Supplementary Information for

Pericytes enable effective angiogenesis in the presence of pro-inflammatory signals

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Supplementary Materials and Methods

Cell culture

Human umbilical vein endothelial cells (HUVECs) were purchased from Yale Vascular Biology Center and cultured in M199 (Gibco) supplemented with 20% FBS (Life Technologies), 1% HEPES (Thermo Scientific), 1% Glutamax (Thermo Fisher), 1% antibiotic-antimycotic (Thermo Fisher), Heparin (25mg/500ml, Sigma Aldrich), and endothelial cell growth supplement (Sigma Aldrich). Human pericytes (PCs) were kindly provide by Dr. Anjelica L. Gonzalez (Yale University, CT, USA). PCs were isolated by explant outgrown from microvessels from de-identified human placenta, characterized using previously established methods(1), and cultured in M199 supplemented with 10% FBS, 1% antibiotic-antimycotic. As angiogenic and inflammatory factor, VEGF (100ng/ml, Gibco) and TNF (100ng/ml, Gibco) were used, respectively. For inducing prolonged and matured vessel formation in 3D angiogenesis model, 40ng/ml of basic fibroblast growth factor (bFGF, Thermo Fisher), 500nM of Sphingosine-1-phosphate (S1P, Sigma Aldrich) and 75ng/ml of phorbol myristate acetate (PMA, Sigma Aldrich) were mixed along with 100ng/ml of VEGF.

Biomimetic 3D angiogenesis model fabrication

The biomimetic 3D angiogenesis model consists of a PDMS chamber, an engineered blood vessel embedded in collagen gel and a cover slip and they were assembled without external jigs. All templates for PDMS and collagen casting were designed by SolidWorks and the cad files were converted to gcode for a commercialized 3D printer (Ultimaker) through Cura (Ultimaker). PLA filament was used for building structures. The fabrication steps are depicted in *SI Appendix*, Fig. S1 and the details are as follows. A single line mold (D: 200 -250 um, L: 10 mm) which has a

semicircular cross-section is deposited on a petri dish. Then the surface of the mold is covered with 3 mg/ml collagen solution (Corning) and the remnant is aspirated gently by pipetting. The collagen is cross-linked in EDC (1-Ethyl-3-(3-dimethylaminopropyl)-carbodiimide)/NHS (N-hydroxysulfoxuccinimide) solution in ethanol (10mg/ml) at room temperature for 1min then the mold is washed with DI water. The same process is repeated three times and the mold is washed with DI water three times at the end. PDMS reservoir is put on the mold and 30 μl of pericytes suspension (6×10^5 cells/ml) is placed in the reservoir. The pericytes seeded mold is incubated at 37 ℃ for 5 hours. For 3D angiogenesis model without pericytes, this process can be skipped. Two holes for channel inlet and outlet, and two holes for collagen injection are drilled with a biopsy punch on the PDMS chamber. And a larger hole with 3 mm in diameter is drilled at the center of the PDMS chamber and the piece was re-plugged into the hole. The PDMS chamber and coverslip are pre-treated with 0.1% (v/v) poly(ethylenimine) (PEI) for 30 min, 1% glutaraldehyde for 1hr. After taking off the reservoir, PDMS chamber is put on the mold. Pre-mixed collagen solution (5mg/ml, Type 1 collagen, BD) according to manufacturer's protocol is injected through the hole (indicated with a red arrow in *SI Appendix*, Fig. S1) and collagen polymerization is induced on ice for 30 min and at 37 ℃ for 1.5 hours. The single line mold is carefully peeled off, then the bottom side of PDMS chamber containing collagen gel is sealed with a glass coverslip. Finally, endothelial cell suspension (1×10^7cells /ml) is injected through the inlet hole (indicated with a red arrow in *SI Appendix*, Fig. S1), then the chip is flipped upside down and incubated at 37 ℃ for 1 hour to allow cells to attach on the lumen. In contrast to the methods proposed in previous works(2-4), this method allows more controlled endothelial cell-pericyte layering. Once HUVECs form a confluent monolayer on the channel surface, fresh medium is injected through the inlet hole and changed every 6 hours before the treatment with pro- or anti-angiogenic factors. The engineered

endothelium was stabilized for 1 day prior to further characterization including the permeability analysis. The re-plugged 3 mm hole at the center of the PDMS chamber is open by taking out the PDMS piece carefully. VEGF is applied on the hole to induce a gradient from the top to the endothelium channel and TNF is directly injected through the endothelium channel as described in Fig. 1C.

Evaluation of 3D Vessel Permeability

The permeability of the engineered endothelium with or without pericytes was measured by introducing a solution of 70 kDa FITC-Dextran (Sigma Aldrich, 30 μg/ml in the cell growth medium) into the parental vessel (based on the prefabricated semi-cylindrical channel). The leakage of Dextran from the vessel into the surrounding collagen was imaged at the point of introduction of the FITC-Dextran solution into the engineered vessel, as well as at 5 min and 10 min afterwards with 20× water-immersion objective attached to Leica scanning disk. The permeability coefficient (P) was calculated as described previously(5).

Co-culture of endothelial cells and pericytes

Endothelial cells and pericytes were cultured on transwell inserts for 6-well plates as described in Fig. 3A. To co-culture endothelial cells and pericytes in a layered configuration, both sides of the insert membrane (Corning), which has 24mm diameter, 0.4 μm pore size, and 10 um thickness, were coated with laminin (10 μg/ml). Pericytes were first seeded onto the bottom side of the membrane and allowed to adhere for 2 hours. Then the transwell insert was placed in a 6-well plate and endothelial cells were seeded on the top side of the membrane. After overnight incubation, TNF and VEGF were added on both apical and basal sides.

Sandwich culture

A 24-well plate was coated with 0.2 ml of collagen mixture (5mg/ml) for each well and incubated for 1 hour at 37 ℃ to form a collagen gel. After gelation, endothelial cells with or without pericytes were seeded on the gel and incubated at 37 ℃ overnight. The total cell number on a gel was set to be 2×10^5 and the ratio of endothelial cells and pericytes was 9:1. On the following day, the culture medium was gently removed and 0.2 ml of collagen mixture was added again and incubated for 1 hour at 37 ℃. After gelation, 1 ml of medium was applied with TNF or VEGF and the samples were incubated up to 48 hours.

