

Astrocytes propel neurovascular dysfunction during cerebral cavernous malformation lesion formation

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Supplementary Materials:

RNA isolation

Total RNA from cultured astrocytes and BMECs were isolated by TRIzol as specified by the manufacturer's protocol (Thermo Fisher Scientific). Briefly, 1ml of TRIzol reagent was added per well. Cell homogenization was completed by pipetting up and down several times throughout the entire surface area where cells were growing. Cell lysates were transferred to Phase Lock Gel 2ml tubes (2302830; VWR). Then, 200 μ l of chloroform (ICN19400290; Thermo Fisher Scientific) was added to each tube and mixed vigorously for 15 seconds, followed by a 3-minute incubation at room temperature prior to centrifugation at 12,000g for 15 minutes at 4°C. The aqueous phase containing RNA was collected and transferred to a 1.5ml DNase/RNase free microfuge tube. To precipitate the RNA, 500 μ l of isopropanol was added, resuspended, and incubated for 10 minutes at room temperature followed by centrifugation at 12,000g for 10 minutes at 4°C. The supernatant was removed, and the pellet was washed with 1ml of 75% ethanol followed by centrifugation at 7,500g for 5 minutes at 4°C. The supernatant was removed, and the pellet was air-dried at room temperature and dissolved in 11 μ l-20 μ l of DNase/RNase free water. To determine the concentration and purity, 1 μ l of each sample was analyzed using UV spectrophotometry at 260 and 280 nm using NanoDrop 1000 Spectrophotometer.

Isolation of primary astrocytes

Vegfa^{tm1.1Nagy} mice or *Hif-1 α* ^{fl/fl} mice with transgenic mice expressing the tamoxifen-inducible recombinase CreERT2 under the control of the astrocyte *Aldh1l1* promoter at postnatal day 5-7 were sacrificed, and their brains were isolated and placed into cold solution A (0.5% bovine serum albumin (BSA) in DMEM and 1 μ g/ μ l glucose, 10mM HEPES, 1x penicillin-streptomycin). Brain cortices were separated from the brain and rolled on dry filter paper to detach and remove the meninges. Cortices from 8-11 mice were pooled and minced with scissors in solution A, and the

tissue was centrifuged at 215g for 5 minutes at 4°C. The tissue pellet was digested with a papain solution (0.7mg/ml papain suspension [LS003126; Worthington], 20units/ml DNase I [11284932001; Sigma-Aldrich], and 0.150µg/ml tosyl-lysine-chloromethyl-ketone [T7254; Sigma-Aldrich]) at 37°C for 25 min with vigorous shaking every 10min. The tissue suspension was triturated using thin-tipped Pasteur pipettes until partially homogenous and centrifuged at 215g for 5 minutes. The pellet was resuspended with solution B (25% BSA in DMEM and 1µg/µl glucose, 10mM HEPES, 1x penicillin-streptomycin) and centrifuged at 1000g for 20min at 4°C. The lighter phase containing astrocytes was extracted, resuspended in 50ml of solution C (DMEM-1µg/µl glucose, 10mM HEPES, 1x penicillin-streptomycin), and centrifuged at 215g for 10 minutes at 4°C. The pellet was resuspended again in 50 ml of solution C and centrifugation was repeated.

Astrocyte culture conditions

The purified primary astrocytes were plated on a Poly-L lysine-coated plate cultured in astrocyte media comprised of 1:1 Neurobasal media and DMEM (1µg/µl glucose) supplemented with the following: 0.1mg/ml BSA, 0.1 mg/ml transferrin, 0.016mg/ml putrescine, 0.025µg/ml progesterone, 0.016µg/ml sodium selenite, 5ng/ml H-BEGF, 5µg/ml N-acetyl cysteine, 1mM sodium pyruvate, 1x penicillin-streptomycin, and 292µg/ml L-glutamine(1). The primary astrocyte culture identity and purity were confirmed by GFAP and integrin β5 immunofluorescence.

Growth surface preparation

Poly-L lysine (P8920-100ml, 0.1% (w/v) in H₂O, Sigma-Aldrich) stock solution was diluted 1 in 10 in Hank's balanced salts solution plus calcium (HBSS+Ca) (14025092, Sigma-Aldrich) and left for 1h at 37°C on the plastic surface of 6-well plate format. Collagen type I (C8919, 0.1% (w/v) in 0.1 M acetic acid) stock solution was diluted 1 in 20 in HBSS+Ca and left for 1h at room temperature (RT) on the plastic surface of 6-well plate format. For experiments that used transwell polyester

membrane inserts (0.4 μm pore, CLS3450 24mm or CLS3460 12mm diameter, Corning Costar), the filters were first coated with Poly-L lysine as described. Coating solutions were removed, and cells were seeded onto the plastic surface or inserts.

Isolation of primary brain microvasculature endothelial cells

To delete *Krit1* in brain microvascular endothelial cells, we used *Krit1^{fl/fl}* mice (loxP-flanked *Krit1* exon 5; generated by Douglas A. Marchuk, Duke University, Durham, NC) crossed with mice expressing an inducible endothelial-specific Cre driver, PDGFb-iCreERT2(2) (*PDGFb-iCreERT2:Krit1^{fl/fl}*). We used littermates and age-matched *Krit1^{fl/fl}* mice as a control on the same C57BL/6 background. To delete *Pdcd10* in brain microvascular endothelial cells, we used *Pdcd10^{fl/fl}* mice (loxP-flanked *Pdcd10* exon 4 and 5; generated by Wang Min, Yale University, New Haven, CT) crossed with mice expressing an inducible endothelial-specific Cre driver, PDGFb-iCreERT2 (*PDGFb-iCreERT2:Pdcd10^{fl/fl}*). We used littermates and age-matched *Pdcd10^{fl/fl}* mice as a control on the same C57BL/6 background and isolation performed as previously described(3). Briefly, Adult mice 2-4 months old were sacrificed, and brains were isolated and placed into cold solution A. Meninges and choroid plexus were detached and removed, and the brains of 5-6 mice were pooled together and minced with scissors in solution A. Brain tissue suspension was centrifuged at 215g for 5 minutes at 4°C. The tissue was digested with a collagenase/dispase solution (1mg/ml collagenase/dispase [10269638001; Sigma-Aldrich], 20 units/ml DNase I [11284932001; Sigma-Aldrich], and 0.150 μg /ml tosyl-lysine-chloromethyl-ketone [T7254; Sigma-Aldrich] in DMEM)] at 37°C for 1h with vigorous shaking every 10min. Then the tissue suspension was triturated using thin-tipped Pasteur pipettes until fully homogenous and centrifuged at 215g for 5 minutes. The pellet was resuspended in cold solution B and centrifuged at 1000g for 20min at 4°C. The lighter phase was discarded and the heavy phase containing the brain microvasculature was digested in collagenase/dispase a second time for 30min at 37°C with vigorous shaking every

10min. After incubation, the suspension was centrifuged (215g for 5min at 4°C) and the pellet was resuspended in BMEC-media that comprised of EBM-2 medium (Lonza) supplemented with the following: 0.025% recombinant human EGF, 0.1% insulin-like growth factor, 0.1% gentamicin, 0.04% ascorbic acid, 0.04% hydrocortisone, and 20% FBS. The BMECs were plated in collagen-coated wells (0.005% collagen in HBSS [C8919, Sigma-Aldrich]) and cultured in 10µg/ml of puromycin for 2 days, followed by 2µg/ml for 2 days(3). Primary BMEC culture identity and purity were confirmed by RNA expression levels of endothelial-specific genes, morphology, and immunofluorescence.

