Differential angiogenesis function of CCM2 and CCM3 in cerebral cavernous malformations

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Object. Loss-of-function mutations in CCM genes are frequently detected in familial cerebral cavernous malformations (CCMs). However, the current functional studies of the CCM genes in vitro have been performed mostly in commercially purchased normal cell lines and the results appeared discrepant. The fact that the cerebral vascular defects are rarely observed in CCM gene-deficient animals suggests the requirement of additional pathological background for the formation of vascular lesions. Consistent with these data, the authors assumed that silencing CCM genes in the endothelium derived from CCMs (CCM-ECs) serves as a unique and valuable model for investigating the function of the CCM genes in the pathogenesis of CCMs. To this end, the authors investigated the role and signaling of CCM2 and CCM3 in the key steps of angiogenesis using CCM-ECs.

Methods. Endothelial cells (ECs) derived from CCMs were isolated, purified, and cultured from the fresh operative specimens of sporadic CCMs (31 cases). The CCM2 and CCM3 genes were silenced by the specific short interfering RNAs in CCM-ECs and in control cultures (human brain microvascular ECs and human umbilical vein ECs). The efficiency of gene silencing was proven by real-time reverse transcriptase polymerase chain reaction. Cell proliferation and apoptosis, migration, tube formation, and the expression of phospho-p38, phospho-Akt, and phospho-extracellular signal-regulated kinase–1 and 2 (ERK1/2) were analyzed under CCM2 and CCM3 silenced conditions in CCM-ECs.

Results. The CCM3 silencing significantly promoted proliferation and reduced apoptosis in all 3 types of endothelium, but accelerated cell migration exclusively in CCM-ECs. Interestingly, CCM2 siRNA influenced neither cell proliferation nor migration. Silencing of CCM3, and to a lesser extent CCM2, stimulated the growth and extension of sprouts selectively in CCM-ECs. Loss of CCM2 or CCM3 did not significantly influence the formation of the tubelike structure. However, the maintenance of tube stability was largely impaired by CCM2, but not CCM3, silencing. Western blot analysis revealed that CCM2 and CCM3 silencing commonly activated p38, Akt, and ERK1/2 in CCM-ECs.

Conclusions. The unique response of CCM-ECs to CCM2 or CCM3 siRNA indicates that silencing CCM genes in CCM-ECs is valuable for further studies on the pathogenesis of CCMs. Using this model system, the authors demonstrate a distinct role of CCM2 and CCM3 in modulating the different processes of angiogenesis. The stimulation of endothelial proliferation, migration, and massively growing and branching angiogenic sprouts after CCM3 silencing may potentially contribute to the formation of enriched capillary-like immature vessels in CCM lesions. The severe impairment of the tube integrity by CCM2, but not CCM3, silencing is associated with the different intracranial hemorrhage rate observed from CCM2 and CCM3 mutation carriers. The activation of p38, ERK1/2, and Akt signal proteins in CCM2- or CCM3-silenced CCM-ECs suggests a possible involvement of these common pathways in the pathogenesis of CCMs. However, the specific signaling mediating the distinct function of CCM genes in the pathogenesis of CCMs needs to be further elucidated. (DOI: 10.3171/2010.5.FOCUS1090)

Key Words • cerebral cavernous malformation • CCM2 • CCM3 • short interfering RNA • angiogenesis

Cerebral cavernous malformations are major vascular anomalies in the CNS that occur sporadically or as a familial form with an incidence of approximately 10%–20% in European cases. Familial CCM, an autosomal dominant disorder attributable to loss-of-function mutations in any of the 3 CCM genes (CCM1, CCM2, and CCM3), commonly show multiple lesions and an increased risk of recurrent intracranial hemorrhages that may cause headaches, seizures, focal neurological deficits, or even death.7,21

All 3 CCM genes/proteins are expressed in a variety of cell types including neurons and astrocytes and in endothelium.18,23 Recent studies indicated that endothelia-specific ablation of CCM2 led to midgestation embryonic death due to failed angiogenesis, and these
vascular defects are endothelially autonomous, suggesting that any of the genes causing CCMs are needed in the endothelium. Supporting these findings, a complete inactivation of CCM proteins or somatic mutations of CCM genes are found in the affected ECs of all forms of familial CCMs. These data indicate the crucial role of endothelial CCM genes both in the vascular development and in the pathogenesis of CCMs.

