Supporting Information

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SI Text

Methods

Cells and Reagents We purchased MDA-MB-231 cells from American Type Culture Collection and primary IMR-90, HDF and primary human umbilical vein endothelial cells (HUVEC) from Lonza. MDA-MB-231, IMR-90, HDF, and Lewis lung carcinoma (LLC) cells were cultured in Eagle's minimal essential medium (MEM) from Lonza supplemented with 10% FBS and 1% GlutaMax (both from Invitrogen). Primary HUVEC cells were cultured in endothelial growth medium (EGM-2, Lonza). Matrigel (growth factor-free) was purchased from BD-Bioscience.

General Protocol for Plating and Culture of Cells on Paper Substrates. To prepare paper substrates, we cut chromatography paper (Millipore) by using scissors or a laser cutter (Versa Laser-Universal Laser VL-300, 50 Watt, using the settings for 200-μm, deep-engraving plastic). The papers were rinsed with deionized water, autoclaved, and dried in a laminar flow hood.

To plate the cells on paper substrates, we detached the cells from growth flasks by using treatment with trypsin-EDTA (3–5 min); washed the cells with serum-containing media and pelleted them by centrifugation at 1,000 rpm (Thermo Electron Corp., IEC Centra CL2) for four minutes. The cells were resuspended in cold Matrigel (free of growth factor) to yield a concentration of 10^7 cells/mL. We spotted this suspension of cells on the specific areas in the paper with a hand-held Gilson P10 pipette man or Eppendorf Repeater Plus pipette, placed the spotted paper into a Petri dish filled with warm (36° C) growth media and cultured for 24 h in an incubator at 36° C and an atmosphere of 5% CO_2. Subsequently, we folded the paper by using sterile tweezers and pressed them together by using custom-made plastic or stainless steel holders. The stacks were cultured in 25-mm-deep Petri dish for the desired time at 36° C, in a 5% CO_2 incubator, on an orbital shaker set to 40–60 rpm. We exchanged the media every two days. At a chosen time, each construct was processed (pretreatment, fixation, permeabilization, staining) according to the Table S1.

The paper containing labeled cells was scanned by using a Typhoon gel scanner with the proper setting for each stain. Table S1 lists scanner settings—the laser excitation, emission wavelength, resolution, and value for the photomultiplier tube—for each stain.

The images obtained after scanning were analyzed by using ImageJ or MatLab.

Quantification of Metabolically Active Cells in Eight Layers Stacks by Using Calcein. To investigate diffusion of calcein in eight-layer stacked samples, we separated or stacked the paper layers immediately before the incubation step. See Fig. S3 for results.

1. "Stack → nonstack." We cultured the stacked layers for nine days, then washed the samples with warm HBSS, separated the layers by using sterile tweezers and placed them for less than one minute in a prewarmed solution of calcein AM (4 μM in HBSS). The separated layers were rocked for ten minutes at 36° C, and fixed with cold paraformaldehyde (PFA) solution.

2. "Nonstack → stack." The nonstacked layers were cultured for nine days, washed with warm HBSS, stacked, pressed together with a plastic holder and placed them within one minute in the prewarmed solution of calcein AM (4 μM in HBSS). The separated layers were rocked for ten minutes at 36° C, and fixed with cold PFA solution.

3. "Stack → flip": After nine days of culture, we washed the stacks with warm HBSS media, removed the cellulose acetate layer from the bottom layer (L8), and placed it on top (L1). The resulting stack was placed in a prewarmed solution of calcein AM (4 μM in HBSS) within one minute. The separated layers were rocked for ten minutes at 36° C, and fixed with cold PFA solution.

Quantification of Gene Expression by Using Real-Time PCR. After nine days of culturing, we placed the cell-containing area in a 2-mL Eppendorf tube containing 500 μL of lysis buffer (buffer RLT, RNAeasy Kit, Qiagen). Vortexing the tubes at 4 °C for twenty min turned the paper substrate to a homogenous pulp. Both the supernatant and the pellet were transferred to QiaShredder tubes (Qiagen) and centrifuged at 13,000 rpm (VWR, Galaxy 16DH) for two minutes. The flow-through solution was processed by using RNAeasy kit according to the manufacturer's procedure (Qiagen). Concentrations of RNA were determined by using NanoDrop (Thermo Scientific). The yield of RNA from the multilayered samples correlated with the number of cells in the layers, and ranged from 80 to 2,000 ng/μL (A_{250}/A_{280} ratio of $>$1.9). We detected nearly no degradation in the samples as judged by 28S:18S rRNA bands on agarose gels.

