SPECIFIC AIMS

Cerebral cavernous malformations (CCM) are vascular lesions of the central nervous system that increase the risk of stroke, epilepsy, recurrent headaches, and paralysis. Familial cases of CCM develop multiple lesions following an autosomal dominant inheritance pattern. Mutations in three genes have been shown to cause the familial form of the disease – *CCM1* (*KRIT1*), *CCM2 (malcavernin)* and *CCM3 (PDCD10)*, the first two of which were discovered by our lab. [We have recently found somatic mutations in late-stage human lesions, suggesting that CCM follows a two-hit mechanism where each of the two allelic copies of a CCM gene must be inactivated for lesion genesis to occur]. A major drawback of studying CCM pathogenesis in humans is that the lesion samples available are late-stage, multicavernous and hemorrhagic, containing many alterations from normal physiology that may or may not reflect the primary cause of CCM lesion genesis. What little is known about early-stage lesions is based on autopsy reports where information about how these early lesions may progress to the late-stage entity is purely speculative.

Our lab was the first to create knockout mouse models for two CCM genes. Homozygous knockout of either *Ccm1* or *Ccm2* results in embryonic lethality, but mice heterozygous for these genes show no obvious phenotype. [Following the two-hit mutation hypothesis, the heterozygous mice were crossed into "sensitized" backgrounds (*Trp53*-/- or *Msh2*-/-) to promote somatic mutation throughout the genome. The resultant mice exhibit both early- and late-stage CCM lesions that demonstrate many of the hallmarks of the human disease, thereby becoming the first authentic animal model of CCM. These mouse models provide a unique opportunity, not only to study late-stage CCM lesions, but also to investigate the molecular and genetic mechanisms underlying CCM lesion genesis and progression.]

[Specific Aim 1: To study the effects of ROCK inhibition on mouse CCM lesion progression.]

[RhoA is a regulator of the actin cytoskeleton that is involved in endothelial cell lumen formation and vascular permeability. A downstream effector of RhoA, Rho Kinase (ROCK) shows increased activity in CCM lesion endothelial cells, but it is unclear which pathological features of the mature lesion are direct results of ROCK activation. I hypothesize that ROCK activity is central to CCM lesion progression. I propose to inhibit ROCK in our mouse models using the ROCK inhibitor fasudil and examine the effects on CCM lesion genesis, growth and maturation to evaluate the centrality of Rho and ROCK in CCM pathogenesis. To accomplish this, I will examine CCM lesions from mice treated with fasudil for alterations known to occur in late-stage CCM lesions, such as iron deposition and endothelial cell proliferation. From this work, I will determine if ROCK inhibition can prevent CCM lesion genesis, halt lesion growth at an early stage or prevent particular histopathological phenotypes from developing.]

[Specific Aim 2**:** To investigate the two-hit mutation mechanism in early-stage mouse CCM lesions.]

[All studies of human CCM lesions have used only late-stage, surgically resected lesion tissue. In these samples it is difficult to determine which features are causes of CCM and which are resultant effects. Using our new mouse models, I propose to investigate the two-hit mutation hypothesis in mouse CCM lesions in order to determine if the genetic, somatic mutation is a primary cause of or a secondary event in CCM lesion growth. I hypothesize that, by immunohistochemistry and high-throughput sequencing, I will find evidence of a second-hit mutation in late-stage, multicavernous lesions as well as in early-stage, isolated caverns.]

[Differentiating causes of CCM from derived effects is essential for development of effective treatments. The proposed work will further understanding of CCM lesion pathogenesis by deducing which elements are specific to the disease pathway and which are downstream effects. This knowledge will, in turn, directly inform potential therapies for CCM.]

BACKGROUND AND SIGNIFICANCE

Medical Context and Manifestation: According to the World Health Organization, stroke was the cause of 5.7 million deaths in 2004, representing nearly 10% of all deaths worldwide (WHO 2008). The genetic factors affecting stroke, however, are not fully understood. Cerebral cavernous malformations (CCM) is an inherited form of hemorrhagic stroke which affects approximately 0.5% of the population (Otten, *et al* 1989). Patients with CCM have an estimated 50-70% lifetime risk (around 2.5% per year per lesion risk) of hemorrhage, epilepsy, recurrent headaches, muscle weakness, paralysis, and/or loss of hearing or vision, usually in the third decade of life (Robinson, *et al* 1993; Labauge, *et al* 2000).

CCMs are vascular lesions of the central nervous system. The lesions consist of vascular sinusoids lined by endothelial cells and surrounded by a dense extracellular matrix of collagen and other proteins. The walls of the caverns are devoid of elastin and smooth muscle, and they often are filled with blood (Robinson, *et al* 1993). No intervening brain tissue or glial cells are present within CCM lesions (Robinson, *et al* 1993; Clatterbuck, *et al* 2001). A paucity of tight junctions between endothelial cells, leaving gaps of almost one micron in width, is the likely source of hemorrhage in this disorder (Clatterbuck, *et al* 2001).

Genetics and Molecular Biology: Mutations in three human genes, *CCM1* (*KRIT1*), *CCM2* (*malcavernin*) and *CCM3* (*PDCD10*), have been found to cause CCM, the first two of which were discovered by our lab (Sahoo, *et al* 1999; Liquori, *et al* 2003). Little is known about the overall function of these genes. CCM1 interacts with integrin cytoplasmic domain associated protein-1α (ICAP1α), implicating a role in integrin signaling (Zawistowski, *et al* 2002). CCM2 can interact with KRIT1 and may form a ternary complex with both CCM1 and ICAP1α (Zawistowski, *et al* 2005). It is also known that the mouse ortholog of CCM2 functions as a scaffold for Mekk3 activation of p38 Mapk in response to hyperosmolarity (Uhlik, *et al* 2003). Lastly, *CCM3* has been shown to be involved in apoptotic pathways in endothelial cells (Bergametti, *et al* 2005; Chen, *et al* 2009). All three CCM gene products have been found forming a single complex with other proteins involved in cell signaling (Hilder, *et al* 2007).