Among these 3 genes, the functions of CCM1, CCM2, and CCM3 have been most extensively studied. However, the role of CCM2 in angiogenesis and the signaling affected by CCM2 deficiency appeared discrepant. Fewer studies have focused on the role of CCM3 in angiogenesis. Furthermore, the vascular defects in CCM2-deficient zebrafish or mice occurred exclusively extracerebrally. It is most likely that additional genetic, epigenetic, or local microenvironmental factors determine whether CCM gene mutations predispose the individuals to CCMs. Recent findings that frequent epigenetic alteration in phosphatase and tensin homolog promoter led to deficiency of this protein expression in the ECs of familial CCMs may support this notion. Furthermore, we (unpublished data, 2010) and others have found that ECs derived from CCMs (CCM-ECs) exhibit unique angiogenesis properties, indicating a pathological background of CCM-ECs. Clinically, a different intracranial hemorrhage rate has been observed in CCMs carrying individual CCM gene mutations. Consistent with these data, we assumed that silencing of individual CCM genes in CCM-ECs serves as a valuable model simulating the pathogenesis of familial CCMs and that these disease genes play a distinct role in angiogenesis of CCMs. To this end, the present study investigated the function of CCM2 and CCM3 in regulating the key steps of angiogenesis in CCM-ECs. Furthermore, the signaling pathways affected by CCM2 and CCM3 gene silencing were also studied.

Methods

Cell Culture

Endothelial Cells Derived From CCMs. The patients enrolled in the present study were all diagnosed with sporadic CCMs based on the specific characteristics of MR imaging and histopathological and familial criteria. Patients provided informed consent and the experimental protocol was approved by the local ethics committee. Participants with a possible family history of CCM or with mutations in CCM genes were excluded in the present study. Endothelial cells derived from resected specimens were obtained in 31 patients (mean age 42 years; 18 females and 13 males), who presented with intracerebral hemorrhage or seizure. The cells were cumulatively prepared according to the protocol by Baev and Awad, with some modifications. Briefly, the fresh surgical specimens were incubated with collagenase IV at 37°C for 30 minutes. After the tissue suspension was filtered, cell pellets were collected by centrifugation and suspended in ECGM-MV (PromoCell). The ECs were obtained by purifying the cell suspension using CD31 antibody-labeled Dynabeads (Dynal Biotech ASA) according to the manufacturer’s protocol. The purified ECs were cultured in ECGM-MV at 37°C in a humidified environment supplemented with 5% CO₂ and 95% air. The CCM-ECs from 31 single donors were cultured until passage 3 and then frozen in liquid nitrogen for further experiments after controlling the purity (> 90%) by immunostaining of von Willebrand factor or fluorescence-activated cell sorting (unpublished data, 2010). The CCM-ECs from 3 to 5 donors were pooled in the same proportion for individual experiments. All experiments were reproduced in at least 3 independent pooled CCM-ECs.

Human Umbilical ECs and Human Brain Microvascular ECs. Human umbilical Ecs (PromoCell) and human brain microvascular ECs (Provitro GmbH) were cultured in ECGM and ECGM-MV, respectively, in a humidified incubator containing 95% air and 5% CO₂ at 37°C. Cells were used between passages 3 to 5 for experiments.

Silencing of CCM2 or CCM3 by siRNA

Cells were seeded at a density of 1.0 x 10⁴/cm² in culture dishes or 24-well plates. After 24 hours of culturing, the cells were transfected with 70 nM of CCM2- or CCM3-specific siRNA or negative control siRNA (Neg. C. Ambion) using GeneSilencer (Gene Therapy System). The cells were incubated for different time periods after the transfection as indicated in the individual experiments. The efficiency of silencing was controlled by real-time RT-PCR in all experiments when siRNA transfections were concerned.

Total RNA/Protein Extraction and cDNA Synthesis

Total RNA/protein was extracted using a Nucleospin RNA/protein purification kit (Macherey-Nagel), and cDNA was synthesized using the First Strand cDNA Synthesis Kit (Fermentas) following the manufacturer’s instructions.