Immunoblotting

Endothelial cells were collected using plastic scrapers carefully without damaging the membrane after PBS washing. Immunoblotting (Western blotting) was used for measuring protein expression levels. Cells were lysed with lysis buffer (RIPA buffer) following the manufacturer's protocol. The cell lysates were heated with laemmli buffer at 95 ℃ for 5 min. Then the lysates were loaded to 4-20 % gels (Bio-Rad) for electrophoresis and proteins were transferred to a nitrocellulose membrane. The membrane was blocked with 3% BSA in TBST and incubated with primary antibodies overnight at 4 ℃ and followed by horseradish-peroxidase-coupled secondary antibody for 1 hour at room temperature. Between each step, the membrane was washed three times with TBST for 15min each. Finally, the membrane was incubated with ECL for revealing bands through ChemiDoc XRS (Bio-rad). The bands were quantified with ImageJ and normalized to GAPDH expression. All primary antibodies were used at 1:1000 dilution and secondary antibodies were used at 1:2000 dilution. Antibodies used in this analysis are as follows: anti-Jagged-1 (Cell

Signaling), andi-Dll4 (Cell Signaling), anti-pNFkB (Cell Signaling), anti-NF-kappaB (Cell Signaling), anti-pErk (Cell Signaling), anti-Erk (Cell Signaling), anti-pJNK (Cell Signaling), anti-JNK (Cell Signaling), anti-GAPDH (Cell Signaling), HRP-linked anti-rabbit (GE HealthCare), HRP-linked anti-mouse (GE HealthCare).

Immunofluorescence staining

For all immunofluorescence staining, cells were fixed with 4% (wt/wt) formaldehyde for 20 min, permeabilized with 0.1% triton-X for 10 min, and blocked in 10% goat serum for 1hour. All primary antibodies were used at 1:50 dilution and secondary antibodies were used at 1:100 dilution. The primary and secondary antibodies used in immunofluorescence staining are as follows: Laminin (Abcam), VE-Cad (Santa Cruz), α-SMA (Abcam), anti-mouse 488 (Thermo Scientific), anti-rabbit 594 (Thermo Scientific). Hoechst and and Alexa Fluor 594 phalloidin were used at 1:500 dilution and 1/200 dilution, respectively. Pre-labeling of cells were performed with Vybrant cell-labeling solution (Thermo Fisher) following the manufacturer's protocol.

Image acquisition and analysis

Sandwich culture images were acquired with 20× objective attached to phase contrast microscopy. The resulting tube-like networks were quantified with Angiogenesis Analyzer for ImageJ. Confocal immunofluorescence images were acquired with 20× water-immersion objective attached to Leica scanning disk. Either Leica software or IMARIS was used to merge channels, stack layers for 3D reconstruction and generate longitudinal and transverse cross-sections. IMARIS was used to count the numbers of sprouting and to measure the length of newly formed

sprouts. Four positions closest to the source of VEGF and pro-angiogenic cocktail were used for each condition, with two independent experiments performed unless otherwise stated.

Statistical analysis

Sample populations were compared using one-way ANOVA. $P < 0.05$ was the threshold for statistical significance.

Supplementary Figures

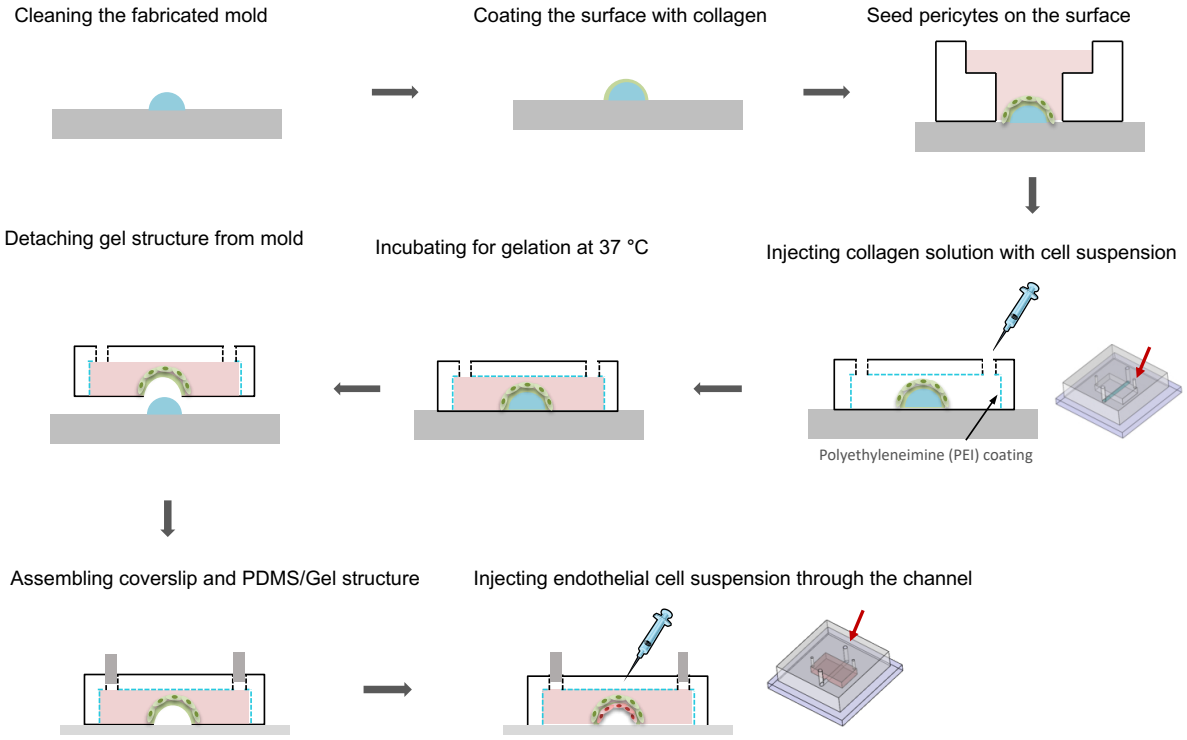

Figure S1. Steps of 3D angiogenesis model fabrication (see the details in *SI Appendix*, Materials and Methods)