[Most notable among the signaling proteins that interact with the CCM proteins is RhoA (Whitehead, *et al* 2009). The Rho family of GTPases regulates reorganization of the actin cytoskeleton, which is involved in endothelial cell lumen formation, migration and vascular permeability (Whitehead, *et al* 2009). A downstream effector of RhoA, Rho Kinase (ROCK) is activated in the endothelial cells of human CCM lesions (Stockton, *et al* 2010). Loss of CCM protein expression, both *in vitro* and *in vivo*, causes phenotypes related to the RhoA pathway, including increases in endothelial cell proliferation (Notelet, *et al* 1997; Sure, *et al* 2001; Whitehead, *et al* 2004; Shenkar, *et al* 2005; Zhang, *et al* 2008) and vascular permeability (Glading, *et al* 2007; Stockton, *et al* 2010). However, it is unknown whether alterations in the RhoA/ROCK pathway directly cause any of the known histopathological features of CCM lesions or if they are downstream effects of CCM lesion growth and maturation.]

From an epidemiological standpoint, cases of CCM can be divided into two classes: sporadic and familial. Sporadic cases most often have single lesions while familial cases tend to present with multiple lesions. This pattern is the same as what Knudson observed for retinoblastoma when he proposed the two-hit mutation hypothesis (Knudson 1971). In this hypothesis, two loss-of-function alleles of a single gene are required for disease pathogenesis (see Figure 1). In sporadic cases, two random somatic mutations occur in the same gene within the same cell. In familial cases, individuals inherit one germline mutation and, thus, only one random somatic second-hit mutation in the same gene is necessary for pathogenesis.

The first evidence for somatic second-site mutations came from work on a single human lesion (Gault, *et al* 2005). The investigators used denaturing high pressure liquid chromatography and sequencing for polymerase chain reaction (PCR) amplifications of each coding exon of *CCM1*. They found a 34bp deletion in exon 12 that was biallelic to the germline mutation (a single base change leading to a premature stop codon in exon 10). Our lab found further evidence for a two-hit mutation mechanism for each of the three CCM genes (Akers, *et al* 2009). Direct sequencing of PCR amplification products of each exon showed no evidence of somatic mutations. [In order to identify sequence alterations that might be present at levels below detection in the bulk PCR-amplified product, each exon was sub-cloned and 48 *individual clones* were sequenced. This number of clones gives 80% power of detecting a somatic mutation in at least two clones out of 48 (half of a micro-titer plate). Potential mutations were validated by re-amplifying using a different polymerase. From this comprehensive procedure, somatic second-hit mutations were found in patients with germline mutations in *CCM1*, *CCM2* and *CCM3*. We also used laser capture microdissection to isolate specific cell types from human lesions, and showed that the somatic mutations occurred exclusively in endothelial cells. Further work in another lab found immunohistochemical evidence for loss of CCM protein expression in patient lesion tissue (Pagenstecher, *et al* 2009). While these investigators were unable to find sequenceable somatic mutations for patients with germline mutations in each of the three CCM genes, they did show a loss of CCM protein immunohistochemical staining in the endothelium of lesion samples compared to nearby, apparently normal vessels. Taken together, these studies suggest that CCM follows a two-hit mutation mechanism as shown at the genetic and protein levels.]

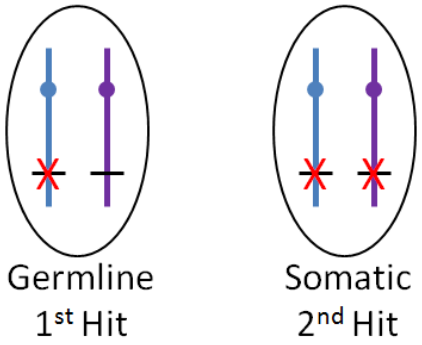

Figure 1: Summary of the Two-Hit Mutation Mechanism. Cells are represented as ovals with one pair of homologous chromosomes (blue and purple). In familial cases of CCM, one germline mutation is inherited as the genetic first-hit. The second-hit occurs as a somatic mutation, inactivating the remaining wildtype copy.

[The specific events and pathways driving lesion genesis, growth and maturation are currently unknown. All of the work done to characterize the genetic and histological alterations within CCMs has been accomplished with late-stage, human lesion tissue. The only means to study the key mechanisms behind CCM lesion pathogenesis, then, is through a robust animal model that captures lesion genesis, growth and maturation.]

PRELIMINARY STUDIES

Mouse Models: To better study this disease, our lab has created mouse models of CCM. The development of the *Ccm1* knockout allele has been described previously (Whitehead, *et al* 2004), as has the gene-trap insertion used to generate the *Ccm2* knockout allele (Plummer, *et al* 2006). [The *Ccm3* knockout allele was also recently generated (He, *et al* 2010). Mice homozygous for the knockout alleles of *Ccm1*, *Ccm2* or *Ccm3* die mid-gestation and heterozygous animal do not show lesions at any appreciable frequency (Whitehead, *et al* 2004; Plummer, *et al* 2006; He, *et al* 2010).] We and others had hypothesized that CCM lesion genesis follows a two-hit mutation mechanism, where a somatic mutation is required in the wild-type copy of the gene mutated in the heterozygous state. Thus, we hypothesized that the lesion penetrance of the heterozygous CCM mice could be increased by crossing the knockout alleles into a genetic background with elevated genetic instability.

