SUPPLEMENTAL MATERIAL

MATERIALS AND METHODS

Human primary cell culture

Dermal microvascular endothelial cells (DMEC), human umbilical vein endothelial cells (HUVEC) or fibroblasts were isolated as described\(^1\). Lymphatic endothelial cells (LEC) were sorted with CD31 and anti-podoplanin antibodies from dermal cell suspensions. Human pulmonary microvascular endothelial cells (HPMEC) were kindly provided by Ronald E. Unger (Johannes Gutenberg University, Mainz)\(^2\). Human bronchial epithelial cells were provided by Harald Renz (Klinikum der Philipps Universität Marburg)\(^3\). All endothelial cells were cultured in the same endothelial growth medium (EGM-2; CC-3156 Lonza) with 15% FCS (F7524 Sigma) and supplements for microvascular cells (CC-4147 Lonza) and were passaged at 90% confluency.

CGN\(^{−/−}\) mice

CGN\(^{−/−}\) mice have been described previously\(^4\). C57Bl6 mice with floxed CGN alleles, obtained from Sandra Citi, University of Geneva\(^5\), were bred with MORE\(^6\) C57Bl6 mice, obtained from Maria Sibilia, Medical University of Vienna. For genotyping the following primers were used for Cre 5'-CATACCTGGAAAATGCTTCTTGCC-3' and 5'-CATCGCTCGACCAGTTTAGTACC-3', and for CGN 5'-GGTTATCTGTGTAAGGAGTGTGA-3', 5' -GGTGATGTCTCTAGGGTAAGG-3', 5' -GCACCTTTCATAATGCAGGCT-3' and 5'-GGAATGCTTCAGGCCTGAGG-3'. In all experiments CGN\(^{−/−}\) mice were used together with wildtype CGN\(^{+/−}\) littermates. All experiments performed in this study have been approved by the Institutional Committee for Animal Research and Care at the Medical University of Vienna (BMWF-66.009/0224-WF/II/3b/2014). Animals were held under standard conditions with unlimited access to water and standard laboratory food.

Isolation of mouse lung endothelial cells

Mouse endothelial cells were isolated by removing the lung lobes from individual euthanized mice. Lung lobes were immersed in ice-cold HBSS (BE10-543F, Lonza) for transfer to a tissue culture hood. Tissue was finely minced using sterile scissors. For digestion, 2 ml of dispase solution (07913, Stemcell) was added and the tissue slurry was incubated at 37°C on a shaker for 1h. The tissue suspension was aspirated with a displacement pipette and passed through a 70µm cell strainer. Cells were washed with PBS (BE17-512F) and centrifuged at 200g for 10 min. Supernatants were aspirated and cells were resuspended in 1ml cold endothelial growth medium (EGM-2, CC-3156, Lonza). Then, rat-anti-mouse CD31 antibody (553369, BD) was added and samples were incubated at 4°C on a rotator for 30min. Cells were washed in endothelial growth medium once and incubated with sheep-anti-rat Dynabeads (11035 Life technologies) at 4°C on a rotator for 30min. Tubes were subjected to magnetic separation. Cells were washed with endothelial growth medium and seeded to gelatin-coated tissue culture plates and cultured at 37°C and 5%CO\(_2\) in endothelial growth medium with 15% FCS (F7524 Sigma) and supplements (CC-4147 Lonza) and were passaged at 90% confluence.

Antibodies, primers and reagents
Primary antibodies used were: cingulin (HPA027586 and HPA027657, Sigma), myc (sc-40, Santa Cruz Biotechnology), GFP (A11122, Life Technologies), β-actin (A2228, Sigma), VE-cadherin (IM1597, Beckman Coulter), ZO-1 (610966, BD), GEF-H1 (ab94348, ab103558, abcam), p114RhoGEF (HPA042689, Sigma) and claudin-5 (35-2500, Invitrogen). Mouse and rabbit IgG controls were purchased from Sigma. Primer sets used for analysis of mRNA expression were Taqman assays from Life Technologies for cingulin, (Hs00430423_m1 and Hs00430416_m1; Mm01263533_m1 and Mm01257792_m1), VE-cadherin (Hs00901463_m1; Mm00486938_m1), claudin-5 (Hs00533949_s1; Mm00727012_s1), JAM-A (Hs00170991_m1; Mm00554113_m1), occludin (Hs00170162_m1) or GEF-H1 (Mm01215879_m1) and GAPDH (Hs99999905_m1) or B2M (Mm00437762_m1) as control.