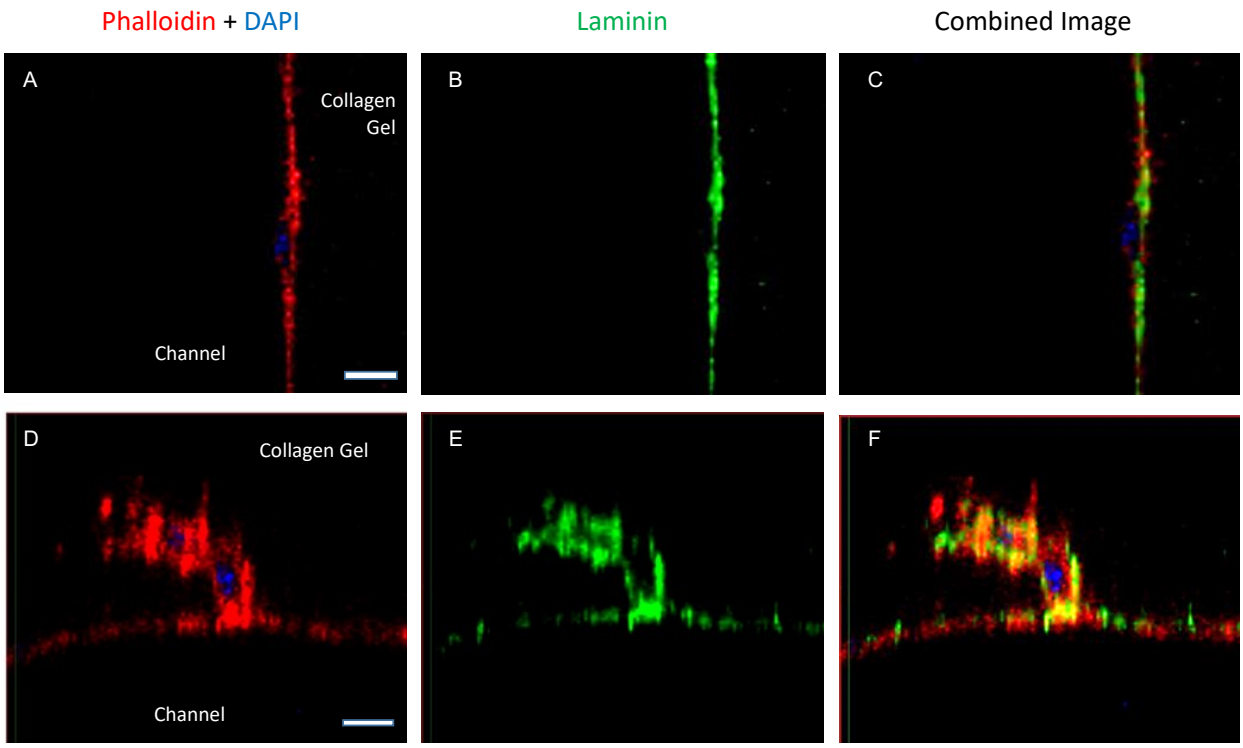

Figure S2. Laminin on the basal side of endothelial cells. Cells lining on the wall of the parental vessel **(A-C)**, and protruding into the lumen **(D-F)**. Red: Phalloidin actin staining, Blue: DAPI nucleic acid staining, scale bar: 25µm.

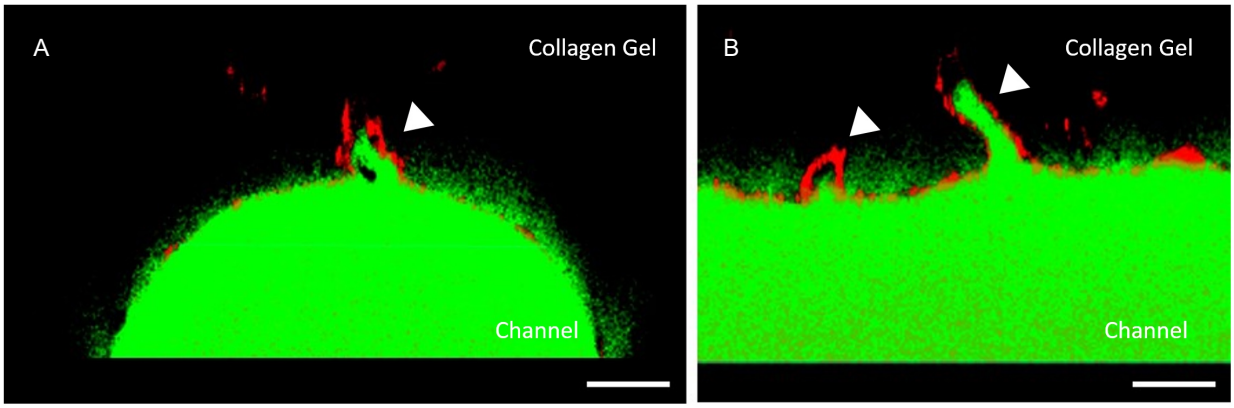

Figure S3. Connection of parental vessel and growing sprouts. FITC-Dextran (70k Da) delivery viewed in cross section **(A)** and longitudinal section **(B)** of 3D angiogenesis model. Red: Phalloidin actin staining, white arrow: lumenized sprout, scale bar: 50μm.

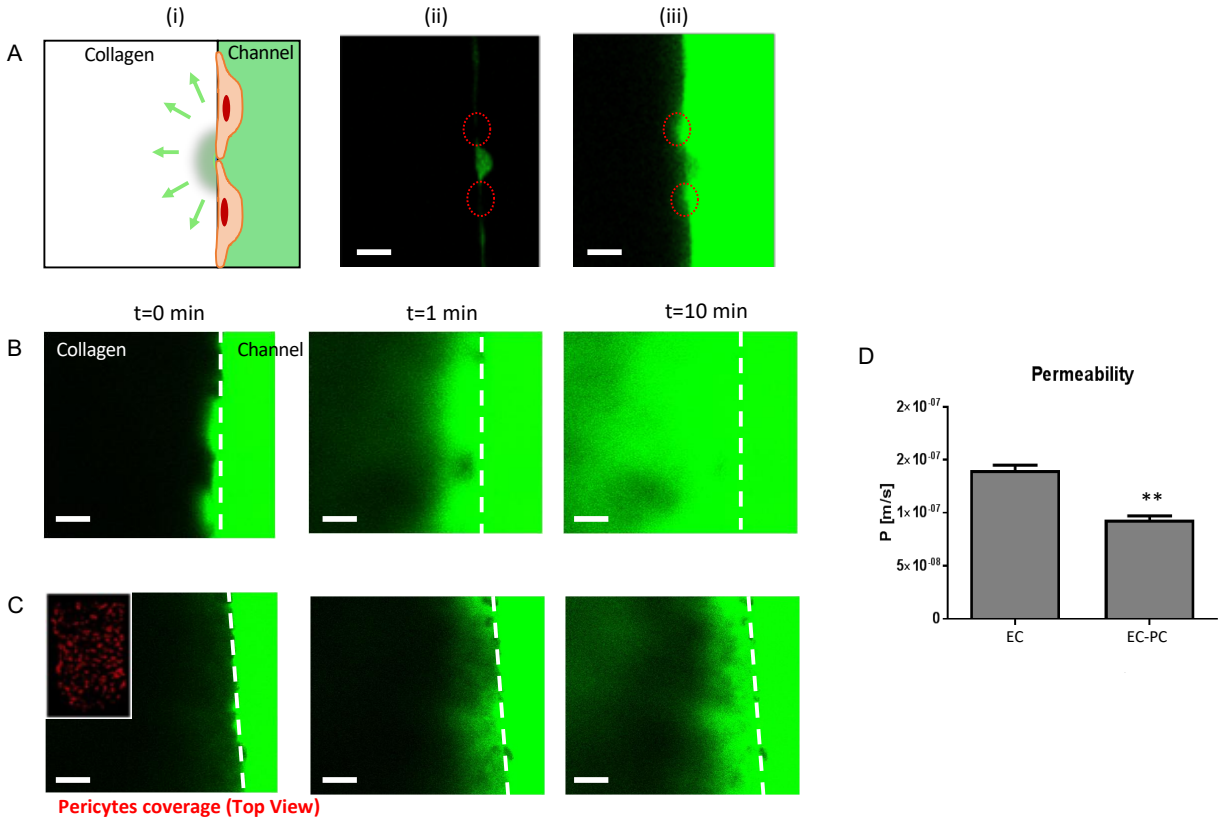

Figure S4. Permeability evaluation with FITC-Dextran (70kDa). (A) Cell culture medium containing FITC-dextran was injected through the channel **(i)** then FITC-Dextran diffused primarily through cell-cell junction **(ii &iii)**. Endothelial cell was pre-labeled with DiO (green). FITC-Dextran diffusion through bare endothelium **(B)** and through endothelium covered with pericytes **(C)**. Pericytes were pre-labeled with DiI (red). Scale bar, 100 μm. **(D)** Permeability evaluation with FITC-Dextran (70 kDa) shows the increase of vessel stabilization by pericytes, resulting in the decrease of permeability. Error bars, s.d. ± mean of experiment values obtained in triplicate. $^{**}P < 0.01$.

