Supplementary Information

A novel 3D human peritubular microvascular system

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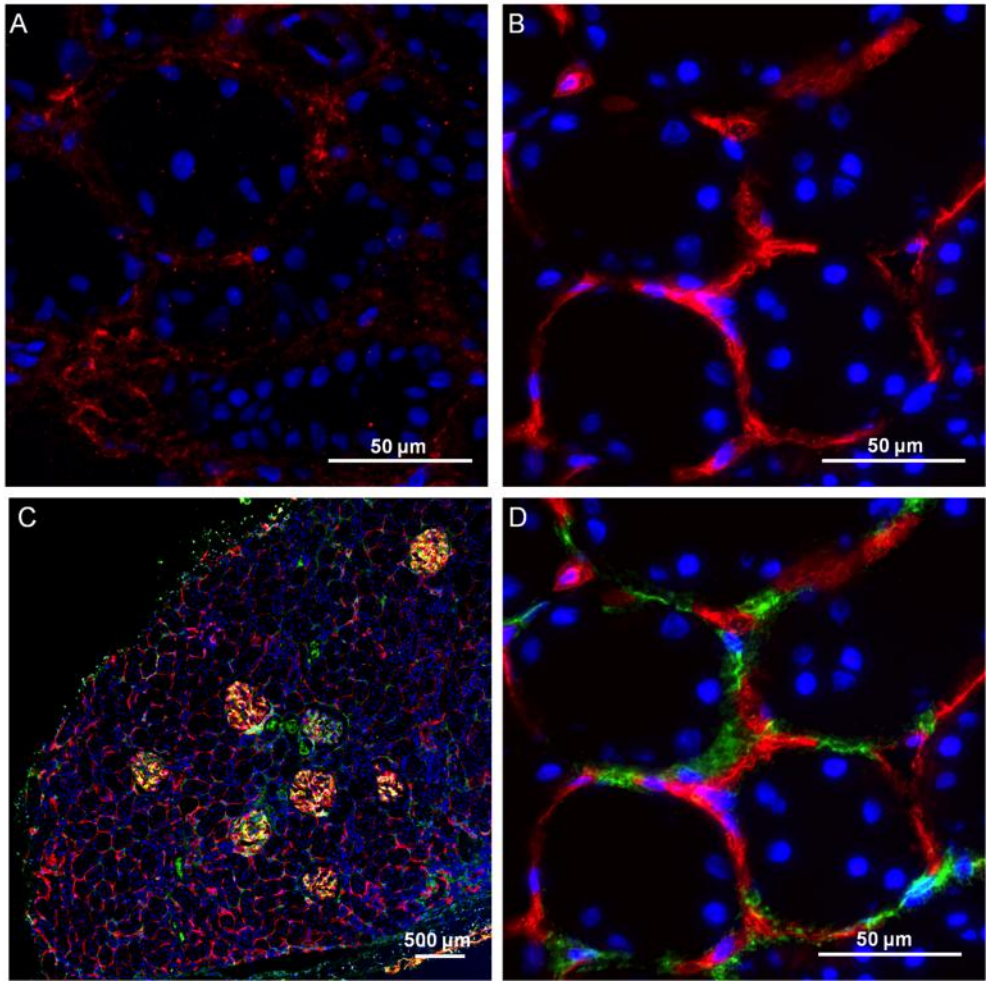
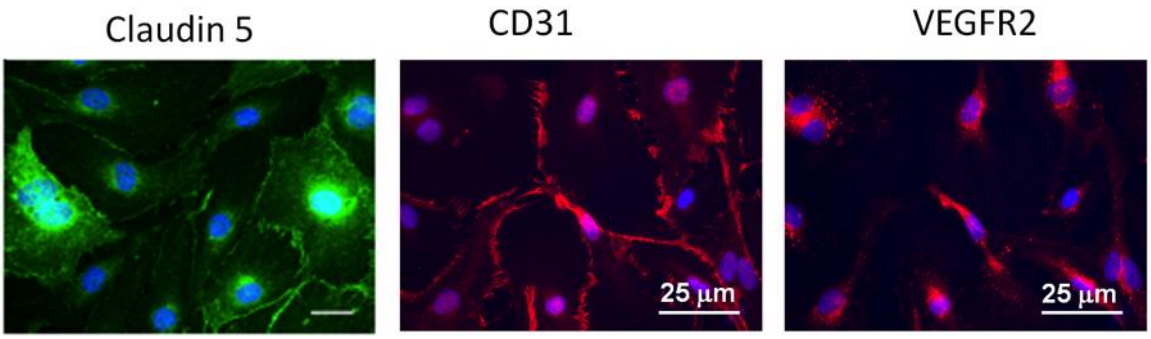
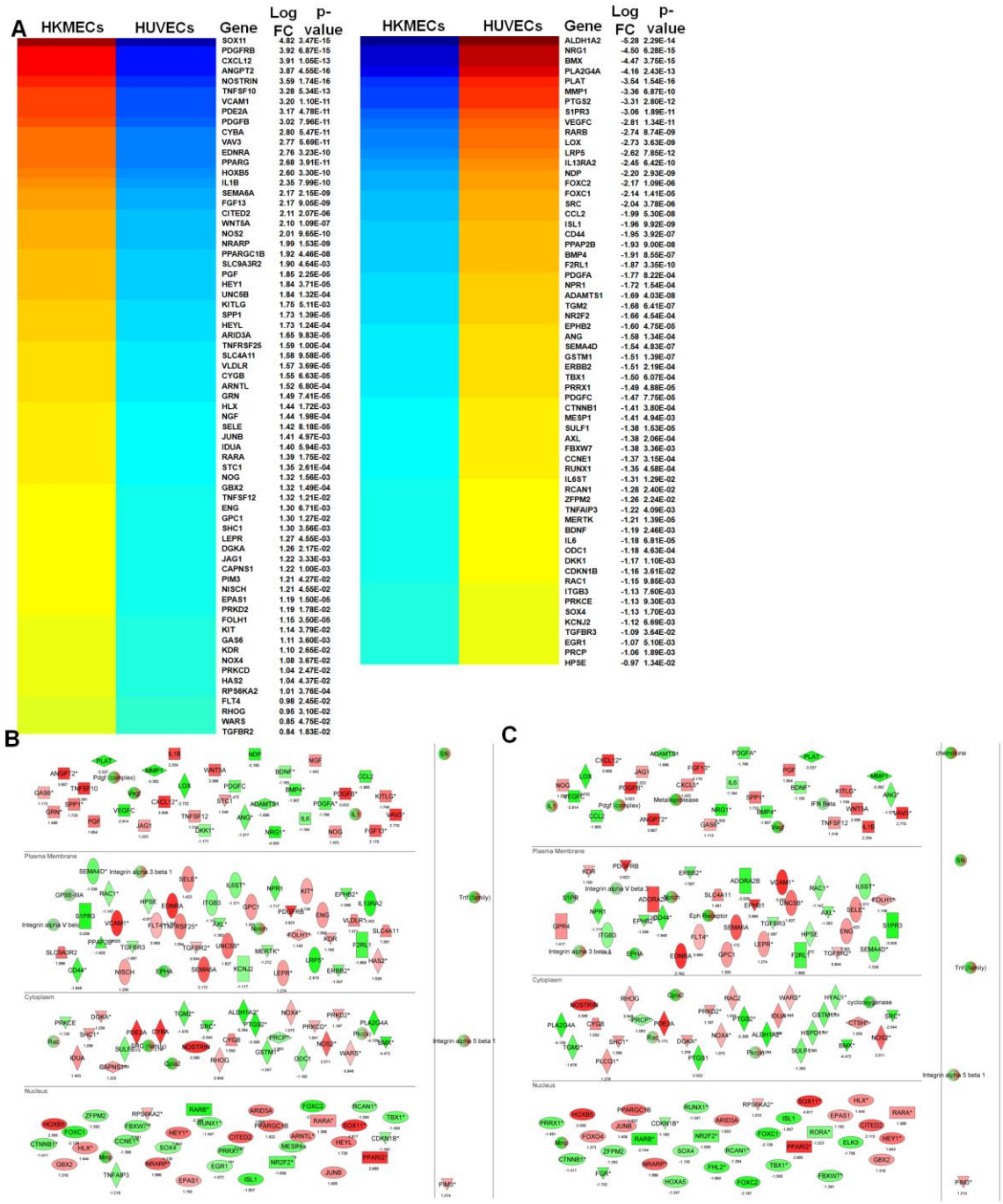
Supplementary Figures:



