Supplemental Material

Sustained inflammation after pericyte depletion induces irreversible blood-retina barrier breakdown

Shuntaro Ogura, Kaori Kurata, Yuki Hattori, Hiroshi Takase, Toshina Ishiguro-Oonuma, Yoonha Hwang, Soyeon Ahn, Inwon Park, Wataru Ikeda, Sentaro Kusuhara, Yoko Fukushima, Hiromi Nara, Hideto Sakai, Takashi Fujiwara, Jun Matsushita, Masatsugu Ema, Masanori Hirashima, Takashi Minami, Masabumi Shibuya, Nobuyuki Takakura, Pilhan Kim, Takaki Miyata, Yuichiro Ogura, and Akiyoshi Uemura

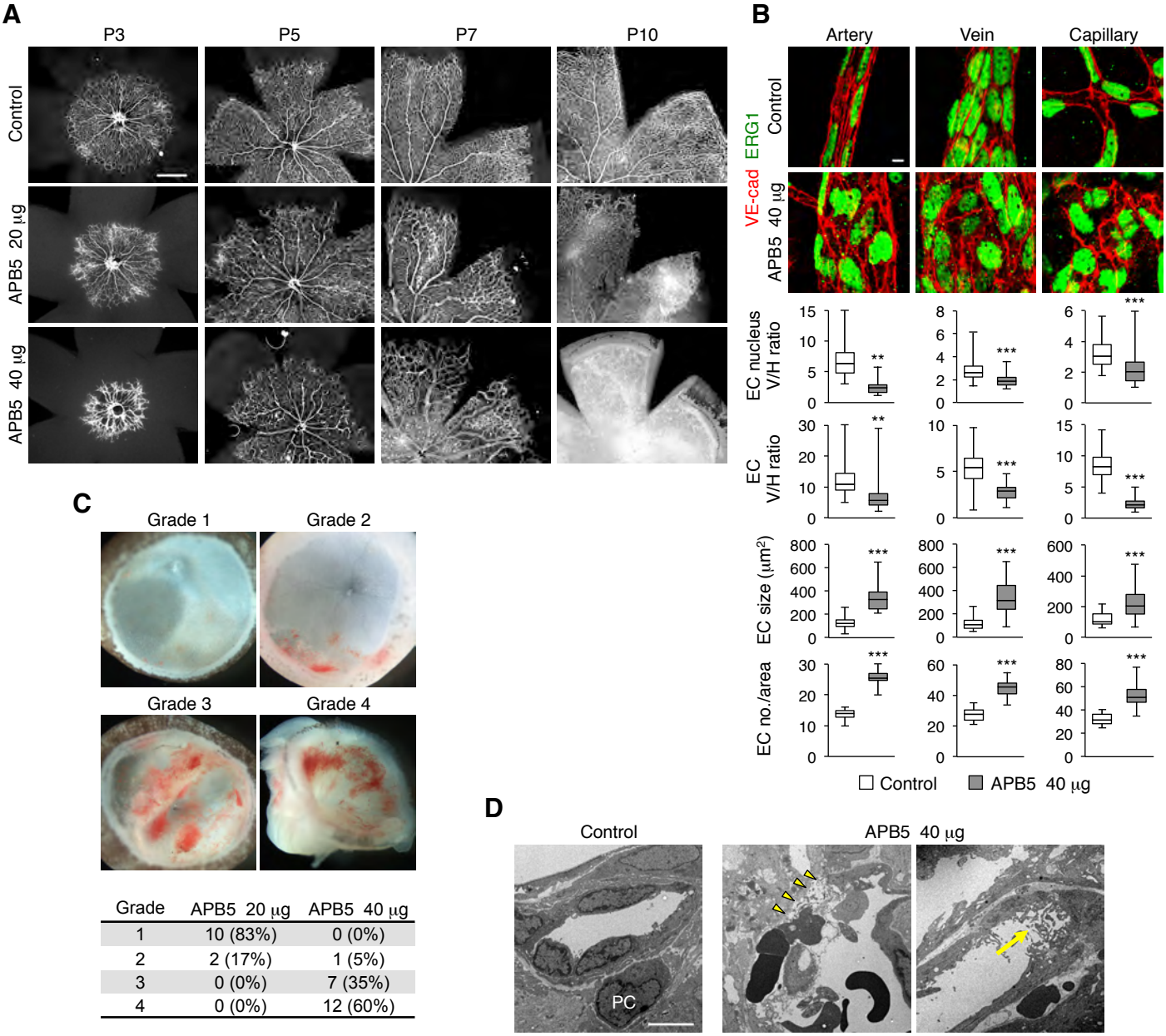
Supplemental Video 1. Aneurysm-like structures in PC-free retinas. 3D movies were reconstructed from confocal microscopy images in P8 whole-mount retinas after i.p. injections of control PBS (left) or 40 μg of APB5 (right) at P1. ECs were labeled for CD31 (green) and ERG1 (red). Note the EC accumulations in the aneurysm-like structures without vertical sprouting.