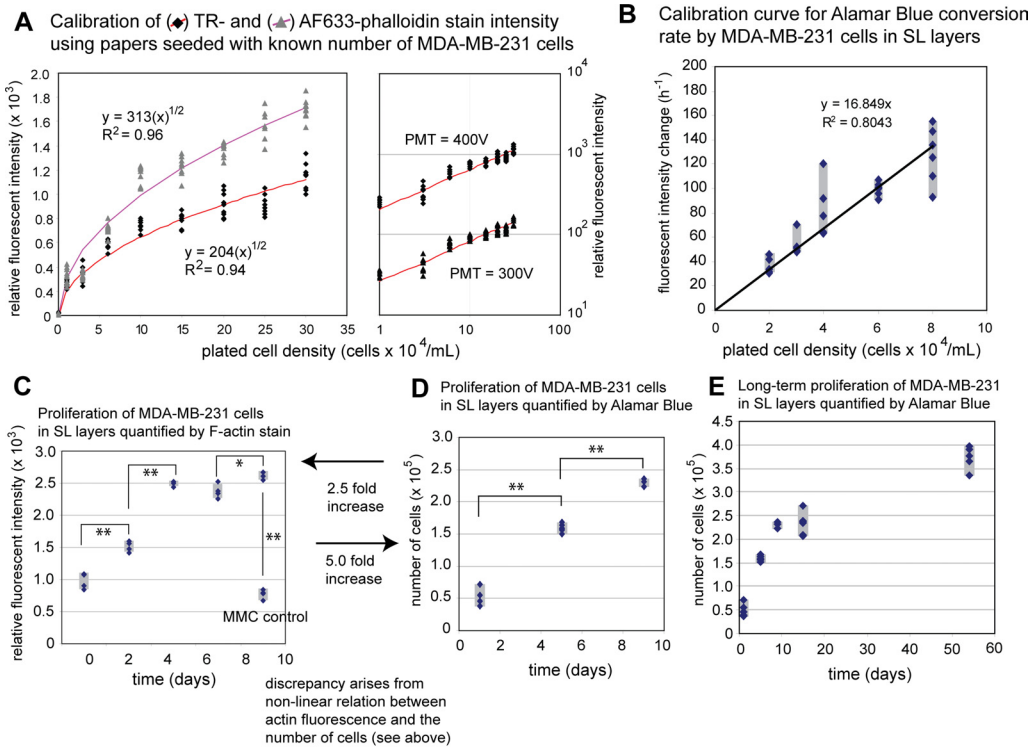
We synthesized cDNA from total RNA by using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, #4368814) using 100 ng of total RNA in 20 μL of the reaction volume. The resulting cDNA solution was diluted tenfold and stored at 80 °C. For PCR, we mixed 5.1 μL of cDNA solution with 7.5 μL of Power SYBR Green PCR Master mix and 1.2 μL of 10-μM solution of forward and reverse primers for human VEGF (forward: 5′-TGFAGTACATCTTCAAGCCATCCTGTGTGC-3′ and reverse 5′-CCTATGTGCTGGCCTTGGTGAGGTTTGAT-3′), human IGFBP3 (forward: 5′-TCTGCGTCAACGCTAGTGC-3′, reverse 5′-GCTCTGAGACTCGTAGTCAACT-3′), human beta-2 microglobulin (forward: 5′-GAATGGAGAGAGAATTGAAAAAGTGGAGCA-3′, reverse 5′-CAATCCAAATGCGGCATCTTCAAAC-3′), human actin (forward: 5-CATGTACGTTGCTATCCAGGC-3′ and reverse 5′-CTCCTTAATGTCACGCACGAT-3′). We performed the reaction on an Applied Biosystems 7900HT Real-Time PCR system by using 40 cycles of a two-step cycling protocol (60° C for one min, 96 °C, 15 seconds)*. The concentration for VEGF, IFGPB3 transcripts was estimated by using the $\Delta\Delta C_t$ approach by using C_t of beta-2 microglobulin (human cells) from the same sample. We performed a pairwise significance test for the samples, calculating P values for $2^{\Delta Ct}$ values by using the Wilcoxon rank sum test. To simplify presentation, all $2^{\Delta Ct}$ were divided by an average $2^{\Delta Ct}$ of L1.

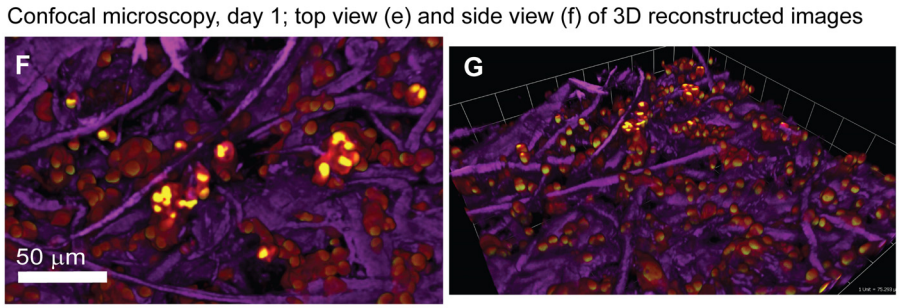
In Vivo Implantation Assay. All animal studies were reviewed and approved by the Animal Care and Use Committee of Children's Hospital Boston. We mixed LLC cells with 1:1 vol/vol mixture of Matrigel and growth medium (10^7 cells/mL), spotted 6 μL of this suspension onto the patterned chromatography paper (size 8 mm × 8 mm, for SL sample) or 8 × 6 μL onto the patterned strip of paper [64 mm × 8 mm, for multilayer sample] (Fig. S4*D*). The samples were incubated in the growth medium at 37 °C overnight, and folded and implanted s.c. on the backs of C57BL/6 mice (left and right side). As controls, we cultured the same samples in growth medium in vitro. After three days, we injected

rhodamine-conjugated ConA into the retro orbital sinus, excised the samples surgically (Fig. S4*E*), fixed them with 4% PFA for 24 h, and destacked the layers for analysis. For sectioning, excised and fixed samples were incubated in 30% sucrose in PBS for 24 h and embedded in optimal cutting temperature (OCT) compound; each sample was cryosectioned from the edge to the middle of the sample (Fig. S4 *F* and *G*). We thawed the remaining halves of the samples, rinsed them with PBS, and destacked the layers for further analysis. Sections and destacked layers were imaged by using fluorescent gel scanner (Fig. 6 *C*–*G*).