Homozygous knockout of *Trp53*, a tumor suppressor gene, increases the overall rate of somatic mutations, so this was used as the genetic sensitizer background to see an increase in lesion burden in our CCM mice. Mice that are heterozygous for either *Ccm1* or *Ccm2* and also have a homozygous knockout of *Trp53* show a highly penetrant increase in cerebral lesions compared to control mice (Plummer, *et al* 2004; Shenkar, *et al* 2008). Lesions can be seen both externally and in coronal cross-sections. MRI and histological analyses have shown these mouse lesions closely resemble lesions surgically removed from humans (Plummer, *et al* 2004; Shenkar, *et al* 2008).

The somatic mutations seen in mice with knockout of *Trp53* are most often gross chromosomal abnormalities and large insertions/deletions, which would not be identified by DNA sequencing. To demonstrate that our CCM mouse model follows a two-hit mutation mechanism as well as bias toward the development of sequenceable mutations (point changes and small insertions/deletions), we have also used homozygous knockout of *Msh2* as a genetic sensitizer. [*Msh2* is a component of the mismatch repair complex and homozygous knockout of this gene causes an increase in somatic point mutations and 1-3 base pair indels (Hegan, *et al* 2006), which are similar to the types of mutations found in human CCM lesions. *Ccm1*+/-,*Msh2*-/- mice showed an increase in lesion penetrance by five months of age compared to controls (McDonald, *et al* 2011). In this model, we have found lesions at all stages of development (see Figure 2). Early-stage lesions appear as grossly dilated, isolated caverns. Late-stage lesions are complex and multicavernous, presenting the same structure as human lesions. Indirect evidence of somatic second-site mutations, determined by loss of CCM protein expression within the lesion endothelium, is apparent in the lesions from these mice.]

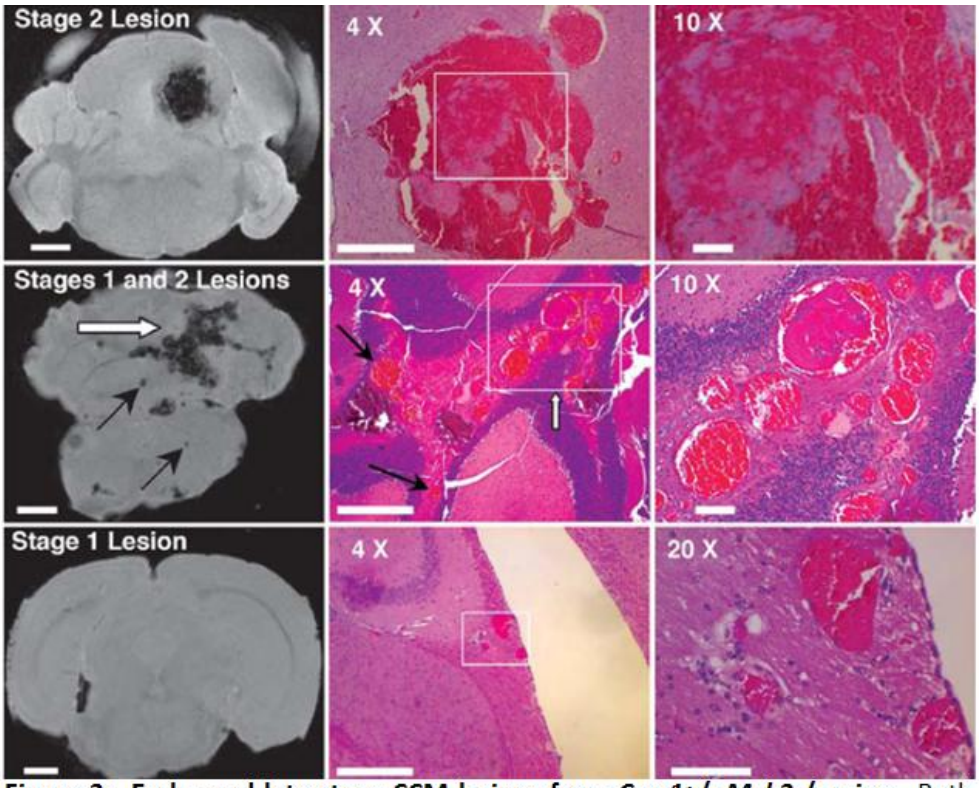

Figure 2: Early- and late-stage CCM lesions from *Ccm1*+/-,*Msh2*-/- mice. Both Stage 1 (early-stage isolated caverns, black arrows) and Stage 2 (clusters of dilated caverns, white arrows) CCM lesions are found in the brains of these mice. Images are shown of coronal sections by MRI (left panels) and H&E staining (center and right panels). Scale bars are 1 mm (left), 0.5 mm (center) and 0.1 mm (right).

[Clear differences between the early- and late-stage lesions have emerged from our CCM mouse models. Late-stage mouse lesions show evidence of immune cell infiltration, endothelial cell proliferation, loss of endothelial cell tight junctions, and recent hemorrhage. Many of these histological features are also observed in human lesions and are related to the known pathology of the disease. By contrast, the early-stage mouse lesions do not show these same phenotypes. Figure 3 illustrates a natural history of CCM lesion genesis, growth

and maturation that has developed from these CCM mouse models. While there are clear stages of lesion genesis and growth, the genetic and molecular mechanisms causing these processes are not well characterized. I propose to examine the somatic second-hit mutation in our CCM mouse models and establish if it is the initiating event in lesion genesis or a downstream consequence of lesion maturation. Furthermore, I will determine if ROCK activation has a causal role in lesion genesis and maturation or if it is a secondary effect of lesion progression. Together, these proposed aims will elucidate the mechanisms driving lesion genesis and maturation, which will directly inform the development of CCM therapies.]

