Developmental timing of CCM2 loss influences cerebral cavernous malformations in mice

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Cerebral cavernous malformations (CCM) are vascular malformations of the central nervous system (CNS) that lead to cerebral hemorrhages. Familial CCM occurs as an autosomal dominant condition caused by loss-of-function mutations in one of the three CCM genes. Constitutive or tissue-specific ablation of any of the Ccm genes in mice previously established the crucial role of Ccm gene expression in endothelial cells for proper angiogenesis. However, embryonic lethality precluded the development of relevant CCM mouse models. Here, we show that endothelial-specific Ccm2 deletion at postnatal day 1 (P1) in mice results in vascular lesions mimicking human CCM lesions. Consistent with CCM1/3 involvement in the same human disease, deletion of Ccm1/3 at P1 in mice results in similar CCM lesions. The lesions are located in the cerebellum and the retina, two organs undergoing intense postnatal angiogenesis. Despite a pan-endothelial Ccm2 deletion, CCM lesions are restricted to the venous bed. Notably, the consequences of Ccm2 loss depend on the developmental timing of Ccm2 ablation. This work provides a highly penetrant and relevant CCM mouse model.

Cerebral cavernous malformations (CCMs) lesions consist of densely packed vascular sinuses lined by a thin endothelium with rare subendothelial cells and no intervening parenchyma (Russel and Rubinstein, 1989; Clatterbuck et al., 2001). Ultrastructural analysis of CCM lesions showed defective tight junctions (TJ) between endothelial cells (ECs). The lesions form predominantly in the central nervous system (CNS), but ~5% of the patients also show lesions in the retina (Labauge et al., 2007; Riant et al., 2010).

The prevalence has been estimated to be up to 0.5% in the general population. CCM occurs as a sporadic (80% of patients) or a familial disease (20% of patients), after an autosomal dominant pattern of inheritance. Sporadic patients most often have a single CCM lesion, whereas patients affected by a hereditary form of the disease have multiple lesions and show a progressive increase in lesion number over time. Symptoms include headaches, seizures, and focal neurological deficits caused by cerebral hemorrhages. The major risk for patients is the recurrence of hemorrhages. Neurosurgery is the only therapy offered today; however, it is not always possible depending on the lesion location within the CNS.

Familial CCM disease is caused by heterozygous germline mutations in any of the three CCM genes identified so far (CCM1/KRIT1, CCM2/LETM1, CCM3/MODY5).

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CCM2/MGC4607, and CCM3/PDCD10; Craig et al., 1998; Laberge-le Couteulx et al., 1999; Liquori et al., 2003; Denier et al., 2004; Bergametti et al., 2005). Recently, a “two-hit” mechanism has been demonstrated in human ECs within the CCM lesions (Gault et al., 2005; Akers et al., 2009; Pagenstecher et al., 2009).

The three CCM genes encode scaffold proteins without any sequence homology. In the recent past, in vitro data obtained in different cell types identified numerous CCM protein partners that form complexes involved in various intracellular signaling pathways (Faurobert and Albuges-Rizo, 2010). Consistent with their involvement in CCM pathogenesis, the three CCM proteins can interact in a cytosolic ternary complex, where CCM2 acts as a bridge between CCM1 and CCM3 (Hilder et al., 2007). However, the in vivo functions of this ternary complex remain unclear.

When associated with the integrin β1-binding protein ICAP-1, KRIT1 shuttles from the cytosol to the nucleus; its function there remains to be determined. CCM2 interaction with KRIT1 modulates KRIT1 trafficking by sequestering KRIT1 in the cytoplasm (Zawistowski et al., 2005). KRIT1 is a Rap1 effector and stabilizes the endothelial cell–cell junctions (Glading et al., 2007). Recently, physical interaction between KRIT1 and CCM2 was proven to be required for CCM protein localization at endothelial cell–cell junctions (Stockton et al., 2010). KRIT1 has also been involved in vascular lumenization and polarization of the endothelial tube in vitro (Lampugnani et al., 2010).

Several studies have provided new insights into CCM protein functions in vitro, including cytoskeletal remodeling, cell–cell junction homeostasis, lumen formation, and polarization. However, it remains to be clarified which protein complexes and signaling pathways are relevant in vivo, and how the loss of function of any of the three CCM proteins might explain the etiology of the CCM disease. To this aim, relevant mouse models for the CCM disease are needed to understand the molecular mechanisms involved in lesion pathogenesis. Moreover, relevant mouse models reproducing CCM lesions would allow development of preclinical therapeutic studies (Leblanc et al., 2009; Yadla et al., 2010).

Over the past few years, several groups reported constitutive or tissue-specific KO mice for all three CCM genes. Homozygous Ccm1-deficient mice die at midgestation with defects in the vascular structure and impaired arterial morphogenesis (Whitehead et al., 2004). We and others have shown that endothelial-specific ablation of Ccm2 results in vascular malformations mimicking human CCM lesions in the CNS (Craig et al., 1998; Soriano, 1999). As assessed by XGal staining, high recombination efficiency was obtained after tamoxifen treatment (Fig. S1, A–F). Unless otherwise mentioned, the genotype of Ccm2-ablated mice (iCCM2 for inducible CCM2 KO) was Cdh5(PAC)-CreERT2; Rosa26-Stopβ-LacZ reporter mice were used to monitor the tamoxifen-induced Cre/Lox recombination (Soriano, 1999). Animals were tamoxifen injected at P1 and were analyzed at different time points, from P6 through 3 wk of age (Table I).

