Published in final edited form as:

Lab Chip. 2012 December 7; 12(23): . doi:10.1039/c2lc40389g.

RhoA Mediates Flow-induced Endothelial Sprouting in a 3-D Tissue Analogue of Angiogenesis

Jonathan W. Song, Julien Daubriac, Janet M. Tse, Despina Bazou, and Lance L. Munn
Edwin L. Steele Laboratory for Tumour Biology, Department of Radiation Oncology,
Massachusetts General Hospital and Harvard Medical School, Charlestown, MA 02129, USA

Abstract

Endothelial cells (ECs) integrate signals from the local microenvironment to guide their behaviour. RhoA is involved in vascular endothelial growth factor (VEGF) - driven angiogenesis, but its role in mechanotransduction during sprouting has not been established. Using dominant negative cell transfections in a microfluidic device that recapitulates angiogenic sprouting, we show that endothelial cells respond to interstitial flow in a RhoA-dependent manner while invading into a 3-D extracellular matrix. Furthermore, RhoA regulates flow-induced, but not VEGF gradient-5 induced tip cell filopodial extensions. Thus, RhoA pathways mediate mechanically-activated but not VEGF- induced endothelial morphogenesis.

Introduction

Endothelial cells (ECs) comprising the inner lining of blood vessels are subjected to hemodynamic forces *in vivo*.^{1, 2} Changes in fluid shear stress acting tangential to the luminal surface of the endothelium activate numerous signalling cascades that determine the shape, cytoskeletal organization and function of ECs. These local changes allow blood vessels to adapt to their fluidic environment and enable vascular remodelling.³ One of the key mediators of mechanotransduction in ECs is the small GTPase RhoA, which is crucial for assembly of focal adhesions and stress fibre complexes,⁴ formation of junction-associated cortical actin cytoskeleton for maintenance and stabilization of vascular barrier function,⁵ activation of integrins,⁶ enhancement of migration and traction force generation,⁷ and induction of polarization⁸ and alignment⁹ in response to shear stress.

In addition to its central role in endothelial mechanobiology, RhoA is emerging as an important mediator acting downstream of vascular endothelial growth factor (VEGF) during angiogenesis.^{10, 11} Inhibition of RhoA reportedly impairs VEGF-driven endothelial migration,^{12, 13} formation of precapillary cords *in vitro*,¹⁴ and vascular development *in vivo*.^{14, 15} In general, previous *in vitro* studies of RhoA in ECs have been performed on rigid, impermeable planar surfaces or in 3D gels without predefined, perfused vessels. Unfortunately, solid substrates do not allow cell-matrix remodelling which is essential for endothelial sprouting^{16, 17} or flow transverse to the endothelial monolayer which has been shown to mediate morphogenesis.¹⁸⁻²⁰ Because of these experimental limitations, the mechanisms of flow-induced vessel morphogenesis are poorly understood.

Microscale engineering technology has enabled unprecedented levels of control of the cellular microenvironment in terms of chemical gradients,^{21, 22} fluid flow,^{23, 24} and

localized 3-D extracellular matrices (ECM),²⁵⁻²⁸ all of which can be integrated into a single system to provide a physiologically-relevant context for angiogenesis. Utilizing a microsystems approach, we previously demonstrated that ECs invading into a 3-D ECM in a controlled mechanochemical setting exhibited dramatic differences in morphogenesis depending on the direction of interstitial flow.²⁹ Here we show that RhoA is involved in flow-induced endothelial sprouting morphogenesis into a 3-D ECM. Dominant-negative RhoA ECs respond to local VEGF signals by producing tip cell protrusions or filopodia^{30, 31} toward a VEGF gradient but exhibit impaired invasion and sprouting in response to interstitial flow through a 3-D ECM. These results suggest that RhoA contributes to flow-induced endothelial morphogenesis and that mechanical signals can act through pathways distinct from those used by growth factors.