Real-Time RT-PCR

The reaction mixture was prepared to a final volume of 25 µl containing 10 µl of the cDNA solution (4 ng/µl), 15 µl of Absolute QPCR SYBR Green Mixes (ABgene), 0.5 µl of forward and reverse primers (10 µM), and RNase-free H₂O. The following primers were used: CCM2 forward 5′-CCCTGTGGAGATGTC-3′ and reverse 5′-AGCGAGACGCAAAGTCTCCT-3′; CCM3 forward 5′-TGCCAGCTGATGTAAGAG-3′ and reverse 5′-TGGTGGCTTTCCGTTTATGTA-3′; and GAPDH forward 5′-AGCCACATCGCTCAGAC-3′ and reverse 5′-GCCAATACGCAAATCC-3′. Real-time RT-PCR was performed using the following settings: initial denaturation at 95°C for 15 minutes; 35 cycles of amplification at 95°C for 30 seconds, 59°C for 20 seconds, and 72°C for 30 seconds; and for the melting curve, 72°C for 30 seconds, 95°C for 1 minute, and 55–95°C with a heating rate of 0.5°C every 10 seconds. Relative mRNA expression (fold of change) for each sample was quantified using the cycle threshold approach, normalized to the reference gene GAPDH. The specificity of amplification was monitored at the end of each reaction by melting curve analysis.

Proliferation Assay

Cell proliferation assay was performed 72 hours after the transfection using WST-1 reagent (Roche Diagnostics).
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according to the manufacturer’s instructions. The absorbance was measured at 450 nm by a plate reader.

**Migration Assay**

After 72 hours of the transfection, cells were trypsinized and resuspended in supplement-free ECGM basal medium and then loaded to the upper part of the transwell insert at identical cell numbers per well. The ECGM basal medium containing 10% fetal calf serum were added to the lower well. After 6 hours of incubation, the nonmigrated cells on the upper side of the inserts were removed by a swab. The migrated cells were stained using Hoechst 33258, and photographed and counted (in a blinded fashion) in 6 random fields per transwell under a microscope at a magnification of 100.

**Tube Formation Assay**

Cells were seeded in Matrigel-coated 96-well plates 72 hours after siRNA transfection and incubated for 6 hours, 24 hours, or 48 hours. Six random fields were blindly photographed from each well and the number of tubes was counted under the microscope at a magnification of 40.

**Induction and Detection of Apoptosis**

To minimize the influence of the proliferation effect of CCM3 siRNA on the apoptosis study, cells were reseeded at the identical density 48 hours after the transfection followed by another 24 hours of incubation. Cells received 100 nM of staurosporine treatment for 5 hours and were then stained with Hoechst 33258. The total number of cells and the number of apoptotic cells were counted (in a blinded fashion) in 6 random fields from each dish. The cells showing condensed nuclei or apoptotic bodies were considered apoptotic cells.

**Western Blot Analysis**

The total protein was extracted from CCM-ECs in parallel with the RNA extraction using a Nucleospin RNA/protein purification kit as described above. Samples containing an equal amount of total protein were loaded on 12.5% sodium dodecyl sulfate-polyacrylamide gels. Following electrophoresis, protein was transferred onto a nitrocellulose membrane. Unspecific binding was blocked by a buffer containing 0.1% Tween-20, 2% bovine serum albumin, and 5% nonfat dry milk in tris-buffered saline. The blots were then incubated overnight at 4°C with the following phosphor-antibodies (1:1000) from Cell Signaling Technology: rabbit antiphospho-p38 (P-P38), rabbit antiphospho-Akt (P-Akt, Ser473), mouse antiphospho-ERK1/2 (P-ERK1/2), or mouse antiantiactin (1:1000, Sigma). After the secondary antibody reaction, the signal was produced by incubating the blots with enhanced chemiluminescence detection solutions.

**Statistical Analysis**

All data are expressed as means ± SDs. The statistical analysis was performed using the WinSTAT program. The differences between the 2 groups were analyzed using ANOVA followed by the Scheffé test. A probability value < 0.05 was considered statistically significant.

**Results**

**Time Course of CCM2 and CCM3 Gene Silencing**

Real-time RT-PCR demonstrated a consistent expression of CCM2 and CCM3 genes in individual CCM-EC cultures as evidenced by the comparable cycle threshold values normalized to the housekeeping gene GAPDH (data not shown), suggesting a homogeneous CCM gene background of the cultures. The time courses of gene silencing were then established in CCM-ECs as well as in HBMECs and HUVECs by siRNA transfection. As shown in Fig. 2A, the expression of CCM2 gene in CCM-ECs declined by more than 70% of the control value at 48 hours and 72 hours after the transfection. The level of CCM2 slowly returned but was still 30% lower than the control at 96 hours after the transfection, whereas CCM3 gene expression decreased by 70%, 90%, and 65% of the control at 48 hours, 72 hours, and 96 hours, respectively, after the transfection. Similar time courses of CCM2 and CCM3 gene silencing were observed in HBMECs (Fig. 1B) and HUVECs (Fig. 1C) after the transfection. Based on these time courses of gene silencing, the functional studies of CCM2 and CCM3 genes were all performed at 72 hours after siRNA transfection. The possible unspecific effect of the siRNAs was ruled out by the detection of CCM2 and CCM3 gene expression after CCM3 and CCM2 siRNA, respectively (data not shown).

