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Investigating the role of increase in hypoxia-inducible factor-1α expression during cerebral cavernous malformations

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Investigating the role of increase in hypoxia-inducible factor-1α expression during cerebral cavernous malformations

A Thesis submitted in partial satisfaction of the

requirements for the degree Master of Science

in

Biology

by

Riya Singh Verma

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2020

The thesis of Riya Singh Verma is approved, and it is acceptable in quality and form for publication on microfilm and electronically:

Co-Chair

Chair

University of California San Diego

2020

DEDICATION

For their never-ending support, love, generosity and guidance this thesis is dedicated to my parents Manish and Shailaja, my sister Riddhi and my partner Boris.

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ACKNOWLEDGEMENTS

This thesis, and my journey, is a product enabled by the support of my community.

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ABBREVIATIONS

BEC – Brain endothelial cell

BBB – Blood-brain barrier

CNS – Central nervous system

CCM – Cerebral cavernous malformation

COX-2 – Cycloxygenase-2

GFAP – Glial fibrillary acidic protein

HIF-1α – Hypoxia inducible factor-1α

KLF2 – Krüppel-like factor 2

KLF4 – Krüppel-like factor 4

NO – Nitric oxide

NVU – Neurovascular unit

NSAID – Nonsteroidal anti-inflammatory drug

ROS – Reactive oxygen species

VEGF – Vascular endothelial growth factor

ABSTRACT OF THESIS

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by

Riya Singh Verma

Master of Science in Biology

University of California San Diego 2020.

Miguel Alejandro Lopez-Ramirez, Chair

Cory Mathew Root, Co-chair

Cerebral cavernous malformations (CCM) are common vascular lesions made of clusters of endothelia filled with blood that primarily affects the central nervous system. Loss-of-function mutations in the genes; *KRIT1* (Krev1 interaction trapped gene 1, *CCM1*), *CCM2*, or *PDCD10* (Programmed cell death protein 10, *CCM3*) propel brain vascular changes marked by loss of endothelial tight and adherens junctions, altered basement membrane composition, increased angiogenesis, and altered number of intercellular signaling pathways (e.g., RhoA/ROCK,

Angiopoietin-2, reactive oxygen species (ROS), anti-coagulation pathway, and endothelial to mesenchymal transition (EndMT)). Moreover, our group, and others, demonstrated that increased vascular endothelial growth factor (VEGF) signaling and associated vascular permeability are significant contributors to CCM disease. Here we show that genetic inactivation of endothelial *Pdcd10* in animals results in normoxic stability of hypoxia-inducible factor 1 α (HIF-1α). Our findings indicate that increase in HIF-1α leads to an upregulation of a hypoxic program with genes implicated in angiogenesis and cell metabolism. Furthermore, we show that an increase in COX-2, a direct HIF-1α target gene, contributes to brain lesion genesis because the administration of COX-2 inhibitor celecoxib significantly prevents CCM lesions in a pre-clinical mice model. Therefore, our findings support the hypothesis that components of the hypoxic program may represent potential therapeutic targets for CCM disease.

1. General introduction

1.1 Central nervous system microvasculature

The central nervous system (CNS), composed of the brain and spinal cord, is the most energetically demanding organ in the body; it consumes about 20% of the body's oxygen while constituting an average of 2% of the body's weight[1]. This is because the neurons in the brain are metabolically demanding cells due to their intense neuronal activity and electrical network formation[2]. For those needs, the CNS has developed a highly vascularized system that provides the brain tissue with sufficient glucose and oxygen (the principal source of energy) while removing waste products, metabolic compounds, and ions out of the CNS to maintain a proper neuronal function[2,3].

The vascular system is divided into the macrovasculature and microvasculature. The macrovasculature is composed of the larger blood vessels such as arteries and veins that supply blood to major organs and the microvasculature is composed of smaller vessels namely arterioles, venules, and capillaries that participate in blood-tissue exchange[3]. The brain vasculature forms a specialized structure termed blood-brain barrier (BBB) that is located at the level of brain endothelial cells (BECs)[4]. Importantly the BBB function and maintenance are regulated by a specialized cellular structure termed neurovascular unit (NVU), which is comprised by BECs, pericytes, the perivascular endfeet of astrocytes, perivascular microglia, nerve terminals of neurons and surrounded by defined extracellular matrix proteins (ECM)[5]. The NVU is highly responsive to physiological and pathological stimuli which give rise to a coordinated response necessary to maintain the CNS homeostasis[4,5].

1.1.1. Brain endothelial cells

The tubular vessels of the brain are composed of a continuous layer of BECs (**Figure 1**)[4]. BECs function, activity, and morphology differ in several ways from endothelial cells present elsewhere in the body. For instance, BECs lack the fenestrations (pores) typical characteristic of capillaries in peripheral tissues[6]. BECs have a high number of mitochondria suggesting an important oxidative metabolic activity but show few pinocytotic vesicles suggesting that there is highly controlled membrane trafficking[4,7]. Importantly, BECs contain a complex tight junction that seals the brain and spinal cord microvasculature in order to form the BBB and preserve the CNS homeostasis[8]. The intercellular interactions between BECs play important roles in regulating the polarization of proteins and lipids (fence function) and restricting the paracellular diffusion of non-ionic hydrophilic and ionic molecules (barrier function)[6,8,9].

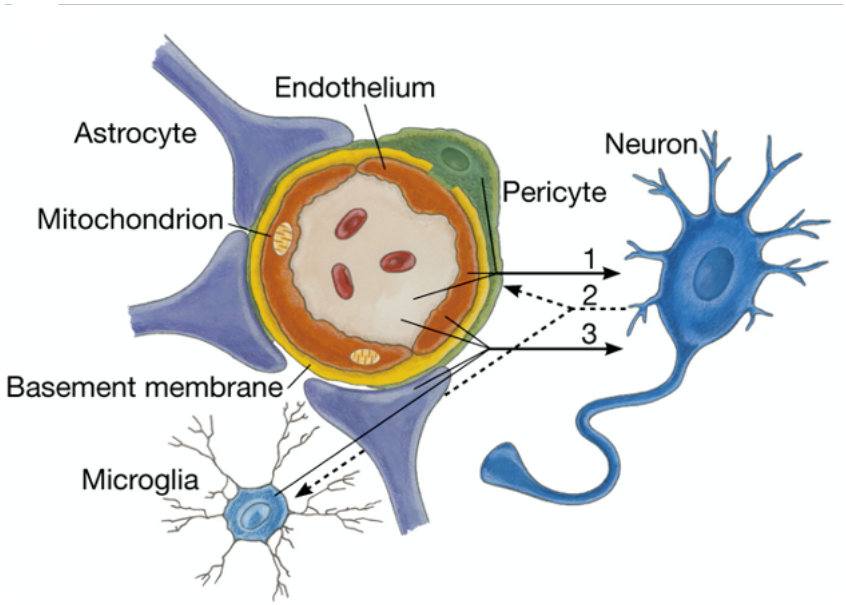

Figure 1: A diagrammatic representation of the NVU depicting the location and relationship of cell types participating in the homeostasis of the BBB[7]; \Image taken from Zlokovic BV. The Blood-Brain Barrier in Health and Chronic Neurodegenerative Disorders. Neuron. 2008;57(2):178–201. doi:10.1016/j.neuron.2008.01.003.

1.1.2. Astrocytes

Astrocytes are star-shaped non-neuronal cells (glial cells) of the CNS that participate in homeostasis through a plethora of biochemical interactions with neighboring NVU cells and they are responsible for maintaining blood flow[10,11]. Additionally, due to proximity to the bloodstream, astrocytes are the middleman for glucose distribution between cells of the NVU[11]. Furthermore, astrocytes are in contact with BECs and thus have the ability to interact and influence BECs differentiation and function[10] (**Figure 1**). For example, the co-culture of astrocytes with BECs leads to improved tight junction formation; interestingly, a similar effect was achieved through incubation of BECs with astrocyte conditioned media[12,13]. Furthermore, astrocytes interact with BECs through secretion of soluble factors such as vascular endothelial growth factor (VEGF) and transforming growth factor-β (TGF-β) that modulate BBB properties[10,13,14]. For example, inhibition of the TGF-β receptor activin like kinase-5 (ALK5) has been shown to increase tight junction protein expression (claudins and occludens) and thereby affecting the BBB permeability[15].

