Electronic Supplementary Information

Cell Culture

Human umbilical vein endothelial cells (HUVECs) were isolated as described previously (12), cultured in fully supplemented endothelial growth medium (EGM-2; Lonza, Walkersville, MD) at 37°C, 5% CO_2 and used at passage 2. Normal human lung fibroblasts (NHLFs; ATCC, Manassas, VA) were cultured in Media 199 (M199; Invitrogen Corporation, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS; Mediatech, Herndon, VA), 1% penicillin/streptomycin (Mediatech), and 0.5% gentamicin (Invitrogen) at 37°C, 5% CO_2, and used up to passage 10. Medium was changed three times per week and cells were harvested at 80% confluency via trypsin-EDTA (Mediatech) treatment.

Fibrin Tissue Assembly

Fibrin-based tissue constructs were assembled as previously described (14). Briefly, Cytodex^{TM}-3 microcarrier beads (Sigma-Aldrich, St. Louis, MO) (diameter of 150 μm) were sterilized and prepared for seeding by autoclaving in a solution of Dulbecco's phosphate buffered saline (PBS; Invitrogen Corporation) followed by a series of four washes in EGM-2. Four million HUVECs were then added to an inverted T-25 culture flask containing 5 ml of EGM-2 and approximately 10,000 Cytodex beads. The inverted flask was then incubated (37°C, 5% CO_2) for four hours, and was gently agitated every 30 minutes during that time period. After four hours, the microcarrier bead solution was transferred to a new T-25 flask along with 5 ml of fresh EGM-2. The flask was then incubated in the standard cell culture position for 24 hours, allowing any suspended cells to attach to the beads at the bottom of the flask. After the 24-hour period, fibrinogen solutions of varying concentrations ranging from 1.0 to 10 mg/ml were prepared in EGM-2 without FBS and sterile filtered. To assemble tissue constructs, HUVEC-coated beads (~25 beads/0.5 ml fibrinogen solution) and 5% FBS were added to the fibrinogen solution. For angiogenic sprouting quantification, tissues were cultured in 24 well plates, where 10 μl of a thrombin (Sigma-Aldrich) solution (50 U/ml) and 0.5 ml of the fibrinogen-bead solution were added to each well and thoroughly mixed. For high resolution imaging, tissues were assembled in 8 well chamber slides (Lab-Tek, Rochester, NY), where 6 μl of thrombin solution and 0.3 ml of the fibrinogen-bead solution were mixed. Tissues were allowed to stand for five minutes before incubating for 20 minutes at the previously stated conditions. After the fibrin crosslinked to form a hydrogel, NHLFs were plated (25,000 cells/well in 24 well plates, 14,000 cells/well in 8 well chamber slides) on top of the fibrin gel. Tissues were then cultured in fully supplemented EGM-2 with the media changed three times per week.

Spatio-Temporal Image Correlation Spectroscopy Analysis

The STICS analysis technique used in this study is a modified version of the original method first described by Hebert *et al.* (20). The key STICS calculation is defined by the following equation:

$$G_{STICS}(\xi,\psi,\Delta T) = \left\langle \frac{\langle \delta i_a(x,y,t)\delta i_b(x+\xi,y+\psi,t+\Delta T)\rangle_{x,y}}{\langle i_a(x,y,t)\rangle_{x,y}\langle i_b(x,y,t+\tau)\rangle_{x,y}} \right\rangle_t ,$$

where i_a and i_b are the intensities at each pixel of images a and b respectively, x and y are the horizontal and vertical spatial coordinates of the images, ξ and ψ are the x and y spatial correlation shifts, t is the frame number and ΔT is the shift between frames. Finally,

$\delta(\ldots) = i - \langle i \rangle$ and $\langle \quad \rangle_{x,y}$ is the average intensity of the image. To improve data processing speed, STICS correlation function is calculated using the Fast Fourier Transform.

For G calculated between two successive images of flowing particles or objects, there will be a peak in the spatial correlation function at a spatial shift equal to the displacement caused by flow. The peak value of the resulting STICS correlation plot is shifted from the center in the direction of flow by an amount that relates to the speed at which the object of interest is traveling. Average velocity can then be extracted by using the following equation:

$$v = \frac{ObservedPixelShift\ x\ Pixel\ Width}{\left(\frac{1}{frameRate}\right)\Delta T},$$

More precise numerical information is extracted from STICS analysis by fitting the experimental STICS plot to a 2-dimensional Gaussian:

$$z = Ae^{-\left[\frac{(x-\mu_x)^2}{2\sigma_x^2}+\frac{(y-\mu_y)^2}{2\sigma_y^2}\right]},$$

where A is the amplitude, σ_x and σ_y are the standard deviation of the Gaussian in the x and y direction respectively and are related to the rate of diffusion. The variables μ_x and μ_y give the displacement of the peak in the x and y direction respectively at each ΔT. The observed pixel shift from average velocity equation can be obtained from a 2-dimensional Gaussian relationship by using the following equation:

$$Observed\ Pixel\ Shift = \sqrt{\mu_x^2 + \mu_y^2},$$

The direction of flow can be calculated by using:

$$\theta = tan^{-1}\frac{\mu_y}{\mu_x},$$

where θ is the angle between the x axis and the direction of flow.

All of the above calculations were performed using SimFCS software from the Laboratory of Fluorescence Dynamics.

