Tortuous Microvessels Contribute to Wound Healing via Sprouting Angiogenesis

Diana C. Chong, Zhixian Yu, Hailey E. Brighton, James E. Bear, Victoria L. Bautch

Objective—Wound healing is accompanied by neoangiogenesis, and new vessels are thought to originate primarily from the microcirculation; however, how these vessels form and resolve during wound healing is poorly understood. Here, we investigated properties of the smallest capillaries during wound healing to determine their spatial organization and the kinetics of formation and resolution.

Approach and Results—We used intravital imaging and high-resolution microscopy to identify a new type of vessel in wounds, called tortuous microvessels. Longitudinal studies showed that tortuous microvessels increased in frequency after injury, normalized as the wound healed, and were closely associated with the wound site. Tortuous microvessels had aberrant cell shapes, increased permeability, and distinct interactions with circulating microspheres, suggesting altered flow dynamics. Moreover, tortuous microvessels disproportionately contributed to wound angiogenesis by sprouting exuberantly and significantly more frequently than nearby normal capillaries.

Conclusions—A new type of transient wound vessel, tortuous microvessels, sprout dynamically and disproportionately contribute to wound-healing neoangiogenesis, likely as a result of altered properties downstream of flow disturbances. These new findings suggest entry points for therapeutic intervention.

Visual Overview—An online visual overview is available for this article. (Arterioscler Thromb Vasc Biol. 2017;37:1903-1912. DOI: 10.1161/ATVBAHA.117.309993.)

Key Words: angiogenesis ■ cell shape ■ intravital imaging ■ microcirculation ■ tortuous microvessels ■ wound healing
relative to normal capillaries in the wound site. High-resolution imaging in vivo revealed that endothelial cells comprising tortuous microvessels have aberrant cell shapes, increased permeability, and suggest altered flow dynamics. Surprisingly, tortuous microvessels have increased sprouting compared with normal capillaries, indicating for the first time that tortuous vessels contribute to sprouting angiogenesis during wound healing. Thus, we identify a new type of tortuous vessel that forms from the smallest capillaries and contributes significantly to sprouting angiogenesis in wounds. Because of its exuberant sprouting, this class of vessel may be a novel interventional target for improved wound healing.

Materials and Methods

Materials and Methods are available in the online-only Data Supplement.

Results

Tortuous Microvessels Are Associated With Wound Healing

To better understand the role of angiogenesis in wound healing, we investigated this process in a mouse ear wound-healing model. We used a Flk1-GFP vascular reporter line and a small ear punch biopsy (0.35 mm) to induce a wound, and we examined angiogenesis and tortuous vessel formation as the wound healed. Using intravital imaging and 2-photon microscopy, we acquired high-resolution images (including the Z plane) of small caliber vessels growing into the wound site of the same mouse ear over an extended time period (≤39 days post-wounding, dpw; Figure 1A through 1C; Figure 1A in the online-only Data Supplement). Image analysis revealed that small-caliber tortuous vessels, which we termed tortuous microvessels, were present in the wound area as early as 3 dpw, alongside normal small capillaries (Figure 1D through 1I).

We defined a tortuous microvessel as having a diameter of 5 to 20 μm and oscillating S curves, kinks, bends, or twists. To accurately quantify tortuous microvessels during wound healing, we applied a tortuosity index to vessel segments by examining the ratio between total segment length (Ls) and the shortest distance between start and end point (Lp), then multiplying the ratio by the number of times the vessel changed direction or curved <160° (Nc; Figure 1J through 1L). We found that tortuosity index values for tortuous microvessels were significantly higher than those for normal nearby microvessels in the wound, verifying that this vessel type has a distinct morphology that can be quantified (Figure 1L). Tracing of all microvessels in the images revealed that the total length of microvessels significantly increased from 3 to 17 dpw, coinciding with the angiogenic phase of wound healing, then leveled off during subsequent times (Figure 1B in the online-only Data Supplement). We then categorized the vessels into normal or tortuous subgroups, which revealed that the percentage of tortuous microvessels also followed this time course initially and peaked at 17 dpw; however, tortuous microvessels then significantly decreased as healing progressed (Figure 1M). Comparison of start, peak, and end points showed that the percentage increase in tortuous microvessels was significant at 17 dpw compared with the beginning and end of the imaging time (Figure 1N). Thus, tortuous microvessel formation increased initially as part of an overall trend, but then decreased while overall microvessel length stabilized, suggesting that tortuous microvessels resolve into normal capillaries over time.

