# Mechanism of a flow-gated angiogenesis switch: Early signaling events at cell-matrix and cell-cell junctions

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#### **5** Characterization of EC monolayer transport properties

#### Diffusional permeability (P<sub>d</sub>)

In order to characterize the functionality of the monolayers cultured in our microfluidic device EC barrier function to dextran was evaluated. For diffusive permeability estimation, monolayers were cultured under static conditions. EC monolayers were formed as described above. Experimental medium in the apical channel was replaced with medium containing FITC-labeled dextran (Fig. S3).

<sup>10</sup> Fluorescent images were obtained using an inverted microscope (Nikon TE300, Nikon Instruments Inc., NY) and micrographs acquired with a Hamamatsu ORCA-ER camera (Hamamatsu, Japan) using OpenLab image acquisition software at least 4 hours following incubation with dextran. We assume that the transport of dextran across the endothelial monolayer was due to passive paracellular transport from the apical channel. With this assumption  $P_d$  was estimated based on the diffusive flux of dextran across the monolayer:

$$J = D_{dextran} * \left(\frac{\Delta C}{\Delta x}\right)_{gel}$$
$$J = P_d * (\Delta C_{EC \ monolayer})$$

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Where  $P_d$  is the diffusional permeability,  $\Delta C_{EC\ monolayer}$  is the concentration difference across the monolayer,  $D_{dextran}$  denotes the diffusion coefficient of dextran,  $\left(\frac{\Delta C}{\Delta x}\right)_{gel}$  the concentration gradient of dextran in the gel region and J the flux from the apical channel across the monolayer. A fluorescence intensity profile was obtained in ImageJ (U.S. National Institute of Health, Bethesda, MD) (Fig. S3B) from which  $\Delta C_{EC\ monolayer}$  and  $\left(\frac{\Delta C}{\Delta x}\right)_{gel}$  were calculated. The diffusion coefficient for 40kDa dextran, 4 x 10<sup>-11</sup> m<sup>2</sup>/s, was used (Vickerman, et al., 2008).

#### Flow rate measurement and Pressure drop Estimation

Fluid velocities in the gel and pressure drop across the EC monolayer were estimated using a bead tracer method (details below). First, the Darcy permeability of collagen gel (k) without an EC monolayer was calculated from Darcy's Equation for flow through a porous <sup>25</sup> matrix.

$$v = -\frac{k}{\mu} \nabla P$$
 Darcy's Equation

where  $\nabla P$  denotes the imposed pressure gradient,  $\mu$  denotes fluid viscosity and v is the fluid velocity which is obtained experimentally <sup>30</sup> from measurements of tracer particle velocity. As before, fluid reservoirs were used to impose a pressure differential across the gel region. To visualize fluid passage through the gel, fluorescent microspheres were added to the upstream reservoirs and tracer velocities, v

determined as a function of a known pressure drop,  $\Delta P_{tot}$ , across the collagen gel alone. Separate experiments were repeated with collagen gels covered by an EC monolayer to obtain new values for v. In these experiments the upstream reservoir was connected to the basal channel. Here, the collagen gel and the endothelial monolayer were modeled as two resistors in series, and the total pressure <sup>35</sup> drop,  $\Delta P_{tot}$ , is the sum of the pressure drops across the gel ( $\Delta P_{gel}$ ) and EC monolayer ( $\Delta P_{EC monolayer}$ ).

$$\Delta P_{tot} = \Delta P_{gel} + \Delta P_{EC monolayer}$$

From Darcy's equation, the resistance,  $R_{gel} = \frac{\mu L}{k}$  where L is the width of the gel in the flow direction. From this, the pressure drop across the EC monolayer,  $\Delta P_{EC \ monolayer}$ , can be estimated.

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$$\Delta P_{EC \ monolayer} = \Delta P_{tot} - v_{gel \ with \ EC \ monolayer} R_{gel}$$

**Bead Tracer Method.** FITC-labeled microspheres (200nm in diameter) were used as fluorescent tracer particles. Fluorescent traces of microspheres flowing through the gel were visualized on a Nikon TE300 inverted microscope and time-lapse micrographs acquired (Hamamatsu, ORCA-ER, Japan) and analyzed using OpenLab image acquisition software. Traces were obtained by using a long exposure during image acquisition which gives rise to streaks indicating bead path through the gel. While the flow is 3-dimensional, we <sup>5</sup> used particles that remained in focus over their entire trajectory, thus ensuring that the flow was primarily in a single plane. Subsequently, tracer bead velocities were calculated from streak lengths and exposure times. Streak lengths were measure in ImageJ (public-domain image processing software, U.S. National Institute of Health, Bethesda, MD). A potential limitation of this method would be channelling of beads between collagen and top or bottom surface of the devices.

### Time-lapse Video Microscopy

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<sup>10</sup> Time-lapse movies were recorded of endothelial cells during B-A flow induced sprouting angiogenesis. Endothelial monolayers were formed on collagen gel and B-A flow established as described in METHODS. EC morphogenesis was visualized with an inverted microscope (Zeiss Axiovert 200, Carl Zeiss, Germany) equipped with an environmental chamber at 37 C and 5% CO2. Images were acquired at 2 min intervals with the AxioCam MRm (Carl Zeiss, Germany) using AxioVision image acquisition software.