The estimated survival median for iCCM2 animals calculated by the Kaplan-Meier test is 17 d (with a P < 0.001 of significance for survival compared with controls; Fig. 1 A). At P8, all iCCM2 animals were alive and grossly undistinguishable from controls. They did not show any significant difference in weight (4.14 ± 0.6 g for iCCM2 versus 4.51 ± 0.7 g for controls). However, from P6 onward, we were able to distinguish the iCCM2 animals upon dissection of the brain (for ~80% of the P6–P7 iCCM2 and 100% from P8 onward) and a clear aggravation of the vascular phenotype over time was observed (Fig. S2 A). P1 deletion of Ccm2 resulted in the development of CCM lesions within the CNS.

### RESULTS

**Endothelial-specific Ccm2 deletion at P1 results in vascular malformations mimicking human CCM lesions in the CNS**

To bypass the embryonic lethality previously encountered in the Tie2Cre;Ccm2 KO mouse model (Boulday et al., 2009), we crossed Ccm2 floxed mice with the endothelial-specific, tamoxifen-inducible Cdh5(PAC)-CreERT2 mice (Wang et al., 2010). Rosa26-Stopβ-LacZ reporter mice were used to monitor the tamoxifen-induced Cre/Lox recombination (Soriano, 1999). As assessed by XGal staining, high recombination efficiency was obtained after tamoxifen treatment (Fig. S1, A–F). Unless otherwise mentioned, the genotype of Ccm2-ablated mice (iCCM2 for inducible CCM2 KO) was Cdh5(PAC)-CreERT2; Ccm2Δ/Δ; Rosa-Stopβ-LacZ (see Materials and methods for details of mouse breeding).

Table I. Number of iCCM2 analyzed after P1 deletion

| Age of analysis | Total numbers of iCCM2 | iCCM2 with cerebral CCM lesions/total number of iCCM2 |
|-----------------|------------------------|------------------------------------------------------|
| P6–7            | 12                     | 10/12                                                 |
| P8–10           | 30                     | 30/30                                                 |
| P11–15          | 10                     | 10/10                                                 |
| P16–19          | 4                      | 4/4                                                   |

*Visible lesions upon dissection.
Ccm2 deletion in the mouse retina mimic the CCM retinal lesions found in patients (Fig. 1 D, right).

We used the same gene targeting strategy to invalidate endothelial Ccm1 or Ccm3 in mice. Tamoxifen-induced deletion of Ccm1 or Ccm3 at P1 resulted in CCM malformations in the CNS of all iCCM1 and iCCM3 animals analyzed (n = 4 in each group). The CCM1 and CCM3 lesions were comparable to the lesions obtained after Ccm2 deletion in terms of location (within the cerebellum and at the periphery of the retina) and phenotype (Fig. S3).

We established a relevant mouse model for the human CCM with a complete penetrance and a fast development of CCM lesions from single to multiple hemorrhagic caverns (Fig. 1 B and Fig. S2, C–F). Moreover, P1 deletion of any of the three Ccm genes resulted in a similar phenotype according to what is established for the human CCM disease.

Endothelial cell–cell junctions are altered in CCM2 KO cells in vitro and in CCM2 lesions

To investigate whether part of the phenotype could be related to altered endothelial cell–cell junctions, we analyzed the expression and organization of junctional proteins in ECs in vitro and in vivo in cerebellar vessels of iCCM2 mice.

We abrogated CCM2 expression by treating ECs derived from Ccm2 fl/fl mice with TAT-Cre recombinase (Fig. S4 A).

Figure 1. Endothelial Ccm2 deletion at P1 results in CCM malformations mimicking the human CCM lesions in the cerebellum and in the retina. All animals were injected at P1 with 10 µl tamoxifen (equivalent to 20 µg) and dissected at the indicated time. Genotypes of inducible CCM2 KO (iCCM2) and control animals were respectively Cdh5(PAC)-CreERT2; Ccm2 Del/fl and Cdh5(PAC)-Cre-ERT2; Ccm2 +/fl. (A) Kaplan–Meier survival curve from the control group (blue line, n = 110) and the iCCM2 group (dotted red line, n = 56). Circles represent censored animals, which were sacrificed for analysis. (B) Control and iCCM2 mouse brains upon dissection (top) and after H&E staining (bottom; n = 6 in each group from 4 different litters, analyzed between P11 and P19). CCM malformations, located in the cerebellum of iCCM2 animals, are composed by single or multiple caverns (asterisks) with extensive hemorrhage (black arrows) around the juxtaposed vascular cavities. Note the dilation of meningeal vessels in the iCCM2 (yellow arrow). (C) Control and iCCM2 mouse retinas at P13 upon dissection (left and middle) and after isolectin-B4 staining (right, n = 7 in each group from 4 different litters, analyzed between P11 and P15). (D) Mouse lesions phenocopy human CCM lesions. (left) Histology of the cerebral lesions in mouse and human. (right) Mouse retina upon dissection and human retinal angiography Bars: 2 mm (B, top); 500 µm (C and D, mouse retina); 100 µm (B [bottom] and D [mouse cerebellum]).
We then analyzed endothelial junction organization in vivo in iCCM2 brain vasculature (Fig. 2). Staining of junctional components supported data obtained in cultured ECs. Indeed, claudin-5 or ZO.1 were correctly expressed in peri-lesion vessels, but strongly reduced in abnormally dilated and hemorrhagic CCM lesions (Fig. 2 B). In addition, the AJ protein VE-cadherin appeared to be disorganized and was not clearly concentrated at intercellular endothelial cell–cell contacts within the lesion, as compared with normal cerebral vessels in iCCM2 animals (Fig. 2 C).

