Research Article

Fibronectin rescues aberrant phenotype of endothelial cells lacking either CCM1, CCM2 or CCM3

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Abstract
Loss-of-function variants in CCM1/KRIT1, CCM2, and CCM3/PDCD10 are associated with autosomal dominant cerebral cavernous malformations (CCMs). CRISPR/Cas9-mediated CCM3 inactivation in human endothelial cells (ECs) has been shown to induce profound defects in cell-cell interaction as well as actin cytoskeleton organization. We here show that CCM3 inactivation impairs fibronectin expression and consequently leads to reduced fibers in the extracellular matrix. Despite the complexity and high molecular weight of fibronectin fibrils, our in vitro model allowed us to reveal that fibronectin supplementation restored aberrant spheroid formation as well as altered EC morphology, and suppressed actin stress fiber formation. Yet, fibronectin replacement neither enhanced the stability of tube-like structures nor inhibited the survival advantage of CCM3−/− ECs. Importantly, CRISPR/Cas9-mediated introduction of biallelic loss-of-function variants into either CCM1 or CCM2 demonstrated that the impaired production of a functional fibronectin matrix is a common feature of CCM1-, CCM2-, and CCM3-deficient ECs.

Keywords
cerebral cavernous malformations, CRISPR/Cas9 genome editing, extracellular matrix

Abbreviations: CCM, cerebral cavernous malformation; CI-huVECs, immortalized human umbilical vein endothelial cells from InSCREENeX; DOC, deoxycholate; EC, endothelial cell; ECM, extracellular matrix; EDA, extra domain A; EDB, extra domain B; FAK, focal adhesion kinase; FC, fold change; FDR, false discovery rate; RNP, ribonucleoprotein.

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1 | INTRODUCTION

Cerebral cavernous malformations (CCMs) belong to the most common cerebrovascular lesions and can be found in the brain and spinal cord. They are characterized by tightly packed convolutes of low-flow leaky vessels that are lined by a single layer of endothelial cells (ECs), lack supporting elastic fibers or vascular smooth muscle cells in their walls, and tend to bleed into the surrounding brain tissue.1-4 CCM can occur in a sporadic or autosomal-dominant familial form (OMIM: 118660, 603284, 603285). The latter often presents with multiple CCMs at a younger age. Protein-truncating variants in either CCM1 [also known as KRIT12,3], CCM2 [MGC4607, OSM4], or CCM3 [PDCD10, TFAR15] can be identified in up to 98% of familial CCM patients.4,5 The CCM proteins act together in a heterotrimeric complex but also independently in different signaling pathways.6-9 Recent reorganization,31-33 and stabilization of cell-cell junctions.34,35

In vitro and in vivo studies have given fascinating new insight into CCM pathobiology and their findings may help to identify a pharmacological approach for CCM management.10-12 EC proliferation, migration, morphogenesis, survival, and vascular stability depend on cell adhesion to the extracellular matrix (ECM), which is a scaffold for vascular organization and acts by transmitting mechanical forces.21 Binding of ECM proteins to integrins can regulate a complex network of intracellular signaling pathways. However, it is not yet fully understood to what extent ECM components contribute to endothelial function and angiogenesis as they can have overlapping functions.22-24 Notably, EC morphogenesis depends on stabilization by laminin-rich matrices but also on reorganization,31-33 and stabilization of cell-cell junctions.34,35

In the present study, we demonstrate that loss of CCM1, CCM2, and CCM3 in human ECs impairs the production of a functional fibronectin matrix, which might trigger CCM formation. We also show that fibronectin replacement suppresses actin stress fiber formation and rescues endothelial dysfunctions of CCM1-, CCM2-, and CCM3-deficient ECs. In particular, a 120 kD cell-binding fragment of fibronectin is critical to restore spheroid formation, cortical actin organization, and EC morphology.

2 | MATERIALS AND METHODS

2.1 | Cell culture

Immortalized human umbilical vein ECs (CI-huVECs, InSCREENeX, Braunschweig, Germany) and human cerebral microvascular ECs (hCMEC/D3; Merck Millipore, Darmstadt, Germany) were cultured at 37°C and 5% of CO2 in EC growth medium (ECGM, PromoCell, Heidelberg, Germany) supplemented with 10% of fetal calf serum (FCS, Thermo Fisher Scientific, Waltham, MA, USA) or in EndoGRO-MV complete medium (Merck Millipore) supplemented with 1 ng/mL of FGF-2 (PromoCell) and 5% of FCS, respectively. For rescue experiments, human plasma fibronectin (F2006, Sigma-Aldrich, St. Louis, MO, USA), human cellular fibronectin (F2518, Sigma-Aldrich), a proteolytic human plasma fibronectin 70 kD fragment (F0287, Sigma-Aldrich), a human fibronectin 120 kD cell attachment fragment (Part Number 175, YO Proteins, Ronninge, Sweden), human type IV collagen (C5533, Sigma-Aldrich), recombinant peristin (rPOSTN; RPH339Hu01, CloudClone Corp., Katy, TX, USA), and recombinant fibulin-5 (rFBLN5; 9006-FB-050, R&D Systems, Minneapolis, MN, USA) were either supplemented to the ECGM or used to coat cell culture plates with the indicated concentrations. If not stated otherwise, the cells were cultured for 48 hours with ECM protein supplementation. Cells cultured on uncoated, tissue culture treated plates served as controls.

