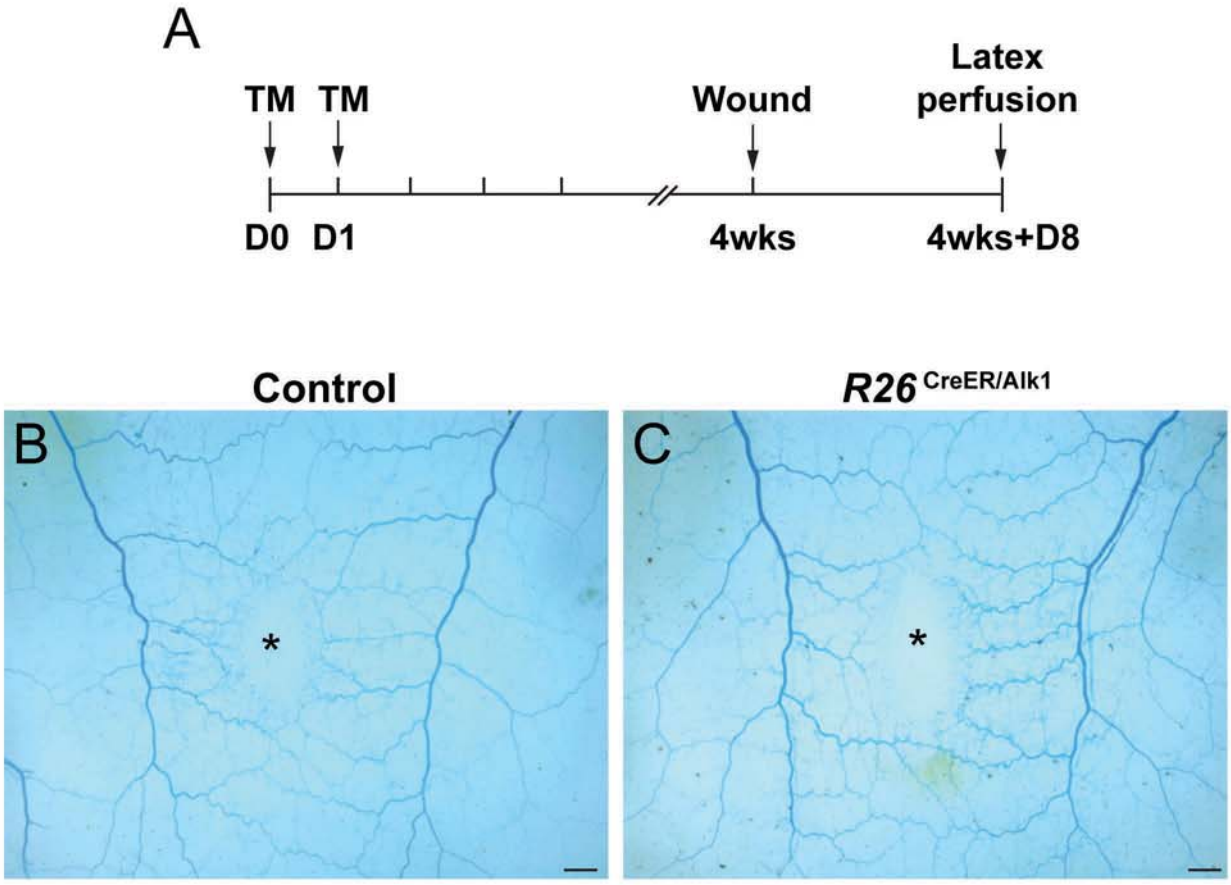
Online Figure V. Endothelial cell-specific ALK1-OE by SclCreER driver. Tamoxifen was administered four-times from PN1 through PN4 by intragastric injection and tissues were collected at PN7. **A-H**, Fluorescence stereomicroscopic images on brains (**A-D**) and lungs (**E** and **H**) of control (*Scl*CreER-negative;ALK1-OE, **A, E** and **G**) and *Scl*CreER;ALK1-OE (**B-D** and **F** and **H**) mice. **I-R,** whole-mount immunofluorescent staining of EMCN (blue), SMA (red), and ERG (white) on skin tissues of control (**I-M**) and *Scl*CreER;ALK1-OE (**N-R**). GFP-positive ALK1-OE cells were shown green. a, artery; v, vein. Scale bars: 100 μm.



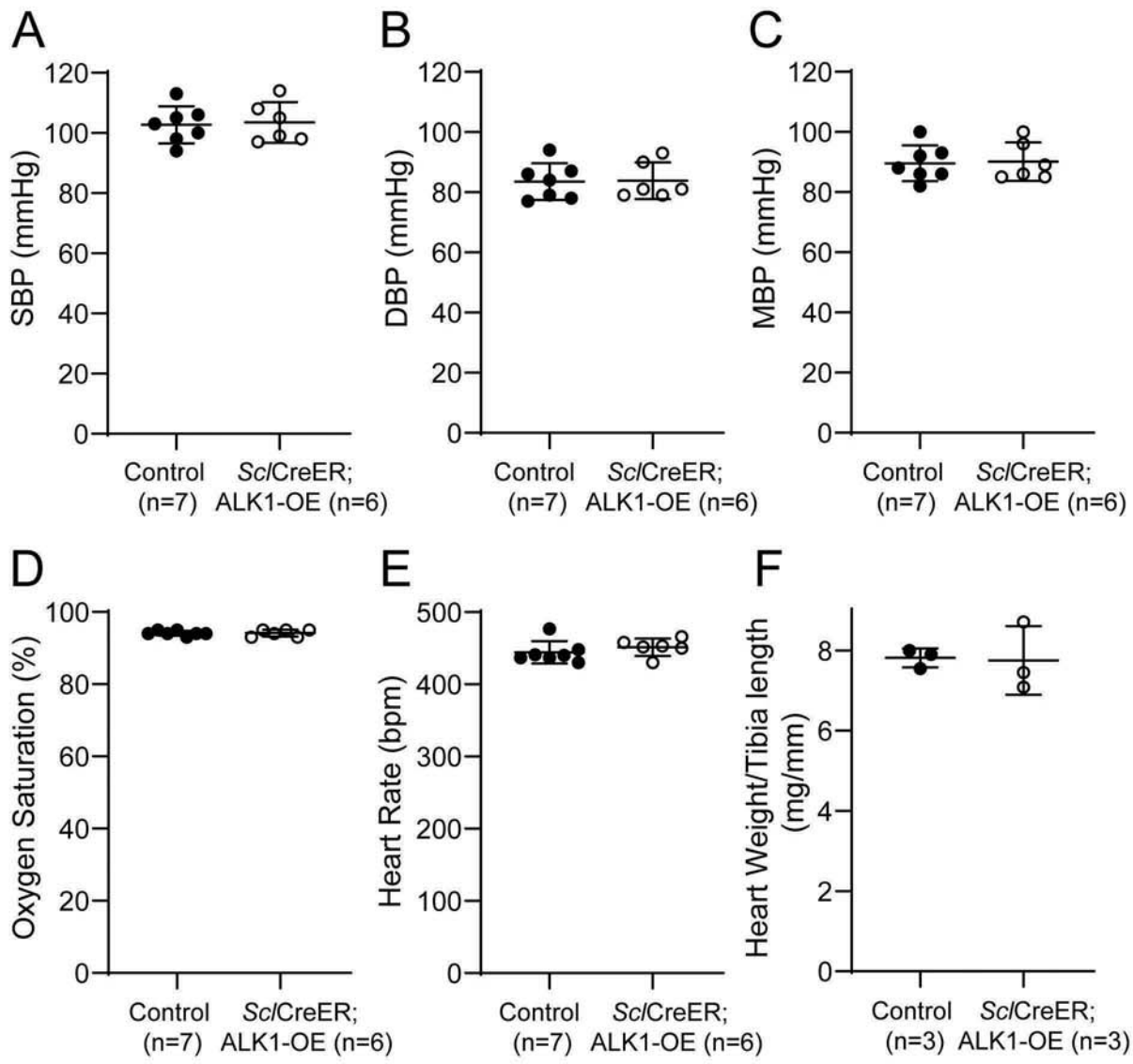
Online Figure VI. Latex-infusion images of ALK1-OE in pan-endothelial cells or smooth muscle cells. Latex dye-perfused blood vessels (upper panels) and processed images (lower panels) on wounded-skin of control (*Scl*CreER-negative;ALK1-OE, **A-H**), *Scl*CreER;ALK1-OE (**I-P**), and *Myh11*CreER;ALK1-OE (**Q-X**) mice. Wound sites are indicated by asterisks. Scale bars: 1 mm.