Material and Methods

Microfluidic device fabrication

The microfluidic device for reproducing sprouting angiogenesis under different fluid flow and VEGF conditions was fabricated as previously described (Figure 1A).²⁹ Briefly, each device consisted of two layers of poly(dimethylsiloxane) (PDMS, Sylgard 184, Dow Corning; mixed 12:1, base:curing agent). The top layer was constructed using soft lithography³² to form a layer with negative relief features ~50 μm in height. The negative relief features were irreversibly sealed against a planar PDMS layer (~2mm thick) via treatment with plasma oxygen (Harrick, Ithaca, NY) for 60 sec, heated to 60°C for 30 min, and then sterilized by exposure to UV light for ~30 min. At least 2 h after plasma oxygen treatment, a mixture of collagen gel (3 mg/ml, rat tail-type I, BD Biosciences, Franklin Lakes, NJ) and fibronectin (10 $\mu\text{g}/\text{ml}$, BD Biosciences) was then introduced into the central channel using vacuum at 4°C to prevent immediate collagen polymerization. The collagen gel solution was kept hydrated and allowed to polymerize at 37°C for 48-72 h. Subsequently, the two flanking channels, which did not contain collagen gel, were coated with a fibronectin (FN) solution (Figure 1, green regions; 10 $\mu\text{g}/\text{ml}$ for 3 h).

HUVEC preparation and seeding

HUVECs were acquired from the Center for Excellence in Vascular Biology, Brigham & Women's Hospital, Harvard Medical School, Boston, MA and maintained in EGM medium (2% FBS, brain bovine extract, heparin, hEGF, and hydrocortisone) (Lonza). HUVECs were transduced with enhanced green fluorescent protein (EGFP) (HUVEC-CTRL) using a previously described protocol.^{33, 34} For the dominant-negative RhoA HUVECs (HUVEC-T19N), the full-length RhoA DN gene was cut out from the pcDNA3-eGFP-RhoAT19N vector purchased from Addgene (plasmid 12967) and cloned into a retroviral pBMN-I-GFP vector backbone.³⁵ HUVECs were then transduced with the RhoT19N retrovirus particles three times. The transduction efficiency was assessed by a RhoA activation pull-down experiment using the Rho Activation Assay Biochem Kit (Cytoskeleton) according to the manufacturer's instructions. The pulled-down proteins and total proteins were analyzed by Western Blot for active RhoA and total RhoA, respectively (Figure 1B).

A concentrated solution of transfected HUVECs (~10⁷ cells/ml) at passage number 5-10 was introduced into the FN coated channels (Figure 1A), allowed to attach on all surfaces of the channels, and grown to confluence (24-48 h after seeding). To induce HUVECs to invade into the collagen gel region, vascular endothelial growth factor (VEGF) (R&D Systems, Minneapolis, MN) was added to EGM media at a concentration of 50 ng/ml.

Control of fluid flow

The described microsystem enables *i*) precise application of physiological levels of shear stress (3 dyn/cm²), *ii*) transendothelial interstitial flow (2.5–35 µm/s) oriented either apical-to-basal (a-b) or basal-to-apical (b-a), and *iii*) specification of VEGF gradient either a-b (invasion away from the VEGF source) or b-a (invasion towards the VEGF source); Figure 1A. Flow of EGM was controlled with a programmable syringe pump (Harvard Apparatus, Holliston, MA) with both positive (or push) and negative (pull) pressure flow capabilities. To introduce flow, clear polypropylene barbed elbow fittings (1/16", Cole-Parmer, Vernon Hills, IL) connected to silicone tubing (Saint-Gobain) were inserted into the inlet and outlet ports of the HUVEC-lined channels. Shear stress levels in the microfluidic channels and interstitial flow across the collagen gel were measured experimentally by tracking fluid displacement over time with 1 µm fluorescent beads.²⁹

The microsystem can be reconfigured to impart different combinations of fluid flow and VEGF gradient conditions. To isolate the effect of interstitial flow without significant shear stress in the HUVEC-lined microchannels, we connected both ports of one of these microchannels to the syringe pump and pulled media (28.5 µm/s) through the intervessel matrix from reservoirs connected to the opposing HUVEC-lined microchannel. In this configuration, the VEGF concentration is uniform.²⁹ To generate stable gradients of VEGF across the collagen gel without interstitial flow, we applied identical flow conditions to both HUVEC-lined channels but only one of the inlets was supplemented with VEGF. With this flow configuration, the slow flow applied minimal shear stress to the endothelium (~0.1 dyn/cm²) to replenish nutrients and maintain a stable biochemical gradient but not inhibit morphogenesis.²⁹ The biochemical gradient profiles for all the described flow conditions were previously characterized experimentally in HUVEC-lined channels using TRITC-BSA (MW 66 kDa) dissolved in cell culture medium as a fluorescent surrogate for VEGF.²⁹ The gradients were stable 30 min after initiation of the flow conditions.