Inactivation of *Krit1* or *Pdcd10* gene in primary BMECs

After 5 days in culture at 37°C in 95% air and 5% CO₂, primary BMEC from mice bearing *Pdcd10^{fl/fl}* or *Pdgfb-iCreERT2;Pdcd10^{fl/fl}* were passaged to equal confluency (~ 2.5 x 10⁵ cells) on collagen-coated 6-well plates. On day 6-7 from initial culture, *Pdgfb-iCreERT2;Pdcd10^{fl/fl}* BMECs were treated with 5µM of 4-hydroxy-tamoxifen (H7904; Sigma-Aldrich) for 48h to delete *Pdcd10* (*Pdcd10^{ECKO}*). *Pdcd10^{fl/fl}* BMECs were also treated with 4-hydroxy-tamoxifen and used as a control. The deletion of *Pdcd10* in *Pdcd10^{ECKO}* BMECs was verified by RT-qPCR analysis. The medium was replaced with fresh BMEC-media (2.5 Isolation of primary brain microvasculature endothelial cells) and changed again every two days(3).

Co-culture of BMECs and astrocytes

Pdcd10^{ECKO} and *Pdcd10^{fl/fl}* BMECs at passage 1-3 were plated on collagen-coated 6-well plates and maintained in BMEC-media for 15-20 days, while mouse primary astrocytes (~3.5 x 10⁵ cells) were seeded on poly-L-lysine coated transwell filters (3450; Sigma-Aldrich) and maintained in astrocyte-media. Astrocytes were maintained for 3 days before transwells were placed onto the

BMEC wells containing astrocyte media supplemented with 500 μ M L-arginine. BMEC and astrocytes were maintained in co-culture for the time indicated in each experiment.

Immunofluorescence microscopy

Astrocytes were grown on poly-L-lysine coated 12-well transwell filters (CLS3460; Sigma-Aldrich) or cover glasses (12-545-81; Thermo Fisher Scientific). For β -gal staining, cells were fixed for 5min at RT in a β -gal fixation solution (5mM EGTA, 2.5mM MgCl₂, 0.2% Glutaraldehyde, 1.3% PFA in PBS) and washed for 5 min at RT with β -gal washing buffer (2mM MgCl₂, 0.02% NP-40 in PBS). A β -gal staining was performed at 37°C for 3h in 0.02% X-Gal, 5mM K₃Fe(CN)₆, 5mM K₄Fe(CN)₆, 2mM MgCl₂ in PBS. After staining, astrocytes were fixed again with 4% PFA for 10 min at RT, pH 7.4, and permeabilized with 0.5% Triton X-100 in PBS for 5min. For astrocytes not undergoing β -gal staining, the cells were fixed for 10 min at RT with 4% PFA in PBS, pH 7.4, and permeabilized with 0.5% Triton X-100 in PBS for 5 min. The cells were blocked with 0.5% BSA for 30min and incubated with rat polyclonal antibodies anti-GFAP (1:80; 13-0300; Thermo Fischer Scientific), sheep polyclonal antibodies anti-IT β 5 (1:40, R&D Systems), rabbit polyclonal antibodies anti-HIF-1 α (1:150; NB100-134; Novus Biologicals), and goat polyclonal antibodies anti-SOX-9 (1:40; AF3075; R&D Systems) overnight at RT. Cells were washed four times with PBS and incubated with anti-rabbit Alexa Fluor 594, and anti-rat Alexa Fluor 488 secondary antibodies (1:300; Thermo Fischer Scientific) or anti-Goat Alexa Fluor 594, anti-rabbit Alexa fluor 488 secondary antibodies (1:300; Jackson ImmunoResearch) in PBS for 1h at RT. Astrocyte nuclei were stained with DAPI and mounted with Fluoromount-G mounting medium (SouthernBiotech). Human tissue was obtained after informed consent from patients undergoing lesion resection, under protocol #10- 295-A, approved by the University of Chicago Institutional Review Board. Human tissue, CCM lesions, and lesion-free brain tissues were snap-frozen and sectioning using a cryostat (Leica). Specimens were air dry for 15 min and fixed in 4% PFA at

room temperature for 15 min and washed three times in PBS. The specimens were blocked and permeabilized using permeabilization buffer for 2 h and incubated with rabbit polyclonal antibody anti-eNOS (1:200; PA1-037; Thermo Fisher Scientific) and goat polyclonal antibody anti-collagen IV (1:100, AB769; Millipore) in PBS at room temperature overnight. Preparations were washed four times in PBS and incubated at room temperature for 1 h with suitable secondary anti-rabbit Alexa Fluor 594 and anti-goat Alexa Fluor 488 antibodies (1:300; Thermo Fisher Scientific) in PBS. Cell nuclei were stained with DAPI (SouthernBiotech).

Immunohistochemistry

Brains from *Pdcd10^{ECKO}* and littermate control *Pdcd10^{fl/fl}* mice at postnatal day 10 were isolated and fixed in 4% PFA at 4°C overnight. After cryoprotection in 30% sucrose dissolved in PBS, brains were embedded and frozen in O.C.T compound (23-730-571; Fischer Scientific). Cerebellar tissues were cut into 12- μ m coronal sections onto Superfrost Plus slides (12-550-15; VWE International). Sections were blocked and permeabilized in a permeabilization solution (0.5% Triton X-100, 5% goat serum, 0.5% BSA, in PBS) for 2h and incubated in rabbit polyclonal antibodies against eNOS (1:200; PA1-037; Thermo Fisher Scientific), rabbit polyclonal antibodies against GFAP (1:250; GA524; Agilent Dako), mouse monoclonal antibody against MBP (1:500; SMI99; Biolegend), rat polyclonal antibodies against CD31 (1:80; 553370; BD Biosciences), rat monoclonal antibody against CD34 (1:100; 119302; Biolegend) in PBS at room temperature overnight. Preparations were washed one time in brain-Pblec buffer (PBS, 1mM CaCl₂, 1mM MgCl₂, 0.1 mM MnCl₂, and 0.1% Triton X-100) and incubated with isolectin B4 FITC conjugated (1:80, L2895; Sigma-Aldrich) in brain-Pblec buffer at 4C overnight. Tissue sections were washed four times in PBS and incubated with suitable Alexa Fluor coupled secondary antibodies (1:300, Thermo Fisher Scientific) in PBS for 1h at RT. Cell nuclei were stained with DAPI and mounted with Fluoromount-G mounting medium (SouthernBiotech). For β -gal staining, brains were fixed

for 5min at RT in PFA 2% and washed with PBS. After cryoprotection in 15% sucrose dissolved in PBS, brains were embedded and frozen in O.C.T compound. A β -gal staining was performed at 37°C for 6h in 0.02% X-Gal, 5mM $K_3Fe(CN)_6$, 5mM $K_4Fe(CN)_6$, 2mM $MgCl_2$ in PBS and tissue sections were washed with cold PBS and fixed in 4% PFA at RT for 30 min. Immunohistochemistry was performed after the β -gal staining. Except for immunohistochemistry for SOX-9 in which a β -gal staining was performed at 37°C for 3h followed by fixation with PFA 4% for 30 min at RT and incubation with unmasking antigen solution (927901; Biolegend). Sections were blocked and permeabilized in a permeabilization solution for 2h and incubated in goat polyclonal antibodies against SOX-9 (1:40; AF3075; R&D Systems) and rabbit polyclonal antibodies against GFAP (1:250; GA524; Agilent Dako) overnight at RT. Tissue sections were washed four times in PBS and incubated with suitable Alexa Fluor coupled secondary antibodies (1:300, Thermo Fisher Scientific) in PBS for 1h at RT. The slides were viewed with a high-resolution SP8 confocal microscope (Leica Microsystems), and the images were captured with Leica application suite software (Leica Microsystems).

In Situ Hybridisation

Brains from *Pdcd10*^{EC^{KO}} and littermate control *Pdcd10*^{fl/fl} mice at postnatal day 10 were isolated and fixed in 4% PFA at 4°C overnight. Brains were washed in cold RNase free PBS and after cryoprotection in 30% sucrose dissolved in RNase free PBS, brains were embedded and frozen in O.C.T compound (23-730-571; Fischer Scientific) and processed for in situ hybridization (ISH) as described previously(4). Immunohistochemistry was performed after the hybridization using rabbit polyclonal antibodies against GFAP (1:200; GA524; Agilent Dako) overnight at RT. Preparations were washed one time in brain-Pblec buffer and incubated with isolectin B4 FITC conjugated (1:80, L2895; Sigma-Aldrich) in Pblec buffer at 4C overnight. Tissue sections were

washed four times in PBS and incubated with suitable Alexa Fluor coupled secondary antibodies (1:300, Thermo Fisher Scientific) in PBS for 1h at RT.

Primary	Host Species	Company	Identifiers	Dilution
eNOS	Rabbit polyclonal	Thermo Fisher Scientific	PA1-037	WB (1:500)
				IF (1:200)
HIF-1 α	Rabbit polyclonal	Novus Biologicals	NB100-134	WB (1:150)
				IF (1:100)
COX-2	Rabbit monoclonal	Cell Signaling	12282	WB (1:100)
COX-2	Goat polyclonal	Thermo Fisher Scientific	PA1-9032	WB (1:100)
ACTB	Mouse monoclonal	Sigma-Aldrich	A5441	WB (1:5,000)
GFAP	Rat polyclonal	Thermo Fisher Scientific	13-0300	IF (1:80)
GFAP	Rabbit polyclonal	Agilent Dako	GA524	IF (1:200)
CD34	Rat monoclonal	BioLegend	119302	IF (1:80)
ColIV	Goat polyclonal	Sigma-Aldrich/Millipore	AB769	IF (1:100)
MBP	Mouse monoclonal-647 Alexa Fluor	BioLegend	808408	IF (1:150)
SOX-9	Goat polyclonal	R&D Systems	AF3075	IF (1:50)
INTGB5	Sheep polyclonal	R&D Bioscience	AF3824	IF (1:50)
CD31	Rat monoclonal	BD Pharmigen	553370	IF (1:100)

Supplemental 1

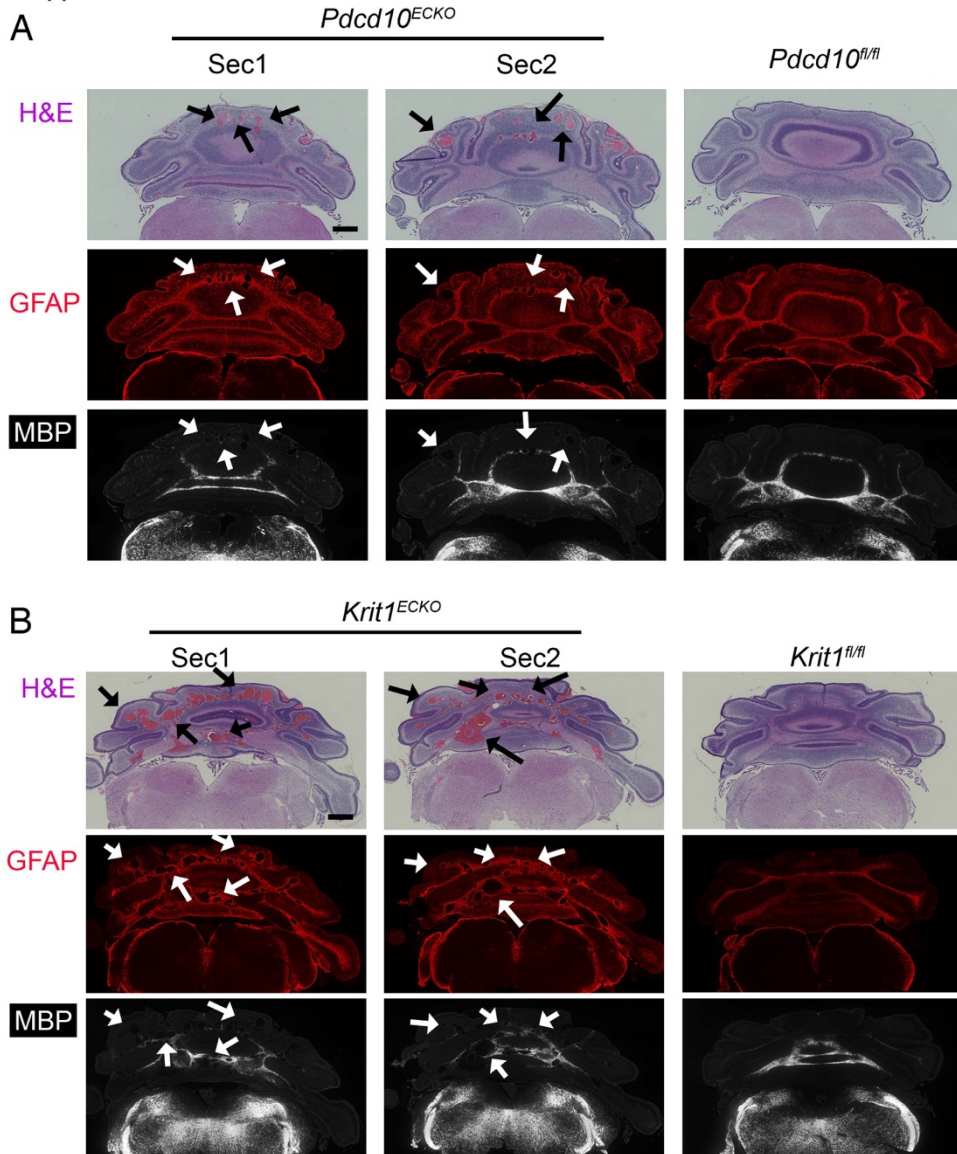


Fig. S1. CCM lesions spatially developed on fibrous astrocytes. (A) Histological analysis of cerebellar sections from P9 *Pdcd10^{ECKO}* and littermate control *Pdcd10^{fl/fl}* mice. Low magnification of CCM lesions detected in sections stained by hematoxylin and eosin. CCM lesions spatially developed on fibrous astrocytes areas positive to GFAP immunostaining (red) and white matter positive to MBP (white). Arrows indicate CCM lesions. (B) Histological analysis of cerebellar sections from P8 *Krit1^{ECKO}* and littermate control *Krit1^{fl/fl}* mice. Low magnification of CCM lesions detected in sections stained by hematoxylin and eosin. CCM lesions spatially developed on fibrous astrocytes areas positive to GFAP immunostaining (red) and white matter positive to MBP (white). Arrows indicate CCM lesions. Scale bar: (A and B) 500 μ m.

Supplemental 2

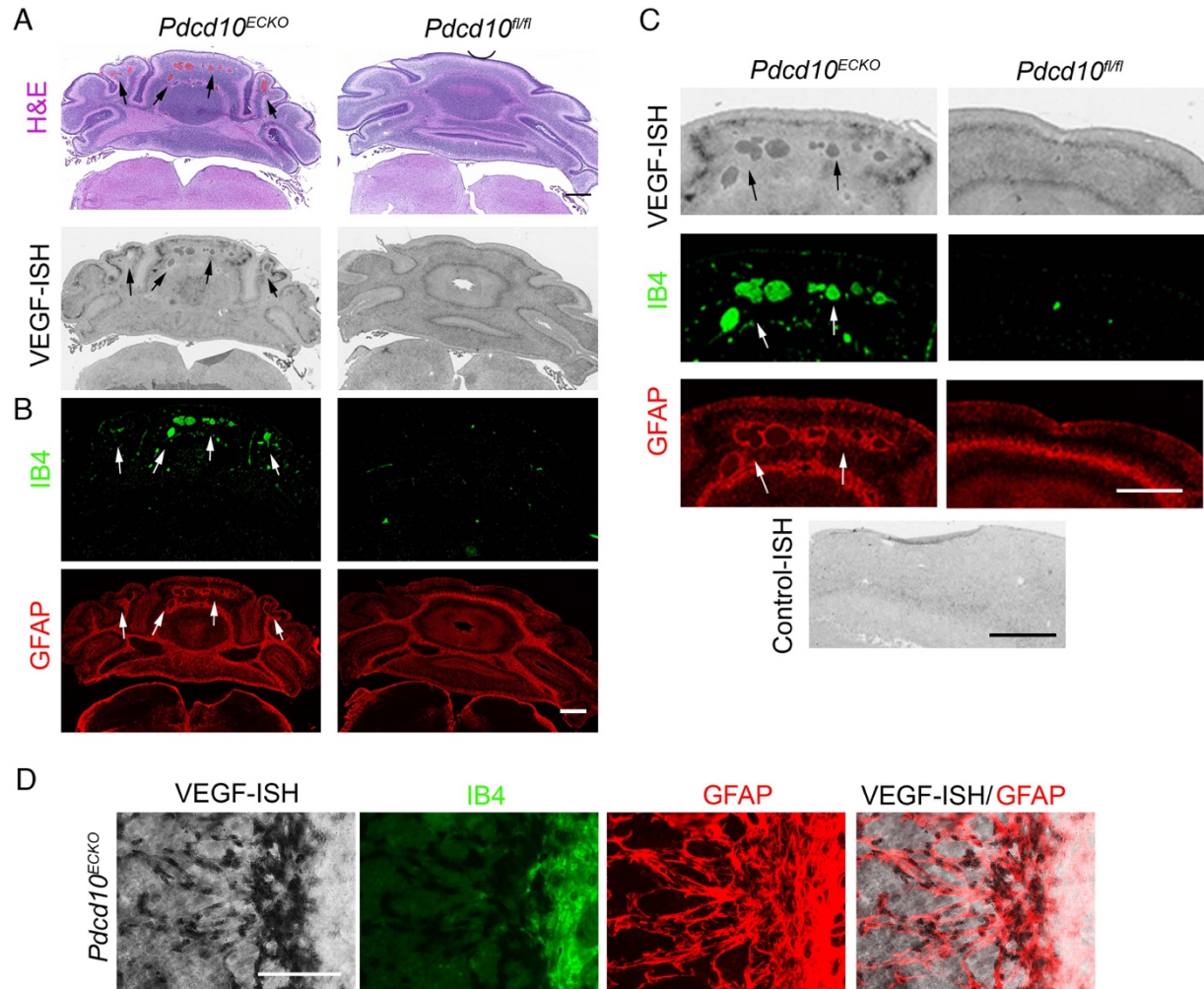


Fig. S2. VEGF increases during cerebral cavernous malformations development. (A) Histological analysis of cerebellar sections from P10 *Pdc10^{ECKO}* and littermate control *Pdc10^{fl/fl}* mice. Low magnification of CCM lesions detected (Arrows) in sections stained by hematoxylin and eosin. (B) ISH for VEGF (black) combined with immunohistochemistry to identify GFAP-positive astrocytes (red), endothelial marker isolectin B4 (IB4; green) in a serial section from A. (C) high magnification of B and control probe for ISH. (D) ISH for VEGF (black) combined with immunohistochemistry to identify GFAP-positive astrocytes (red), endothelial marker isolectin B4 (IB4; green) in P10 *Pdc10^{ECKO}* retinas (n=2 or 3). Scale bars: (A, B and C) 500 μ m, (D) 100 μ m.

Supplemental 3

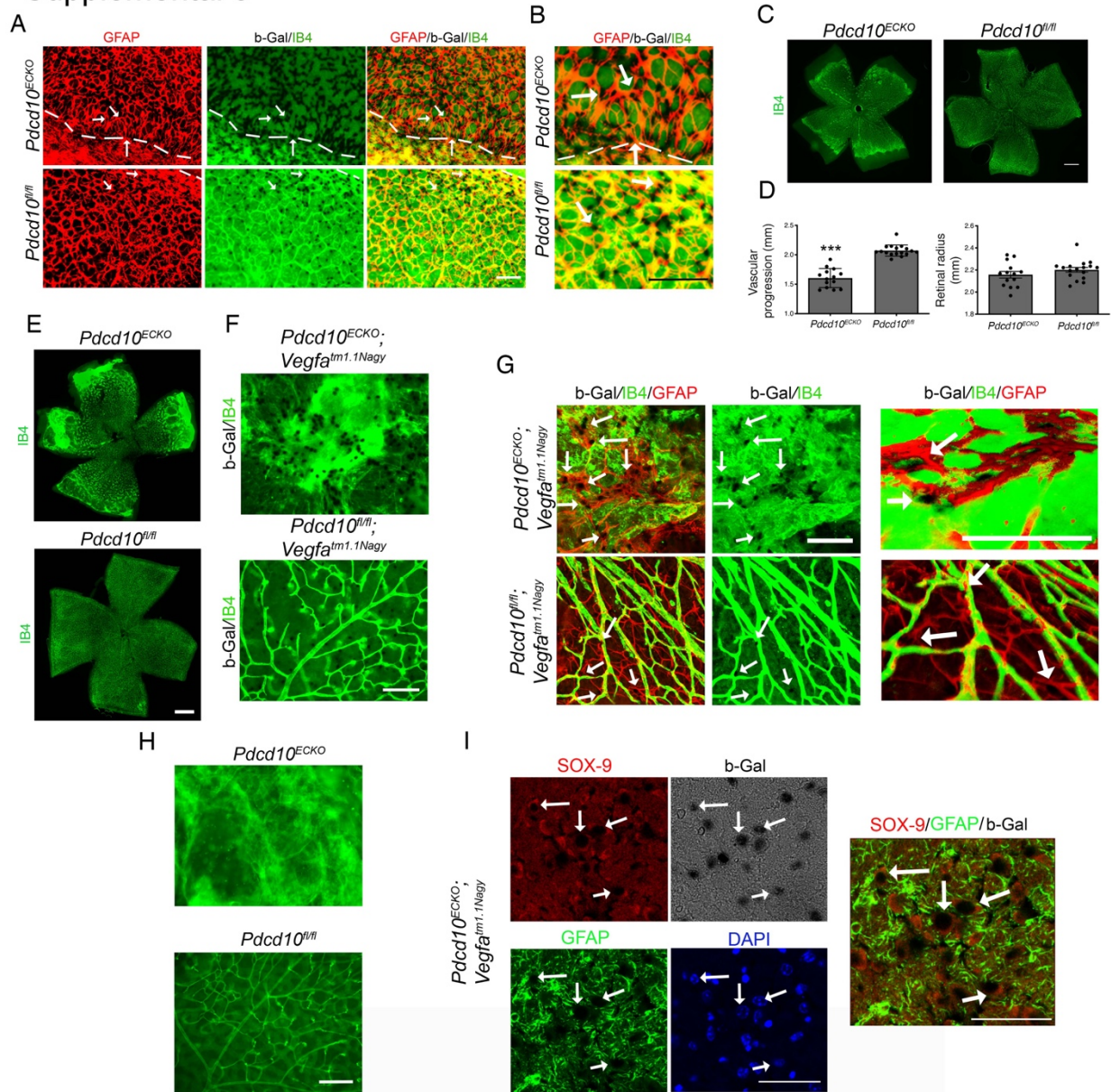


Fig. S3. VEGF increases in retinas during cerebral cavernous malformations. (A) Whole-mount retinal vasculature at the angiogenic growth front stained for GFAP (red), Isolectin B4 (green), and β -gal/VEGF expression detected by X-gal staining (black) in P9 *Pdcd10^{ECKO};Vegfa^{tm1.1Nagy}* mice and *Pdcd10^{fl/fl};Vegfa^{tm1.1Nagy}* littermate control. Dotted line indicated angiogenic front. (B) Magnified whole-mount retinal vasculature in A. Arrows indicate β -gal/VEGF in GFAP-positive astrocytes. (C) Isolectin B4-stained *Pdcd10^{ECKO}* and control *Pdcd10^{fl/fl}* P9 retinas and in (D) the quantification of vascular parameters of retinas (SEM, $n = 14$ or 17 mice in each group). (E) Whole-mount retinal vasculature by isolectin B4-stained *Pdcd10^{ECKO}* and control *Pdcd10^{fl/fl}* P12 retinas. (F) Magnified whole-mount retinal vasculature indicate β -gal/VEGF.

expression in P12 *Pdcd10*^{ECKO};*Vegfa*^{tm1.1Nagy} mice and *Pdcd10*^{fl/fl};*Vegfa*^{tm1.1Nagy} littermate control. **(G)** Correspond to β -gal control in P12 *Pdcd10*^{ECKO} mice and *Pdcd10*^{fl/fl} littermate control. **(H)** Maximum-intensity projection of whole-mount P12 retinal vasculature from *F* stained for GFAP (red), Isolectin B4 (green), and β -gal/VEGF (black). Arrows indicate β -gal/VEGF in GFAP-positive astrocytes. Surface reconstruction of β -gal/VEGF in GFAP-positive astrocytes (n=3 or 4). **(E)** Confocal microscopy of cerebrum cortex from *Pdcd10*^{ECKO};*Vegfa*^{tm1.1Nagy} stained for β -gal/VEGF expression, SOX-9-positive astrocytes (red), GFAP-positive astrocytes (green), and DAPI for nuclear DNA (blue). Data are mean \pm SEM. ***, P<0.001; determine by Student's *t* test. Scale bars: **(A, B and H)** 100 μ m; **(F and G)** 200 μ m; **(C and E)** 500 μ m.

Supplemental 4

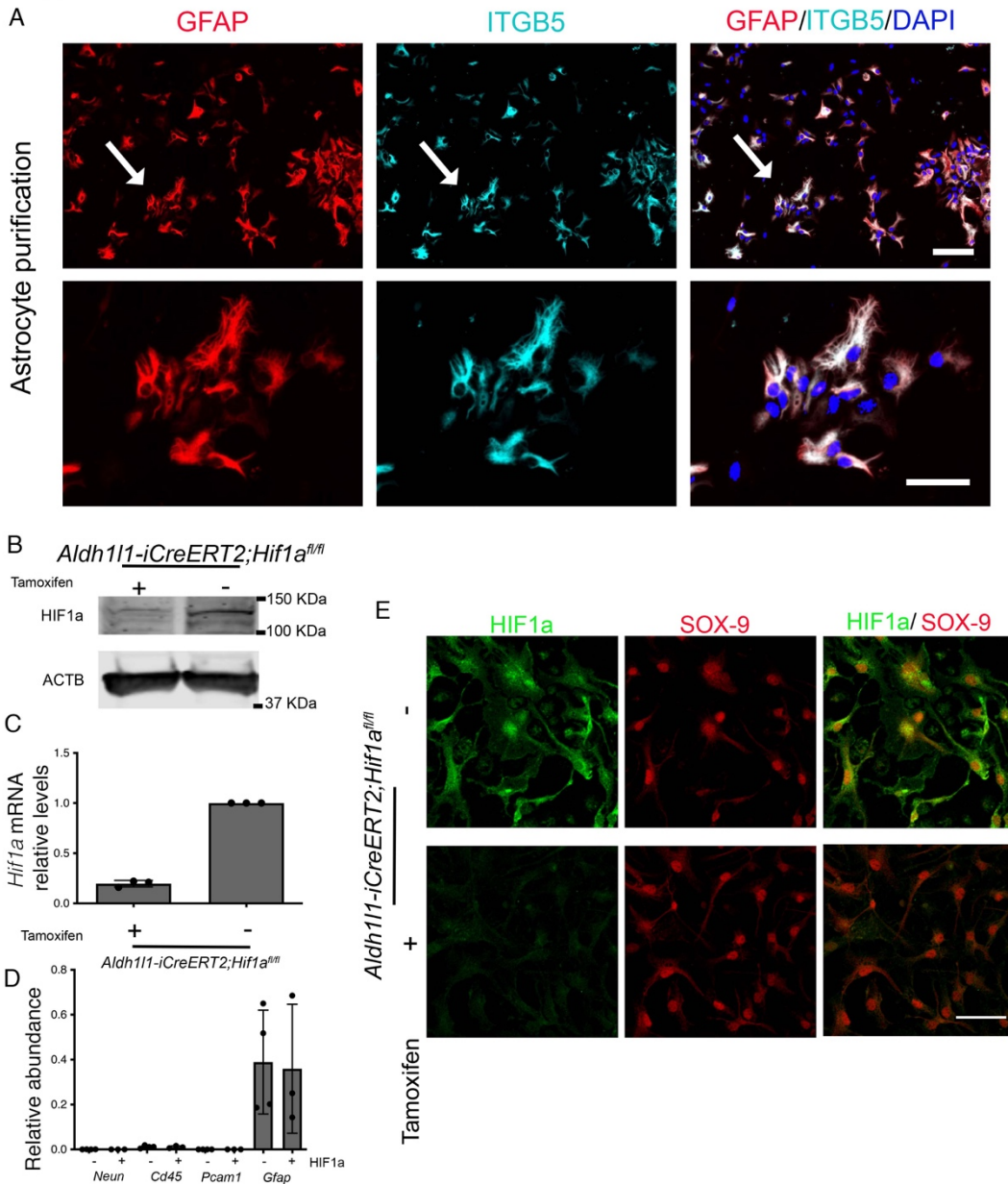


Fig. S4. Mouse primary astrocyte culture characterization and purity. Astrocytes isolated from cortex of postnatal day 5-7 mice. **(A)** Purity of the isolation was confirmed by the presence of GFAP and ITGB5 astrocyte markers visualized by immunofluorescence (n=5). **(B)** the HIF-1 α antibody specificity by Western blot analysis of primary astrocyte cultures was determined using *Aldh111-iCreERT2;Hif1 α ^{fl/fl}* astrocytes in the presence and absence of tamoxifen (n=2). **(C)** Inactivation of HIF-1 α was further validated by RT-qPCR that showed ~80% reduction in HIF-1 α mRNA levels in tamoxifen-treated-*Aldh111-iCreERT2; Hif-1 α ^{fl/fl}* cells (n=3). **(D)** Gene expression of neuronal marker *Neun*; leucocyte marker *Cd45*; endothelial cell marker *Pcam1*; astrocyte markers *Gfap* from C (n=3 or 4). **(E)** HIF-1 α antibody specificity by immunocytochemistry of astrocytes was performed by increasing HIF-1 α expression by adding 100 μ M of CoCl₂ for 24 h

to the culture medium. SOX-9 immunostaining was used as a nuclear marker for astrocytes. Diffuse and nuclear accumulation of HIF-1 α immunostaining in non-tamoxifen treated-*Aldh1l1-iCreERT2; Hif-1 α ^{fl/fl}* cells that is significantly reduced by tamoxifen treatment astrocytes (n=2). Scale bars: (A and E) 100 μ m.