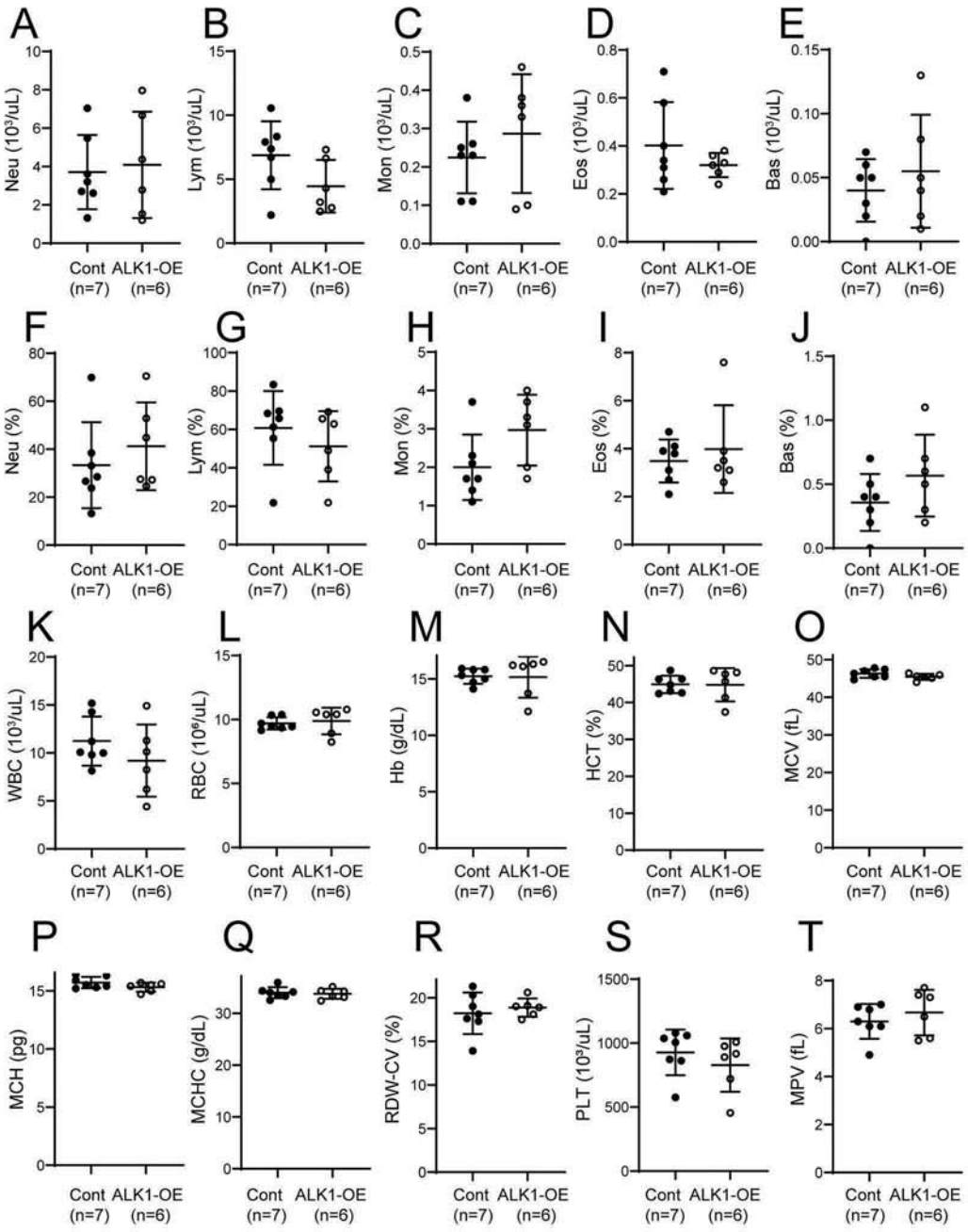
Online Figure VII. Long-term expression of HA-ALK1 in pan-endothelial cells or smooth muscle cells does not induce formation of AV-shunts. A, Schematic overview of the experimental set up for assessing the effect of long-term ALK1 overexpression. Tamoxifen was intraperitoneally injected five times. 4 mm-wounds were punched at 4weeks, then latex dye was perfused 8 days after wounding. **B,** Hb levels over time in control (SclCreER-negative;ALK1-OE, n=7), *Scl*CreER;ALK1-OE (n=4) or *Myh11*CreER;ALK-OE (n=3) mice. Two-way ANOVA with Tukey's correction was used for statistical analyses. **C-H,** Visualized vessels by latex infusion (**C-E**) and processed images (**F-H**) on wounded-dorsal skins of control (**C** and **F**), *Scl*CreER;ALK1-OE (**D** and **G**) or *Myh11*CreER;ALK1-OE (**E** and **H**) mice. Wound sites are indicated by asterisks. All data are means ± SDs. Scale bars: 1 mm.



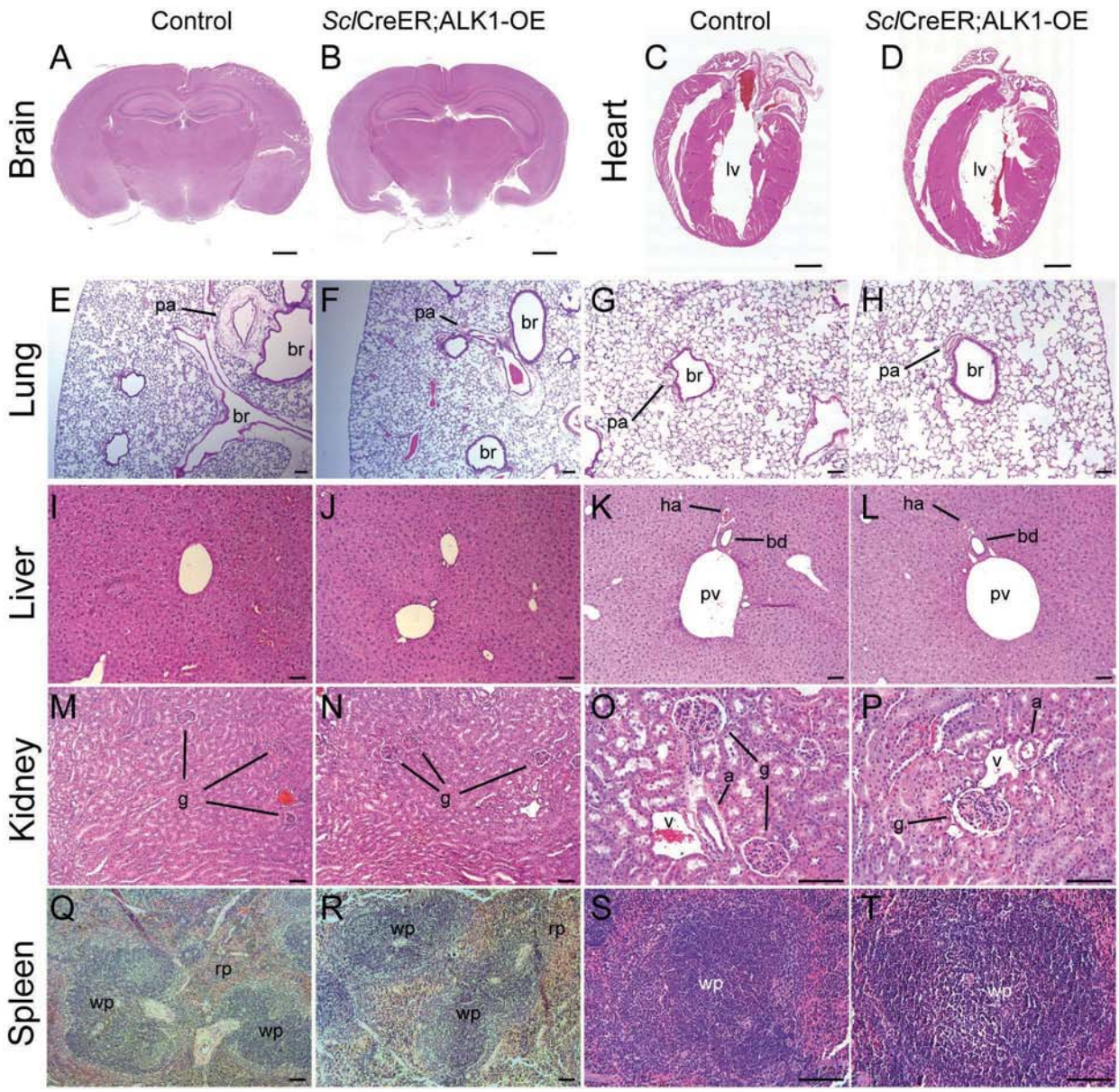
Online Figure VIII. Global ALK1-OE for 5-weeks does not provoke AVMs. A, Scheme of the long-term experiments. Tamoxifen was intraperitoneally injected twice. 4 mm-wounds were made in the dorsal skin 4 weeks after last tamoxifen injection, then latex dye was infused 8 days after wounding. **B** and **C**, Latex perfusion images of control (R26+/+; ALK1-OE,**B**) and $R26^{CreER}$;ALK1-OE (**C**) mice. Wound sites are indicated by asterisks. Scale bars: 1 mm.