We stained the sections (18-μm thick) with hematoxylin and eosin (HE) (Fig. 6 *H*–*K*) as described previously (1, 2). For immunostaining, the sections were hydrated with PBS (10 min), permeabilized with PBS containing 0.1% Triton-X100 (30 min) and blocked with PBS containing 10% FCS and 0.1% Triton X-100 (30 min). We used 1:100 dilution of primary antibody (anti-CD31 or anti-CD45, overnight at 4 °C), washed with PBS (3 × 5 min), and incubated with 1:100 dilution of Alexa Fluor 633-conjugated secondary antibody. We mounted the samples by using Vectashield+DAPI and performed four-color imaging (blue, DAPI; green, GFP; red, rhodamine ConA; far red, immunostaining) by using a Carl Zeiss ApoTome or Leica laser scanning confocal microscope (Fig. 6 *I*–*P* and Fig. S4).

1. Yung CW, et al. (2007) Transglutaminase crosslinked gelatin as a tissue engineering scaffold. *J Biomed Mater Res A* 83A:1039–1046.
2. Mammoto A, Huang S, Moore K, Oh P, Ingber DE (2004) Role of RhoA, mDia, and ROCK in cell shape-dependent control of the Skp2–p27(kip1) pathway and the G(1)/S transition. *J Biol Chem* 279:26323–26330.
3. Carrilho E, Martinez AW, Whitesides GM (2009) Understanding wax printing: A simple micropatterning process for paper-based microfluidics. *Anal Chem*, 10.1021/ac901071p.





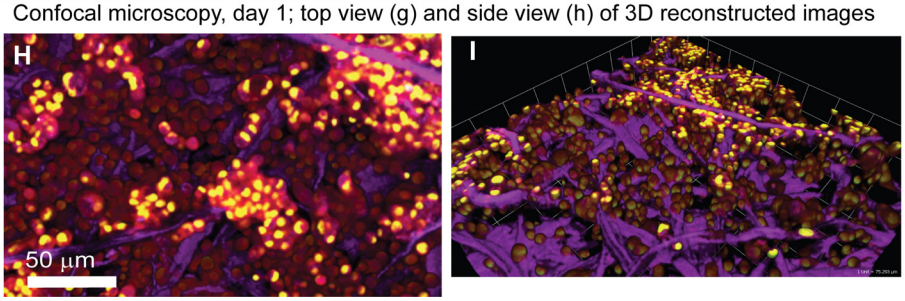


Fig. S1. Calibration of the staining protocols. (*A*) To relate the fluorescent intensity of F-actin to the number of MDA-MB-231 cells inside the paper, we spotted 4 μL of a suspension of MDA-MB-231 cells in Matrigel ($10^6 - 3 \times 10^7$ cells/mL) onto filter paper, incubated the samples for two hours in growth medium, fixed them, and stained them with Texas Red- or Alexa Fluor 633-conjugated phalloidin. Similar to the previous results (Fig. 1*I*), we observed a nonlinear relation between F-actin fluorescence and the number of cells in paper. The nature of the dependence ($y = ax^{1/2}$) was similar for the two dyes we tested. Nonlinearity is not the result of the detector saturation because we observed similar trends with different photomultiplier voltages applied to the detector. Both datasets on log–log plot fit a line with slope equal to 0.5. (*B*) We also quantified the number of MDA-MB-231 cells in paper by using the rate of turnover of metabolic reagent (Alamar Blue). We incubated papers presenting different number of cells with growth medium containing 10% of Alamar Blue. Growth medium was sampled at different times (one, two, three, and six hours); fluorescence of the media at these times was determined, and the rate-of-change of fluorescent intensity (units per hour) was plotted against the number of cells. The observed linear correlation between Alamar Blue turnover and the number of cells was used as a calibration curve in Figs. 3 and 5. (*C*) We cultured MDA-MB-231 cells in a paper–Matrigel matrix for one, three, five, seven, or nine days, and on those days we removed one sample from the medium and fixed it with 4% PFA solution. At the end of the experiment, samples from different days were stained with fluorescently labeled phalloidin and quantified by using a fluorescent gel scanner and ImageJ. (*D*) We cultured MDA-MB-231 cells in a paper–Matrigel matrix for nine days; on days 1, 5, or 9, the media was supplemented with Alamar Blue. After measuring the reagent turnover, the media was changed back to normal growth media. Both F-actin stain intensity (*C*) and Alamar Blue turnover rate (*D*) increased over time, but the results from F-actin stain did not match that of Alamar Blue because of the nonlinear relation between F-actin fluorescence and the number of cells. The discrepancy can be resolved when F-actin fluorescence is converted to the number of cells by using $y = x^{1/2}$ calibration. The resulting $(2.5)^2 \sim$ 6-fold increase of F-actin stain over nine days resembles the result obtained by Alamar Blue (5-fold/9 days). Growth rate obtained from F-actin stain is higher because this staining method detects both metabolically active and inactive cells. (*F*) Long-term proliferation of MDA-MB-231 cells monitored by Alamar Blue. (*F–I*) Confocal microscopy images of MDA-MB-231 cells on day 1 (*F* and *G*) and day 9 (*H* and *I*) confirm the results from Alamar Blue, and F-actin and show that the number of cells in paper indeed increase by five- to sixfold over nine days. We fixed the cells with PFA, stained with AF-633 phalloidin and Sytox to visualize F-actin (red) and nuclei (green), and imaged them by using a laser scanning confocal microscope (Leica) with 20× objective. Cellulose fibers were detected via reflection (purple). 3D reconstruction was performed by using Volocity. The inside of the cell appear yellow because of overlap of red (F-actin) and green (nucleus) channels in the 3D reconstructed images.