Overall, these data indicate that CCM2 is required for proper cell–cell junction architecture in ECs in vitro and that CCM2 deletion in vivo results in impaired EC junction architecture in CCM2 lesions.

To investigate the mechanism of the strong reduction in claudin-5 expression upon CCM2 ablation, we evaluated the major regulators of claudin-5 expression, including the Akt–FoxO1 signaling pathway (Brunet et al., 1999; Burgering and Kops, 2002; Zhang et al., 2002; Daly et al., 2004; Taddei et al., 2008), the transcription factor Sex-determining region Y-box-18 (SOX18), and the ETS ternary complex ELK3 (Fontijn et al., 2008). Phosphorylation of Akt was enhanced in confluent CCM2 KO ECs compared with control ECs, accompanied by a strong phosphorylation of FoxO1 (Fig. S5 A). These data excluded Akt–FoxO1 pathway involvement in claudin-5 reduction in CCM2-null ECs. In contrast, SOX18 expression was found to be reduced by 50% by quantitative RT-PCR in CCM2 KO versus control ECs, whereas ELK3 expression was not modified (Fig. S5 B). This result supports the idea that alteration in SOX18 expression...
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The Wnt–β-catenin pathway is not involved in CCM2 lesion development in vivo

β-Catenin participates in the formation and stabilization of cadherin-based adhesion by forming a connection to the actin cytoskeleton (Dejana, 2010). β-Catenin is also a key element of the canonical Wnt (wingless and Int-1) signaling that promotes the nuclear localization of β-catenin by blocking the β-catenin destruction complex. Binding of β-catenin to cadherins can antagonize Wnt signaling by sequestering β-catenin at the membrane. Alteration of AJ promotes β-catenin nuclear translocation and the subsequent activation of T cell factor (TCF)/lymphoid enhancer factor (LEF) transcriptional complexes. KRIT1 is localized at the endothelial cell–cell junction and can be co-precipitated with β-catenin (Glading et al., 2007). Depletion of endothelial KRIT1 in vitro has been described to induce β-catenin delocalization from the membrane to the nucleus leading to increased β-catenin transcriptional activity (Glading and Ginsberg, 2010).

Interestingly, in CCM2 KO ECs in vitro, we also observed a delocalization of β-catenin from the membrane to the nucleus, suggesting β-catenin transcriptional activity (unpublished data). However, TCF/LEF-β-catenin transcriptional activity, as detected using the TOPFlash reporter, which includes six TCF-binding sites, was not significantly modified in CCM2-null cells compared with controls (Fig. 3 A), suggesting that the Wnt–β-catenin pathway is not disregulated in CCM2-null ECs in vitro.

To investigate whether the Wnt–β-catenin signaling pathway is involved in vivo in the CCM pathogenesis, we assessed the β-catenin transcriptional activity in the absence of CCM2 in mice. Animals were crossed with the BAT-Gal reporter mouse (see Materials and methods for mating details), which drives β-galactosidase expression under the control of multimerized TCF/LEF binding sites for activated nuclear β-catenin. iCCM2; BAT-Gal mice were injected at P1 with tamoxifen to ablate Ccm2. Consistent with the previous study by Maretto et al. (2003), the β-catenin signaling was activated in the mouse cerebellum at P8 (Fig. 3, B–I). We found a similar activation of the β-catenin signaling in controls or iCCM2 cerebellum, mostly located in the Purkinje cell layer, and in some ECs within the white matter. In contrast, the endothelium lining the CCM malformations did not show any evidence of β-catenin signaling pathway activation at P8, neither in the cerebellum nor in the retina (Fig. 3, G–I; and not depicted), suggesting an absence of Wnt–β-catenin pathway involvement in iCCM2 lesions at the time of analysis.

CCM lesions specifically affect the venous bed and not the arterial compartment

Retinal vessels develop after birth in the mouse, after a very well defined and organized pattern (Dorrell and Friedlander, 2006; Fruttiger, 2007). Within the first week of life a vascular plexus develops at the inner surface of the retina, from the central retina toward the periphery. At this stage, the strict