Immunohistochemistry and Immunofluorescence

Formalin-fixed human tissues were retrieved by a punch biopsy with a diameter of 0.6 mm taken from the paraffin blocks of patient samples according to a protocol approved by the Vienna ethics committee (180/11/2013-EK405/2006). Native material for frozen sections was taken from tumor-surrounding normal tissue of surgical specimens according to ethics committee #136/02/2014-EK1150/2011. Formalin fixed paraffin embedded or freshly frozen tissue blocks were cut to 6 µm sections and subjected to immunohistochemistry or immunofluorescence staining as described1 and analyzed by conventional microscopy or by a confocal laser scan microscope (LSM 700; Zeiss) with a pinhole set to one airy unit. To evaluate co-localization, images were subjected to deconvolution with the software package Huygens Essential (Scientific Volume Imaging). Co-localization of cingulin with junctional markers was quantified by calculating Pearson’s correlation coefficient in Coloc2 within the FIJI software package7.

Electron microscopy

Electron microscopy was performed as described previously8. HUVEC were grown to confluence and immediately fixed for electron microscopy. Mice were euthanized to obtain small tissue samples of about 3mm diameter from mouse lung. All specimens were incubated in fixative used for routine electron microscopy containing 2% paraformaldehyde/2.5% glutaraldehyde granting adequate ultrastructural morphology and rinsed in 0.1 M cacodylate buffer. After treatment with 2% osmium tetroxide and uranyl acetate, dehydrated samples were embedded in EPON 812. Ultrathin sections were collected on copper grids and stained with uranyl acetate/lead citrate. Finally, sections were examined and micrographed using a JEM 1010 electron microscope (JEOL). To determine the number of junctional structures, electron micrographs were taken for a minimum total distance of 16µm of adjacent endothelial cell membranes for each sample type. Results were summarized as mean number of junctions per µm of adjacent endothelial membrane.

Quantitative RT-PCR

Cells were seeded in 6-well-plates and incubated for 4 or 7 days. At respective time-points cells were rinsed with PBS and lysed with 350 µl RLT buffer with 1% β-mercaptoethanol. Tissues and blood vessels for qPCR were explanted from euthanized wildtype or CGN−/− C57Bl6 mice and immediately submersed in RNA later. All tissues were transferred to RLT buffer with β-mercaptoethanol, ball milled. All tissues were processed in the same way. Samples were processed for isolation of RNA with Qiashredder (79656; Qiagen) and RNeasy Mini Kit (74106; Qiagen).
according to manufacturer’s instructions and reverse transcribed using RevertAid H Minus First Strand cDNA Synthesis Kit (K1632 Thermo Scientific). 200ng cDNA and Taqman PCR master mix were used with Taqman gene expression assays for respective targets. The Ct threshold was set to 0.08 for all samples. Ct values at threshold value were recorded and normalized to GAPDH control. Fold changes were calculated with respect to fibroblasts.