2.2 | CRISPR/Cas9 genome editing

Following our established protocol,20 crRNA:tracrRNA:Cas9 ribonucleoprotein (RNP)-mediated genome editing was used to inactivate CCM1, CCM2 or CCM3, respectively. In addition to the previously identified CRISPR/Cas9 target region in CCM3 exon 3 [LRG_651; 5′-CTTGTATGAGGTGATGTTG-3′; (crRNA CCM3)], target sequences located in CCM1 exon 10 [LRG_665; 5′-CCCCGATCCTGGTCTGTTCC-3′; (crRNA CCM1)], and CCM2 exon 4 [LRG_664; 5′-GGTCTGCCAGCCAGTGGCC-3′; (crRNA CCM2)] were selected using the Alt-R crRNA design tool (Integrated DNA Technologies, Leuven, Belgium). CI-huVECs and hCMEC/D3 cells were transfected with crRNA:tracrRNA:Cas9 RNP-complexes and clonally expanded as described before.20 CCTop-CRISPR/Cas9 target online predictor36 was used to identify potential off-target sides. The following criteria were used: (1) ≤ 4 crRNA mismatches and (2) ≤ 2 crRNA mismatches in the core of the first twelve nucleotides next to the PAM. Two exonic off-target regions were selected for each crRNA. T7EI assay was used to analyze off-target sides in crRNA:tracrRNA:Cas9 RNP-treated cell mixtures [off-target CCM1: 5′-CCA-TACCTTTGTCGTTCTGAGG-3′ (ARVC); 5′-CCA-TACCTTTGTCGTTCTGAGG-3′ (ARVE)]; off-target CCM2: 5′-GGAAAGTTAAGGTAATCGCA TACC-AGG-3′ (PAPS2), 5′-GATCAGCTACGTCTTAATCGCA ATGCC-TGG-3′ (KDM4D)]. Sanger sequencing was used to screen for off-target mutations in clonally expanded CCM± cells [off-target CCM3: 5′-CCG-CTGTTTAAATG AGGTGACAGC-3′ (ZNF256), 5′-CCG-CTGTTTAAATG AGGTGACAGC-3′ (ZNF256)].
GACTAGATG-3’ (ZNF586)]. PCR primers used to amplify the CRISPR/Cas9 target and predicted off-target regions for Sanger sequencing or T7EI assays were as follows: CCMI: forward: 5′-ACAGAGAAACTGCAAAGGCTA-3′, reverse: 5′-AAGTGATGCTTCTTTATCCACTCACACCACCCG-3′; CCMI: forward: 5′-CCATATTAGCGTGTATTTGCT-3′, reverse: 5′-GGATGGATACTCAGTATGCTGCAAGC-3′; CCM2: forward: 5′-GTGGTGTAGACATCGGAGCTAAGCT-3′, reverse: 5′-CTTCTAGTACTTCATACACCCACCTGTCC TTC-3′; ARVC: forward: 5′-GCCCTGAGAGACCTGACCTGACCTGC-3′, reverse: 5′-GAGGACCAACAGTGAAGCG-3′; PAPSS2: forward: 5′-AGGATGTTCATTGGCAGTGGT GTGATGACAAAGCTCTTAATGGGT-3′; reverse: 5′-GCACTTTTCAAGGCGACATTTGCGCAGCTGC-3′.

2.3 | T7EI assay and amplicon deep sequencing

T7EI analyzes were performed as described before. To analyze the CRISPR/Cas9-induced mutational spectrum, amplicon deep sequencing libraries were prepared with a custom two-step PCR approach following our established protocol. PCR products were pooled and purified with Agencourt AMPure XP beads (Beckman Coulter, Pasadena, USA). The library was sequenced on a MiSeq instrument with 2 × 150 cycles (Illumina, San Diego, USA). The SeqNext software was used for data analysis (JSI Medical Systems, Ettlingen, Germany). Only variants with combined read frequencies ≥ 1% and quality score ≥ 25 were called. The following primers with adapter sequences were used for specific target enrichment: CCMI: forward: 5′-ATCGGGAAAGGCTAAGGCTAAC TGGAGATTAAACCGA-3′, reverse: 5′-ATCGCGAGGT TAGTGATGACAAAGCCTCTTATGGGTGTGCTTCTTTATCCACCCACACCCG-3′; CCMI: forward: 5′-ATCGGGAAAGGCTAAGGCTAAC TGGAGATTAAACCGA-3′, reverse: 5′-ATCGCGAGGT TAGTGATGACAAAGCCTCTTATGGGTGTGCTTCTTTATCCACCCACACCCG-3′; CCM2: forward: 5′-ATCGGGAAAGGCTAAGGCTAAC TGGAGATTAAACCGA-3′, reverse: 5′-ATCGCGAGGT TAGTGATGACAAAGCCTCTTATGGGTGTGCTTCTTTATCCACCCACACCCG-3′; CCMI: forward: 5′-ATCGGGAAAGGCTAAGGCTAAC TGGAGATTAAACCGA-3′, reverse: 5′-ATCGCGAGGT TAGTGATGACAAAGCCTCTTATGGGTGTGCTTCTTTATCCACCCACACCCG-3′. The second step, barcoded reverse primers and universal forward primer were used to generate the final sequencing library.