To more closely examine the relationship between tortuous microvessels and wound closure, we measured microvessel location relative to the wound. Circular regions were outlined every 100 μm outward from the wound center and used to calculate the percentage of tortuous microvessels in these regions at each time point (Figure 2A and 2B) for 31 days. We found that the majority of vessels near the wound boundary (100–300 μm from centroid) were tortuous. Because most tortuous microvessels were found close to the wound boundary, we next asked how tortuous microvessels formed in new tissue that is initially avascular. We outlined the avascular area (including the biopsy punch hole) around the wound at 1 dpw and used this constant area to assess new angiogenesis at subsequent time points (Figure 2C; Figure 1A and 1B in the online-only Data Supplement). The area remained avascular until 5 dpw, and subsequent vascularization consisted mainly of tortuous microvessels at early time points (7–17 dpw; Figure 2D). However, as the wound remodeled, the total length of the tortuous microvessels decreased, while the total length of the normal capillaries in this area increased, and the 2 lengths were equal by 31 to 39 dpw. To determine whether the predominant vascularization by tortuous microvessels at the early time points was because of angiogenesis, tissue migration into the wound area, or a combination of both, we tracked the location of distinct vessels in this region over time. We found that the wound area initially showed some mass movement, suggesting that microvessels may move in because of larger tissue displacement at early time points (Figure 2B in the online-only Data Supplement). However, we tracked individual patterns over time and found that starting around 7 to 9 dpw, mass movement was minimal, and new microvessels appeared to form via sprouting angiogenesis into the avascular region (Figure 2B in the online-only Data Supplement). These findings suggest that sprouting angiogenesis is the driving force behind vascularization of new tissue after a short burst of tissue displacement and that sprouting angiogenesis is active at the wound border where tortuous microvessels are concentrated.

We next hypothesized that the shift from tortuous to normal microvessels in the avascular area resulted at least in part from resolution of tortuous vessels, and we temporally tracked individual tortuous microvessels over time to test this hypothesis (Figure 2E and 2F). We found that 69% of tortuous microvessels normalized, while 27% of microvessels remained tortuous over the time course of wound healing, suggesting that tortuous microvessel formation and resolution is a dynamic process associated with the angiogenic phase of wound healing.

Tortuous Microvessel Endothelial Cells Have Distinct Properties

We took advantage of high-resolution intravital imaging to interrogate the endothelial cells that line tortuous microvessels. The topology of tortuous microvessels suggested that
similar to larger tortuous vessels, they experience disturbed flow, and as a result, the endothelial cells may change shape and acquire other properties consistent with changes that promote vessel sprouting, defined as a state of activation. We acquired images from live intravital imaging while injecting small-diameter (100 nm) fluorescent microspheres to visualize blood flow. Before injection, there were no red fluorescent microspheres present in any vessels (Figure 3A; Movie in the online-only Data Supplement). On injection, the microspheres moved through the imaging field quickly in areas with

Figure 1. Tortuous microvessels during wound healing. A–C, Wound and surrounding vasculature at indicated times. Yellow stippled line, wound punch and avascular area; boxes, areas magnified below. D–F, High magnification of blue boxed areas showing normal microvessels. G–I, High magnification of red boxed areas showing tortuous microvessels. J and K, Examples of normal and tortuous microvessel segments used to calculate tortuosity index. L, Quantification of normal and tortuous microvessel segments. M, Quantification of percentage of tortuous microvessels over 39 days, n=3 ears. N, Graph representing the ratio of tortuous to normal microvessels at indicated times, n=3 ears. Error bars, mean±SEM; Statistical comparisons by 2-way ANOVA with Tukey multiple comparison test; *P≤0.05; ****P≤0.0005. Scale bars: 200 μm (A), 50 μm (D). Vessels pseudocolored using Photoshop. ANOVA indicates analysis of variance; and dpw, days post-wounding.

linear capillaries, and microsphere patterns changed completely from one time frame to the next (Figure 3B through 3F, blue boxes). In contrast, some microspheres appeared to stick in the curved area of tortuous microvessels, and these became more prevalent with time (Figure 3B through 3G, red boxes), while the linear regions lost association with microspheres over time as they were presumably cleared from the circulation. Analysis revealed that on average 5 beads per tortuous microvessel remained stationary post-injection, while no beads were stationary in linear microvessels (Figure 3H).

