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Supplemental Information

Derivation of Brain Capillary-like Endothelial Cells from Human Pluripotent Stem Cell-Derived Endothelial Progenitor Cells

Catarina Praça, Susana C. Rosa, Emmanuel Sevin, Romeo Cecchelli, Marie-Pierre Dehouck, and Lino S. Ferreira

SUPPLEMENTAL INFORMATION

5 Supplemental data:

- S1, related to Figure 2.
- S2, related to Figure 3.
- S3 and S4, related to Figure 4.
- Table S1, related to Figure 3.

Supplemental Experimental Procedures

Supplemental References, related to Supplemental Experimental Procedures

3 Supplemental Tables

- Table S2, sequence of primers used in this study.
- Table S3, primary and secondary antibodies used in this study.
- Table S4, main products used in this study.

Supplemental data items

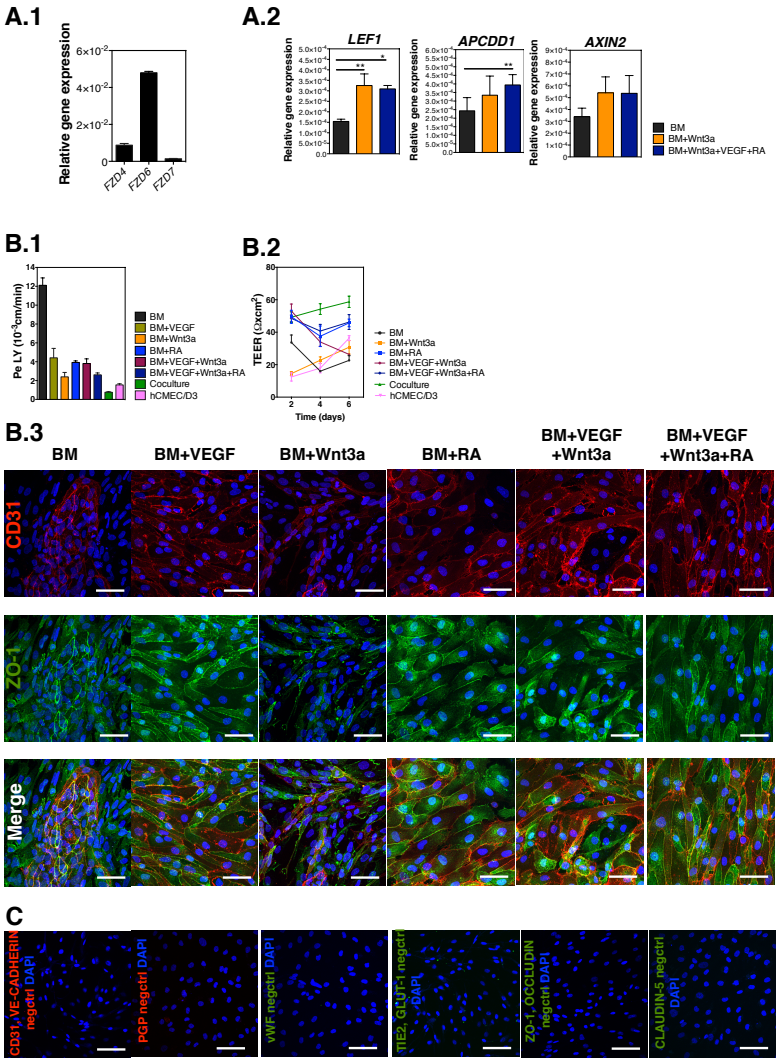

Figure S1 – Gene expression of WNT pathway genes and functional characterization of non-purified endothelial cells after 6 days of culture in a transwell insert (related to Figure 2).
(A.1). Relative gene expression of frizzled receptors (*FZD4*, *FZD6* and *FZD7*) in CD31-ECs at passage 1. Genes were normalized against the control gene *ACTB*. Mean ± SEM (n=1 independent experiment, 3 technical replicates per experimental condition).
(A.2) Relative expression of *LEF1*, *AXIN2* and *APCDD1* genes in CD31^{+} cells at passage 1 exposed to basal medium (BM) or to Wnt3a (BM+Wnt3a or BM+VEGF+Wnt3a+RA; 10 ng/mL of Wnt3a) for 24 h. Genes were normalized against the control gene *ACTB*. Mean ± SEM (n=3 independent experiments, 3 technical replicates per experimental condition). **P<0.05* and ***P<0.01* using one-way ANOVA followed by Dunnett's multiple comparison test.
(B.1) Paracellular permeability of cells to LY after 6 days of culture on Matrigel-coated filters. As controls we have used the hCMEC/D3 cell line and human hematopoietic progenitor cells derived ECs cocultured with pericytes (Coculture condition). Values are Mean ± SEM (n=2 independent experiments, at least 3 transwell inserts per independent experiment and experimental condition).
(B.2) TEER at different time points for all the conditions tested on Matrigel-coated filters. As controls we have used the hCMEC/D3 cell line and human hematopoietic progenitor cells derived ECs cocultured with pericytes (Coculture condition). Values are Mean ± SEM (n=2 independent experiments, at least 3 transwell inserts per independent experiment and experimental condition).
(B.3) Co-localization of CD31 and ZO-1 markers in the cells after 6 days of culture on Matrigel-coated filters. Scale bar is 50 µm.
(C) Controls (without primary antibodies) for immunostaining analyses. Scale bar is 50 µm.

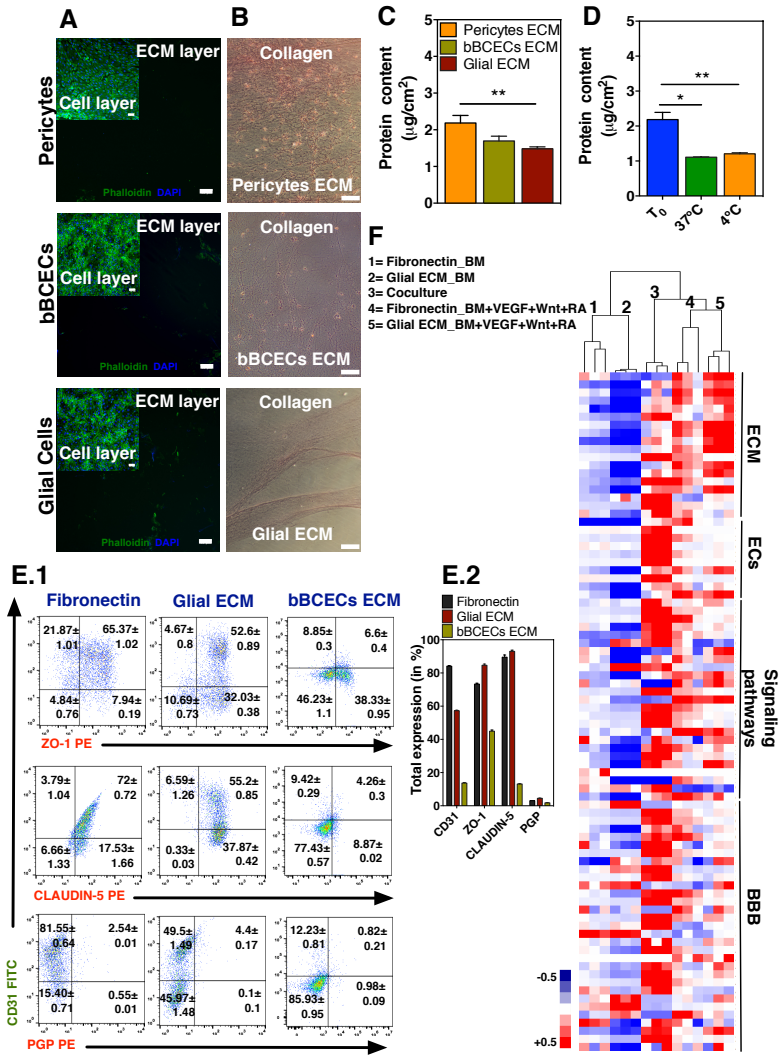

Figure S2 – Isolation and characterization of decelullarized ECM from cells of the neurovascular unit and characterization of BCLECs maturated in these matrices in the presence of soluble factors (related to Figure 3).
(A) Evaluation of the decellularization protocol efficacy by staining with DAPI and phalloidin. Scale bar is 50 µm.
(B) Evaluation of collagen by Sirius red assay. Scale bar is 100 µm.
(C) Comparison of ECM production from bovine pericytes, bovine brain capillary endothelial cells (bBCECs) and rat glial cells at day 8. Values are Mean ± SEM (n= 3 independent experiment, 3 individual wells per experimental condition). ***P<0.01* using one-way ANOVA followed by Tukey's multiple comparison test.
(D) Stability of the native pericytes ECM at 37°C and 4°C in terms of maintenance of the collagen content. Values are Mean ± SEM (n= 3 independent experiment, 3 individual wells per experimental condition). **P<0.05* and ***P<0.01* using One-way Anova followed by Tukey's multiple comparison test.
(E.1) Expression by flow cytometry of BBB markers (ZO-1, CLAUDIN-5 and PGP) co-localized with CD31 marker in cells maturated on fibronectin or decellularized matrices at passage 4. Percentages of positive cells were calculated based in the isotype controls (1%).
(**E.2**) Total expression of each cell marker in the different assessed conditions. Values are Mean ± SEM (n=1 independent experiment, 3 individual wells per experimental condition).
(**F**) Heatmap and hierarchical clustering dendogram in gene expression results obtained by Fluidigm**.** Relative gene expression levels on cells cultured on BM or BM+VEGF+Wnt3a+RA either on fibronectin or glial ECM (positive control was "coculture" condition, i.e., human hematopoietic stem/progenitor-derived ECs cocultured with pericytes, please see ref (Cecchelli et al., 2014) followed by their plating on Matrigel-coated transwell inserts for 6 days. Genes were normalized against the control gene *ACTB* (n=1 independent experiment, 3 technical replicates per experimental condition). For each gene, the normalized values were between +0.5 and -0.5 (please see Supplementary Information for further information). Gene expression that was above the gene expression median for all the experimental groups was shown in red, the expression below the median in blue and the expression similar to the median in white.