2.4 | RNA isolation, RNA sequencing, and qPCR

The PepGold TriFast reagent (Peqlab-VWR, Radnor, PA, USA) and Direct-zol RNA MiniPrep Plus Kit (Zymo Research, Irvine, CA, USA) were used to extract and purify total RNA. The integrity of the RNA samples was checked on a 2100 Bioanalyzer using the Agilent RNA 6000 Nano Kit (Agilent, Santa Clara, CA, USA). RNA concentrations were measured on a Qubit 2.0 (Thermo Fisher Scientific) with the Qubit RNA HS Assay Kit (Q32852, Thermo Fisher Scientific). Sequencing libraries were prepared with the TrueSeq Stranded mRNA Kit according to the manufacturer’s instructions (Illumina, San Diego, CA, USA). Pooled libraries were sequenced as 100 bp or 150 bp paired-end runs on a HiSeq 4000 instrument (Illumina). Reads that mapped to annotated genes were quantified with HTseq-count and the Relative Log Expression (RLE) normalization, which is implemented in the R Bioconductor package DESeq2 was used to normalize gene counts. RNA sequencing data were uploaded to the Gene Expression Omnibus (GEO) database (record number: GSE138431). Transcripts with a llog2FC > 2 and Padj < .05 were subjected to a PANTHER overrepresentation test (version 13.0; http://pantherdb.org/). The PANTHER GO-Slim Cellular Component annotation data set was used as reference list. The First Strand cDNA Synthesis Kit (Thermo Fisher Scientific) was used to transcribe mRNA into cDNA. Deregulated gene expressions of FN1, POSTN, and FBLN5 were validated by SYBR Green-based qPCR on a Roche Light Cycler 480 instrument (Roche, Mannheim, Germany). The housekeeping gene RPLPO (ribosomal protein lateral stalk subunit P0) was used as an endogenous control. For detection of FN1, POSTN, and FBLN5, PrimeTime qPCR Primers were purchased from Integrated DNA Technologies (Hs.PT.58.40005963, Hs.PT.58.4452022, Hs.PT.58.14576443). The following primer pair were used for RPLPO qPCR: 5′-ATCGGAAATGGCACGATCTAC-3′ and 5′-ATCCCGGTCTCCACACAGAACAGC-3′.

2.5 | Western blot analyzes

Proteins were extracted with PepGold TriFast reagent (Peqlab-VWR) and solubilized in buffer containing 8 M of urea, 2 M of Thio-Urea, and 20 mM of Tris for CCM3 immunoblotting. Proteins of cell culture supernatant were precipitated with 20% of trichloroacetic acid and washed two times with ethanol before solubilization in buffer containing 8 M of urea, 2 M of Thio-Urea, and 20 mM of Tris. For deoxycholate (DOC) assays, cells were treated as described before. In brief, 200 000 cells were cultured on a 6-well plate, washed with phosphate-buffered saline (PBS, PromoCell), lysed with 500 μL of DOC buffer [2% of DOC, 20 mM of Tris-HCl (pH 8.8), 2 mM of
EDTA, 1× Halt protease and phosphatase inhibitor cocktail (Thermo Fisher Scientific) and scraped from the cell culture plate. After centrifugation, the insoluble fraction was solubilized in SDS buffer [1% of SDS, 25 mM of Tris-HCl (pH 8.2), 2 mM of EDTA, 1× Halt protease, and phosphatase inhibitor cocktail]. Quantification was performed with the Qubit Protein Assay Kit (Thermo Fisher Scientific) on a Qubit 4 Fluorometer (Thermo Fisher Scientific) or with the Micro BCA Protein Assay Kit (Thermo Fisher Scientific). About 10 or 20 µg total protein were separated on 7.5% or 10% of TGX stain-free gels (Bio-Rad, Hercules, California, USA), transferred to PVDF membranes (Roche, Basel, Switzerland), immunostained with monoclonal mouse anti-human fibronectin (1:5000, MAB19182, R&D systems) or rabbit anti-CCM3 (1:150; IG-626, ImmunoGlobe, Himmelstadt, Germany), HRP conjugated mouse IgG light chain binding protein (1:30 000, sc-516102, Santa Cruz Biotechnology, Dallas, Texas, USA) or anti-rabbit HRP secondary antibody (1:30 000, sc-2357, Santa Cruz Biotechnology), Precision Protein StrepTactin-HRP Conjugate (1:15 000, Bio-Rad) and detected with Clarity Western ECL (Bio-Rad). A ChemiDoc XRS+ (Bio-Rad) imager was used for blot documentation of Stain-Free total protein and chemiluminometric signal detection. To semi-quantify relative CCM3 and fibronectin protein expression, normalized band intensities were calculated with the ImageLab software (v6.0, Bio-Rad). Since total protein can be used as reliable loading control, the volume intensities of the detected protein bands were normalized to the volume intensities of the total protein fraction. For normalization of the DOC-insoluble fractions, total protein volume intensities of the DOC-soluble fractions were used. For relative fibronectin quantification in cell culture supernatants, the volume intensities of the detected protein bands were normalized to the cell number per 0.5 × 0.5 mm growth area, which was documented right before sample preparation.