These findings indicate that endothelial cells in tortuous microvessels differ from those in linear regions and suggest that reduced or disturbed blood flow is associated with tortuous microvessels, which could ultimately lead to differences in endothelial cell properties between normal and tortuous microvessels.

Because activated endothelial cells are often round rather than being spindle-shaped and elongated, we hypothesized that endothelial cells in tortuous microvessels had abnormal cell shapes. To test this hypothesis, we generated mosaic mouse lines

Figure 2. Tortuous microvessel location, formation, and resolution. A, Example of 3 dpw wound illustrating defined regions every 100 μm (not to scale). Note avascular area (wound punch+tissue) in segments close to the wound. B, Dot plot representing percentage of tortuous microvessels in each region over time. Warmer colors (red/orange), elevated tortuous microvessels; cooler colors (blue/purple), reduced tortuous microvessels. C, Avascular wound area+punch, defined at 1 dpw and analyzed at subsequent time points. D, Quantification of normal and tortuous microvessel length in new wound tissue (same areas measured for all time points). Error bars, mean+SEM. Statistical comparisons, 2-way ANOVA with Sidak multiple comparisons test. *P≤0.05, **P≤0.005. E, Example of a tortuous microvessel over time, showing normalization. F, Percentage of vessels that normalized, remained tortuous, or outcome unknown during 39 day wound healing time course. n=86 tortuous microvessels. Vessels pseudocolored using Photoshop. ANOVA indicates analysis of variance; and dpw, days post-wounding.
that expressed GFP in endothelial cells along with a vascular-selective inducible Cre driver, Cdh5-CreERT2, and a tdTomato excision reporter. The reporter was activated in a small subset of endothelial cells (10%–30%) using low-dose tamoxifen prior to wound healing, and individual groups of endothelial cells were followed during an abbreviated time course (21 days) that, nevertheless, encompassed the major angiogenic period in our model. Endothelial cells in normal capillaries maintained a stereotypical spindle shape, while those in tortuous microvessels were spindle-shaped at 1 dpw, but became more rounded over time, coincident with increased tortuosity (Figure 4A and 4B). The shapes of individual endothelial cells were measured at 11 dpw in fixed samples stained for PECAM-1 (platelet endothelial cell adhesion molecule 1), an endothelial cell border marker (Figure 4C). The longitudinal/transverse axis ratio was significantly reduced in endothelial cells from tortuous microvessels relative to control endothelial cells in normal microvessels (Figure 4D). These data confirm the visual appearance of endothelial cells in tortuous microvessels as rounded and suggestive of activation.

To determine whether the endothelial cells in tortuous microvessels had other distinct properties, we examined the expression of several markers associated with a proinflammatory phenotype. Expression of ICAM-1 (intercellular adhesion molecule 1), an intercellular adhesion molecule involved in leukocyte extravasation, showed robust expression at 15 dpw in tortuous vessels near the wound (Figure IIIA in the online-only Data Supplement). Likewise, staining for P-selectin, which is associated with postcapillary venules that are competent for extravasation, was positive in areas of tortuous microvessels (Figure IIIB in the online-only Data Supplement).