**Immunoprecipitation and western blotting**

All endothelial cells were cultured in the same conditions using endothelial growth medium (EGM-2; CC-3156 Lonza) with 15% FCS (F7524 Sigma) and supplements for microvascular cells (CC-4147 Lonza) and were seeded in parallel to samples for qRT-PCR. Cells were lysed with Tris-buffer containing 1% NP-40 and 1% Triton X-100. Lysates were centrifuged at 10000 rpm and 4°C for 4 min. For sequential lysis, supernatants of the Tris-buffer lysis were transferred into a new vial and the pellets were lysed with RIPA-buffer (50 mM TrishCl, 150 mM NaCl, 1 % NP-40, 0.5% sodium deoxycholate, 0.1 % SDS, 1 mM EDTA and protease inhibitors). RIPA lysates were centrifuged once more and the remaining pellet was lysed with SDS sample buffer (Laemmli) buffer. Proteins were separated by SDS-PAGE, transferred onto a nitrocellulose membrane and blots were stained with indicated antibodies.

For immunoprecipitation, cells were lysed with RIPA buffer. Protein G-Sepharose pre-cleared cell lysates were incubated with Protein G-Sepharose fast flow (P3296 Sigma) bound with the ZO-1 or IgG1 control antibody. After washing with Tris buffer the agarose bound proteins were resuspended in 50 µl SDS sample buffer. Samples were incubated at 96°C for 5 minutes and separated by SDS-PAGE. Proteins were transferred onto a nitrocellulose membrane and western blots were incubated with the ZO-1, GEF-H1 and cingulin antibodies.

**Cloning and lentiviral transfection**

pcDNA3.1-GFP-cingulin-myc construct was described earlier and re-cloned into pWPT vector (Addgene) for lentiviral transfection. pWPT with GFP only was used as a control vector. 293T cells served as a production cell line for viral particles and were transfected with either cingulin or control plasmid and helper plasmids (pMD2.G and psPAX2 Addgene). Primary endothelial cells were transduced with virus supernatant and transfected cells were sorted for GFP expression.

**Proliferation assay**

Cells were seeded to 24-well plates and grown in endothelial growth media. After 1, 3 or 5 days cells were washed in PBS and lysed in 0.9% Triton X-100 for 30 min at room temperature. Samples were frozen to -20°C and analysed in parallel by picogreen assay (P7581 Life Technologies) to determine the amount of dsDNA in each sample.

**Transendothelial electrical resistance (TEER)**

Electrical impedance of endothelial cells was measured using the ECIS system (Applied Biophysics). Cells were seeded on gelatine-coated plates at a subconfluent density of 4000 cells per 0.8 cm² and resistance was monitored at 250 and 32000 Hz over time.

**Permeability assay**

Endothelial cells were seeded into transwells (PISP12R48 Millipore) and cultured until confluence. Then, 376 Da (NaFluorescein, F6377 Sigma) or 70,000 Da
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Cingulin regulates endothelial barrier function

(Dextrane-TRITC, D1818 Life Technologies) tracers were added to the transwell. After indicated times, fluorescence was measured in the well below the transwell.

**Vascular permeability in CGN\(^{-/-}\) mice**

CGN\(^{-/-}\) mice of 8–10 weeks and wild type (WT) littermates were injected with biotin into the tail vein (100 μg/ mouse, 21217 Thermo Scientific), 60 min later, animals were sacrificed and organs immediately taken for further analysis. Tissues for immunohistochemistry were formalin fixed, paraffin embedded and biotin was visualized by avidin-HRP stainings. Biotin-positive Purkinje cells were counted blinded to the conditions in 3 high power fields/mouse. Blood vessels for qPCR were placed in RNAlater (AM7021, Ambion), lysed with RLT buffer (Quiagen) using a ball mill and RNA was isolated as described above. For protein analysis tissues (lungs or vena cava and aorta) were subjected to milling, suspended in Laemmli buffer and subjected to SDS-PAGE and western blotting.

**Statistical analysis**

The statistical evaluation was blinded to the conditions. Data were analyzed with SPSS v. 21 software package (IBM Corp). Statistical significance was assessed by Student’s t-test. A p-value below or equal to 0.05 was considered statistically significant. Bonferroni-corrected p-values were considered, if more factors were compared from the same sample. An ANOVA corrected for repeated measurements was used to evaluate significance of time-resolved data from proliferation and resistance measurements.