1.1.3. Pericytes

Pericytes are mural cells that surround endothelial cells of the microvasculature that consists of arterioles, capillaries, and venules[16] (**Figure 1**). Pericytes are considered to be essential for healthy microvasculature stability and function[17]. They communicate with BECs through signal cascades such as platelet-derived growth factor β (PDGF-β) and angiopoietin[18,19]. PDGF-β is the paracrine responsible for endothelial to mural cell signaling, and PDGF-β deficient mice lacked vascular recruitment of pericytes and had

impaired capillary wall development[18,20]. Conversely, angiopoietins have been shown to play a role in vascular remodeling through signaling from mural to endothelial cells[18]. Furthermore, during development, pericytes play a key role in the protein composition of the basal lamina (see 1.1.6) by secretion of basement membrane proteins[21]. In this context, pericyte-endothelial communications are essential for the healthy formation of brain vasculature and BBB properties[7,18,20].

1.1.4. Microglia

Microglia cells (**Figure 1**) are detected within the perivascular space at irregular intervals in the NVU[22]. Microglia cells are the resident immunocompetent cells of the CNS that enter the brain during embryonic development[22]. Perivascular microglial cells survey the brain parenchyma and respond to neuroinflammation and vascular dysfunction to maintain BBB integrity[22,23].

1.1.5. Neurons

Crosstalk between neurons and cells of the CNS has been previously shown as necessary for the establishment of healthy vasculature[24] (**Figure 1**). Neurons relay vascular communication using paracrine signaling through astrocytes and other glial cells to regulate cerebral blood flow (neurovascular coupling)[25,26]. Thus, neurons interact with astrocytes and play a role in maintaining BBB properties and the formation of blood vessels in the brain[5,24,25].

1.1.6. Basal lamina

The basal lamina is a non-cellular layer comprised of collagen type IV, fibronectin, perlecan, and laminins that provides an interface for cells of the NVU to adhere to[27]. The physical position of cells of the NVU is maintained by proteins of the integrins and dystroglycans family[27,28]. There are two main basal lamina – the endothelial (from BECs) and the parenchymal (from astrocytes and pericytes) basement membranes[27]. Furthermore, not only does the basal lamina not only serve to anchor cells of the NVU but also have a high affinity for molecules like VEGF and basic fibroblast growth fiber that modulate vascular growth (bFGF)[29]. Additionally, the basement membrane adds to the barrier function of the BBB by blocking infiltrating leukocytes[30,31].

1.2. Cerebral cavernous malformation disease

Cerebral cavernous malformations (CCMs) are common low flow neurovascular lesions made of a collection of vascular channels[32]. These lesion areas are filled with blood and surrounded by astrogliosis that intermingles with granulation tissue creating the appearance of mulberry like lesions[32–34] (**Figure 2)**. CCMs occur in about 0.5% of the general population, causing a lifelong risk of brain hemorrhage and neurologic deficit for which there is no currently effective pharmacologic therapy[35,36].

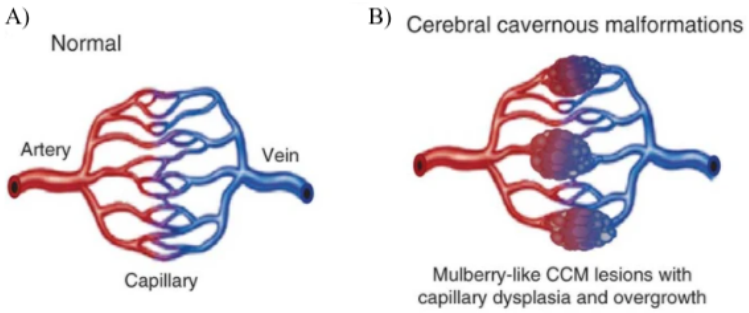

Figure 2: Diagrammatic depiction of healthy vasculature network
A) A healthy feeder artery (in red) normal capillary network and venous drainage (in blue).
B) Contrasted with mulberry like low-flow sinusoids of CCMs.
Image taken and adapted from Storkebaum E, Quaegebeur A, Vikkula M, Carmeliet P. Cerebrovascular disorders: molecular insights and therapeutic opportunities. Nat Neurosci. 2011 Nov;14(11):1390–7.

CCMs are caused by loss-of-function mutations in one of three genes, Krev interaction trapped 1 (*KRIT1 or CCM1*), *CCM2* (Malcavernin), and Program cell death gene 10 (*PDCD10 or CCM3*). CCMs can be presented as a familial (germline loss-of-function mutations) or sporadic (somatic loss-of-function mutations) form[37]. Hallmarks of the disease include increased risk for hemorrhagic strokes, dysfunctional NVU, BBB dysfunction, and excessive angiogenesis[35,37,38].

1.2.1. Sporadic cerebral cavernous malformations

The sporadic form of CCM is considered to be more benign since they usually present as a solitary CNS lesion and may remain undiagnosed in the patients (~40% of affected individuals may never experience symptoms)[35,39]. However, sporadic CCMs have been associated with developmental venous anomalies, which is a common congenital malformation of venous drainage[40]. Commonly, individuals affected with CCMs present a wide variety of symptoms, including headaches, and neurological deficits like blurred vision and weakness in limbs[41]. Most often, symptoms are managed through medications as surgery possess the possibility of higher risks of symptomatic intracranial hemorrhages over the long run[34,36,42]. That being said, patients with an unmanageable symptomatic and

lesion require surgery if the location and depth of the lesion is not at high risk for physiological defects post-surgery (for example, the brain stem)[35].

1.2.2. Familial cerebral cavernous malformations

Familial cases of CCMs makeup about 20% of the cases and are commonly characterized as a more aggressive form of the disease due to its early onset and multiple cavernoma pathology[35,43]. Patients affected with the familial form of CCM typically present multiple lesions recur throughout their lifetime, possibly accompanied by cerebral hemorrhagic strokes[37,39]. CCMs develop in an autosomal dominant pattern with incomplete penetrance and variable expressivity and patients with familial CCMs usually have multiple lesions[36,43]. Additionally, in familial cases of CCMs, heterozygous loss of *KRIT1*, also known as *CCM1*, occurs in about 40% of the affected patients[37,39,44].

1.2.3. Cerebral cavernous malformations proteins

The proteins responsible for cavernoma formation in the CCM disease are the CCM proteins, namely- *KRIT1*, *CCM2,* and *PDCD10*. Previous work implicates the potential formation of a CCM protein complex[45]. This protein complex plays an integral role in junctional integrity of endothelial cells[46].

1.2.3.1. Krev interaction trapped-1 (*KRIT1)*

KRIT1 is an 84 kDa protein located on chromosome 7 that localizes in cell-cell junctions, cytoplasm, nucleus, and microtubules[44,47,48]. On the plasma membrane, KRIT1 binds and activates Rap1, a proangiogenic GTPase essential for vessel formation, and is

important in maintaining the integrity of blood vessels[49]. In addition, KRIT1 can stabilize ICAP-1, which represses the activity of β1 integrin driven angiogenesis[50,51]. Additionally, KRIT1 depletion has been shown to reduce the drive cell surface β-catenin stabilization of adheren junctions which contributes to disruption in endothelial cell junction integrity[52]. Furthermore, loss of KRIT1 has been shown to increase the formation of actin stress fibers and disruption of the endothelial barrier by disassembly of cell-to-cell junctions[49].

1.2.3.2. *CCM2* (Malcavernin)

CCM2 is a 48kDa integral scaffolding protein located on chromosome 7 that codes for the formation of the CCM complex as it interacts with both KRIT1 and PDCD10[53]. KRIT1 and *CCM2* association is essential for RAF-mitogen activated protein kinase (MAPK) activity and integrin-mediated cell-adhesion[54]. Disruption of the KRIT1-CCM2 interaction fails to suppress Rho-associated protein kinase (ROCK), which contributes to increased permeability and augmented vascular leakage in CCM pathology[54,55].

1.2.3.3. Program cell death gene (*PDCD10)*

PDCD10, a 94 kDa conserved protein located on chromosome 3, is involved in multiple cellular processes including the formation of the CCM protein complex, promotes Golgi assembly and has been shown to regulate apoptosis and cell migration pathology[53,56,57]. Furthermore, studies in zebrafish have shown that loss of *pdcd10* leads to vascular defects starkly different from those caused by loss of *krit1* or *ccm2*[58]. Previous work done by Fidalgo et al., demonstrates that levels of *CCM3* regulate activation of a Golgi center kinase III (GCKII) called Mst4 that is protective during oxidative stress

response[57]. Upon exposure to reactive oxygen species (ROS), Mst4 phosphorylates proteins downstream that are protective against unnecessary cellular apoptosis[57,59]. Additionally, *CCM3* inhibits the exocytosis of angiopoietin-2 (*Angpt-2*), which is shown to lead to disruption of endothelial cell junctions, dissociation of endothelial cells, and pericytes as well as vessel dialation[60].

1.2.4. Molecular pathogenesis of CCMs

As mentioned above, CCM protein complex in endothelial cells regulates multiple signaling pathways and function in endothelial cells that have been validated in mouse CCM models and observe in human CCM including – reactive oxygen species (ROS)[61], RhoA-ROCK signaling[55], KLF2 and 4 signaling[62], integrin activation[63], VEGF signaling[64], NOTCH signaling[65], angiopoietin-2 signaling[60], endothelial-to-mesenchymal transition[66], autophagy[67], and anticoagulation pathway[68,69].

1.2.4.1. Rho-ROCK signaling

CCM protein complex stabilizes endothelial cell-to-cell contact and, therefore, barrier function by suppressing the activation of the RhoA-Rho-associated kinase (ROCK) signaling[55]. Loss of endothelial *KRIT1* results in an increase in RhoA and its downstream effector ROCK that leads to actomyosin contractility that affects the integrity of the endothelial barrier by negative regulation of cell-cell junctions[49,55]. ROCK-induced actomyosin contractility involves both direct phosphorylation of myosin light chain (MLC) by activation of the myosin light chain kinase (MLCK), and indirect regulation of MLC by inhibiting the MLC phosphatase[55]. Current clinical trials involve the use of atorvastatin (as

shown on clinicaltrials.gov). Atorvastatin belongs to the statin cholesterol of drugs that is shown to inhibit ROCK activation and has the potential to lower the number of lesions[70].

1.2.4.2. Krüppel-like factor 2 and 4 (KLF2 and KLF4) signaling

Transcription factors KLF2 and KLF4 are expressed in endothelial cells and regulate genes, like endothelial nitric oxide (*Nos3*) and thrombomodulin (*Thbd*), that are involved in inflammation, vascular tone, and vascular development[71,72]. Importantly, the gain of endothelial MAPK/ERK kinase kinase 3 (MEKK3) (MEKK3) activity has been associated with the upregulation of KLF4 and KLF2 in the CCM disease[62,73–75]. MEKK3 interacts with the CCM protein complex by binding directly to CCM2[76]. Loss of CCM proteins results in MEKK3 activation and the subsequent cascade of events that increase MEK5-ERK5-MEF2 module that in turn, upregulates KLF2 and KLF4[62,73–75]. In addition, the MEKK3-KLF signaling has also shown to increase the activity of Rho kinase, which contributes to increased actin stress fibers formation[49,62,77,78]. Furthermore, previous work in our lab has demonstrated that KLF2/KLF4-driven suppression of endothelial thrombospondin1, TSP1, a potent endogenous angiogenesis inhibitor with a key role in CCM pathogenesis[68]. More recently, our group has shown that CNS hemorrhage in CCM is associated with locally elevated expression of the anticoagulant endothelial receptors thrombomodulin (TM) and the endothelial protein C receptor (EPCR) that generate activated protein C (APC) to form an anticoagulant vascular domain that might contribute to the bleeding-induced morbidity in CCMs[69].

1.2.4.3. Integrin activation

Integrins are transmembrane proteins that are involved in ECM adhesion, and upon ligand binding are responsible for initiating signal transduction pathways that modulate receptor expression on the cell membrane, the lifecycle of the cell, and arrangement of the cytoskeleton[26]. One such protein, that is necessary for adhesion in BMECs is integrin cytoplasmic associated protein-1 (ICAP1), which has been known to be a suppressor of integrin activation and is often competing with activators like talins[47,48]. It has been shown that *KRIT1* can directly bind to ICAP1 and acts as an antagonist to the ICAP1 mediated suppression of integrin activation[48]. Therefore, it has been suggested that during CCMs there is an increase in integrin signaling and vascular growth that contributes to CCM formation[48,49].

1.2.4.4. Angiogenesis

Previous studies have demonstrated elevated levels of angiogenic factor VEGF in CCM lesions, and in the plasma of individuals with the hereditary and sporadic form of the disease[79,80]. VEGF signaling mediates disruption of the brain endothelial barrier by disassembly of inter-endothelial junctions[81,82] and can cause hemorrhages[83], all prominent features of neurovascular lesions caused by CCMs[34,37,55]. Several studies support the hypothesis that CCMs are hypersensitive to angiogenesis due to an increase in the secretion of angiopoietin-2[60], deregulation of Notch signaling[65], loss of an anti-angiogenic checkpoint protein TSP1[68], and increase in VEGF signaling[46,84,85]. These studies suggest that strong drivers of angiogenesis may exacerbate neurovascular lesion and brain bleeding during CCMs. In addition, cell migration due to disruption of endothelial apical-basal polarity and

endothelial-mesenchymal transition have been reported to be hallmarks of CCMs[66]. However, the hierarchy of signaling events leading to altered angiogenic phenotype and subsequent endothelial dysfunction during CCM disease is not, as yet, completely understood.

1.3. Hypoxia inducible factor-1α (Hif-1α) regulation

Hypoxia-inducible factor-1a (HIF-1α) is a transcription factor that maintains cellular homeostasis by regulating genes implicated in angiogenesis, metabolism, and inflammation during conditions of low oxygen. During normal normoxic conditions (oxygen tension between 10-21%), prolyl-hydroxylase enzymes (PHD) induce hydroxylation of HIF-1α leads to binding to von Hippel–Lindau (VHL) ligase, directing HIF-1α for protein ubiquitination and degradation[86]. In addition, part of the HIF1α activity under normoxic conditions is also decreased by the factor inhibiting HIF-1 protein (FIH-1) by hydroxylation[87,88]. Therefore, PHD and FIH-1 tightly regulate HIF-1α activity in the presence of normal oxygen levels[86,89]. In hypoxic conditions, low O_2 availability (low oxygen tension 1-5 % O_2) limit PHD hydroxylation and FIH-1 activity which prevents degradation of HIF-1α and subsequent translocation to the nucleus by interacting with HIF1b[86].

1.3.1. Hif-1α in angiogenesis

HIF-1α is stabilized during conditions of low oxygen; consequently, the angiogenic pathway is activated in order to adapt to lower cellular oxygen supply [86] **(Figure 3)**. Upon stabilization, Hif-1α translocates into the nucleus and, along with other relocated factors,

via the Hypoxia Response Element, enhances hypoxia-inducible gene transcription[90]. One of these genes is VEGF, which is an important inducer of angiogenesis[91]. Additionally, the von Hippel-Lindau protein, a regulator of Hif-1α, has also been shown to degrade VEGF protein in normoxic conditions; this suggests a strong case for hypoxic meditated loss of VHL contributing to angiogenesis[92]. Furthermore, HIF pathways also regulate a plethora of angiogenic signaling pathways such as angiopoietin-1, angiopoietin-2, Tie2, platelet-derived growth factor (PDGF), basic fibroblast growth factor (bFGF), and monocyte chemoattractant protein–1 (MCP-1)[86]. Consequently, Hif-1α mediated expression of the aforementioned factors leads to the execution of their specific angiogenic processes like endothelial cell proliferation, migration, adhesion, and increase in vascular permeability[82,86,93,94] **(Figure 3)**.

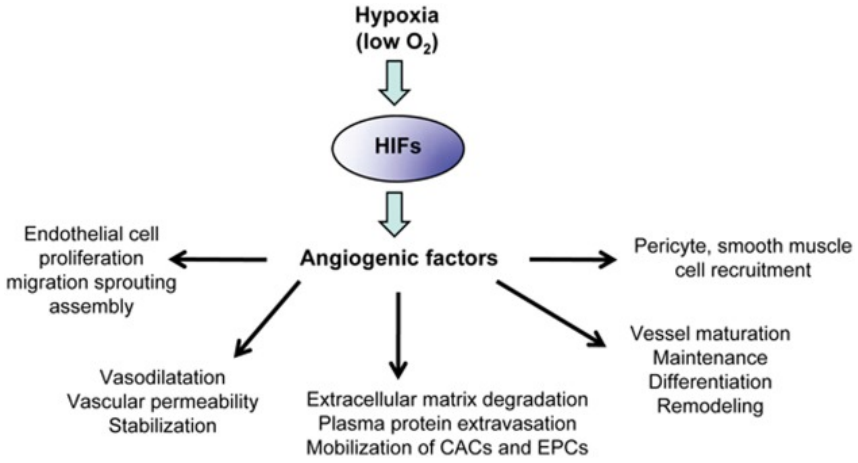

Figure 3: A schematic depicting the pathways activated in hypoxia and regulated by Hif-1α. CACs: circulating angiogenic cells; EPCs: endothelial progenitor cells. Image taken from Krock BL, Skuli N, Simon MC. Hypoxia-Induced Angiogenesis: Good and Evil. Genes & Cancer. 2011;2(12):1117–1133. doi:10.1177/1947601911423654

1.4. Objectives of the present study

CCMs are common brain vascular lesions causing a lifelong risk of hemorrhage and neurologic deficits for which there is no current effective pharmacologic therapy. Our group and others demonstrated that increased VEGF signaling and associated vascular leakage are significant contributors to CCM disease. We found that proliferative astrocytes play a critical role in CCM pathogenesis by serving as a major source of VEGF during CCM lesions formation. We observed that an increase in astrocyte VEGF synthesis is regulated by endothelial nitric oxide (NO) produced as a consequence of elevation in endothelial nitric oxide synthase (eNOS) in CCM endothelium in normoxic conditions. Moreover, increased NO in CCM endothelium stabilizes hypoxia-inducible factor-1 alpha (HIF-1α) in astrocytes, resulting in increased VEGF production and expression of a "hypoxic" program under normoxic conditions.

Hence, the aims of the present work are:

1) To establish a CCM animal model.
2) To quantify changes in HIF-1α expression during CCM lesion formation.
3) To analyze HIF-1α target genes by RT-qPCR in cerebellar tissue from animals that develop CCM lesions.
4) To determine the effect of COX-2, a direct HIF-1α target gene, inhibition on CCM lesion formation.

2. Materials and Methods

2.1 Genetically modified mice slco1-CRE and *Pdcd10*

Pdcd10-null mice that were endothelial-specific conditional knockouts were generated by crossing a *Pdgfb* promoter-driven, that was tamoxifen induced, Cre recombinase[95,96] (*iCreERT2*) that contained loxP flanking *Pdcd10* (*Pdcd10*$^{fl/fl}$; generous gift from Wang Min, Yale University; *Pdgfb-iCreERT2;Pdcd10*$^{fl/fl}$) mice. On postnatal day 3, mice were administered 50 μg of tamoxifen [T5648; Sigma-Aldrich] by intragastric injection. This induced genetic inactivation of the endothelial *Pdcd10* gene in littermates with *iCreERT2* (*Pdcd10*^{ECKO}). *Pdcd10*$^{fl/fl}$ mice were used as littermate controls. Brain endothelial-specific conditional *Pdcd10*-null mice were generated by crossing a *Slco1c1* promoter-driven tamoxifen-regulated Cre-recombinase[97]. (iCreERT2, generous gift from Markus Schwaninger) with *Pdcd10*$^{fl/fl}$ mice. On postnatal day 5, mice were administered 50 μg of tamoxifen [H7904; Sigma-Aldrich] by intragastric injection to induce genetic inactivation of endothelial *Pdcd10* gene in littermates with *Slco1c1*-iCreERT2; (*Pdcd10*^{BECKO}), and *Pdcd10*$^{fl/fl}$ mice were used as littermate controls. *Vegfa*$^{tm1.1Nagy}$ mice, expressing a β-galactosidase (LacZ) reporter gene inserted into the 3' untranslated region of the *Vegfa* gene[98], were obtained from the Jackson Laboratory. The GFAP-TK mice line was used to selectively ablate proliferative astrocytes[99,100]. Animal experiments were carried out in accordance of animal care protocols established by the University of California San Diego.

2.2 Immunohistochemistry

Whole brain tissue samples were isolated from mice at days P10. The collected tissue sample was fixed in 4% paraformaldehyde for 5 minutes. After fixation, tissue samples were washed in Phosphate Buffered Saline 1x (PBS) three times. Samples were then placed 30% D-sucrose

(prepared in PBS 1x) for roughly 24 hours. Samples are then frozen in optimal cutting temperature compound (23-730-571; Fischer Scientific) and stored at -80°C. Tissue samples are sectioned into 12 μm sections on Superfrost Plus slides (12-550-15; VWE International). Prepared slides were then left to thaw for 30 minutes followed by 3 washes of 1X PBS to remove OCT. For β-gal staining was performed at 37°C for overnight in 0.02% X-Gal, 5mM $K_3Fe(CN)_6$, 5 mM $K_4Fe(CN)_6$, 2 mM $MgCl_2$ in PBS. After staining, tissue was fixed again with 4% PFA for 10 minutes at RT, pH 7.4. Next slides were washed and placed at room temperature in blocking and permeabilization solution (0.5% BSA, 0.5% Triton X-100, 5% goat serum, PBS) for 2 hours. Preparations were washed place in primary antibodies for CD34 (1:100; Cell signaling) and anti-GFAP (1:100; 13-0300; Thermo Fischer Scientific). Cell nuclei were stained with DAPI and mounted with Fluoromount-G mounting medium (SouthernBiotech). Tissue sections were washed four times in PBS and incubated with suitable anti-rabbit Alexa Fluor 594/488-coupled secondary antibody (1:300, Thermo Fischer Scientific) in PBS for 1 hour at RT.

2.3 Hematoxylin and eosin.

Frozen slides were left thawing at room temperature for at least 30 minutes. Slides were then washed in PBS 1x thrice to remove OCT. Next slides were placed in acetone for 10 minutes. Slides were then placed in Hematoxylin for 3.5 minutes, followed by bluing 30 seconds in acidic alcohol (1% HCL in 70% EtOH) and then in Scott's solution (2% sodium bicarbonate, 0.35% magnesium sulfate in ddH_2O) for 30 seconds. Then, slides are then eosin for 7 minutes and rinsed. The slides are then dehydrated in 75% → 80% → 90% → 100% ethanol (30

seconds each) then cleared in xylene. Lastly, sealed with xylene based cytoseal mounting media.

2.4 Western blots

Whole tissue samples of hindbrains or spinal cords are collected and immediately snap frozen in liquid nitrogen. Tissue samples are then cold pulverized and placed in 300 µl RIPA buffer (25 mM Tris-HCl, pH 7.6, 150 mM NaCl, 1% Nonidet P-40, 1% sodium deoxycholate, and 0.1% SDS) and a mixture of inhibitors (11836170001; Roche) and 1 mM sodium orthovanadate. Samples were then left shaking in 4°C for 1 hour, followed by centrifugation for 20 minutes at 4°C to collect lysis supernatant. Protein concentration was determined using a Micro BCA protein assay kit (500-0116; Thermo Scientific). Tissue lysates were diluted in 2x Laemmli's buffer (1610737; Biorad 4% SDS) and denatured at 95°C for 5 min. The samples were then resolved on 4% to 20% gradient sodium dodecyl sulfate-polyacrylamide gel electrophoresis (XP04200, Thermo Fischer Scientific) and run with a molecular marker (1610374, BioRad). The running buffer for the western blot was composed of 125mM Trizma base, 1M glycine, and 0.01% (v/v) SDS in ddH2O. A wet overnight transfer system was used to transfer proteins to nitrocellulose membranes (Amersham) in transfer buffer (50mM Trizma base, 40mM, 20% (v/v) methanol in ddH2O. Membranes were then blocked for 1 hour at room temperature in a blocking solution (0.1% tween-20, 10% fat-free dried milk dissolved in TBS at pH 7.4). After blocking, membranes were incubated for HIF-1a (1:150; NB100-134; Novus Biologicals) or monoclonal rabbit (1:100; 12282; Cell Signaling) at 4°C overnight. The membranes were then washed multiple times in wash buffer (0.1% tween-20 dissolved in TBS at pH 7.4) and placed in

corresponding secondary antibodies IRDye/Alexa Fluor-coupled secondary antibodies (1:10,000, 926-68070; 926-32211; Li-COR) for 1h at RT. As a protein loading control, a mouse monoclonal antibody against beta-actin (1:5000; A5441; Sigma-Aldrich) was used. Finally, blots were imaged and quantified using Odyssey CLx Infrared Imaging (Li-COR).