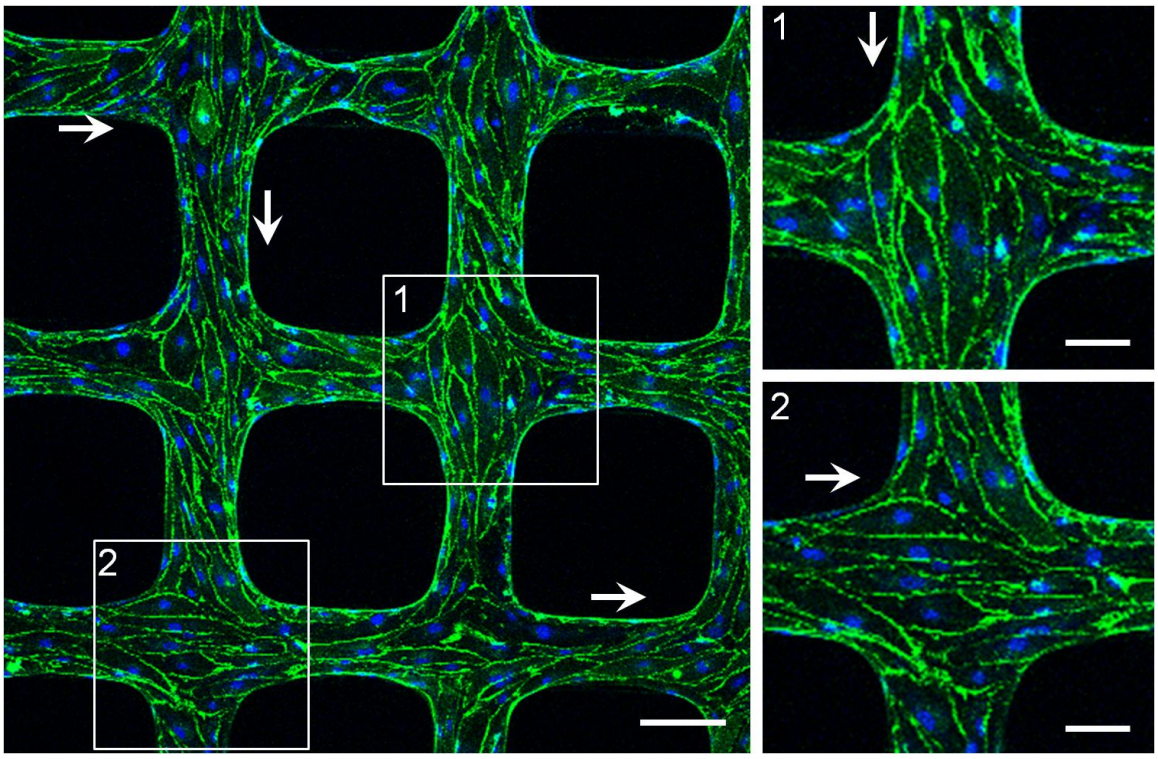
Supplementary Fig. 1. *Immunohistochemistry of the peritubular microvasculature in human adult kidney tissues.* **A.** VE-Cadherin (red) and nuclei (blue). **B.** CD34 (red) and nuclei (blue). **C-D.** CD34 (red), PDGFRβ (green) and nuclei (blue).