**Role of CCM2 and CCM3 in Endothelial Proliferation and Migration**

As shown in Fig. 2A, silencing CCM3 significantly promoted cell proliferation as evidenced by a 39%, 36%, and 24% increase in the absorbance generated by proliferating CCM-ECs, HBMECs, and HUVECs, respectively (p < 0.001 in CCM-ECs and HBMECs; p < 0.05 in HUVECs). However, the cell proliferation was not altered by CCM2 silencing in all 3 types of ECs.

Subsequently we checked cell mobility after CCM2 and CCM3 silencing by the transwell migration assays. Surprisingly, a significantly higher number of migrated cells were detected after CCM3 silencing only in CCM-ECs (p < 0.05; Fig. 2B and C), but not in HBMECs and HUVECs. Furthermore, the change of cell mobility was not observed in CCM-ECs nor in HBMECs and HUVECs after CCM2 siRNA transfection (Fig. 2B).

**Influence of CCM2 and CCM3 Silencing on Sprout Growth and Tube Formation**

To examine the role of CCM2 and CCM3 genes in the process of tube formation, HUVECs and CCM-ECs were seeded on Matrigel 72 hours after the siRNA transfection. Interestingly, dramatic growth and extension of sprouts were observed exclusively in CCM-ECs after CCM3 silencing and to a lesser extent after CCM2 silencing (Fig. 3), which even made the quantification of the number of tubes difficult. Therefore, the quantification of tube formation was performed in HUVECs (Fig. 4). The tubelike structure was similarly observed in HUVECs transfected with negative control siRNA, CCM2 siRNA, or CCM3 siRNA at 6 hours after seeding cells on Ma-
However, the tubelike structures collapsed at 24 hours and were completely lost at 48 hours after CCM2 siRNA transfection, whereas the tubes remained intact at 24 hours and were slightly injured at 48 hours in cells transfected with negative control and CCM3 siRNA (Fig. 4 upper). Quantitative analysis confirmed a significant reduction of the tube numbers at 24 hours after CCM2, but not CCM3, siRNA transfection (p < 0.001; Fig. 4 lower).

**Influence of CCM3 on Endothelial Apoptosis**

The CCM3 gene, alternatively referred to as the programmed cell death gene, codes for a protein related to apoptosis. It is thus highly interesting to investigate the role of the CCM3 gene in endothelial apoptosis. Stauroporine, a broad spectrum kinase inhibitor and a widely used apoptosis inducer, induced a pronounced apoptosis in CCM-ECs, HBMECs, and HUVECs (p < 0.001). Of note, the percentage of apoptosis was significantly reduced by approximately 15% after CCM3 gene silencing in all 3 cell types (p < 0.001 in CCM-ECs; p < 0.01 in HBMECs and HUVECs; Fig. 5).

**Effect of CCM2 and CCM3 Silencing on p38, Akt, and ERK1/2 Activation in CCM-ECs**

To identify the signaling pathway possibly affected by CCM2 or CCM3 silencing, the protein and RNA were extracted in parallel for detection of protein levels of P-p38, P-Akt, and P-ERK1/2 and for detection of the expression of the CCM2 and CCM3 genes, respectively, at 72 hours after the transfection. Western blot analysis revealed a clear upregulation of P-p38, P-Akt, and P-ERK1/2 after CCM2 and CCM3 siRNA transfection (Fig. 6 upper). Real-time RT-PCR confirmed a more than 90% downregulation of CCM2 and CCM3 gene expression in the
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same sourced cells (Fig. 6 lower), indicating an association of the increased activation of these signaling proteins and an efficient CCM2 and CCM3 silencing.