We next asked whether tortuous vessels had altered barrier function. To test for permeability, we injected smaller
Figure 4. Cellular morphology in tortuous microvessels. Mosaic analysis of microvessels during 21 day wound healing time course in mice of indicated genotype. A, Normal microvessel endothelial cell (EC) have stereotypical spindle morphology throughout time course (blue arrowheads, red cell in diagram). B, Tortuous microvessel ECs initiate as spindle-like and become rounded with time and tortuosity (blue arrowheads, red cell in diagram). C, PECAM (platelet endothelial cell adhesion molecule) staining of Flk1-GFP mice at 11 dpw, highlighting individual cell borders. D, Ratio of longitudinal to transverse axis of EC in normal and tortuous microvessels; long line and error bars, mean±SEM. Statistical comparisons, Student t test (unpaired, 2-tailed); ****P≤0.0001. Scale bar, 10 μm (C and D). dpw indicates days post-wounding.
fluorescent microsphere beads (20 nm) via tail vein injection at 15 dpw and collected samples after 20 minutes (Figure 5A and 5B). Line scan measurements revealed that microspheres were significantly increased in the areas adjacent to tortuous microvessels relative to normal nearby capillaries (Figure 5C through 5E). Interestingly, we also noted increased microspheres within the tortuous microvessels, consistent with our earlier finding that larger microspheres were enriched in tortuous areas. These results indicate that tortuous vessels have increased permeability, consistent with the cell shape changes. Taken together, these findings show that endothelial cells in tortuous microvessels are distinct from cells in normal capillaries by multiple criteria.

Tortuous Microvessels Sprout Robustly

The altered morphology and properties of tortuous microvessel endothelial cells suggest that they may be more prone to sprouting, than endothelial cells in normal capillaries. Thus, we hypothesized that tortuous microvessel endothelial cells sprout more frequently than normal capillaries, and we used our high-resolution longitudinal data set from the complete time course (39 days) of wound healing to test this idea. Analysis of blood vessel sprouts, defined as new extensions of at least 10 μm that were unconnected (blind-ended) and that initiated during the wound healing time course, revealed that tortuous microvessels sprouted exuberantly (Figure 6A through 6G). For example, a microvessel near the wound that appeared normal at 1 dpw subsequently became tortuous at 5 dpw, and sprouts emanated from curved regions (Figure 6A and 6B). At 7 dpw, the main microvessel remained tortuous, and the sprouts continued to extend (Figure 6C). By 9 dpw onward, the main microvessel began to normalize, while the sprouts persisted and made connections to each other or to other vessels (Figure 6D through 6F). When analyzing new sprout initiations, we found that tortuous microvessels sprouted at a significantly higher frequency than normal capillaries from 5 to 13 dpw (Figure 6G).

To determine whether sprout initiations from tortuous microvessels were more or less stable than sprout initiations from normal vessels, we quantified how often sprouts from each type of vessel formed a connection or retraction (Figure IVA and IVB in the online-only Data Supplement). Our analysis showed no significant differences between connections and retractions of sprouts from tortuous or normal microvessels (Figure IVC in the online-only Data Supplement), suggesting that sprouting from tortuous vessels leads to stable connections and expands the vascular plexus near the wound.
We asked whether sprouting from tortuous microvessels was a resolution mechanism and found that 36% of tortuous microvessels normalized after sprouting, while 54% normalized without sprouting, suggesting that sprouting is not required for vessel normalization (Figure IVD in the online-only Data Supplement).

To better understand the sprouting behavior of tortuous microvessels, we examined when sprout initiations occurred relative to acquisition of microvessel tortuosity (Figure 6H through 6L). This analysis showed that significantly more sprouts formed after microvessels became tortuous compared with sprouts initiating coincident with tortuosity (Figure 6L), indicating that sprouting is downstream of tortuous vessel formation. This data suggest that tortuous vessel formation promotes endothelial cell sprouting. To determine whether some regions within tortuous microvessels were more prone to sprouting, we measured sprout initiations from curved regions versus noncurved regions of tortuous microvessels. Our data revealed a significant increase in sprout frequency from the apex of the curved region of tortuous microvessels versus other areas of the vessel (Figure 6M through 6O), suggesting that these curved areas are the most sprout-prone regions of tortuous microvessels. Taken together, these data support our hypothesis that endothelial cells in tortuous microvessels are distinct, and the enhanced sprouting frequency combined with enhanced overall tortuosity suggest that tortuous microvessel formation is a key player during the angiogenic phase of wound healing.

