Trends in Molecular Medicine

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Review

Blocking Signalopathic Events to Treat Cerebral Cavernous Malformations

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Cerebral cavernous malformations (CCMs) are pathologies of the brain vasculature characterized by capillary-venous angiomas that result in recurrent cerebral hemorrhages. Familial forms are caused by a clonal loss of any of three *CCM* genes in endothelial cells, which causes the activation of a novel pathophysiological pathway involving mitogen-activated protein kinase and Krüppel-like transcription factor KLF2/4 signaling. Recent work has shown that cavernomas can undergo strong growth when CCM-deficient endothelial cells recruit wild-type neighbors through the secretion of cytokines. This suggests a treatment strategy based on targeting signalopathic events between CCM-deficient endothelial cells and their environment. Such approaches will have to consider recent evidence implicating 'third hits' from hypoxia-induced angiogenesis signaling or the microbiome in modulating the development of cerebral hemorrhages.

Highlights

Cerebral cavernous malformations (CCMs) are caused by the clonal loss of any of three *CCM* genes within endothelial cells of lowly perfused venous capillary vessel beds.

In mouse models of CCM, cavernoma growth is spurred when CCM-deficient clones of vessel-resident endothelial progenitor cells recruit wild-type neighboring endothelial cells.

Increasing evidence suggests that non-genetic 'third hits' cause an activation of quiescent CCM-deficient cells; this may provide novel avenues for drug treatments directed at blocking acute phases of the CCM pathology.

Pathophysiology of Cerebral Cavernous Malformations: Unresolved Questions

Cerebral cavernous malformations (CCMs) are a group of **capillary-venous cavernoma** (see Glossary) pathologies that mainly affect lowly perfused venous vessel beds and can cause bleeding within the central nervous system (Figure 1). CCM lesions may be deeply situated within the brain or spinal cord and thus pose major therapeutic challenges (Box 1). The more common form of CCM occurs sporadically with a prevalence of about 0.5% in the general population while familial forms are much rarer. These latter forms are autosomal dominant diseases caused by the loss of CCM1 [also known as Krev interaction trapped protein 1 (KRIT1)] [1], CCM2 (also known as malcavernin) [2], or CCM3 proteins [also known as programmed cell death 10 (PDCD10)] [3].

The etiopathogeny of CCMs remains largely unclear. We still lack a good understanding of the molecular and physiological triggers that cause acute phases of CCM lesion growth and bleeding. Other questions revolve around why this pathology is restricted to lowly perfused venous capillary beds, and which are the best targets for interventions to suppress lesion formation and bleeding (see Clinician's Corner). Answering these questions will require thoroughly characterizing the molecular heterogeneity of affected endothelial cell types. One approach would be to analyze CCM-related gene programs that cause endothelial cells to change from a **quiescent** state to one that is proliferative and pathological. Here, we review recent insights into the mechanisms by which external physiological or molecular stimuli activate CCM-deficient endothelial cells. A plethora of pathways that may contribute to this pathology have been implicated by a combination of functional studies, transcriptomics, and pharmacological suppression screens, which suggest novel therapeutic approaches in addition to current clinical trials with statins [4,5]. Another question to be resolved is to determine whether pathological signaling mechanisms are shared by CCMs and other vascular pathologies, such as arterial-venous malformations (AVMs) or moyamoya.

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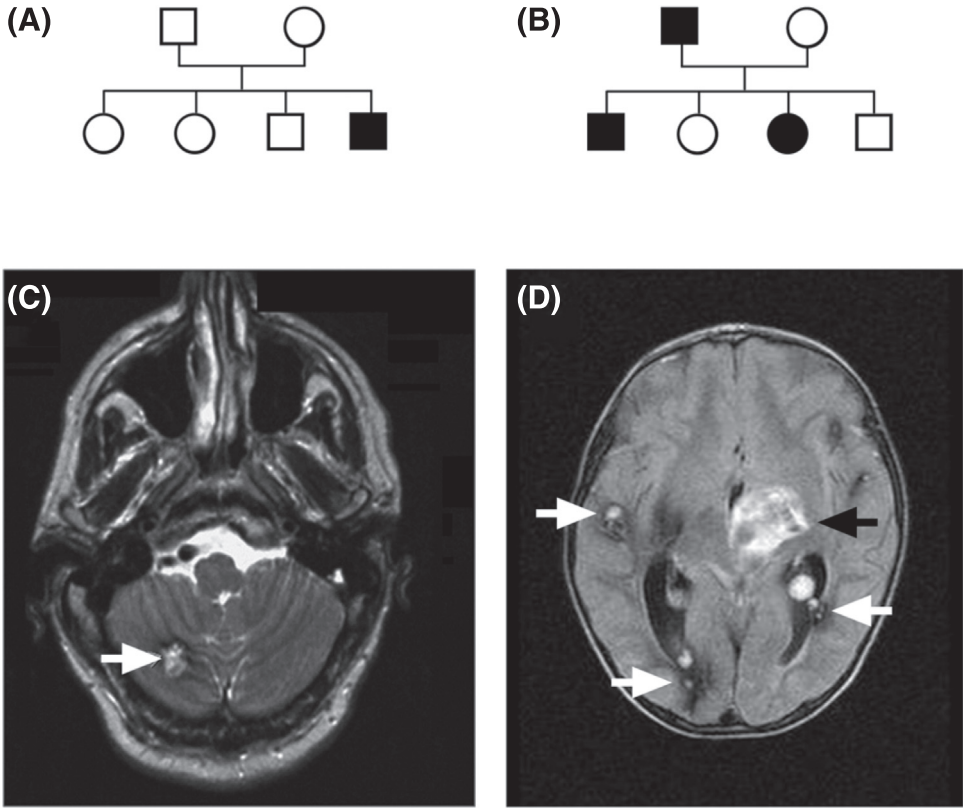

Figure 1. Brain Magnetic Resonance Imaging (MRI) Images from a Sporadic Case and a Familial Case of Cerebral Cavernous Malformations (CCMs). Schematics of family trees (A) of the common sporadic form of CCMs versus (B) the rare familial form. (C) MRI image of a patient with the common sporadic form shows a single CCM lesion (arrow). (D) MRI image of a patient with the familial form of CCMs shows multiple lesions (white arrows). The dark arrow points at a cerebral hematoma.