RESEARCH DESIGN AND METHODS

[*Specific Aim 1: To study the effects of ROCK inhibition on mouse CCM lesion progression.]*

[Rationale: The human CCM samples available for study are late-stage, grossly dilated, highly hemorrhagic, multicavernous lesions. Thus, it is impossible to determine which of the characteristics observed in these lesions are directly related to lesion genesis and which characteristics are downstream effects related to lesion growth and maturation. Only through studying early-stage CCM lesions will we be able to elucidate the initial causes of lesion genesis and growth. Our CCM mouse models develop late-stage lesions similar to those obtained from patients as well as early-stage, isolated caverns (Shenkar, *et al* 2008; McDonald, *et al* 2011). These mice are a novel tool for studying CCM lesion pathogenesis *in vivo* because we can capture lesion genesis and progression at different stages of growth. We have extensively characterized the lesions from these mice, and we were the first to show that many of the well established features of CCM lesions, including hemosiderin deposits, endothelial cell proliferation, loss of tight junctions, and immune cell infiltration, are observed in the mature, late-stage lesions but not at the earlier stages (McDonald, *et al* 2011). While these histopathological phenotypes directly relate to clinical outcomes of CCM (stroke, neurological deficits, etc.), they appear to be downstream events in lesion development; however, these histopathological features can be used as indicators of disease progression to assess the effectiveness of possible therapeutics that target specific pathways.]

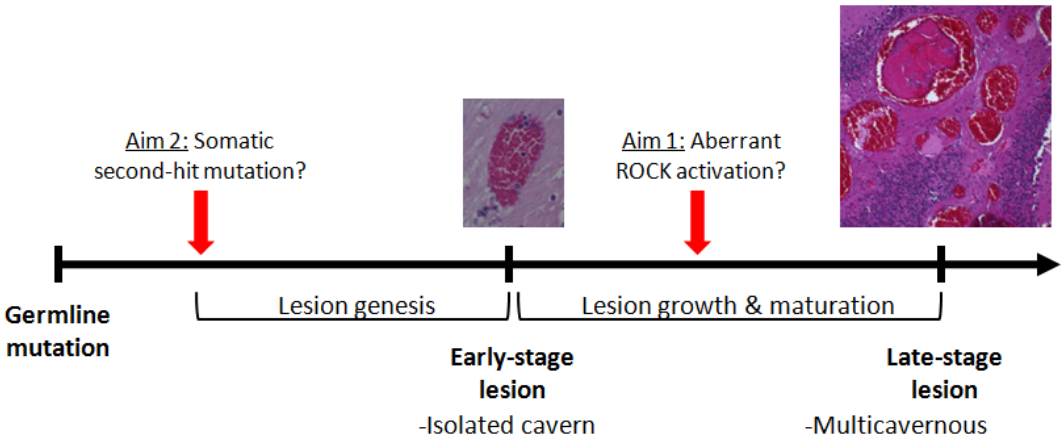

Figure 3: Schematic representation of lesion genesis, growth and maturation in inherited cases of CCM. The line represents the relative progression of a CCM lesion starting with a germline mutation and developing into a late-stage lesion. Characteristics of the early- and late-stage lesions are shown. The images of lesions are taken from our CCM mouse models.

[RhoA is a small GTPase which regulates endothelial cell migration, lumen formation and vascular permeability (Whitehead, *et al* 2009). We recently observed increased activity of the RhoA effector Rho Kinase (ROCK) in endothelial cells of mouse (McDonald, *et al* 2011) and human CCM lesions (Stockton, *et al* 2010). This *in vivo* work supports the growing *in vitro*

evidence connecting the loss of CCM proteins to activation of the RhoA pathway (Whitehead, *et al* 2009; Borikova, *et al* 2010; Stockton, *et al* 2010). However, it is still unknown if these alterations in RhoA signaling are directly involved in CCM pathogenesis or if they are downstream effects. Since our mouse model is the only means of studying CCM lesions at different stages of development, we have a unique opportunity to answer this question. I propose to evaluate the centrality of RhoA/ROCK signaling to CCM pathogenesis by examining the effects of ROCK inhibition on the known histopathological features of mouse CCM lesions.]

[Hypothesis: I hypothesize that ROCK activation is an upstream event in CCM pathogenesis that causes the downstream, pathological characteristics that occur during lesion development (see Figure 3). Inhibition of ROCK will, therefore, prevent the development of the histopathological phenotypes associated with the mature CCM lesion. To test this hypothesis, I will treat our mouse models of CCM with a specific ROCK inhibitor. After these mice are aged appropriately, I will examine their brains for alterations known to occur in CCM lesion pathogenesis. Specifically, I will examine ROCK activity, hemosiderin deposition (an indicator of hemorrhage), endothelial cell proliferation, and immune cell infiltration using immunohistochemistry as we did previously (McDonald, *et al* 2011). Furthermore, I will examine the lesions for ultrastructural changes by electron microscopy.]

[Mice: I will primarily use the *Ccm1*+/-,*Msh2*-/- and *Ccm2*+/-,*Trp53*-/- mouse models that develop CCM lesions with high penetrance (Shenkar, *et al* 2008; McDonald, *et al* 2011). Additionally, we have recently obtained mice bearing a knockout allele of *Ccm3* (He, *et al* 2010), and I am currently generating *Ccm3*+/-,*Msh2*-/- mice using a similar breeding scheme as our other models (Shenkar, *et al* 2008; McDonald, *et al* 2011). All animals will be sacrificed between four and five months of age and their brains will be collected. With these mice, I will examine the effects of ROCK inhibition on CCM lesions in models using all three known CCM genes.]

[Fasudil Treatment: Fasudil is a specific ROCK inhibitor that is currently in use in Japan to treat cerebral vasospasm. *Ccm1*+/- and *Ccm2*+/- mice show increased vascular permeability compared to wildtype mice, but treatment with fasudil eliminates this difference (Stockton, *et al* 2010). I propose to orally treat our three mouse models (*Ccm1*+/-,*Msh2*-/- mice, *Ccm2*+/-,*Trp53*-/- mice and *Ccm3*+/-,*Msh2*-/- mice) with fasudil at a dosage of 100 mg/kg/day in their drinking water. Previous studies have shown oral fasudil treatment in this manner to be effective (Ying, *et al* 2006; Slotta, *et al* 2008; Huentelman, *et al* 2009; Meyer-Schwesinger, *et al* 2009), and this dosage was chosen because it is one of the highest published dosages in the literature (Ying, *et al* 2006). These mice, along with controls receiving drinking water only, will be treated from weaning until sacrifice at five months of age. To ensure the effectiveness of the ROCK inhibition therapy, staining of phosphorylated myosin light chain (pMLC), an indicator of ROCK activity, in mouse CCM lesions and normal capillaries will be compared for each genotype between the two treatment groups.]