Figure 6. Tortuous microvessels have increased sprouting. Analysis of sprouting from normal and tortuous microvessels over 39 day wound healing time course. A-F, Example of a normal microvessel near wound that became tortuous and initiated sprouting. A, Normal microvessel near wound at 1 dpw. B, At 5 dpw, the main microvessel became tortuous and initiated sprouting (blue arrows) from the apex of curved regions (yellow arrowheads). C, Main microvessel remained tortuous and sprouts persisted at 7 dpw. D-F, From 9 to 15 dpw, the main tortuous microvessel was normalized, and the sprouts formed new connections (green arrows). G, Quantification of sprout initiations from normal and tortuous microvessels during wound healing, n=3 ears. Error bars, mean±SEM; Statistical comparisons: 2-way ANOVA with Sidak multiple comparisons test. *P≤0.05; **P≤0.005; ***P≤0.0005. H-K, Example of sprout initiation after tortuous microvessel formation. Note that sprout initiation does not occur until 9 dpw (K, yellow arrow). L, Quantification of sprout initiations subsequent to tortuous microvessel formation relative to sprout initiations simultaneous with tortuous microvessel formation. Error bars, mean±SEM; Statistical comparisons: Student t test (unpaired, 2-tailed); **P≤0.005. M, Example of sprout initiation from apex of curve on tortuous microvessel. N, Example of sprout initiation from noncurved region of tortuous microvessel. O, Quantification of sprout initiations from the apex of tortuous microvessels relative to noncurved regions. Error bars, mean±SEM; Statistical comparisons: Student t test (unpaired, 2-tailed); **P≤0.005. P, Model of tortuous microvessel initiation, sprouting, and resolution during wound healing. Scale bar, 50 μm (A). Vessels pseudocolored using Photoshop. dpw indicates days post-wounding.
Discussion

Here, we identify tortuous microvessels for the first time and present a longitudinal, in-depth analysis of microvessel tortuosity during wound healing. Microvessel tortuosity increases over a reproducible time course that largely correlates with increased overall angiogenesis. We show that sprouting is promoted from tortuous microvessels, and this exuberant sprouting likely contributes significantly to wound angiogenesis and overall healing. Our data are consistent with a model whereby tortuosity alters flow parameters and leads to endothelial cell activation that promotes sprouting in these regions (Figure 6P). Thus, the emergence of tortuous microvessels near a wound site may be a mechanism to efficiently expand wound angiogenesis and promote healing.

Although tortuous vessels have been observed during wound healing and in diseases such as cancer, small vessels of the caliber (5–20 μm) analyzed here were not resolved by most of these studies. Yet the bulk of the vasculature in most vessel beds comprised small-caliber capillaries, and these capillaries are usually the source of sprouts that contribute to neoangiogenesis. While some studies analyzed static images of wound vessels, our approach allowed for high spatiotemporal resolution of microvessels and longitudinal analysis of this critical component of wound healing. Our finding that tortuous microvessels contribute significantly to overall sprouting angiogenesis in the wound environment, and our results showing that these sprouts often lead to new connections and conduits, provide a novel link between vessel tortuosity and wound healing via sprouting angiogenesis.

Sprouting from tortuous microvessels occurs at a higher frequency than from normal vessels, and these sprouts often initiate from the apex of curved regions of tortuous microvessels. While we do not have the resolution to interrogate junctional integrity, our finding that tortuous microvessels are leakier than normal capillaries, and that microspheres become leakier than normal vessels, and these sprouts often lead to new connections and conduits, provide a novel link between vessel tortuosity and wound healing via sprouting angiogenesis.

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What triggers tortuous microvessel formation during wound healing? It is likely a complex combination of local environmental cues and other changes associated with wound healing because the tortuous microvessels resolve over time. The peak frequency of tortuous microvessels roughly corresponds to the angiogenic phase of wound healing, and it is known that proangiogenic cytokines such as VEGF (vascular endothelial growth factor) induce tortuosity. Although the highest window of VEGF expression in the wound occurs from 3 to 7 days post-wounding, it is likely that other proangiogenic growth factors, such as bFGF (basic fibroblast growth factor), contribute to tortuous vessel formation at later stages. Tortuous microvessels are enriched close to the wound border, which is the most hypoxic area of the wound, consistent with proangiogenic influences. However, proangiogenic signals are not sufficient to induce vessel tortuosity because most developmental angiogenesis occurs independent of tortuosity. Wound healing is also associated with transient inflammation, so proinflammatory cytokines may also contribute to microvessel tortuosity. Additionally, cytoskeletal rearrangements accompany endothelial cell shape changes and sprouting migration, and growth factors induce cytoskeletal changes, so the elevated growth factors found in the wound healing environment likely contribute in complex ways to microvessel tortuosity. In any case, it is likely that local changes in the wound healing environment promote tortuosity, and this work shows that vessel tortuosity contributes substantially to microvessel sprouting and neoangiogenesis that promotes wound healing.