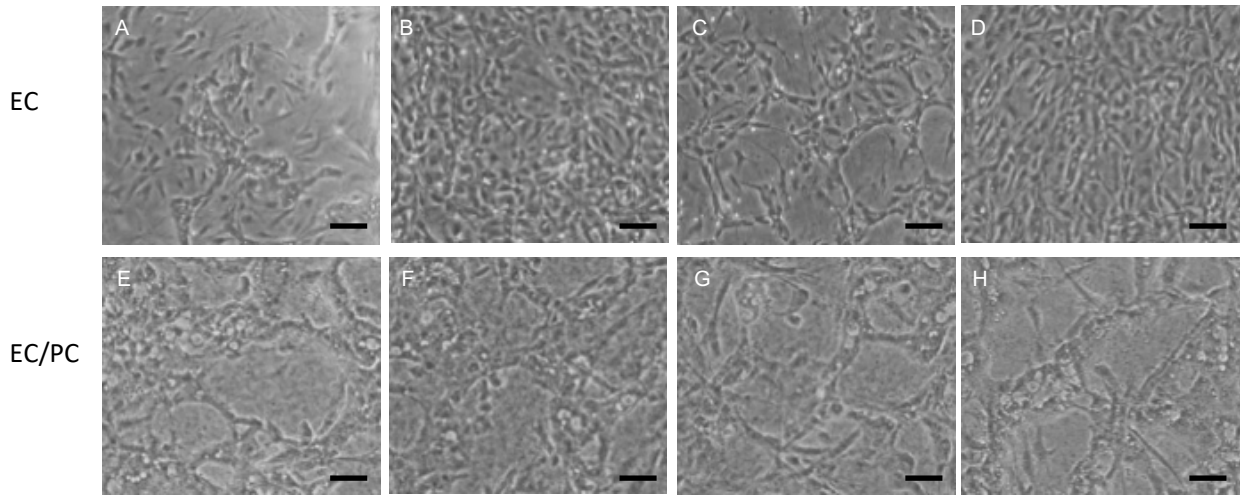

Figure S5. Vasculogenesis on sandwich culture. Formation of tube-like structure with normal medium **(A)**, with 100 ng/ml of TNF **(B)**, with 100 ng/ml of VEGF **(C)**, with 100 ng/ml of TNF and 100ng/ml of VEGF **(D)** on EC monolayer, formation of tube-like structures with normal medium **(E)**, with 100ng/ml of TNF **(F)**, with 100 ng/ml of VEGF **(G)**, with 100 ng/ml of TNF and 100 ng/ml of VEGF **(H)** on EC/PC mixed monolayer. Scale bar, 100 μm

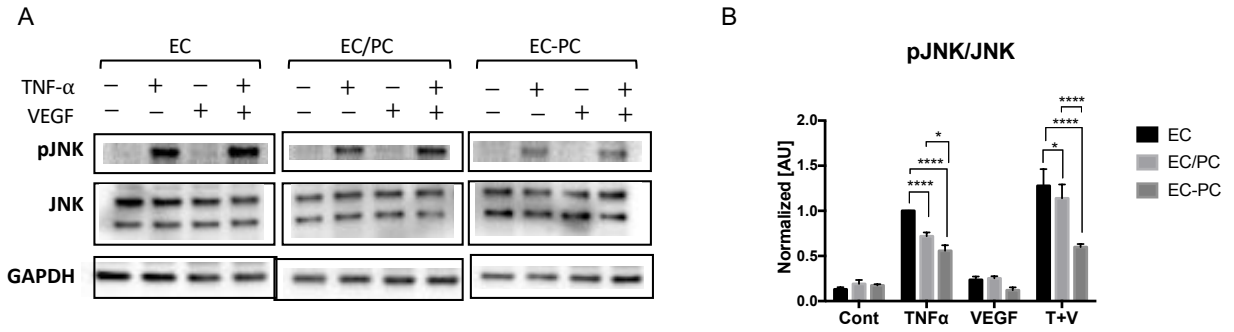

Figure S6. Analysis of JNK expression. (A) Western blot images of phospho-JNK and JNK, **(B)** quantified data from the images. Error bars, s.d. ± mean of experiment values obtained in triplicate. **P* < 0.05, ***P* < 0.01, ****P* < 0.001, *****P* < 0.0001.