A Clonal Origin of Cerebral Cavernous Malformations

Genetic studies of familial forms of CCM have revealed that those affected bear heterozygous loss-of-function **germline mutations** in one of the three *CCM* genes [1–3]. Lesions only develop when a second, **somatic mutation** [6] affects the remaining wild-type allele within endothelial cells [7–9]. This has raised a number of questions regarding disease etiology, including whether dispersed lesions have a clonal relationship. Efforts have been made to establish animal disease models that reflect the human pathology. In zebrafish and mouse models, a complete knockout of any of the three *Ccm* genes causes severe cardiovascular malformations and embryonic lethality [10–18]. This has hampered mechanistic studies of the CCM pathology using conventional gene knockouts.

To circumvent this problem, alternatives have included conditional endothelial-specific knockout models in mice. This has led to two types of disease models. *Ccm1–3* knockouts induced during early postnatal stages lead mice to develop widespread vascular lesions throughout the cerebellum and retina within a few days. This 'acute CCM model' reduced the long-term survival of animals. In a 'chronic model', *Ccm3* knockouts were induced with a lower dose of tamoxifen or at a later stage. This lead to fewer lesions, allowing more animals to survive into adulthood [19,20]. Here, lesions were distributed in a mosaic fashion throughout the entire

Glossary

Angiogenesis: developmental growth process of the vasculature that involves sprouting from pre-existing vessels.
Capillary-venous cavernoma: dilated and malformed venous capillary blood vessels that frequently form lesions.
Cytokines: peptides or proteins with important roles in cell signaling. In the context of this review cytokines are also meant to include growth factors.
Endothelial-to-mesenchymal transition (endMT): process by which endothelial cells break down cell adhesion, loose cell polarity, and become migratory mesenchymal cells.
Germline mutation: permanent mutation present within germline cells that can be transmitted to offspring.
Hypoxia: physiological condition of oxygen deprivation within the entire body or regions of the body.
Krüppel-like transcription factors 2/4 (KLF2/4): zinc finger transcription factors implicated in many developmental and physiological processes including endothelial cell biomechanical signaling.
Mitogen-activated protein kinase (MAPK) pathway: signal transduction cascade that includes Ras–Raf–MEK–ERK proteins with roles in many developmental and pathophysiological process including tumorigenesis.
Quiescent state (of endothelial cells): a reversible and nonproliferative state of endothelial cells.
Somatic mutation: permanent mutation that is present only within somatic tissue and that is not propagated to offspring.
SRY-related HMG-box 18 (SOX18): transcription factor with many developmental roles including in blood vessel development and angiogenesis.
Striatin-interacting phosphatase and kinase (STRIPAK) complex: evolutionarily highly conserved multiprotein scaffolding and signaling complex with many cellular roles in cell cycle control, apoptosis, vesicular trafficking, Golgi assembly, cell polarity, cell migration, and vascular development. This complex has also been connected to cerebral cavernous malformation.
Third hit: a nonmutational third parameter that is needed in addition to two mutations (one for each allele of a gene) to cause a phenotype.

Box 1. Cerebral Cavernous Malformation Patient Phenotypes and Clinical Features

Cerebral cavernous malformations, also called cavernous angioma or cavernoma, are vascular lesions characterized by abnormally enlarged capillary cavities without intervening brain parenchyma. Most of them are located within the central nervous system but they can also affect the retina or the skin. CCMs occur both as a sporadic and as a familial condition [87]. Most sporadic cases showing a single lesion on cerebral magnetic resonance imaging (MRI) do not carry a germline mutation in any of the three *CCM* genes. Familial cases present most often with multiple lesions whose numbers increase with the patients' age.

Main clinical manifestations are epileptic seizures and intracerebral hemorrhages, the most feared clinical manifestation. Most sporadic cases with a solitary lesion remain asymptomatic throughout the entire life. By contrast, familial cases with multiple lesions are symptomatic in around 50–60% of the cases. The average age of onset in symptomatic individuals is around age 30 but symptoms can start during childhood and in elderly people. A number of studies have been conducted to estimate the annual or 5-year risk of intracerebral hemorrhages and to characterize the natural history of CCM [88].

The familial CCM pattern of inheritance is autosomal dominant with an incomplete clinical and neuroradiological penetrance. The severity of CCM is highly variable from one patient to another. The identification of the three *CCM* genes provided a unique opportunity to compare clinical and MRI features of genetically homogeneous groups of *CCM* mutation carriers. CCM3 patients are more prone than CCM1 and CCM2 patients to develop cerebral hemorrhages at a young age [71]. In addition, a highly specific association with multiple meningiomas has been observed in CCM3 patients [89].

Currently available therapies include symptomatic treatment of epileptic seizures with antiepileptic drugs and surgical resection of accessible lesions. However, deeply seated lesions are poorly accessible to neurosurgery. Radiosurgery is sometimes discussed for these deep-seated lesions that are unaccessible to neurosurgery but its indication is not clearly established.

cerebral vasculature, including the hippocampus and the olfactory bulb. This chronic model revealed important aspects of the clonal nature of CCM lesions. Genetic labeling of Ccm3-deficient cells revealed that single lesions appeared to arise from clones of single cells. This did not reveal whether multiple and regionally dispersed lesions have a common clonal origin, but it showed that when lesions grow to larger sizes, wild-type cells and mutant cells become intermingled. This suggests that Ccm3-deficient endothelial cells can recruit wild-type cells into lesions, contributing to their growth. Moreover, a therapeutic strategy might be developed to limit lesion size by preventing the recruitment of wild-type cells (Figure 2).

