Supporting Information

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SI Materials and Methods

Cell Culture. Primary human umbilical endothelial cells (HUVECs; Lonza) were maintained on 0.1% (wt/vol) gelatin-coated dishes in EGM-2 (Lonza). C3H10T1/2 cells (ATCC) were maintained in low-glucose DMEM containing 10% (vol/vol) FBS (Atlanta Biologicals), 100 U/mL penicillin, and 100 mg/mL streptomycin (Invitrogen). Primary human hepatocytes from a 1-y-old female Caucasian donor (Lot Hu8085; CellzDirect) were maintained in high-glucose DMEM (Cellgro) containing 10% (vol/vol) FBS (Gibco), 1% (vol/vol) ITS (insulin, transferrin, sodium selenite) supplement (BD Biosciences), 0.49 pg/mL glucagon, 0.08 ng/mL dexamethasone, 0.018 M Hepes, and 1% (vol/vol) penicillin–streptomycin (pen-strep; Invitrogen). Primary rat hepatocytes were isolated as described previously (1–4) and maintained in high-glucose DMEM containing 10% (vol/vol) FBS, 0.5 U/mL insulin (Lilly), 7 ng/mL glucagon (Bedford Laboratories), 7.5 μg/mL hydrocortisone (Sigma–Aldrich), and 1% (wt/vol) pen-strep. J2-3T3 fibroblasts (gift from Howard Green, Harvard Medical School, Boston) were maintained in high-glucose DMEM containing 10% (vol/vol) bovine serum and 1% (wt/vol) pen-strep. Human hepatoma (Huh-7.5) cells (gift from Charles Rice, The Rockefeller University, New York) were maintained in high-glucose DMEM containing 10% (vol/vol) FBS (Atlanta Biologicals), 100 U/mL penicillin, and 100 mg/mL streptomycin (Invitrogen) and used for in vitro imaging of hepatic constructs instead of primary hepatocytes.

In Vivo Implantation of Constructs. All surgical procedures were conducted according to protocols approved by the University of Pennsylvania or Massachusetts Institute of Technology Institutional Animal Care and Use Committee. To preserve geometry during implantation, constructs were embedded in a gasket cut from a polypropylene surgical mesh (Davol). Eight-week-old female Nu/nu nude mice (Charles River) or NCr nude mice (Taconic) were anesthetized using isoflurane, and the constructs were sutured to the mesenteric parametrial fat pad. For "cords + hepatocytes – excised" animals, engineered tissue and attached mesentery were cut from the remainder of the mesentery via an upstream excision so that the tissue and attached mesentery were isolated from host circulation. The incisions were closed aseptically, and the animals were administered 0.1 mg/mL buprenorphine every 12 h for 3 d following surgery.

In Situ Imaging. To enable noninvasive imaging of function, hepatocytes were transduced with a lentiviral vector expressing firefly luciferase under the human albumin promoter (pTRIP.Alb.IVSb.IRES.tagRFP-DEST; gift of Charles Rice, The Rockefeller University, New York). For luminescence imaging, mice were injected i.p. with 250 μL of 15 mg/mL D-luciferin (Caliper Life Sciences) and then imaged using the IVIS Spectrum system (Xenogen). To visualize perfused vessels, a solution of 20 mg/mL FITC-labeled dextran (150 kDa; Sigma) in PBS was injected i.v. via the tail vein. To visualize mouse vs. human vessels, a solution of 500 μg/mL lectin from *Helix pomatia* agglutinin (HPA) conjugated to Alexa 488 (Sigma–Aldrich), and 100 μg/mL lectin from *Ulex europaeus* agglutinin (UEA-1) conjugated to TRITC (Vector Laboratories) in PBS was injected i.v. via the tail vein. These lectins previously were demonstrated to bind specifically to mouse or human endothelial cells, respectively (5, 6). Perfused vessels subsequently were imaged using a Zeiss 710 laser scanning confocal microscope.

Tissue Harvesting, Processing, and Histology, and Immunohistochemistry. Animals were killed at various time points, and tissue was harvested from the i.p. space. Explants were fixed in 4% (vol/vol) paraformaldehyde (PFA) for 48 h at 4 °C, dehydrated in graded ethanol (50–100%), embedded in paraffin, and sectioned using a microtome (6 μm) for immunohistochemical staining. For gross visualization of tissue, sections were stained with hematoxylin and eosin (H&E). For identification of cords composed partially of collagen, sections were stained with Sirius red (collagen) and fast green (other tissue elements). For identification of vessels containing human endothelial cells, mouse endothelial cells, smooth muscle cells, and erythroid cells, sections first were blocked using M.O.M. Blocking Reagent (Vector Laboratories) and normal goat serum and then immunostained using primary antibodies against human CD31 (1:20; Dako), mouse CD31 (1:50; BD Biosciences), Ter-119 (1:100; BD Biosciences), and alpha-smooth muscle actin (1:100, Abcam), respectively. Signal was visualized after incubation with secondary goat anti-IgG1–Alexa 555, goat anti-rat–Alexa 488, and donkey anti-rabbit–Alexa 647 antibodies (Jackson ImmunoResearch). For identification of primary hepatocytes adjacent to vessels containing RBCs, sections were blocked using normal donkey serum then incubated with primary antibodies against arginase 1 (ARG-1, 1:400; Sigma–Aldrich) and Ter-119 and followed with species-appropriate secondary antibodies conjugated to Alexa 488 and 555. Images were obtained using a Zeiss 710 laser scanning confocal or Nikon 1AR Ultra-Fast Spectral Scanning confocal microscope.