2.6 Celecoxib preparation

Mice of the litter were weighed and dosed with 40mg/Kg celecoxib (dissolved in 20% dimethyl sulfate) (Pfizer) or vehicle (0.5% methylcellulose and 0.0025% Tween 20). 100 µl celecoxib or vehicle was administered by intragastric injection on P6, P7, P8, P9 and animals were sacrificed at P13. Adults received a dose of 40mg/Kg celecoxib for 15 days.

2.7 Data analysis

Data mean was graphed using prism with standard error mean. Statistical analysis was done using a two-tailed students t-test with significance set at p-value of 0.05.

Sections 2 and 3, in part, is being prepared for publication in - Lopez-Ramirez, Miguel; Soliman, Shady; Hale, Preston; Lai, Catherine; Pham, Angela; Verma, Riya; Sun, Hao. “Non cell-autonomous effect of astrocytes on cerebral cavernous malformations”. The thesis author is the primary author of this paper.

3. Results

3.1 Loss of endothelial *Pdcd10* CCM gene results in malformations in hindbrains of neonatal mice

The murine embryonic loss of *Pdcd10* gene consist of severe defects in vascular growth, resulting in lethality[96]. Therefore, our group and others have shown that tamoxifen treatment of neonatal endothelial-specific inactivation of murine *Pdcd10* (Pdgfb-iCreERT2;$Pdcd10^{fl/fl}$) (see methods 2.1) producing $Pdcd10^{ECKO}$ mice are not immediately lethal and results in cerebellar vascular lesions similar to those in CCM patients[69,96]. This murine model of CCM has been very useful to investigate molecular mechanisms underlying the pathogenesis of CCM disease[60,69]. The genetic inactivation of the *Pdcd10* gene using the 4-hydroxy-tamoxifen injections (**Figure 4A**) administration by intragastric injection in neonatals at postnatal day 3 (P3) results in dilated blood vessels (in red) in the hindbrains of $Pdcd10^{ECKO}$ mice (**Figure 4A**) but not in littermate $Pdcd10^{fl/fl}$ controls (**Figure 4A**). Hematoxylin and eosin staining in coronal sections enables the visualization of lesions (arrows) in the cerebellum (**Figure 4B).**

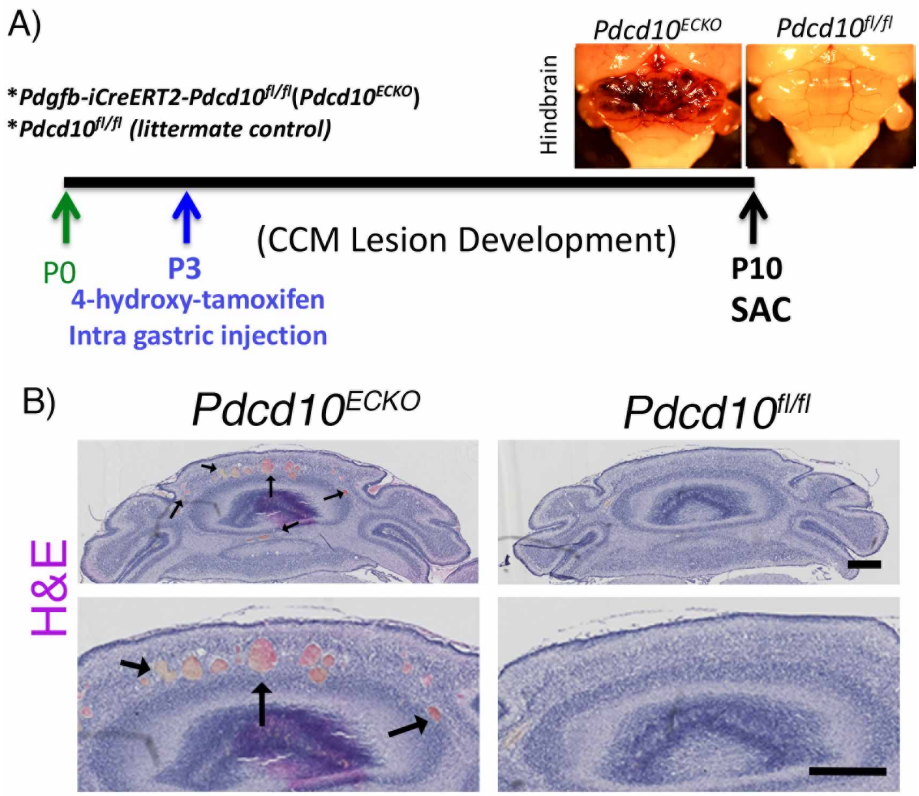

Figure 4: Loss of Pdcd10 leads to CCM pathology in neonatal mice.
A) Timeline of intra-gastric tamoxifen injections with whole brain images showing bleeding in the hindbrain of P10 mice due to cavernoma formations in *Pdcd10*^{ECKO} (right) compared to no pathology in *Pdcd10*$^{fl/fl}$ (left) hindbrains.
B) Histological analysis of cerebellar sections from *Pdcd10*^{ECKO} compare to *Pdcd10*$^{fl/fl}$ mice. CCM lesions detected in sections stained by hematoxylin (purple) and eosin (pink). Arrows depict dilated blood vessels forming lesions. Scale bar is 500 μm.

In order to further investigate lesion pathology, we studied coronal sections of the cerebellum **(Figure 5A)**. Using antibodies specific against CD34, a vascular endothelial cell marker, allow us to visualize grossly dilated blood vessels that are characteristic of CCM pathology[39,101]**(Figure 5B, C)**. Moreover, we notice that using antibodies specific against glial fibrillary acidic protein (GFAP, in red) a marker for astrocytes, show that lesions are in close proximity to astrocytes in *Pdcd10*^{ECKO} hindbrains[102,103]**(Figure 5B, C)**.