Figure 3. Ccm2 deletion has no significant effect on Wnt–β-catenin signaling pathway in CCM2 KO ECs in vitro and in iCCM2 lesions. (A) TCF/LEF-β-catenin transcriptional activity in CCM2 WT and null ECs in vitro was determined by transfecting CCM2 WT and KO ECs with the TOP-TK-Luc or the FOP-TK-Luc reporter constructs (containing WT or mutant Tcf/Lef binding sites, respectively, and a basal TK promoter, upstream a luciferase gene). Columns are means ± SD of triplicates from a representative experiment out of three performed. (B–I) Animals were bred with the BAT-Gal reporter mouse to assess β-catenin activation (see Materials and methods for breeding details). All animals were injected with tamoxifen at P1. XGal staining, performed on control and iCCM2 cerebellum, is shown (n = 8 in each group, from 3 different litters). White arrows show the CCM lesions in iCCM2 animals (in C, E, and G). In F–I, H&E staining was performed on cerebellum sections, after XGal staining. The box in G is magnified in I. H shows a CCM lesion composed of multiple juxtaposed caverns. Gcl, granular cell layer; ml, molecular layer; pcl, Purkinje cell layer; wm, white matter. Bars: 1 mm (B and C); 500 µm (D and E); 100 µm (F and G); 50 µm (H and I).
In utero Ccm2 deletion led to vascular anomalies in the cerebral hemispheres, affecting cerebral rhinal veins and the surrounding capillaries (Fig. 4 B). In contrast, middle cerebral arteries remained normal. To further confirm that the arterial compartment is not affected by CCM lesion, we compared vessels from mice mated with either the Rosa26-Stopfl-LacZ reporter mouse (to visualize all the vessels), or with the artery-specific EphrinB2lacZ reporter mouse (Fig. 4, A [right] and C; see Materials and methods for breeding details). XGal stainings showed that endothelium of the retinal and cerebral CCM lesions did not express the arterial-specific EphrinB2 marker. In addition, P1 deletion also resulted in dilatation of vessels running along the cerebellar folia at the meningeal surface corresponding to dorsal cerebellar veins (Fig. 1 B; Nonaka et al., 2002).

Collectively, our results showed that Ccm2 deletion affects the venous bed and leads to capillary-venous malformations.

Natural history of CCM lesions

A great advantage of animal models is the possibility to explore the etiology of the vascular malformations at very early stages to determine herein the natural history of the CCM lesion over time. We followed lesion development in the retina with an isolectin-B4 staining (Fig. 5). As early as P7, the vascular plexus was thicker at the venous leading edge of the retina in iCCM2 animals. Quantification of the vascular coverage at the venous leading edge showed a 29% increase in iCCM2 retinas compared with controls (Fig. 5 B; 0.56 ± 0.01 in controls versus 0.76 ± 0.01 in iCCM2; P = 0.0004; n = 4). By P9, the main veins were dilated in iCCM2 animals with abnormal capillaries at the periphery of the vascular plexus. In 3-wk-old control mice, the retinal vasculature was fully established, with 3 distinct plexuses. In contrast, at the periphery of iCCM2 retinas, it was not possible to distinguish different vascular plexuses. The vasculature from the lesion was composed by bubblelike vascular structures packed together. A 2,000-kD intracardiac FITC-Dextran injection confirmed that those abnormal vascular structures were lumenized (unpublished data).

To investigate the mechanisms of the increase in diameter of iCCM2 retinal vessels at P7, we assessed whether EC proliferation was altered in the absence of CCM2. Using a phospho-histone-3 staining, we could not detect any significant increase in EC proliferation in iCCM2 compared with controls at P6 (Fig. 5 C). These data suggest that EC proliferation is not the primary event leading to CCM malformations and does not contribute to the early vascular phenotype induced by Ccm2 deletion.
Collectively, our data on \textit{Ccm2} deletion during embryogenesis, the postnatal period, and at 3 wk of age strongly suggested that the effects of \textit{Ccm2} deletion on CCM lesion development were restricted to key time points temporally related to the angiogenic process.

**DISCUSSION**

In the present paper, we used an inducible, EC-specific deletion of the \textit{Ccm2} gene, to bypass the embryonic lethality encountered after constitutive or endothelial-specific \textit{Ccm2} ablation (Boulday et al., 2009; Whitehead et al., 2009).

**The pathological consequences of \textit{Ccm2} ablation are restricted to key time points temporally related to intense angiogenesis**

\textit{Ccm2} deletion at P1, a time of intense angiogenesis in the mouse retina and cerebellum (Yu et al., 1994; Plate, 1999; Acker et al., 2001; Dorrell and Friedlander, 2006) resulted in CCM lesions in both organs within a week after deletion. To assess whether the effect of \textit{Ccm2} deletion is temporally correlated to the angiogenic process we compared deletion of \textit{Ccm2} at P1 with deletion either at 3 wk of age, after establishment of retinal and cerebellar vessels (Acker et al., 2001; Dorrell and Friedlander, 2006), or during embryogenesis, when cerebral angiogenesis is at an intensive stage (Fig. 6; Yu et al., 1994; Plate, 1999). Tamoxifen-induced recombination efficiency was confirmed for the different protocols used (Fig. S1).

\textit{Ccm2} deletion in 3-wk-old animals did not lead to any gross cerebrovascular phenotype, as observed on iCCM2 brains upon dissection at 2 mo of age (\textit{n} = 4; Fig. 6 A, middle). Histology performed on iCCM2 brains did not reveal any vascular lesion or any sign of hemorrhage in the cerebral hemispheres and cerebellum (unpublished data). Moreover, isolecitin-B4 stainings of retinal vessels showed similar vasculature in iCCM2 and control animals (Fig. 6 B). These data show that \textit{Ccm2} deletion at 3 wk did not lead to CCM in the mouse, suggesting that endothelial \textit{Ccm2} is dispensable for mature vessels.