2.6 | Phospho-kinase detection assay and immunofluorescence imaging

Relative phosphorylation levels of different human protein kinases were determined using the Proteome Profiler Human Phospho-Kinase Array Kit (ARY003B, R&D Systems) according to the manufacturer’s instructions. In detail, approximately 1 × 10^7 cells/mL were solubilized in lysis buffer, and the total protein concentration was measured with the Qubit Protein Assay Kit (Thermo Fisher Scientific). About 600 µg were used per array set (A + B). A ChemiDoc XRS+ (Bio-Rad) imager was used for documentation, and pixel densities were determined using ImageJ software. For immunofluorescence analysis, 1 × 10^4 cells/well were cultured on a 96-well plate followed by fixation, permeabilization, and immunostaining as described previously. In brief, cells were fixated with 4% paraformaldehyde at room temperature, permeabilized with 0.1% of Triton X-100 for 15 min, and blocked with 2% of normal goat serum for 1 hour. DNA and F-actin were visualized with DAPI (D9542, Sigma-Aldrich) and iFluor 488-conjugated phalloidin (ab176753, Abcam, Cambridge, UK), respectively. To analyze actin stress fiber formation, at least 120 cells per replicate were manually counted. Cells with short F-actin bundles spanning the nucleus were counted as stress fiber-positive. For fibronectin staining, cells were fixed with ice-cold (−20°C) methanol for 10 minutes. Primary mouse anti-human fibronectin antibodies (1:160, MAB19182, R&D Systems) and Alexa Fluor 488-conjugated secondary goat anti-mouse IgG antibodies (1:200, A-11029, Life Technology, Carlsbad, CA, USA) were incubated at RT for 1 hour. After adding Ibidi Mounting Medium (Ibidi, Martinsried, Germany), image acquisition was performed for each sample of an experiment with the same settings.

2.7 | Cell morphology, spheroid, and tube formation assay

CI-huVECs were seeded with 1 × 10^4 cells per well on a 96-well plate that had been coated with fibronectin to study cell morphology. Uncoated, tissue culture treated plates served as controls. After 48 hours, at least 300 cells were manually counted to determine the proportion of cells with a compact morphology. Approximately 400 cells were cultured for 24 hours with or without ECM protein supplementation in methocel containing medium (2.4 g/L) as hanging drop (25 µL) to study spheroid formation. The ImageJ software was used to analyze the spheroid cross-sectional areas and circularities of at least 10 spheroids per replicate. In brief, we manually traced the perimeters of each individual spheroid in ImageJ, calculated the lengths of the perimeters (l) in pixels as well as the areas (A) of the selections in square pixels and finally converted the values into millimeters or square millimeters by spatial calibration of the images. The following equation was used to calculate the circularity of the spheroids: \( \frac{4\pi A}{\pi l^2} \). To analyze tube formation, 2 × 10^4 cells were cultured on Matrigel (Corning, Kaiserslautern, Germany) in 96-well plates or Angiogenesis µ-Slides (Ibidi). The number of master segments (number of tubes), total master segments length (tube length), and mesh numbers were quantified after 20 hours with the Angiogenesis Analyzer ImageJ plugin. For these assays, bright-field monochrome images were acquired with the EVOS FL Cell Imaging System at 4× (AMEP4680) or 10× (AMEP4681) magnification.
2.8 | Caspase-3 activity and ECM cell adhesion assays

After 72 hours of fibronectin supplementation, CI-huVECs were incubated for 2 hours with 1 μM of staurosporine to induce apoptosis. The Caspase-3 DEVD-R110 Fluorometric HTS Assay Kit (Biotium, Fremont, CA, USA) was used according to the manufacturer’s instructions.

The ability of *CCM3*+/− and *CCM3*−/− CI-huVECs to bind to fibronectin, type I collagen, type II collagen, type IV collagen, laminin, tenascin, and vitronectin was quantified with a fluorometric ECM Cell Adhesion Array Kit (ECM545, Sigma-Aldrich) according to the manufacturer’s instructions. In brief, CI-huVECs were resuspended in assay buffer and seeded in precoated 96-well plates with 1.5 × 10^5 cells/well. After 2 hours at 37°C and 5% of CO₂, nonadherent cells were aspirated and the wells were washed three times with assay buffer. Finally, the sample wells were incubated for 15 minutes with a solution of 4X cell lysis buffer and CyQuant GR dye, which shows an enhancement of fluorescence when bound to cellular nucleic acids. Fluorescence intensity was measured in relative fluorescence units (RFU) at Ex/Em (nm) = 470/510-580 nm. Bovine serum albumin coated wells served as control.