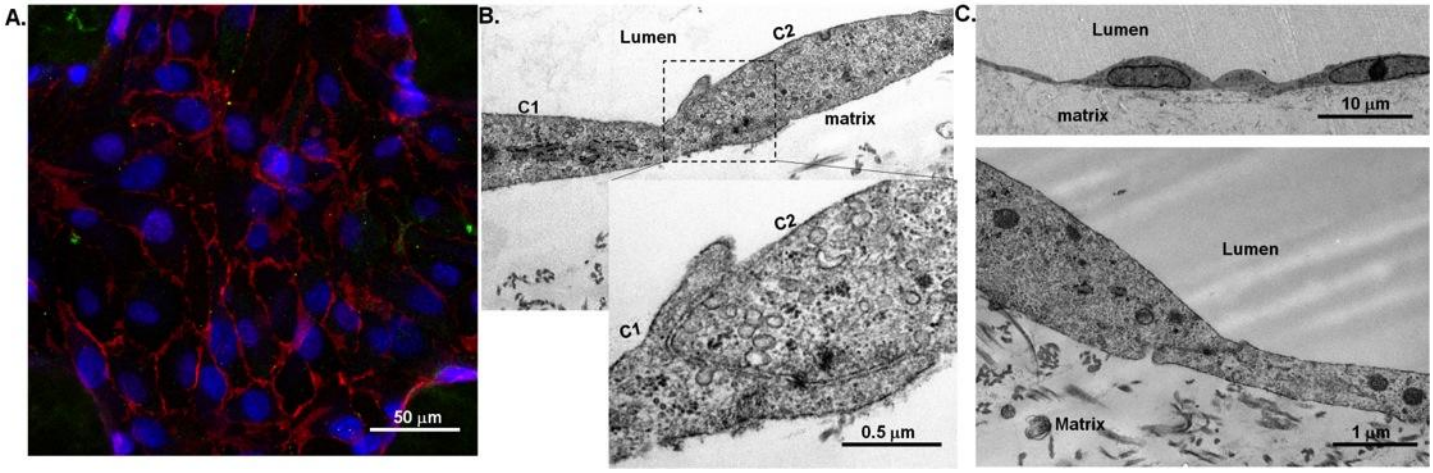
Supplementary Fig. 2. Isolated HKMECs showed strong expression of Claudin 5 (left panel), CD31 (middle panel) and VEGFR2 (right panel).



Supplementary Fig. 3. *Microarray analysis comparing HKMECs with HUVECs*. A. Expression panels for genes involved in angiogenesis function with significant changes comparing HKMECs vs. HUVECs. **B**. Ingenuity Pathway Analysis (IPA) for tubulogenesis function with genes categorized on subcellular domains. Red: upregulation, and green: downregulation. **C**. IPA analysis for angiogenesis function with genes categorized on subcellular domains. Red: upregulation, and green: downregulation.



Supplementary Fig. 4. *Engineered kidney peritubular microvascular network*. Projection of confocal image of a vessel network formed by mouse KMECs with magnified view of boxes 1 and 2 in right panels. Green: CD31 and blue: nuclei. Scale bar: 100 μm (left panel) and 50 μm (right panels).



Supplementary Fig. 5. *Engineered HUVEC microvessels*. **A**. z-stack projection of confocal image of half of a vessel network formed by HUVECs at a cross junction. The culture conditions were the same as with HKMECs including 20ng/mL of VEGF. Red: CD31, green: PV1, and blue: nuclei. **B-C**. Transmission electron micrograph of HUVEC microvessels showing a continuous endothelial cell membrane and focal junctions near the cell-cell contacts between two neighboring cells C1 and C2.