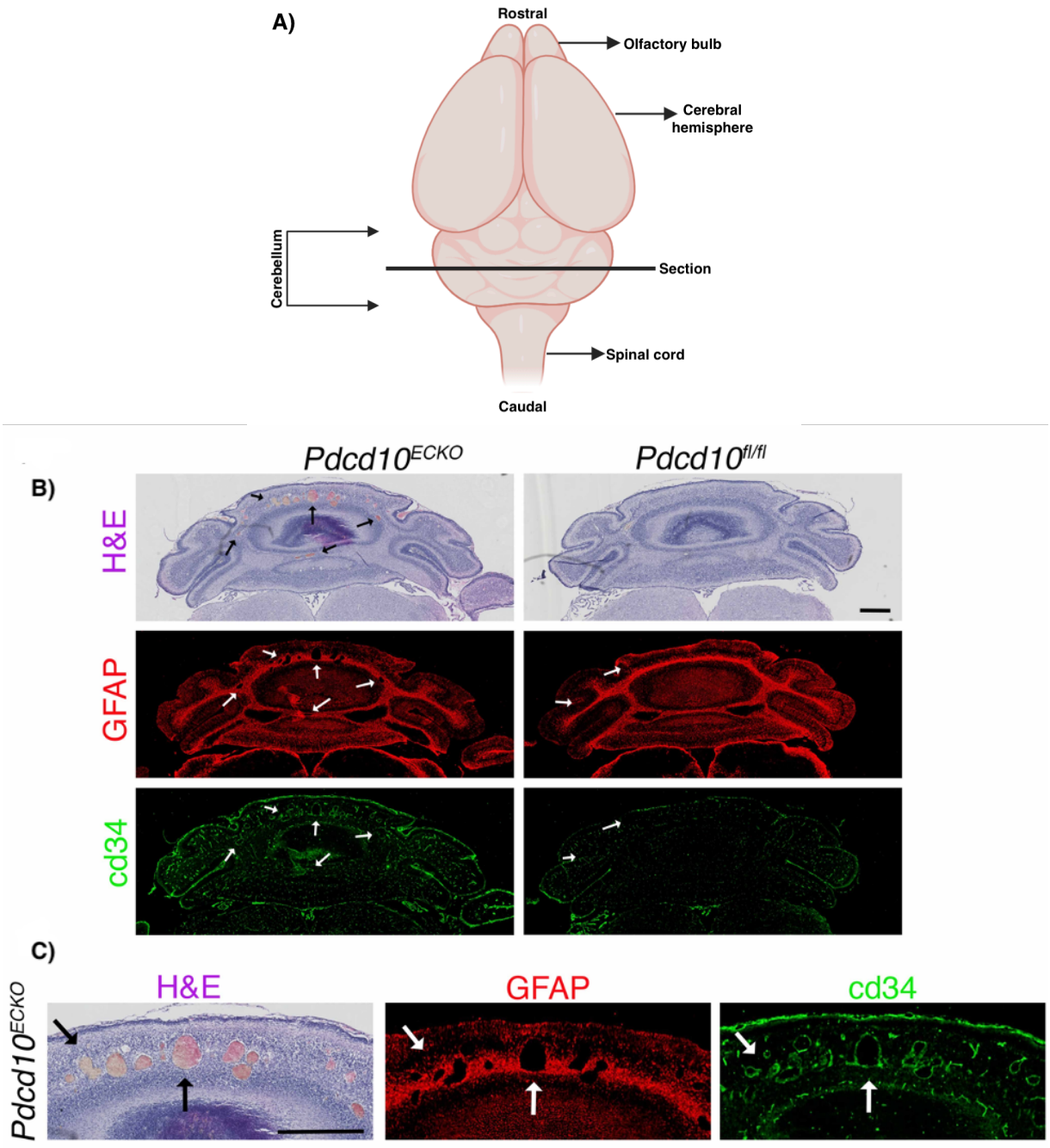

Figure 5: CCM lesions spatially developed on fibrous astrocytes.
A) Diagrammatic dorsal view of whole mouse brain depicting anatomy and section regions. Image made with biorender.
B-C) Histological analysis of cerebellar sections from endothelial inactivated *Pdcd10* in hindbrains that are analyzed at P10, coronal sections in two regions for $Pdcd10^{ECKO}$ (left) compared to $Pdcd10^{fl/fl}$. Staining depicts proliferative astrocytic marker GFAP and vascular brain endothelial cell surface molecule CD34 (green). Scale bar is 500 µm. Arrows depict lesions.

3.2 Astrocytic derived VEGF contributes to lesion formation in endothelial inactivated *Pdcd10* neonatal mice

In order to characterize the role of astrocytes in lesion formation, we aimed for selective ablation of proliferative astrocytes in *Pdcd10*^{ECKO} mice. We used a GFAP driven promoter of herpes simplex virus thymidine kinase (HSV-TK) transgene (mice referred to as *GFAP-TK*)[100]. This allowed for a time-dependent induction of cellular depletion in proliferating astrocytes by using the antiviral ganciclovir (GCV) dosed at 20mg/Kg in P7 mice. Here we observed that mice at P9 that were GCV-treated *Pdcd10*^{ECKO}; *GFAP-TK* lead to a visual decrease in hindbrain lesion formation when compared to GCV-treated littermates *Pdcd10*^{ECKO} controls **(Figure 6A)**. Furthermore, contrast-enhanced, high-resolution, and x-ray micro-computed tomography (micro-CT) allowed us to measure lesion volumes using semiautomated software[104]. In order to quantify lesion volumes in P9 mice hindbrains we conducted blind measurements using micro-CT. We found that lesion volumes in GCV-treated *Pdcd10*^{ECKO}; *GFAP-TK* mice was significantly reduced when compared to control GCV-treated *Pdcd10*^{ECKO} littermates **(Figure 6B)**. Therefore, this implicated that in our murine model, proliferative astrocytes participate in the cavernoma formation.

Previous work has shown that CCMs have increased VEGF signaling due to dysregulation of anti-angiogenic checkpoints[68]. In order to investigate whether astrocytes could be considered a potential source for VEGF, we used a VEGF reporter CCM murine model (*Pdcd10; Vegfa*$^{tm1.1Nagy}$) that contains a knock-in for lac operon β-galactosidase (LacZ). *Vegfa*$^{tm1.1Nagy}$ carries a nuclear-localized beta-galactosidase (β-gal) knock-in at the 3' UTR of the *Vegfa* gene locus that permits monitoring of VEGF expression[98]. Thus, by using X-gal staining for β-gal we follow VEGF expression (see methods 2.2). We observed that in P10

Pdcd10^{ECKO}; *Vegfa*$^{tm1.1Nagy}$ mice, there was increased β-gal/VEGF expression in areas surround lesions **(Figure 6C)**. Furthermore, we found that most of β-gal/VEGF+ cells colocalized with proliferative astrocytic marker GFAP; primarily, this colocalization occurred in fibrous astrocytes and the Bergman glia[103] **(Figure 6C)**. We utilized RT-qPCR to quantify *Vegfa* mRNA expression. Consistent with results observed using β-gal/VEGF reporter mice, *Pdcd10*^{ECKO} hindbrains exhibited ~1.7-fold increase in *Vegfa* mRNA levels when compared with littermate *Pdcd10*$^{fl/fl}$ control. Moreover, we observed that the depletion of proliferative astrocytes prevented an increase in *Vegfa* mRNA levels in *Pdcd10*^{ECKO} **(Figure 6D)**. These data suggest that increased expression of astrocyte-derived VEGF could account for a contribution of astrocytes in CCM lesion formation.