2.9 | Microscope image acquisition

An EVOS FL Cell Imaging System (Thermo Fisher Scientific; Sony ICX445 monochrome CCD; EVOS Light Cube DAPI, GFP, RFP; 10x AMEP4681, 40x AMEP4683) or a Zeiss LSM 510 META Confocal Microscope (Carl Zeiss AG, Oberkochen, Germany; Zeiss LSM Software 3.5 plus; meta detector; laser: 405 nm, 488 nm; major beam splitter: HFT 405/488/561 nm; band-pass filter: 505-550 nm; Plan-Neofluar 40x/1.3 Oil DIC) were used for image acquisition.

2.10 | Statistical analysis

GraphPad Prism software (v.8.0.1, GraphPad Software, LA Jolla, CA, USA) was used for data analyzes. Data are presented as mean and single data points if not stated otherwise. Two-tailed, Student’s t tests, multiple t tests, and two-way ANOVA with Holm-Šidák’s multiple comparisons tests were used for two or more groups, respectively. RNA sequencing data were analyzed with Wald test and the Benjamini-Hochberg procedure for multiple testing, while Fisher’s Exact test with false discovery rate (FDR) multiple test correction was used for PANTHER overrepresentation analysis. P-values <.05 and q-values < .05 were regarded as statistically significant.

3 | RESULTS

We have recently demonstrated that CRISPR/Cas9-induced *CCM3* inactivation in human ECs induces profound morphological and functional changes. In our present study, we used genome-wide RNA sequencing of *CCM3*+/− and *CCM3*−/− CI-huVECs to get a better understanding of the underlying molecular mechanisms. About 569 upregulated or downregulated genes (log₂FC > 2 and p < .05) were identified in clonally expanded *CCM3*-deficient ECs (Figure 1A,B). A gene set enrichment analysis revealed an overrepresentation of cell junction, extracellular region, and plasma membrane components (Table 1).

We focused our further analyzes on FNI (log₂FC = −2.3; p = 2.10 × 10^−99), POSTN (log₂FC = −8.5; p = 2.68 × 10−226), and FBLN5 (log₂FC = −8.2; p = 2.44 × 10−197) since they were either the most downregulated genes in *CCM3*−/− CI-huVECs (POSTN and FBLN5) or highly expressed in wild-type *CCM3*+/+ but significantly downregulated in *CCM3*−/− CI-huVECs (FNI). Expression differences of the three genes that encode for the ECM glycoprotein fibronectin or the matricellular proteins periostin and fibulin-5, respectively, were validated by RT-qPCR (Figure 1C). Fibronectin is important for proper ECM assembly and the highest endothelial FNI expression has been found in the brain, suggesting that it might support the integrity of the neurovascular unit. Therefore, we decided to study the role of fibronectin in CCM pathogenesis in more detail. In Western Blot analyzes, we observed less fibronectin in the cell culture supernatants of *CCM3*−/− CI-huVECs and reduced DOC-insoluble fibronectin aggregates upon *CCM3*-inactivation (Figure 1D). No fibronectin was detected in the DOC-soluble fractions.

To test whether *CCM3*−/− CI-huVECs have a cell-to-ECM adhesion defect, we used cell culture plates coated with fibronectin, type I, II, and IV collagens, laminin, tenascin, and vitronectin in a fluorometric cell adhesion array approach. Of note, binding of *CCM3*-deficient ECs to exogenous fibronectin was intact and no major cell binding abnormalities were observed for the other ECM components (Figure 1E). These results confirmed that long-term *CCM3* inactivation impairs FNI expression but not the adhesion of CI-huVECs to fibronectin. This is consistent with the observation that supplementation of plasma fibronectin enhanced fibronectin fiber assembly in the ECM of *CCM3*−/− CI-huVECs (Figure 1F). Therefore, we next examined whether exogenous fibronectin supplementation could rescue the endothelial dysfunction of *CCM3*-deficient ECs.

3.1 | Fibronectin replacement improves spheroid formation and prevents actin stress fiber assembly

