MATERIALS AND METHODS

Mouse Strains

All experiments involving animals were performed with approval of the University of North Carolina, Chapel Hill Institutional Animal Care and Use Committee. Flk1-GFP mice [Kdr\textsuperscript{tm2.1Jrt}/J, JAX #017006] express GFP in endothelial cells as previously described \textsuperscript{1}. R26R tdTomato [Gt(ROSA)26Sortm14(CAG-tdTomato)Hze/J, JAX #007914] mice have a STOP cassette flanked by loxP sites followed by tdTomato \textsuperscript{2}. Cdh5(PAC)-CreERT2 transgenic mice were generated by microinjecting a transgene containing a genomic Cdh5(PAC) promoter fragment fused to a CreERT2 cDNA into zygotes \textsuperscript{3}. Cre was induced by one application (7 days prior to biopsy punch) of a 10nM solution of tamoxifen dissolved in DMSO to the mouse ear for 10 min, then rinsing with ethanol.

Intravital Imaging

Flk1-GFP mice were commercially obtained from Jackson Laboratory and bred into a CD1 background. Mice were anesthetized using 2% isoflurane and a 0.35mm biopsy punch (Fine Science Tools) was used to create a small wound. To perform intravital imaging, we adhered to a protocol established by Chan et al. \textsuperscript{4}. Briefly, mice were placed in an induction chamber, anesthetized with 3-4% isoflurane, and then placed on a heating pad with continuous 2% isoflurane inhalation. Multiphoton imaging was performed on an Olympus FV1000MPE microscope mounted on an upright BX-61WI microscope, using a 25x objective, with a custom-built aluminum clamp to immobilize the ear during imaging. A coupling gel composed of 300 mM D-sorbitol (Sigma-Aldrich) and 0.5% Carbomer 940 (Spectrum Chemical) adjusted to pH 7.4, was placed between the objective and coverslip. We used a 910nm excitation wavelength to image GFP, and a 960nm excitation wavelength to simultaneously image GFP and tdTomato. Images were obtained using the Multi Area Time Lapse tool on the Olympus Fluoview software in a 5x5 grid (~1.5mm x 1.5mm) of the wound and the surrounding area. Images were processed using Fiji and were stitched together using the Grid/Stitching Plugin. Wounds were imaged every other day for 39 days in experiments using Flk1-GFP mice, and for 21 days in experiments using Flk1-GFP; Cdh5-CreERT2; Rosa-tdTomato.

Tortuosity Index

To calculate tortuosity index, a modified equation from Bullitt et al \textsuperscript{5} was used. We measured the ratio between the shortest distance of the start and end point of a vessel segment, noted as the geodesic distance (L\textsubscript{G}), over the total distance of a vessel segment, noted as Euclidian distance (L\textsubscript{E}). We then multiplied this ratio by the number of inflection points, or the number of times the vessel changes direction with a degree of < 160\textdegree\ (N\textsubscript{C}). Statistical significance was quantified using GraphPad Prism software and an unpaired t-test where p<0.05 was significant. To determine the cut-off value for tortuosity, a receiver operating characteristic (ROC) curve was used to calculate 3.2 as the value where there is 100% specificity and 94% sensitivity.
**In Vivo Flow Experiments**

To visualize *in vivo* blood flow, we injected 100 nm, red fluorescent, FluoSpheres® Carboxylate-Modified Microspheres (Invitrogen) at 7dpw in Flk1-GFP female mice. Mice were anesthetized with 3% isoflurane, and a tail vein catheter was used to administer a solution of 10% microspheres in sterile PBS. To simultaneously image GFP and red spheres, an excitation wavelength of 960 nm was used. For live capture, we imaged using an Olympus FV1000MPE microscope and acquired images at frame rate of 1.1 frames/sec using a 25x objective. Images were processed using Fiji.

**Permeability Experiment and Analysis**

To measure permeability, we injected 100 µl of 20 nm, red fluorescent, FluoSpheres® Carboxylate-Modified Microspheres (Invitrogen) at 15dpw via tail vein injection in Flk1-GFP female mice. Mice were euthanized 20 min after injection and perfused with 10 ml 4% PFA. Ears were dissected and post-fixed for an additional 2 hr with 4% PFA, rinsed in PBS, and then mounted on slides with Vectashield Hard Set Mounting Media (Vectashield). To quantify signal, the radius for each vessel segment was measured and a line (10 pixels wide) was drawn from the middle of the vessel segment extending outward two radius lengths, so that the radius plus an equivalent distance outside the vessel was measured. Plot Profile in Fiji was used to quantify fluorescence signal along the line for both green and red channels. An average of 3 lines was used to calculate fluorescence signal for each vessel segment.

**Antibody Staining**

Mice were euthanized at 11dpw or 15dpw and perfused with 4% PFA. Ears were removed and dissected to expose vessels and fixed an additional 2 hr with 4% PFA. Tissue was then rinsed with PBS, permeabilized with a 1% Triton X-100/PBS solution for 1 hr, then blocked with 5% Serum/0.5% Triton X-100/1% BSA/ PBS (blocking solution). Tissue was incubated with PECAM (BD, 1:100), ICAM-1 (BD, 1:50) or P-selectin (BD, 1:50) in blocking solution, overnight at 4°C. The samples were rinsed with PBS, incubated with Alexa-Fluor 555 secondary antibody for 2 hr, then rinsed with PBS and mounted onto slides using Vectashield Hard Set Mounting Media (Vectashield). Images were acquired on an Olympus FV1200 using a 40x long working distance objective and processed using Fiji. For ICAM1, the AND function in Fiji was used to isolate ICAM1 co-expression with Flk1-GFP.
Vessel Length and Sprout Analysis

Vessels segments were traced and isolated using a mask function in Fiji. Tracings were skeletonized using the skeletonize plugin in Fiji and analyzed using the Analyze Skeleton Plugin in Fiji. Total traced segments were used to determine the percentages of tortuous microvessels and normal capillaries for each time point. For sprouting, images were stacked to make a time series of the 39 day wound healing time course. The Cell Counter Plugin was used to annotate sprout initiations, connections, and retractions from normal and tortuous sprouts. Significance was determined using Two-Way ANOVA, with p≤0.05 being significant.
SUPPLEMENTAL REFERENCES

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