G-LISA Sample Preparation

A RhoA G-LISA assay (Cytoskeleton, Denver, CO) was performed using HUVEC lysates to confirm the expected up- or down-regulation of RhoA activity induced by expressing mutant forms of RhoA via adenoviral gene delivery (Fig. S1). Given that our 3D angiogenesis model is a co-culture, a specialized procedure was developed to separate the two cell types and isolate the HUVECs for the G-LISA assay. A 24 well plate's worth of tissues was used for each condition and time point. Gels were twice washed with phosphate buffered saline (PBS) before adding 100 µl of concentrated trypsin solution (2.5%; Sigma-Aldrich) for 30-60 seconds to remove the monolayer of NHLFs. Trypsin was neutralized with 200 µl of M199 containing 20% FBS, 1% penicillin-streptomycin per well and the resulting solution aspirated off the top of the gels. To isolate HUVECs from NHLF-free tissues, gels were again washed with PBS, dislodged from their wells with a spatula and subsequently dissolved by addition of 500 µl of trypsin solution (2.5%) and incubation at 37ºC for 20 minutes with periodic agitation. Once the fibrin

clots were dissolved, 1 ml of M199 containing 20% FBS, 1% penicillin-streptomycin was added per well to neutralize trypsin. The contents of each well were pooled together, then beads allowed to settle, and the supernatant extracted and centrifuged to collect HUVECs. Resulting cell pellets were suspended in RNA lysis buffer (Qiagen, Valencia, CA) before analysis with the G-LISA kit as specified by the manufacturer.

Supplementary Figure Captions

Movie S1: Representative videos of 2.5 mg/ml fibrin tissues containing HUVECs expressing A) V14-RhoA, B) N19-RhoA, or C) GFP. Each video contains 250 images, each 15 seconds apart; image size is 105 x 105 μm.

Figure S1: G-LISA results to quantify RhoA activity within 3D constructs in A) 2.5 mg/ml, B) 5.0 mg/ml, and C) 10 mg/ml fibrin tissues at all time points. HUVECs were induced to express mutant forms of RhoA via adenoviral gene delivery. N=3, error bars represent standard deviations, asterisks indicate statistical significance between associated control and test sample, such that $p < 0.05$.

Figure S2: STICS analysis of average matrix velocities surrounding vessel tips in cultures treated with various inhibitors of actomyosin contractility in A) 2.5 mg/ml, B) 5.0 mg/ml, or C) 10 mg/ml 3D fibrin tissues at all time points. N=10, error bars represent standard deviations, asterisks indicate statistical significance between associated control and test sample, such that $p < 0.05$.

Figure S3: ECM velocity as a function of vessel proximity. A) Analysis diagram indicating partition of each data set into 4 fragments for which average velocities were obtained alongside the vessel and B) surrounding the vessel tip; image size 211 x 211 μm. Average velocity data for 2.5 mg/ml fibrin C) alongside the vessel, and D) surrounding the vessel tip. N=3; error is represented as standard deviation. In panel C), * indicates statistical significance ($p < 0.05$) between fragment 1 and each test sample. In panel D), * indicates statistical significance ($p < 0.05$) between fragment 4 and each test sample.

Figure S1:

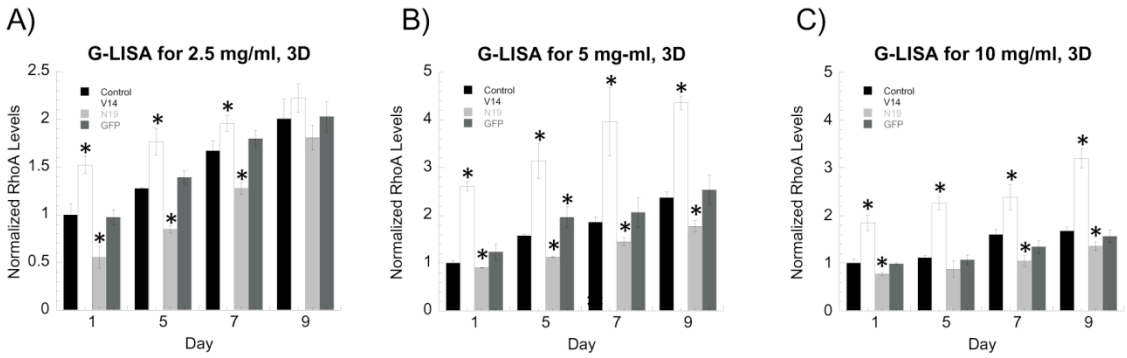

Figure S2:

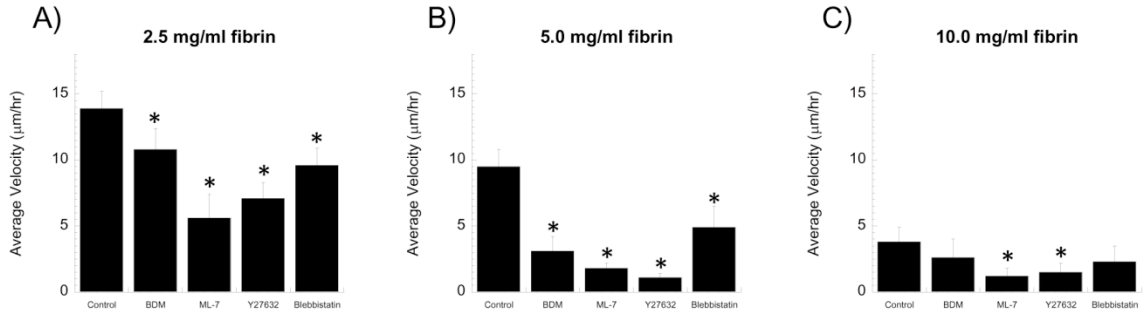

Figure S3: