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Supplemental Information

Closing the Mitochondrial Permeability Transition Pore in hiPSC-Derived Endothelial Cells Induces Glycocalyx Formation and Functional Maturation

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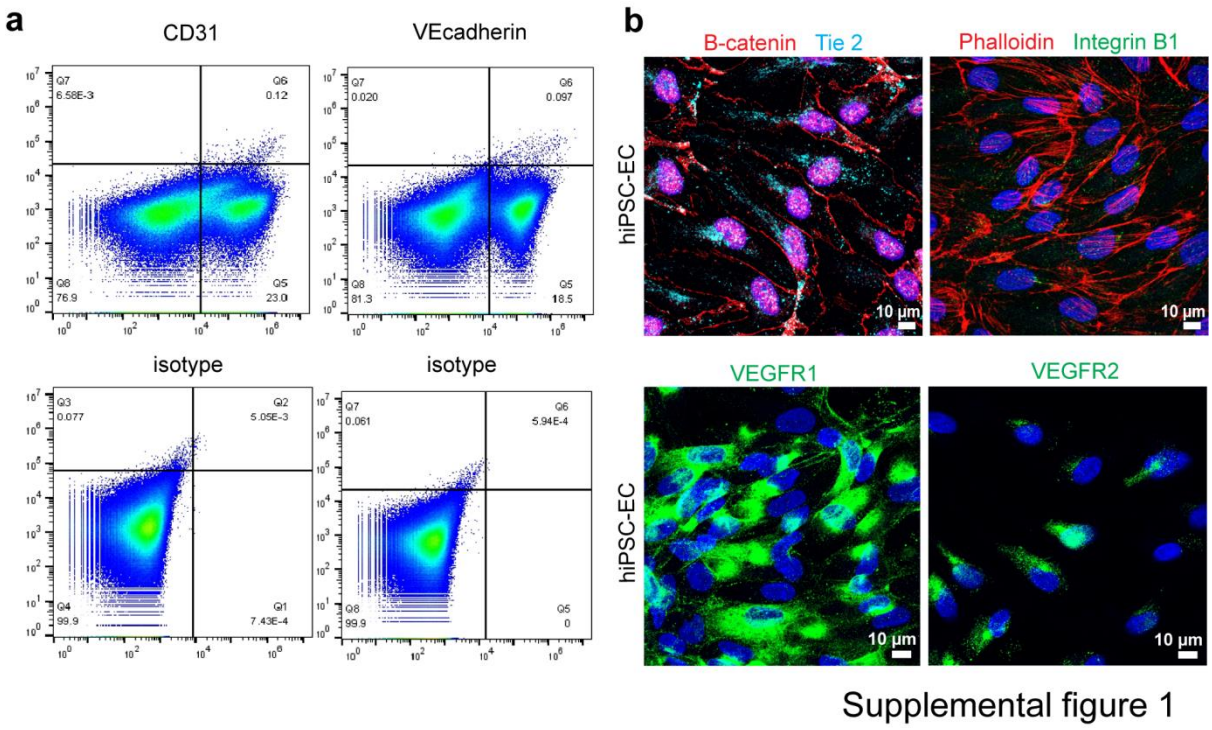
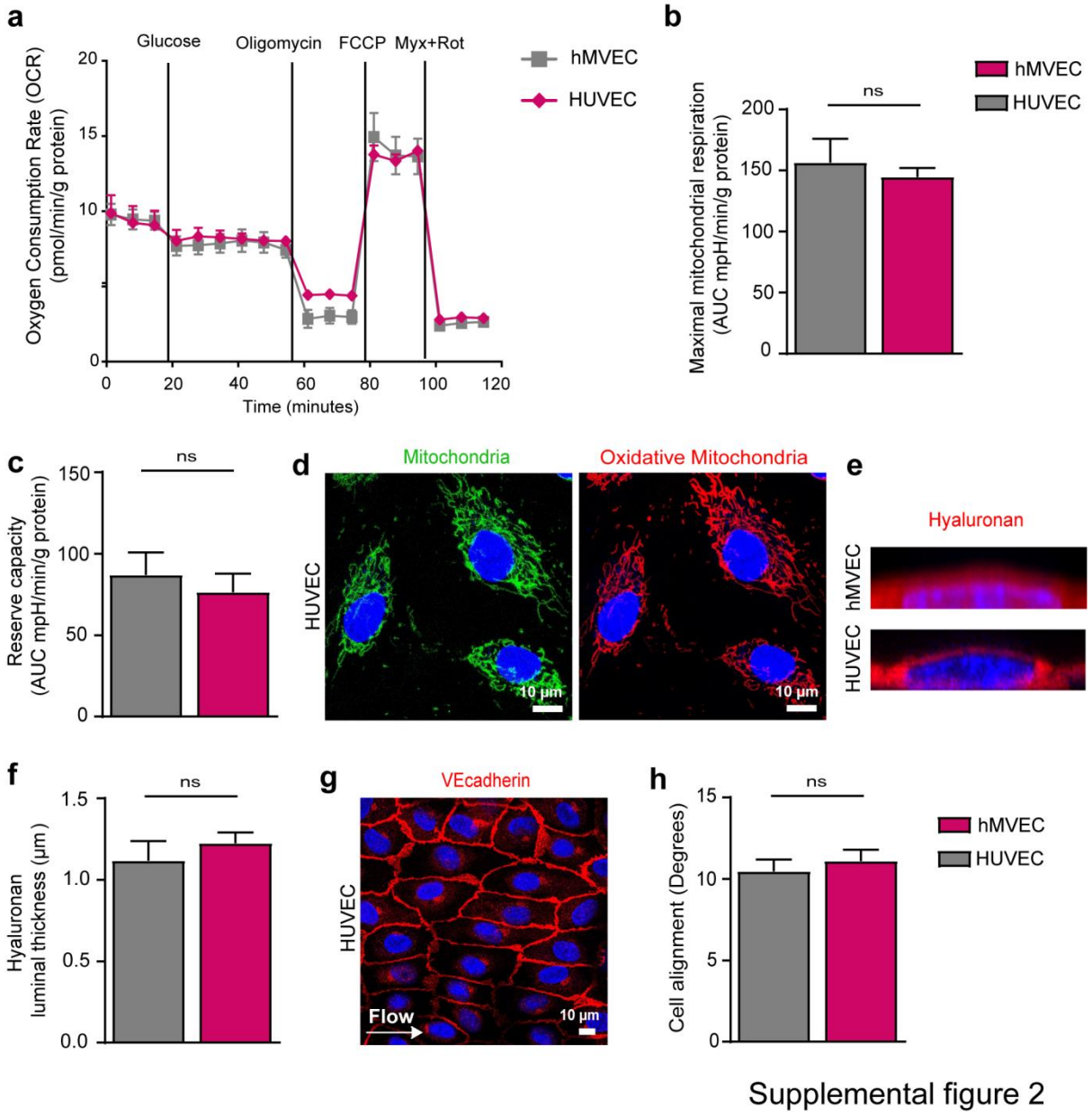
Supplemental Information

Supplemental data



S1. **Characterization of hiPSC-ECs. Related to figure 1.**

(a) FACS analysis of cells after differentiation before CD31 bead selection shows a distinct ECs population positive for CD31 (23%) and VEcadherin (18.5%). **(b)** Representative cross-sectional confocal images stained for Tie-2 (cyan), B-catenin (red) upper left, Phalloidin (red), Integrin (green) upper right, VEGFR1 (green) lower left, VEGFR2 (green) lower right of hiPSC-ECs NCRM1.



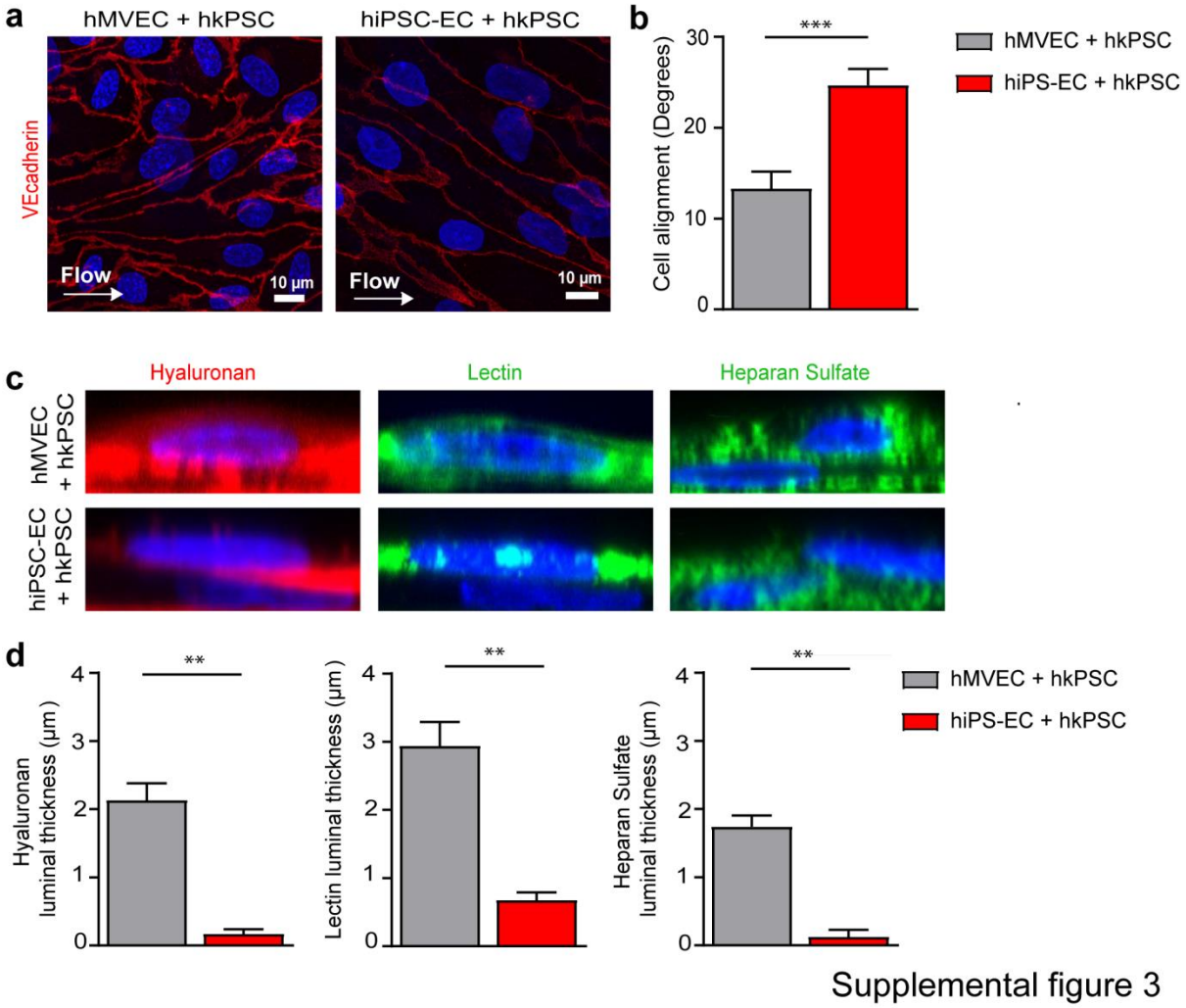
Supplemental figure 2

S2. **Comparison of hMVECs with HUVECs. Related to figure 1.**

Using a Seahorse XF flux analyzer **(a)** the oxygen consumption rate revealed no difference in mitochondrial function between hMVECs and HUVECs. Both **(b)** maximal mitochondrial respiration and **(c)** mitochondrial reserve capacity were not significantly different. **(d)** Representative cross-sectional confocal images stained for Mitotracker Red (oxidative mitochondria) and Mitotracker Green (mitochondria) of HUVECs. **(e)** Representative side view confocal images stained for Hyaluronan (Neurocan, red) after 4 days of laminar flow culture of hMVECs and HUVECs **(f)** Quantification of luminal thickness of Hyaluronan after 4 days of laminar flow of hMVECs and HUVECs. (12-20 cells/group) indicating similar glycocalyx thickness. **(g)** Representative cross sectional confocal images of VEcadherin (green) and Hoechst (blue) show the alignment of cells to flow.

(h) Quantification of cell alignment after 4 days of laminar flow culture of hMVECs and HUVECs shows similar alignment to shear stress. (100 cells/group).