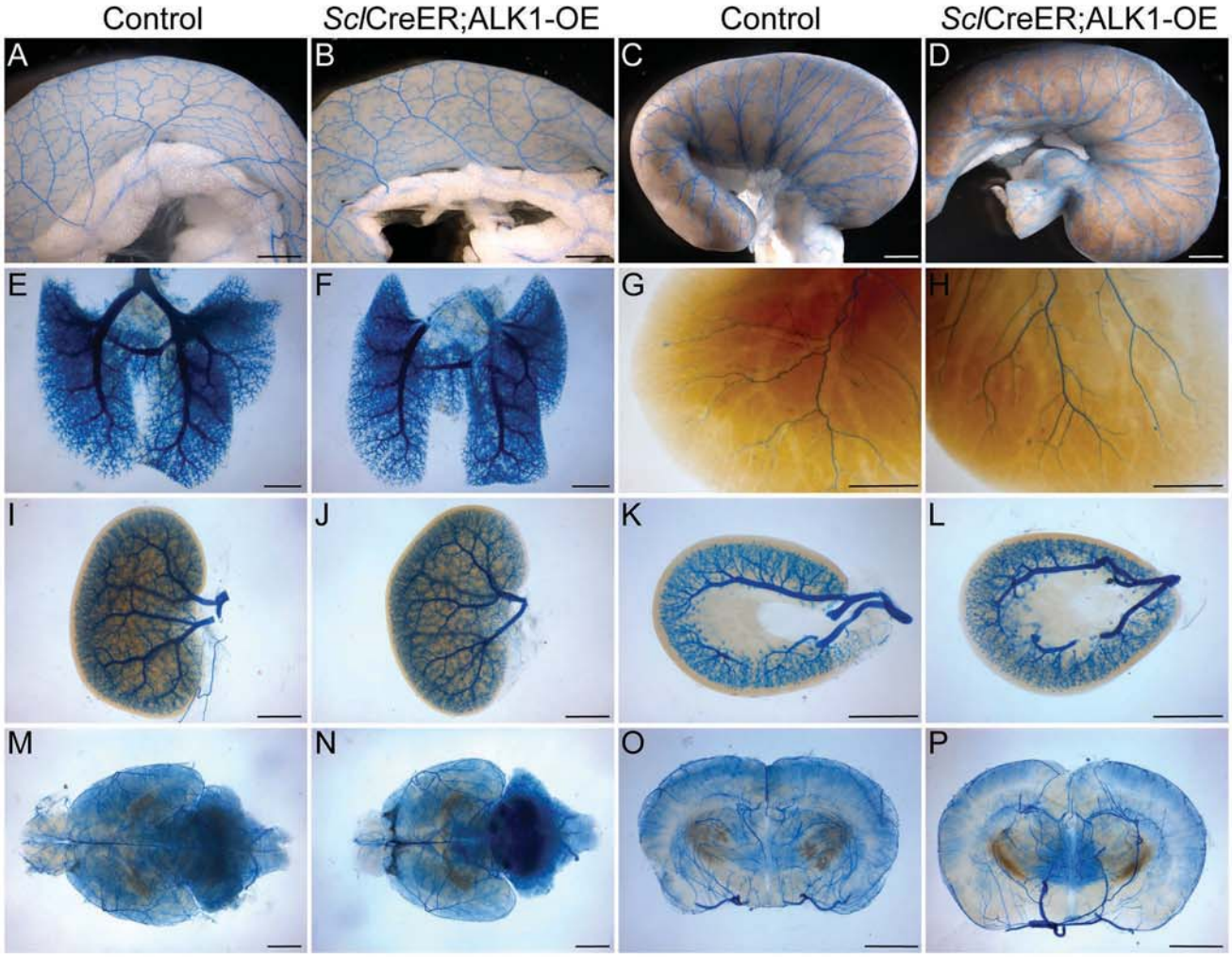
Online Figure IX. ALK1-OE in endothelial cells for 12 weeks does not result in the alteration of blood pressure and heart function. Tamoxifen was intraperitoneally injected five times daily. Blood pressures, oxygen saturation, heart beating and heart sizes were examined at 12 weeks after tamoxifen treatment. **A-C,** Systolic (**A**, unpaired t-test), diastolic (**B,** Mann-Whitney test) and mean (**C,** unpaired t-test) blood pressures on control (*Scl*CreER-negative;ALK1-OE, n=7) and *Scl*CreER;ALK1-OE mice (n=6). **D,** Oxygen saturation on control (n=7) and *Scl*CreER;ALK1-OE mice (n=6). Mann-Whitney test. **E,** Heart rates of control (n=7) and *Scl*CreER;ALK1-OE mice (n=6). bpm, beats per minute. Mann-Whitney test. **F,** Comparison of heart sizes of *Scl*CreER;ALK1-OE mice (n=3) with those of controls (n=3). Heart weight was normalized with tibia length. Unpaired t-test for statistical analyses. All data are means±SDs.