Statistical Analysis and Quantification of Vascularization Parameters. Quantification was performed manually on imaged H&E sections using FIJI Open Source software. Blood area was quantified by measuring the total area of tissue containing blood within a cord. Measurements were normalized to average cord area to compensate for oblique cutting angles. Vessel number was quantified by counting individual vessels within a cord and then normalized to the average cord area. Vessel diameter was quantified by measuring the diameter of individual vessels within a cord. Sections for quantification were chosen from the center of the constructs, and a minimum of three sections at least 150 μm apart were quantified per cord. All data are expressed as the mean ± SE. Statistical significance was determined using a one-way ANOVA followed by Tukey's post hoc test for group comparisons.

1. March S, Hui EE, Underhill GH, Khetani S, Bhatia SN (2009) Microenvironmental regulation of the sinusoidal endothelial cell phenotype in vitro. *Hepatology* 50(3): 920–928.
2. Chen AA, et al. (2011) Humanized mice with ectopic artificial liver tissues. *Proc Natl Acad Sci USA* 108(29):11842–11847.
3. Seglen PO (1976) Preparation of isolated rat liver cells. *Methods Cell Biol* 13:29–83.
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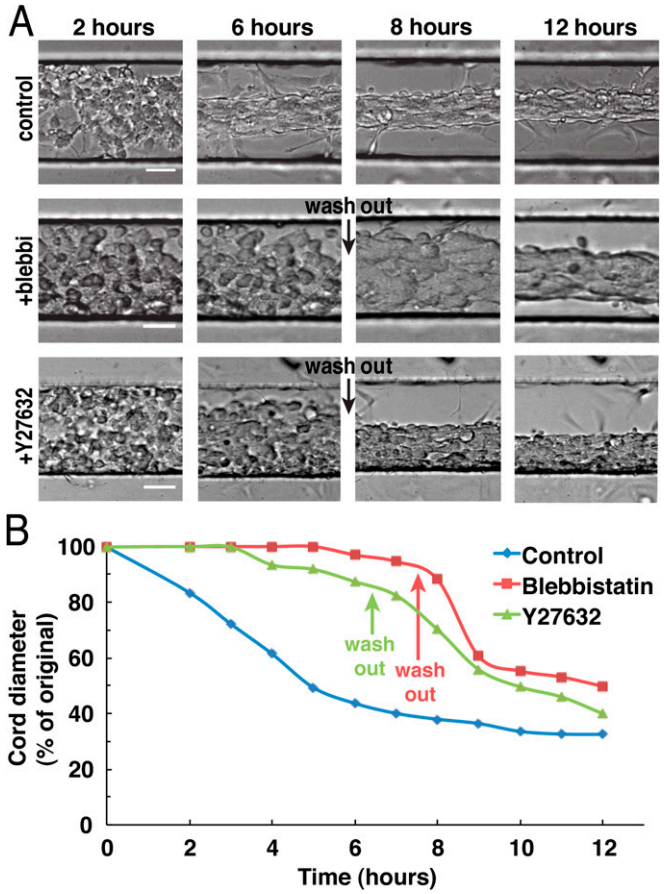

Fig. S1. Cytoskeletal tension is required for cord contraction. (*A*) Time-lapse imaging depicting cord formation in samples treated with vehicle, blebbistatin, or Y27632 (bar, 50 μm). (*B*) Quantification of cord contraction reveals rapid increase in contraction after wash-out of contractility inhibitors. Constructs were treated with 20 μM blebbistatin or 25 μM Y27632 at 7.5 or 6.5 h.

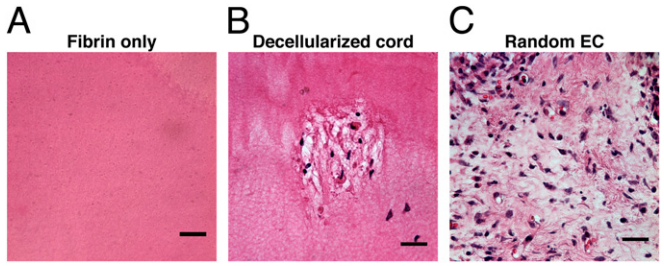

Fig. S2. Cells are required for vascularization. (*A*) H&E staining of fibrin gel (no cells) implanted and resected 5 d PI. (*B*) H&E staining of fibrin gel containing decellularized endothelial cell (EC) cords 5 d PI (bars, 25 μm). (*C*) Fibrin gel containing a mixture of HUVECs and 10T1/2s used in making EC cords. Cells were allowed to organize into networks in vitro for 2 d before implantation and resected 5 d PI.