Supplemental Video 2. Ex vivo retina imaging of P8 *Cx3cr1-GFP* mice. ECs, microglia, and MPs were labeled with isolectin B4 (red), after i.p. injections of control PBS (left) or 50 μg of APB5 (right) at P1. Scale bar, 100 μm. The time is shown in h and min.

Supplemental Video 3. Intravital retina imaging in 4-wk *Cx3cr1-GFP* mice. Blood vessels were fluorescently labeled by i.v. perfused Evans Blue dye (red), after i.p. injections of control PBS (left) or 40 μg of APB5 (right) at P1. The skeletal structure and the centroid of each GFP^+ cell in the raw time-series images (upper) were automatically analyzed by using IMARIS software (lower). Scale bar, 100 μm. The time is shown in h and min.

Supplemental Video 4. Ex vivo retina imaging of P8 *Cx3cr1-GFP* mice combined with VEGF Trap treatment. ECs, microglia, and MPs were labeled with isolectin B4 (red), after i.p. injections of 50 μg of APB5 at P1. After 3-h imaging (left), the retinas were incubated with nonspecific human IgG (upper) or VEGF Trap (lower), and further monitored for 3 h (right). Scale bar, 50 μm. The time is shown in h and min.

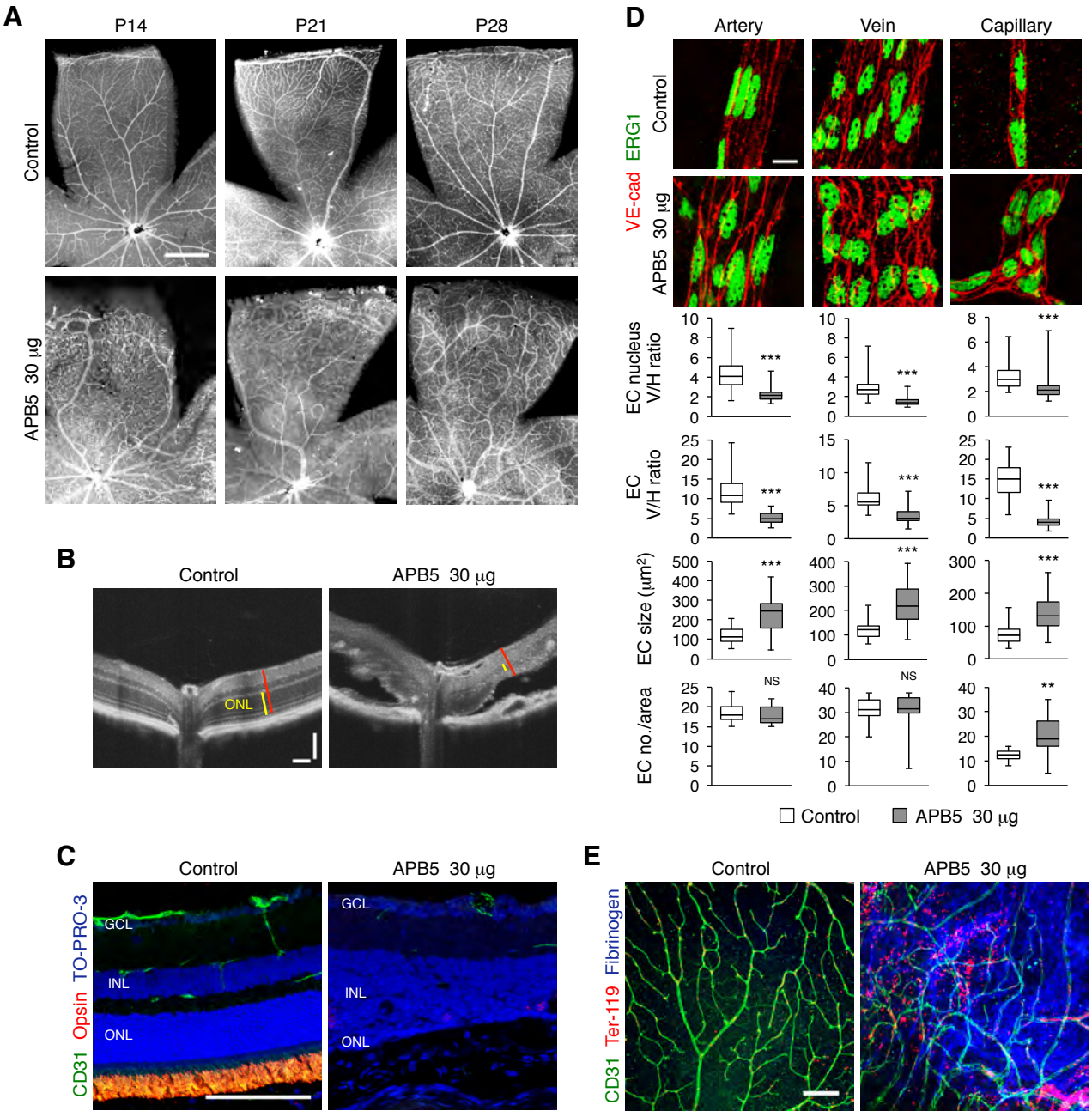
Supplemental Figure 1



Grade	APB5 20 μg	APB5 40 μg
1	10 (83%)	0 (0%)
2	2 (17%)	1 (5%)
3	0 (0%)	7 (35%)
4	0 (0%)	12 (60%)