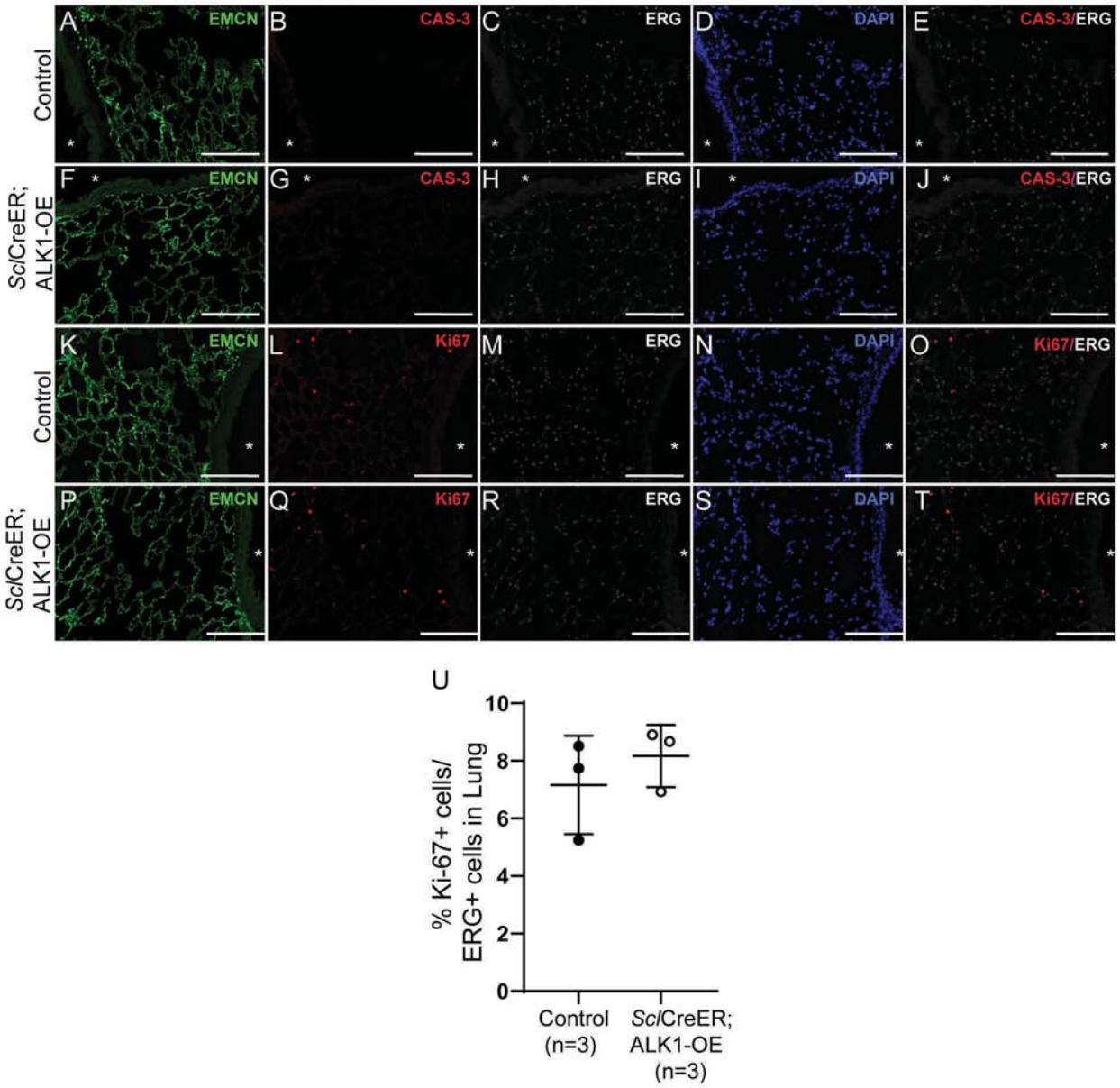
Online Figure X. Endothelial-specific ALK1-OE for 12 weeks does not change the blood components. Tamoxifen was intraperitoneally injected five times daily, then complete blood counts were carried out after 12 weeks. Note that any significant differences were not detected in *Scl*CreER;ALK1-OE mice (n=6) compared to control mice (*Scl*CreER-negative; ALK1-OE, n=7). Neu, neutrophil; Lym, lymphocyte; Mon, monocyte; Eos, eosinophil; Bas, basophil; WBC, white blood cell; RBC, red blood cell; Hb, hemoglobin; HCT, hematocrit; MCV, mean corpuscular volume; MCH, mean corpuscular hemoglobin; MCHC, mean corpuscular hemoglobin concentration; RDW-CV, Red blood cell distribution width; PLT, Platelet; MPV, mean platelet volume. Unpaired *t*-test (A, B, C, E, F, G, H, J, K, L, N, O, P, Q, R, S, and T) and. Welch t-test (D and M) were used for statistical analyses. All data represent means±SDs.



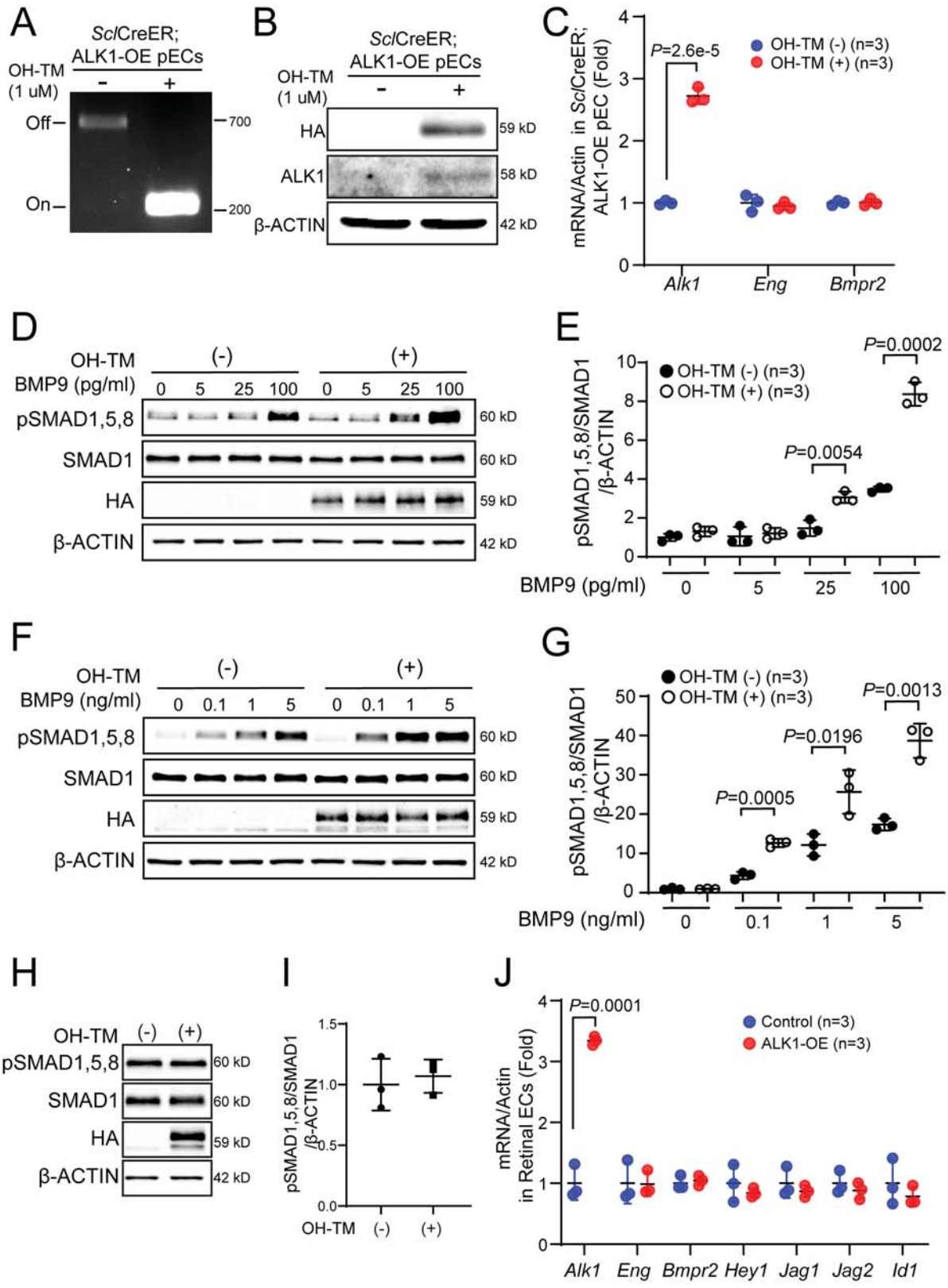
Online Figure XI. Similar histological features between control and ALK1-OE mice. Hematoxylin and eosin staining was carried out on the sections of brain (**A** and **B**), heart (**C** and **D**), lung (**E** and **H**), liver (**I** and **L**), kidney (**M** and **P**) and spleen (**Q** and **T**) of control (*Scl*CreER-negative;ALK1-OE, n=3) and *Scl*CreER;ALK1-OE mice (n=3), 12 weeks after tamoxifen administrations. Note that obvious abnormal histopathological signs were not detectable in ALK1-OE mice. a, artery; bd, bile duct; br, bronchus; g, glomerulus; ha, hepatic artery; lv, left ventricle; pa, pulmonary artery; pv, portal vein; rp, red pulp; v, vein; wp, white pulp. Scale bars: A-D, 1 mm; E and T, 100 μm.