Supplementary Table: Primer designs for human genes used for RT-PCR and real time q-PCR.

Human Genes for RT-PCR	**Forward Primer**	**Reverse Primer**
PECAM	5' GAA GTC CGG ATC TAT GAC TCA G 3'	5' GTG AGT CAC TTG AAT GGT GCA 3'
VE-Cad	5' TGG AGA CTC CTT CCA GCT TCA 3'	5' GCT TCC ACC ACG ATC TCA TAC 3'
VEGFR2	5' CTG GCG GCA CGA AAT ATC CTC TTA 3'	5' GGC CGG CTC TTT CGC TTA CTG TTC 3'
VWF	5' ATG ATT CCT GCC AGA TTT GC 3'	5' GGC TCA CTC TCT TGC CAT TCT GG 3'
PDGFBB	5' AAG TGT GAG ACA GTG GCA GCT GC 3'	5' GCT TGA ATT TCC GGT GCT TGC CC 3'
CD45	5' CAT GTA CTG CTC CTG ATA AGA C 3'	5' GCC TAC ACT TGA CAT GCA TAC 3'
CD146	5' AAG GCA ACC TCA GCC ATG TCG 3'	5' CTC GAC TCC ACA GTC TGG GAC 3'
Tie2	5' TGT TCC TGT GCC ACA GGC TG 3'	5' CAC TGT CCC ATC CGG CTT CA 3'
Robo4	5' CCC TGT GCT TGG AAC TCA GTG 3'	5' CGC TGA TGT ACC CAT AGG TGG 3'
E-Cad	5' TGC TCT TGC TGT TTC TTC GG 3'	5' TGC CCC ATT CGT TCA AGT AG 3'
α-SMA	5' TGG CTA TTC CTT CGT TAC TA 3'	5' CGA TCC AGA CAG AGT ATT TGC 3'
β-actin	5' CCT CGC CTT TGC CGA TCC 3'	5' GGA ATC CTT CTG ACC CAT GC 3'
Human Genes for Real time q-PCR	**Forward Primer**	**Reverse Primer**
MMP1	5'- CAT GAC TTT CCT GGA ATT GG -3'	5'- CCT GCA GTT GAA CCA GCT AT -3'
RGS5	5'- GAA AGG GCC AAG GAG ATT -3'	5'- TCA AAG CTG CTC AGG GAA -3'
CD34	5'- AAT GAG GCC ACA ACA AAC AT -3'	5'- ACA GGC TAG GCT TCA AGG TT -3'
ANGPT2	5'- GAT GAT CCG ACC AGC AGA TT -3'	5'- GGT CTT GCT TTG GTC CGT TA -3'
CXCL12	5'- GTG ATT GCC TCT GAA GCC TA -3'	5'- AAT GTC ACC TTG CCA ACA GT -3'
DLL4	5'- AGA TGC AAT ACC CTT CCA CA -3'	5'- GCC ACT CTC TCT GGA AAA CA -3'
JAM2	5'- TAC CAC CAC ACC TGG CTA AT -3'	5'- TCA GTG GGT GCT TAA TCC AT -3'
VEGFR2	5'- GTT CTT GGC TGT GCA AAA GT -3'	5'- GTC TTC AGT TCC CCT CCA TT -3'
PDGFB	5'- TTC CTC CCC ATA CTC CAC TC -3'	5'- CCC TGG CCT CTA GTC TTC TG -3'
PLVAP	5'- TGA CCA AGG AGC TCA ACT TC -3'	5'- GCA GCT CTT GTT CAT GTC CT -3'
β-ACTIN	5'- GGA CTT CGA GCA AGA GAT GG -3'	5'-AGC ACT GTG TTG GCG TAC AG -3'