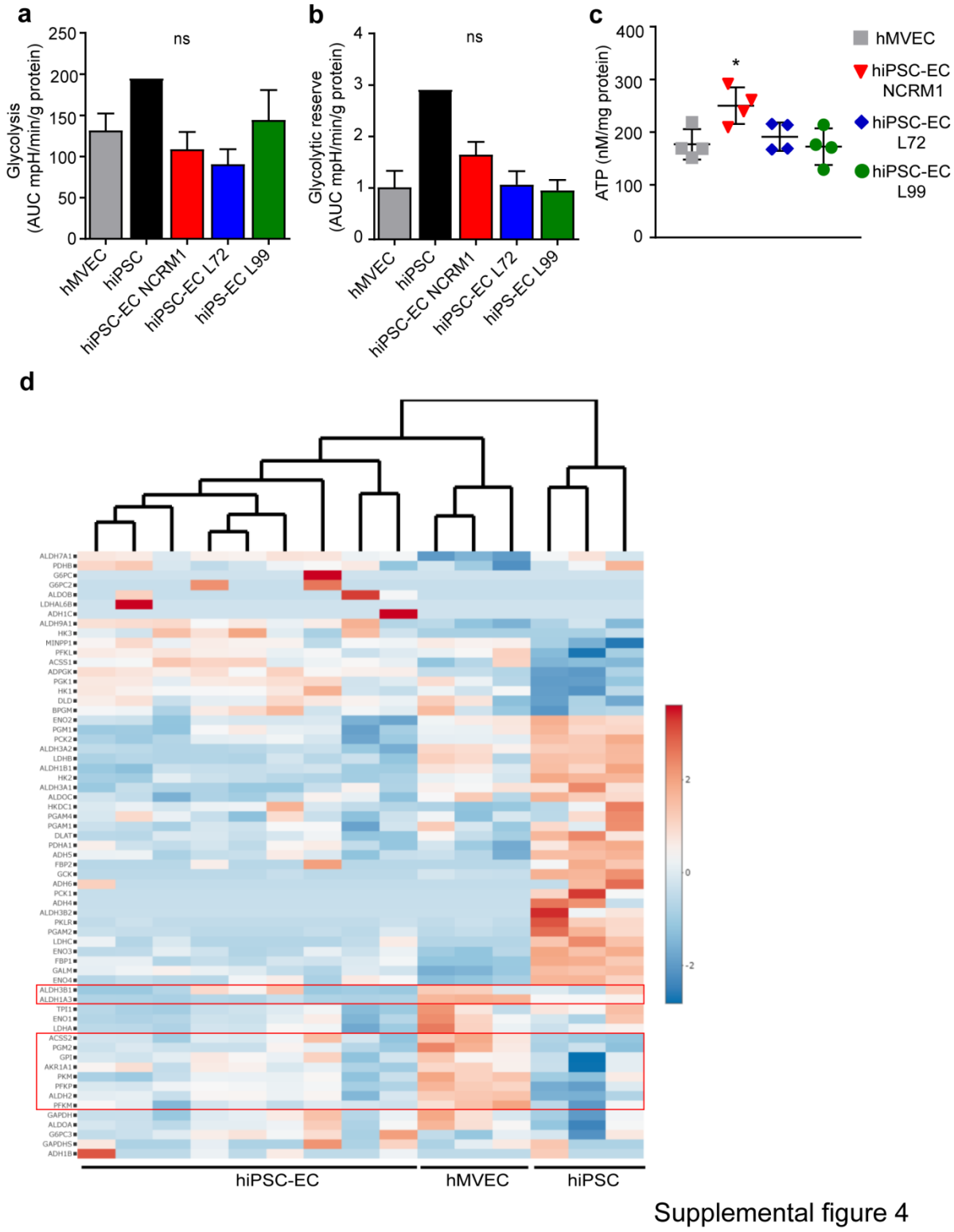
Values are given as mean ± SEM of n=3-4 independent experiments. Non-paired 2-tailed Student's t-test was performed; ns = non-significant.



S3. **No maturation after co-culture of hiPSC-ECs NCRM1 and hMVECs with human kidney pericytes (hkPSC). Related to figure 1.**

(a) Representative cross-sectional confocal images stained VE-cadherin (red) and Hoechst (blue) after 4 days of laminar flow co-culture of hMVECs and hiPSC-ECs + hkPSC. **(b)** Quantification of cell alignment after 4 days of laminar co-culture of hMVECs and hiPSC-ECs. (100 cells/group). **(c)** Representative side view confocal images stained for Hyaluronan (Neurocan, red), Lectin (LEA, green), and Heparan Sulphate (JM403, green) after 4 days of laminar flow culture of hMVECs and hiPSC-ECs with hkPSC. **(d)** Quantification of luminal thickness of Hyaluronan, Lectin Lycopersicon esculentum, and Heparan sulfates after 4 days of laminar flow of hMVECs and hiPSC-ECs with hkPSC. (8-16 cells/group).

Values are given as mean ± SEM of n=4 independent experiments. Non-paired 2-tailed Student's t-test was performed; *P < 0.05, **P < 0.001, ***P < 0.0001.



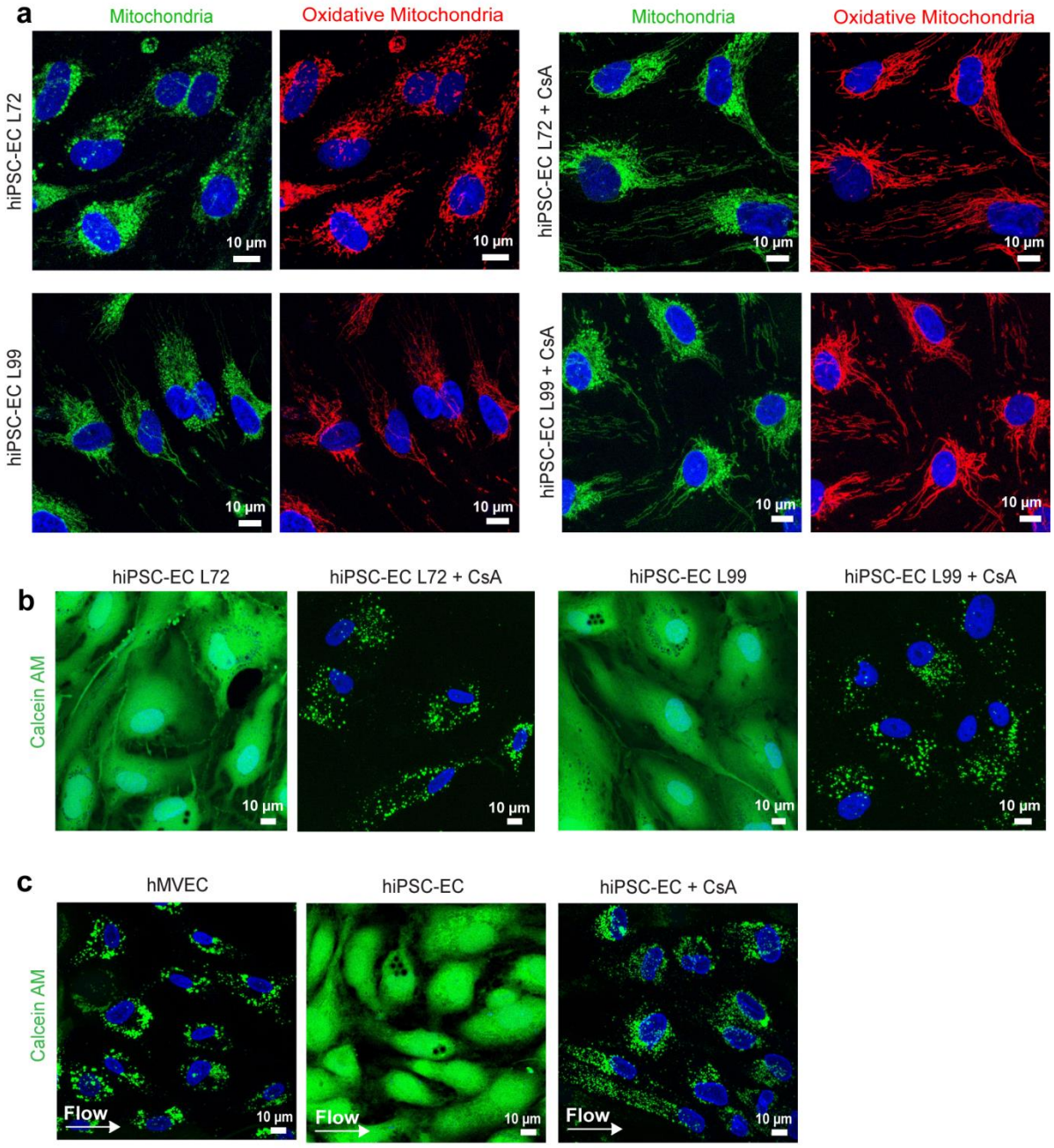
Supplemental figure 4

S4**. No functional differences in glycolysis and ATP production between hMVECs and hiPSC-ECs. Related to figure 2.**

Using a Seahorse XF flux analyzer the extracellular acidification rate (ECAR), an indicator of cellular lactate production revealed similar **(a)** glycolysis and **(b)** glycolytic reserve of tree different hiPSC-ECs cell lines and hMVECs. **(c)** ATP luciferase assay measures nM ATP per mg protein. **(d)** Heatmap of RNA sequencing results

of metabolic genes involved in the glycolysis. Scale bar represents Z-scores: blue indicates lower gene expression and red, a higher gene expression.

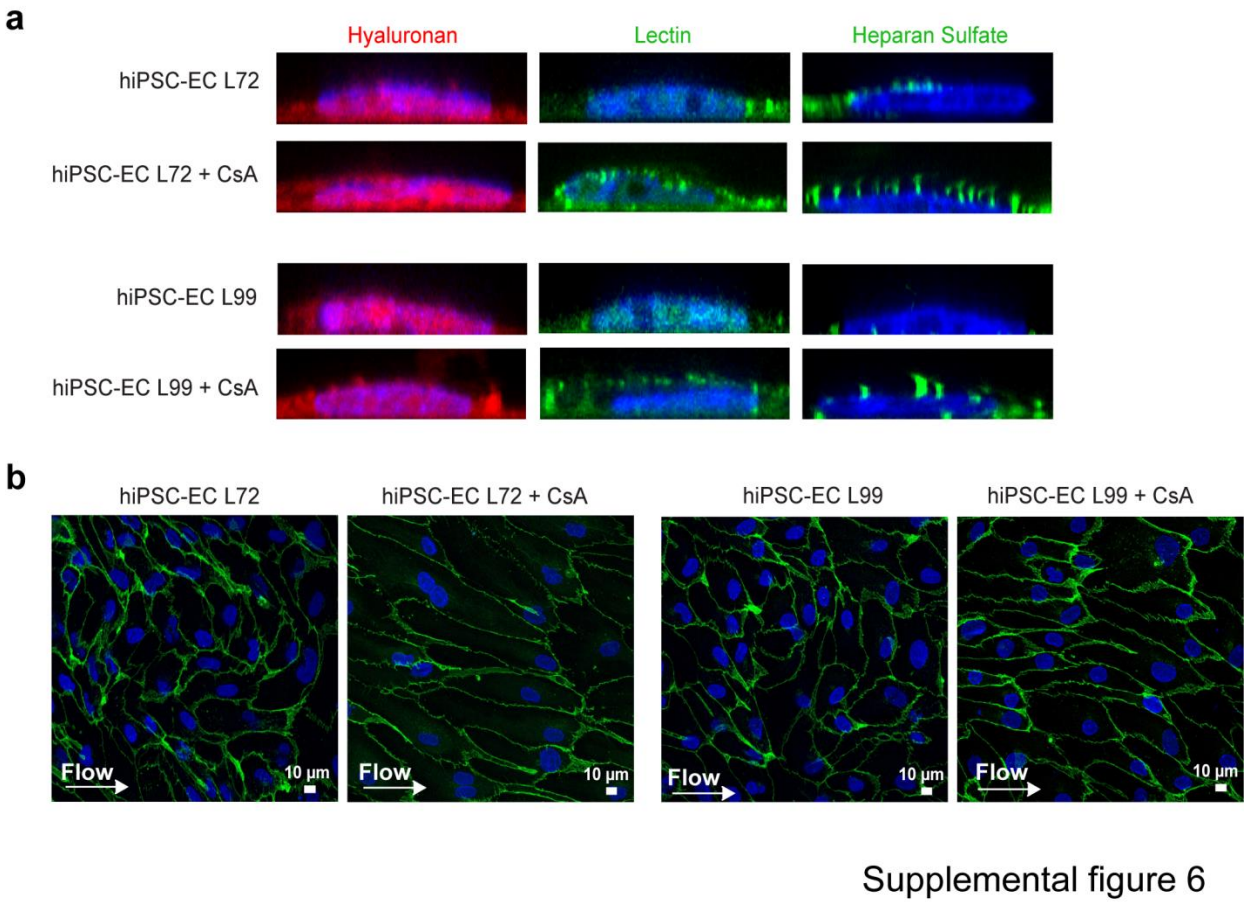
Values are given as mean ± SEM of n=3-4 independent experiments. one-way ANOVA was performed; *P < 0.05, **P < 0.001, ***P < 0.0001.



Supplemental figure 5

S5. **Treatment with cyclosporine-A results in closure of the mPTP and subsequent maturation of the mitochondria. Related to figure 4**

(a) Representative cross-sectional confocal images stained for Mitotracker Red (oxidative mitochondria) and Mitotracker Green (mitochondria) of hiPSC-ECs L72/L99 treated with CsA. **(b)** To determine the state of the mPTP in hiPSC-ECs, the cobalt/calcein AM (green) quenching method was used. hiPSC-ECs L72/L99 treated with CsA for 30 minutes prevented calcein leakage, indicating that CsA closed the mPTP. **(c)** Assessment of the state of the mPTP with Calcein AM (green) staining after 4 days flow shows prolonged effect of CsA.



Supplemental figure 6

S6. **Treatment with cyclosporine-A restores the glycocalyx and improves alignment to flow. Related to figure 6.**

(a) Representative side view confocal images stained for Hyaluronan (Neurocan, red), Lectin (LEA, green) and Heparan Sulphate (10e4, green) after 4 days of laminar flow culture of hiPSC-ECs L72/L99 Control and hiPSC-ECs L 72/L99 treated with 500 nM CsA during differentiation. **(b)** Representative cross sectional confocal images of hiPSC-ECs L72/L99 stained for VEcadherin (green) and Hoechst (blue) show the alignment of cells to flow.

Supplemental Experimental Procedures

Primary human umbilical vein endothelial cells (HUVECs) culture

HUVECs were freshly isolated, by perfusion and subsequent incubation (20 minutes) of trypsin at 37°C, from different donors as described (Jaffe et al., 1973)(with approval of the Medical Ethical Commission of the Leiden University Medical Center, and informed consent from all subjects) and used between passage 1 and 3. Freshly isolated HUVECs were cultured on 0.5% gelatin–coated plastic flasks in endothelial basal medium medium (CCK3121; Lonza), supplemented with human epidermal growth factor, vascular endothelial growth factor, human fibroblast growth factor, R3KIGFKI, ascorbic acid, heparin, and 10% human serum (Lonza) supplemented with antibiotics (100 IU/mL penicillin and 100 μg/mL streptomycin).

Human kidney-derived perivascular stromal cells (hkPSC) culture

hkPSC were isolated from transplant grade kidneys discarded for as surgical waste (Leuning et al., 2017). In short, kidneys were perfused with collagenase (2500 units, NB1, Serva) and DNAse (2.5 mL Pulmozyme, Genentech) at 37 °C with a flow of 300mL/min via the renal artery. After approximately 30 minutes, the cell suspension was washed in DMEM-F12 (Invitrogen, Gibco) containing 10% fetal calf serum and further cultured in alphaMEM (Lonza) containing 5% platelet lysate, glutamine (Lonza) and penicillin/streptomycin and cells were cultured in tissue culture flasks until confluence was reached. At passage 1, cells were trypsinized (1X, BE02-007E, Lonza) and the $NG2^+$ cell population selected using anti-human NG2 according to manufacturer's protocol (Miltenyi Biotech).