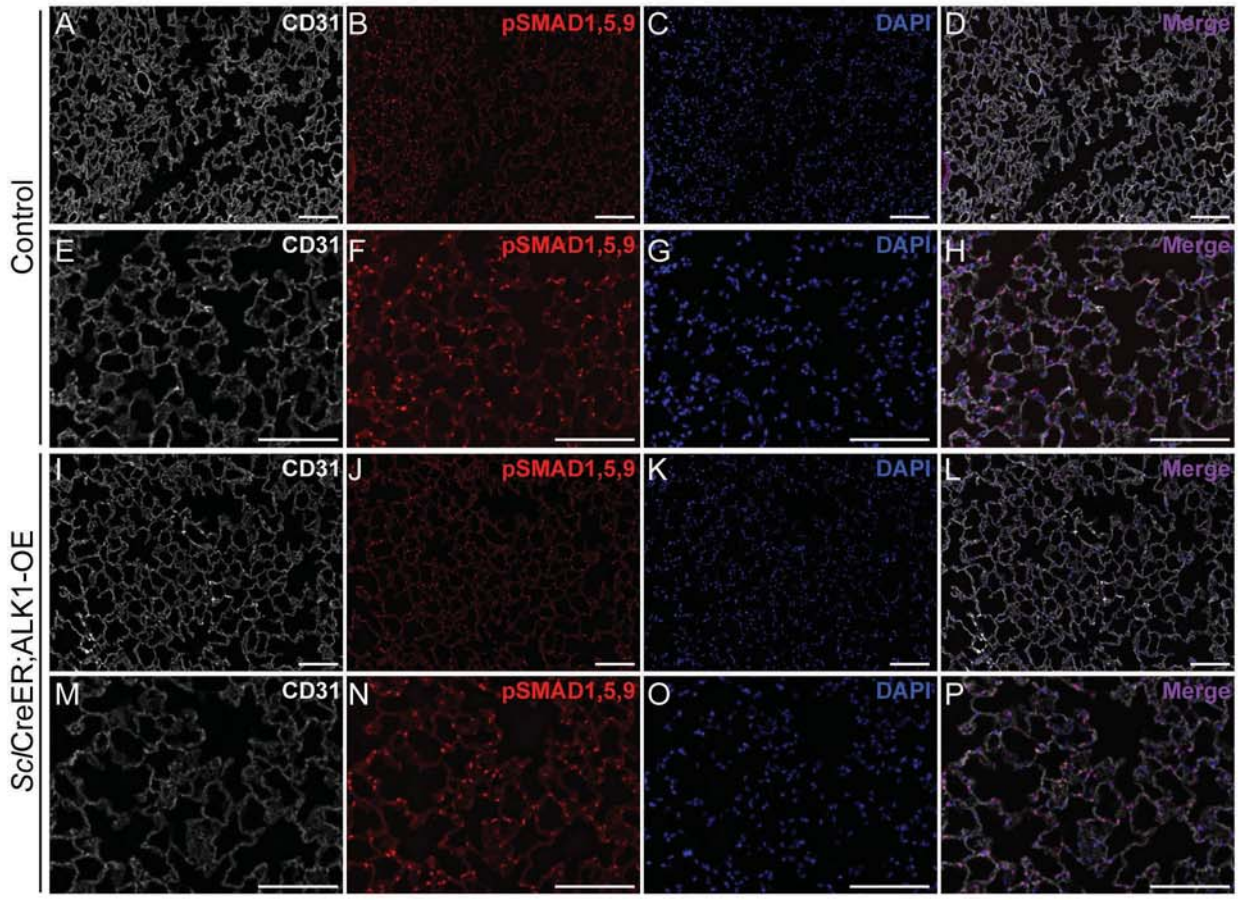
Online Figure XII. Long-term endothelial-specific ALK1-OE does not induce AVMs in visceral organs. Tamoxifen was intraperitoneally treated five times daily, then latex dye was infused into both left and right ventricles of control (*Scl*CreER-negative ALK1-OE, n=4) and *Scl*CreER;ALK1-OE (n=3) mice after 12 weeks. Latex perfusion images of small intestine (**A** and **B**), cecum (**C** and **D**), lung (**E** and **F**), liver (**G** and **H**), kidney (**I-L**), and brain (**M-P**). Scale bars: 2mm.



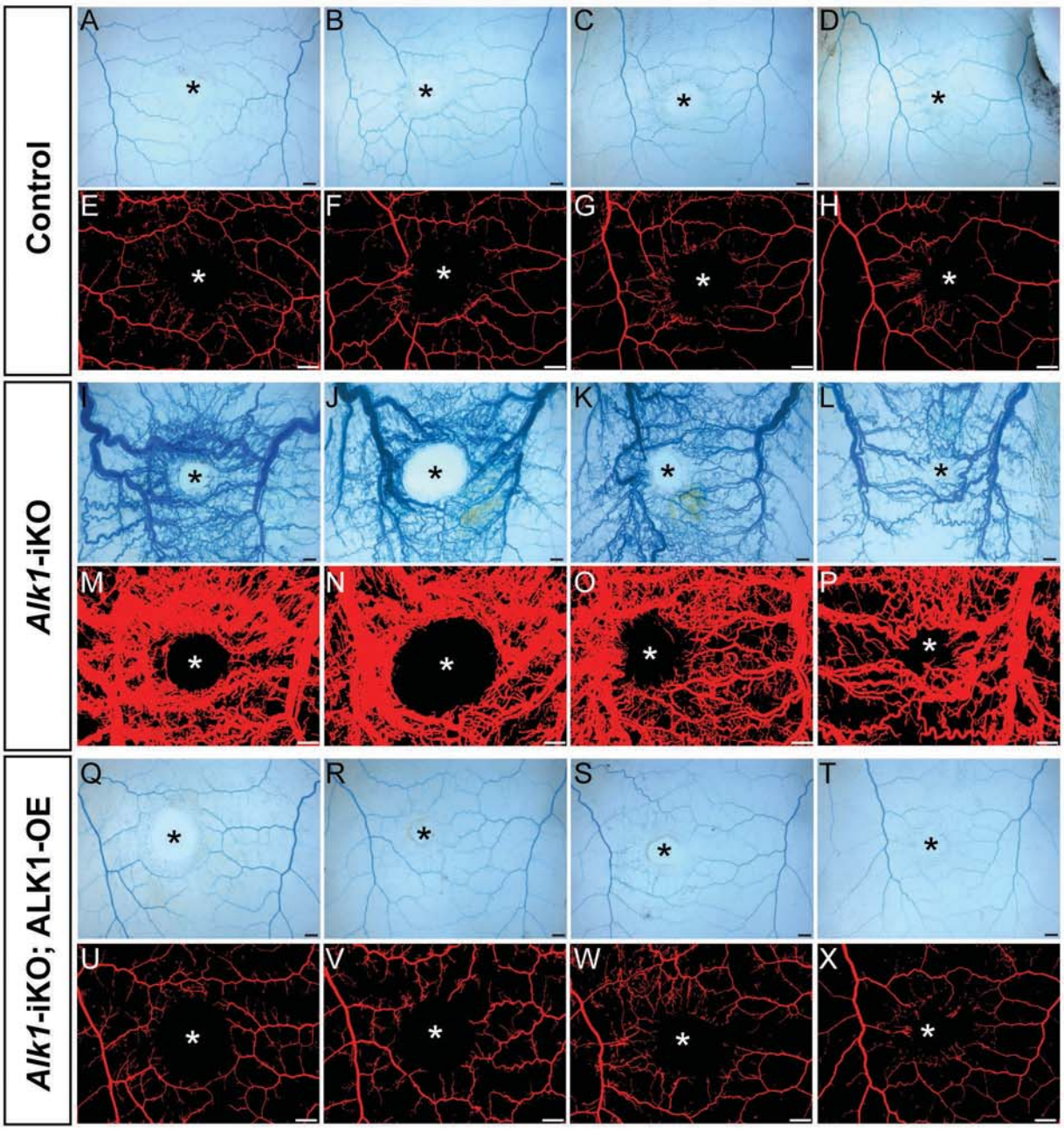
Online Figure XIII. Long-term ALK1-OE in endothelial cells does not lead to abnormal apoptotic or proliferative activities. A-J, Immunofluorescence staining of EMCN (**A** and **F**, red), cleaved caspase3 (**B** and **G**, red), and ERG (**C** and **H**, white) on l lung tissues of control (*Scl*CreER-negative ALK1-OE) mice (**A-E**; n=3) and *Scl*CreER;ALK1-OE (**F-J**; n=3) mice. Nuclei are stained with DAPI (blue). Bronchi are marked by asterisks. Denote that cleaved caspase3-positive apoptotic ECs were barely detected in both control and ALK1-OE mice. **K-T,** Immunofluorescent staining of Ki67 (red) with lung tissues of control (**K-O**; n=3) and *Scl*CreER;ALK1-OE (**P-T**; n=3) mice. ECs were marked with EMCN (green) and ERG (white). Nuclei are stained with DAPI (blue). Bronchi are marked by asterisks. **U**, Quantification of Ki67-positive proliferating ECs in lung tissues of control and *Scl*CreER;ALK1-OE mice. Ki67-positive cells were normalized with total endothelial cell numbers marked with ERG in the given fields. n=3 per each group. Unpaired *t*-test was used for statistical analysis. All data are means±SDs. Scale bars: 100 μm.