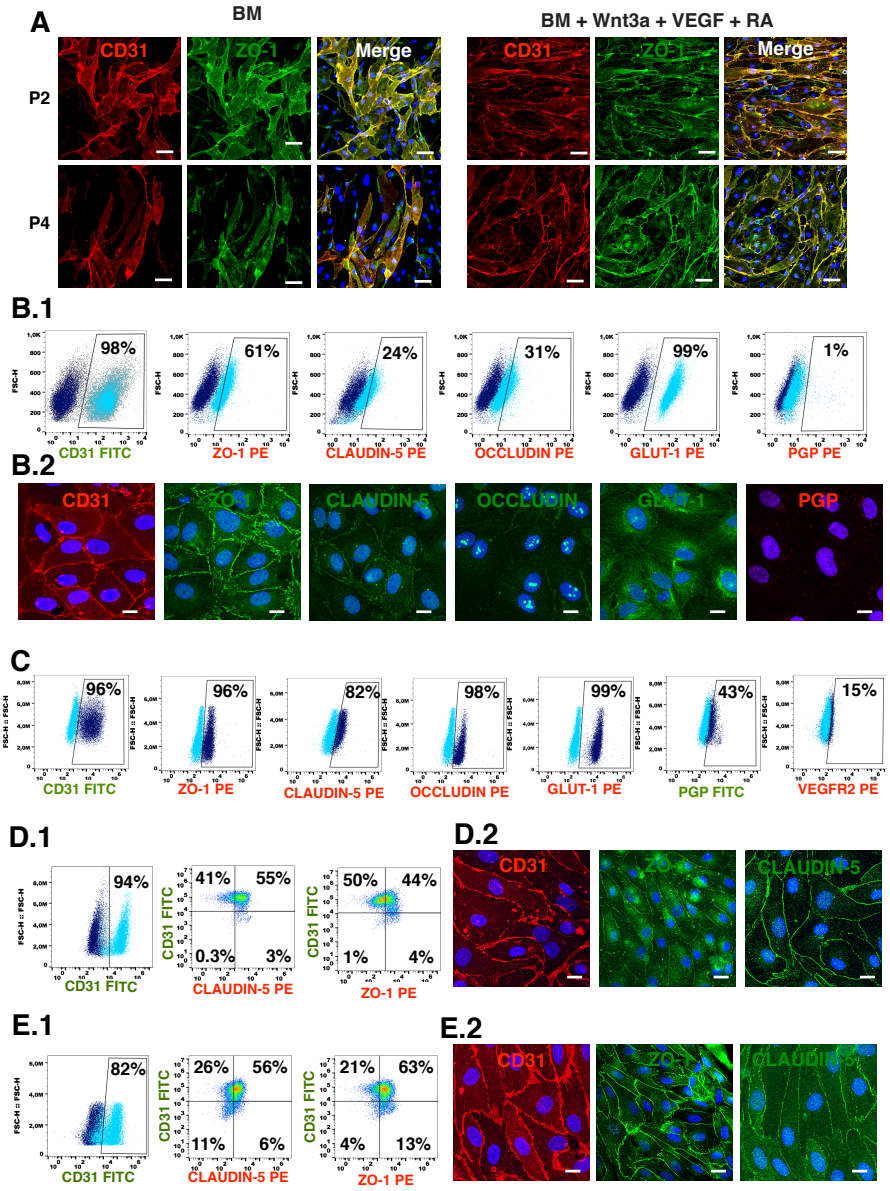

Figure S3 – Characterization of BCLECs and control cells (related to Figure 4).
(A) Immunofluorescence analyses for the co-localization of CD31 with ZO-1 proteins in BCLECs at passage 2 and 4 on fibronectin-coated plates. Scale bar is 50 µm.
(B.1) Expression of BCEC markers in HUVECs by flow cytometry. Percentage of positive cells were calculated based in the isotype controls (1%; dark blue scatter plot).
(B.2) Immunofluorescence analyses of BCEC markers in HUVECs. Scale bar is 50 µm.
(C) Expression of BCEC markers in hCMEC/D3 by flow cytometry. Percentage of positive cells were calculated based in the isotype controls (1%; light blue scatter plot).
(D.1) Characterization of BCLECs derived from a human embryonic stem cell line (hESCs-NKX2-$^{5eGFP/W}$) by flow cytometry. Cells were cultured in BM+VEGF+Wnt3a+RA media for 4 passages. Percentages of positive cells were calculated based in the isotype controls (1%).
(D.2) Characterization of BCLECs derived from a human embryonic stem cell line (hESCs-NKX2-$^{5eGFP/W}$) by immunofluorescence. Immunofluorescence analyses for CD31, ZO-1 and CLAUDIN-5 on cells platted on Matrigel-coated filters. Scale bar is 50 µm.
(E.1) Characterization of BCLECs derived from iPSCs cell line (HGPS-iPSCs) by flow cytometry. Cells were cultured in BM+VEGF+Wnt3a+RA media for 4 passages. Percentages of positive cells were calculated based in the isotype controls (1%).
(E.2) Characterization of BCLECs derived from a iPSCs cell line (HGPS-iPSCs) by immunofluorescence. Immunofluorescence analyses for CD31, ZO-1 and CLAUDIN-5 on cells platted on Matrigel-coated filters. Scale bar is 50 µm.

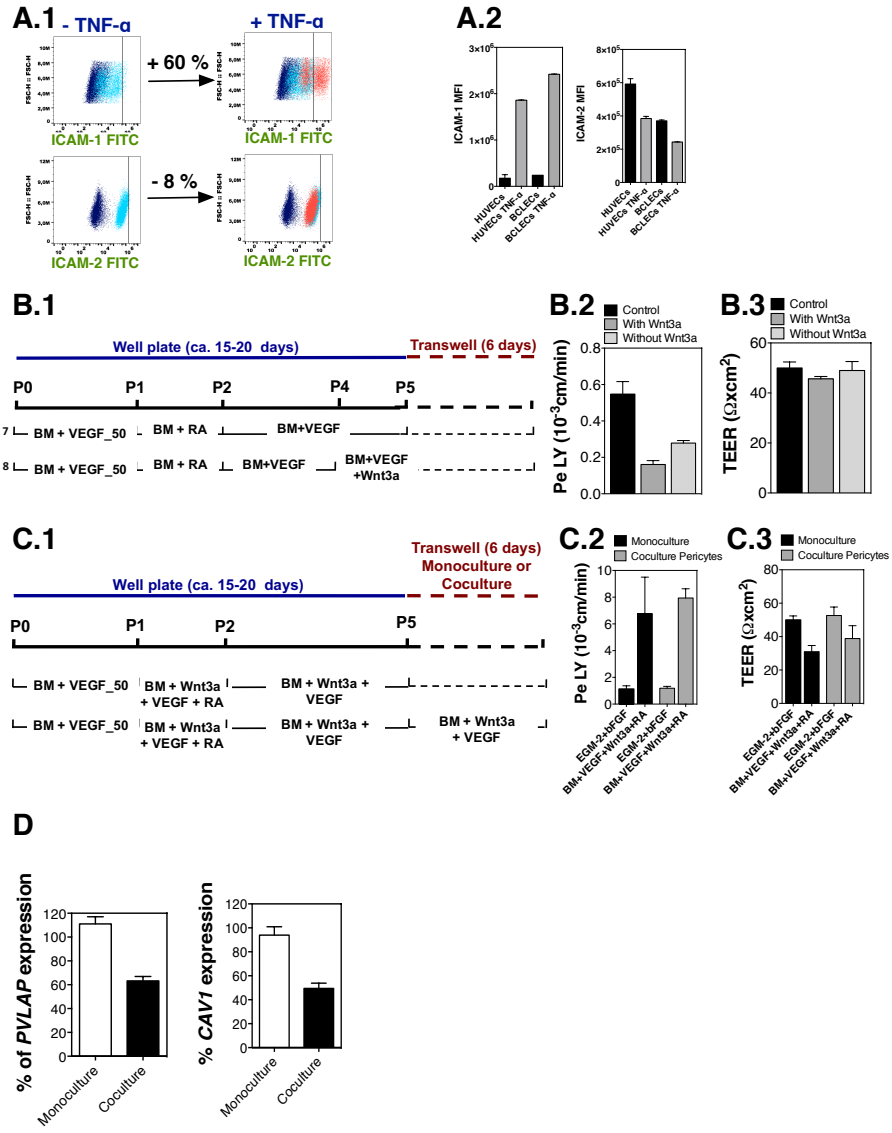

Figure S4 – Functional Characterization of BCLEC (related to Figure 4).

(**A.1**) Expression of adhesion molecules (ICAM-1 and ICAM-2) in HUVECs at basal level and after exposure to TNF-α (10 ng/mL) for 24 h, by flow cytometry. Percentage of positive cells were calculated based in the shift of the basal expression for each marker (1%; light blue scatter plot).

(**A.2**) Expression of ICAM-1 and ICAM-2 by mean intensity fluorescence (MFI; flow cytometry) in HUVECs and BCLECs at basal level and after exposure to TNF-α (10 ng/mL) for 24 h. Results are Mean ± SEM (n=1 independent experiment, 3 technical replicates per experimental condition).

(**B.1**) Protocol used to assess the impact of Wnt3a at later stages of the specification.

(**B.2**) Paracellular permeability to LY at day 6 in the transwell system. Results are Mean ± SEM (n=1 independent experiment, with 3 transwell inserts per experimental condition). Control are BCLECs derived with protocol 6.

(B.3) TEER at day 6 in the transwell system. Results are Mean ± SEM (n=1 independent experiment, with 3 transwell inserts per experimental condition). Control are BCLECs derived with protocol 6.

(C.1) Protocol used to assess the impact of pericytes coculture.

(**C.2**) Paracellular permeability to LY at day 6 in the transwell system. Results are Mean ± SEM (n=1 independent experiment, with 3 transwell inserts per experimental condition).

(C.3) TEER at day 6 in the transwell system. Results are Mean ± SEM (n=1 independent experiment, 3 filters per condition).

(**D**) Relative gene expression of *PVLAP* and *CAV1* in BCLECs (cells differentiated with BM+VEGF+Wnt3a+RA) in filters in monoculture or coculture with pericytes at day 6. Data was normalized by day 3 in filters for each condition. Genes were normalized against the control gene *ACTB*. Results are Mean ± SEM (n=1 independent experiment, 3 technical replicates per experimental condition).

Table S1 – Relative gene expression data for heatmap (in Excel format). The gene expression was normalized against the endogenous control *(ACTB*) and presented as relative gene expression**.** Related with Figure 3.

SUPPLEMENTAL EXPERIMENTAL PROCEDURES

Cultivation of pluripotent stem cells and differentiation into EPCs.
Pluripotent stem cells were kindly donated from several collaborators. K2-iPSC cell line generated from human cord blood (passages: 30-40) (Haase et al., 2009) and Hutchinson-Gilford Progeria Syndrome (HGPS) cell line generated from fibroblasts (passages: 43-51) (Nissan et al., 2012). For certain experiments the human embryonic stem cell line hESCs-NKX2-5$^{eGFP/W}$ (passages: 40-45) (Elliott et al., 2011) was used. Pluripotent stem cells were grown on mitomycin C-inactivated mouse embryonic fibroblast feeder layer in undifferentiating culture medium [(knockout Dulbecco's Modified Eagle Medium; DMEM (GIBCO)], 20% knockout serum replacer (Gibco), non-essential amino acids (1%, GIBCO), β–mercaptoethanol (0.1 mM, Sigma), L-glutamine (1 mM, Sigma), Pen/Strep (50 U/mL:50 μg/mL, Lonza) and fibroblast growth factor-basic (bFGF; 5 ng/mL, Preprotech). iPSCs (at passages 27-35) were passaged every 3-4 days with collagenase IV (1mg/mL, Gibco) at a typical split ratio of 1:4 or 1:6. To initiate the differentiation, iPSCs (27×10^3 – 45×10^3 cells per cm^2) were treated for 45-60 min with collagenase IV and plated on fibronectin-coated dishes (1 μg/cm^2; Calbiochem) in a chemically defined medium (CDM) (Vallier et al., 2009) containing Iscove's Modified Dulbecco's Medium (IMDM; 50% (v/v), Gibco), F12 (50%, Gibco), BSA (5 mg/mL, Sigma), β-mercaptoethanol (0.1 mM, Sigma), Pen/Strep (50 U/mL:50 μg/mL, Lonza), transferrin (15 mg/mL, Sigma) and insulin (7 mg/mL, Sigma). Immediately after cell seeding, to induce mesoderm differentiation, cells were exposed to bone morphogenetic protein 4 (BMP4, 10 ng/mL, Peprotech) for 1.5 days and then BMP4 (50 ng/mL, Peprotech) with bFGF (20 ng/mL, Peprotech) for 3.5 days. To differentiate mesoderm progenitor cells into endothelial cells, cells were cultured with vascular endothelial growth factor ($VEGF_{165}$;50 ng/mL, Peprotech) and thymosin β4 (Tβ 4; 100 ng/mL, Caslo peptide synthesis). CD31$^+$ cells were isolated by Magnetic-Activated Cell Sorting (MACS; Miltenyi Biotec) (Rosa et al., 2019). CD31$^+$ enrichment was confirmed by flow cytometry analyses using a different anti-CD31 antibody (eBiosciences), with a positivity superior to 90%.