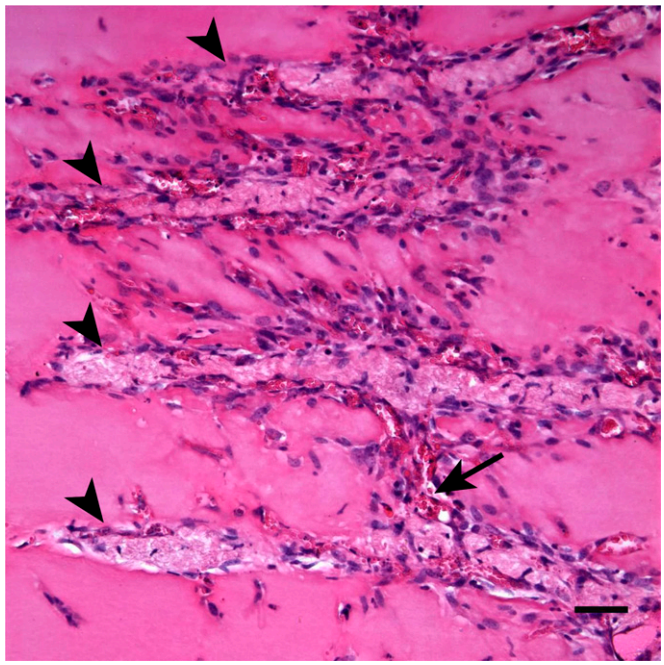

Fig. S3. Evidence of sprouting between capillaries within adjacent cords. H&E staining of longitudinal cord cross-sections at day 7 PI revealed evidence of capillary sprouts (arrow) between adjacent cords (arrowheads) (bar, 25 μm).

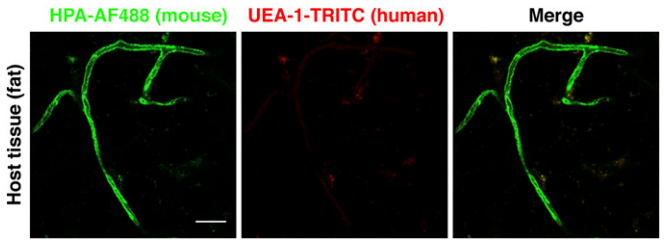

Fig. S4. Mouse and human lectin cross-reactivity. Human-specific (UEA-1) lectin and mouse-specific (HPA) lectin were perfused at 14 d following implantation of constructs. Imaging of surrounding host adipose tissue demonstrated minimal cross-reactivity between UEA-1 and mouse vessels (bar, 25 μm).

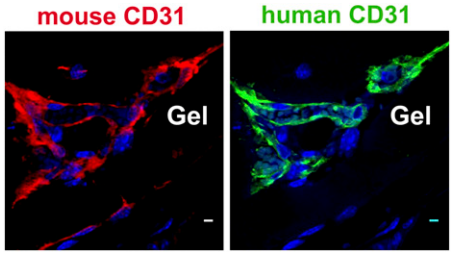

Fig. S5. Contribution of mouse vs. human endothelial cells to capillaries. Adjacent tissue sections were immunostained using antibodies specific for either mouse (*Left*) or human (*Right*) CD31. Capillaries in the grafts contained both human and mouse endothelium at 14 d PI (bar, 10 μm).

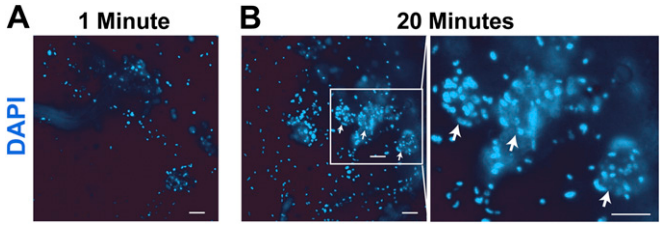

Fig. S6. Diffusion of small-molecule DAPI through ligated constructs. Constructs containing both hepatic aggregates and cords were implanted using procedures identical to those of the "EC Cord Ligated" control. Following implantation, the animals were injected i.p. with DAPI using the same volume and concentration typically used for luciferin administration. (*A*) Single dispersed DAPI-positive cells could be identified in constructs as early as 1 min after DAPI injection. (*B*) By 20 min post injection, DAPI-positive hepatic aggregates were identified throughout the constructs. These studies demonstrate that a small molecule similar in size to luciferin permeates the construct and stains hepatic aggregates within 20 min post injection within time scales relevant for bioluminescence imaging (bar, 50 μm).