## RESEARCH STRATEGY

### 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 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 1993; Labauge 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 cavern walls are devoid of elastin and smooth muscle. No intervening brain tissue or glial cells are present within CCM lesions, and they are often filled with blood (Robinson 1993; Clatterbuck 2001).

Genetics and Molecular Biology: Germline 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 1999; Liquori 2003). Until recently, little was known about the overall function of these genes. All three CCM gene products form a single complex with other proteins involved in cell signaling (Hilder 2007). Most notable among the signaling proteins that interact with the CCM proteins is RhoA (Whitehead 2009). The Rho family of GTPases regulates reorganization of the actin cytoskeleton and vascular permeability (Whitehead 2009). A downstream effector of RhoA, Rho Kinase (ROCK), is activated in the endothelial cells of human CCM lesions (Stockton 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 1997; Sure 2001; Whitehead 2004; Shenkar 2005; Zhang 2008), lumen formation (Borikova 2010) and vascular permeability (Glading 2007; Stockton 2010). However, it is unknown whether alterations in the RhoA/ROCK pathway directly cause CCM lesion genesis or if they are downstream effects of lesion maturation and growth.

Epidemiologically, cases of CCM can be divided into two classes: sporadic cases which have single lesions and familial cases which tend to present with multiple lesions. This same pattern is 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. 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 was found in a single CCM1 lesion (Gault 2005). The investigators found a 34bp deletion in exon 12 of *CCM1* that was biallelic to the germline nonsense mutation in exon 10. Our lab found evidence for a two-hit mutation mechanism for each of the three CCM genes in lesions isolated from patients with germline CCM mutations (Akers 2009). Loss of CCM protein expression in patient lesion tissue has also been detected by immunohistochemistry (IHC; Pagenstecher 2009). While these investigators were unable to find sequenceable somatic mutations, they did show a loss of CCM protein IHC staining in lesion endothelium compared to nearby, apparently normal vessels. Taken together, these studies suggest that CCM follows a two-hit mutation mechanism as shown at the DNA and protein levels.

The specific events and biochemical/signaling pathways driving lesion genesis and growth are currently unknown. CCM lesions are removed only after they become highly hemorrhagic and cause clinical symptoms. All of the work characterizing the genetic and histological alterations within CCMs has been accomplished with surgically resected lesion tissue, meaning these large, multicavernous lesions were already in the late-stage of development at the time they were removed. Since early-stage human lesions are effectively clinically silent, they are not surgically resected and are unavailable for study. 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 especially at the earliest stages.

### PRELIMINARY STUDIES

Mouse Models: To better study this disease, our lab has created mouse models of CCM. The *Ccm1* and *Ccm2* knockout alleles have been described (Whitehead 2004; Plummer 2006). The *Ccm3* knockout allele

was also recently generated by others (He 2010). Mice homozygous for the knockout alleles of *Ccm1*, *Ccm2* or *Ccm3* die mid-gestation and heterozygous animals do not show lesions at an appreciable frequency (Whitehead 2004; Plummer 2006; He 2010). We and others 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, or *Msh2*, a mismatch repair gene, increases the rate of somatic mutations, so these genetic sensitizer backgrounds were used to increase lesion burden in our CCM mice. *Ccm1*+/-,*Trp53*-/- mice, *Ccm2*+/-,*Trp53*-/- mice and *Ccm1*+/-,*Msh2*-/- mice show CCM lesions with high penetrance (Plummer 2004; Shenkar 2008; McDonald 2011). We extensively analyzed CCM lesions from the *Ccm1*+/-,*Msh2*-/- mice as they have a lower attrition rate than mice with *Trp53* as the sensitizer (McDonald 2011). In the *Ccm1*+/-,*Msh2*-/- mice, we have found lesions at all stages of development (see Figure 1). Late-stage lesions are multicavernous while early-stage lesions appear as grossly dilated, isolated caverns. Importantly, this model offers the <u>first</u> opportunity to study early-stage lesions. Clear differences between the early- and late-stage lesions have emerged from our CCM mouse models. Similar to human lesions, late-stage mouse lesions show immune cell infiltration, endothelial cell proliferation, loss of endothelial cell tight junctions, and recent hemorrhage (McDonald 2011). By contrast, the early-stage mouse lesions do not show these phenotypes. These studies were the first molecular analysis of early-stage CCM lesions.

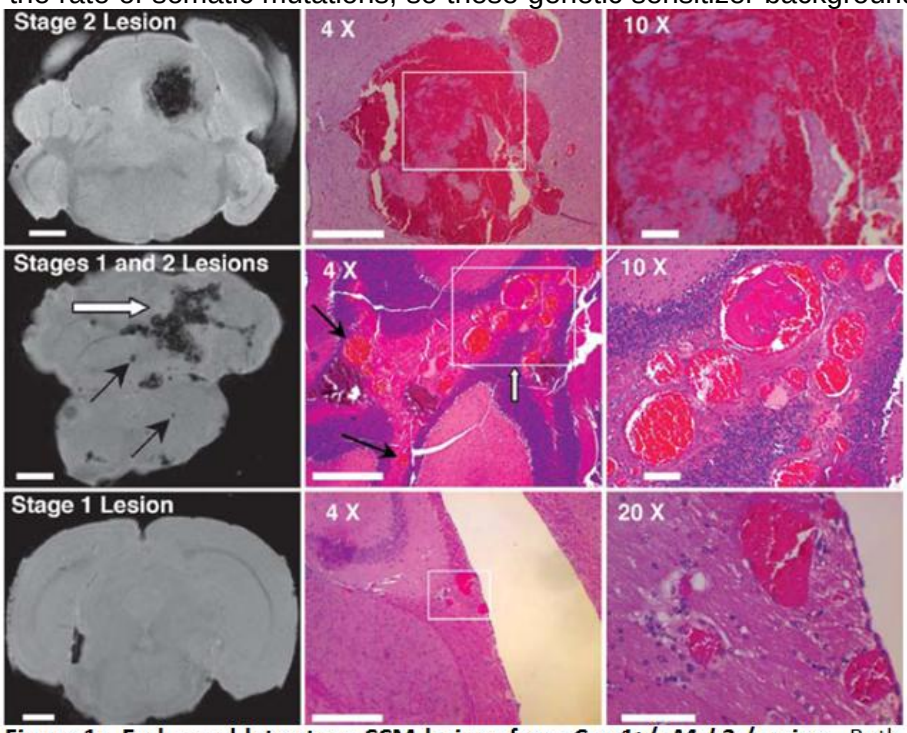


**Figure 1: Early- and late-stage CCM lesions from *Ccm1*+/-,*Msh2*-/- mice.** Both Stage 1 (early-stage isolated caverns, black arrows) and Stage 2 (clusters of dilated caverns, white arrows) CCM lesions are found in the brains of these mice. Images are shown of coronal sections by MRI (left panels)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

Figure 2 illustrates a natural history of CCM lesion genesis, growth and maturation that has developed from these CCM mouse models. Thus far, 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 APPROACH

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

<u>Rationale:</u> The human CCM samples available for study are late-stage, grossly dilated, highly hemorrhagic, multicavernous lesions. Thus, it is impossible to determine which characteristics observed in these lesions are directly related to lesion genesis and which are downstream effects of lesion 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 2008; McDonald 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 mature, late-stage CCM lesions are not observed at the earlier stages (McDonald 2011). While these histopathological phenotypes directly relate to clinical outcomes of CCM (i.e. stroke), they

appear to be downstream events in lesion development; however, these phenotypes can be used as indicators of disease progression to assess the effectiveness of possible therapeutics.

RhoA is a small GTPase which regulates endothelial cell migration and vascular permeability (Whitehead 2009). We recently observed increased activity of the RhoA effector Rho Kinase (ROCK) in endothelial cells of mouse (McDonald 2011) and human CCM lesions (Stockton 2010). This *in vivo* work supports the growing *in vitro* evidence connecting the loss of CCM proteins to activation of the RhoA pathway (Crose 2009; Whitehead 2009; Borikova 2010; Stockton 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 growth, 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 phenotypes of mouse CCM lesions.