Fibronectin supplementation significantly attenuated the aberrant phenotype of *CCM3*−/− CI-huVECs. In particular, they
Figure 1: Deregulation of extracellular matrix components after long-term CCM3 inactivation. A, Western Blot analyzes verified complete CCM3 inactivation in clonally expanded CCM3−/− CI-huVECs used in this study (clones I-IV). Expression levels normalized to the CCM3+/+ control group are given below the panel. B, RNA-Seq data of CCM3+/+ control (x-axis) and CCM3−/− CI-huVECs (y-axis) are presented as scatter dot plot. FPKM = fragments per kilobase of exon model per million mapped reads. C, Expression levels of FN1, FBLN5, and POSTN were validated by qPCR. D, Western Blot results revealed less fibronectin in cell culture supernatants of CCM3−/− CI-huVECs and reduced DOC-insoluble fibronectin aggregates upon CCM3-inactivation. Expression levels normalized to the CCM3+/+ control group are given below the subpanels. E, Fluorometric cell adhesion assays demonstrated no major cell binding abnormalities of CCM3−/− CI-huVECs to ECM components. F, A reduced fibronectin expression was observed in immunofluorescence imaging of 1 × 10⁴ cells/well cultured on a tissue culture treated 96-well plate after 48 hours. Plasma fibronectin supplementation promoted fibronectin matrix assembly. Scale bars ≈ 100 µm in the left and 50 µm in the right panels. Images were acquired using the same setting for each sample and no changes were implemented. ND = not detected, RFU = relative fluorescence units, ctrl = CCM3+/+ control cells, Col I = type I collagen, Col II = type II collagen, Col IV = type IV collagen, FN = fibronectin, LN = laminin, TN = tenasin, VN = vitronectin, and Neg = bovine serum albumin. Data are presented as mean and single data points (n = 3-4). Multiple t tests were used for statistical analyzes. ****P < .0001.
lost their rounded morphology and recovered a spindle-like cell shape when cultured on plates, which had been coated with human plasma fibronectin (Figure 2A). Moreover, fibronectin replacement suppressed actin stress fiber formation and rescued the inability to form round and demarcated spheroids in 3D culture. When cultured in plasma fibronectin-containing hanging drops, the circularity of spheroids formed by CCM3−/− cells was not rescued by fibronectin supplementation (Figure 2E). As expected, fibronectin supplementation in general (CCM3−/− versus wild-type cells) increased significantly and their cross-sectional area decreased to nearly normal levels when compared to spheroids formed by CCM3+/+ wild-type cells (Figure 2B). Besides, normalization of cortical actin filaments and less actin stress fibers were observed in CCM3−/− CI-huVECs that had been cultured on fibronectin-coated plates (Figure 2C; Figure S1). As expected, fibronectin supplementation had no adverse effects on CCM3+/+ CI-huVECs that had been cultured on fibronectin-coated plates (Figure 2C; Figure S1). The cell-binding fragment of fibronectin is essential to rescue the altered endothelial phenotype

Plasma fibronectin exists as a soluble dimer of two covalently linked approx. 250 kD subunits with a complex structure of various functional domains. It differs from cellular fibronectin by the two extra domains A (EDA) and B (EDB). As verified by our RNA sequencing data, both isoforms are expressed by CI-huVECs (Figure S2). With two proteolytic plasma fibronectin fragments, we were able to narrow down the region that is crucial to suppress reorganization of the actin cytoskeleton to stress fibers and rescue the dysfunction of CCM3-deficient ECs. The 70 and 120 kD fragments, as well as the less-soluble cellular fibronectin isoform reduced actin stress fiber assembly in CCM3+/+ CI-huVECs (Figure 4A) but only the 120 kD cell attachment fragment with its RGD domain and major integrin interaction sites also normalized spheroid formation (Figure 4B). The N-terminal 70 kD region of fibronectin does not contain the assembly domain, but can also stimulate outside-in signaling. Interestingly, it has already been demonstrated that the N-terminal 70 kD fragment can bind α5β1 integrins and control actin assembly. This might be an explanation

### Table 1: Gene set enrichment analysis

| PANTHER GO-slim cellular component | # Genes | FC | FDR |
|-----------------------------------|---------|----|-----|
| Neuronal cell body                | 3       | 8.27 | 0.0479 |
| Cell junction                     | 10      | 4.44 | 0.0036 |
| Extracellular region              | 30      | 1.88 | 0.0117 |
| Integral to membrane             | 39      | 1.77 | 0.0098 |
| Plasma membrane                   | 56      | 1.64 | 0.0060 |
| Membrane                         | 43      | 1.55 | 0.0407 |
| Intracellular                     | 102     | 0.79 | 0.0421 |
| Organelle                        | 58      | 0.6  | 0.0004 |
| Nucleus                           | 24      | 0.5  | 0.0045 |
| Mitochondrion                    | 3       | 0.24 | 0.0239 |

**Note:** Over- and underrepresented terms in PANTHER cellular component analysis are marked in green and red, respectively. Abbreviations: FC, fold change; FDR, false discovery rate.
FIGURE 2 Restored endothelial function of $CCM3^{-/-}$ CI-huVECs by fibronectin replacement. A, $CCM3^{-/-}$ CI-huVECs cultured on fibronectin coated plates (5 µg/cm²) regained a typical endothelial morphology. Black arrowheads indicate a compact cell shape, while white arrowheads indicate cells that show a spindle-shaped morphology. $CCM3^{+/+}$ and $CCM3^{-/-}$ CI-huVECs were seeded with $1 \times 10^4$ cells/well on a 96-well plate. Scale bar $\equiv 200 \mu$m. B, Fibronectin supplementation (32 µg/mL) significantly improved the spheroid organization of $CCM3^{-/-}$ CI-huVECs. The circularity and the cross-sectional area of the spheroids were determined. The manually traced perimeter of the shown spheroid is depicted in the upper right corner. Scale bar $\equiv 100 \mu$m. C, $CCM3^{-/-}$ CI-huVECs cultured on fibronectin-coated plates demonstrated a reduced actin stress fiber formation ($1 \times 10^4$ cells/well; 96-well plate). Confocal microscopy was used for image acquisition. Phalloidin-iFluor 488 and DAPI staining are shown in green and blue, respectively. The brightness was adjusted equally for all images to show the relevant structures of F-actin formation. Original images are shown in Figure S1. Scale bar $\equiv 50 \mu$m. D, The reduced ability of $CCM3^{-/-}$ CI-huVECs to form tube-like structures could not be rescued by fibronectin supplementation (32 µg/mL). Scale bar $\equiv 1 \text{mm}$. E, Neither fibronectin coating (5 µg/cm²) nor supplementation to the culture medium (32 µg/mL) had an effect on staurosporine-induced Caspase-3 activity. F, Representative Phospho-Kinase array membranes are shown for $CCM3^{-/-}$ CI-huVECs cultured without and with fibronectin supplementation (5 µg/cm²). Spots showing the detection of phosphorylated forms of Src and FAK are marked in green or red, respectively. G and H, RNA-Seq data of $CCM3^{+/+}$ control cells without (x-axis) and $CCM3^{-/-}$ CI-huVECs with 5 µg/cm² fibronectin supplementation (y-axis) (G) or $CCM3^{-/-}$ CI-huVECs without (x-axis) and with (y-axis) fibronectin supplementation (H) are presented as scatter dot plot. FPKM = fragments per kilobase of exon model per million mapped reads. ctrl = $CCM3^{+/+}$ control cells, FN = fibronectin, Nb = Number. Data are presented as mean and single data points ($n = 3-5$). Two-way ANOVA with Holm-Šidák’s multiple comparisons test, multiple $t$ test or Student’s $t$ test were used for statistical analyzes: *$P < .05$; **$P < .01$; ****$P < .0001$
for the actin cytoskeleton reorganization that we observed in CCM3−/− CI-huVECs. However, the 70 kD fragment is also a well-known inhibitor of fibronectin matrix polymerization, and therefore, might not be able to facilitate organization of spheroids formed by CCM3−/− CI-huVECs.