We and others have reported that endothelial-specific \textit{Ccm2} deletion resulted in early embryonic death around embryonic day 10.5 (E10.5; Boulday et al., 2009; Whitehead et al., 2009). In the present work, tamoxifen-induced endothelial-specific \textit{Ccm2} deletion was performed at E14.5 to bypass the early embryonic lethality. At E19.5, iCCM2 mice were distinguishable from their hemorrhagic skin (unpublished data). Upon dissection, iCCM2 brains showed vascular anomalies located on the cerebral hemispheres with irregular and tortuous rhinal cerebral veins surrounded by abnormal capillaries (Fig. 6 A, right; \textit{n} = 8).

To get some insight into the tightness of the time-window for \textit{Ccm2} deletion-mediated vascular effects, \textit{Ccm2} was ablated at different postnatal time points. P4 inactivation (\textit{n} = 4) resulted within 10 d after induction, in a milder vascular phenotype compared with P1 inactivation, which was still clearly visible upon dissection (Fig. S6 B, to be compared with Fig. S2 A). Histology of the brain showed single isolated caverns located within the cerebellum, without sign of hemorrhage (Fig. S6 E). Lesions were also observed at the periphery of the retinal vasculature (Fig. S6 H). After P8– (\textit{n} = 5) and P15– (\textit{n} = 2) \textit{Ccm2} ablation, no obvious vascular phenotype was detected upon dissection in the cerebellum, at P16 and P34, respectively (Fig. 6 D and not depicted). However, histology analysis revealed vessel dilations that might precede CCM lesion development (Fig. S6 F). Isolecitin-B4 stainings also showed vessel dilations at the periphery of the P8-ablated iCCM2 retinas (Fig. S6 J).

Collectively, our data on \textit{Ccm2} deletion during embryogenesis, the postnatal period, and at 3 wk of age strongly suggested that the effects of \textit{Ccm2} deletion on CCM lesion development were restricted to key time points temporally related to the angiogenic process.

**DISCUSSION**

In the present paper, we used an inducible, EC-specific deletion of the \textit{Ccm2} gene, to bypass the embryonic lethality encountered after constitutive or endothelial-specific \textit{Ccm2} ablation (Boulday et al., 2009; Whitehead et al., 2009).
Early post-natal deletion of Com2 resulted in vascular lesions strikingly recapitulating human CCM lesions in the brain as well as in the retina, in 100% of Com2-deleted animals. In this robust and relevant mouse model for the human CCM disease, we showed that CCM lesions affect only the venous bed and that CCM development is restricted to key time periods temporally related to intense angiogenesis.

In the past few years, different groups working in the field of CCM in vivo models concluded to a vascular, EC-specific, autonomous function of the 3 Com genes (Hogan et al., 2008; Boulday et al., 2009; Whitehead et al., 2009; He et al., 2010). Recently, a paper challenged these results showing CCM-like lesions after neuroglial-specific loss of Com3. In contrast to what was previously published, Louvi et al. (2011) demonstrated a cell autonomous effect of Com3 in astrocytes, resulting in increased cell proliferation and cell survival, as well as a cell nonautonomous effect, resulting in a vascular phenotype. This data suggest that Com genes play a role in vascular and non vascular cells within the CNS, pointing out the importance of the communication between cells composing the neurovascular unit, which may partly explain the CNS restriction of the CCM lesions.

Another approach to obtain a mouse model for human CCM has been developed using genetic sensitizers, attempting to increase the rate of somatic mutation of CCM genes and obtain vascular lesions in heterozygous Com1+/− or Com2+/− mice. On a tumor repres- sor Trp53-null background, 30% of the Com1−/− mice developed lesions (Plummer et al., 2004), but the frequency of early onset malignacies was a limitation of this model. With a similar approach, 50% of the Com1−/− mice with a mismatch repair complex null background (Msh2−/−; Com1−/−) developed lesions (McDonald et al., 2011). No lesion was obtained in the Msh2−/−; Com2−/− mice.

This sensitized mouse model, as well as the model from Louvi et al. (2011), has the advantage of progressing slowly, with a relative normal lifetime for the animal. In addition, according to what is thought to happen in human, lesion development in the heterozygous McDonald’s model occurs as a stochastic event throughout the brain (McDonald et al., 2011). In contrast, in the model described herein, a very efficient loss of both Com2 alleles is obtained in ECs, providing a more aggressive model. iCCM2 animals after P1-induction show a rapid onset of the disease, with lesion development restricted to some specific locations of the CNS, followed by an early death caused at least in part by severe hemorrhages within the CNS. However, our results using later postnatal Com2 deletion (Fig. S6) suggested that a milder mouse model, useful for long term studies, could be obtained that might allow lesion development within the brain hemispheres. This will be the subject for further analysis. Finally, it would be unrealistic to expect one animal model to fully recapitulate a human phenotype and we believe that the different CCM mouse models available so far will be complementary for mechanistic exploration.

In our study, AJ and TJ organization was strongly affected in CCM2-deficient ECs in vitro and in the CCM2 lesions in vivo, consistent with the impaired TJs described in human CCM lesions. CCM2 deletion caused complex changes in AJ and TJ organization, which included down-regulation of
juncti onal components and alteration of their distribution at intercellular contacts. Surprisingly, endothelial cell–cell TJs were maintained in the Msh2+/−; Ccm1+/− mouse model as assessed by electronic microscopy (McDonald et al., 2011). The apparent discordance between this model and our data may come from the different approaches used by the two groups. In our model, we cannot exclude that, even though we observed a down-regulation and alteration of some AJ and TJ components using immunostaining approaches, the TJ ultrastructure could be preserved. Previous studies (Whitehead et al., 2009; Stockton et al., 2010) showed that in CCM2-deficient ECs, cortical actin cytoskeleton was severely affected. This effect required the association of CCM2 with CCM1/Krit. Data presented here add to these observations and show that deletion of CCM2 causes complex changes in AJ and TJ organization, which include down-regulation of junctional components and alteration of their distribution at intercellular contacts. In a previous paper, we showed (Lampugnani et al., 2010) that similar junctions’ alterations could also be observed in CCM1-depleted ECs, further supporting the idea of a physical and functional interaction between CCM1 and CCM2. One of the most striking effects of CCM2 depletion observed in our study was the strong reduction of claudin-5 expression, which is likely the cause of altered TJ organization. This effect may explain, to a good extent, the defect in permeability control of CCM2 KO ECs in vitro and in vivo (Fig. S4; Stockton et al., 2010). Furthermore, as already mentioned, TJ are severely affected in the vascular lesions of CCM patients. Genetic deletion of claudin-5 is known to be associated to defects in blood–brain barrier (Morita et al., 1999), which leads to death immediately after birth.

Another functional consequence of alterations in AJ or TJ architecture is defective cell polarity. We previously reported that Ccm1 silencing altered VE-cadherin and AJ organization and inhibited the localization of the polarity complex at cell–cell junctions (Lampugnani et al., 2010). As a consequence, the polarized expression of apical (podocalyxin) and basal (collagen IV) proteins was affected. In this study, although junctions are altered in vivo in CCM2 lesions, apical and basal proteins seem to be correctly distributed (Fig. S2 H). It is reasonable that because CCM1, but not CCM2, also interacts with integrins and modulates their functions, this additional property may be required for cell polarity (Zovein et al., 2010).

Our results clearly showed that despite pan–endothelial Ccm2 ablation, CCM lesions did not affect all vascular beds. CCM lesions developed only in the cerebellum and the retina after P1 ablation. At the time of analysis, other highly vascularized organs, such as the heart and lungs, did not show evidence of CCM lesions upon dissection, even though Cre-mediated recombination was confirmed in those organs (unpublished data). Thus, our data clearly demonstrate that loss of Ccm2 is not sufficient to induce CCM lesions. In addition to the complete endothelial absence of CCM2, additional factors, possibly specific for the neurovascular microenvironment, might be necessary to cause the CCM disease.

Within the brain and the retina, CCM lesions affect only the venous bed. This is consistent with what is observed in human retinal CCM lesion when using retinal angiography. The bubblelike vascular structures composing the CCM retinal lesion are the last vessels to be filled up by the fluorescent dye, suggesting that lesions are composed by pocket-like capillaries connected to the venous system. In zebrafish, Ccm1 or Ccm2 knockdown using morpholinos did not affect dorsal aorta and intersomitic vessel development, but resulted in abnormal morphogenesis with major dilation of the posterior cardinal vein and the caudal vein (Hogan et al., 2008). In our study, mechanisms explaining the venous restriction of CCM2 lesions remain to be elucidated. The venous-specific effect of CCM2 ablation cannot be explained by a difference in the timing of excision that would affect a vascular bed with a later development, because veins and arteries of the superficial vascular plexus develop concomitantly in the retina (Dorrell and Friedlander, 2006; Fruttiger, 2007). We then excluded a difference in recombination efficiency between veins and arteries. As assessed by XGal staining on retinas or cerebral hemispheres, P1-tamoxifen–induced recombination was clearly affecting arteries and veins to the same extent (Fig. S1, A and B). Another trivial explanation would be a venous restriction of Ccm2 expression during late embryogenesis and the postnatal period. In a previous work, we detected a moderate labeling for all three Ccm transcripts in the heart, arterial, and venous large vessels by E14.5, decreasing at late embryonic stages (Petit et al., 2006). To further address this issue, we compared CCM2 mRNA and protein expression in mesenteric arteries and veins. No significant difference in Ccm2 gene expression level was observed in these two vascular beds at the perinatal period (unpublished data). CCM2 protein expression was also confirmed in both types of vessels (unpublished data). The venous specificity of CCM lesions could also reflect a different level of TJ component expression (i.e., claudin-5 expression) in veins versus arteries. However, claudin-5 expression, evaluated by quantitative RT-PCR was similar in mesenteric arteries and veins (unpublished data). Thus, additional work is needed to clarify what differs between veins and arteries that could explain the specific response of venous EC to Ccm2 deletion.

The main characteristic feature of affected veins at early stages of lesion development in retinas of the iCCM2 mice was an increase in the size of the veins. To understand the mechanisms of this venous dilation, we analyzed EC proliferation before lesion formation. We did not find any enhancement in EC proliferation, suggesting that proliferation is not the primary event leading to lesions. This is consistent with what was found by other groups in the mouse as well as in the zebrafish (Hogan et al., 2008; McDonald et al., 2011). In iCCM2 cerebellum, the endothelium lining the already formed CCM lesions (single or multicavernous) did not show any increase in cell proliferation compared with endothelium from controls, as assessed by stainings for the proliferation-associated nuclear protein Ki67 at P8 and P14 (Fig. 2 J and not depicted). Our results contrast with other data, showing...
an increase in proliferation of EC lining multiple mature caverns as compared with single, early cavernous lesions (McDonald et al., 2011). It is possible that the relatively short median survival in our mouse model may be a limit for analyzing EC proliferation in mature, multicavernous CCM lesions. Interestingly, loss of Ccn1 in zebrafish resulted in impaired EC morphology rather than increase in EC proliferation, with a progressive spreading and thinning of the ECs forming the dilated vessel (Hogan et al., 2008). We do hypothesize that such a mechanism could explain the phenotype described in our CCM2 mouse model.