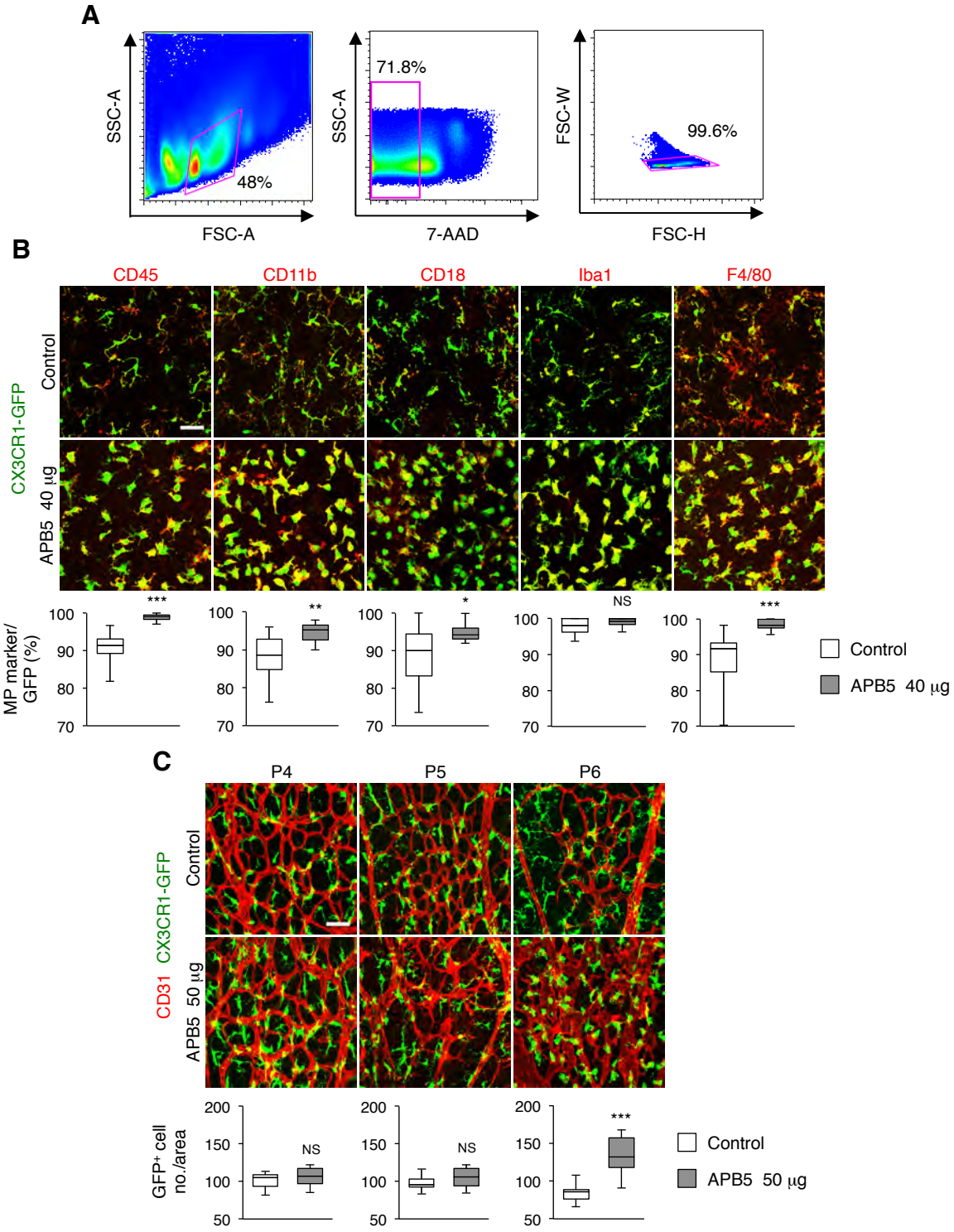
Supplemental Figure 1. Defective retinal vascular development after transient PDGFRβ blockade. (**A**) Panoramic views of retinas labeled for CD31. Note the intense autofluorescence due to elevated vascular leakage and hemorrhage in P10 retinas after APB5 injections. (**B**) IHC for VE-cadherin and ERG1 in P8 retinal vessels. The graphs show quantification of the vertical/horizontal (V/H) ratio of the nucleus ($n = 40$) and cell body ($n = 40$), the squared cell size ($n = 40$), and the cell number ($n = 16$ areas) of ECs. $^{**}P < 0.01$, $^{***}P < 0.001$ (two-tailed Student's *t*-test). (**C**) Representative retina cups at P10. Severity scores of the retinal edema and hemorrhage are defined as follows: Grade 1, no hemorrhage and edema; Grade 2, local hemorrhage and/or mild edema; Grade 3, edema up to half of the whole retina; Grade 4, collapse of the retina. (**D**) TEM of P8 retinas, without biotin tracer perfusion. Note the rupture (arrowheads) and villous protrusions (arrow) of EC luminal surfaces in APB5-treated retinas. Scale bars, 500 μm (**A**); 5 μm (**B** and **D**).