Supplemental 5

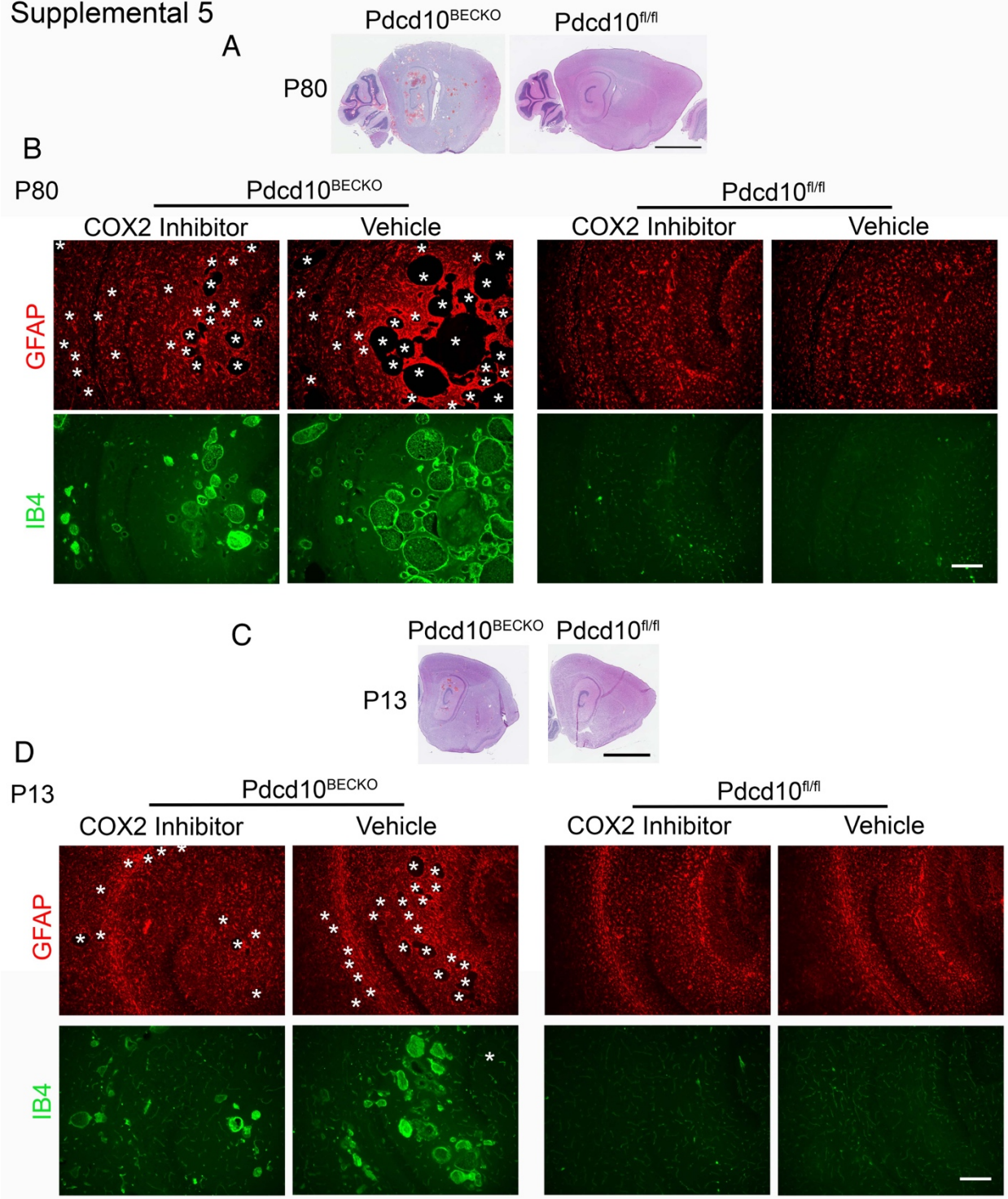


Fig. S5. COX-2 inhibition prevent CCM lesions in acute and chronic CCM mouse models. (A) Histological analysis of brain sections from P80 *Pdcd10^{BECKO}* and littermate control *Pdcd10^{fl/fl}* mice. Low magnification of CCM lesions detected throughout the brain sections stained by hematoxylin and eosin in P80 *Pdcd10^{BECKO}* mice. More extensive and complex lesions were prevalent in the hippocampal area (n=3). (B) Oral gavage administration of 40 mg/Kg celecoxib or vehicle for fifteen consecutive days P55 to P70. GFAP astrocyte marker (red) staining and IB4 endothelial marker (green) of mouse hippocampal region at P80. CCM lesions' high propensity to develop surrounded by GFAP+ astrocytes in the hippocampal region in vehicle-treated *Pdcd10^{BECKO}* mice. Significant decrease in CCM lesions' density and GFAP-immunoreactivity in celecoxib-treated *Pdcd10^{BECKO}* mice. Arrows indicate CCM lesions (n=3). (C) Histological analysis of brain sections from P13 *Pdcd10^{BECKO}* and littermate control *Pdcd10^{fl/fl}* mice. Low magnification of CCM lesions detected throughout the brain sections stained by hematoxylin and eosin in P13 *Pdcd10^{BECKO}* mice. Extensive lesions were prevalent in the hippocampal area (n=3). (D) Intragastric administration of 40 mg/Kg celecoxib or vehicle for four consecutive days P6 to P9. GFAP astrocyte marker (red) staining and IB4 endothelial marker (green) of mouse hippocampal region at P13. CCM lesions' high propensity to develop surrounded by GFAP+ astrocytes in the hippocampal region in vehicle-treated *Pdcd10^{BECKO}* mice. Significant decrease in CCM lesions' density in celecoxib-treated *Pdcd10^{BECKO}* mice (n=3). Asterisks, vascular lumen of CCM lesions. Scale bars: (B and D) 200 μ m.