Supplementary Information - Methods

Kidney tissue process. Fresh kidney tissue was finely cut in a 100 mm petri dish using a razor blade in 1 mL of cold Dulbecco's modified Eagle's medium **(**DMEM/F12 Medium, GIBCO) containing 1% penicillin-streptomycin (PS, GIBCO), 0.2 mg/mL Liberase DL (Roche Applied Science) and 100 U/mL DNase I (Roche Applied Science) then transferred in 50 mL tubes and incubated at 37 °C for 30 min in a shaking water bath. The enzymatic digestion was inactivated by adding 2.5 mL of DMEM/F12 medium containing 10% fetal bovine serum (FBS, GIBCO). The single cell suspension was passed two times through a 40 µm cell strainer (Fisher) to remove glomeruli and multicellular debris. Cells were then centrifuged at 1300 rpm at 4 °C for 10 min, washed once in isotonic phosphate buffered solution (PBS) and resuspended in 0.5 mL of MACS buffer (miltenyibiotec). The single cell suspension was then depleted of epithelial cells using magnetic beads conjugated with antibodies against the epithelial cell marker CD326 (Miltenyibiotec). Epithelial depletion was performed according to the manufacturer's instructions (Miltenyibiotec). After epithelial depletion, remaining cells were cultured in T75 flasks coated with 0.2% gelatin (Sigma) in endothelial cell medium EBM-2 (GIBCO) containing 1% antibiotic-antimycotic (Life Technologies), 10% FBS, 100 µg/mL endothelial cell growth supplements (ECGS) (Sigma), 50 µg/mL Heparin (Sigma), and 40 ng/mL VEGF-A (R&D). Cells were then cultured for five days in a humidified incubator at 37 °C supplied with 5% O_2 and 5% CO_2 before proceeding with flow-cytometric sorting.

Flow Cytometric Analysis and Sorting. Human kidney endothelial cells were analyzed and sorted by using multicolor fluorescence-activated cell sorting (FACS). For analysis, the single cell suspension after tissue processing was prepared to obtain a cell pellets of the entire cell population. Cell pellets were resuspended in FACS buffer (1% BSA, 0.5 µM EDTA (pH7.4) in PBS) and incubated with -Fc blocking reagent (Biolegend) for 10 min on ice. Directly conjugated anti-CD45 (Biolegend), and anti-PECAM (BD Pharmingen, clone WM59) were added to the cell mixture at 1:100 dilutions and incubated for 20 min on ice. The stained samples were then fixed in 4% formaldehyde and ready for flow-cytometric analysis using BD FACS Aria II (BD Biosciences).

To sort the HKMECs, cell mixtures grown in T75 flasks were detached using 0.05% trypsin/EDTA, centrifuged and washed once in cold PBS. Cell pellets were then resuspended in FACS buffer (1% BSA, 0.5 μM EDTA (pH7.4) in PBS) and incubated with Fc blocking reagent (Biolegend) for 10 min on ice. Directly conjugated anti-CD45 (Biolegend), and PECAM (BD Pharmingen, clone WM59) were added to the cell mixture at 1:100 dilutions and incubated for 20 min on ice. After incubation, cells were washed with ice-cold FACS buffer twice, followed by FACS sorting using BD FACS Aria II (BD Biosciences).

Primary Cell cultures. Primary HKMECs were cultured in T25 flasks coated with 0.2% gelatin and maintained in EBM-2 basal medium containing 1% antibiotic-antimycotic (Life Technologies), 10% FBS, 100 μg/mL ECGS, 50 μg/mL Heparin, and 20 ng/mL VEGF (R&D). Human umbilical vein endothelial cells (Lonza) were cultured in the same condition as HKMECs. Primary cells used in all experiments were between passages 0 and 5.

Collagen preparation. Collagen I stocks were prepared at 15 mg/mL in 0.1% acetic acid, as described previously[1]. Briefly, tendons were extracted from the rat tails, and dissolved in 0.1% acetic acid, followed by centrifugation and lyophilization to obtain a dry collagen powder. The dry collagen was resuspended in 0.1% acetic acid to reach a 15 mg/mL collagen stock solution. The neutralized liquid collagen solution was prepared on ice by mixing the neutralizing diluents, composed of 10X M199, 1N NaOH and supplemented 1X EBM, with the desired mass of collagen to reach the targeted concentration.