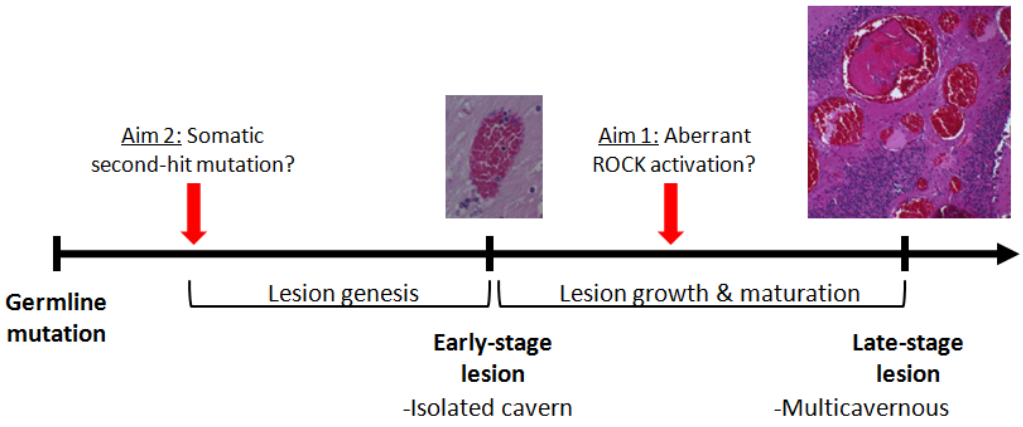


**Figure 2: Schematic representation of lesion genesis, growth and maturation in inherited cases of CCM.** The line represents the relative progression of a CCM lesion starting with a germline mutation and developing into a late-stage lesion. Characteristics of the early- and late-stage lesions are shown. The images of lesions are taken from our CCM mouse models.

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 2). Inhibition of ROCK will, therefore, prevent the development of phenotypes observed in the mature CCM lesion. To test this hypothesis, I will treat our mouse models with a specific ROCK inhibitor. I will examine the mouse brains for alterations known to occur in CCM lesion pathogenesis, including recent hemorrhage, endothelial cell proliferation, and immune cell infiltration using IHC as we did previously (McDonald 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 2008; McDonald 2011). Additionally, we have recently obtained mice bearing a knockout allele of *Ccm3* (He 2010), and I am currently generating *Ccm3*+/-,*Msh2*-/- mice using a similar breeding scheme as our other models (Shenkar 2008; McDonald 2011). All animals will be sacrificed between four and five months of age and their brains will be collected.

Fasudil Treatment: Fasudil specifically inhibits ROCK by binding to the ATP-binding cleft (Yamaguchi 2006), and this drug is currently in use in Japan to treat cerebral vasospasm (Iwabuchi 2011). *Ccm1*+/- and *Ccm2*+/- mice show increased permeability in normal vasculature in all organs compared to wildtype mice, but treatment with fasudil abrogates this effect (Stockton 2010). I propose to 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. Similar oral doses of fasudil have effectively inhibited ROCK activity in other model systems (Ying 2006; Slotta 2008; Meyer-Schwesinger 2009). The CCM mice receiving fasudil or control drinking water will be treated from weaning until sacrifice. A pilot study shows that the fasudil-treated mice have a reduced lesion burden compared to controls (data not shown), and further work is necessary to confirm these results in a larger group of mice and investigate how ROCK inhibition affects the phenotypes observed in the mature CCM lesion. 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 between the two treatment groups.

IHC: Slides of control vasculature and CCM lesions will be stained using our standard IHC protocols (McDonald 2011). Iron deposits, indicative of cerebral vessel hemorrhage, will be visualized by Perl's Prussian stain. 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 with biotinylated secondary antibodies and the Vectastain ABC kit (Vector Laboratories, Burlingame, CA, USA) to visualize alterations in lesion growth 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 2011).

Electron Microscopy: Previous studies of human CCM lesions found defective tight junctions between endothelial cells (Wong 2000; Clatterbuck 2001; Tu 2005) and our mouse models recapitulate this phenotype (McDonald 2011). I will use electron microscopy to investigate how ROCK inhibition affects the loss of the endothelial tight junctions during lesion progression. Slides will be prepared from brains of *Ccm1*+/-,*Msh2*-/- mice, *Ccm2*+/-,*Trp53*-/- mice and *Ccm3*+/-,*Msh2*-/- mice using our previously published procedures (McDonald 2011).

Defining Stages of Lesion Development: CCM lesions will be classified according to the system we recently outlined (McDonald 2011). Early-stage lesions (Stage 1, isolated caverns) 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 lesions (Stage 2) 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 2011). Based on this work, ten *Ccm1*+/-,*Msh2*-/- mice, ten *Ccm2*+/-,*Trp53*-/- mice and ten *Ccm3*+/-,*Msh2*-/- mice in both treatment (ROCK inhibitor) and control (vehicle) groups will suffice for these experiments. If unforeseen problems arise with *Msh2*-sensitized mice, *Trp53*-sensitized mice will be substituted for these experiments. We favor the *Msh2*-sensitized mice where possible because they are the most well characterized (McDonald 2011).