[Sample Preparation and Immunohistochemical Staining: *Ex vivo* MRI will be performed by our collaborators under the direction of Issam Awad, M.D., at the University of Chicago. Imaging will be performed on a 14.1T Bruker Advance imaging spectrometer (Bruker, Milton, Canada) using a T2*-weighted gradient recall echo MRI program. We have used this method previously to identify lesions in brains from *Ccm1*+/-,*Msh2*-/- (McDonald, *et al* 2011) and *Ccm2*+/-*Trp53*-/- mice (Shenkar, *et al* 2008). Using this MRI technique on *ex vivo* mouse

brains, we can identify lesions at sizes as small as 0.2mm in diameter. These magnetic resonance images will provide stereotactic localization of both large and small lesions that can be used for immunohistochemical staining. If finer detail is needed to identify the earliest lesions, we will use a susceptibility-weighted imaging technique which will provide finer detail to the magnetic resonance images (de Souza, *et al* 2008). After MRI, the mouse brains will be embedded in paraffin and cut into coronal slices.]

[Slides of control vasculature and CCM lesions will be stained using our standard immunohistochemical protocols (McDonald, *et al* 2011). Perl's Prussian staining method will be used to visualize iron deposits, indicative of cerebral vessel hemorrhage. Primary antibodies against CD11b (anti-macrophage), B220 (anti-B-cell), CD138 (anti-plasma cell), Ki-67 (cell proliferation marker), and pMLC (ROCK activation marker) will be used along with the appropriate biotinylated secondary antibodies and the Vectastain ABC kit (Vector Laboratories, Burlingame, CA, USA) to visualize alterations in lesion development and pathogenesis. Slides of lesions from control and treated mice will be compared for differences in ROCK activity, hemosiderin deposition, cell proliferation, and immune cell infiltration using statistical methods described in our recent publication on these CCM mouse models (McDonald, *et al* 2011).]

[Electron Microscopy: Previous studies of human CCM lesions have found defective tight junctions between endothelial cells in the lesion (Wong, *et al* 2000; Clatterbuck, *et al* 2001; Tu, *et al* 2005). In lesions from our CCM mouse model, the endothelial cell tight junction proteins appear normal by electron microscopy, but large gaps between the lesion endothelial cells are evident (McDonald, *et al* 2011). To investigate how ROCK inhibition affects the loss of the endothelial tight junctions during lesion progression we will employ electron microscopy using standard procedures. Slides will be prepared from brains from *Ccm1*+/-,*Msh2*-/- mice, *Ccm2*+/-,*Trp53*-/- mice and *Ccm3*+/-,*Msh2*-/- mice using our previously published procedures (McDonald, *et al* 2011).]

[Defining Stages of Lesion Development: CCM lesions will be classified according to the system we recently outlined (McDonald, *et al* 2011). Early-stage (Stage 1, isolated caverns) lesions will be defined as dilated capillaries having a diameter of at least 25 red blood cells and not connected to any other lesion. Late-stage (Stage 2) lesions will be defined as multicavernous structures composed of the union of two or more caverns.]

[Power Estimates: We have demonstrated that ten CCM mice were sufficient to show measurable differences in histopathological characteristics between early-stage and late-stage lesions (McDonald, *et al* 2011). Based on this previous work, ten *Ccm1*+/-,*Msh2*-/- mice, ten *Ccm2*+/-,*Trp53*-/- mice and ten *Ccm3*+/-,*Msh2*-/- mice in both treatment (receiving the ROCK inhibitor) and control (receiving only vehicle) groups will be sufficient for these experiments. Using approximately ten to fifteen cages, I will be able to generate these mice and treat them with ROCK inhibitor or vehicle control during Year 1.]

[Expected Outcomes: I hypothesize that all of the downstream histopathological phenotypes observed in the late-stage CCM lesions of our mouse models will be abrogated by ROCK inhibition. The mice receiving the ROCK inhibitor may still develop CCM lesions, but if those lesions show fewer indications of lesion growth and maturation compared to controls (e.g. reduced proliferation, maintenance of tight junctions, reduced immune cell infiltration, etc.), then the RhoA pathway is central to CCM lesion development. This result would indicate that ROCK activation is necessary for lesions to transition from early-stage, isolated caverns to late-stage, multicavernous structures (See Figure 3). Moreover, inhibition of this pathway

would prove to be an effective therapeutic strategy as it would prevent many of the lesion characteristics that cause clinical phenotypes (e.g. stroke and seizures).]

[Alternatively, ROCK inhibition could prevent lesion genesis altogether. If ROCK inhibition causes decreased penetrance of lesions or reduces lesion burden, ROCK inhibitors would be an invaluable therapy for CCM patients. Conversely, if ROCK inhibition has no effect on the lesion genesis, growth, or the development of the downstream pathogenic characteristics, then increased Rho/ROCK signaling is just a consequence of CCM lesion pathogenesis rather than a cause. Many different groups are investigating the roles of RhoA and ROCK in CCM pathogenesis (Whitehead, *et al* 2009; Borikova, *et al* 2010; Stockton, *et al* 2010). If these experiments provide a negative result, then it would be important to show that the Rho/ROCK pathway is not directly involved with CCM lesion growth and maturation so that other signaling pathways could be investigated.]