Studies based on the chronic *Ccm3* mouse model have also revealed that vessel-resident endothelial progenitor cells are present in lesions at early stages of their formation. When *Ccm3* was inactivated specifically in cells expressing *Protein C Receptor*, a characteristic marker of this progenitor cell type [21,22], lesions formed that resembled those of the standard chronic Ccm3 model. This finding is particularly intriguing because the number of vessel-resident endothelial progenitor cells decreases with age [23]. This may explain why the later onset of induction in chronic CCM models causes fewer CCM lesions, and also hints that endothelial cells might not have the same potential to initiate growth of vascular malformations. Intriguingly, there are similarities between tumor-initiating cells that cause metastatic dissemination in cancers and tissue-resident stem cells. In tumors, neighboring wild-type cells are also recruited into the affected tissue, similar to what is observed during CCM formation. What may make tumor biology even more relevant to CCM pathology is the finding that the cavernoma-initiating endothelial cells of *CCM* mutants harbor some benign oncogenic properties [20]. These studies highlight two avenues for therapeutic applications based on either blocking signalopathic events between cavernoma-initiating cells and their wild-type neighbors or specifically targeting vessel-resident endothelial progenitor cells. These findings also illustrate that effective interventions will depend on a deeper understanding of the cell types and molecular events involved in the formation of lesions.

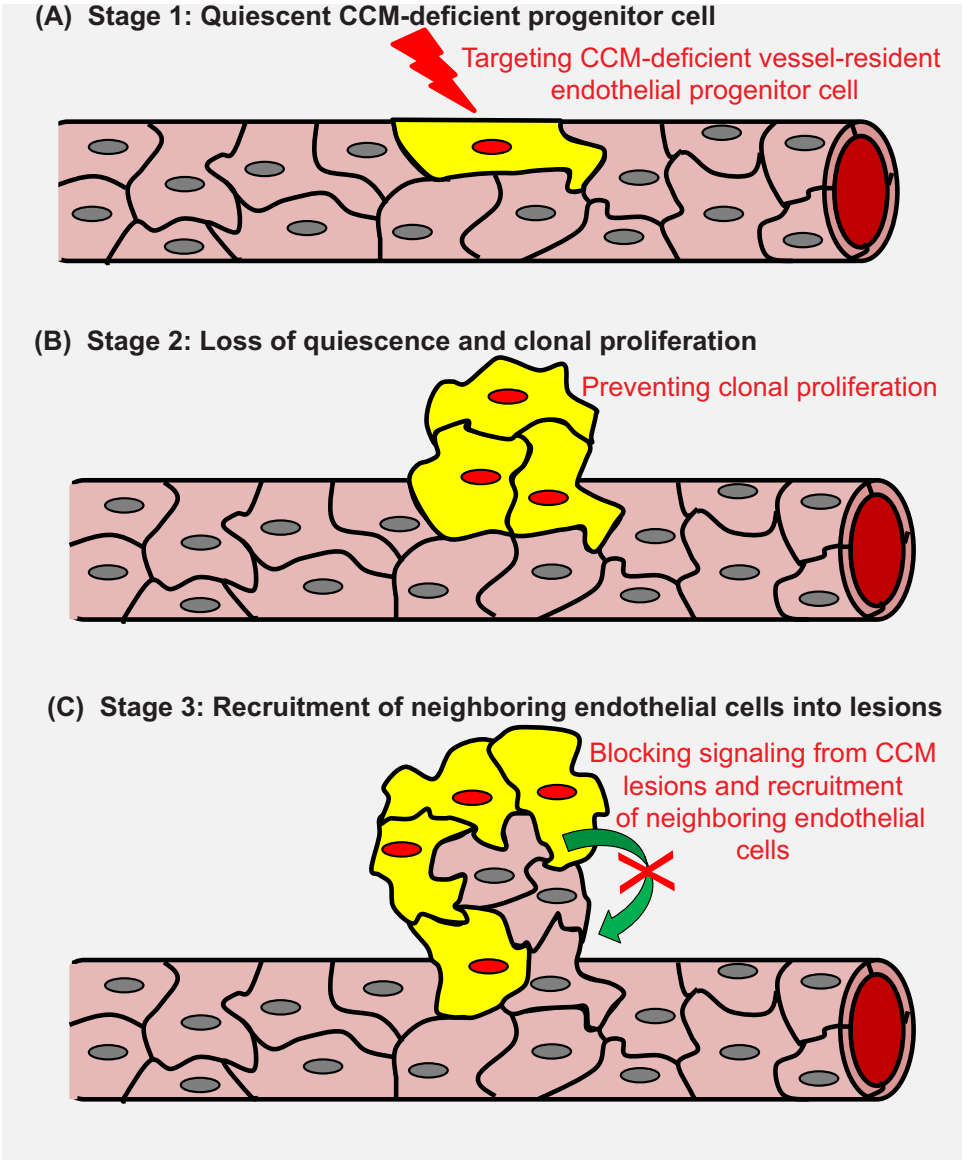

Figure 2. Strategies for Preventing the Growth of Cerebral Cavernous Malformations. Based on evidence from 'chronic' cerebral cavernous malformation (CCM) models in mouse, potential therapies may be directed at different stages of CCM progression. (A) Initially, a 'second hit' affects a vessel-resident endothelial progenitor cell (yellow). Specifically targeting this cavernoma-initiating cell (red bolt) could be one therapeutic approach (Stage 1). (B) Clonal proliferation of the CCM-deficient cavernoma-initiating cell results in the formation of a small venous capillary growth (Stage 2). (C) Growth of the cavernoma is strongly spurred when cavernoma-initiating cells recruit neighboring wild-type cells into the outgrowth. The pharmacological blockade of such signalopathic events (green arrow) between cavernoma-initiating cells and their wild-type neighboring endothelial cells may provide promising therapeutic strategies (Stage 3).

Diverse Pathways Are Affected in Cerebral Cavernous Malformations

A number of molecular pathways have been implicated in CCM through combinations of unbiased pharmacological suppression screens, comparative molecular studies, and functional studies. Most of this work had been carried out in *Caenorhabditis elegans*, *Drosophila*, zebrafish, mouse, and human cells. Comparative transcriptome studies have shown that the pathways

disrupted by a loss of *CCM* gene function are highly conserved across phyla [24]. Work with different model organisms has also revealed a critical role for **mitogen-activated protein kinase (MAPK) pathway** signaling involving Mekk3 and Erk5 [18,25–29]. They activate a signaling output through the **Krüppel-like transcription factors KLF2/4**, which are mediators of biomechanical responses within endothelial cells [17,18,25,27,29–31]. The pharmacological inhibition of MAPK signaling as well as endothelial-specific knockouts of Mekk3 [25] and Klf2/4 suppressed vascular lesion formation in CCM-deficient mice [18,25,27]. This suggests an involvement of a novel Mekk3–Erk5–Klf2/4 pathophysiological signaling axis in CCM (Figure 3). Intriguingly, KLF2 also plays a role in **endothelial-to-mesenchymal transition** (**endMT**) processes that have been implicated in the formation of CCM lesions. During endMT, endothelial cells break down cell–cell junctions, causing a loss of endothelial integrity, and acquire mesenchymal properties. This may cause affected blood vessels to bleed [30,32,33].