Preparation of decellularized ECM.
We have prepared decellularized ECM from three different primary cells types: bovine pericytes, bovine brain capillary endothelial cells (bBCECs) and rat glial cells. Cells were platted on fibronectin-coated dishes (25 μg/mL; 1.7 μg/cm^2 of fibronectin adsorbed to the culture dish as quantified by immunocytochemistry) at a cell density of $3x10^4$ cells per cm^2 during 8-12 days to allow extracellular matrix deposition. Cell layers were then decellularized using a solution of 20 mM ammonium hydroxide (Sigma) in PBS supplemented with 0.5% Triton X-100 (Fluka). After 1 min with agitation in contact with the solution, the resulting ECM layers were washed 2x with PBS and 1x with EGM-2. ECM layers were either used immediately or stored at 4°C. Approximately 10% (i.e. 0.17 μg/cm^2 of fibronectin) of the initial bovine fibronectin was still adsorbed to the cell culture dish after the decellularization (data not shown).

Endothelial permeability measurements.
To perform the assay, 10 $x10^4$ cells were seeded on a 12-well 0.4 μm filters (Costar) coated with Matrigel and kept in culture with EGM-2 supplemented with bFGF (1 ng/mL) for 6 days. Before initiating the permeability experiment, EBM-2 was added to empty wells of a 12-well plate. Filter inserts containing the BCLECs were placed in the multi-well and filled with EBM-2 containing the fluorescent integrity marker Lucifer yellow (LY; 20 μM, Sigma). The plates were placed on an orbital shaker for 1 h and then withdraw from the receiver compartment. The fluorescence of the samples (inserts with cells and without cells) was quantified using the wavelengths 430/530 (excitation/emission). The permeability values were generated through the blue-norna brain exposure simulator (http://www.blue-norna.com).

TEER analyses.
BCLECs TEER (Ohmxcm^2) on Transwell filters was measured using the Millicell-ERS 2 (Electrical Resistance System, Millipore). The resistance of Matrigel-coated inserts was subtracted from the resistance obtained in the presence of the endothelial cultures according to the followed equation: TEER = [(TEER, cells)-(TEER, insert)×A], where A is the area of the filter (cm^2).

Dil-acLDL uptake
Cells were platted at a density of 10 $x10^4$ cells and 24 h later Dil-acLDL (Harbor Bio-Products, 20 μg/mL) was added to the cells for 4 h at 37ºC. At the end, Hoechst dye (0.25μg/ml) was added to stain cell nuclei. Cells were washed and kept in EGM-2. Images of the cells were acquired by a InCell Analyser HCA System.

Immunocytochemistry analyses.
Cells were fixed in cold methanol/acetone (50%/50%, v/v) for 5 min or with 4% (v/v) paraformaldehyde (PFA; electron Microscopy Science) for 10 min at room temperature and permeabilized with Triton X-100 (0.1%, v/v, Fluka) for 10 min, whenever required. The cells were then blocked with BSA (1%, w/v, Sigma) solution for at least 30 min followed by incubation with primary antibody (Table S3) during 1 h at RT. After washing, the cells were stained with secondary antibody (Table S3) for 30 min in dark at room temperature. The nuclei of the cells were counterstained with DAPI

(Sigma) and cells mounted with cell-mounting medium (DAKO). All the photos were taken using confocal microscopy (Zeiss) with an 40x oil objective.

Flow cytometry analyses.
Cells were dissociated from the culture plate by exposure to Cell Dissociation Buffer (Life Technologies) for 10 min and gentle pipetting, centrifuged and ressuspended in PBS supplemented with 5% (v/v) FBS (GIBCO). The single cell suspensions were aliquoted, fixed with ice-cold methanol/acetone (50%/50% v/v) or 1% (v/v) PFA and permeabilized with 0.5% (v/v) Tween, whenever necessary. The cells were stained with specific primary antibodies (Table S3). Cells were further incubated with the secondary antibody when necessary. For the co-localization experiments, ZO-1 and claudin-5 primary antibodies were conjugated with a R-Phycoerythrin (Abcam) dye to facilitate the setup of the experiment. Percentages of positive cells were calculated based in the isotype controls (1% of overlap with the isotype scatter plot). FACS Calibur and Accuri C6 were used for the acquisition and FlowJo was used for data analysis.

Expression of adhesion molecules in the BCLECs.
The expression of the adhesion proteins was assessed by flow cytometry. After the maturation of the cells for 4 passages in the presence of all soluble factors, the cells were purified for CD31 marker and plated on Matrigel-coated filters. After 5 days in the filters, the cells were exposed to Tumor Necrosis Factor-α (TNF-α) (10 ng/mL) for 24 h. The subsequent protocol was performed as previously described for flow cytometry experiments. The single cell suspensions were aliquoted and cells were stained with specific primary antibodies (Table S3). Cells were further incubated with the secondary antibody when necessary (Table S3). Non-treated cells were used to determine the basal expression of adhesion proteins. HUVECs were used as a control.