Image acquisition and processing

Phase and corresponding fluorescent images were acquired with an epi-fluorescence microscope (Olympus IX70, PRIOR automated stage, OpenLab software). Images were stitched together with Adobe Photoshop and used to quantify the area of HUVECs invading into the 3-D ECM gel (see below). Confocal fluorescence stacks were acquired with an Olympus BX61WI microscope and 20X water immersion lens (Fluoview software) with 1 µm slice thickness. Projections of these confocal z-stacks were processed with either Volocity (PerkinElmer, Waltham, MA) or ImageJ (NIH, Bethesda, MA) software and used to quantify filopodia formation (see below). Confocal z-stacks were captured at multiple time points using an inverted confocal microscope (Olympus IX81, PRIOR automated stage) fitted with an on-stage cell culture incubator system (Tokai).

Quantification of Cellular Alignment and Elongation

Changes in cell morphology in response to shear stress were quantified using angles of orientation and Shape Indices (SI).³⁶ Briefly, the angle of orientation is defined as the angle between the cell's major axis and the direction of flow where 0° is a cell aligned perfectly in the direction of flow and 90° is a cell aligned orthogonal to the direction of flow. The angle of orientation of a population of randomly oriented cells is ~45°. The SI is a dimensionless measure of the elongation of a cell defined as:

$$SI = \frac{4\pi A}{P^2}$$

where A is the area of the cell and P is the perimeter of the cell. The SI ranges from 0 to 1 where 0 is a straight line and 1 is a perfect circle. Cells were segmented manually and measured using ImageJ.

Immunofluorescence

HUVECs in the microchannels were washed with Phosphate Buffered Saline (PBS), fixed with 3% formaldehyde for 30 min, washed three times with PBS and blocked with 5% donkey serum/0.1% Triton in PBS for 60 min. Actin filaments were labelled with phalloidin conjugated with Alexa Fluor 546 (Molecular Probes) for 30 min. Cell nuclei were stained with DAPI nuclear stain (Invitrogen, 1:200 dilution) and washed three times with PBS prior to confocal microscopy.

Area of invasion and filopodia quantification

The normalized area of invasion of HUVECs into the collagen gel was determined by calculating the difference between the projected area of HUVECs in the collagen gel at $t = n$ (A_n) and the area at $t = 0$ (A_0); this difference in area was then normalized by the area of the collagen region (A_c) and expressed as a percentage. Projections from z-resolved confocal stacks were used to analyze filopodia formation by sprouting HUVECs following a previously described protocol.³⁷ Images were anonymized and analyzed by two independent reviewers, then averaged. Only filopodia from sprouting HUVECs that were directed toward the opposite side with acute angles (not rounded) were counted. Cells that were surrounded by collagen gel, and not connected to either side were excluded. Filopodia counts were normalized to the HUVEC sprouting area. Data for the area of invasion and filopodia counts were obtained from devices with both HUVEC channels lined with cells of the same type.

Statistical Analysis

Sample populations were compared using either student t-test or ANOVA. T-test was used for simple comparison of two populations (i.e. a single value). ANOVA was used for comparison of two or more populations over a course of multiple days. $p < 0.05$ was the threshold for statistical significance (indicated on graphs with a “*”). “**” denoted $p < 0.001$; “***” denoted $p < 0.0001$. ‘ns’ denoted $p > 0.05$. Data points on the graphs represent mean values and error bars depict standard error (s.e.m.).

Results

Imaging temporal dynamics of sprout morphogenesis *in vitro*

To capture the dynamics of endothelial extension into the 3-D collagen gel, we utilized high-resolution confocal timelapse microscopy. Figure 2 shows two nearby vessel segments comprised of HUVEC-CTRL cells extending within a VEGF gradient. Both of the segments project filopodia toward the VEGF source and against b-a interstitial flow, while one segment initiates vessel branching (Figure 2, Supplementary Movie 1[†]). This behaviour of rapid extension and filopodia projection is analogous to the vessel dynamics seen in the zebrafish model³⁸ or in the mouse retina^{31, 37} during the development of blood vessel networks.