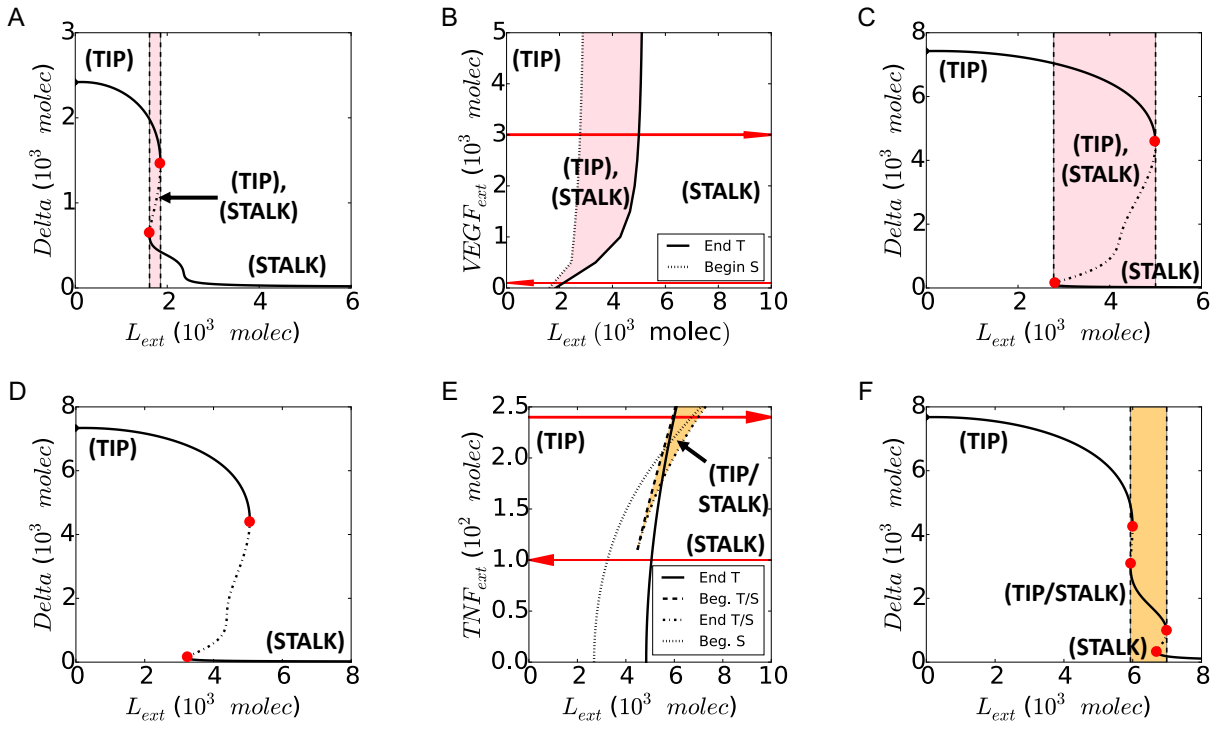

Figure S7 Effect of external signals on cell fate determination in the single-cell model. (A) Bifurcation diagram of intra-cellular Delta with the external Notch ligand ($L_{ext} = D_{ext} + J_{ext}$) as control parameter and low exposure to external VEGF signal ($VEGF_{ext} = 0$ molecules, see red arrow in panel **(B)**). Continuous lines indicate stable fixed points, while dotted lines indicate unstable fixed points. **(B)** Phenotype phase diagram of the single cell exposed to external Notch ligands (x-axis, L_{ext}) and external VEGF signal (y-axis, $VEGF_{ext}$). Tip (T) and Stalk (S) phenotypes coexist for a strong VEGF signal (pink-shaded area indicates bi-stability). **(C)** Same as **(A)** for a high VEGF external signal ($VEGF_{ext} = 3000$ molecules, see top red arrow in panel **(B)**). **(D)** Bifurcation diagram of intracellular Delta with the external Notch ligands (L_{ext}) as control parameter and a low exposure to external TNF signal ($TNF_{ext} = 0$ molecules, see bottom red arrow in panel **(E)**). **(E)** Phenotype phase diagram of the single cell exposed to external Notch ligands (x-axis, L_{ext}) and TNF signal (y-axis, TNF_{ext}). TNF signal introduces a hybrid Tip/Stalk

phenotype (T/S) (orange-shaded area). **(F)** Same as **(D)** for high TNF external signal ($TNF_{ext} = 240$ molecules, see top red arrow in panel **(E)**). In panels **(A)**, **(B)** and **(C)**, $TNF_{ext} = 0$ molecules, while in panels **(D)**, **(E)** and **(F)** $VEGF_{ext} = 2000$ molecules. Bifurcation diagrams of all other variables corresponding to **(A)-(C)-(D)-(F)** are presented in Fig. S8-S9.

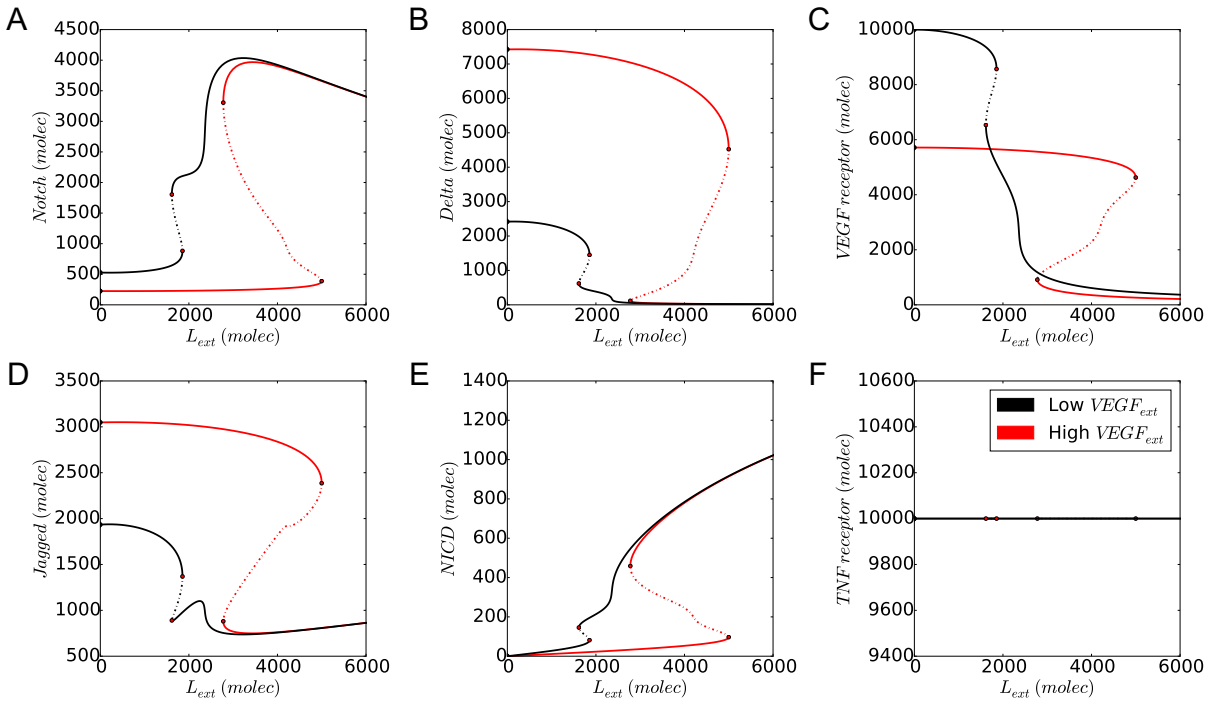

Figure S8. **Bifurcation diagram of the model's variables for low and high exposure to VEGF signaling.** Bifurcation diagram of **(A)** Notch, **(B)** Delta (Dll4), **(C)** VEGF receptor, **(D)** Jagged (Jag1), **(E)** NICD and **(F)** TNF receptor with external Notch ligands ($L_{ext} = D_{ext} + J_{ext}$) as control parameter. Black curves indicate low exposure to external VEGF signaling (as of Fig. S7A) while red lines indicate high exposure (as of Fig. S7C). $TNF_{ext} = 0$ molecules in this simulation.