Discussion

Recently, several studies have revealed a pivotal role of endothelial CCM genes in vascular development and in disease states. It is noteworthy, however, that the vascular defects occurred exclusively extracerebrally in Ccm2 deficient animals and that the typical CCM-like vascular lesions were rarely observed in heterozygote Ccm1+/- or Ccm2+/- mice. These findings raised the assumption that additional factors may determine whether CCMs occur in CCM gene mutation carriers. Although this proposal remains speculative, one could imagine that the endothelium in CCM lesions harbors certain unique properties because the lesions have been exposed to this abnormal, yet undefined, microenvironment. Within this context and considering that the functional study of CCM genes in culture has been performed exclusively in cell lines or in commercially purchased normal ECs (HUVECs and HBMECs), silencing CCM genes in CCM-ECs established in the present work may simulate...
some aspects of the diseased endothelium in the CCM lesions, and thus may serve as a unique and valuable model for investigating the function of CCM genes in the pathogenesis of CCMs.

Cerebral cavernous malformations occur either sporadically (in approximately 80% of cases) or in a familial pattern. The former usually appears as a single lesion, whereas the latter occurs as multiple lesions along with a family history of CCMs and/or an autosomal dominant mode of inheritance. In the present study, we prepared CCM-ECs from sporadic CCMs. Because sporadic CCMs account for the majority of cases, there are more specimens available than from familial CCMs. More importantly, sporadic CCMs do not harbor CCM gene mutations and express comparable levels of CCM genes in individual cases (unpublished data, 2010), which allows us to manipulate individual CCM gene expression in CCM-ECs under a more homogeneous genetic background. Technically, the CCM-ECs from our laboratory exhibited the following advantages in comparison with the previous protocol: 1) a purification step using EC marker (CD31) antibody-labeled Dynabeads was performed during the preparation, thereby ensuring the purity (90–95%) of ECs and minimizing the responses/effects derived from non-ECs; and 2) the CCM-ECs used in all experiments were pooled at the same proportions from multiple donors in the present study. The pooled CCM-ECs displayed very similar properties as the single donor (unpublished data, 2010), but provided more efficient work with more representative participants.

Angiogenesis is the primary mode of vascularization for the brain, requiring the precise coordination of multiple different steps. Based on the aberrant angiogenic features of CCMs, we focused on the role of CCM2 and CCM3 genes in angiogenesis of CCM-ECs, and in addition, these functional studies were also performed in HBMECs.
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and in HUVECs as a parallel comparison. Furthermore, all individual functional studies were performed only when the real-time RT-PCR confirmed efficient gene silencing in the sister culture. We demonstrated a significantly increased cell proliferation in CCM-ECs as well as in HBMECs after CCM3 silencing, whereas this effect was only slightly observed in HUVECs, suggesting the physiological variability between the ECs derived from cerebral microvessels (CCM-ECs and HBMECs) and from veins (HUVECs; Fig. 2A). Interestingly, CCM3 silencing accelerated cell migration exclusively in CCM-ECs, but not in HBMECs and HUVECs (Fig. 2B), indicating a unique property of CCM-ECs. The migration effect of CCM3 siRNA observed exclusively in CCM-ECs does not appear to be a simple consequence of the enhanced cell proliferation because CCM3 silencing promoted proliferation in all 3 types of tested cells (Fig. 2A). Furthermore, the migration assay was performed after reseeding cells at identical cell numbers per transwell 72 hours after the transfection, followed by only 6 hours of incubation. Interestingly, knockdown of CCM2 influenced neither cell proliferation nor migration in all 3 types of ECs. These results indicate a distinct role of CCM2 and CCM3 in regulating endothelial proliferation and migration.

Tube formation is another key step involved in angiogenesis. In the present study, we studied tube formation after CCM2 and CCM3 silencing in both CCM-ECs and HUVECs. The CCM2 or CCM3 silencing did not significantly affect the formation of tubelike structures as evidenced by the similar formation of tubelike structures in negative control-, CCM2 siRNA-, and CCM3 siRNA-treated HUVECs at 6 hours after pooling cells on Matrigel (Fig. 4 upper). Interestingly, silencing CCM2, but not CCM3, largely impaired the tubelike structure at 24 hours and 48 hours after cell growth on Matrigel (Fig. 4 upper and lower), indicating an essential role of CCM2 for maintenance of tube integrity. Our findings are partially in agreement with the recent report that knockdown of CCM1–3 inhibited tube formation in the EC line derived from mouse brain.3 The variant results from the tube formation study may be a result of using different types of ECs and due to a broader time course of the tube formation assay in the present study.

