Supplementary Materials for

Low-flow intussusception and metastable VEGFR2 signaling launch angiogenesis in ischemic muscle

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The PDF file includes:

Figs. S1 to S5
Legends for movies S1 to S7

Other Supplementary Material for this manuscript includes the following:

Movies S1 to S7
**Figure S1: Differential effect of VEGFR2 inhibition on formation of endothelial sprouts and pillars**

Graphs depicting the density of intraluminal pillars and sprouts in the newly vascularized extensor digitorum longus muscle of mice 5 days after femoral artery excision. On day 4.5, mice were injected with the VEGFR2 tyrosine kinase inhibitor (TKI), ZM323881, or vehicle (mean ± SE; n=4 and 3 mice).
Figure S2: The anti-VEGFR2 antibody, DC101, is biased toward inhibiting ligand-mediated signaling rather than VEGFR2 mechano-transduction

a. Photomicrographs of HUVECs cultured either under static conditions or subjected to shear stress (3 dyn/cm² for 15 minutes), immunostained for activated VEGFR2 (p-VEGFR2). Junctional VEGFR2 activity is induced by flow (arrows). The VEGFR2 tyrosine kinase inhibitor cabozantinib completely blocked flow-induced junctional VEGFR2 activity whereas DC101 did not. b. Graph depicting activation of VEGFR2 by VEGFA and by flow. Signal is expressed relative to the p-VEGFR2 signal in the absence of the respective stimulus. VEGFA-induced VEGFR2 activity is completely abrogated by DC101 whereas flow-induced VEGFR2 activity is only partially inhibited by the antibody. Data are median, interquartile range, and individual cell data.
Figure S3: Local activation of VEGFR2 on endothelial pillars in primordial vessels

Confocal micrographs of planar-projected optical sections of a primordial vessel in extensor digitorum longus muscle 5 days after ischemic injury, immunostained for VEGFR2 (cyan) and p-VEGFR2 (red). The primordial vessel wall and a hollowing intraluminal pillar (arrows) is delineated by the VEGFR2 signal. The p-VEGFR signal is also evident on the wall endothelial cells and on the pillar (arrow), but the latter has a more focused distribution than total VEGFR2.
Figure S4: Microfluidic device

Schematic depiction (top) and photograph (bottom) of a microfluidic chamber that can be fully lined with endothelial cells, which can then be subjected to ultra-low fluid shear stress.
Figure S5: Morphology of endothelial cells with and without VEGFR2 knock-down co-cultured under static conditions

Confocal microscopy maximum intensity projection and XZ and YZ orthogonal views of endothelial cells differentially transfected with VEGFR2 siRNA or control siRNA in static planar cultures. Control-siRNA cells express GFP and VEGFR2-siRNA cells express RFP. Nuclei were stained with DAPI. XZ and YZ views show no apical protrusions. However, the VEGFR2-knockdown cell displays multiple atypical lateral protrusions that span across control cells (arrows). The number of cell protrusions that crossed at least 50% of a neighboring cell body was significantly higher for VEGFR2-knockdown cells (1.6±0.05 vs. 0.2±0.02 p=0.0003, n=3 experiments, mean ± SE).
**Movie S1.** Red blood cell (RBC) transit in the native external digitorum longus muscle microvasculature visualized by blue light epi-illumination real-time video microscopy. RBCs are seen in relief against the bright plasma that has been labeled with high molecular-weight FITC-labeled dextran. The video entails a larger field of view displayed in Fig. 2b.

**Movie S2.** Real-time video microscopy sequence showing RBC transit in the early (5-day) regenerated external digitorum longus muscle microvasculature. The wide primordial vessels display slow flow and high hematocrit.

**Movie S3.** Real-time video microscopy sequence showing slow RBC transit in day-5 primordial vessels. Within the middle vessel there is a small obstruction to flow (arrow), consistent with a pillar, with RBCs deflecting around it.

**Movie S4.** Real-time video microscopy sequence showing RBC transit in day-5 primordial vessels, corresponding to Fig. 3f, also showing multiple slit-like obstruction to flow indicative of the progression of an intussusceptive splitting process.

**Movie S5.** Video presentation of serial 0.5 µm sections of a skeletal muscle neovessel 5 days after femoral artery excision, stained with basic fuchsin and methylene blue. The video steps through 9 µm of primordial vessel length, revealing two separate intussusceptive events. The first, on the left, is a finger-like pillar / septum that produces a short, separated side-lumen. The second, on the right, is a thicker projection comprised of two opposing endothelial cell bodies, each with their nucleus.

**Movie S6.** 3D volume projection of a HUVEC-lined microfluidic device subjected to ultra-low flow (shear, 0.3 dyn/cm²) and stained with Alexa Fluor 488-phalloidin. Four discrete, transluminal endothelial cell bridges can be seen, including 3 adjacent pillars within ~20 µm of each other.

**Movie S7: 3D volume projection of a HUVEC-lined microfluidic device subjected to ultra-low flow (shear, 0.3 dyn/cm²), stained with Alexa Fluor 488-phalloidin and immunostained for VE cadherin with Dylight 550 visualization. A discrete, transluminal endothelial cell pillar can be seen. There is VE cadherin signal at the lower third of the pillar, suggesting end-to-end intraluminal connection with a second endothelial cell.