[Potential Problems and Alternative Approaches: In order to establish the proper fasudil treatment regimen, I am currently running a pilot experiment to find a dose of fasudil that will effectively inhibit ROCK activity in the cerebral vasculature. Thus far, the mice have tolerated fasudil at a dose of 100 mg/kg/day with no apparent side effects. However, during the course of this study, if problems arise where the fasudil treatment protocol is ineffective at inhibiting ROCK activity, there are multiple means to fine-tune ROCK inhibition within this system. I can use other dosages of fasudil or different treatment durations. For instance, the duration can be shortened to a single month, or I can treat pregnant mothers with fasudil such that the mice are exposed to the drug *in utero*. Lastly, there are many other routes of treatment that are available, including intraperitoneal injection, oral gavage, or implanting subcutaneous osmotic pumps (Alzet, Cupertino, CA). While the methods I have proposed are based on previously reported dosages and effective routes of treatment, the means of inhibiting ROCK *in vivo* in our CCM mice are quite flexible.]

[Statins have been used as a RhoA inhibitor *in vivo* (Kranenburg, *et al* 1997, Collisson, *et al* 2002; Whitehead, *et al* 2009) because they disrupt RhoA isoprenylation (Park, *et al* 2002; Zeng, *et al* 2005), a process necessary for targeting RhoA to the cell membrane for proper function and signaling. If fasudil proves to be ineffective at inhibiting ROCK *in vivo*, then statins could be used instead. Statins target the cholesterol biosynthesis pathway, which indirectly inhibits ROCK. Since statins inhibit a metabolic pathway, there is an increased possibility that statin treatment will alter circulating cholesterol levels and liver function. Statins are a viable alternative ROCK inhibitor, but I favor fasudil for these experiments because it inhibits ROCK directly (Uehata, *et al* 1997).]

[Specific Aim 2: To investigate the two-hit mechanism in early-stage mouse CCM lesions.]
[Rationale: Four recent papers, including one from our lab, have provided evidence for a two-hit mechanism in human CCM lesion tissue (Gault, *et al* 2005; Akers, *et al* 2009; Gault, *et al* 2009; Pagenstecher, *et al* 2009). All of these studies utilized human CCM samples from surgically resected lesion tissue, meaning these large, multicavernous lesions were already in the late-stage of development at the time they were removed. CCM lesions are removed only after they become highly hemorrhagic and cause clinical symptoms. Since early-stage lesions are effectively clinically silent, they are not surgically resected and are unavailable for study.]

[We have recently shown immunohistochemical evidence of a somatic loss of CCM1 protein in the endothelial cells surrounding CCM lesions from *Ccm1*+/-,*Msh2*-/- mice (McDonald, *et al* 2011; see Figure 4). However, this work only examined a limited number of lesions from one of our mouse models. More rigorous study of both early- and late-stage lesions from these mice, as well as *Ccm2*+/-,*Trp53*-/- mice and *Ccm3*+/-,*Msh2*-/- mice, is still

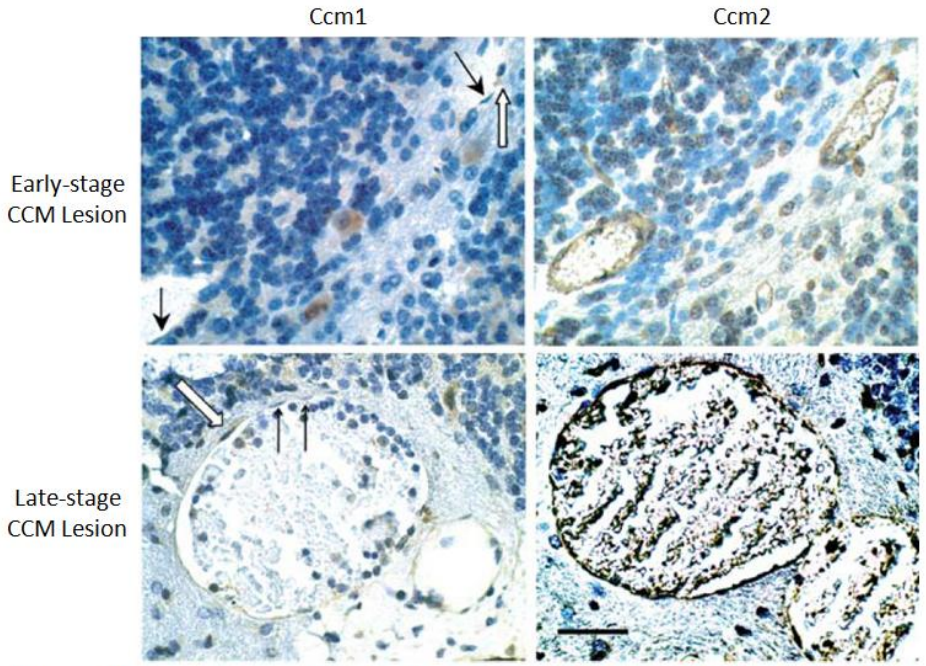

Figure 4: Reduced Ccm1 expression in mouse CCM lesion endothelium. Endothelial cells lining early-stage lesions show normal staining for Ccm2 (right), but either reduced (white arrow) or no staining (black arrow) for Ccm1 (left). Scale bar is 50 µm.

needed to determine if the two-hit mutation mechanism is common to all three known CCM genes. It is unclear if the somatic mutations directly cause CCM lesion growth or if they are one of many characteristics that occur during lesion growth (see Figure 3). Either result has important implications for developing CCM therapies. If somatic second-hit mutations cause CCM lesion genesis, then CCM patients carrying a germline CCM mutation should avoid environmental risk factors that can cause somatic mutations. For instance, these patients would have to limit radiation exposure, such as that used in cancer treatment and X-ray radiography. I propose to investigate the two-hit hypothesis in mouse CCM lesions in order to determine if somatic second-hit mutations occur in the early lesion or if they are only in late-stage lesions as a result of CCM lesion growth.]

