ONLINE RESOURCES

Contact-dependent signaling triggers tumor-like proliferation of CCM3 knockout endothelial cells in co-culture with wild-type cells

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Online Resource 1 Verification of CCM3 knockout in CI-huVECs. Western Blot results are given for one wild-type CI-huVEC (WT) and seven $CCM3^{-/-}$ CI-huVEC cell lines (KO). No CCM3 protein was detected in the KO cell lines. Pure His-tagged CCM3 protein was used as a positive control (+). ND = not detected

Online Resource 2 Increased cell survival rates of $CCM3^{+/+}$ CI-huVECs under staurosporine stress. $CCM3^{+/+}$ and $CCM3^{-/-}$ CI-huVECs were treated with either 0.05 µM or 0.25 µM staurosporine to induce apoptotic cell death. After 24 hours, the number of cells per mm$^2$ was determined and normalized to the initial cell count (n = 3 per genotype). Students two-tailed $t$ tests were used for statistical analyses: **P<0.01
Online Resource 4 CCM3−/− CI-huVECs demonstrate increased proliferative activity only in co-cultures with low mutant:wild-type ratios. CCM3−/− CI-huVECs were co-cultured with CCM3+/+ CI-huVECs with high (left subpanels) or low (right subpanels) mutant:wild-type ratios, respectively (n = 3 per condition). The ratios are given in the upper left corner of each subpanel. Mutant allele frequencies were determined by amplicon deep sequencing. Cell counts were quantified with the CyQUANT cell proliferation assays. The proliferation rates of CCM3−/− and CCM3+/+ CI-huVECs were calculated by combining the mutant allele frequencies and the cell numbers in co-culture at the indicated time points. Students two-tailed t tests were used for statistical analyses: *P<0.05
Online Resource 5 AICS-0036 and AICS-0054 hiPSCs. **a** AICS-0036 hiPSCs are characterized by cytoplasmic mEGFP expression. AICS-0054 hiPSCs with an mTagRFP-tag localized to the plasma membrane were used as controls. **b** The presence of the most common chromosomal abnormalities was excluded in CCM3⁻/⁻ AICS-0036 hiPSC. Shown is a representative result for one CCM3⁻/⁻ AICS-0036 hiPSC clone. * The parental hiPSC line (WTC/AICS-0) had been established from fibroblasts of a male donor. Therefore, the copy number for Xp is 1.
Online Resource 6 hiPSC-derived hBMEC-like cells express tight junction proteins and can form capillary-like structures. Representative images of the morphology (upper left panel, scale: 200 µm), tube formation assay (upper right panel, scale: 500 µm), and the immunofluorescence analyses of the tight junction proteins occludin and claudin-5 (lower panels, scale: 50 µm) for AICS-0036 CCM3⁻/⁻ and AICS-0054 CCM3⁺/+ derived hBMEC-like cells. Brightness was adjusted for occludin (AICS-0054 CCM3⁺/+ derived hBMEC-like cells) for clarity.
Online Resource 7  Deregulated \textit{ITGB4} gene expression in different CCM models. Listed p-values were adjusted for multiple testing (adj. p-value) by the authors. For Chernaya et. al 2018 differential gene expression was calculated using GFOLD (0.01), no p-values were included in the datasets.

| Gen | Model     | Strategy   | Analysis   | ITGB4 log2FC | adj. p-value | Reference                  |
|-----|-----------|------------|------------|--------------|--------------|----------------------------|
| CCM3| CI-huVEC  | knockout   | RNA-seq    | 4.28         | 2.57E-44     | Schwefel et al. 2020       |
| CCM1| BMEC      | knockout   | RNA-seq    | 2.50         | 6.07E-23     | Lopez-Ramirez et al. 2017 |
| CCM2| zebrafish | knockout   | RNA-microarray | 3.99         | 0.0104       | Renz et al. 2015          |
| CCM3| HCAEC     | knockdown passage 7 | RNA-microarray | 1.96         | 0.0050       | Guerrero et al. 2015      |
| CCM3| HCAEC     | knockdown passage 11 | RNA-microarray | 2.81         | 0.000014     |                            |
| CCM1| HBMVEC    | knockdown   | RNA-seq    | 3.66         | 0.00005      | Abou-Fadel et al. 2019    |
| CCM2| HBMVEC    | knockdown   | RNA-seq    | 0.09         | 0.00005      |                            |
| CCM1| zebrafish | knockout   | RNA-seq    | 0.96         | 0.3872       |                            |
| CCM2| zebrafish | knockout   | RNA-seq    | -2.71        | 0.0031       |                            |
| CCM1| HUVEC     | knockdown   | RNA-seq    | 3.46         |              | Chernaya et al. 2018      |
| CCM2| HUVEC     | knockdown   | RNA-seq    | 3.48         |              |                            |
| CCM3| HUVEC     | knockdown   | RNA-seq    | 1.71         |              |                            |
| CCM1| HUVEC     | overexpression | RNA-microarray | -0.56         | 0.0039       | Wüstehube et al. 2010     |