Supplemental Figure 2



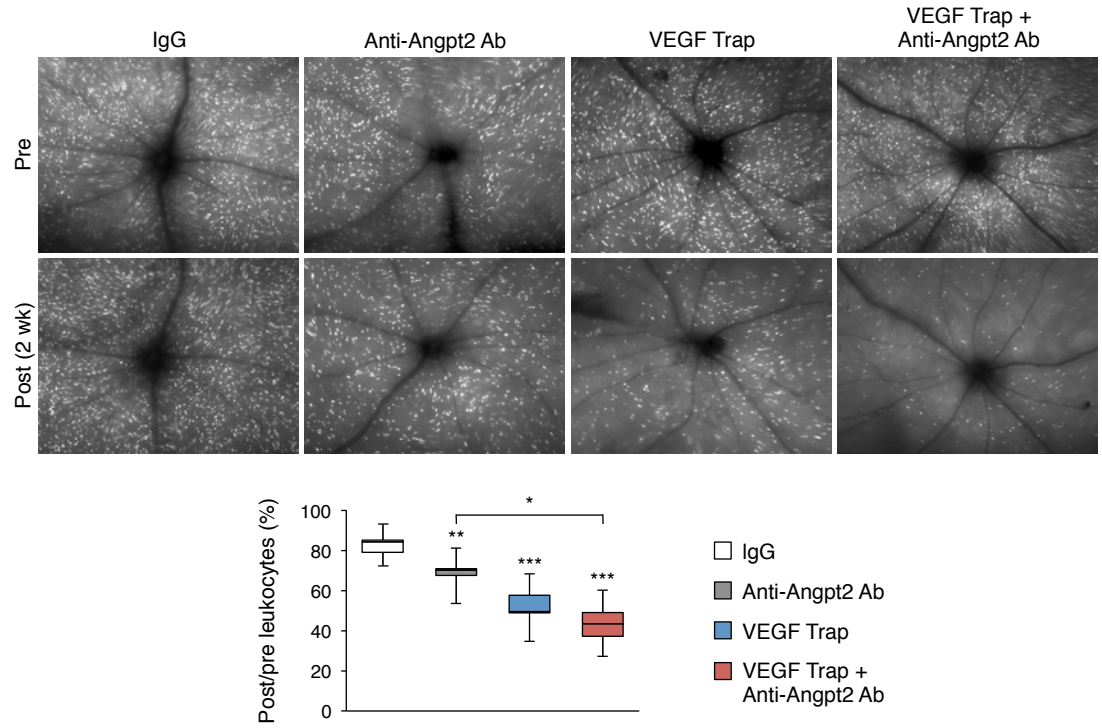
Supplemental Figure 2. Sustained retinal abnormalities after transient inhibition of PC recruitment. (**A**) Panoramic views of retinas labeled for CD31. (**B**) OCT of 4-wk retinas. The thicknesses of the retinas (red lines) and the ONL (yellow lines) were measured. (**C**) IHC for CD31 and opsin with nuclear labeling in cryosections of 4-wk retinas. Note the absence of the intermediate and deep vascular layers in the APB5-treated retina. (**D**) IHC for VE-cadherin and ERG1 in 4-wk retinal vessels. The graphs show quantification of the V/H ratio of the nucleus (n = 40) and cell body (n = 40), the squared cell size (n = 40), and the cell number (n = 16 areas) of ECs. (**E**) IHC for CD31, Ter-119, and fibrinogen in 4-wk retinas. $^{**}P < 0.01$, $^{***}P < 0.001$ (two-tailed Student's t-test). Scale bars, 500 μm (**A**); 100 μm (**B**, **C**, and **E**); 10 μm (**D**).

Supplemental Figure 3



Supplemental Figure 3. Inflammation in PC-deficient retinas. (**A**) The gating scheme of FACS analyses in P8 retinas. Putative live cells were gated as shown in the left panel. After removal of dead cells labeled with 7-aminoactinomycin D (7-AAD), the doublet cells were eliminated utilizing forward scatter height (FSC-H) versus forward scatter width (FSC-W) gates. (**B**) IHC for leukocyte and MP markers in retinas of P8 *Cx3cr1-GFP* mice. The graphs show the proportions of individual marker expression in GFP$^+$ cells (n = 12 areas). (**C**) Labeling for CD31 and GFP in retinas of *Cx3cr1-GFP* mice. Note the significant increase of amoeboid MPs in the APB5-treated retina at P6. The graphs show the number of GFP$^+$ cells per area (n = 12). $^*P < 0.05$, $^{**}P < 0.005$, $^{***}P < 0.001$ (two-tailed Student's t-test). Scale bars, 50 μm (**B** and **C**).