In this paper, we showed that the timing of Ccm2 deletion (E14.5, P1, and 3 wk of age) defines the cerebral (or retinal) EC response to CCM2 loss. We first excluded differences in tamoxifen-induced recombination efficiency that could explain the disparate temporal and spatial responses to Ccm2 deletion. XGal staining confirmed a high recombination efficiency at E14.5, P1, and 3 wk of age, in all the cerebral and retinal vessels (Fig. S1, A–F). Mice induced at 3 wk, after vessel deletion, did not develop CCM lesion in the CNS. In contrast, mice induced at P1 showed CCM lesions in the cerebellum and the retina, whereas late in utero Ccm2 deletion elicits vascular malformations in the cerebral hemispheres. In those two situations, the location of CCM lesions in the CNS corresponds to specific places undergoing intense angiogenesis at the time of deletion (Plate, 1999; Acker et al., 2001; Dorrell and Friedlander, 2006). Thus, our results comparing the different timing of Ccm2 deletion strongly suggest that angiogenesis might be the extra trigger leading to CCM lesion development. Interestingly, in human CCM patients, the number of lesions increases significantly with age, particularly after 50 yr old (Denier et al., 2006; Labauge et al., 2007). In addition, it has been shown that angiogenesis can occur in human adult brain in response to cerebral ischemia (Beck and Plate, 2009). We speculate that an increase in CCM lesion number over 50 yr old may be related to proangiogenic stimuli that may be caused by hypoxic events that occur with aging.

The mechanisms of this restricted temporal CCM competence are thus far unknown. In some aspects, it is reminiscent of the previously reported restricted temporal cystogenic competence of renal epithelial cells. The autosomal dominant polycystic kidney disease is characterized by a progressive increase in renal tubular diameter followed by multiple cysts formation. A key postnatal developmental switch has been involved in this cystogenic process and has been related to abnormal planar cell polarity signaling (PCP; Fischer et al., 2006; Piontek et al., 2007; Karner et al., 2009; Verdeguer et al., 2010). PCP controls, through the coupling of cell division and morphogenesis, the growth and the size of the normal renal tube. Interestingly, in the normal developing retinal vasculature, orientation of mitosis along the vessel axis was also reported (Zeng et al., 2007), suggesting that vessel growth is determined by PCP.

While this manuscript was in revision, two other studies were published (Chan et al., 2011; Cunningham et al., 2011) that independently validated the use of inducible Ccm KO approaches to obtain mouse models for the CCM disease. All these very recent complementary in vivo studies show similarities but also differences, most likely linked to distinct methodological approaches, which will be useful to decipher the mechanisms of CCM development.

In this study, we describe a relevant and robust mouse model for CCM disease with a complete penetrance in the CNS. We believe this model to be of importance in better deciphering molecular mechanisms involved in the CCM pathogenesis. Moreover, the rapid onset of the disease in the iCCM2 mouse model makes it particularly suitable for therapeutic preclinical evaluation, especially for a fast first screening of novel agents targeting lesion genesis. Indeed, prevention of lesion development/progression/bleeding, or induction of lesion regression are now the real challenge to pursue for the CCM disease (Yadla et al., 2010).

Herein, analysis of the iCCM2 model suggests that the loss of Ccm2 is required and sufficient for the development of CCM lesions but only in a restricted spatial and temporal manner. We propose that, within an appropriate time window, a pro-angiogenic stimulus in the neurovascular unit microenvironment provides a permissive signal for venous EC from the CNS to eventually form CCM lesions.

MATERIALS AND METHODS

In vivo tamoxifen-induced deletion. Tamoxifen (Sigma-Aldrich) was diluted in sunflower oil-10% ethanol at 10 mg/ml, and subsequent dilutions were performed in sunflower oil when necessary. For postnatal deletion, pups were injected at P1 with a single intragastric injection of 20 µg tamoxifen. Deletion at 3 wk of age was performed by repeated i.p. injections of 1 mg tamoxifen for 4 consecutive days. For deletion during embryogenesis, pregnant females were injected once i.p. with 1 mg tamoxifen at E14.5, and a cesarean was performed at E19.5.

Mouse lines. The strategy used to target the Ccm2 gene in mice (Cm2 fl/fl and Cm2 deleted alleles) was previously described (Boulday et al., 2009). Ccn1 and Cm3 flxed mice were made by Taconic. The Cdh5(PAC)-CreERT2 mouse line was previously reported (Wang et al., 2010), The Rosa26-StopK-LacZ (Soriano, 1999), EphrinB2-cre2 (Wang et al., 1998), and BAT-Gal (Maretto et al., 2003) mice have been purchased from The Jackson Laboratory. Mice were all bred on a C57BL/6 background.