[Hypothesis: In accordance with a two-hit mutation mechanism, I hypothesize that the somatic second-hit mutation causes lesion genesis (see Figure 3). Once the biallelic somatic mutation occurs, the affected endothelial cell will effectively have no functional protein from the altered CCM gene, initiating the growth of the CCM lesion. I will investigate this hypothesis using an indirect immunohistochemical staining method for loss of CCM protein staining in a large sample of mouse CCM lesions. Additionally, I will use high-throughput sequencing of DNA isolated from mouse CCM lesions to directly identify somatic second-hit mutations. I will use lesions at different stages of development to determine if the somatic second-hit mutations occur as an early event in lesion growth or as a later, downstream event.]

[Mice & Sample Preparation: As in Specific Aim 1, I will use lesion samples from *Ccm1*+/-,*Msh2*-/- mice, *Ccm2*+/-,*Trp53*-/- mice and *Ccm3*+/-,*Msh2*-/- mice. The mice will be sacrificed between four and five months of age and their brains will be collected. For immunohistochemistry samples, the brains will be fixed in 10% formalin, then embedded in paraffin and cut into coronal slices. For high-throughput sequencing, DNA will be isolated from brains immediately after sacrifice or after fixing in 70% ethanol.]

[Immunohistochemical Staining: If somatic second-hit mutations cause lesion genesis, they should inactivate the single wildtype copy of the particular CCM gene in our mouse models. After the somatic second-hit mutation occurs, the mutant endothelial cell will lose CCM protein expression, which should be evident by immunohistochemical staining even as the mutant cells proliferate and remodel. In order to indirectly determine the stage at which the somatic

second-hit mutation occurs, I will examine early- and late-stage CCM lesions for loss of CCM protein expression by immunohistochemistry. While our lab has experience with immunohistochemical staining of mouse CCM lesions (Plummer, *et al* 2004), some of the best work has been carried out by Rebecca Stockton, Ph.D., at the University of California, San Diego (Stockton, *et al* 2010; McDonald, *et al* 2011). During the summer of 2011, I will travel to San Diego to work with Rebecca to learn the very sensitive staining required to visualize the mosaic pattern of CCM protein expression expected in the CCM lesions (see Expected Outcomes).]

[Our lab possesses antibodies raised against specific epitopes of mouse Ccm1 (Bethyl Laboratories, Inc., Montgomery, TX) and Ccm2 (Aves Labs, Inc., Tigard, OR). Anti-mouse Ccm3 antibodies are also commercially available (Proteintech, Chicago, IL). Using these primary antibodies, as well as biotinylated secondary antibodies and the Vectastain ABC kit (Vector Laboratories, Burlingame, CA, USA), I will stain the brain sections from our CCM mouse models.] Eosin will be used as a counterstain according to standard protocols. As shown in the Preliminary Data section, this procedure works well for staining the endothelial cell layer of blood vessels in the brain. After staining, the lesions from these sections will be observed for staining intensity. Early-stage lesions will be compared to normal vessels from the same slide (an internal control) as well as lesions at later stages of development, including large multicavernous CCMs, identified by differences in size and gross architecture (see Defining Stages of Lesion Development in Specific Aim 1). Cerebral vessels from control animals will also be used to confirm the staining protocol works. Staining for CD31 (PECAM-1) will be used as an endothelial cell marker control.

[High-Throughput Sequencing: While immunohistochemistry can demonstrate a loss of CCM protein expression, it cannot provide direct genetic evidence of a somatic second-hit mutation. Previous work in our lab to identify somatic mutations from human CCM lesions used a cloning and sequencing procedure where each exon of a particular CCM gene was cloned and sequenced to 48X coverage (Akers, *et al* 2009). In a pilot study, I have used this cloning and sequencing strategy on a limited number of mouse CCM lesion samples, but I did not have the depth of sequence coverage necessary to differentiate *bona fide* mutations from PCR errors.]

[Next-generation sequencing technology can achieve upwards of 1000X coverage per exon in a more time- and cost-efficient manner. I will use high-throughput sequencing available at the Duke Institute for Genome Science and Policy DNA Sequencing Facility to look for evidence of somatic second-hit mutations in early- and late-stage mouse CCM lesion samples. I have recently worked with Lisa Bukovnik, manager of the DNA Sequencing Facility, and Jean Qin, a bio-informatician, to establish a protocol for high-throughput sequencing of our mouse CCM samples. In a pilot experiment using four mouse brain samples, I amplified each of the coding exons of *Ccm1* from each sample using primers tagged for high-throughput sequencing. The DNA Sequencing Facility then analyzed the samples using the Roche/454 GS-FLX Fusion high-throughput sequencer. The yield of the runs was over 1400X coverage of each amplicon (approximately 30-fold more coverage than the cloning and sequencing strategy used in Akers, *et al* 2009) with a median length of 246 base pairs. Using this same procedure, I propose to search for evidence of somatic second-hit mutations within mouse CCM lesion samples.]

[I will analyze both early-stage isolated CCM caverns as well as late-stage, multicavernous lesions for somatic mutations using this method. Possible mutations will be limited to variants that severely affect the mature CCM protein (altering the splice sites, creating premature stop codons, etc.) since those are the types of somatic mutations observed in human CCM (Gault, *et al* 2005; Akers, *et al* 2009; Gault, *et al* 2009). It should be noted that

we previously examined late-stage human CCM lesions for somatic epigenetic changes, but we found no evidence that epigenetic modifications were a common type of somatic second-hit. Variants that are predicted to severely alter the mature CCM protein will be validated by our published cloning and sequencing method (Akers, et al 2009) or by SNaPshot assay (Hoogendoorn, *et al* 2000; Bujalkova, *et al* 2008; Akers, *et al* 2009). Briefly, the SNaPshot assay (Applied Biosystems, Carlsbad, CA) quantifies the frequency of variants at a particular position in an amplicon. We previously used the SNaPshot assay to validate a sequence variant found by cloning and sequencing (Akers, *et al* 2009). Since SNaPshot quantitatively measures the frequency of a variant in millions of amplicons (though it can only measure one base at a time), it is the most sensitive means of confirming that the variants found by high-throughput sequencing are *bona fide* somatic second-hit mutations.]