The finding that CCM-deficient cell clones recruit wild-type cells into cavernomas has raised the question of whether this process is mediated by secreted **cytokines** or other cell–cell signaling

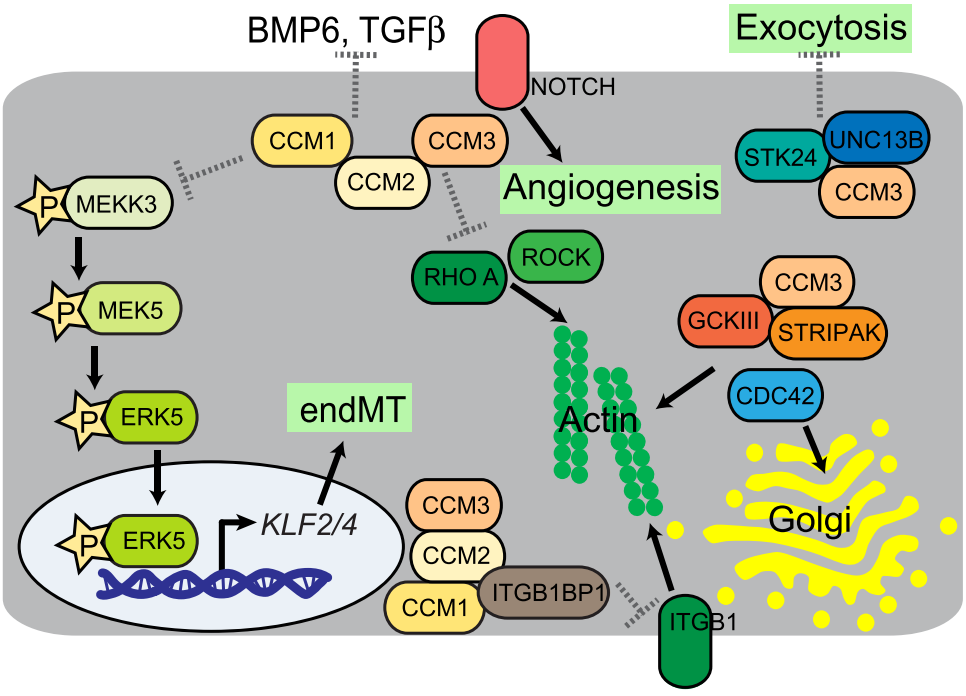

Figure 3. Model of Cerebral Cavernous Malformation (CCM) Pathological Signaling Pathways.

For a Figure360 author presentation of Figure 3, see the figure legend at https://doi.org/10.1016/j.molmed.2020.03.003. CCM proteins affect a number of cellular functions. The CCM1–3 protein complex attenuates mitogen-activated protein kinase (MAPK) pathway signaling via mitogen-activated protein kinase kinase kinase 3 (MEKK3) and extracellular-signal-regulated kinase 5 (ERK5) within endothelial cells. In turn, MAPK signaling activates expression of the transcription factors KLF2/4. The overexpression of Klf2/4 causes lesion formation and an increased endothelial-to-mesenchymal transition (endMT) in endothelial-specific *Ccm* mouse knockout models and cardiovascular defects in zebrafish or mouse *Ccm* mutants. Increased endMT has also been linked with an enhanced secretion of bone morphogenic protein 6 (BMP6) and transforming growth factor β (TGFβ) upon loss of CCM1–3. The same CCM1–3 protein complex also affects actin fiber formation via regulating the activity of RHO/ROCK or integrin signaling. Integrin-β1 (ITGB1) signaling is attenuated by the CCM-associated protein integrin-β1 binding protein 1 (ITGB1BP1). The loss of CCM proteins has been associated with increased angiogenesis signaling. CCM3 protein mainly associates with the STRIPAK (striatin-interacting phosphatase and kinase) complex and is engaged in additional signaling pathways including endocytic trafficking and Golgi assembly. CCM3 together with UNC13B and STK24 inhibits exocytosis.

pathways. Molecular and genetic studies have shown that in CCM the expression of a number of cytokines is characteristically changed. These include enhanced **angiogenesis** signaling due to a suppression of the antiangiogenic factor thrombospondin [34]. Similarly, an increased secretion of angiopoietin-2 detected in a CCM3 model [35] has a destabilizing effect on blood vessels and can promote angiogenesis. Other studies reported increased signaling via the Notch receptor [36–42], bone morphogenetic proteins (BMPs)/transforming growth factor β (TGFβ) [30,43], or wingless-related integration site (WNT) ligands [32,43–45]. Several of these growth factors also affect endMT processes or angiogenesis signaling.

Experiments mainly based on tissue culture systems have shown that *CCM* gene loss alters a number of other basic cellular functions, including rearrangements of actin fibers due to a strong stimulation of Rho/Rock activity [46–49] and increased β1-integrin signaling [17,36,50]. Changes in the composition of the extracellular matrix have been linked to the strong activation of the degrading metalloproteases Adamts 4/5 [18]. CCM-deficient endothelial cells have also been shown to experience higher oxidative stress [51] and defective autophagy [52]. The involvement of such highly diverse pathways in CCM raises the question of whether some are epistatic over others, or are activated secondarily as a consequence of a malformed vasculature.