Microarray and PCR analysis. Total RNA was extracted from endothelial cells cultured in 6-well dishes using RNeasy kit (Qiagen). RNA was purified to reach 400ng at concentration above 50ng/mL for microarray studies using Illumina BeadChip Arrays (HumanHT-12 v4.0 Expression BeadChip Kit, BD-103-0204). Raw data (Idat files) were read into the R statistical software using the Bioconductor beadarray package [2]. The data

were then background corrected using negative control probes and quantile normalized using the neqc function from the Bioconductor limma package [3]. Comparisons between HKMECs and HUVECs were made by first fitting a weighted analysis of variance model, and then computing empirical Bayes adjusted contrasts, using the Bioconductor limma package[4]. Genes were selected based on a multiplicity-adjusted p-value[5] < 0.05, and an absolute fold change of 1.5. The core analysis feature of the Ingenuity Pathway Analysis software (http://www.ingenuity.com/) was used to identify canonical pathways and other functional groups in the differentially expressed genes (>1.5-fold differential expression, false discovery rate =0.05). The microarray data has been submitted to NCBI's GEO data base and the reviewers can access the data via the following URL: http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?token=yjibeggsjxutbqv&acc=GSE65848.

For RT-PCR, cDNA was synthesized with SuperScript II reverse transcriptase (Invitrogen). PCR was performed for 30 cycles at 58 °C annealing temperature with Taq polymerase (Invitrogen), and PCR products were electrophoresed on 2% agarose gels. For real time q-PCR, total RNA was isolated from cells cultured in a well of six-well plate using an RNeasy mini kit (QIAGEN) with on-column DNase digestion (QIAGEN) to remove genomic DNA. 2ul of total RNA was applied onto NanoDrop 1000 Spectrophotometer for quantification. First-strand cDNA was synthesized by iScript reverse transcription supermix (Bio-Rad Laboratories). Next, 10ng of cDNA was used in a 10ul reaction with Power SYBR® Green PCR Master Mix [Applied Biosystems (ABI)] to determine mRNA level of specific genes using β-Actin as an internal control. using a model 7900HT Real-Time PCR System [Applied Biosystems (ABI)]. Comparative CT method was used to analyze the relative quantification of gene expression between the samples. The primers used for PCR are listed in the Supplementary Table. Each set of oligonucleotides was designed to span two different exons to avoid PCR products from genomic DNA contamination.

Immunofluorescence staining and analysis. *Cell immunostaining*: Endothelial cells were plated on coverslips and cultured in 24-well dishes for 48 h. Cells were then fixed in 4% PFA for 10 min, washed several times with PBS and in the case of PV1 and Cav1,

blocked and incubated with anti-PLVAP (Abcam), anti-Cav1 (Abcam) overnight, otherwise permeabilized in 0.1% Triton X-100 for 10 min. Cells were then blocked in a buffer containing 2% BSA, for one hour and incubated with the antibodies against the endothelial cell marker CD31 (Abcam), VE cadherin (Abcam), claudin-5 (Abcam) overnight at 4^{o}C and then washed in PBS several times. Following rinsing, cells were then incubated in Alexa Fluor 647-phalloidin (Life Technologies), or the secondary antibodies PE-conjugated anti-VEGFR2 (R&D Systems), FITC-conjugated anti-VWF (Abcam), goat anti-mouse IgG Alexa488 (Invitrogen) and goat anti-rabbit IgG Alexa568 (Invitrogen), and Hoechst (Life Technologies) as a nucleic counterstain, all diluted in blocking buffer. Coverslips were mounted on slides and were analyzed by immunofluorescence microscopy.