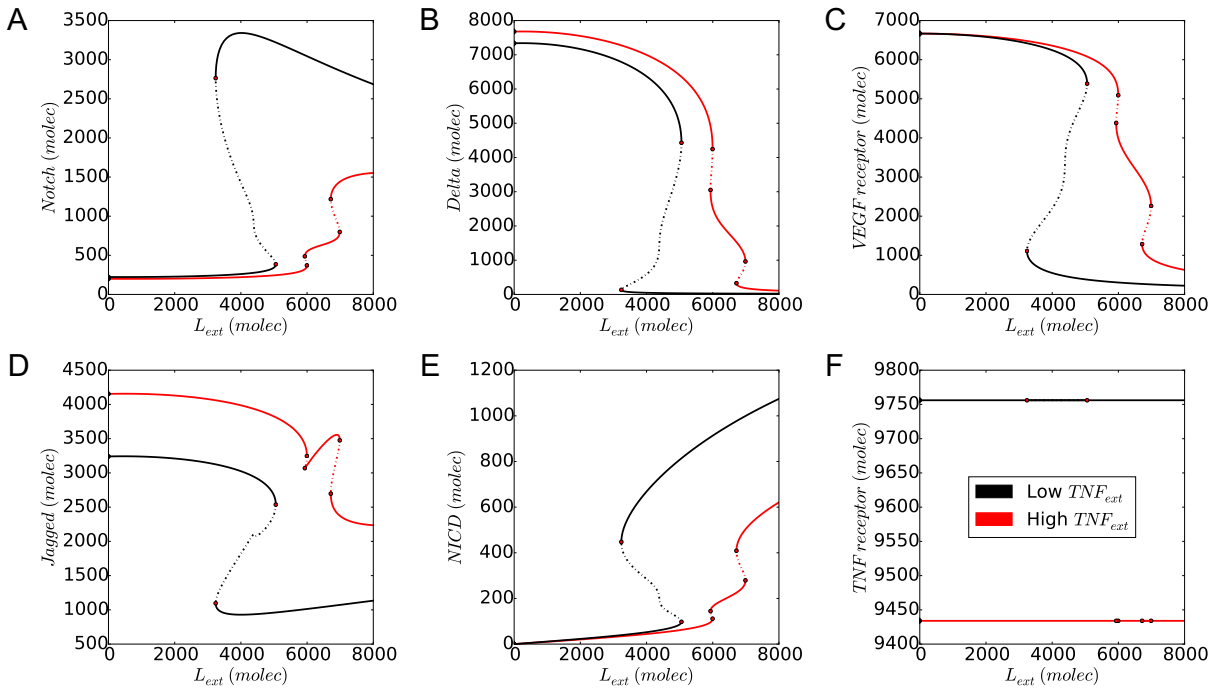

Figure S9 Bifurcation diagram of the model's variables for low and high exposure to TNF signaling. Bifurcation diagram of **(A)** Notch, **(B)** Delta (Dll4), **(C)** VEGF receptor, **(D)** Jagged (Jag1), **(E)** NICD and **(F)** TNF receptor with external Notch ligands ($L_{ext} = D_{ext} + J_{ext}$) as control parameter. Black curves indicate low exposure to external TNF signaling (as of Fig. S7d) while red lines depict high exposure to external TNF signaling (as of Fig. S7F). $VEGF_{ext} = 2000$ molecules in this simulation.

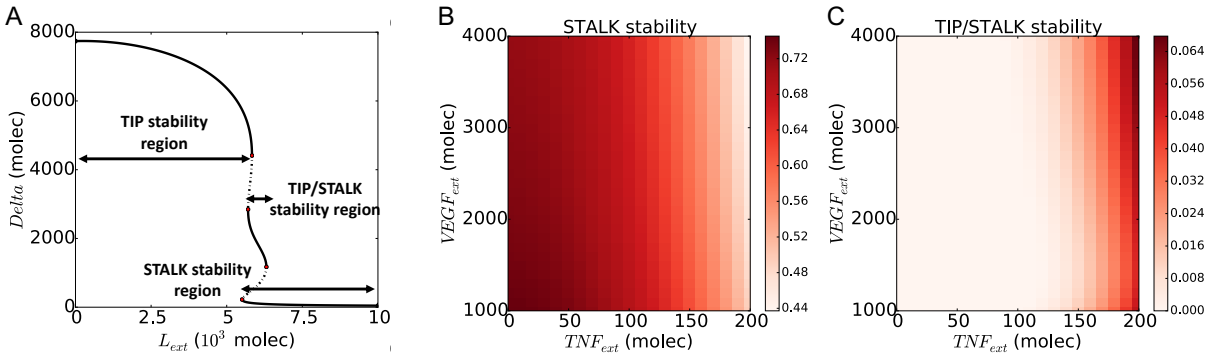

Figure S10 Stabilization/destabilization of each state by VEGF and TNF signaling. (A) Intra-cellular Delta (Dll4) as a function of external Notch ligands, the stability of STALK **(B)** and TIP/STALK **(C)** depending on the level of $VEGF_{ext}$ and TNF_{ext} in each region of **(A)**. The stability of a certain cell state corresponds to the range of the parameter L_{ext} on the x-axis that allows that state, divided by the total range of the x-axis.