Studies in *C. elegans,* which lacks a cardiovascular system, have helped shed some light on the relevance of some of these pathways for basic cell biological processes. An unbiased genetic screen in *C. elegans kri-1* (*CCM1*) mutants uncovered the entire extracellular-signal-regulated kinase 5 (ERK5) pathway because loss-of-function mutations in *mekk-3* (*MEKK3*), *mek-5* (*MEK5*), and *mpk-2* (*ERK5*) completely suppressed the *kri-1* mutant phenotype [29]. This finding supports evidence that the activation of the MEKK3–ERK5 pathophysiological signaling axis is a direct consequence of a loss of CCM proteins, from work in other model organisms. Genetic analyses in *C. elegans* have also contributed to a better understanding of the diverse molecular roles of CCM3 in this pathology (Figure 3). Since CCM3 is predominantly associated with the **striatin-interacting phosphatase and kinase complex**, it likely engages additional signaling pathways distinct from those downstream of CCM1/2 [16,53–55]. For example, studies in mammalian endothelial cells, *C. elegans*, and *Drosophila* have revealed a role for CCM3 in endocytic trafficking [55–59] and studies in both *C. elegans* mutants and murine models have revealed a key role for Cdc42 [31,54].

The number of molecular pathways affected by CCM gene loss raises challenges for developing pharmacological therapies. Besides, there are further differences between CCM1/2 and CCM3 downstream signaling mechanisms. These studies have identified characteristic molecular signatures of this pathology, but we still lack a solid understanding of their relevance to disease pathophysiology in patients. However, their conservation across phyla provides excellent opportunities to clarify mechanisms relevant to the disease in genetically tractable model organisms. For example, systems biology approaches in *C. elegans* uncovered a role for mouse protein-25 in the regulation of CCM3 function that was shown to be conserved in human endothelial cells [54]. This underscores the power of using different model organisms to define common CCM signalopathic mechanisms that can be exploited therapeutically to slow lesion formation in patients.

Pharmacological Interventions against Cerebral Cavernous Malformations

To date, most pharmacological approaches to suppress CCM signaling pathways have been based on hypothesis-driven molecular studies. The CCM disease state will likely be susceptible to drugs because its pathology arises through the overactivation of pathways. Based on observations that a loss of CCM proteins leads to the hyperactivation of ROCK, Stockton and

colleagues [60] showed that the ROCK inhibitor fasudil can prevent stress fiber formation and vascular leakage in *Ccm1* and *Ccm2* mutant mice. Similarly, because the loss of Pdcd10 stimulates the transcriptional activity of β-catenin, Bravi *et al.* [44] tested known inhibitors of β-catenin signaling and showed that sulindac sulfonate could reduce the size and number of lesions in mice in which Ccm3 has been depleted in brain endothelial cells. More recently, the ROCK inhibitors fasudil and atorvastatin showed suppressive effects in reducing lesion burden in Ccm3-deficient mice [61], indicating that the CCM1/2 and CCM3 pathways converge on the actin cytoskeleton.

Several unbiased small-molecule suppression screens in recent years have produced other compounds with promising effects in animal models of CCM. For example, a screen of 2100 drugs and bioactive compounds in endothelial cells deficient for CCM2 yielded vitamin D_3 and the free radical scavenger tempol. Both compounds alleviated the lesion burden in a *Ccm2* mouse model [62].

Although the effectiveness of statins in suppressing CCM phenotypes have yielded conflicting results, an unbiased small-molecule screen in Ccm3-deficient mouse astrocytes uncovered the 3-hydroxy-3-methylglutaryl (HMG)-CoA reductase inhibitor fluvastatin [63]. A literature search for compounds that act synergistically with fluvastatin revealed that zoledronic acid, an inhibitor of protein prenylation, has potent effects in both human astrocytes and *Drosophila* glial cells deficient in Ccm3 [63]. Although statins had not demonstrated suppressive effects in previous mouse CCM models [62], this may have been due to the lack of a synergizing compound such as zoledronic acid. This also highlights the importance of using multiple *in vivo* systems to evaluate potential therapeutics and suggests that treatments for CCM might require combinations of drugs.

Recently, *C. elegans*, zebrafish embryos, and human umbilical vein endothelial cells (HUVECs) deficient in CCM proteins were used to screen 5268 compounds from the LOPAC/Selleck, Spectrum, and GlaxoSmithKline protein kinase inhibitor libraries [43]. This led to the identification of 32 compounds that suppressed CCM phenotypes in both *C. elegans* and zebrafish, five of which prevented actin stress fiber formation in HUVECs depleted of CCM1 [43]. These compounds have been predicted to affect diverse biological processes including angiogenesis (ENMD-2076), phospholipid metabolism (DL-erythro-dihydrosphingosine), PI3K (phosphatidylinositol-3-kinase)/Akt (protein kinase B)/mTor (mammalian target of rapamycin) signaling (ridaforolimus), acetylcholine signaling (DL-homatropine hydrobromide), and hormone signaling (13-*cis*-retinoic acid). Indirubin-3-monoxime had suppressive effects in zebrafish and HUVECs and reduced lesion number and size in an 'acute model' of *Ccm2* and *Ccm3* in mice. Molecular studies demonstrated that indirubin-3-monoxime reduced the activating phosphorylation of ERK5 in CCM-deficient HUVECs and suppressed the elevated expression levels of *klf2a* messenger RNA in zebrafish *ccm2* mutants [43]. Analyses of known and predicted targets of indirubin-3-monoxime (and other compounds) have revealed multiple cellular targets [43], highlighting the importance of genetic validations of predicted therapeutic targets. Curiously, a number of drugs identified in both *C. elegans* and zebrafish have antihypertensive and antiangiogenic effects. Since *C. elegans* lacks a circulatory system, the drugs appear to exert effects that go beyond the well-established physiological functions of these molecular pathways in the cardiovascular system of vertebrates. These findings emphasize the importance of defining precise mechanisms of action when repurposing candidate therapeutics for diseases such as CCM. For example, the non-selective β-adrenergic blocker propranolol has been reported to suppress CCMs in patients [64–67]. However, recent work has shown that this drug contains a mixture of R(+) and S(–) enantiomers: the R(+) enantiomer is active in suppressing vascular bleeding by targeting the