RhoA-mediated behaviour of HUVECs in response to shear stress

Expression of active RhoA-GTP in HUVEC-CTRL cells was greatest 5 min after VEGF application and remained high at 24 h (Figure 1B) which is consistent with previously

[†]Electronic Supplementary Information (ESI) available: Video of sprout extension and branching *in vitro*. See DOI: 10.1039/b000000x/

reported results.³⁹ In comparison, RhoA-GTP expression in HUVEC-T19N cells treated with VEGF was highly attenuated (Figure 1B).

We next assessed morphological changes in control and RhoA dominant negative cells exposed to shear stress. ECs in a 2-D confluent monolayer are known to orient with the direction of flow in these conditions.⁴⁰ After reconfiguring the device to impart physiological shear stress in the microfluidic vessel lumen (Figure 1A, 3 dyn/cm²), HUVEC-T19N cells were less able to align and elongate in the direction of flow compared to HUVECCTRL cells (Figures 3A-D, $p < 0.0001$), in accordance with previously reported results.⁶ Little invasion into the 3-D ECM was seen for either cell type when exposed to this level of shear stress (3 dyn/cm²), regardless of VEGF or interstitial flow conditions (Figure 4, red bars). This inhibition of EC invasion by normal levels of shear is consistent with previous results²⁹ and suggests that shear can be a stabilizing signal for blood vessels. Thus, it is interesting that RhoA attenuation interfered with the ability of the cells to align with flow, but still allowed shear-induced attenuation of 3-D invasion.

We stained the actin cytoskeleton with phalloidin to further assess the morphological response of HUVECs lining the microchannels in response to different VEGF and shear stress conditions. Previous results from static 2-D cultures have shown that VEGF promotes mobilization of cortical actin away from endothelial cell junctions.¹³ Our HUVEC-CTRL cells exhibited this response upon stimulation with VEGF (50 ng/ml) for 48h (Figure 3E). With application of fluid shear (3 dyn/cm²), the HUVEC-CTRL cells formed stress fibres that aligned with the direction of flow regardless of VEGF stimulation (Figure 3E). In contrast, the actin fibres of the HUVEC-T19N cells did not reorganize in response to shear stress, but remained prominent at the periphery of cells. Collectively, these results support that RhoA is crucial for mediating actin reorganization and cellular remodelling in response to shear stress.

RhoA is involved in interstitial flow-guided endothelial invasion

Interstitial flow is known to augment 3-D endothelial morphogenesis,^{18, 19, 29} so we assessed the role of RhoA in interstitial flow-guided invasion of HUVECs. In the absence of fluid flow, uniform VEGF stimulation induced invasion of HUVEC-CTRL and HUVEC-T19N cells into the 3-D collagen gel to comparable levels (Figure 4, black/white bars, $p = 0.56$). Application of a VEGF gradient oriented either a-b (cells invading away from the VEGF source) or b-a (invasion toward the VEGF source) (Figure 1A), in the absence of interstitial flow, and at negligible shear stress levels in the microfluidic vessel lumen (~ 0.1 dyn/cm²) triggered comparable levels of invasion of HUVEC-CTRL and HUVEC-T19N cells (Figure 4, gray bars, $p > 0.10$).

Application of either a-b or b-a interstitial flow in a uniform VEGF field resulted in more invasion by HUVEC-CTRL compared with HUVEC-T19N cells (Figure 4, blue bars, $p < 0.037$). This difference was maintained when either an a-b or b-a VEGF gradient was co-administered with interstitial flow (Figure 4, orange bars, $p < 0.015$). There were no differences in invasion for HUVEC-T19N cells in response to interstitial flow alone compared to interstitial flow combined with a VEGF gradient ($p > 0.13$). These results show that inhibiting RhoA impairs the morphogenic invasion of HUVECs in response to interstitial flow.