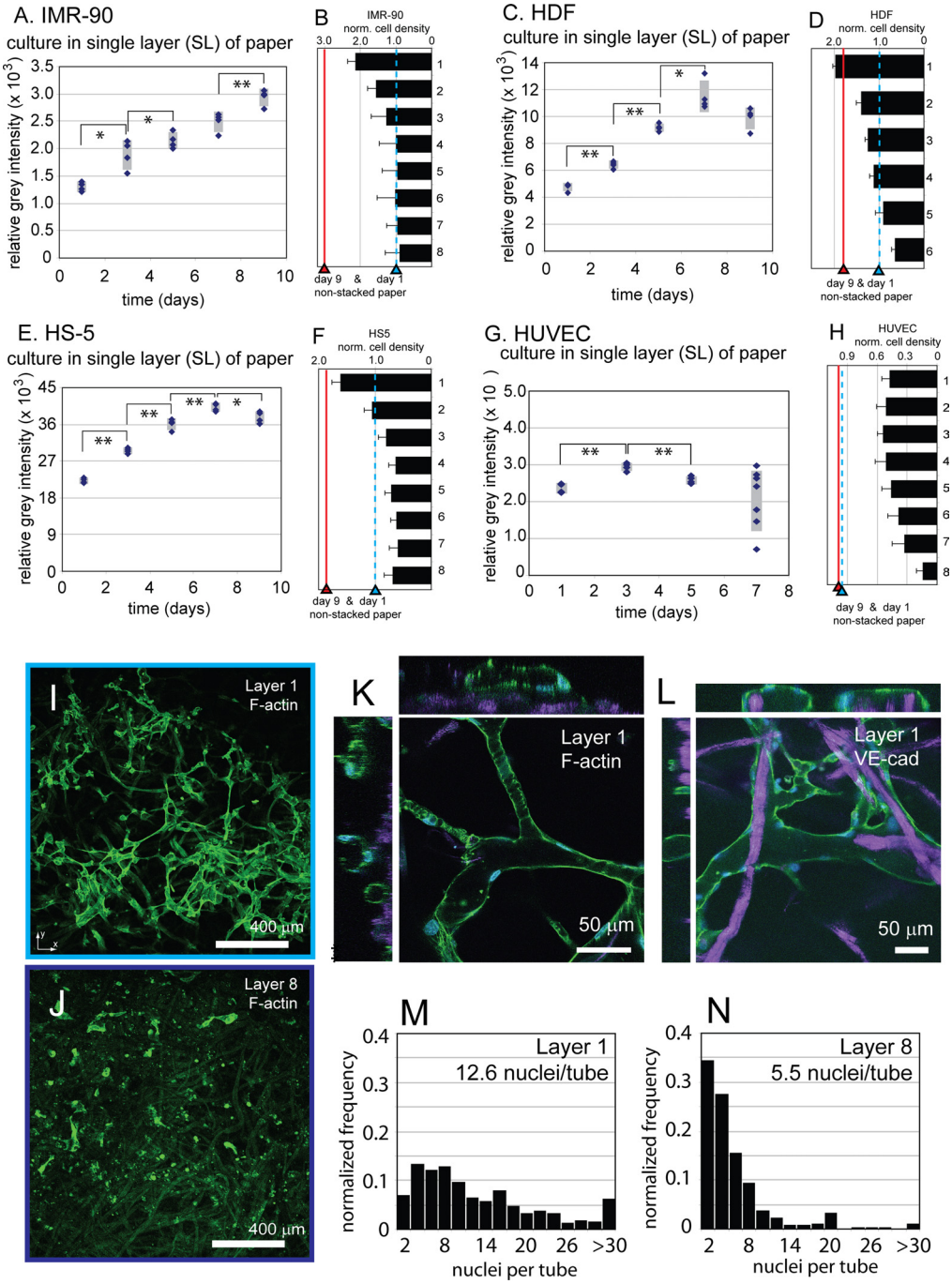
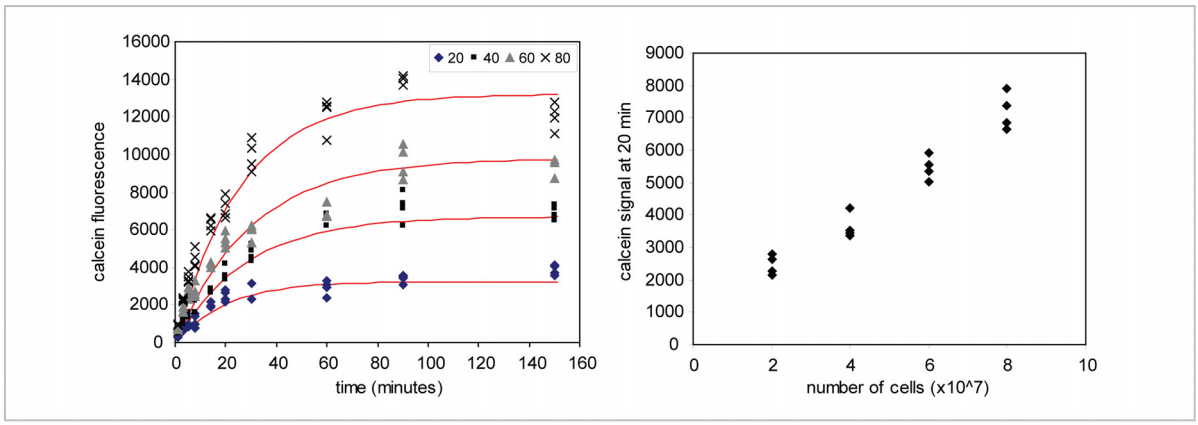
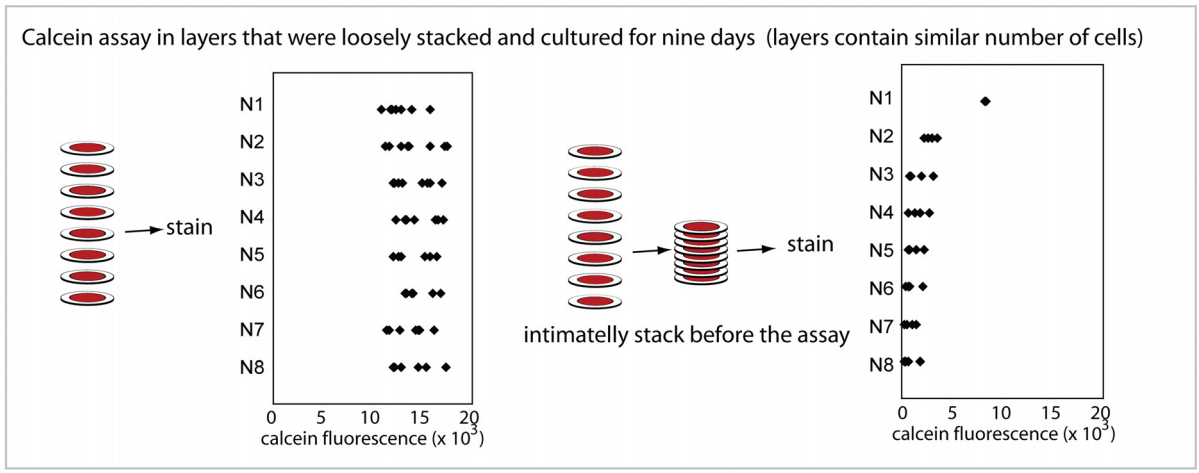