It is noteworthy to mention that supplementation of type IV collagen increased the circularity of CCM3−/− CI-huVECs but did not reduce actin stress fiber assembly (Figure 4A,B). Periostin replacement stabilized tube-like structures but did not attenuate dysfunctional cytoskeletal dynamics, and fibulin-5 treatment reduced stress fiber formation but induced severe morphological changes in CCM3−/− CI-huVECs (Figure 5).

3.3 Dysregulation of the fibronectin matrix is a common feature of CCM1/2/3-deficiency

We next asked the question of whether inactivation of CCM1 and CCM2 in human ECs also impairs fibronectin expression. Thus, CRISPR/Cas9 genome editing was used for CCM1 and CCM2 gene disruption in CI-huVECs. Eight days after RNP-transfection, average T7EI cleavage efficiencies of 32% (n = 3; range: 31%-33%) and 17% (n = 3; range: 15%-19%) were observed, respectively. After thirteen days, these had increased to 43% (CCM1; range: 39%-48%) and 53% (CCM2; range: 47%-54%). These results are in line with the survival benefit of CCM3- and CCM1-deficient human ECs that we have described before.

We could not identify commercial antibodies specific to CCM1 or CCM2 in this study. Nevertheless, the results of our deep sequencing analysis indicated protein inactivation in the vast majority of cells: A high proportion of CCM3−/− CI-huVECs (Figure 5). As expected, disruption of the CCM1 and CCM2 genes reduced fibronectin expression (Figure 6). Furthermore, supplementation of human plasma fibronectin rescued the impaired spheroid formation, the reorganization of the actin cytoskeleton, and the atypical rounded cell morphology that were observed in cell mixtures treated with CCM1- or
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Ccm2-specific crRNA:tracrRNA:Cas9 RNP-complexes, respectively (Figure 7). Together, these results indicate overlapping effects and regulation of endothelial fibronectin expression by CCM1, CCM2, and CCM3. In addition, alterations in potential off-target sites after crRNA:tracrRNA:Cas9 RNP-treatment targeting CCM1, CCM2, and CCM3 could be excluded in all cell mixtures and clonal cell lines of CI-huVECs and hCMEC/D3 cells (Figure 6 and Data not shown).

4 | DISCUSSION

In this study, we show that CCM1, CCM2, and CCM3 regulate the production of a functional fibronectin matrix by human ECs. Our results also demonstrate that supplementation of fibronectin rescues morphological changes as well as impaired endothelial spheroid organization and normalizes actin stress fiber formation upon CRISPR/Cas9-induced inactivation of either CCM1, CCM2 or CCM3.

The formation of cavernous vascular malformations upon endothelial CCM3 inactivation has recently been associated with impaired dispersion of ECs in vivo.6 The results of Castro and colleagues suggest that CCM3−/− ECs might be unable to build or remodel their ECM, which serves as a scaffold for coordinate migration. In addition to an important role in angiogenesis, the CCM proteins also contribute to the maintenance and regulation of the vascular endothelial barrier function.57,58 Therefore, studying cytoskeletal remodeling, cell-cell, and cell-matrix interactions is useful to get a better understanding of the mechanisms leading to increased vascular permeability and recurrent bleeding events in CCM patients.