RhoA selectively regulates flow-induced but not VEGF gradient-induced filopodia formation

In our system, we see two modes of invasion into the gel: lateral migration of the vessel wall without formation of filopodial projections, which is analogous to vessel dilation, and

migration of tip cells that extend filopodia, which is equivalent to vessel sprouting.²⁹ Next, we specifically assessed the role of RhoA in endothelial sprouting initiated by VEGF and fluid forces. In the absence of interstitial flow, both HUVEC-CTRL and HUVECT19N cells entering the 3-D collagen gel extended significantly more tip cell processes or filopodia^{30, 31} up the (b-a) VEGF gradient versus down the (a-b) gradient (Figure 5A, E, $p < 0.02$). Furthermore, both HUVEC-CTRL and HUVEC-T19N cells prominently extended tip cells when invading up the (b-a) VEGF gradient and against (b-a) interstitial flow (Figure 5B, F). Conversely, few tip cell extensions were produced by either cell type when invading down the (a-b) VEGF gradient and with (a-b) interstitial flow; under these conditions, the vessel analogues migrated into the gel as a coordinated sheet, as previously described²⁹ (Figure 6C, F, $p < 0.033$).

When isolating the effects of interstitial flow under uniform VEGF concentration (no gradient),²⁹ invading HUVEC-T19N cells produced fewer filopodia compared to HUVEC-CTRL cells for both interstitial flow directions (Figure 5D, G, $p < 0.033$). Furthermore, HUVEC-CTRL cells extended more filopodia when extending against interstitial flow compared to with the flow (193 ± 34 versus 103 ± 26 respectively, $p = 0.038$), but HUVECT19N cells extended filopodia to the same extent, regardless of flow direction (58 ± 15 against b-a interstitial flow versus 34 ± 15 with a-b interstitial flow, $p = 0.25$).

Discussion

ECs in blood vessels serve as the interface for hemodynamic forces to influence vascular tone, remodelling and ultimately the efficient delivery of blood, oxygen, and nutrients.³ RhoA has been shown to be a key regulator of shear-induced remodelling of EC morphology.^{6, 7, 9} For instance, in the 2-D *in vitro* setting, it was demonstrated that RhoA is crucial for laminar shear stress induced cell polarization⁸ which is a pre-requisite for alignment of ECs with the direction of flow. Yet, it is difficult to establish a direct link with results from the 2-D setting to understand how RhoA orchestrates endothelial sprouting and morphogenesis in 3-D.

Using our microfluidic model of angiogenesis,²⁹ we previously demonstrated that ECs invading into a 3-D ECM exhibit dramatic differences in morphogenesis based on the direction of interstitial flow.²⁹ Moreover, recent work has shown that the direction of interstitial flow can elicit differential responses in the remodelling of endothelial adherens junctions and the actin cytoskeleton.⁴¹ These results suggest that, similar to laminar shear stress in 2-D, interstitial flow can induce polarization of sprouting ECs in 3-D, as evidence by filopodia projections. Here we show that RhoA is not necessary for VEGF-driven 3-D morphogenesis, but is involved in the morphogenic response to interstitial flow. Dominant-negative RhoA ECs responded to local VEGF signals by producing filopodia towards a VEGF gradient (Figure 5) but were impaired in their ability to invade and sprout in response to interstitial flow through a 3-D extracellular matrix (ECM) (Figure 4). Consistent with the conclusion that these cells had a defective shear-sensing mechanism, the RhoA dominant negative cells were unable to align with flow in the channels (Figure 3). However, their invasion into the gel was still inhibited by shear (Figure 4), suggesting that some mechanosensing pathway(s) remained active. Thus, it is likely that independent mechanosensing pathways exist, for example at cell-cell junctions and cell-substrate adhesions, only some of which are RhoA-dependent.

Given its critical role in cytoskeletal organization and dynamics, RhoA has been proposed as a target for modulating angiogenesis.⁴² Our results show that the function of RhoA during angiogenesis is dependent on the context of signals present in the microenvironment and suggest that RhoA is specifically critical for flow-guided neovascularization but not VEGF

gradient- driven vessel guidance. An important outstanding question is how RhoA ties into the mechanotransduction machinery to participate in the morphogenesis. A better understanding of how ECs integrate signals from fluid forces and local gradients to coordinate sprouting should lead to more effective therapeutic strategies for targeting pathological angiogenesis.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This study was supported by grants from the National Institutes of Health: R01CA149285 (LLM) and T32CA073479.