A
Scl/CreER; ALK1-OE pECs
OH-TM (1 uM)
-
+
Off
On
700
200
B
Scl/CreER; ALK1-OE pECs
OH-TM (1 uM)
-
+
HA
ALK1
β-ACTIN
59 kD
58 kD
42 kD
C
mRNA/Actin in Scl/CreER; ALK1-OE pEC (Fold)
P=2.6e-5
OH-TM (-) (n=3)
OH-TM (+) (n=3)
Alk1
Eng
Bmpr2
D
OH-TM
(-)
(+)
BMP9 (pg/ml)
0 5 25 100 0 5 25 100
pSMAD1,5,8
SMAD1
HA
β-ACTIN
60 kD
60 kD
59 kD
42 kD
E
pSMAD1,5,8/SMAD1 /β-ACTIN
OH-TM (-) (n=3)
OH-TM (+) (n=3)
P=0.0002
P=0.0054
BMP9 (pg/ml)
0
5
25
100
F
OH-TM
(-)
(+)
BMP9 (ng/ml)
0 0.1 1 5 0 0.1 1 5
pSMAD1,5,8
SMAD1
HA
β-ACTIN
60 kD
60 kD
59 kD
42 kD
G
pSMAD1,5,8/SMAD1 /β-ACTIN
OH-TM (-) (n=3)
OH-TM (+) (n=3)
P=0.0013
P=0.0196
P=0.0005
BMP9 (ng/ml)
0
0.1
1
5
H
OH-TM (-) (+)
pSMAD1,5,8
SMAD1
HA
β-ACTIN
60 kD
60 kD
59 kD
42 kD
I
pSMAD1,5,8/SMAD1 /β-ACTIN
OH-TM (-) (+)
J
mRNA/Actin in Retinal ECs (Fold)
P=0.0001
Control (n=3)
ALK1-OE (n=3)
Alk1
Eng
Bmpr2
Hey1
Jag1
Jag2
Id1

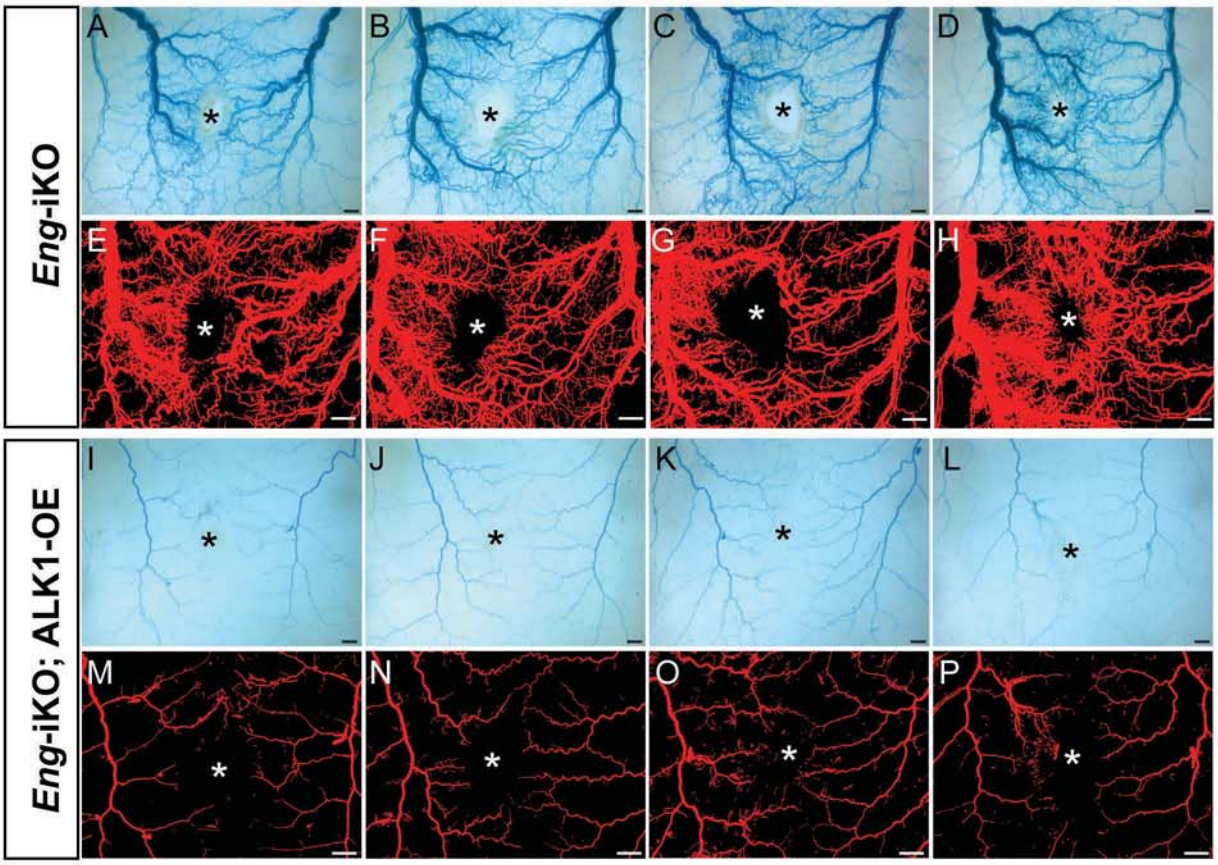
Online Figure XIV. ALK1-OE does not affect the signaling pathways in wild-type endothelial cells despite the overexpression of functional ALK1. One μM of 4-hydroxyl-tamoxifen (OH-TM) was treated to induce HA-ALK1 expression in hTERT-immortalized *Scl*CreER;ALK1-OE pECs.
A, Conversion of genotypes from $R26^{Alk1\text{-}off}$ to $R26^{Alk1\text{-}on}$ upon introduction of OH-TM.
B, OH-TM-induced ALK1-OE in *Scl*CreER;ALK1-OE pECs. Ectopic HA expression and increased ALK1 level by OH-TM treatment were detected with Western blot analyses.
C, Quantitative RT-PCR on *Alk1*, *Eng* and *Bmpr2* transcripts in OH-TM-untreated (n=3) and -treated (n=3) *Scl*CreER;ALK1-OE pECs. Actin was used for normalization. N=3 per each group. Unpaired *t*-test for each gene.
D and **F**, Western blot analyses of phosphorylated SMAD1,5,8 (pSMAD1,5,8) and HA in *Scl*CreER;ALK1-OE pECs with or without OH-TM treatment. Various concentration of BMP9 were treated for 30 mins after serum-starvation.
E and **G**, Quantification of relative pSMAD1,5,8 levels normalized with total SMAD1 and β-ACTIN. N=3 per each group. Unpaired t-test for each concentration of BMP9. Significant *p*-values in the same BMP9 concentration group were labelled.
H, Western blot analyses of pSMAD1,5,8 and HA in *Scl*CreER;ALK1-OE pECs with or without OH-TM in normal culture condition. **I**, Quantification of pSMAD1,5,8 levels. pSMAD1,5,8 levels normalized with total SMAD1 and β-ACTIN. n=3 per each group. Unpaired *t*-test. **J,** Quantitative RT-PCR analysis of *Alk1, Eng, Bmpr2, Hey1, Jag1, Jag2* and *Id1* in retinal ECs purified from control (*Scl*CreER-negative ALK1-OE, n=3) and *Scl*CreER;ALK1-OE (n=3) mice. The mRNA levels of the genes are normalized with Actin. N=3 per each group. Unpaired *t*-test (*Alk1, Eng, Hey1, Jag1 and Jag2*). Mann-Whitney test (*Bmpr2* and *Id1*) were used for statistical analyses.
All data are means±SDs.