Supplemental 6

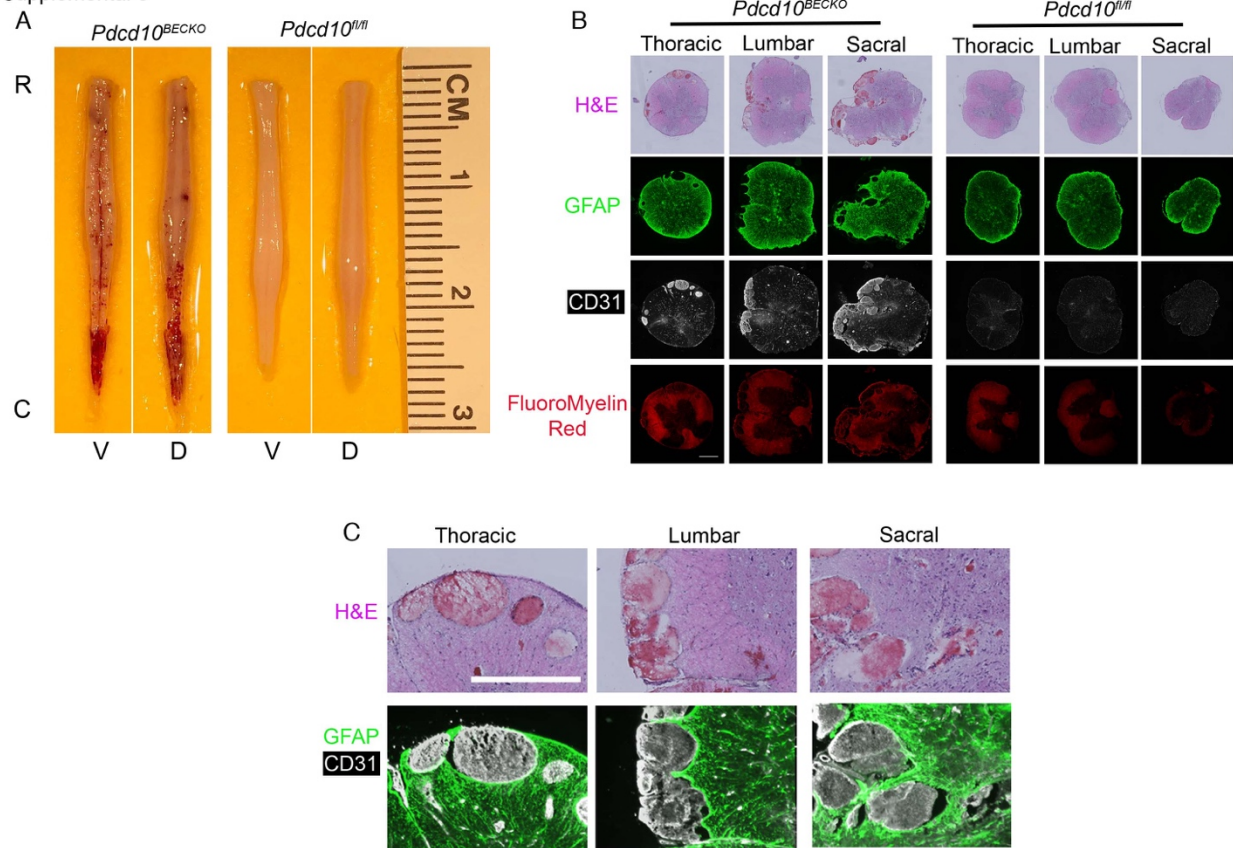


Fig. S6. Cavernous malformations in the spinal cord of chronic CCM mouse model. (A) Prominent lesions are present along the spinal cord of P80 *Pdc10^{BECKO}* mice. R=rostral, C=caudal, V=ventral, D=dorsal (n=14). **(B)** Histological analysis of serial sections of spinal cords from P80 *Pdc10^{BECKO}* and littermate control *Pdc10^{fl/fl}* mice. Spinal cord sections stained by hematoxylin and eosin, GFAP astrocyte marker (green), CD31 endothelial marker (white), and myelin staining (red) of a mouse at P80 (n=2). **(C)** High magnification of CCM lesions, in **B**, present in the thoracic, lumbar, and sacral region of a spinal cord, shown a high propensity to develop surrounded by GFAP+ astrocytes (n=2). Scale bars: **(B and C)** 500 μ m.

Supplemental 7

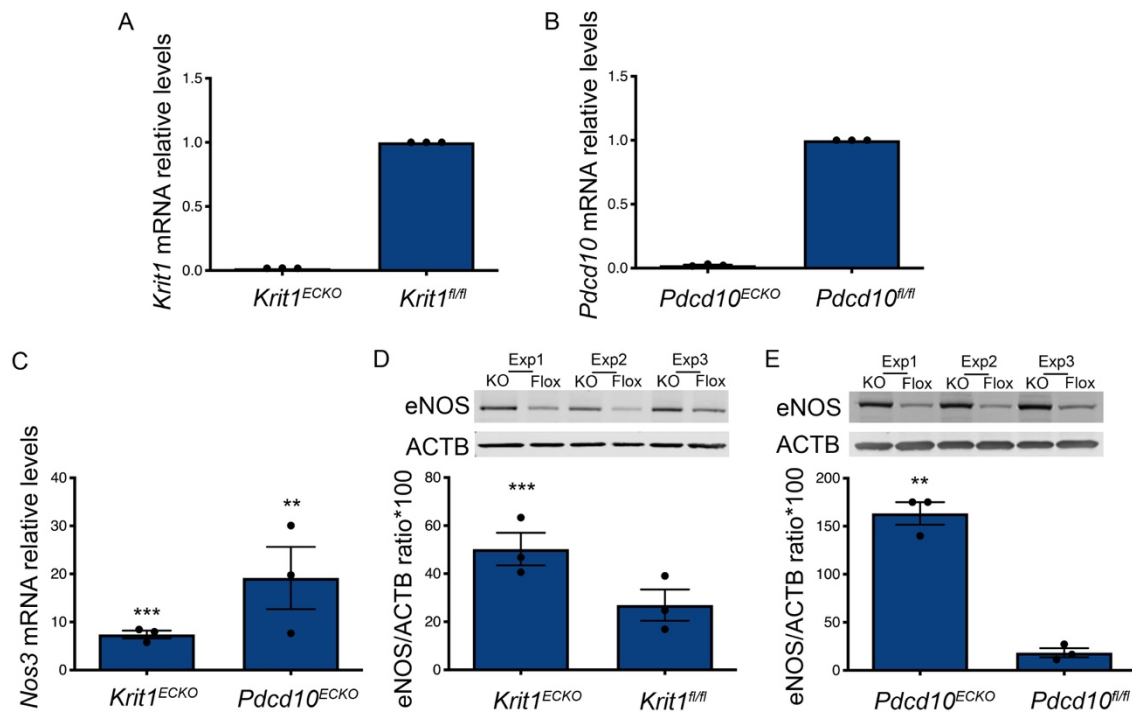


Fig. S7. Loss of brain endothelial *Krit1* or *Pcd10* increases the expression of eNOS. (A) Analysis of *Krit1* mRNA levels by RT-qPCR in *Krit1^{ECKO}* BMEC and *Krit1^{fl/fl}* BMEC control (SEM, $n=3$). (B) Analysis of *Pcd10* mRNA levels by RT-qPCR in *Pcd10^{ECKO}* BMEC and *Pcd10^{fl/fl}* BMEC control (SEM, $n=3$). (C) Analysis of *Nos3* mRNA levels by RT-qPCR in *Krit1^{ECKO}* BMEC and *Pcd10^{ECKO}* BMEC, as compared to *Krit1^{fl/fl}* BMEC or *Pcd10^{fl/fl}* BMEC control, respectively (SEM, $n=3$). (D) Quantification of eNOS protein in *Krit1^{ECKO}* BMEC compared with *Krit1^{fl/fl}* BMEC control (SEM, $n=3$). (E) Quantification of eNOS protein in *Pcd10^{ECKO}* BMEC compared with *Pcd10^{fl/fl}* BMEC control (SEM, $n=3$). Data are mean \pm SEM. **, $P<0.01$, ***, $P<0.001$; determine by Student's *t* test.