Supplemental Figure 4



Supplemental Figure 4. Serial monitoring of retinal inflammation after intravitreal drug injections. Acridine orange labeling in retinas before and 2-wk after intravitreal drug injections to 4-wk mice treated with 20 μg of APB5 at P1. The graph shows the pre/post proportion of the number of leukocytes ($n = 6$ areas). $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ (one-way ANOVA).

Supplemental Table 1. Upregulated and downregulated genes encoding transporters and channels in PC-free ECs.

Probe Set ID	Gene Symbol	Gene Title	Fold Change	local FDR
Upregulated				
170276_at	*Slc25a37*	solute carrier family 25, member 37	7.26	0.08
116709_at	*Slc7a11*	solute carrier family 7 (cationic amino acid transporter, y^+ system), member 11	6.19	0.184
168633_i_at	*Slc25a29*	solute carrier family 25 (mitochondrial carrier, palmitoylcarnitine transporter), member 29	5.88	0.001
96955_at	*Atp6v0e2*	ATPase, H^+ transporting, lysosomal V0 subunit E2	5.28	0.039
105215_at	*Slc37a1*	solute carrier family 37 (glycerol-3-phosphate transporter), member 1	4.82	0.056
114264_at	*Abcf1*	ATP-binding cassette, sub-family F (GCN20), member 1	4.44	0.115
115314_at	*Slc35c1*	solute carrier family 35, member C1	4.04	0.115
92798_at	*Atp5c1*	ATP synthase, H^+ transporting, mitochondrial F1 complex, gamma polypeptide 1	3.95	0.227
160577_at	*Atp9a*	ATPase, class II, type 9A	3.94	0.112
134712_at	*Abca3*	ATP-binding cassette, sub-family A (ABC1), member 3	3.66	0.051
95744_at	*Atp6v1a*	ATPase, H^+ transporting, lysosomal V1 subunit A	3.32	0.141
163836_at	*Slc22a16*	solute carrier family 22 (organic cation transporter), member 16	3.18	0.153
113876_at	*Slc16a6*	solute carrier family 16 (monocarboxylic acid transporters), member 6	3.11	0.193
96365_at	*Slco6c1*	solute carrier organic anion transporter family, member 6c1	2.91	0.115
93330_at	*Aqp1*	aquaporin 1	2.88	0.22
139269_at	*Clcn2*	chloride channel 2	2.74	0.135
113013_s_at	*Slc4a4*	solute carrier family 4 (anion exchanger), member 4	2.48	0.105
166422_at	*Slc35f1*	solute carrier family 35, member F1	2.44	0.163
170096_r_at	*Slc35d3*	solute carrier family 35, member D3	2.42	0.088
105012_at	*Atp7a*	ATPase, Cu^{++} transporting, alpha polypeptide	2.41	0.012
103274_at	*Abcc8*	ATP-binding cassette, sub-family C (CFTR/MRP), member 8	2.33	0.03
97730_at	*Abca4*	ATP-binding cassette, sub-family A (ABC1), member 4	2.32	0.085