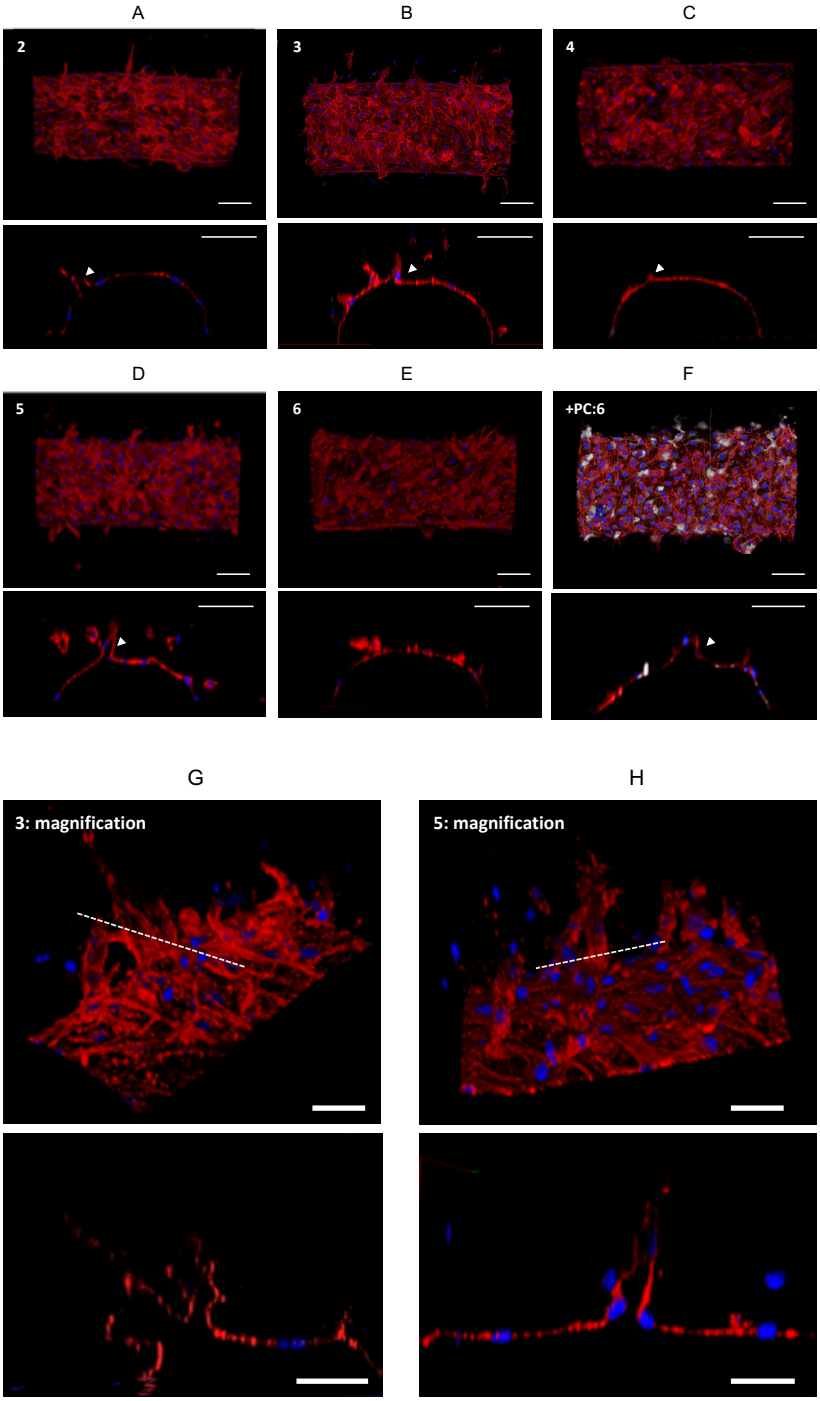

Figure S11. Angiogenesis in 3D angiogenesis model in response to gradient of VEGF and local TNF. Confocal images of sprouting and tube formation in response to the condition of 2 **(A)**, 3 **(B)**, 4 **(C)**, 5 **(D)**, 6 **(E)**, and 6 with pericytes **(F)** indicated in Table 1. Scale bar, 100 μm. The magnified cross-section images of condition 3 **(G)** and 5 **(H)** show highly branched and irregular

tube formation and elongated single tube formation, respectively. Scale bar, 25 μm. Actin filaments of endothelial cells and pericytes were stained with phalloidin (red) and pericytes were pre-labeled with DiD (white).

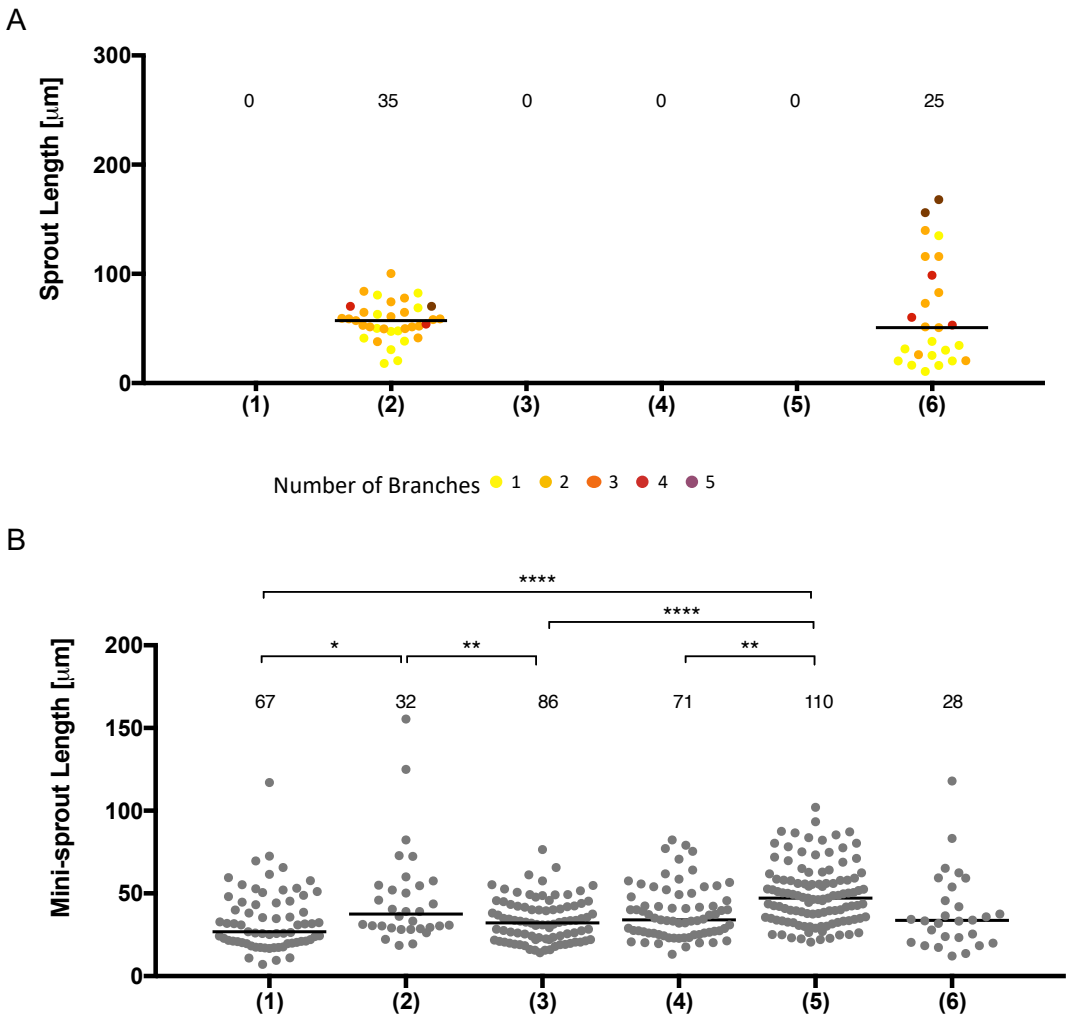

Figure S12. Angiogenic response at condition 3 in table 1. (A) Quantification of lumenized sprout formation. Color codes from 1 to 5 of sprouts indicate the number of branches. **(B)** Quantification of single cell-sized mini-sprouts formation. Data were acquired from four positions with two independent experiments for each condition. Samples were treated with VEGF and TNF for 3 days.

Supplementary Mathematical Modeling

Mathematical model of the Notch-VEGF-TNF circuit

The model of the Notch-VEGF-TNF circuit comprehends six variables: Notch receptor (N), Delta (D) and Jagged (J) ligands, Notch IntraCellular Domain (NICD, I), VEGF receptor (V_R) and TNF receptor (T_R). Here, Delta and Jagged correspond to Dll4 and Jag1, the ligand subtypes considered in the experimental system. The temporal dynamics of the intra-cellular levels of the six variables were described via a system of ordinary differential equations:

$$\frac{dN}{dt} = N_0 H^{S+}\left(I, \lambda_{I,N}\right) - N[(k_C D + k_T D_{ext}) + (k_C J + k_T J_{ext})] - \gamma N$$

$$\frac{dD}{dt} = D_0 p_D H^{S-}\left(I, \lambda_{I,D}\right) H^{S+}\left(k_T V_R V_{ext}/\gamma_S, \lambda_{V,D}\right) - D[k_C N + k_T N_{ext}] - \gamma D$$

$$\frac{dJ}{dt} = J_0 p_J H^{S+}\left(I, \lambda_{I,J}\right) H^{S+}\left(k_T T_R T_{ext}/\gamma_S, \lambda_{T,J}\right) - J[k_C N + k_T N_{ext}] - \gamma J$$