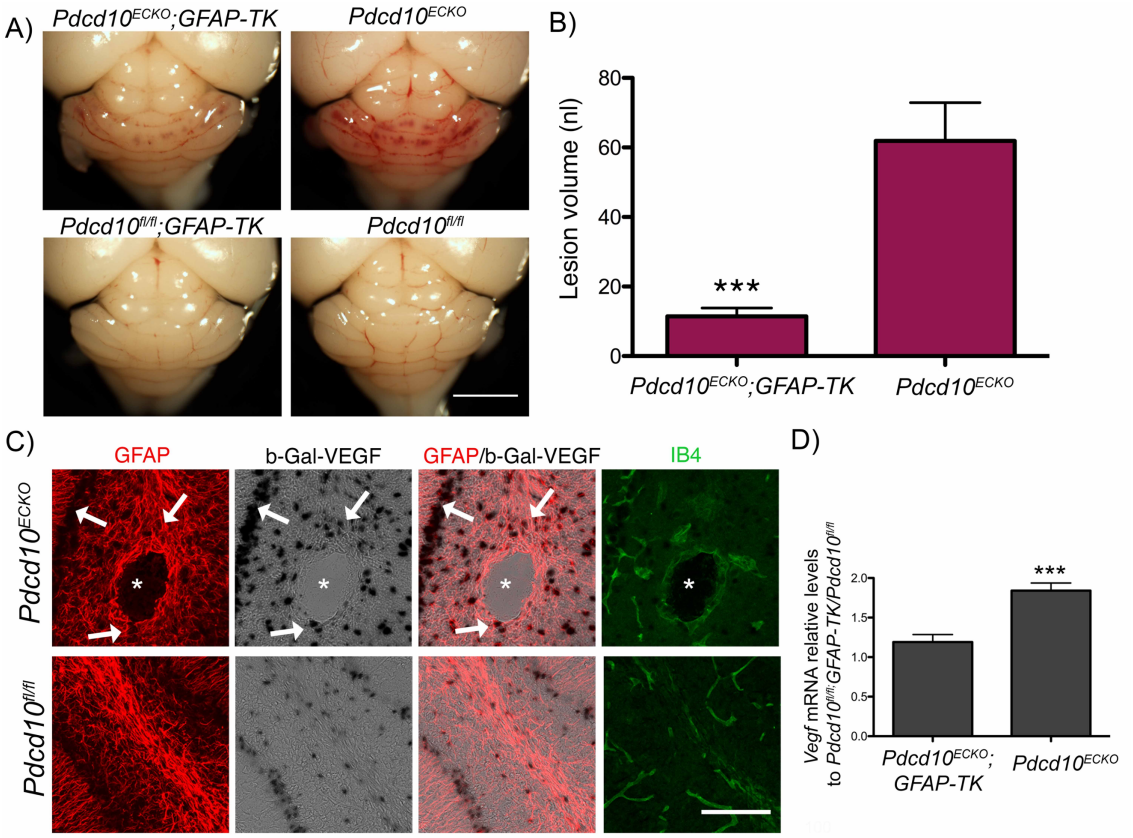

Fig 6. Astrocytes contribute to cerebral cavernous malformations development.
A) Images of hindbrains (cerebellum) depicting decreased lesion pathology in P9 *Pdcd10^{ECKO}*; *GFAP-TK* mice compared to GCV-treated littermate *Pdcd10^{ECKO}*. Mice were administered GCV (20 mg/Kg) at P7. Scale bar is 2 mm.
B) Quantification using bar graph of mean lesion volumes by micro-CT analysis from mice in **A**). Data graphed is mean±SEM, n=8 or 9 in each group. Statistical significance determined with students t-test (*** p<0.001).
C) Immunohistochemical analysis of hindbrain sections depicting β-gal/VEGF (black) expression in areas surrounding CCM lesions in P10 *Pdcd10^{ECKO}; Vegfa$^{tm1.1Nagy}$* compared to *Pdcd10$^{fl/fl}$; Vegfa$^{tm1.1Nagy}$* littermate control with GFAP marker of fibrous astrocytes (red) and endothelial marker IB4 (green). Asterisks indicate lesion lumen. Arrows indicate β-gal/VEGF+ cells colocalized with the GFAP marker. Scale bar is 100 µm.
D) Bar graph of RT-qPCR quantification of *Vegfa* mRNA levels in *Pdcd10^{ECKO}* hindbrains that is reduced in *Pdcd10^{ECKO}*; *GFAP-TK* and *Pdcd10$^{fl/fl}$; GFAP-TK* were used as controls. Data graphed is mean±SEM, n=5 or 7 in each group. Statistical significance determined with students t-test (*** p<0.001)

3.3 Increase in normoxic Hif-1α stabilization during CCM

HIF-1α is a transcription factor known to regulate VEGF expression[91] (see 1.3.1). Additionally, previous *in vitro* work in our lab shows that co-cultures of astrocytes with *Pdcd10^{ECKO}* BECs lead to the upregulation of HIF-1α (Lopez-Ramirez et al., in revision). Thus, we sought to explore the effect Hif-1α has *in vivo* and further develop the role of the

hypoxia proteins in the pathophysiological development of CCMs. For this analysis, we utilized western blots from total protein lysates from *Pdcd10*^{ECKO} hindbrains and littermate *Pdcd10*$^{fl/fl}$ controls at P10. We identified an almost 1.7- fold increase in Hif-1α protein expression in actin normalized *Pdcd10*^{ECKO} hindbrains compared to littermate *Pdcd10*$^{fl/fl}$ controls (**Figure 7A**). Furthermore, RT-qPCR of cerebral tissue identified increased mRNA levels of hypoxic protein Hif-1α target genes (as identified with cDNA library of genes with hypoxia response elements). (**Figure 7B**). We observed an increase in genes that lead to a hypoxia response. This includes metabolic reprogramming through an expression of lactic acid and pyruvate transporter solute carrier family 16 (*Slc16a3*), also known as monocarboxylate transporter 4 (*mct4*)[105] (**Figure 7B**). Additionally, there was an upregulation of inflammatory proteins like monocyte chemoattractant protein-1 (MCP-1) and cyclooxygenase-2 (COX-2)[94,106] (**Figure 7B**). In addition, we also observed increase genes involved in angiogenesis, including a cell-surface glycoprotein (Cd44), angiopoietin-like 4 (Angptl4), proangiogenic enzyme lysl oxidase-like 2 (Loxl2)[107–111](**Figure 7B**). Collectively, these results suggest that CCM endothelium can increase HIF-1α stabilization and expression of a "hypoxic" program under normoxic conditions.

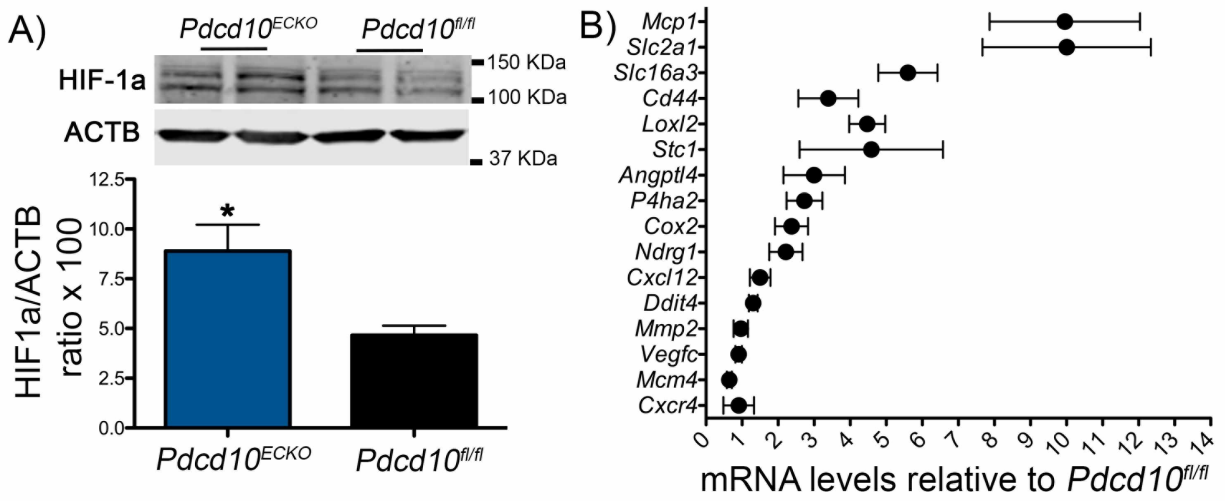

Figure 7: Increase in normoxic HIF1α stabilization and hypoxic program during CCM.
A) Quantification of HIF-1α in cerebellar tissue in P10 *Pdcd10*^{ECKO} control *Pdcd10*$^{fl/fl}$ littermates, as assessed by Western blot. Protein levels normalized with actin (SEM, n= 4 mice in each group). Statistical significance determined with students t-test (* $p<0.05$).
B) Analysis of mRNA levels of HIF-1α-target genes (hypoxic program) by RT-qPCR in cerebellar tissue from P10 *Pdcd10*^{ECKO} relative to control *Pdcd10*$^{fl/fl}$ littermates (SEM, n=5 or 7 mice in each group).

3.4 COX-2 inhibition ameliorates lesion formation in brain endothelial inactivated *Pdcd10* mice

Celecoxib is an FDA approved non-steroidal inflammatory drug (NSAID) that inhibits the activity of COX-2[112]. In order to best characterize the role of COX-2, we utilized a genetic model that inactivated *Pdcd10* only in brain endothelial cells (*Pdcd10*^{BECKO}) as opposed to globally (*Pdcd10*^{ECKO}) (see methods 2.1). Our aim is to investigate the role COX-2 has on the pathophysiology of lesions. Here, we administered the celecoxib to inhibit the activity of COX-2 through intragastric gavaging for 15 consecutive days (40mg/Kg) in adult mice the mice were sacked at day P70 and brains were collected for analysis. In the neonatal model, mice were treated by intragastric injections (40mg/Kg) for four consecutive days, P6 to P9, with celecoxib and mice were sacked at P13. The drug was administered to normoxic *Pdcd10*^{BECKO} and vehicle *Pdcd10*^{BECKO} controls. Consequently, we were able to see a visual

decrease in surface bleedings of *Pdcd10*^{BECKO} hindbrains that were treated with celecoxib when compared to vehicle-treated **(Figure 8A).** Additionally, using micro-CT (see results 3.2) in neonatal mice treated with celecoxib we saw a significant decrease in lesion volume **(Figure 8A)**. Therefore, these results suggest that elevation of the enzyme COX-2, may contribute to lesion formation during CCM disease.