Tissue immunostaining: fresh tissues embedded in OCT were frozen by immersion in methanol cooled in dry ice. Sections (8μm) were cut on a cryostat and dried for 20 min at room temperature (RT). Sections were then fixed in 4% PFA (Sigma) for 10 min, washed three times in PBS and in the case of PV1 and Cav1, blocked and incubated with anti-PLVAP, anti-Cav1 at 4^{o}C overnight, or permeabilized in 0.1% Triton X-100 (Sigma) for 10 min and then blocked with blocking buffer containing 2% BSA in PBS for 1 h at RT. Primary antibodies against CD31 (Abcam), VE cadherin (Abcam), and PDGFRβ (Abcam) were then incubated at 4^{o}C overnight. After rinsing, sections were incubated for 1 hour at room temperature with the secondary antibodies PE-conjugated anti-CD34 (BD Biosciences), FITC-conjugated anti-VWF (Abcam), goat anti-mouse IgG Alexa Fluora 488 (Invitrogen) and goat anti-rabbit IgG Alexa568 (Invitrogen) diluted in blocking buffer. Nuclei were stained with Hoechst (Molecular Probes) diluted in blocking buffer.

3D gel immunostaining: In the studies of tubulogenesis and angiogenesis assays, fixed 3D gel cultures in PDMS wells were washed with PBS for three times, followed by incubation in buffer with blocking and membrane permeabilization reagent (2% BSA and 0.1% Triton X-100) for 30 min. Following rinse, the bulk gel were incubated with working solutions of Alexa Fluora 488 Phalloidin (Life Technologies) and Hoechst (Life Technologies) for 2 hours to stain the cytoskeleton and nuclei in the tubulogenesis assay.

The gels for angiogenesis assays were stained against junction protein CD31 following standard staining procedure described above and nuclei was costained with Hoechst.

Microvessel immunostaining: After each experiment, microvessels were perfused with 4% formaldehyde for 20 min to perform histological analyses. Staining of microvascular networks followed standard immunohistochemistry methods described above, utilizing the inlet/outlet for solution exchange. Primary antibodies against CD-31 (Abcam), VE cadherin (Abcam), PLVAP (Abcam), and Cav1 (Abcam) were used. Vessels were incubated in Alexa Fluor 647-phalloidin (Life Technologies), or the secondary antibodies PE-conjugated anti-VEGFR2 (R&D Systems), FITC-conjugated anti-VWF (Abcam), goat anti-mouse IgG Alexa488 (Invitrogen) and goat anti-rabbit IgG Alexa568 (Invitrogen), and Hoechst (Life Technologies) as a nucleic counterstain.

Transmission electron microscopy preparation and imaging: Microfluidic vascular networks were fixed by perfusing with half-strength Karnovsky's solution (2% paraformaldehyde/2.5% glutaraldehyde in 0.2 M cacodylate buffer) for 20 min followed by full immersion in the same fixative solution overnight. These samples were rinsed in 0.1 M cacodylate buffer then post-fixed using 2% OsO_4 in 0.2 M cacodylate buffer followed by another rinse with 0.1 M cacodylate buffer. Sample dehydration was performed using immersions in graded solutions of ethanol, then propylene oxide (PO), before 1:1 PO/Epon 812 (Ted Pella Inc., Redding, CA) immersion overnight. Fresh Epon 812 was then exchanged for 2 h after which the blocks were cured for 48 h at 60 ^{o}C. Ultrathin sections (70 nm) were cut from blocks using a diamond (Diatome US, Hatfield, PA) blade on a Leica EMUC6 ultra-microtome and placed onto grids. Grids were stained with uranyl acetate for 2 h and lead citrate for 5 min. Sections were imaged using a JEOL JEM-1400 Transmission Electron Microscope (JEOL Ltd., Japan) using a typical acceleration voltage around 100 kV. Images were acquired with a Gatan Ultrascan 1000XP camera (Gatan, Inc., Pleasanton, CA).

References:

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