Notes and references

1. Davies PF. Physiological Reviews. 1995; 75:519–560. [PubMed: 7624393]
2. Stapor P, Wang W, Murfee W, Khismatullin D. Cardiovascular Engineering and Technology. 2011; 2:124–136.
3. Tzima E. Circ.Res. 2006; 98:176–185. [PubMed: 16456110]
4. Ridley AJ, Paterson HF, Johnston CL, Diekmann D, Hall A. Cell. 1992; 70:401–410. [PubMed: 1643658]
5. Spindler V, Schlegel N, Waschke J. Cardiovascular research. 2010; 87:243–253. [PubMed: 20299335]
6. Tzima E, del Pozo MA, Shattil SJ, Chien S, Schwartz MA. Embo J. 2001; 20:4639–4647. [PubMed: 11532928]
7. Shiu Y-T, Li S, Marganski WA, Usami S, Schwartz MA, Wang Y-L, Dembo M, Chien S. Biophysical journal. 2004; 86:2558–2565. [PubMed: 15041692]
8. Wojciak-Stothard B, Ridley AJ. The Journal of Cell Biology. 2003; 161:429–439. [PubMed: 12719476]
9. Li S, Chen BP, Azuma N, Hu YL, Wu SZ, Sumpio BE, Shyy JY, Chien S. The Journal of clinical investigation. 1999; 103:1141–1150. [PubMed: 10207166]
10. Bryan BA, D'Amore PA. Cell Mol Life Sci. 2007; 64:2053–2065. [PubMed: 17530172]
11. Mammoto A, Mammoto T, Ingber DE. Curr Opin Hematol. 2008; 15:228–234. [PubMed: 18391790]
12. Bryan BA, Dennstedt E, Mitchell DC, Walshe TE, Noma K, Loureiro R, Saint-Geniez M, Campaigniac J-P, Liao JK, D'Amore PA. The FASEB Journal. 2010; 24:3186–3195.
13. van Nieuw Amerongen GP, Koolwijk P, Versteilen A, van Hinsbergh VWM. Arteriosclerosis, thrombosis, and vascular biology. 2003; 23:211–217.
14. Hoang MV, Whelan MC, Senger DR. Proceedings of the National Academy of Sciences of the United States of America. 2004; 101:1874–1879. [PubMed: 14769914]
15. Park H-J, Kong D, Iruela-Arispe L, Begley U, Tang D, Galper JB. Circ.Res. 2002; 91:143–150. [PubMed: 12142347]
16. Davis GE, Senger DR. Circ.Res. 2005; 97:1093–1107. [PubMed: 16306453]
17. van Hinsbergh VWM, Koolwijk P. Cardiovascular research. 2008; 78:203–212. [PubMed: 18079100]
18. Helm CL, Fleury ME, Zisch AH, Boschetti F, Swartz MA. Proceedings of the National Academy of Sciences of the United States of America. 2005; 102:15779–15784. [PubMed: 16249343]
19. Hernandez Vera R, Genove E, Alvarez L, Borros S, Kamm R, Lauffenburger D, Semino CE. Tissue engineering. 2009; 15:175–185. [PubMed: 18636940]
20. Tada S, Tarbell JM. American Journal of Physiology - Heart and Circulatory Physiology. 2000; 278:H1589–H1597. [PubMed: 10775138]