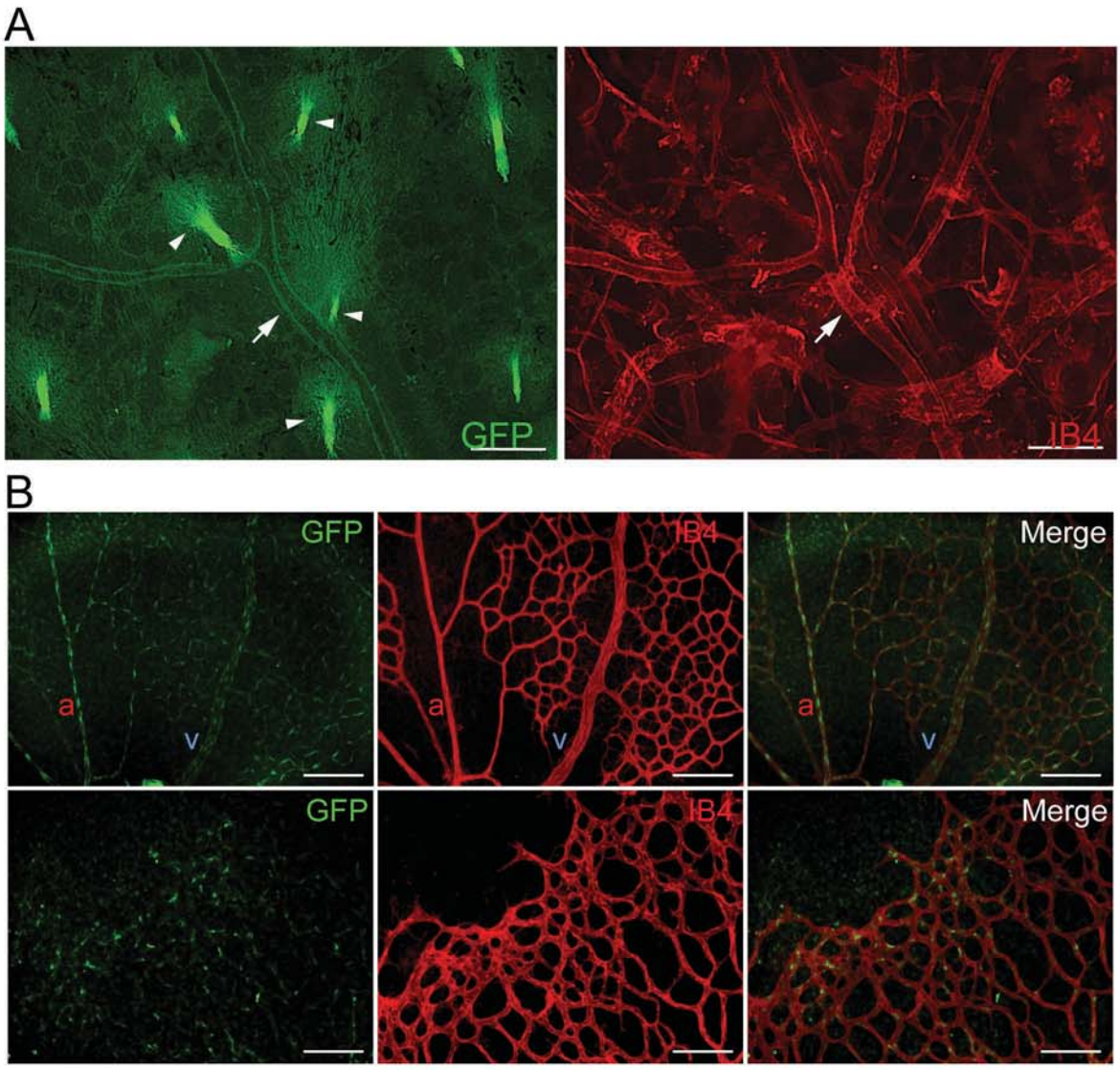
Online Figure XV. Long-term ALK1-overexpression does not affect SMAD1,5,8 phosphorylation in comparison with wild-type endothelial cells. Immunofluorescence staining of CD31 (white) or pSMAD1,5,8 (red) on lung sections of control (**A-H**, *Scl*CreER-; ALK1-OE) and *Scl*CreER+;ALK1-OE (**I-P**) mice. Nuclei are stained with DAPI (blue). ALK1-HA was overexpressed for 12 weeks by tamoxifen treatment. Scale bars: 100 µm.



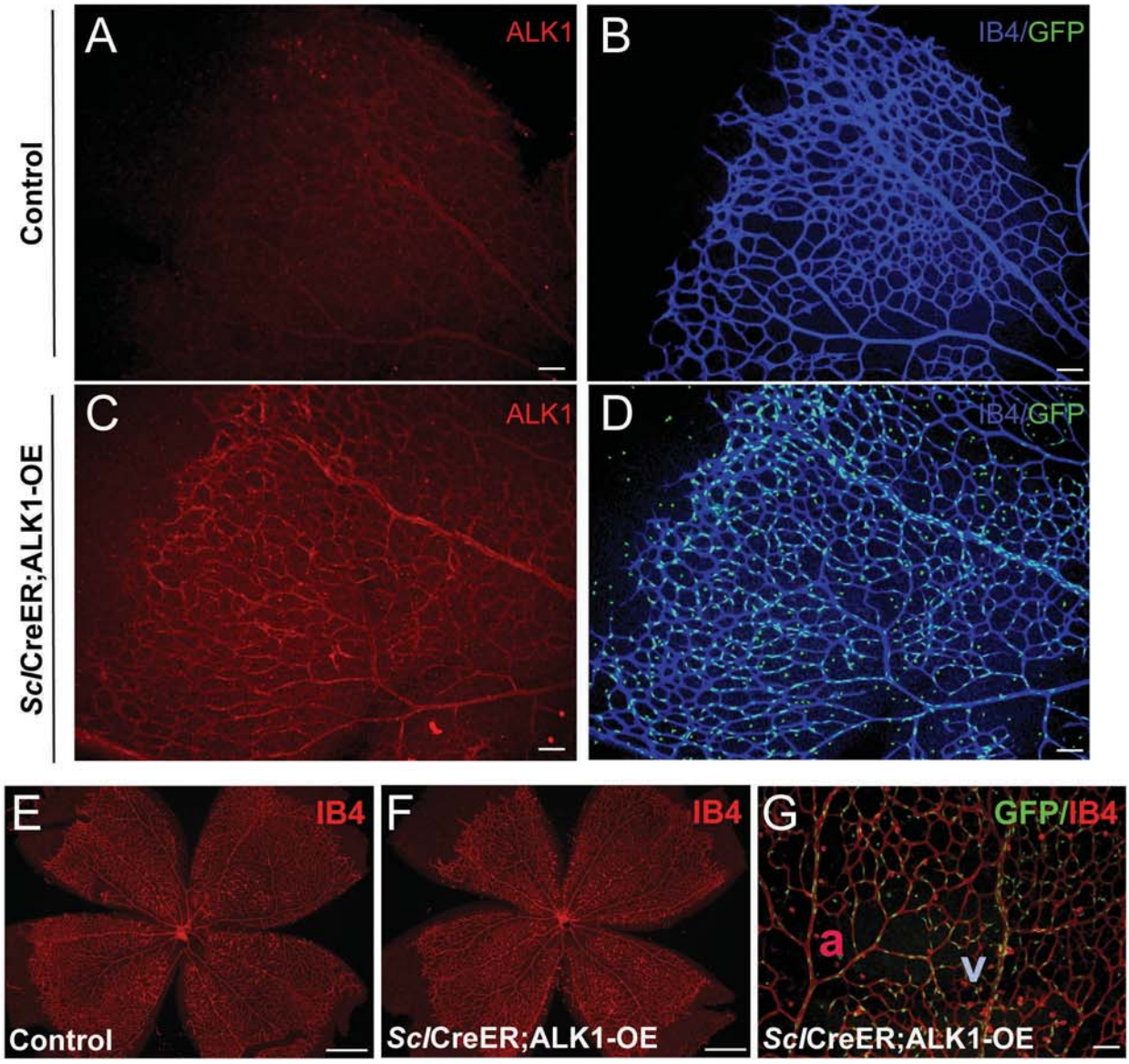
Online Figure XVI. ALK1-OE completely rescues the vascular anomalies caused by *Alk1*-iKO in ECs. Latex dye-perfused blood vessels (upper panels) and processed images (lower panels) on wounded-skin of control (*Scl*CreER-negative ALK1-OE, **A-H**), *Scl*CreER; *Alk1*-iKO (**I-P**) and *Scl*CreER;*Alk1*-iKO;ALK1-OE mice (**Q-X**) 8 days after wounding. Wound sites are indicated by asterisks. Scale bars: 1 mm.