Fig. S2. Investigation of the rate of proliferation of various cell types in a paper-supported Matrigel matrix. (*A–H*) We spotted a suspension of cells in Matrigel (4 μL, 10^7 cells/mL) onto filter paper and allowed the cells to equilibrate for 24 h in growth media. The stacks of eight or six layers were cultured nine days. SL controls were cultured for one, three, five, seven, or nine days, and on those days we removed one sample from the growth medium and fixed it with 4% PFA. At the end of the experiment, L1–L8 samples and SL samples from different days were stained with fluorescently labeled phalloidin and quantified by using a fluorescent gel scanner and ImageJ. The blue line represents actin stain intensity in SL samples on day 1; the red line represents stain intensity in SL on day 9. In *B*, *D*, *F*, and *H*, an average of four to six measurements are presented, and the error bar is one standard deviation. In *A*, *C*, *E*, and *G*, all data are presented, and the overlaying gray bar is equal to 2× (standard deviation). We calculated *P* values (*, $P < 0.05$; **, $P < 0.01$) by using the two-tailed, two-sample unequal variance *t* test. (*I* and *L*). Confocal images and 3D reconstruction of the confocal images of the endothelial lumens formed on day 7 by HUVEC cells in the top (L1) layer, and the small lumens in the bottom (L8) layer of the eight-layered stack of HUVECs. (*I–K*) Cells were stained with AF488-phalloidin or anti-VE-cadherin antibody (L) and imaged by using laser scanning confocal microscope. Color schemes are the following: purple, cellulose fibers (detected via reflection); blue, nuclei; green, F-actin (*I–K*) or VE-cadherin (*J*). (*L* and *M*) The number of nuclei per tube was counted in 11 images for layer 1 and six images for layer 8. Counting was performed manually by three independent participants. Layer 1 has a significantly higher fraction of large tubes, with more cells per tube (3–30 nuclei per tube); whereas much smaller tubes (1–5 nuclei per tube) are observed in layer 8.