Table 1. Compounds and Drugs Tested for the Treatment of CCM

Compound or drug	Targeted molecular pathway	Drug application	Model	Refs
Propranolol	β-Adrenoceptor receptor	Antihypertension	Patients, zebrafish *ccm2* embryo	[43,65–67]
Sorafenib		Multikinase inhibitor, antiangiogenesis	HUVECs *shCCM1*	[39]
NVP-BHG712	Ephrin receptor B4		HUVECs *shCCM3*; transplants of HUVECs *shCCM3* into nude mice	[41]
Rapamycin	mTor	Antineoplastic	Mouse *Ccm1* knockout (k.o.)-derived embryonic fibroblasts; mouse *Ccm1* k.o.-derived endothelial cells; human cerebral microvascular endothelial cells *siCCM1*	[52]
Torin1	Mammalian target of Torin1 (mTor)	Antineoplastic	Mouse *Ccm1* k.o.-derived embryonic fibroblasts; mouse *Ccm1* k.o.-derived endothelial cells; human cerebral microvascular endothelial cells *siCCM1*	[52]
DMH1	TGFβ		Mouse *Ccm1* endothelial k.o. (acute model)	[30]
LY364947	TGFβ		Mouse *Ccm1* endothelial k.o. (acute model)	[30]
SB431542	TGFβ		Mouse *Ccm1* endothelial k.o. (acute model)	[30]
Sulindac sulfone/sulfide	TGFβ, Wnt	Nonsteroidal anti-inflammatory	Mouse *Ccm3* endothelial k.o.(acute model)	[44]
Fasudil	RhoA	Vasodilation	Mouse $Ccm3^{+/-}$;$Trp53^{-/-}$ or $Ccm3^{+/-}$;$Msh2^{-/-}$ k.o. models; mouse $Ccm1^{+/-}$ or $Ccm2^{+/-}$ vascular leakage	[60,61]
Atorvastatin	RhoA	Hypercholesterolemia	Patients; mouse $Ccm3^{+/-}$;$Trp53^{-/-}$ or $Ccm3^{+/-}$; $Msh2^{-/-}$ k.o. models	[4,61]
Simvastatin	RhoA	Hypercholesterolemia	Patients; Mouse $Ccm3^{+/-}$;$Trp53^{-/-}$ or $Ccm3^{+/-}$; $Msh2^{-/-}$ k.o. models; mouse $Ccm2^{+/-}$ vascular leakage	[5,49,61]
Tak242 (Resatorvid)	TLR4 and mitogen-activated protein kinase kinase kinase 3 (MEKK3)-KLF2/4	Sepsis	Mouse *Ccm1* endothelial k.o. (acute model)	[74]
Anti-BR3 antibody	B cells	B-cell depletion	Mouse $Ccm3^{+/-}$;$Trp53^{-/-}$ or mouse $Ccm3^{+/-}$ k.o. models	[76]
Indirubin-3 monoxime	ERK5-KLF2		Zebrafish *ccm 2* embryo, *shCCM2* HUVEC, mouse *Ccm2* and *Ccm3* endothelial k.o. (acute models)	[43]
BA-1049	ROCK2		Mouse $Ccm3^{+/-}$;$Trp53^{-/-}$, $Ccm1^{+/-}$;$Msh2^{-/-}$; or $Ccm3^{+/-}$;$Trp53^{-/-}$ k.o. models	[90]
XMD17-109	ERK5		*siCCM1* HUVEC	[25]
BIX02189	MEK5		*siCCM1* HUVEC	[25]
Ponatinib	MEKK3		Mouse *Ccm1/2* endothelial k.o.	[91]
Vitamin D_3		Vitamin	Human adult dermal microvascular endothelial cells *siCCM2*; mouse *Ccm2* endothelial k.o. (acute model)	[62]
Tempol		Antioxidant; anti-inflammatory	Human adult dermal microvascular endothelial cells *siCCM2*; mouse *Ccm2* endothelial k.o. (acute model)	[62]
Fluvastatin	3-Hydroxy-3-methylglutaryl-CoA reductase	Hypercholesterolemia	Mouse *Ccm3* brain primary astrocytes; *Drosophila ccm3* glial cells; mouse acute and chronic glial-specific *Ccm3* k.o.	[63]
Zoledronic acid	Protein prenylation	Bone diseases, osteoporosis	Mouse *Ccm3* brain primary astrocytes; *Drosophila ccm3* glial cells; mouse acute and chronic glial-specific *Ccm3* k.o.	[63]

Table 1. (continued)

Compound or drug	Targeted molecular pathway	Drug application	Model	Refs
Ridaforolimus	mTor, PI3K, AKT	Immune suppressant	*Caenorhabditis elegans kri-1; ccm-3* double mutants; zebrafish *ccm2* embryo; *shCCM2* HUVEC	[43]
ENMD-2076		Multikinase inhibitor, antiangiogenesis	*C. elegans kri-1; ccm-3* double mutants; zebrafish *ccm2* embryo; *shCCM2* HUVEC	[43]
DL-Erythro-dihydrosphingosine	Protein kinase C, phospholipase A2, D-sphingosine precursor		*C. elegans kri-1; ccm-3* double mutants; zebrafish *ccm2* embryo; *shCCM2* HUVEC	[43]
DL-Homatropine hydrobromide	Muscarinic acetylcholine receptor antagonist		*C. elegans kri-1; ccm-3* double mutants; zebrafish *ccm2* embryo; *shCCM2* HUVEC	[43]
13-*cis*-Retinoic acid		Acne	*C. elegans kri-1; ccm-3* double mutants; zebrafish *ccm2* embryo; *shCCM2* HUVEC	[43]

SRY-related HMG-box 18 (SOX18) transcription factor [68]. SOX18 plays an important role in vascular development and affects the expression of angiopoietin-like 4, a regulator of angiogenic growth and vascular permeability [69]. This raises the question of whether R(+) propranolol exhibits its effects on CCM through the KLF2/4 transcription factors, or whether it indicates a possible link between SOX18 and the pathogenic mechanisms of this disease. In summary, several unbiased pharmacological CCM suppression screens and functional studies have uncovered a number of potential avenues for treating CCM in patients (Table 1).

Third Hits Trigger the Pathogenesis of Cerebral Cavernous Malformations

Despite progress on avenues for treating CCM in patients, we still lack a good understanding of what causes the growth of CCMs in patients and why they may remain asymptomatic for long periods before acute bleeding occurs. There are strong indications that factors beyond genetics are required to activate quiescent CCM-deficient endothelial cells. Identifying triggers that cause such nongenetic, **third hits** may provide novel avenues for drug treatments that directly target acute phases of CCM pathology (Figure 4).