FACS confirmed homogeneous NG2 positive hkPSC populations between passages 4-8 and hMVECs between passages 6-8, cultured in EC-SFM full medium at 37 °C and 5% CO_2.

Confocal immunofluorescence microscopy

After exposure to flow (at day 4), hMVECs and hiPSC-ECs were fixed in freshly made 4% paraformaldehyde (Alfa Aesar) in HBSS (Invitrogen, Gibco) for 10 minutes at room temperature, washed twice with HBSS /1% BSA (Sigma-Aldrich) and then blocked for 30 minutes with 3% BSA in HBSS at room temperature. 0.2% Triton (Sigma-Aldrich) was added during fixation to permeabilize the cells. Cells are incubated overnight (16 hours) at 4 °C with primary antibodies: Ncan-dsRed (1:7), VE-cadherin (1ug/mL, R&D Systems, MAB9381), JM403 (1.1mg/mL, gift from dr. van der Vlag and dr. Kuppevelt (Nijmegen Centre for Molecular Life Sciences,

Radboud University Medical Centre, Nijmegen, The Netherlands)) or the appropriate control IgG, IgG1, IgM isotype antibodies (Dako, X0931; Abcam, Ab18400) diluted in HBSS/1% BSA. After washing three times with HBSS/1% BSA, cells are incubated with Hoechst 33258 (Life Technologies, H3569), LEA FITC, Lectinfrom Lycopersicon esculentum (10 ug/mL, Sigma-Aldrich, L2895) and the appropriate secondary antibody (1:500) goat-α-mouse labeled with Alexa 488/568 (Molecular Probes, IgG1: A11001, A11004; IgG: A21121, A21124, Invitrogen, IgM: A21042) for 1 hours at room temperature. Cells were imaged using a Leica SP8 White light laser confocal immunofluorescence microscopy.

Sequential 16-bit confocal images (xyz dimensions, 0.142×0.142×0.3µm, 0.142×0.142×1µm or 0.116x0.116×1µm) were recorded using LASX Image software (Leica) and analysed with the open-source image-analysis program ImageJ (developed by W.Rasband, National Insitutes of Health, Bethesda, MD; http://rsb.info.nih.gov/ImageJ). From each independent experiment (n=4) 6-100 cells were analysed.

Detection of hyaluronan

To detect hyaluronan, a specific binding peptide from the hyaluronan binding protein neurocan conjugated to green fluorescent protein was used. Ncan-eGFP (Gift from J. Kappler) (Zhang et al., 2004) was modified in which eGFP was replaced with dsRed (Ncan-dsRed) to create a red fluorescent hyaluronan binding probe.

Transmission electron microscopy

Cell were grown in sterile cell culture dishes (PS, 35x10mm, with vents, Greiner Bio-One GmbH). Just before fixation, the medium was removed and 2.5% glutaraldehyde (Electron Microscopy Sciences)/2% paraformaldehyde (Electron Microscopy Sciences, EMS)/2mM $CaCl_2$ (Merck) in 0.15M sodium-cacodylate (Sigma-Aldrich) buffered solution was applied gently on top of the cells. Fixation was for 2 hours at room temperature. Cells were then rinsed 2x with 2mM $CaCl_2$ in 0.15 M sodium-cacodylate buffered solution, and post-fixed for 1 hour on ice with a mixture of 1% osmium tetroxide (EMS), 1.5% potassium ferrocyanide (Merck) and 2mM $CaCl_2$ in 0.15 M sodium-cacodylate buffer. Samples were further rinsed 2x with 2 mM $CaCl_2$ in 0.15M sodium-cacodylate buffer, dehydrated overnight in 70% ethanol, followed by 80% ethanol (10 min), 90% ethanol (10 min), and 100% ethanol absolute (2x 15 min; 1x30 min). The cells were then permeated with a mixture of epon LX-112 (Ladd Research) and propylene oxide (Electron Microscopy Sciences) (1:1) for 1 hour, followed by polymerization in pure epon.. Ultrathin sections (90 nm) were collected onto copper slot grids (Storck Veco) covered by Formvar film and a thin carbon layer, then stained with an aqueous solution of 7% uranyl acetate for 20 minutes, followed by Reynold's lead citrate for 10 minutes. Samples were analysed at

an acceleration voltage of 120 kV using an FEI Tecnai 12 (BioTWIN) transmission electron microscope (Thermo Fisher Scientific), equipped with a 4k Eagle CCD camera (Thermo Fisher Scientific). Automated data acquisition and stitching software was used to record virtual slides of the cells (Faas et al., 2012). Images were captured at 11.000x magnification, with binning 2, corresponding to a 2 nm pixel size at the specimen level.