21. Mosadegh B, Huang C, Park JW, Shin HS, Chung BG, Hwang SK, Lee KH, Kim HJ, Brody J, Jeon NL. *Langmuir*. 2007; 23:10910–10912. [PubMed: 17910490]
22. Shamloo A, Heilshorn SC. *Lab on a chip*. 2010; 10:3061–3068. [PubMed: 20820484]
23. Gu W, Zhu X, Futai N, Cho BS, Takayama S. *Proceedings of the National Academy of Sciences of the United States of America*. 2004; 101:15861–15866. [PubMed: 15514025]
24. Unger MA, Chou H-P, Thorsen T, Scherer A, Quake SR. *Science*. 2000; 288:113–116. [PubMed: 10753110]
25. Chaw KC, Manimaran M, Tay FE, Swaminathan S. *Biomedical microdevices*. 2007; 9:597–602. [PubMed: 17505887]
26. Huang CP, Lu J, Seon H, Lee AP, Flanagan LA, Kim H-Y, Putnam AJ, Jeon NL. *Lab on a chip*. 2009; 9:1740–1748. [PubMed: 19495458]
27. Song JW, Bazou D, Munn LL. *Integrative Biology*. 2012
28. Vickerman V, Blundo J, Chung S, Kamm R. *Lab on a chip*. 2008; 8:1468–1477. [PubMed: 18818801]
29. Song JW, Munn LL. *Proceedings of the National Academy of Sciences of the United States of America*. 2011; 108:15342–15347. [PubMed: 21876168]
30. De Smet F, Segura I, De Bock K, Hohensinner PJ, Carmeliet P. *Arteriosclerosis, thrombosis, and vascular biology*. 2009; 29:639–649.
31. Gerhardt H, Golding M, Fruttiger M, Ruhrberg C, Lundkvist A, Abramsson A, Jeltsch M, Mitchell C, Alitalo K, Shima D, Betsholtz C. *The Journal of Cell Biology*. 2003; 161:1163–1177. [PubMed: 12810700]
32. Duffy DC, McDonald JC, Schueller OJA, Whitesides GM. *Analytical chemistry*. 1998; 70:4974–4984. [PubMed: 21644679]
33. Au P, Tam J, Fukumura D, Jain RK. *Blood*. 2008; 111:4551–4558. [PubMed: 18256324]
34. Schechner JS, Nath AK, Zheng L, Kluger MS, Hughes CCW, Sierra-Honigmann MR, Lorber MI, Tellides G, Kashgarian M, Bothwell ALM, Pober JS. *Proceedings of the National Academy of Sciences of the United States of America*. 2000; 97:9191–9196. [PubMed: 10890921]
35. Tse JM, Cheng G, Tyrrell JA, Wilcox-Adelman SA, Boucher Y, Jain RK, Munn LL. *Proceedings of the National Academy of Sciences of the United States of America*. 2012; 109:911–916. [PubMed: 22203958]
36. Song JW, Gu W, Futai N, Warner KA, Nor JE, Takayama S. *Analytical chemistry*. 2005; 77:3993–3999. [PubMed: 15987102]
37. Suchting S, Freitas C, le Noble F, Benedito R, Brant C, Duarte A, Eichmann A. *Proceedings of the National Academy of Sciences*. 2007; 104:3225–3230.
38. Isogai S, Lawson ND, Torrealday S, Horiguchi M, Weinstein BM. *Development (Cambridge, England)*. 2003; 130:5281–5290.
39. Beckers CM, van Hinsbergh VW, van Nieuw Amerongen GP. *Thromb Haemost*. 2010; 103:40–55. [PubMed: 20062930]
40. Levesque MJ, Nerem RM. *Journal of biomechanical engineering*. 1985; 107:341–347. [PubMed: 4079361]
41. Vickerman V, Kamm RD. *Integrative biology : quantitative biosciences from nano to macro*. 2012
42. van der Meel R, Symons MH, Kudernatsch R, Kok RJ, Schiffelers RM, Storm G, Gallagher WM, Byrne AT. *Drug discovery today*. 2011; 16:219–228. [PubMed: 21262381]

Figure 1.

A, Microsystem with multiple apertures allowing endothelial invasion into a central collagen gel. Expanded view depicts a single aperture where adjacent HUVECs are exposed to combinations of shear stress (solid arrows), transendothelial interstitial flow (dashed arrow), and VEGF gradients (blue gradient bar). Interstitial flow can be applied to the endothelial cells lining the collagen with orientation either apical-to-basal (a-b; flow leaving the vessel) or basal-to-apical (b-a; flow entering the vessel). **B**, Western blot showing RhoA activation pull-down of HUVECs transduced with either T19N RhoA dominant negative vector (T19N) or GFP only vector (CTRL) and in response to VEGF stimulation (50 ng/ml). LPA, a strong inducer of RhoA-GTP, served as a positive control.

Figure 2.