Murine 'acute' endothelial-specific knockouts of *Ccm* reveal a crucial role for such third hits. When a pan-endothelial knockout strategy was used, lesions occurred primarily within cerebellar and retinal vessel beds that undergo strong angiogenesis during early postnatal stages [70]. This suggests that angiogenesis signaling might trigger lesion formation in mouse models of CCM. In adult animals, angiogenesis is strongly activated by **hypoxia**. Interestingly, there is a significant increase in lesion number with age in human CCM patients, particularly in CCM2 patients after 50 years of age, which might correlate with increasingly hypoxic conditions [71]. Angiogenesis signaling has also been identified as an important functional term in comparative transcriptomics, as well as in pharmacological and functional studies of pathways affected by *CCM* gene loss [24,43,72].

In comparison with adult onset in CCM1/2 patients, CCM3 patients frequently develop a more severe form of the pathology and early onset during childhood [73]. Here, too, there are indications of 'third hit' modifiers in the etiology of the disease. Two recent studies identified a connection between the gut microbiome and brain lesions in mouse models of CCM [74,75]. One study showed that injection of Gram-negative bacteria or lipopolysaccharide, which mimics the outer membrane composition of Gram-negative bacteria, triggered a strong activation of bleeding within the brain vasculature of *Ccm1* mutant mice. This finding points to an involvement of the innate immune system in this pathology. Consistent with this, pharmacological suppression as well as endothelial-specific knockout of the innate immunity receptor Toll-like receptor 4 (Tlr4)

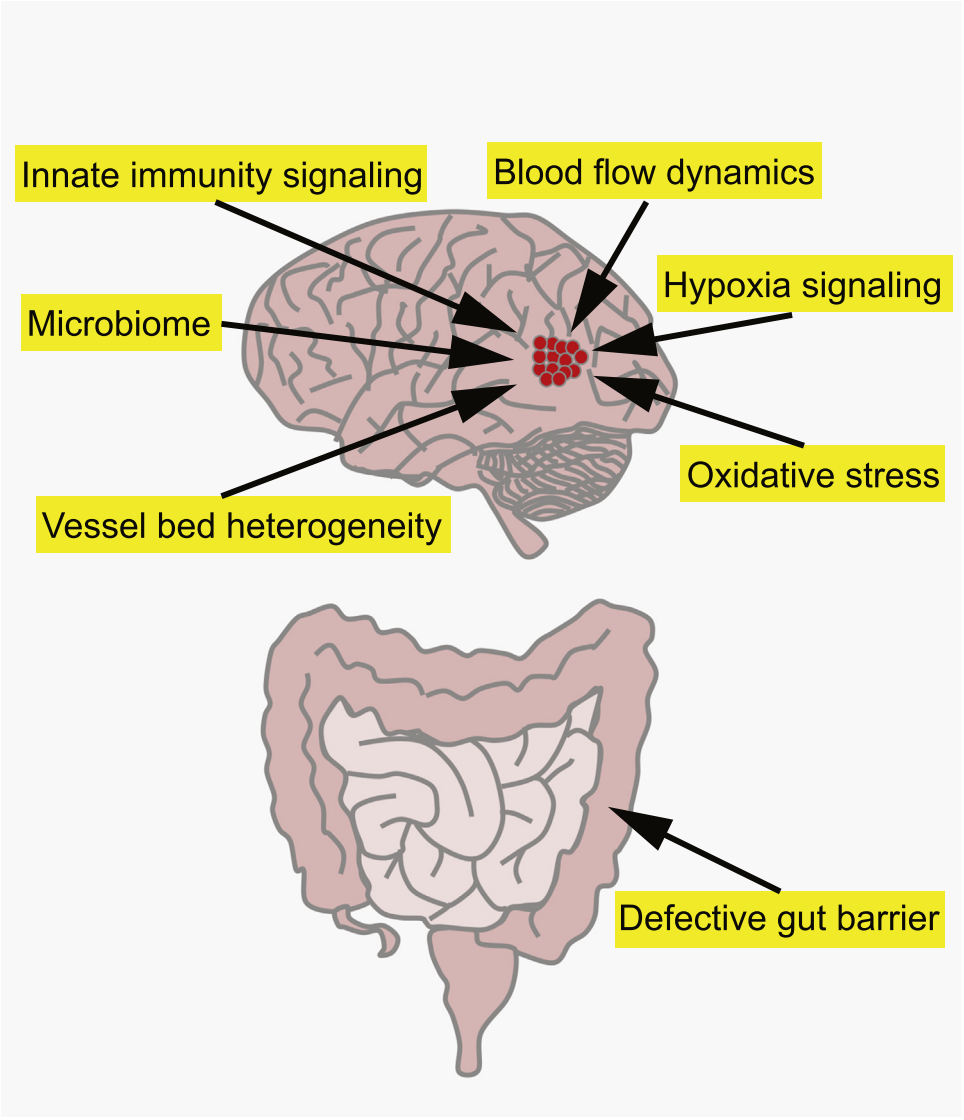

Figure 4. Third Hit Triggers in the Pathogenesis of Cerebral Cavernous Malformations (CCMs). Not only genetics but also a number of nongenetic factors are currently being discussed as potential triggers that may activate quiescent CCM-deficient endothelial cells. The scheme summarizes those factors that may be causative in activating asymptomatic cavernomas. Effective therapeutic approaches should take into consideration that preventing such nongenetic third hits may provide novel avenues for drug treatments that prevent acute phases of the CCM pathology.

Clinician's Corner

Currently available therapies for CCM include symptomatic treatment of epileptic seizures with antiepileptic drugs and surgical resection of accessible lesions. However, deep-seated lesions are poorly accessible to neurosurgery. Radiosurgery is sometimes discussed for these deep-seated lesions that are unaccessible to neurosurgery but its indication is not clearly established.

Signalopathic events involved in CCM lesion development have been identified in various CCM cellular and animal models including nematode, zebrafish, and mouse models.

Novel treatment strategies currently in clinical trials include statins and propranolol.