Total RNA extraction and quantitative real-time polymerase chain reaction (qRT-PCR) analyses.
Total RNA was isolated with a RNeasy Micro Kit (Quiagen) and quantified by a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Inc., USA) at 260 nm. The cDNA was reverse transcribed from total RNA using TaqMan reverse transcription reagents kit (Invitrogen) according to manufacturer instructions. qPCR reactions (10 µL) were prepared using NZY Speedy qPCR Green Master Mix (2x, 5 µL) (Nzytech), primers at a final concentration of 400 nM (1 µL each sequence), water (2 µL) and cDNA (1 µL). The qPCR was run in CFX Connect Real-Time System (BioRad). qPCR reactions in triplicate were performed for each qPCR experiment. Ct data were obtained using Bio-Rad CFX Manager Software. Relative quantification of the target was calculated relative to the housekeeping gene *ACTB*. The $2^{-\Delta Ct}$ values (relative to the housekeeping gene) were used for the heatmap and hierarchical clustering analyses. The genes and primers sequences are given in Table S2.

qPCR using the high-throughput platform BioMarkTM HD System –Fluidigm
The oligos were designed for human transcripts and were synthesized by Sigma. Each RNA sample was diluted to the same concentration (15 ng/µL) and 1 µL was used to perform Retro transcription reactions. Fluidigm Reverse Transcription Master Mix (1 µL) was added to diluted RNA sample (1 µL) and water (3 µL) to a final volume of 5 µL. To perform the Pre-amplification (PA) of the cDNA samples a pool of primers was prepared. The primers were dissolved at a concentration of 100 µM in water. For each assay, a Primer Pair Mix was prepared containing 50 µM Forward Primer and 50 µM Reverse Primer. In order to prepare 10 × Pre-amplification Primer Mix (500 nM each primer), 10 µL of each of the 96 Primer Pair Mixes (50 µM each primer) was mixed with 40 µL buffer consisting of 10 mM Tris–HCl, pH 8.0; 0.1 mM EDTA; 0.25% Tween-20. In order to prepare 10× Assay (5 µM each primer) each Primer Pair Mix was diluted by mixing 10 µL Primer Pair Mix (50 µM each primer) with 90 µL buffer consisting of 10 mM Tris–HCl, pH 8.0; 0.1 mM EDTA; 0.25% Tween-20. PA reaction for each cDNA sample was prepared by mixing PreAmp Master Mix (1 µL, Fluidigm), pooled primers (500 nM, 0.5 µL), H_20 (2.25 µL) and cDNA (1.25 µL). Thermal cycling was performed according to the manufacturer for 18 PA cycles. The samples were then treated with Exonuclease (New England Biolabs) to remove non-hybridized primers. The Fluidigm® 96.96 Gene expression IFC was used with EvaGreen chemistry. After a prime of the chip, a 10× assay mix and sample mix were prepared and pipetted into the inlets. The qRT-PCR on the preamplified samples was then carried out with a single pair of primers specific for the target, so non-specific interactions were likely negligible. The chip was loaded and data was collected using the BioMark HD™. Data were analyzed using Fluidigm® Real Time PCR Analysis v2.1 software and genes were normalized against *ACTB*. Genes with melting curves displaying more of one peak (amplification of non-specific products) were not included in the analysis. The genes and primers sequences are given in Table S2.

Heatmap and hierarchical clustering analyses
The data processed by the Cluster 3.0 program was initially centered and then normalized. First, each individual gene expression ($2^{-\Delta Ct}$) (e.g. gene A) was subtracted to the gene expression median (e.g. gene A) for all experimental conditions. At the end, this creates a range of values for each gene so that the median value for all the experimental conditions is 0. Second, the centered values of each gene were normalized, by multiplying each centered value by a scale factor

(calculated by the program; a separate scale factor is computed for each gene) so that the sum of the squares of each value, for all experimental conditions, is 1.0. Therefore, for each gene the normalized values were between +1 to -1. The expression that is above the median is shown in red, the expression below the median in blue, and the expression similar to the median in white. The assembling of the data in terms of heatmap was performed in Java TreeView v1.1 software. The relative gene expression per condition for Figure 3B is given in Table S1.

Stain for actin cytoskeleton and nuclear DAPI.
To visualize the actin cytoskeleton and nuclear DAPI in a confluent cell layer and in ECM layer post decellularization, we fixed the plates with 4% PFA and permeabilize with 0.1% Triton X-100. Samples were then incubated with phalloidin-fluorescein (50 µg/mL, Sigma) for 40 min and washed twice. The nuclei were counterstained with DAPI and kept in mounting medium. All the photos were taken with the objective of 20x in the In Cell Analyzer 2200.

Stain for collagen and non-collagenous proteins.
Total amount of collagen in the decellularized ECM was quantified using Sirius Red/Fast Green Collagen staining kit (Chondrex) according to the manufacturer's instructions. The amount of sulfated glycosaminoglycans (sGAG) was quantified by staining with 1,9-dimethylmethylene blue dye (Blyscan Glycosaminoglycan Kit, Biocolor).

Coculture experiments.
Bovine pericytes, characterized elsewhere (Cecchelli et al., 2014), were immortalized using a SV40 large T antigen strategy (P7-P10). Immortalized cells were defrosted into a 100-mm gelatin-coated petri dish and cultured in Dulbecco's Modified Eagle's Medium (DMEM; Sigma) supplemented with FBS (20%, v/v), L-glutamine (2 mM), geneticin (200 μg/mL, G418, Sigma) and bFGF (1 ng/mL). Pericyte culture was confluent within 2-3 days. Once cells reached confluency, 45 $x10^3$ were seeded into each well of 12-well plates (Costar). Endothelial cells cultured in 100-mm fibronectin-coated dishes for 4 passages with protocol 6 were trypsinized and purified using CD31 MACS beads. Cells were seeded at a cell density of 10 $x10^4$ in 0.4 µm filters (Costar) coated with Matrigel and kept in culture with EGM-2 supplemented with bFGF (1 ng/mL) for 6 days.