Dynamics of sprout extension and branching *in vitro*. Time-lapse images of confocal projections of HUVECs sprouting in the 3-D matrix towards a VEGF source, in the presence of b-a interstitial flow. The vessel segment on the left extends (purple arrow) from the purple dot reference point with filopodia projections at the leading edge. From the vessel segment on the right (green diamond reference point), branching is initiated at the 0:42 timepoint (green arrowhead) and fully formed at the 1:03 time point. Subsequently, one of these branches extends more rapidly (green arrow). The elapsed time from the first frame of each successive image is shown in the format hours:minutes. Dashed arrow indicates direction of interstitial flow. Gradient blue bar indicates VEGF gradient in the collagen gel. See Supplementary Movie 1[†] for the complete time-lapse sequence.

Figure 3.

Morphological response to 2-D fluid shear stress. **A, B** HUVEC-CTRL cells align with fluid shear. Quantification showing alignment (angle of orientation; **A**) and elongation (Shape index; **B**) of HUVEC-CTRL cells exposed to flow for 48h. ***, $p < 0.0001$, $n = 293-421$ cells per condition. **C, D** HUVEC-T19N cells do not align with fluid shear. The angle of orientation (**C**) and Shape index (**D**) are not affected in HUVEC-T19N cells exposed to flow. ***, $p < 0.0001$, $n = 281-294$ cells per condition. **E**, Confocal projections of actin filaments stained with phalloidin (red) in HUVECs lining the microchannels; the cells were exposed to VEGF (50 ng/ml) and/or fluid shear (3 dyn/cm²). Images were recorded 48h after initiation of experiments. Cell nuclei were labelled with DAPI (blue). Scale bar: 50 μm.

Figure 4.

Normalized area of invasion in the 3-D collagen gel of HUVEC-CTRL and HUVEC-T19N cells. Uniform VEGF or a VEGF gradient triggers invasion of HUVEC-CTRL (black bars) and HUVEC-T19N cells (uniform VEGF: black/white bars; VEGF gradient: gray bars) but is significantly attenuated by application of shear stress of 3 dyn/cm² (HUVEC-T19N, red bars). In the absence of shear stress, interstitial flow induced significantly more invasion by HUVEC-CTRL compared with HUVEC-T19N cells (blue bars) for both b-a and a-b flow directions. This difference was maintained when either an a-b or b-a VEGF gradient was co-administered with the interstitial flow (HUVEC-T19N, orange bars). n = 21-49 per day per condition. The statistical bars indicate comparisons between HUVEC-CTRL and HUVEC-T19N cells over the timecourse using ANOVA. ns, p > 0.08, *, p < 0.05, **, p = 0.001.

Figure 5. RhoA mediates filopodia formation initiated by interstitial flow but not VEGF gradients. **A** Both HUVEC-CTRL and HUVEC-T19N cells preferentially extend filopodia in response to a VEGF gradient oriented b-a versus a-b. **B**, Both HUVEC-CTRL and HUVEC-T19N cells prominently extend filopodia when invading up the b-a VEGF gradient and against b-a interstitial flow (2.5 $\mu\text{m/s}$). **C**, In contrast to **(B)**, HUVEC-CTRL and HUVEC-T19N cells migrate as a coordinated sheet down the a-b VEGF gradient and with a-b interstitial flow (35 $\mu\text{m/s}$). **C**, HUVEC-CTRL and HUVEC-T19N cells exhibit differential responses to isolated effects of interstitial flow (28.5 $\mu\text{m/s}$) in the absence of a VEGF gradient. **E-G**, Quantification of the number of filopodia per sprouting area produced by the HUVEC-CTRL and HUVEC-T19N cells **(E)** in the presence of a VEGF gradient only, **(F)** with a VEGF gradient co-administered with interstitial flow, and **(G)** with interstitial flow under uniform VEGF concentrations. Dashed arrows indicate direction of interstitial flow.

Gradient blue and solid blue VEGF bars indicate VEGF gradient and uniform VEGF concentration, respectively, in the collagen gel. . Scale bars: 100 μm . n = 7-36. ns, p > 0.12, *, p < 0.038, **, p < 0.0033, ***, p < 0.0002. For **B**, **C**, and **F**, the flow configuration required shear flow (3 dyn/cm²) in one of the HUVEC channels and static conditions in the other channel. Data for filopodia counts were obtained only from the static side which was permissive for gel invasion (Figure 4).