ONLINE MATERIALS AND METHODS

Genetic experiments and pharmacological inhibition

All animal procedures were performed in accordance with Yale University and University of North Carolina Institutional Animal Care and User Committee guidelines. The following mouse strains were used: *Alk1*$^{fl/fl}$ [1], *Alk2*$^{fl/fl}$ [2], *Alk3*$^{fl/fl}$ [3], *Bmpr2*$^{fl/fl}$ [4], and *Cdh5(Pac)Cre*^{ERT2} [5], and BRE-gfp[6] mice. For endothelial-cell-specific loss-of-function of BMPRs, mice carrying homozygous floxed alleles of BMPR were interbred with *Cdh5(Pac)Cre*^{ERT2} mice. All of the mouse strains were back-crossed into the C57Bl/6J background. Genetic modifications were induced by intraperitoneal injections with 30 or 100μg tamoxifen (Sigma) at P1 and P2.

Isolation of mouse endothelial cell and manipulation of mouse retina

Mouse lung endothelial cells (MLECs) for qRT-PCR analyses were isolated as described previously (Magee et al. 1994; Sakurai et al. 2002). In total, six P6 pups were euthanized by decapitation. The lungs were removed and placed in a 1.5mL eppendorf tube. Lungs were finely minced with sharp scissors, then the tissue was digested with 2mg/mL collagenase type I in PBS at 37˚C for 45 min. The digest was passed through a 75μm cell strainer to remove undigested tissue fragments. Cells were pelleted at 400 g for 5 minutes, resuspended in 2% FBS containing PBS with magnetic beads-coated rat anti-mouse CD31 (PECAM-1) antibody (Ab) (BD PharMingen). After incubation on room temperature for 12 min, the cells were placed on the magnet for 5 min and unbound cells were removed. The bound cells were resuspended in medium and plated onto a 0.1% gelatin-coated dish. Mouse retina dissection and manipulation was performed as previously reported[7].

Immunofluorescence (IF)

The following antibodies were used: anti-ALK1 (1:100, R&D Systems), anti-ALK2 (1:100, Proteintech), anti-ALK3 (1:100, Thermo), anti-BMPR2 (1:100, BD), anti-PECAM-1 (1:200, BD) and isolectin-B4 (1:100, BD). Alexa Fluor 488, 555 and 647 donkey secondary antibodies were from Invitrogen. Retina immunostaining was carried out with littermates processed simultaneously under the same conditions. To analyze and quantify the retina vascular phenotype, the eyes were fixed in 4% PFA for 2 hours at 4˚C and dissected as previously reported (REF). After washing the retinas were permeabilized using PBS-T (0.1% Triton X-100 containing PBS) and incubated overnight at 4˚C in PBS-T containing biotinylated isolectin B4 (IB4) and the primary antibodies. Then the retinas were incubated with fluorophore-conjugated antibodies and with Alexa Fluor 488/555/647 (Invitrogen), and mounted with Fluorescence Mounting Medium (Dako). Confocal microscopy was performed with Leica SP5 confocal microscopes. ImageJ (NIH) was used for the data analysis. The figures were assembled using Adobe Photoshop. The only adjustments used in the preparation of the figures were for brightness and contrast.

RT-PCR

For gene expression analysis, the total RNA was isolated with RNeasy Mini kits (QIAGEN) and 1 μg total RNA was reverse transcribed using High-Capacity cDNA Reverse Transcription Kit (Thermofisher scientific) following the manufacturer instructions. The cDNAs were amplified using the FG Power SYBR Green PCR Master Mix (Applied Biosystems) in an Eppendorf Mastercycler gradient. The relative expression differences represent the means of the results obtained from at least three-independent experiments.

Statistical analysis

Data are expressed as mean ± SEM. Comparisons between groups were made using a two-tailed Student t test. Prior to the student t test, we have used the Levene's test to examine whether the assumptions and conditions for t test were violated, and therefore, could not be used to determine the differences between groups. We found that knockout mice and phenotypic wild-type siblings are statistically indistinguishable groups for a number of unrelated parameters. In all parameters we used, the F values were significantly smaller than the Table F value at α=0.001. Our statistical analyses demonstrated that knockout mice and their phenotypic wild-type siblings have similar population

variances, and therefore, the assumptions and conditions for t test was not violated. This result supports our approach to employ t test to perform statistical analyses.

ONLINE MATERIALS AND METHODS REFERENCES

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