[Power Estimates: In previous work, ten CCM mice provided a sufficient number of lesion samples for histopathological and genetic analysis (McDonald, *et al* 2011; unpublished data). Based on this work, at least ten *Ccm1*+/-,*Msh2*-/- mice, ten *Ccm2*+/-,*Trp53*-/- mice and ten *Ccm3*+/-,*Msh2*-/- mice will provide sufficient lesion samples for these experiments. Using approximately ten to fifteen cages, I will be able to generate these mice during Year 1.]

[Expected Outcomes: I expect to observe cerebral endothelial cells in the mouse lesions lacking staining for one of the CCM proteins (i.e. loss of staining for CCM1 in lesions from *Ccm1*+/-,*Msh2*-/- mice). Similar to the large, multicavernous lesions for patients (Pagenstecher, *et al* 2009; Stockton, *et al* 2010), I expect both early- and late-stage mouse CCM lesions to show a widespread lack of immunohistochemical staining in the endothelium. This result would indicate that the somatic second-hit mutation precedes lesion genesis (see Figure 3). A portion of the endothelial cells will likely still express either Ccm1, Ccm2 or Ccm3, so there may be a mosaic staining pattern in these lesions, reflecting the fact that only a subset of cells harbor the somatic mutation and lose CCM protein expression. Though the same somatic mutation is found in multiple endothelial cells, possibly from clonal expansion of the original cell bearing the somatic mutation, mutated cells may not cluster together due to remodeling of existing endothelial cells. However, I would still expect all of the cells derived from this original CCM mutant endothelial cell to lose expression of functional CCM protein and have a mosaic staining pattern for the CCM proteins.]

[In the high-throughput sequencing experiment, I expect to find evidence of somatic second-hit mutations both in early-stage isolated CCM caverns as well as in late-stage lesions. These data would demonstrate that the somatic mutation occurs before lesion genesis, which would be the first genetic evidence of a two-hit mutation mechanism in mouse CCM lesions. Furthermore, the presence of somatic second-hit mutations within the early-stage lesions would suggest that somatic inactivation of the wildtype allele is an early, **causative** event in CCM pathogenesis and not a secondary effect of CCM growth.]

[One key corollary to the two-hit mutation hypothesis is that two independent lesions should bear two separate, unique somatic mutations. This point has not been investigated in human CCM lesions because it is extremely rare to obtain two independent human CCM lesion samples from the same patient. I will be the first to investigate this implication of the two-hit hypothesis because many of our CCM mice show multiple, independent lesions and I can easily analyze each lesion for somatic mutations. If, in the proposed work, a particular mouse shows two stereotactically-independent lesions, I expect each of those lesions to harbor a different somatic mutation. This result would provide important evidence for a two-hit mechanism **causing** CCM.]

Potential Problems and Alternative Approaches: [In the event that immunohistochemical staining of Ccm1, Ccm2 or Ccm3 proteins in mouse CCM lesions produces a weak signal compared to the background, immunofluorescence could be substituted, using co-staining with antibodies against PECAM-1, an endothelial cell marker.] We have successfully used immunofluorescence on mouse CCM lesion samples (Plummer, *et al* 2004), so I could use this procedure to produce a stronger signal. Another potential pitfall of using immunohistochemistry is that inconsistent staining of the lesions could be misinterpreted as a loss of CCM protein. Examining normal vessels on the same slide as the lesion tissue will ensure that the changes in CCM protein expression within the endothelium are due to *bona fide* loss of CCM protein expression and not a technical problem in the immunostaining protocol. Lesions can often be cut into multiple slides as well, so that consistency of staining (or lack thereof) between slides can be examined.

Timetable: Throughout the first year of this project, CCM mice will be bred, treated with ROCK inhibitor or control (Specific Aim 1 only), and aged to allow lesions to grow and progress. The immunohistochemical staining for downstream histological characteristics of lesions as well as for the CCM proteins will require a year to complete in full, starting midway through Year 1 and finishing midway through Year 2. Lastly, samples will be prepared for high-throughput sequencing, the resultant data will be analyzed and variants will be validated during the second year.

[Table 1: Timeline.]

Aim	Objective	Year	0.5	1	1.5	2
1	Fasudil treatment		X	X		
	Immunohistochemistry			X	X	
2	Obtain mouse CCM samples (slides & DNA)		X	X		
	Immunohistochemistry for CCM proteins			X	X	
	High-throughput sequencing & validation				X	X

[Future Directions: From this project, we will gain an understanding of the molecular and genetic mechanisms underlying lesion genesis and maturation. By investigating the two-hit mutation mechanism in mouse CCM lesions, we will know if the somatic second-hit mutation initiates lesion genesis. By inhibiting ROCK in our CCM mouse models, we will determine if the activation of the RhoA/ROCK pathway causes CCM lesions to mature or if it is a downstream effect of lesion progression. Furthermore, treating mice with ROCK inhibitors will be the first *in vivo* experiment to treat CCM. The results of this work will direct the development of novel therapeutic strategies for CCM.]

ETHICAL ASPECTS OF THE PROPOSED RESEARCH

This proposed project involves the use of vertebrate animals for research purposes. These methods have been designed to ensure strict adherence to the Guide for the Care and Use of Laboratory Animals as well as to prevent any stress or discomfort in our animals. Additionally, all animal procedures will be approved by the Duke Institutional Animal Care and Use Committee.