### Transmission Electron Microscopy (TEM)

- <sup>15</sup> A method for processing microfluidic samples for TEM was developed. Briefly, glass coverslips were coated with a thin layer of PDMS. Coated glass slides were used in place of glass coverslips which are used to seal the PDMS device. All other setup and experimental procedures remain unchanged. All samples were fixed and processed for TEM *in situ* by perfusion of fixative and solutions via microfluidic channels. At the end of experiment, samples were fixed with Karnovsky's fixative (2.5% glutaraldehyde and 2% paraformaldehyde in phosphate buffer, pH 7.4). Samples were post-fixed with 1% osmium tetroxide (Electron Microscopy Sciences, Utifield PA) and 16%, extension for the speciment situation of the setup o
- <sup>20</sup> Hatfield, PA) and 1.5% potassium ferrocyanide (Fisher Scientific Company, New Jersey) for 1 hour, dehydrated in cold ethanol and embedded in Epon-Araldite (Electron Microscopy Sciences, Hatfield, PA). Samples embedded in plastic were removed from the PDMS device and re-embedded in Epon-Araldite. Ultrathin sections were cut with an ultramicrotome, counterstained with uranyl acetate (Fisher Scientific Company, New Jersey) and imaged by TEM (Model 300, Philips Eindhoven, The Netherlands).

## p-FAK Y397 and FAK Antibody Pair Validation

- <sup>25</sup> HMVECs were used in all antibody validation experiments. HMVECs were cultured on glass bottom dishes, fixed with 4% PFA and double stained (see METHODS) with pFAK and FAK antibody pair. Four different primary antibody pairs were evaluated to find a "compatible" pair (Table S1). Compatibility was determined by visual inspection with selection criteria which were based on the presence of the classic staining pattern and colocalization of pFAK with FAK in focal adhesion complexes. Stained samples were imaged (Nikon TE300, Nikon Instruments Inc., NY; Hamamatsu Orca ER, Japan) and analyzed (OpenLab Software, Improvision, MA). Higher
- <sup>30</sup> resolution images were obtained with a confocal microscope (LSM 510 Zeiss Axiovert 200M v4.0). Additionally, a requirement that the antibody binding epitope did not occur in the region of Tyr 397 was also imposed. Where available, information regarding binding epitope was obtained from the product supplier. The selected antibody pair was then subjected to a secondary cross-reaction test. Here HMVEC samples were processed for immunolabeling according to METHODS, labeled with primary antibody "A" and incubated with the secondary antibody for primary antibody "B". For e.g. EC labeled with mouse anti-FAK was incubated with a Goat anti-rabbit <sup>35</sup> secondary.

# Supplementary movie: Dynamics during B-A transendothelial flow induced sprouting from an intact EC monolayer



Fig. S1 Expression profile of adherens (VE-Cadherin, RED) and tight (ZO-1, GREEN) junctional proteins in HMVEC cultured on glass substrate (static culture).

Frequency ■HUVEC B ≈HMVEC ន្មី ន្មី ន្មី ន្មី ន្ដី ន្ដី ន្ដី Sprout Length (µm) 

Fig. S2: Sprouting response of microvascular (HMVEC) and macrovascular (HUVEC) endothelial cells to B-A flow. (A) Confocal images of HMVEC (LEFT) and HUVEC (RIGHT) (B) Distribution of sprout lengths for HMVEC and HUVEC monolayers subjected to B-A flow.



<sup>5</sup> **Fig. S3**: Estimation of EC Monolayer Diffusional Permeability ( $P_d$ ). Barrier function to macromolecule was evaluated using a 40 kDa fluorescentlytagged dextran. (A) Schematic of diffusional permeability experimental setup. Medium containing FITC-tagged dextran is introduced into the apical channel. Intensity profiles are obtained perpendicular to the monolayer (black dashed-line) (B) A typical concentration profile of dextran along a line perpendicular to monolayer used to calculate  $P_d$ .

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Fig. S4: Bead Tracer Method for Flow Characterization. (A) Low resolution fluorescent micrograph of gel cage region during a typical bead tracer experiment. White arrow indicates fluid flow direction and white spots are fluorescently labeled microbeads. (B) High resolution micrograph taken at long exposure time showing typical fluorescent streaks for flow velocity calculation.

			Anti-FAK	Anti-FAK (Y397)
			Pair 1	
Antibody Pair #	Anti-FAK antibodies	Anti- FAK (Y397) antibodies		
1	Mouse monocional ab105917	Rabbit polyclonal ab4803	Pair 2	
2	Rabbit monoclonal ab76496	Mouse monoclonal Millipore 05-1140		
3	Rabbit monoclonal ab40794	Mouse monoclonal Millipore 05-1140	Pair 3	
4	Mouse monocional Millipore clone 4.47	Rabbit polyclonal ab4803		
-	_		Pair 4	
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5 Table S1: FAK and p-FAK Y397 Antibody Evaluation. (LEFT) Antibody pairs screen. (RIGHT) Fluorescent micrographs of HMVEC stained for FAK and p-FAK Y397 proteins using four different antibody pairs.



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Fig. S5: Negative controls and secondary antibody cross reaction test. (A) Confocal images of HMVEC monolayer incubated with Alexa Fluor 488 and 568 secondary antibodies and DAPI (BLUE) (Negative control test). (B) Confocal images HMVEC monolayer for antibody cross reaction test. (TOP ROW) Mouse anti-FAK antibody incubated with Rabbit secondary and (BOTTOM ROW) Rabbit p-FAK Y397 antibody incubated with Mouse secondary antibody.