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 growth and maturation compared to controls (i.e. reduced hemosiderin deposition), 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 2). Moreover, inhibition of this pathway would be an effective therapeutic strategy as it would prevent many of the lesion characteristics that cause clinical phenotypes (i.e. stroke and seizures).

Alternatively, ROCK inhibition could prevent lesion genesis altogether. If ROCK inhibition decreases lesion penetrance 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 downstream pathogenic characteristics, then increased ROCK activity is just a consequence of CCM lesion pathogenesis rather than a cause. Many different groups, including our own, have shown that RhoA and ROCK play a role in CCM pathogenesis (Whitehead 2009; Borikova 2010; Stockton 2010). If these experiments provide a negative result, then it would be important to show that this pathway is not directly involved with CCM lesion growth and maturation so that other signaling pathways could be investigated as an approach to therapy.

Potential Problems and Alternative Approaches: To establish a fasudil treatment regimen, I am currently conducting a pilot experiment to determine a fasudil dosage 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 for 4 months with no apparent side effects. Although fasudil treatment effectively inhibits ROCK *in vivo* (Ying 2006), the timing of treatment may be important in our mouse models. If treatment needs to start earlier to see a therapeutic effect, then fasudil can be given to pregnant mothers to expose mice *in utero*. Conversely, it may be prudent to treat mice for shorter time periods to test the effects of ROCK inhibition on preexisting CCM lesions, in which case I will start treating mice at a later age.

Statins have been used as RhoA inhibitors *in vivo* (Kranenburg 1997, Collisson 2002; Whitehead 2009) because they disrupt RhoA isoprenylation (Park 2002; Zeng 2005), which is necessary for RhoA function. If

fasudil is ineffective at inhibiting ROCK *in vivo*, then statins could be used instead. Since statins target the cholesterol biosynthesis pathway and inhibit ROCK indirectly, there is an increased possibility that statin treatment will have off-target effects. Statins are a viable alternative ROCK inhibitor, but I favor fasudil for these experiments because it inhibits ROCK directly and specifically (Yamaguchi 2006).

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

Rationale: We have recently shown IHC evidence of a somatic loss of CCM1 protein in the endothelial cells surrounding lesions from *Ccm1*+/-,*Msh2*-/- mice (McDonald 2011; see Figure 3). 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 from *Ccm2*+/-,*Trp53*-/- mice and *Ccm3*+/-,*Msh2*-/- mice, is still needed to determine if the two-hit mutation mechanism is common to all three known CCM genes. It is also 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 2). Either result has important implications for CCM patients. If somatic second-hit mutations cause lesion genesis, then patients carrying a germline CCM mutation should avoid environmental risk factors that can cause somatic mutations, such as radiation exposure in X-ray radiography. I propose to investigate the two-hit hypothesis in mouse CCM lesions in order to determine if somatic second-hit mutations precede the early lesion or if they are a result of CCM lesion growth.

Hypothesis: I hypothesize that the somatic second-hit mutation causes lesion genesis (see Figure 2). Once the biallelic somatic mutation occurs, the affected endothelial cell will effectively have no functional protein from the altered CCM gene, initiating CCM lesion growth. I will investigate this hypothesis using IHC to examine a large sample of mouse CCM lesions (from *Ccm1*+/-,*Msh2*-/- mice, *Ccm2*+/-,*Trp53*-/- mice and *Ccm3*+/-,*Msh2*-/- mice) for loss of CCM protein staining. Additionally, I will use deep resequencing of mouse lesion DNA to directly identify somatic second-hit mutations. I will examine lesions at different stages of growth to determine if somatic second-hit mutations precede lesion growth or occur as a downstream event.

IHC Staining: After a somatic second-hit mutation occurs, loss of CCM protein expression will be evident by IHC 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 IHC. Our lab has previous experience with IHC staining of mouse CCM lesions (Plummer 2004), and we possess 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. As shown in the Figure 3, 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 assessed 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 (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.