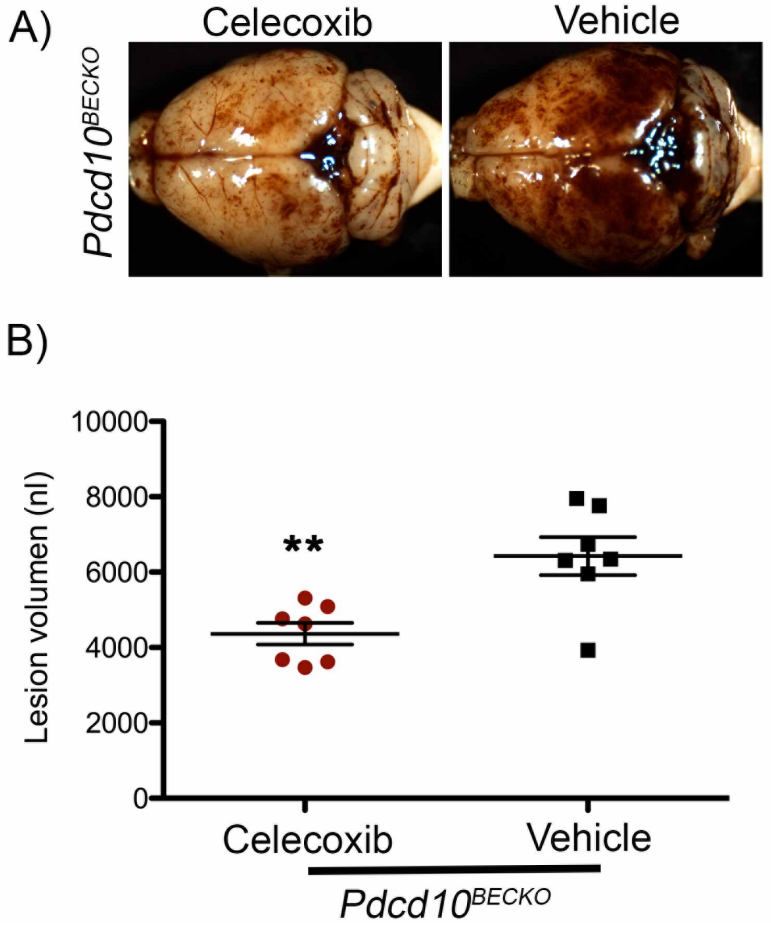

Figure 8: COX-2 inhibition reduces CCMs lesions.
A) P5 tamoxifen injected *Pdcd10*^{BECKO} treated with 40 mg/Kg celecoxib or vehicle for 15 consecutive days in adults *Pdcd10*^{BECKO} mice.
B) Intragastric administration of 40 mg/Kg celecoxib for four consecutive days P6 to P9, quantification of lesion volumes by micro-CT analysis from *Pdcd10*^{BECKO} mice at P13 treated with celecoxib or vehicle (SEM, n=7 mice in each group). Data graphed is mean±SEM, n=7 in each group. Statistical significance determined with students t-test (** $p<0.01$)

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4. Discussion

CCMs are vascular anomalies associated with disruption of angiogenic checkpoints, altered cell signaling in RhoA-ROCK[55], KLF2/KLF4[62], integrin activation[63], reactive oxygen species (ROS)[61], VEGF[64], NOTCH[65], angiopoietin-2[60], endothelial-to-mesenchymal transition[66], autophagy[67], and anticoagulation pathway that contributes to vascular leakage and hemorrhagic strokes[68,69]. Here we show that the genetic inactivation of *Pdcd10* in murine endothelial cells leads to CCM lesions burden in acute and chronic mouse models. Our study shows that astrocytes respond to CCM endothelium and serve as a major source of VEGF during CCM lesion formation. An increase in astrocyte VEGF synthesis was associated to a normoxic stabilization of HIF-1α in CCM tissue. Furthermore, we show that the inhibition of COX-2, a direct HIF-1α target gene, ameliorates the lesion pathology in our $Pdcd10^{BECKO}$ murine model.

CCMs have been recognized as an endothelial cell-autonomous disease because of endothelial-specific inactivation of murine *Pdcd10, Krit1, or Ccm2* results in brain and retinal vascular lesions similar to those in CCM patient[68,113]. These murine studies are also supported with the finding of a "second hit" on the normal *PDCD10* or *KRIT1* allele in the human CCM endothelium[43]. However, although it is a disease that affects the neurovascular unit, we know little about whether neural cells influence CCM pathogenesis[114,115]. Recent work in the lab has shown that an increase in astrocyte VEGF synthesis is driven by endothelial nitric oxide (NO) generated as a consequence of elevation in endothelial nitric oxide synthase (eNOS or *Nos3* gene) in CCM endothelium. Production of NO in CCM endothelium stabilizes hypoxia-inducible factor-1 alpha (HIF-1α) in astrocytes, resulting in increased VEGF production and expression of a "hypoxic" program under normoxic conditions (Lopez-Ramirez et al., in revision).

CCMs are hypertensive to angiogenesis due to disruption of anti-angiogenesis regulators like TSP1, and increase in VEGF signaling[22,25,46,68]. Additionally, in the plasma of CCM patients, increased levels angiogenic VEGF have been documented[79,80]. Reactive astrocytes have been shown to influence BBB properties through increase in VEGF-A secretions in inflammatory CNS disease like multiple sclerosis[81]. Increased VEGF leads to BBB breakdown by reducing the expression of tight junction proteins claudin and occludins[81,83]. Therefore, we demonstrate that astrocyte derived VEGF may contribute to lesion pathology in *Pdcd10*^{ECKO} mice.

The increase in HIF-1α stabilization in CCM lesions may be associated with augmented production of NO or an increase in ROS during CCM[116]. NO can stabilize HIF-1α protein from degradation by several mechanisms such has through prolyl hydroxylase (PHD) and factor inhibiting HIF (FIH) activity by interacting with enzyme bound Fe^{2+} [89,117]. HIF-1α can translocate to the nucleus where it dimerizes with HIF-1β and binds to the hypoxia-responsive element in the promoter region of several target genes such as VEGF[118,119]. Furthermore, we see HIF-1α driven increased expression of hypoxia program proteins that lead to metabolic reprogramming, angiogenesis and inflammation. For example, increased glycolysis with GLUT-1 expression that guarantees rapid energy production, and an increase in MCT4 that promotes efficient removal of lactic acid, an end product of glycolysis metabolism[118]. Elevated HIF activity in CCM tissue was also associated with increases in several pro-angiogenic and inflammatory factors produced by glial cells, including ANGPTL4 that can induce vascular dysfunction in CNS pathologies by triggering angiogenesis[120,121] and COX-2 an enzyme that catalyzes the biosynthesis of prostanoids during inflammation[122].

Previous work in vitro has shown that COX-2 mediated PGE2 secretion in endothelial cells is necessary for angiogenesis and increased of angiopoetin-2 signaling[123,124]. Furthermore, in

tumor models, genetic deletion of COX-2 attenuated tumorigenesis by impeding tumor associated vascularization[125]. Our results show that pharmacological inhibition of COX-2 reduced vascular lesion formation in *Pdcd10*^{ECKO} mice. Quantification of lesion volumes in *Pdcd10*^{BECKO} also depicted a significant decrease in lesion volume of celecoxib treated mice when compared to vehicle treated animals. In line with these results, a recent retrospective cohort study reported that the use of NSAIDs was correlated with lower risk of hemorrhage among patients affected by CCMs[126]. Therefore, our findings here not only implicate the role of HIF-1α mediated COX-2 expression in cavernoma formation but also provide insight into potential therapeutic approaches.

However, COX-2 inhibitors must be investigated for safety and effectiveness in prospective controlled trials, and specific dose-effect and duration of treatment must be carefully defined. A platform for trial readiness, exploring proof of concept effect on lesions bleeding in human subjects, is currently being developed, and can efficiently be applied to repurposed drugs, like NSAIDs[127]. The precise role of NSAIDs in reducing CCM risk of hemorrhage version lesion formation remains incompletely understood. However, the COX-2-prostaglandin pathway has been implicated in angiogenesis by induction of VEGF, and selective COX-2 inhibitors confer antiangiogenic properties[128]. Whether COX-2 inhibitors prevent an increase in astroglia-derived VEGF in response to CCM endothelium needs further investigation.

Nonetheless, collectively our data are consistent with the notion that there is reciprocal communication between CCM endothelium and astrocytes that drives CCM lesion formation and contributes to neurovascular dysfunction. Future studies should focus on whether long-term COX-2 inhibition is effective in reducing VEGF secretions and ameliorating CCM lesion burden. Additionally, genetic inactivation of HIF-1α in genetically inactivated CCM mice could further support the role of the hypoxia program on pathophysiological development of CCM disease.

These observations point to the possibility of designing therapeutic approaches aimed at preventing endothelial dysfunction and astroglia activation as an intervention to reduce the burden of CCM disease in humans.

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