Metabolic assays

To measure ATP, the Promega CellTiter-Glo reagent was used. Cells were cultured in a 96 well plate (20.000 cells per well) and the CellTiter-Glo 2.0 reagent was added 1:1 to the culture medium and mixed on an orbital shaker for 2 minutes. After 10 minutes incubation, the luminescence was recorded. A standard curve of ATP was used to quantify the total amount of ATP produced.

To determine mitochondrial activity, ECs were seeded at a density of 20.000 cells in a 24 well plate and left to adhere for 16 hours. Subsequently, cells were treated with MTT (5mg/mL, Sigma Aldrich) 1:10 in culture medium and incubated for 2 hours at 37 °C. Afterwards, MTT was removed and isopropanol/0.04M HCL added and measured at 570 nm by Molecular Devices Spectramax i3x. Cellular protein content was determined with a BCA-protein kit from Pierce (Thermo Fischer Scientific) and the data shown as MTT normalized to protein. Each measurement was averaged from triplicate wells.

RNA sequencing

RNA isolation was performed after cells in a 6-well plate reached a confluent state using a RNAeasy mini kit (Qiagen). Samples from 3 independent experiments were used for RNA sequencing. For each sample, an indexed cDNA library was prepared from 1µg total RNA using the KAPA stranded mRNA-seq kit (Sopachem). Clusters were generated using the Cbot2 system (Illumina) and amplified cDNA fragments were sequenced on a HiSeq 4000 system (Illumina) as follows: 51 cycles for read 1 and 8 cycles for index 1. The raw sequenced reads were mapped to the human reference genome build GRCh38 using STAR (Dobin et al., 2013). Mapped reads were quantified using RSEM (Li and Dewey, 2011) for accurate quantitation resulting in, on average, 34,740,890 ± 8,771,147 counts per sample. After auto-scaling, the resulting data were first summarized by principal component analysis (PCA) using the *flash*PCA (R package). Plotly was used to generate interactive graphs (2D and 3D plots). Either all genes were considered or the subset of metabolic genes present in KEGG metabolic pathways (https://www.genome.jp/kegg/) and in the Recon3D model (http://vmh.life). Heatmap analysis was performed using the *heatmaply* package. KEGG metabolic pathways were used. RNA-sequencing

data are available in ArrayExpress (https://www.ebi.ac.uk/arrayexpress/experiments/E-MTAB-8392) under accession E-MTAB-8392.

Mitochondrial DNA qPCR

DNA isolation was performed after cells reached confluence using a DNAeasy mini kit (Qiagen). qPCR for mitochondrial DNA was performed as previously described (Rooney et al., 2015). The forward and reversed mitochondrial DNA primer sequences that were used are CACCAAGAACAGGTTTGT (forward), TGGCCATGGGTATGTTGTTA (reverse). The Ct value of mtDNA is normalized by nuclear DNA, with the following sequence: TGCTGTCTCCATGTTTGATGTATCT (forward) and TCTCTGCTCCCCACCTCTAAGT (reverse).

Supplemental References

Dobin, A., Davis, C.A., Schlesinger, F., Drenkow, J., Zaleski, C., Jha, S., Batut, P., Chaisson, M., and Gingeras, T.R. (2013). STAR: ultrafast universal RNA-seq aligner. Bioinformatics (Oxford, England) *29*, 15-21.

Faas, F.G.A., Avramut, M.C., M. van den Berg, B., Mommaas, A.M., Koster, A.J., and Ravelli, R.B.G. (2012). Virtual nanoscopy: Generation of ultra-large high resolution electron microscopy maps. The Journal of cell biology *198*, 457.

Jaffe, E.A., Nachman, R.L., Becker, C.G., and Minick, C.R. (1973). Culture of human endothelial cells derived from umbilical veins. Identification by morphologic and immunologic criteria. The Journal of clinical investigation *52*, 2745-2756.

Leuning, D.G., Reinders, M.E., Li, J., Peired, A.J., Lievers, E., de Boer, H.C., Fibbe, W.E., Romagnani, P., van Kooten, C., Little, M.H., *et al.* (2017). Clinical-Grade Isolated Human Kidney Perivascular Stromal Cells as an Organotypic Cell Source for Kidney Regenerative Medicine. Stem cells translational medicine *6*, 405-418.

Li, B., and Dewey, C.N. (2011). RSEM: accurate transcript quantification from RNA-Seq data with or without a reference genome. BMC bioinformatics *12*, 323.

Rooney, J.P., Ryde, I.T., Sanders, L.H., Howlett, E.H., Colton, M.D., Germ, K.E., Mayer, G.D., Greenamyre, J.T., and Meyer, J.N. (2015). PCR based determination of mitochondrial DNA copy number in multiple species. Methods in molecular biology (Clifton, NJ) *1241*, 23-38.