99909_at	*Trpc6*	transient receptor potential cation channel, subfamily C, member 6	2.23	0.234
134666_at	*Slc35d2*	solute carrier family 35, member D2	2.18	0.074
170010_r_at	*Slc25a33*	solute carrier family 25, member 33	2.18	0.111
112210_at	*Slc25a40*	solute carrier family 25, member 40	2.18	0.092
140487_r_at	*Kcnq5*	potassium voltage-gated channel, subfamily Q, member 5	2.15	0.11
93414_at	*Abcb1b*	ATP-binding cassette, sub-family B (MDR/TAP), member 1B	2.09	0.144
108568_at	*Slc35f5*	solute carrier family 35, member F5	2.07	0.139
162569_at	*Slc43a1*	solute carrier family 43, member 1	2.06	0.171
115576_at	*Slc4a8*	solute carrier family 4 (anion exchanger), member 8	2.05	0.089
164693_r_at	*Atp2a2*	ATPase, Ca^{++} transporting, cardiac muscle, slow twitch 2	2.04	0.211
Downregulated				
136025_at	*Slco1c1*	solute carrier organic anion transporter family, member 1c1	0.07	0.168
108891_at	*Cacna1e*	calcium channel, voltage-dependent, R type, alpha 1E subunit	0.24	0.067
117035_at	*Atp8a1*	ATPase, aminophospholipid transporter (APLT), class I, type 8A, member 1	0.25	0.132
133759_at	*Tmc7*	transmembrane channel-like gene family 7	0.36	0.143
100943_at	*Slc1a4*	solute carrier family 1 (glutamate/neutral amino acid transporter), member 4	0.36	0.102
110839_at	*Slc30a1*	solute carrier family 30 (zinc transporter), member 1	0.37	0.061
110292_at	*Slc38a3*	solute carrier family 38, member 3	0.38	0.196
166012_at	*Slc38a2*	solute carrier family 38, member 2	0.38	0.144
115753_at	*Slc22a8*	solute carrier family 22 (organic anion transporter), member 8	0.41	0.16
166840_r_at	*Cacnb4*	calcium channel, voltage-dependent, beta 4 subunit	0.42	0.054
113865_at	*Slc43a2*	solute carrier family 43, member 2	0.42	0.091
107490_at	*Kctd13*	potassium channel tetramerisation domain containing 13	0.43	0.144
109069_at	*Slc40a1*	solute carrier family 40 (iron-regulated transporter), member 1	0.43	0.162
164152_at	*Slc6a11*	solute carrier family 6 (neurotransmitter transporter, GABA), member 11	0.44	0.047
137348_at	*B830032F12 /// Kcnq3*	uncharacterized protein B830032F12 /// potassium voltage-gated channel, subfamily Q, member 3	0.47	0.171
138577_at	*Atp6v1b2*	ATPase, H^+ transporting, lysosomal V1 subunit B2	0.47	0.172
162738_at	*Tfrc*	transferrin receptor	0.48	0.004
166353_s_at	*Atp9a*	ATPase, class II, type 9A	0.48	0.114
105008_at	*Slc30a7*	solute carrier family 30 (zinc transporter), member 7	0.48	0.098
111439_at	*Atp11c*	ATPase, class VI, type 11C	0.49	0.136