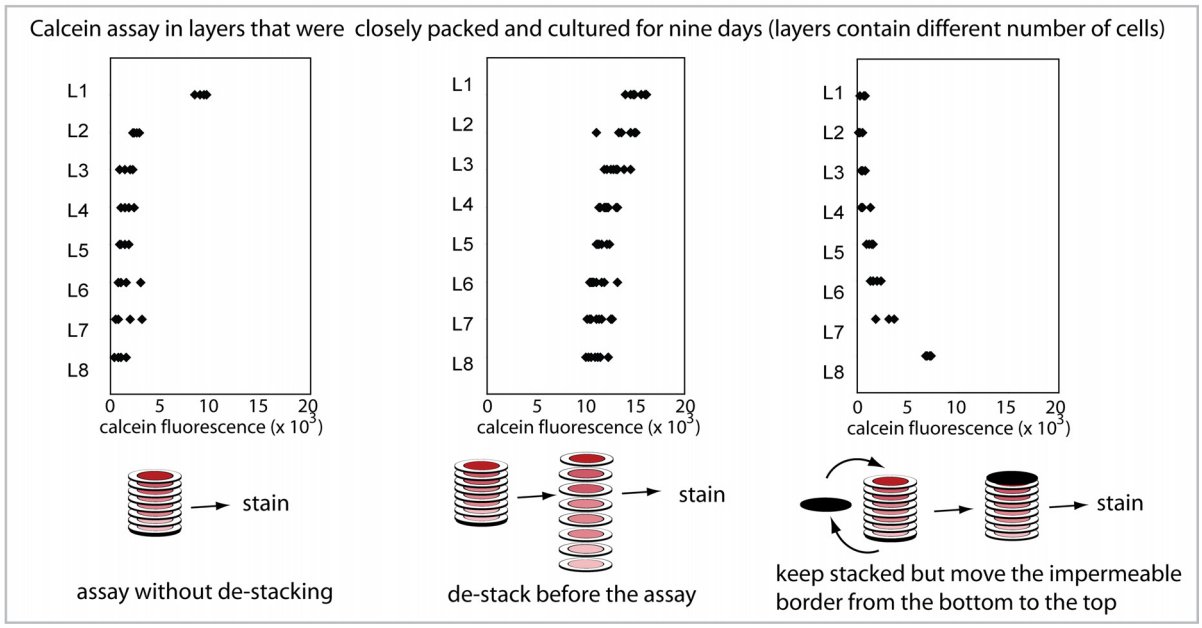


Fig. S3. Quantification of metabolically active cells by using calcein dye. The outcome of the calcein assay depended on the time and the concentration of cells and could be described by using first-order kinetics. The red line demonstrates the fit to the curve $C = C_0(1-e^{-kt})$. At a defined time point (e.g., 20 min), the signal was proportional to the number of cells. In 3D cultures, however, the outcome of calcein analysis was also determined by the balance of diffusion rate and reaction rate. To address this issue, we investigated several stacked and nonstacked geometries and determined that staining of thick 3D cultures led to the development of the calcein signal only in layers exposed to medium (even though live cells reside in every single layer). This discrepancy was resolved when the layers were separated before the assay to allow for unrestricted diffusion of calcein to cells in each layer.