More interestingly, we observed a massive growth of sprouts after CCM3 and, to a lesser extent, CCM2 siRNA in CCM-ECs, again indicating the unique angiogenic property of CCM-ECs. These sprouts appear to undergo the formation of a microtube-like structure (Fig. 3). Whether this phenomenon is associated with the increased cell proliferation and migration remains questionable, because CCM3 silencing promoted proliferation in all 3 types of cells tested (Fig. 2A), but the formation of these sprouts was exclusively observed in CCM-ECs. Furthermore, CCM2 siRNA influenced neither cell proliferation (Fig. 2A) nor migration (Fig. 2B). Activation of notch signaling has been previously shown in sprouting ECs.15 Nevertheless, the mechanism for the sprout growth after CCM2 and CCM3 silencing in CCM-ECs needs to be further elucidated.

Intracerebral hemorrhage is a consistent pathological feature of CCMs. The severe impairment of the tube structure integrity after endothelial CCM2 silencing is closely associated with the clinical observation that CCM2 mutation-related familial CCMs presented with a higher incidence of hemorrhages in comparison with CCM1 and CCM3 forms of familial CCMs.3,8,24 The role of CCM2 in maintenance of vascular integrity has been proposed through regulating the activity of Ras homolog gene family member A. The mechanism of this regulation has been recently addressed by Crose et al.9

The CCM3 gene is suggested to be involved in apoptosis14,16 and tumor signaling.14 We found that loss of CCM3 led to a significant resistance of all 3 tested types of ECs to apoptotic stimuli (Fig. 5), suggesting a dominant apoptotic function of CCM3 in ECs. Considering apoptosis and proliferation as two conflicting cellular events, CCM3 silencing-mediated promotion of endothelial proliferation (Fig. 2A) could be at least partially a consequence of the increased antiapoptotic potency of endothelium. If this is true, targeting endothelial apoptosis could be a possible therapeutic strategy for CCMs, particularly for CCM3 mutation carriers.

The signaling proteins p38, Akt, and ERK1/2 are all importantly involved in the pathways necessary for regulating cell growth, proliferation, and apoptosis, as well as cell migration. The CCM2 gene has been defined as a scaffold for p38 activation in response to hyperosmotic stress induced by sorbitol in mouse CCM2 cell line.27 However, CCM2 silencing in HBMECs did not affect the activation of p38 and ERK1/2,25 The different studies also showed various regulations of p38 activation in response to CCM3 silencing.5,14 These data suggest that CCM genes trigger cellular signaling pathways in a cell typespecific manner. We demonstrated that CCM2 or CCM3 silencing commonly activated p38, ERK1/2, and Akt in CCM-ECs, consistent with the current opinion that these 3 CCM genes interact as an intracellular complex and share some common signaling pathways.11 However, the individual CCM proteins may have a specific function,3 and thus it cannot be ruled out that the diverse cellular functions of individual CCM genes signal independently besides sharing some common pathways.10 Our finding that silencing CCM2 or CCM3 differently regulates the key steps of angiogenesis in CCM-ECs supports this notion. The specific signaling that accounts for the different function of CCM genes needs to be further studied.

Conclusions

Performing a functional study of CCM genes in CCM-ECs derived from familial CCMs would be an ideal strategy. However, this strategy is limited practically by the rarity of cases and by the availability of the genetic diagnosis. The present study, silencing CCM genes in CCM-ECs derived from sporadic CCMs, established an in vitro model simulating certain aspects of familial CCMs. The unique response of CCM-ECs to CCM2 or CCM3 silencing indicates that this model could be valuable for further studies on the pathogenesis of CCMs. The distinct role of CCM2 and CCM3 in modulating the different processes of angiogenesis may be associated with the different clinical penetrance rates observed in CCM2 and CCM3 mutation carriers. The identification of the activation of p38, ERK1/2, and Akt signal proteins in CCM2- or CCM3-silenced CCM-ECs suggests a possible involvement of these signal-
ing pathways in the pathogenesis of CCMs that harbor the individual CCM gene mutations.

Disclosure
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Author contributions to the study and manuscript preparation include the following. Conception and design: Zhu. Acquisition of data: Wu, Xu. Analysis and interpretation of data: Zhu, Miller, Sandalcioglu. Drafting the article: Zhu. Critically revising the article: Zhang, Sure. Reviewed final version of the manuscript and approved it for submission: Zhu, Zhang, Sure. Statistical analysis: Miller. Study supervision: Zhu, Sure.

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