In tumor microenvironments, microvessels readily become tortuous, which may decrease antitumor drug efficacy. The wound healing process is a natural physiological response, but wound healing is compromised in some diseases as a result of defective vascularization. For example, wound healing is often defective in diabetic patients and sometimes results in limb amputation, and a defective angiogenic response from the microcirculation contributes to this dysfunction. Furthermore, with time and increasing vessel dysfunction, tortuosity subsides in larger vessels of diabetic patients but increases in capillary-sized vessels. The microcirculation comprises capillaries that make up the bulk of the vasculature and readily respond to signaling cues. Here, we identify a new class of vessel, a tortuous microvessel, that is associated with wound healing and contributes significant angiogenic sprouting to the process. Knowledge of the contribution of tortuous microvessels to angiogenic sprouting and wound healing will be useful in designing new therapeutic approaches to accelerate and improve wound healing.

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Disclosures

None.

References

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Online Figure I. Time course for wound healing in mouse ear. Wound incurred at 0dpw. Compressed Z-stack image frames of wound area expressing Flk-GFP (vascular, white) were stitched together for each time point and used for analysis. Yellow stippled line, avascular area + punch wound at each time point. dpw, days post wounding. *, p<0.05; **, p<0.005
Online Figure II. The avascular area of the mouse ear wound. (A) Image (compressed Z-stack) of wound, with avascular area outlined at 1dpw and used to analyze subsequent time points. (B) Defined 1 dpw area at subsequent time points (compressed Z-stacks). Some segments pseudo colored with Photoshop to highlight the same regions in each image. dpw, days post wounding.
Online Figure III. ICAM1 and P-selectin expression in tortuous vessels of 15 dpw mouse ears (compressed Z-stacks). (A) ICAM1 expression is enriched in curved areas of tortuous vessels (arrows). (B) P-selectin is expressed in tortuous and sprouting vessels (arrows). Scale bar, 10µm
Online Figure IV. Comparison of sprout dynamics. (A) Example of sprout initiation from a tortuous microvessel (yellow arrowhead) that connected to another vessel segment (blue vessel). (B) Example of a sprout initiation from a tortuous microvessel (yellow arrowhead) that retracted. (C) Percentage of sprout initiations from normal (blue) or tortuous (red) microvessels that resulted in connections or retractions. (D) Percentage of tortuous microvessels that exhibited sprouting and normalized (blue), exhibited sprouting and did not normalize (red), or normalized absent sprouting (yellow). Vessels pseudo colored using Photoshop. dpw, days post wounding. Error bars, mean ± SEM; NS, not significant. Scale bars, 20µm.
SUPPLEMENTAL MOVIE LEGEND

Online Movie. Microspheres injected into circulation. Live-imaging of 100 nm, fluorescent microspheres (red) injected through a tail vein catheter into Flk1-GFP (green vessels) mouse at 7dpw. Image acquisition, 1.1 frame/sec.
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 \( [Kdr^{tm2.1Jrt/J}, JAX \#017006] \) express GFP in endothelial cells as previously described \(^1\). R26R tdTomato \( [Gt(ROSA)26Sor^{tm14(CAG-tdTomato)Hze/J}, JAX \#007914] \) mice have a STOP cassette flanked by \( loxP \) sites followed by tdTomato \(^2\). 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 \(^3\). 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. \(^4\). 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 \(^5\) 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 \( (L_G) \), over the total distance of a vessel segment, noted as Euclidian distance \( (L_E) \). We then multiplied this ratio by the number of inflection points, or the number of times the vessel changes direction with a degree of \(< 160^\circ \) \( (N_C) \). 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 960nm 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 1hr, 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 \( p \leq 0.05 \) being significant.
SUPPLEMENTAL REFERENCES