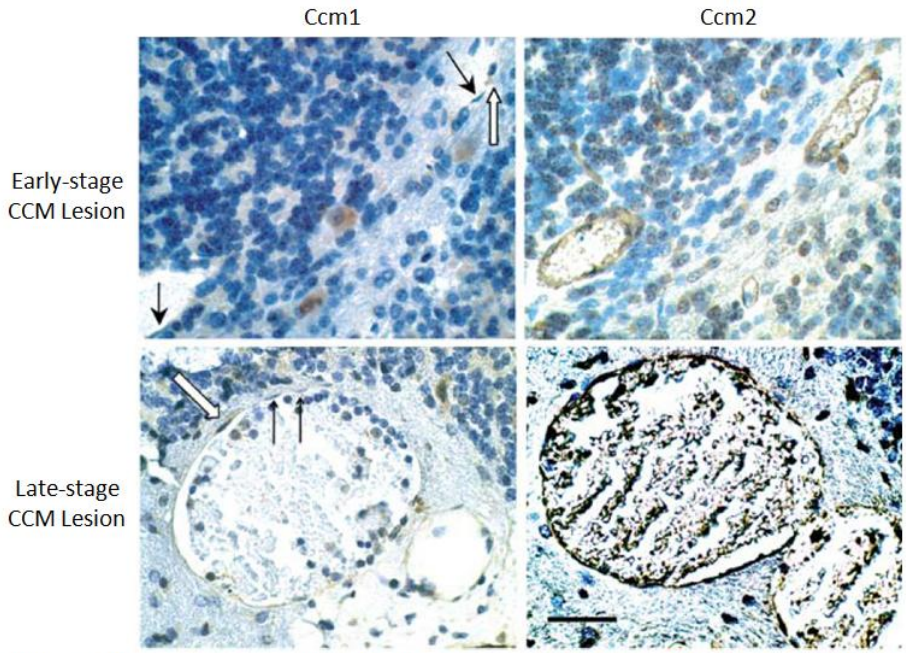


**Figure 3: Reduced Ccm1 expression in mouse CCM lesion endothelium.** Endothelial cells lining early-stage lesions show normal staining for Ccm2 (right), but either reduced (white arrow) or no staining (black arrow) for Ccm1 (left). Scale bar is 50 µm.

Deep Resequencing: While IHC can demonstrate 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 procedure where each exon of a particular CCM gene was cloned and sequenced to 48X coverage (Akers 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 greater sequence coverage in a more time- and cost-efficient manner. I will use deep resequencing at the Duke DNA Sequencing Facility to look for somatic second-hit mutations in early- and late-stage mouse CCM lesions. In a pilot experiment using 4 mouse brain samples, I amplified each of the coding exons of *Ccm1* and the DNA Sequencing Facility analyzed the samples with the Roche/454 GS-FLX Fusion high-throughput sequencer. The yield was over 1400X coverage of each amplicon (30-fold more coverage than the cloning and sequencing strategy used in Akers 2009).

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 (altered splice sites, premature stop codons, etc.) since those are the types of somatic mutations observed in human CCM (Gault 2005; Akers 2009; Gault 2009). Variants meeting this criterion will be validated by SNaPshot assay (Hoogendoorn 2000; Bujalkova 2008; Akers 2009). Since SNaPshot (Applied Biosystems, Carlsbad, CA) quantitatively measures the frequency of a variant in millions of amplicons, it is the most sensitive means of confirming that variants 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 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.

Expected Outcomes: Similar to human studies in late-stage lesions (Pagenstecher 2009; Stockton 2010), I expect both early- and late-stage mouse CCM lesions to show a widespread lack of IHC staining in the endothelium for one of the CCM proteins (i.e. loss of CCM1 staining in lesions from *Ccm1*+/-,*Msh2*-/- mice). This result would indicate that the somatic second-hit mutation precedes lesion genesis (see Figure 2). 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 because only a subset of cells harbor the somatic mutation.

In the deep resequencing 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 initiates lesion genesis. 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.

Potential Problems and Alternative Approaches: In the event that IHC staining of CCM proteins in mouse 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 2004), so I could use this procedure to produce a stronger signal. Another potential pitfall of using IHC is that inconsistent staining of the lesions could be misinterpreted as loss of CCM protein. Examining normal vessels on the same slide as the lesion tissue will ensure that *bona fide* changes in CCM protein expression are identified. Lesions can often be cut into multiple slides as well, so that consistency of the staining pattern between slides can be examined.

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.