CI-huVEC = immortalized human umbilical vein endothelial cells  
BMEC = human blood-brain barrier endothelial cells  
HCAEC = human coronary artery endothelial cells  
HBMVEC = human brain microvascular endothelial cells  
HUVEC = Human umbilical vein endothelial cells

1. Schwefel K, Spiegler S, Kirchmaier BC, Dellweg PKE, Much CD, Pané-Farré J, et al. Fibronectin rescues aberrant phenotype of endothelial cells lacking either CCM1, CCM2 or CCM3. The FASEB Journal. 2020.
2. Lopez-Ramirez MA, Fonseca G, Zeineddine HA, Girard R, Moore T, Pham A, et al. Thrombospondin1 (TSP1) replacement prevents cerebral cavernous malformations. J Exp Med. 2017;214(11):3331-46.
3. Renz M, Otten C, Faurobert E, Rudolph F, Zhu Y, Boulagay G, et al. Regulation of beta1 integrin-Klf2-mediated angiogenesis by CCM proteins. Dev Cell. 2015;32(2):181-90.
4. Guerrero A, Iglesias C, Raguz S, Floridia E, Gil J, Pombo CM, et al. The cerebral cavernous malformation 3 gene is necessary for senescence induction. Aging Cell. 2015;14(2):181-90.
5. Abou-Fadel J, Vasquez M, Grajeda B, Ellis C, Zhang J. Systems-wide analysis unravels the new roles of CCM signal complex (CSC). Heliyon. 2019;5(12):e02899.
6. Chernaya O, Zhurikhina A, Hladyshev S, Pilcher W, Young KM, Ortner J, et al. Biomechanics of Endothelial Tubule Formation Differentially Modulated by Cerebral Cavernous Malformation Proteins. iScience. 2018;9:347-58.
7. Wüstehube J, Bartol A, Liebler SS, Brutsch R, Zhu Y, Felbou U, et al. Cerebral cavernous malformation protein CCM1 inhibits sprouting angiogenesis by activating DELTA-NOTCH signaling. Proc Natl Acad Sci U S A. 2010;107(28):12640-5.
Online Resource 8  

**Online Resource 8 a** CCM3<sup>−/−</sup> CI-huVECs show increased membrane-bound laminin on laminin-332-coated plates. Representative images of CCM3<sup>−/−</sup> and CCM3<sup>+/+</sup> CI-huVECs cultured on laminin-332-coated and non-coated plates (control) for 6 days (scale: 50 µm). **b** High purity of sorted ITGB4<sup>high</sup> CCM3<sup>neg</sup> and ITGB4<sup>low</sup> CCM3<sup>pos</sup> CI-huVEC populations. Western blot analysis verified complete CCM3 knockout in sorted ITGB4<sup>high</sup> cells (n = 3 per condition). CCM3 transcript levels were also significantly reduced in ITGB4<sup>high</sup> CCM3<sup>neg</sup> cells (n = 3 per condition). Students two-tailed t tests (b) were used for statistical analyses: **P<0.01
Online Resource 10 Effect of NSC59984 on \( CCM3^{--} \) and \( CCM3^{+/+} \) CI-huVECs in mono- and co-culture. 

NSC59984-treated \( CCM3^{--} \) and \( CCM3^{+/+} \) CI-huVEC mono-cultures were analyzed by RNA sequencing. Significantly up- or downregulated genes were subjected to gene ontology analyses. Shown are the top 20 of significantly enriched biological process GO terms (\( n = 3 \) per group). 

\( \text{c, d} \) p53 protein levels and transcription factor binding activities were analyzed in sorted \( \text{ITGB4}^{\text{high}} \text{CCM3}^{\text{neg}} \) and \( \text{ITGB4}^{\text{low}} \text{CCM3}^{\text{pos}} \) CI-huVEC populations. The Red line (d) indicates the value of the kit-provided transcription factor p53 positive control. 

\( \text{e} \) NSC59984-treated \( CCM3^{--} \) CI-huVECs were subjected to targeted RNA panel sequencing (QIAseq targeted RNA human apoptosis panel).
and cell death panel). The results are depicted as volcano plot. FC = fold change (NSC59984 vs. DMSO treatment). Transcripts with a $|\log_2 FC| \geq 1$ and an adjusted p-value < 0.05 are highlighted in red. f, g Upregulation of CDKN1A (p21) was verified on RNA (f) but not on protein level (g). black bar = DMSO control, grey bar = NSC59984 treatment (n = 3 per group). h A MAPK phosphorylation antibody array was used to study MAPK/ERK signaling in sorted ITGB4$^{\text{high}}$ CCM3$^{\text{neg}}$ and ITGB4$^{\text{low}}$ CCM3$^{\text{pos}}$ CI-huVEC populations (n = 3 per genotype). Representative array membranes are shown for both genotypes. Green rectangles mark phosphorylated p53 (pS15). No significant differences were found for any markers. Fisher exact test (a, b), Students two-tailed t test (d, f), and DESeq2 normalization method (e) were used for statistical analyses: *P<0.05