CCM mouse models with a long survival are now available to test treatments for attenuating the growth and bleedings from pre-existing lesions or for driving their regression.

Data obtained in CCM mouse models have established the role of microbiome in modifying the severity of the CCM disease. Analysis of CCM patients' microbiome will be of major interest to determine if there is any correlation with CCM severity in humans.

suppressed CCM lesion formation in mice. Of note, in *Ccm1* mutants, stimulation of Tlr4 with lipopolysaccharide resulted in a strong activation of the pathological Mekk3–Klf2/4 signaling pathway [74]. Another study revealed that Ccm3, but not Ccm1, is required to maintain the colonic mucosal barrier of the gut [75]. A gut epithelium-specific knockout of *Ccm3* increased the severity of CCM lesion formation in a murine CCM model. Treatment of these animals with dexamethasone, which has anti-inflammatory effects, suppressed brain lesions. Consistent with a role of the immune system in CCM, B-cell depletion also suppressed lesion formation in mouse models [76]. Taken together, these studies point to a gut–brain axis in the CCM pathology that involves a loss of protective antimicrobial gut barrier properties and an increase in innate

immunity signaling via the TLR4 receptor within brain endothelial cells. Whether this finding is relevant to lesion burden in CCM patients is still unknown.

Blood Flow Matters in Cerebral Cavernous Malformations

Several studies suggest that a loss of CCM proteins causes pathological changes in biomechanical signaling, which has severe consequences for cardiovascular development and physiology. Blood flow and the biomechanical forces it triggers play important roles in cardiovascular (patho)physiology [77,78]. That blood flow may be an important factor in the etiology of CCM pathology stems from a number of observations in human patients and diverse CCM animal models. In patients, lesions are mostly restricted to venous capillary beds, which only experience low levels of fluid shear stress.

Two recent studies investigated the role of blood flow for CCM formation. In zebrafish, a complete loss of Ccm proteins causes severe cardiac defects that result in a lack of blood flow. Without it, these mutants exhibit vascular anomalies in major blood vessels including the lateral dorsal aorta. However, when Ccm1 was rescued specifically within the heart, blood flow was restored in *ccm1* mutants. This prevented overgrowth of the lateral dorsal aorta and suggests that blood flow has a vasoprotective role within strongly perfused blood vessels in CCM [79].

Another recent study directly addressed the impact of blood flow on the transcriptional activation of target genes in CCM. When human endothelial cells depleted of CCM1 or CCM2 were subjected to different strengths of fluid shear stress, only low fluid shear stress conditions promoted the transcriptional activation of genes related to CCM signaling [80]. By contrast, the transcriptional responses under high shear stress conditions did not drastically differ in wild-type and CCM-deficient endothelial cells. These experiments suggest a hypothesis that can explain the lack of vascular anomalies within major blood vessels of CCM patients.

Concluding Remarks

CCM is one of the best characterized vascular pathologies. Still, questions remain regarding the heterogeneity of the endothelial cell types affected in CCM (see Outstanding Questions). Clones of CCM-deficient endothelial cells also acquire oncogenic properties, and cells homozygous for these mutations influence the behavior of neighboring wild-type endothelial cells. This suggests that an effective therapeutic approach might be to target cell–cell signaling events between these endothelial cell populations. Shielding wild-type cells from such signalopathic events might prevent lesion formation from reaching an acute stage.

Collectively, these findings may have important implications beyond CCM. Recent studies of a number of other vascular pathologies have provided important new insights into the molecular and cellular mechanisms by which different types of malformations develop. Several other vascular pathologies that cause malformations or bleeding also have a clonal origin of mutant cells. These include venous malformations caused by mutations in the angiogenesis receptor Tie2 [81] and AVMs due to abnormal BMP9/10 signaling via the Alk1 receptor [82]. Still other vascular pathologies share molecular hallmarks of CCM. For instance, moyamoya is characterized by mutations in the Ras–MAPK pathway [83]. Capillary malformations have been linked to mutations in *RASA1*, which encodes a suppressor of Ras [84], and several AVMs have also been linked to the Ras–MAPK pathway [85,86]. Therefore, findings from studies in CCM may also be of relevance for these pathologies as well.

We need a much better understanding of why specific vascular pathologies affect distinct vessel beds, why pathologies arise at a different age of onset, and how characteristically different

Outstanding Questions

Why is the CCM pathology restricted to lowly perfused venous capillary beds and does the molecular heterogeneity of affected endothelial cell types play a role in the pathophysiology of CCM? Why is it restricted essentially to brain venous capillary beds?

What determines the differences in age of onset in different vascular pathologies, and how do the characteristically different morphologies of vascular anomalies evolve?

Is the CCM pathology related to other vascular anomalies that share some of the molecular hallmarks but affect other vessel beds? Will therapeutic approaches to suppress CCM be of relevance for curative approaches toward these pathologies as well? May some of the effective therapeutic strategies used against other vascular malformations also be effective against CCM?

What will be the best strategy for combinatorial drug treatments based on the improved understanding of the multiple pathways deregulated in CCM?

What is the best strategy for intervention to suppress CCM lesion formation, growth, and bleeding? Will patients have to be treated constantly or only during acute phases of the disease? Will it be effective to treat acute phases of lesion formation by shielding wild-type endothelial cells from signalopathic recruitment by CCM-deficient cells? Will these therapies also be useful for sporadic patients with unique but threatening deep-seated lesions?

Are molecular and physiological 'third hit' triggers causing acute phases of CCM lesion growth and bleeding? What is the potential role of the microbiome in the disease presentation and is it also possible that gene variants in different individuals are involved?

vascular morphologies develop. Given the number of signaling pathways affected by CCM gene loss, it is also possible that specific variants of genes belonging to these pathways may influence lesion formation and development. For example, different patients with the same CCM gene mutation can experience a range of numbers and severity of lesions. While external factors such as oxidative stress, hypoxia conditions, or the microbiome are potential modifiers of disease presentation, gene variants may also be involved. Genome-wide sequencing studies of large patient cohorts and evaluations of the functions of specific variants in animal models will be necessary to test this hypothesis.

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Disclaimer Statement

Two patent applications are pending for S.A-S. related to the treatment of CCM.

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