Multidrug resistance accumulation assay.
Six days after being plated on polycarbonate filters, endothelial cell monolayers were washed with pre-warmed HEPES-buffered Ringer´s (RH) solution (NaCl 150 mM, KCl 5.2 mM, $CaCl_2$ 2.2 mM, $MgCl_2$ 0.2 mM, $NaHCO_3$ 6 mM, Glucose 2.8 mM, HEPES 5 mM) with 0.1 % human serum albumin (Sigma). Cells were incubated with RH solution containing Rhodamine123 at a final concentration of 5 µM with or without Pgp inhibitor (0.5 µM elacridar). After 2 h in shaking conditions, Transwell filter with monolayer cells were placed on ice and the cells were washed five times with ice-cold RH solution. Cells were then lysed with RIPA lysis buffer (Millipore) for 3 min at 37°C and 200 µL were transferred to a 96 well-plate for measurements. The fluorescence of the samples was quantified using the wavelengths 501/538 (excitation/emission). Data were normalized against the control (cells without elacridar).

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SUPPLEMENTAL TABLES

Table S2 - Details of primers for qRT-PCR and Fluidigm. Related to Figure 2, Figure 3, Figure 4, Figure S1 and Figure S4.

GENE	SENSE	ANTISENSE
ABCB1	TGAATCTGGAGGAAGACATGAC	CCAGGCACCAAAATGAAACC
ABCC1	AATAGAAGTGTTGGGCTGAG	CGAGACACCTTAAAGAACAG
ABCC2	ATATAAGAAGGCATTGACCC	ATCTGTAGAACACTTGACCA
ABCC4	AATCTACAACTCGGAGTCCA	CAAGCCTCTGAATGTAAATCC
ABCC5	ATTCTGATGTGAAACTAACAG	TTCCTATGTCGATATCCTTC
ABCC8	GATCATTGTGGGTGTGATTC	AGCCAGTAGAATGATGACAG
ABCG2	CAAGATGATGTTGTGATG	GATTCGTCATAGTTGTTG
ACTB	CGTCTTCCCCTCCATCGT	GATGGGGTACTTCAGGGTGA
APCDD1	GGAGTCACAGTGCCATCACAT	CCTGACCTTACTTCACAGCCT
AXIN2	AAAGAGAGGAGGTTCAGATG	CTGAGTCTGGGAATTTTTCTTC
CADH5	CGCAATAGACAAGGACATAAC	TATCGTGATTATCCGTGAGG
CAV1	GATTGACTTTGAAGATGTGATTG	AGAGAATGGCGAAGTAAATG
CCND3	AGACCAGCACTCCTACAG	GGCTTAGATGTGGTGTGG
CD31	AGATACTCTAGAACGGAAGG	CAGAGGTCTTGAAATACAGG
CD34	TGAAGCCTAGCCTGTCACCT	CGCACAGCTGGAGGTCTTAT
CDK4	TACCTGAGATGGAGGAGTC	GCAGAGATTCGCTTGTGT
CDK6	GAAAAGTGCAATGATTCTGGA	GAAGCGAAGTCCTCAACA
CLDN1	GAAAGACTACGTGTGACA	GGTCCTAATGTTAATGATAGTATC
CLDN3	ATCACGTCGCAGAACATC	TACACCTTGCACTGCATCTG
CLDN5	TTAACAGACGGAATGAAGTT	AAGCGAAATCCTCAGTCT
COL4A1	AAAGGGAGATCAAGGGATAG	TCACCTTTTTCTCCAGGTAG
COL4A2	AAAAGGAGATAGAGGCTCAC	GTATTCCGAAAAATCCAGCC
DAAM1	GAAGAAGAAAAGCATTCCTCAG	CAGTTTGTTCTCGGGCAG
ENOS	AACGTGGAGATCACCGAG	GGGCAGAAGGAAGAGTTC
SELE	AGCTTCCCATGGAACACAAC	CTGGGCTCCCATTAGTTCAA
FN1	CCATAGCTGAGAAGTGTTTTG	CAAGTACAATCTACCATCATCC
FZD4	TACCTCACAAAACCCCCATCC	GGCTGTATAAGCCAGCATCAT
FZD6	TCGTCAGTACCATATCCCATG	CCCATTCTGTGCATGTCTTTT
FZD7	GATGATAACGGCGATGTGA	AACAAAGCAGCCACCGCAGAC
HES1	GCCTATTATGGAGAAAAGACG	CTATCTTTCTTCAGAGCATC
HEY1	CCGGATCAATAACAGTTTGTC	CTTTTTCTAGCTTAGCAGATCC
INSR	TGTTCATCCTCTGATTCTCTG	GCTTAGATGTTCCCAAAGTC
HO1	GAAAAGCACATCCAGGCAAT	GCTGCCACATTAGGGTGTCT
HSPG2	CCACTACTTCTATTGGTCCC	GTATTGGATTGGTGGAGATTAC
ITGA1	CAGGTTGGAATTGTACAGTATG	TGTCTATTCCAAGAGCTGTC