$$\frac{dI}{dt} = k_T N[D_{ext} + J_{ext}] - \gamma_S I$$

$$\frac{dV_R}{dt} = V_{R0} H^{S-}\left(I, \lambda_{I,V_R}\right) - k_T V_R V_{ext} - \gamma V_R$$

$$\frac{dT_R}{dt} = T_{R0} - k_T T_R T_{ext} - \gamma T_R$$

In the model, all variables are expressed in number of molecules. $N_0, D_0, J_0, V_{R0}, V_{T0}$ are the basal cellular production rate constants of N, D, J, V_R and T_R. Since only the molecular copy number is considered in the model, these rate constants account for both transcription and translation. All quantities degrade at the same rate γ, while I degrades at a faster rate γ_S. The transcriptional

regulation that I exerts on any other component X (N, D, J or V_R) was modeled via shifted Hill functions $H^{S+/-}$ defined as:

$$H^{S+/-}\left(I, I_0, n_{I,X}, \lambda_{I,X}\right) = \frac{1}{1+\left(\frac{I}{I_0}\right)^{n_{I,X}}} + \lambda_{I,X}\frac{\left(\frac{I}{I_0}\right)^{n_{I,X}}}{1+\left(\frac{I}{I_0}\right)^{n_{I,X}}}$$

where +/- stands for activation (N, J) or inhibition (D, V_R) and I_0 is a half-maximal level of I. The Hill coefficient $n_{I,X}$ relates to the steepness of the regulation with respect to I and $\lambda_{I,X}$ is a fold-change in the production rate of X due to I ($\lambda_{I,X} < 1$ for inhibition, $\lambda_{I,X} > 1$ for activation)(6). The binding of V_R to external VEGF molecules (V_{ext}) leads to activation of D. This interactions was described via the shifted Hill function $H^{S+}\left(k_T V_R V_{ext}/\gamma_S, \lambda_{V,D}\right)$, where the amount of active intra-cellular VEGF signaling $k_T V_R V_{ext}/\gamma_S$ is explicitly derived in the following paragraph. Similarly, J is activated upon binding of T_R to external TNF molecules (T_{ext}). This interaction was modeled via the shifted Hill function $H^{S+}\left(k_T T_R T_{ext}/\gamma_S, \lambda_{T,J}\right)$, where $k_T T_R T_{ext}/\gamma_S$ is the level of active intra-cellular TNF signaling (see next paragraph). N_{ext}, D_{ext} and J_{ext} represent a constant level of external Notch receptor and ligands in the single cell model or the level of Notch receptor and ligands in the neighboring cell for the 2-cell model. In the latter case, each cell is described by its own set of variables for the Notch, VEGF and TNF pathways. V_{ext} and T_{ext} are the levels of external VEGF and TNF ligands, and are assumed to be constant for both the single cell and the 2-cell model. k_C and k_T are the cis- and trans- receptor-ligand binding rate constants. In the model, only N can bind with ligands produced in the same cell (cis-interaction), leading to degradation of the complex. V_R and T_R can only bind to external molecules (trans-interaction). p_D and p_J are

numerical factors that decrease the production rates of D and J in presence of pericytes. In this case, the production rate constants of D and J are simply rescaled as:

$$D_0 \rightarrow p_D D_0$$

$$J_0 \rightarrow p_J J_0$$

Since pericytes have an inhibitory effect on D and J, p_D and p_J can be varied between 0 (complete shutdown of D, J production) and 1 (control case with no effect of pericytes).

Determination of active VEGF/TNF intracellular signaling

The level of active signaling A (valid for both VEGF or TNF) is described by the equation:

$$\frac{dA}{dt} = k_T A_R A_{ext} - \gamma_S A$$

where k_T is the binding rate of the receptor A_R with the external signaling molecule A_{ext} and γ_S is a degradation rate constant. By assuming fast equilibration $\frac{dA}{dt} = 0$, the steady state level of active signaling becomes:

$$A = \frac{k_T A_R A_{ext}}{\gamma_S}$$

therefore, $\frac{k_T V_R V_{ext}}{\gamma_S}$ and $\frac{k_T T_R T_{ext}}{\gamma_S}$ represent the level of active VEGF and TNF signaling in the cell, respectively.

Parameters estimation

The parameters of the Notch-VEGF axis were taken from the original model of Boareto et al(7) without modifications. In the original model, the values of the production rates of N, D, J and V_R ensured an order of magnitude of ~ 10000 molecules at cell surface(8). The Hill function parameters were estimated from seminal experimental data. The Supplementary Information of

Boareto et al(7) provides to the interested reader the detailed derivation and discussion of the pre-existing parameters. The degradation rate of T_R is equal to the degradation rate of N, D, J and V_R ($\gamma = 0.1$, typical of protein degradation(9)). The production rate T_{R0} equals the production rate of VEGF (1000 mRNA h^{-1}) to ensure a similar receptor level. Similarly, the ligand-receptor binding rate for TNF equals the trans-interaction rate of N and V_R ($k_T = 5\ 10^{-4}\ h^{-1}$). The fold-change in the production of J due to TNF ($\lambda_{T,J} = 5$) was inferred from the experimental data presented in this paper (Fig .3G), while the Hill coefficient was assumed to be $n_{T,J} = 2$. The threshold level of active TNF signal ($T_{0,J} = 400$ molecules) was chosen to enable a tangible effect on J, given the baseline level of T_R in the model. The fold-changes p_D and p_J were estimated from the experimental data presented in this paper (Figs. 3G & H). All parameters are listed in Table S1.

Parameter group	**Parameter**	**Value**	**Dimensions**
Degradation	γ, γ_S	$0.1, 0.5$	h^{-1}
Production	$N_0, D_0, J_0, V_{R0}, T_{R0}$	$1200, 1000, 800, 1000, 1000$	mRNA h^{-1}
Hill coefficient	$n_{I,N}, n_{I,D}, n_{I,J}, n_{I,V}, n_{V,D}, n_{T,J}$	$2, 2, 5, 2, 2, 2$	Dimensionless
Hill fold-change	$\lambda_{I,N}, \lambda_{I,D}, \lambda_{I,J}, \lambda_{I,V}, \lambda_{V,D}, \lambda_{T,J}$	$2, 0, 2, 0, 2, 5$	Dimensionless
Hill threshold	$I_{0,N}, I_{0,D}, I_{0,J}, I_{0,V}, V_{0,D}, T_{0,J}$	$200, 200, 200, 200, 200, 400$	molecules
Binding rate constants	k_T, k_C	$5\ 10^{-4}, 2.5\ 10^{-5}$	h^{-1}
Pericytes	p_D, p_J	$0.6, 0.6$	Dimensionless

Table S1. Parameters of the Notch-VEGF-TNF circuit.

Simulation details

The bifurcation and phase diagrams of the single cell model were calculated using the Python numerical library PyDSTool(10). The code for the calculations of the 2-cell model was developed by the Federico Bocci, and it is available upon request. The system of ODEs that describes the 2-cell system was solved using an explicit Eulerian numerical integration scheme, and the level of active VEGF signaling in the two cells were taken upon equilibration.

Supplementary References

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