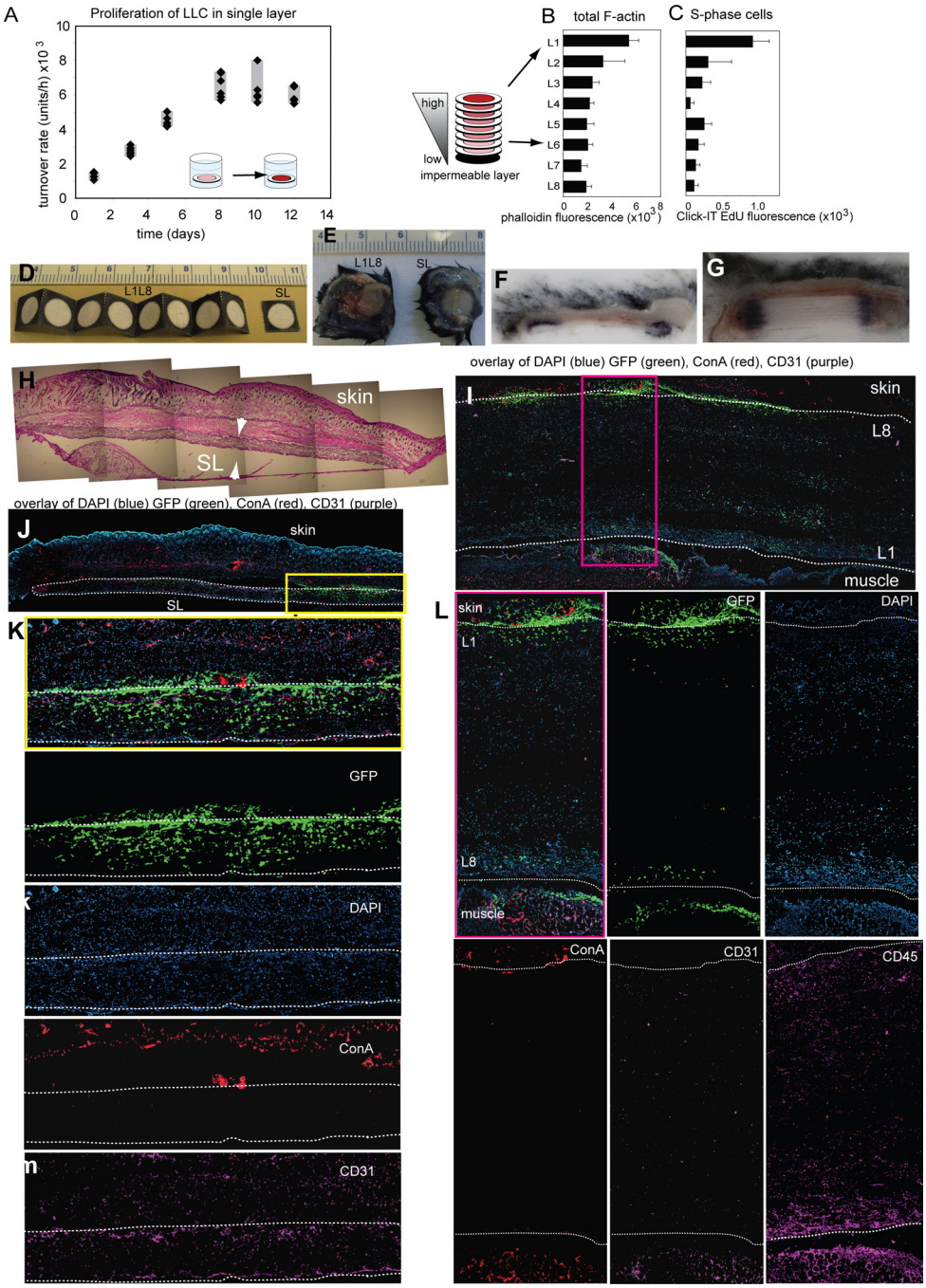


Fig. S4. Analysis of paper-Matrigel substrates cultured in vivo. (*A*) LLC cells proliferate normally when cultured inside paper–Matrigel substrates in vitro as demonstrated by Alamar Blue. In this experiment, we generated suspension of LLC cells in 1:1 vol/vol mixture of Matrigel and growth medium (10^7 cells/mL) and spotted 4 μL of this suspension onto the 200-μL-thick chromatography paper. At a designated time, we supplemented the medium with 10% of Alamar Blue, measured the rate of change of fluorescence, and substituted back to the complete growth medium. (*B* and *C*) After nine days of culturing in eight layers of paper, the distribution of cells (as judged by F-actin stain (*B*) and distribution of S-phase cell (as determined by Click-iT EdU stain) (*C*) resembles that of MDA-MB-231 cells (Fig. 2 *B* and *D*) and other cell lines (Fig. S2). (*D*) Patterned chromatography paper before cell seeding and implantation. Black areas are patterned with printer ink (3) and form a hydrophobic, impermeable border; white areas are unmodified paper. (*E*) Samples after three days of implantation and excision. (*F* and *G*) Light microscopy (2×) image of the OCT-embedded samples sectioned to the middle of the sample. (*H*) Eighteen-μm-thick sections of implanted SL sample stained with HE. (*I*–*L*) We stained the sections from SL samples (*J* and *K*) and L1-L8 samples (*I* and *L*) with anti-CD31 to detect endothelial cells or anti-CD45 antibody to detect differentiated hematopoetic cells and appropriate secondary antibody conjugated to AlexaFluor633. Functional vasculature was labeled by intravenously injected Rhodamine-ConA. Confocal imaging was performed by using a Carl Zeiss ApoTome equipped with 10× objective, automated stage, and mosaic imaging capability. An average of 20 (SL) to 80 (L1-L8) images were collected and stitched to obtain the overview images. We used the following color scheme: green, GFP; red, rhodamine ConA; blue, DAPI; purple, CD31 or CD45 (as noted).