Supplemental Table 2. List of antibodies and reagents.

Antibody/reagent	Clone	Manufacturer	Cat. No.	Host	Dilution/ dosage
IHC					
Angiopoietin-2	4H10	G.Y. Koh (KAIST)	NA	Humanized	1:200
αSMA Cy3	1A4	Sigma-Aldrich	C6198	Mouse	1:1000
Biotin Cy3	BN-34	Sigma-Aldrich	C5585	Mouse	1:1000
CD11b	M1/70	BD Biosciences	557394	Rat	1:500
CD18	M18/2	eBioscience	14-0181	Rat	1:500
CD31	Mec13.3	BD Biosciences	553370	Rat	1:500
CD31	2H8	Abcam	ab119341	Ar. Hamster	1:1000
CD45	30-F11	eBioscience	14-0451	Rat	1:500
Claudin-5 Alexa 488	4C3C2	Thermo Fisher Scientific	352588	Mouse	1:1000
Cleaved Caspase-3	5A1E	Cell Signaling Technology	9664S	Rabbit	1:500
ERG1	EPR3864	Abcam	ab92513	Rabbit	1:1000
ERG1 Alexa 488	EPR3864	Abcam	ab196374	Rabbit	1:200
F4/80	BM8	Abcam	ab16911	Rat	1:50
FOXO1	C29H4	Cell Signaling Technology	2880	Rabbit	1:100
ICAM-1	YN1/1.7.4	BioLegend	116102	Rat	1:500
Opsin	RET-P1	Sigma-Aldrich	O4886	Mouse	1:1000
PLVAP	MECA-32	BD Biosciences	550563	Rat	1:30
Ter-119 Biotin	TER-119	eBioscience	13-5921	Rat	1:500
Tie2 Biotin	TEK4	Abcam	ab95721	Rat	1:1000
TNFα	MP6-XT22	BioLegend	506308	Rat	1:200
VE-cadherin	11D4.1	BD Biosciences	555289	Rat	1:200
VEGFR2	Avas12a1	eBioscience	14-5821	Rat	1:500
Collagen IV	polyclonal	Cosmo Bio Co.	LB-1403	Rabbit	1:1000
Desmin	polyclonal	Abcam	ab15200	Rabbit	1:500
Esm1	polyclonal	R&D Systems	AF1999	Goat	1:200
Fibrinogen	polyclonal	Dako	A0080	Rabbit	1:200
GFP Alexa 488	polyclonal	Thermo Fisher Scientific	A-21311	Rabbit	1:1000
Iba1	polyclonal	Abcam	ab107159	Goat	1:1000
NG2	polyclonal	Merck Millipore	AB5320	Rabbit	1:1000
Pimonidazole	polyclonal	Hypoxyprobe Inc.	Pab2627	Rabbit	1:200
Tie1	polyclonal	R&D Systems	AF619	Goat	1:200
Isolectin B4 Biotin	NA	Sigma-Aldrich	L3759	NA	1:1000
TO-PRO-3 Iodide	NA	Thermo Fisher Scientific	T3605	NA	1:2000
FACS					
CD16/32	93	BioLegend	101330	Rat	1 μl/test
CD11b BB515	M1/70	BD Biosciences	564454	Rat	2.5 μl/test
CD45 APC/Cy7	30-F11	BioLegend	103115	Rat	2.5 μl/test
Ly-6C BV421	AL-21	BD Biosciences	562727	Rat	2.5 μl/test
Ly-6G/Ly-6C (Gr-1) BV510	RB6-8C5	BioLegend	108437	Rat	5 μl/test
Tie2 PE	TEK4	eBioscience	12-5987	Rat	10 μl/test
VEGFR1 APC	141522	R&D Systems	FAB4711A	Rat	10 μl/test
IP/IB					
Phosphotyrosine	PY20	Enzo Life Sciences	BML-SA240-1000	Mouse	1:1000
Tie2	polyclonal	Santa Cruz Biotech	sc-324	Rabbit	1:500
Tie2	polyclonal	R&D Systems	AF762	Goat	1 μg/sample

Supplemental Table 3. List of pRT-PCR primers.

Gene Symbol	Sense (5′ - 3′)	Antisense (5′ - 3′)
Angpt2	TCCAAGGCACTGAGAGACACC	AGCACTTCCTGATGTGGAAAGG
Ccl2	TAAAATTTCTTAAATGCAAGGTGTGGATCC	TGTTGAATCTGGATTCACAGAGAGGG
Il6	TGCCTAAGCATATCAGTTTGTGGAC	CAGGATATATTTTCTGACCACAGTGAGG
Pgf	TCCCTGCTGGTACCTACCCTC	CGTCATTCAGCAGGGACGAGTC
Tbp	CCCCCTCTGCACTGAAATCA	GTAGCAGCACAGAGCAAGCAA
Tnf	CCACTCTGACCCCTTTACTCTGAC	GATACAGACTGGGGCTCTGAG
Vegfa	TCCCTCGTGGGACTGGATTC	GGCTTGGCGATTTAGCAGCAG
Tie1	GGCCGGAGCAAATTGCTTCCAC	CAGAGGTGAGAAGGGTCCAAAG
Tie2	TGTGCTGTGTTTGGGACCCTC	GAGTCAGAACACACTGCAGATCC

Tbp, TATA box-binding protein