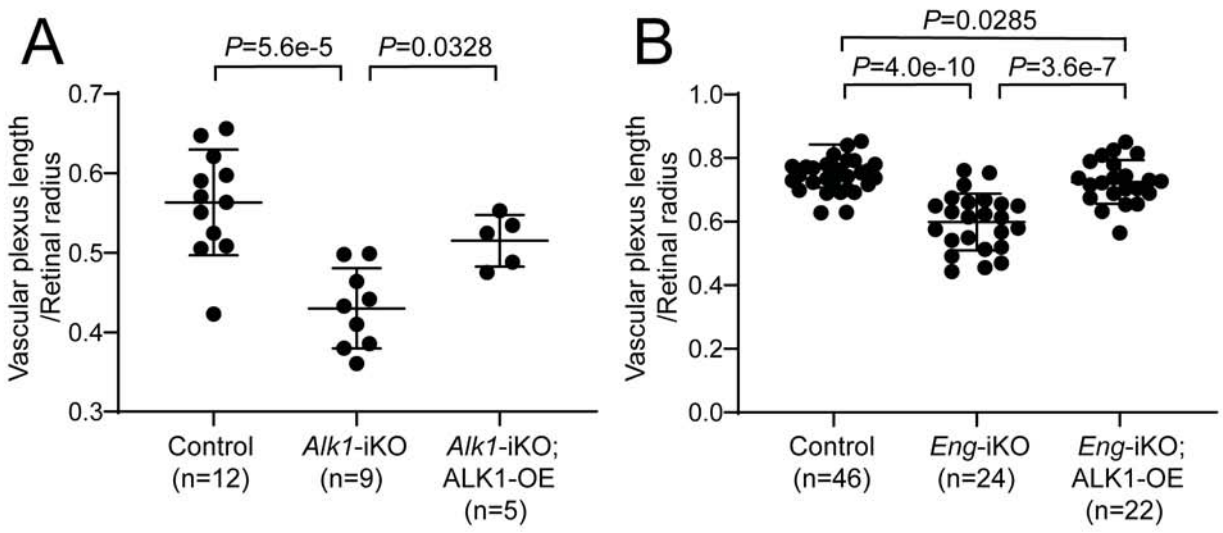
Online Figure XVII, ALK1-OE effectively prevent the abnormal AV-shunt formation caused by *Eng*-iKO in ECs. Blood vessels containing latex dye (upper panels) and the processed images (lower panels) on wounded-skin of *Scl*CreER;*Eng*-iKO (**A-H**) and *Scl*CreER;*Eng*-iKO;ALK1-OE mice (**I-P**) 8 days after wounding. Wound sites are indicated by asterisks. Scale bars: 1mm.



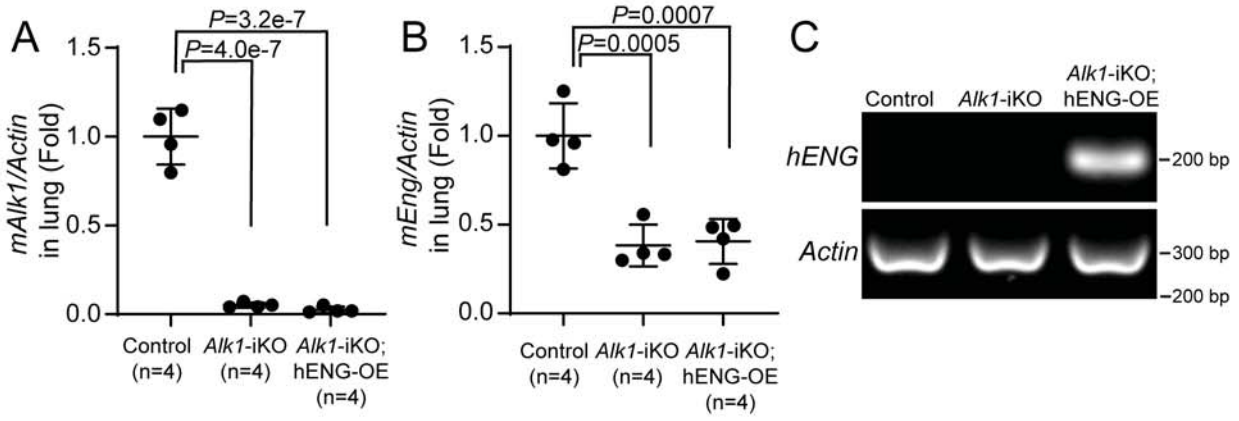
Online Figure XVIII. Alk1 expression is artery specific in adult mice with wounded-skin but is detected in all blood vessel types in developing retina. *Alk1* expression patterns in vasculatures of wounded skin and developing retinas were determined using *Alk1*$^{+/GFP}$ reporter mice in which GFP cassette is knocked-in into *Alk1* allele. **A,** Immunofluorescence staining of GFP (left panel) and IB4 (right panel) on wounded-ear isolated from *Alk1*$^{+/GFP}$ mice. Arrow indicates a GFP-positive arterial vessel and arrowheads mark non-specific background staining. **B,** Endogenous GFP reporter expression in developing retinas of *Alk1*$^{+/GFP}$. IB4 staining marks retinal vasculatures. a, artery; v, vein. Scale bars: 100 μm.



Online Figure XIX. ALK1-OE in developing retinal ECs does not lead to the vascular abnormalities. **A-D**, Immunofluorescence staining of ALK1 (red) and IB4 (blue) in PN7 retinas of controls (*Scl*CreER-negative ALK1-OE, **A** and **B**) and *Scl*CreER;ALK1-OE (**C** and **D**). ALK1 staining (**A** and **C**) and merged images of IB4 and bicistronically expressed GFP (**B** and **D**). **E-G,** IB4 staining (red) in PN7 retinas of control (**E**) and *Scl*CreER;ALK1-OE (**F** and **G**). The merged image indicates *Scl*CreER-driven GFP reporter expression in *Scl*CreER;ALK1-OE retinas (**G**). a, artery; v, vein. Scale bars: **A-D** and **G**, 100μm; **E** and **F**, 500 μm.



Online Figure XX. ALK1-OE restores impaired radial expansion of retinal vasculature caused by *Eng*-iKO as well as *Alk1*-iKO. Vascular outgrowth towards the vascular front was measured by the ratio of the vascular radius and the retina's radius in **Figure 3**. **A**, Retinal vasculature expansion of Control (n=12), *Scl*CreER;*Alk1*-iKO (n=9), and *Scl*CreER;*Alk1*-iKO;ALK1-OE (n=5) for PN5 retinas. **B**, Retinal vasculature expansion of Control (n=46), *Scl*CreER;*Eng*-iKO (n=24), and *Scl*CreER;*Eng*-iKO;ALK1-OE (n=22) for PN7 retinas. Note that ALK1-OE rescued the reduced vascular outgrowth resulting from ALK1-deficiency (**A**) and ENG-deficiency (**B**). One-way ANOVA with Tukey's correction was used for statistical analyses. All data are means±SDs.



Online Figure XXI. hENG-OE does not influence the expression of mouse Alk1 and Eng in *Alk1*-iKO mice. **A** and **B**, Quantitative RT-PCR on Alk1 (**A**) and Eng (**B**) transcripts in control, R26^{CreER};*Alk1*-iKO (*Alk1*-iKO) and R26^{CreER};*Alk1*-iKO; hENG-OE (*Alk1*-iKO;hENG-OE) mouse lungs. The mRNA levels of the genes are normalized with Actin. n=4 per each group. **C,** Induced hENG transcription in the lung of R26^{CreER};*Alk1*-iKO;hENG-OE mice. One-way ANOVA with Tukey's correction was used for statistical analyses. All data are means ± SDs.