Supplementary 8

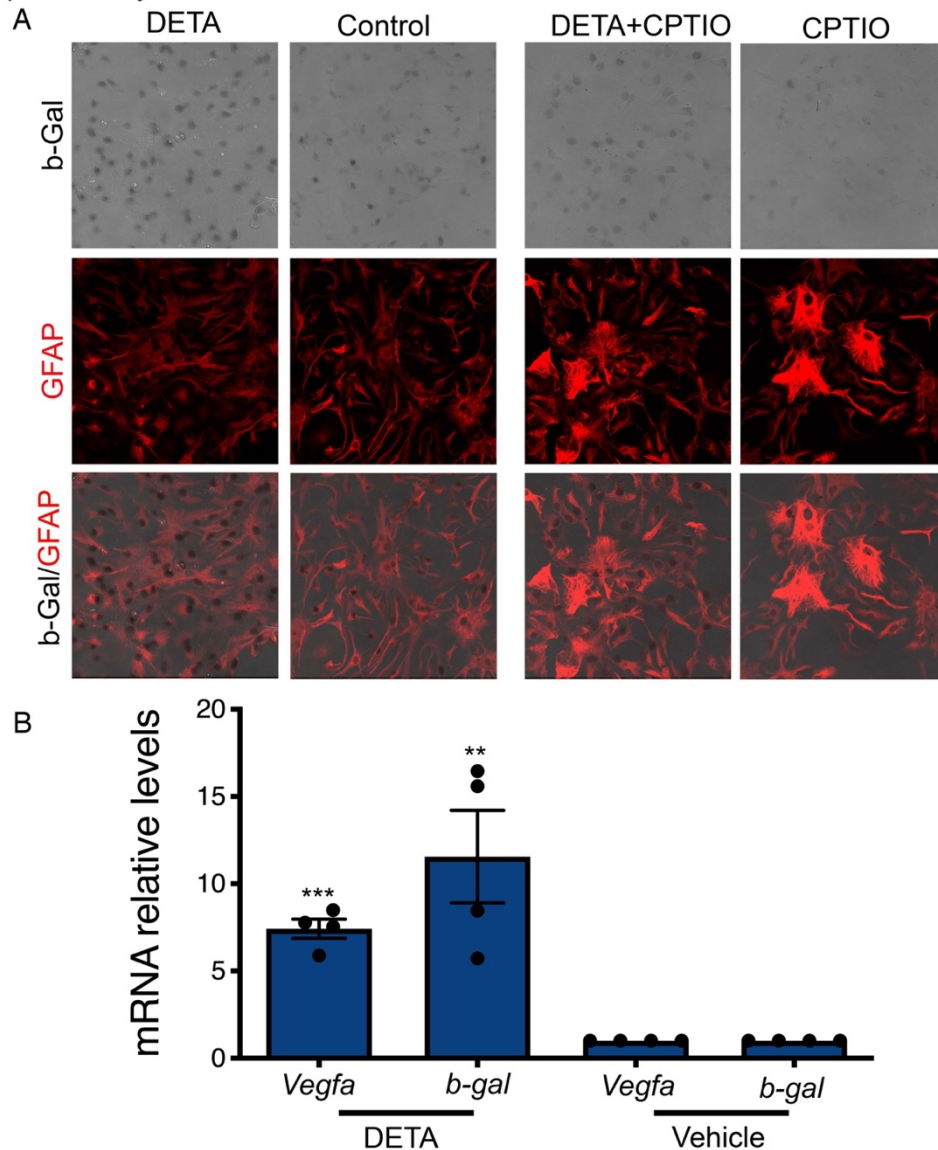


Fig. S8. Primary cultures of astrocytes respond to elevation of NO. (A) Increase in β -gal/VEGF expression, as shown by X-gal staining (black), in primary cultured astrocytes (GFAP-positive cells, red) treated with 0.5mM DetaNONOate (NO donor) for 24h when compared with vehicle-treated astrocytes. Astrocytes pre-treated with 15 μ M CPTIO, a NO scavenger, prevented DetaNONOate-induced increase in β -gal/VEGF expression in astrocytes. (B) DETANONOate in astrocyte culture media induced an ~7 fold increase in astrocyte *Vegfa* mRNA and an ~11.50 fold increase in astrocyte *β -gal* mRNA levels, an effect that was prevented in astrocytes pre-treated with CPTIO. Data are mean \pm SEM. **, P<0.01, ***, P<0.001; determine by Student's *t* test.

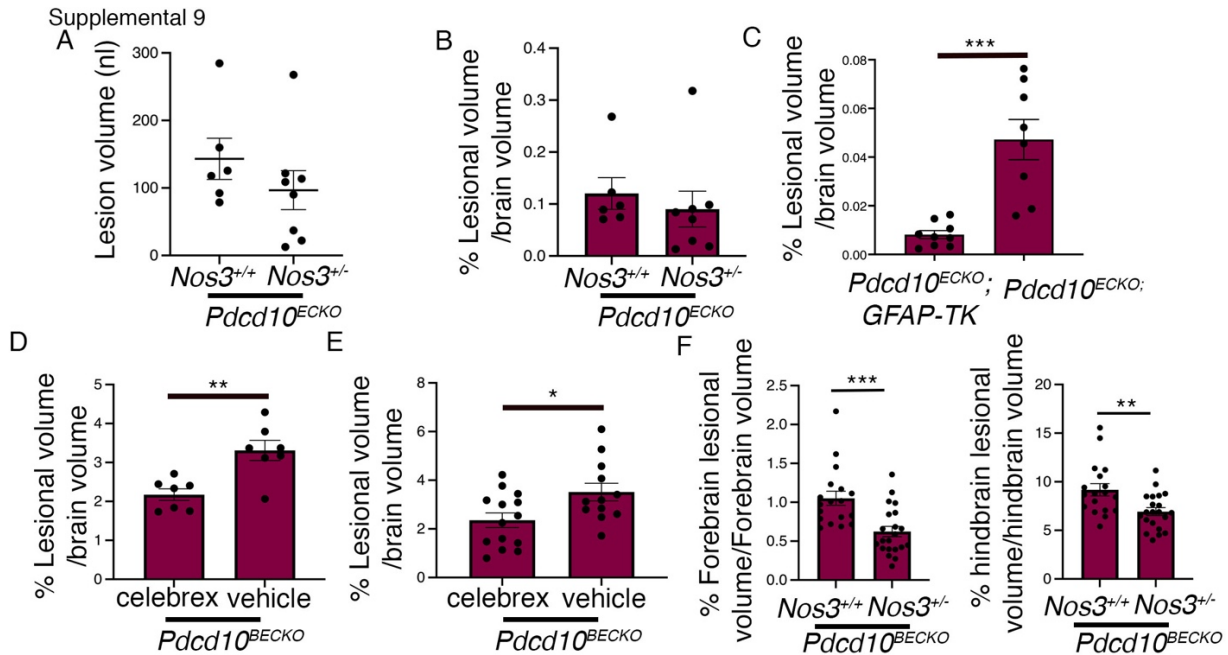


Fig S9. Quantification of CCM lesion and brain volume by micro-CT analysis. A) Quantification of lesion volumes by micro-CT analysis from mice at P9 in $Pdcd10^{ECKO};Nos3^{+/-}$ and littermates $Pdcd10^{ECKO};Nos3^{+/+}$ (SEM, n=6 or 8 mice in each group). Quantification analysis of lesional volume per brain volume per animal. B) from mice at P9 in $Pdcd10^{ECKO};Nos3^{+/-}$ and littermates $Pdcd10^{ECKO};Nos3^{+/+}$. Analysis of the same brains depicted in Fig. S9A. (SEM, n=6 or 8 mice in each group). C) from mice at P9 $Pdcd10^{ECKO};GFAP-TK$ and littermate control $Pdcd10^{ECKO}$ mice. Analysis of the same brains depicted in Fig. 1D. (SEM, n=8 or 9 mice in each group). D) from mice at P13 $Pdcd10^{BECKO}$ mice treated with celecoxib or vehicle. Analysis of the same brains depicted in Fig. 5A (SEM, n=7 mice in each group). E) from mice at P80 $Pdcd10^{BECKO}$ mice treated with celecoxib or vehicle. Analysis of the same brains depicted in Fig. 5C (SEM, n=12 or 14 mice in each group). F) from mice at P14 $Pdcd10^{BECKO};Nos3^{+/+}$ and $Pdcd10^{BECKO};Nos3^{+/-}$ mice. Analysis of the same brains depicted in Fig. 8K (SEM, n=18 or 21 mice in each group). Data are mean \pm SEM. *, P<0.05, **, P<0.01, ***, P<0.001, ****, P<0.0001; determine by Student's *t* test.

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