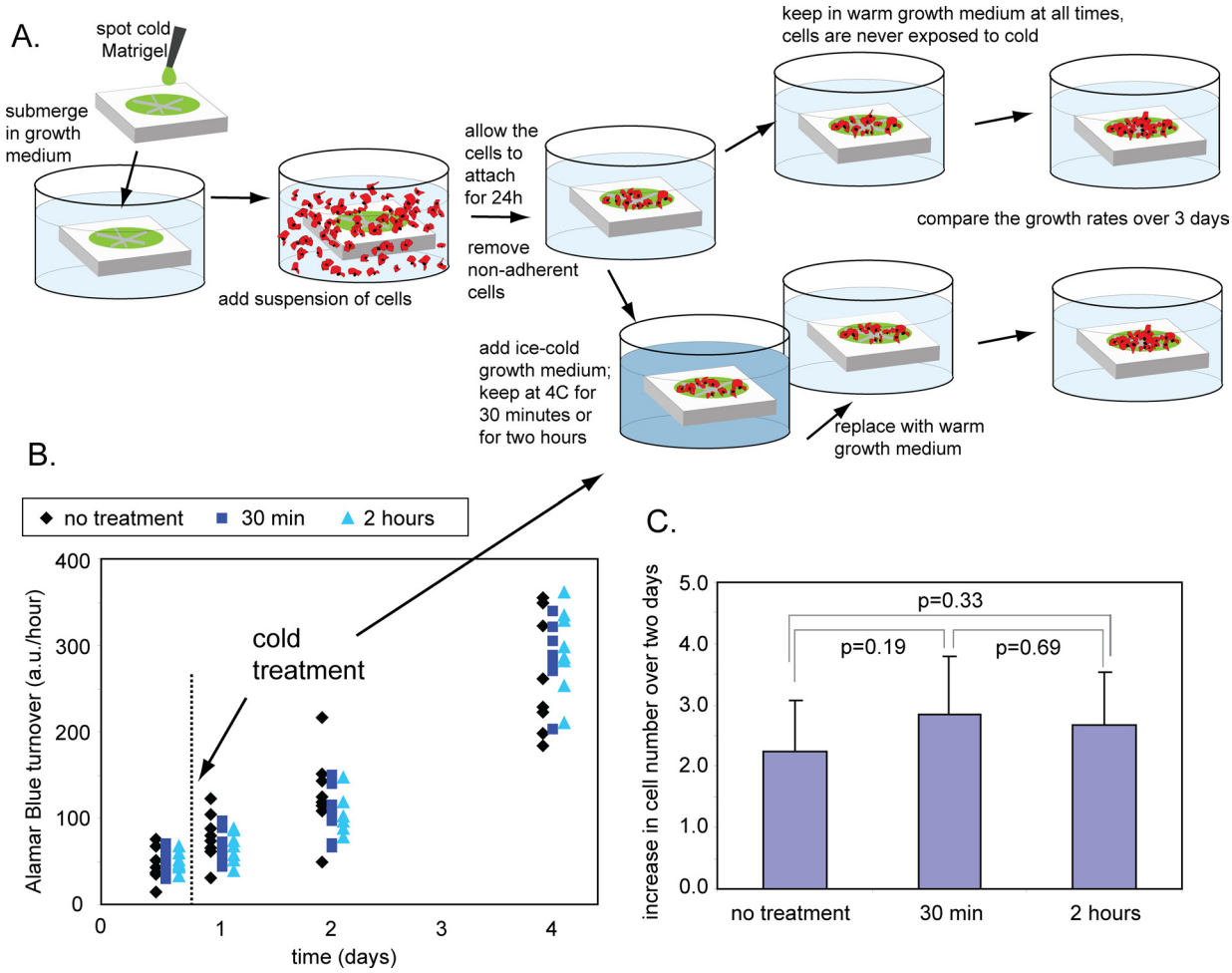


Fig. S5. Assessment of brief cold treatment on long-term proliferation of cells. (*A*) We plated the LLC cells atop paper permeated with Matrigel, and we allowed the cells to adhere for 24 h and exposed one set of samples to ice-cold media (4° C) for 30 min, another set of samples to ice-cold media for two hours, and the third (control) set was never exposed to any cold media ($n = 8$ in each set). We monitored the number of cells in all samples over the next three days by using Alamar Blue. (*B*) A plot of reagent turnover, which is proportional to the number of metabolically active cells, before and after the treatment. All data from all samples is presented. (*C*) Increase in the number of cells from day 2 to day 4 in cold-treated samples is similar to that of control, untreated samples. We calculated the ratio of reagent turnover on day 4 to that on day 2 for each sample and averaged the resulting values ($n = 8$); error bar is one standard deviation. Growth rates are statistically insignificant ($P > 0.05$) as indicated by two-tailed unequal variance *t* test. We conclude that 24-h incubation in 36° C warm media is sufficient to alleviate the effect of brief exposure (<2 h) of cells to cold medium.

Table S1. Pretreatment and staining procedures

Quantification/staining	Pretreatment	Fixation, permeabilization*	Staining protocol (time)	Scanner settings [excitation/emission (nm); resolution (μm); PMT (V)]
Quantification of number of cells in paper by using actin stain	N	Y, Y	Incubate with 1:500 dilution of Alexa Fluor 633 phalloidin in TB-PBS (30 min)	red/670; 50; 450
			Incubate with 1:300 dilution of Texas Red phalloidin in TB-PBS (30 min)	green/610; 50; 500
			Incubate with 1:300 dilution of Alexa Fluor 488 phalloidin in TB-PBS (30 min)	blue/520; 50; 300
Quantification of S-phase cells by using Click-iT EdU stain	Supplement the media with 4-μM EdU for 4 or 24 h at 36° C and 5% CO_2	Y, Y	(*i*) Click-iT EdU reaction (12 h) (*ii*) Wash with TB-PBS (3×5 min) (*iii*) Incubate with 1-μM SYTOX green in TB-PBS (15 min)	EdU: red/670; 50; 400 SYTOX: green/526; 50; 300
Quantification of DNA damage by using Click-iT TUNEL stain	N	Y, Y	(*i*) Incorporate TdT (Invitrogen) (*ii*) Click-iT reaction to conjugate azido-Alexa Fluor 633 (Invitrogen) (*iii*) Wash with TB-PBS (3×5 min) (*iv*) Incubate with 1-μM SYTOX green in TB-PBS (15 min)	TUNEL: red/670; 50; 400 SYTOX: green/526; 50; 300
Quantification of metabolically active cells by using calcein	(*i*) Rinse samples with HBSS (20 min) (*ii*) Incubate in calcein solution (4 μM in HBSS).	Y, N	N	blue/520; 50; 300†

EdU, 5-ethynyl-2′-deoxyuridine; N, no; PMT, photo multiplier tube; TB-PBS, 0.1% Triton X-100 and 0.2% bovine serum albumin-phosphate buffered saline; TdT, terminal deoxynucleotidyl transferase; V, voltage; Y, yes.

*To fix the samples, we incubated them in the solution of formaldehyde (4% PFA in PBS) for 15 min. To permeabilize cell membranes, we incubated them with solution of TB-PBS for 10 min.

†We noticed that prolonged incubation in PFA solution (>24 h) leads to significant loss of fluorescence because of diffusion of the calcein out of the cells. The samples, hence, were scanned by using a Typhoon gel scanner within a few hours after fixation.