ITGA3	AGGTAATCCATGGAGAGAAG	GTAGAAGTTCTCATCCACATC
ITGA4	AAAGCTTGGATCGTACTTTG	CTCTTCCTTCCTCTCTGATG
ITGA5	AAGCTTGGATTCTTCAAACG	TCCTTTTCAGTAGAATGAGGG
ITGA6	AAATACCAAACCAACACAGG	TACTGAATCTGAGAGGGAAC
ITGB3	AATCTGCTGAAGGATAACTGT	CTCTGGGGACTGACTTGA
ITGB4	ATCTGGACAACCTCAAGAAG	GCCAAATCCAATAGTGTAGTC
JAG1	GTCTCAAAGAAGCGATCAG	ATATACTCCGCCGATTGG
LAMA4	GAAATTGCATTTGAAGTCCG	ACCTGTCCATTTTTCATGTG
LAMA5	ATCCTATGACTTCATCAGCC	TTGTTATAGAAGAGGGAGAGG
LAMB1	GTGTGTATAGATACTTCGCC	AAAGCACGAAATATCACCTC
LAMC1	TCTCCTCTACCTTTCAGATTG	GGTTCTGACCATAACTCAAC
LDLR	GCCATTGTCGTCTTTATGTC	AAACACATACCCATCAACGA
LEF1	AAGGAACACTGACATCAATT	TTTGGAACTTGGCTCTTG
LEPR	GGAAATCACACGAAATTCAC	GCACGATATTTACTTTGCTC
LRP1	GACTACATTGAATTTGCCAGCC	TCTTGTGGGCTCGGTTAATG
MFSD2A	CAAACTTATTACTGGCTTCCTC	AGATGGGAATGGTTAAAGTG
NOTCH1	ATCTGAAATAGGAAACAAGTGAA	ATAACCAACGAACAACTACATAA
NOTCH2	AACATCTCATCCATGCTTTG	ACAGTGGTACAGGTACTTC
NOTCH4	ATTGACACCCAGCTTCTTG	GAGGACAAGGGTCTTCAA
OCLN	TTCTGGATCTCTATATGGTTCA	CCACAACACAGTAGTGATAC
P21	CTCTACATCTTCTGCCTTAGT	TCTCATTCAACCGCCTAG
PLVAP	CAATGCAGAGATCAATTCAAGG	ACGCTTTCCTTATCCTTAGTG
RARα	CCATCCTCAGAACTCACAA	ACCAGCGAGAATTAATACCT
RARβ	CACCTAGAGGATAAGCACTT	GGACTCACTGACAGAACA
RARγ	CCACCTTCTTGCTCCTAC	CTTTCACCCTCTGTTCCT
SLC2A1	ACGCTCTGATCCCTCTCAGT	GCAGTACACACCGATGATGAAG
SLC3A2	TTGGCTCCAAGGAAGATT	GAGTAAGGTCCAGAATGACA
SLC6A8	TGAGAGAATGAGATTTCTGCTTGT	TAGGGCTCACAGGGATGG
SLC6A12	AAGGTGGTTTATTTCACAGC	TTCAAGTAGTAGATGATGCCC
SLC7A1	CCTCCTGAGACATCTTTG	CTGGAATATGACGGGAAG
SLC7A5	TTGACACCACTAAGATGAT	GTAGCAATGAGGTTCCAA
SLC16A1	ACACAAAGCCAATAAGAC	ACAGAATCCAACATAGGTA
SLC44A5	TTTCTCCAGAGATGTTTCCC	TACAACACTTCTTGTCCCTC
STRA6	TTTGGAATCGTGCTCTCCG	AAGGTGAGTAAGCAGGACAAG
TLE1	TATTCCAGTCCAAAGAGTCC	AGATGACTTCATAGACTGTAGC
TFRC	ATGCTGACAATAACACAA	CCAAGTAGCCAATCATAA
VEGFR2	GTACATAGTTGTCGTTGTAGG	TCAATCCCCACATTTAGTTC
VWF	TGTATCTAGAAACTGAGGCTG	CCTTCTTGGGTCATAAAGTC
WIF1	AGTTGTTCAAGTTGGTTTCC	TAGCATTTTGAGGTGTTTGG
ZO1	CCTGAACCAGTATCTGATAA	AATCTTCTCACTCCTTCTG

Table S3 - Details of antibodies used for immunofluorescence and flow cytometry. Related to all main Figures and Supplemental figures.

Antibody	Dilution	Technique	Supplier	Catalog Number
CD31	1:50	ICC	DAKO/Labometer	M0823
VE-CADHERIN	1:100	ICC	Life Technology	sc-9989
TIE-2	1:100	ICC	R&D Systems	AF313
vWF	1:100	ICC	Dako	A0082
ZO-1	1:200	ICC	Life Technology	61-7300
CLAUDIN-5	1:100	ICC/Flow cytometry	Life Technology	34-1600
OCCLUDIN	1:200	ICC/Flow cytometry	Life Technology	71-1500
GLUT-1	1:50	ICC/Flow cytometry	Millipore	07-1401
PGP	1:10	ICC	GeneTex	GTX23364
ICAM-1	1:100	Flow cytometry	Santa Cruz Biotechnology	sc-107
***PE-conjugated anti-VEGFR2**	10:200	Flow cytometry	R&D Systems	FAB357P
***FITC-conjugated anti-CD31**	5:200	Flow cytometry	EBioscience	11-0319-42
***PE-conjugated anti-PGP**	5:200	Flow cytometry	Abcam	Ab93590
***FITC-conjugated anti-PGP**	20:200	Flow cytometry	BD Pharmigen	557002
***PE-conjugated anti ICAM-2**	5:100	Flow cytometry	BioLegend	328506
R-PE conjugation KIT		Flow cytometry	Abcam	Ab102918
Phalloidin-fluorescein	50 μg/mL	ICC	Sigma	P5282
Anti rabbit Cy3	1:100	ICC/Flow cytometry	JacksonImmuniResearch	111-165-144
Anti mouse Cy3	1:100	ICC/Flow cytometry	Sigma	C2181
Anti rabbit Alexa 488	1:200	ICC	Life Technology	A11034
Anti mouse Alexa 488	1:200	ICC	Life Technology	A11001
Anti mouse Alexa 555	1:200	ICC	Life Technology	A21422

Table S4 - Details of products used. Related to experimental procedures.

Product	Supplier	Catalog number
EGM-2 bullet kit	Lonza	CABRCC-3162
FGF-b	Peprotech	167100-18B-B
BMP 4	Peprotech	120-05ET
VEGF	Peprotech	100-20
Tβ4	Caslo peptide synthesis	S-1298
Wnt3a	R&D Systems	5036-WN
SB 431542	Tocris	1614
RA	Sigma	R2625
Transferrin	Sigma	T8158
Insulin	Sigma	I9278
TNF-α	Peprotech	300-01A
CD31 MACS beads	Miltenyi Biotec	130-102-608
Lucifer yellow	Sigma	L-0259
Sirius red/fast green collagen staining kit	Chondrex	9046
Blyscan glycosaminoglycan kit	Biocolor	054B1000
Matrigel	BD	354234
Fibronectin	Calbiochem	341631