We here demonstrate that CRISPR/Cas9-induced CCM3 gene disruption in ECs significantly impairs the expression of fibronectin in vitro. Our observation also reflects the situation in human CCM tissues, in which less and irregularly organized fibronectin fibrils have been noticed around CCM microvessels. Interestingly, this was accompanied by an altered dispersion of tight junction proteins.28
Besides, our study disclosed that the inability to produce a functional fibronectin matrix is not limited to CCM3 deficiency but is also a consequence of CCM1 and CCM2 inactivation in human ECs. CCM1, CCM2, and CCM3 are known to act together in the same signaling pathways but also independently from each other.\textsuperscript{59-61} GST pull-down and co-immunoprecipitation studies have demonstrated that the three CCM proteins can form a ternary complex in vitro and that CCM2 acts as a linker molecule between CCM1 and CCM3.\textsuperscript{62,63} The shared phenotype of CCM1-, CCM2-, and CCM3-deficient human ECs indicates that the three CCM proteins regulate the expression of fibronectin in a common pathway.

The formation of actin stress fibers has been consistently observed in CCM1-, CCM2-, and CCM3-deficient ECs.\textsuperscript{20,33,64,65} Therefore, it has been widely used as surrogate marker for endothelial dysfunctions that are induced by CCM1/2/3 inactivation. Noteworthy, stress fiber formation was also a readout parameter in a recent pharmacological high-throughput screen and in one of the first studies that identified Rho kinase inhibition as a potential therapeutic approach in CCM disease.\textsuperscript{31,66} Fibronectin directly participates in mechanosignaling and is linked to the cytoskeleton by the matrix-integrin-cytoskeletal signaling axis.\textsuperscript{67,68} Its sustained binding to integrin receptors can induce RhoA activation and stress fiber assembly.\textsuperscript{48} On the contrary, Rho-dependent cytoskeletal contractility also promotes fibronectin fibrillogenesis.\textsuperscript{69} Therefore, reorganization of the actin cytoskeleton into stress fibers might be a compensatory mechanism to impaired fibronectin expression and lack of a fibronectin-rich ECM upon CCM protein inactivation. The paradoxical effect of fibronectin supplementation on CCM1\textsuperscript{−/−}, CCM2\textsuperscript{−/−}, and CCM3\textsuperscript{−/−} ECs, namely suppression of actin stress fiber formation and restoration of the cortical actin network, supports this hypothesis. Notably, the formation of cortical actin filaments has been associated with enhanced endothelial barrier function\textsuperscript{70} which would be beneficial in CCM pathogenesis.

Cseh and colleagues have demonstrated that supplementation of exogenous fibronectin can restore functional cell-cell junctions between fibronectin-depleted bovine ECs.\textsuperscript{71} In addition, monolayers of porcine brain capillary
ECs supplemented with fibronectin showed increased transendothelial electrical resistance (TEER) indicating that fibronectin supports tight junction formation and barrier function. These observations are interesting since the expression and distribution of cell-cell adhesion proteins is impaired in CCMs. The endothelial spheroid formation assay which was first described in 1998 is a versatile tool to study EC function in a 3D microenvironment. Not only cell-cell but also cell-matrix interactions play important roles in this organization process. Notably, fibronectin fibers can be found at the periphery of endothelial spheroids.

In CCM mouse models, vascular lesions predominantly develop in the brain and retina. Fibronectin is an essential ECM component of retinal blood vessels, and its endothelial-specific inactivation in mice leads to reduced radial growth, less vessel branching, and more vascular regression events. This phenotype is partially reminiscent of the leaky retinal lesions that can be found in CCM mouse models. In the retinas of Ccm1- and Ccm3-knockout mice, a dense network of dysfunctional vessels can be observed at the periphery of the vascular plexus. Furthermore, treatment of aortic explants with a Gly-Arg-Gly-Asp-Ser (GRGDS) peptide, which inhibits the binding of fibronectin to its cognate integrin receptors induced regression of developing microvessels. An altered dispersion of tight junction proteins which are important elements of the blood brain barrier is accompanied by less fibronectin in CCM lesions. In addition, fibronectin may enhance barrier function through its influence on the localization of tight junction proteins. Together, these observations may lead to the hypothesis that disturbance of fibronectin triggers endothelial dysfunction in Ccm1- and Ccm3-knockout mice.

Taken together, we show that fibronectin was able to rescue a broad spectrum of endothelial alterations that were induced by the disruption of CCM1, CCM2, and CCM3 in human ECs. However, its supplementation neither rescued...
aberrant gene expression signatures in $CCM3^{-/-}$ ECs nor inhibited their survival advantage. These observations support the conclusion that the formation and progression of CCM lesions are controlled by a complex network of deregulated pathways. A positive influence on individual signaling cascades and endothelial dysfunctions is not necessarily associated with a complete rescue. Therefore, combinatorial strategies are likely required to prevent CCM formation and progression in a therapeutic setting.

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CONFLICT OF INTEREST
The authors declare no conflicts of interest.

AUTHOR CONTRIBUTIONS
K. Schwefel, S. Spiegler, C.D. Much, and P.K.E. Dellweg performed most of the experiments; K. Schwefel, B. C. Kirchmaier, U. Felbor, and M. Rath contributed to the intellectual conception and the design of the study; M. Rath, K. Riedel, and U. Felbor supervised the experiments; K. Schwefel, S. Spiegler, P.K.E. Dellweg, B.C. Kirchmaier,
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DATA AVAILABILITY STATEMENT

All relevant data are published within the paper and its supporting additional files.

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**SUPPORTING INFORMATION**
Additional Supporting Information may be found online in the Supporting Information section.

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