To obtain the iCCM2 mice, the Cdh5(PAC)-CreERT2 mice were first bred with the Ccm2fl1 animals. The Cdh5(PAC)-CreERT2; Cm2fl1 mice were then crossed with Rosa26-StopK-LacZ; Cm2fl1 animals. Thus, unless otherwise mentioned, the genotype of iCCM2 animals and controls were as follows: Cdh5(PAC)-CreERT2; Cm2fl1; Rosa26-StopK-LacZ, and Cdh5(PAC)-CreERT2; Cm2fl1; Rosa26-StopK-LacZ.

EphrinB2-cre2 and BAT-Gal mice were bred with Cm2fl1 animals before be crossed with Cdh5(PAC)-CreERT2; Cm2fl1 mice. All procedures described in this study were in full accordance with the Institutional Animal Care and Use Committee “Lariboisiere-Villemin” (Committee number 9, Paris, France).

Cell culture. Mouse lung ECs were derived from lungs of 3-mo-old Cm2 fl/+ mice (Boulday et al., 2009) and immortalized as previously described (Dong et al., 1997; Balconi et al., 2000). Conditional deletion of Cm2 in vitro was obtained using TAT-Cre fusion protein which is known to promote the nuclear translocation of Cre recombinase (Petit et al., 2002). As a control, cells were treated either with buffer or with an inactive form of TAT-Cre. Sparse ECs were washed with HyQ ADCF mAb medium (Thermo Fisher Scientific) and treated with 100 μg/ml TAT-Cre for
TOP/FOP assay. The assay was performed using a previously described standard technique (Taddei et al., 2008). In brief, $6 \times 10^5$ CCM2 WT and null cells were plated in 6-well plates to form 80% confluent cultures at time of transfection. Cells were transfected 24 h after seeding using the LipofectAMINE-2000 method (Invitrogen), in accordance with the manufacturer’s instructions. To normalize for transfection efficiency, a pCMV-β-Gal plasmid was co-transfected. 3 μg of either TOP-TK-LUC or FOP-TK-LUC (containing WT or mutant Tcf/Lef binding sites and a basal TK promoter, upstream a luciferase gene, respectively) was used in combination with 1 μg pCMV-β-Gal. Luciferase activity was assayed 48 h after transfection, using the Enhanced Luciferase Assay kit (BD). TCF/LEF β-catenin–mediated gene transcription was defined by the ratio of TOP-TK-LUC/FOP-TK-LUC luciferase activities, where the β-Gal activity of the internal control reporter pCMV-β-Gal was used to correct differences in transfection efficiency.

Western blot analysis. Western blot analysis was performed according to standard protocols. In brief, confluent cells were washed with PBS and lysed by boiling in a modified Laemmli sample buffer (2% SDS, 20% glycerol, and 125 mM Tris-HCl, pH 6.8). Lysates were incubated for 10 min at 95°C to allow protein denaturation. The concentration of protein was determined using a BCA Protein Assay kit (Thermo Fisher Scientific) according to the manufacturer’s instructions. Equal amount of proteins were loaded on gel and separated by SDS-PAGE, transferred to a Protran Nitrocellulose Hybridization Transfer Membrane 0.2 μm pore size (Whatman), and blocked for 1 h at room temperature in TBST (150 mM NaCl, 10 mM Tris-HCl, pH 7.4, and 0.05% Tween)-powdered milk. The membranes were incubated overnight at 4°C in primary antibodies diluted in TBST-5% BSA or in TBST-5% milk. Next, they were incubated for 1 h at RT with horseradish peroxidase–linked secondary antibodies (diluted in TBST-5% milk). Membranes were rinsed 3 times with TBST for 5 min each, and specific binding was detected by the enhanced chemiluminescence system (GE Healthcare) using Hyperfilm (GE Healthcare). The molecular masses of proteins were estimated relative to the electrophoretic mobility of the co-transferred, pre-stained protein marker Broad Range (Cell Signaling Technology).

Histology, β-galactosidase staining, and immunofluorescence. Brains were fixed by immersion overnight in 4% paraformaldehyde, before being paraffin-embedded. For histology analysis, hematoxylin and eosin (H&E) staining was performed on every 10 sections (10 μm each) on half-sagittal brain. Whole-mount staining for β-galactosidase activity was performed by incubation overnight with XGal (1mg/ml solution) after 30 min fixation in cold 0.1% formaldehyde fixation buffer. Immunofluorescence on frozen sections was performed after fixation in aceton or cold methanol. Sections were counterstained with DAPI and mounted in a fluorescent mounting medium (Dako). The following antibodies were used for immunohistochemistry and/or immunofluorescence: rat anti-PECAM (MEC13.3, BD); rat anti–VE-cadherin (BD); rabbit anti–ZO1 (Zymed); rabbit anti-claudin-5 (from H. Wolburg, Institute of Pathology, University of Tubingen, Tubingen, Germany); rabbit anti-phosphohistone H3 (Abcam); peroxidase-conjugated anti–rat (Jackson Immunoresearch Laboratories); Alexa Fluor S94–conjugated anti–rat (Invitrogen); Alexa Fluor 488–conjugated donkey anti–rabbit (Invitrogen); FITC–conjugated anti–rabbit (Jackson Immunoresearch Laboratories).

Immunofluorescence on whole-mount retinas was performed as previously described (Pitulescu et al., 2010). Staining of retinal vessels was obtained by incubations with biotin-conjugated isocitrate-B (AbCys) and Cy3-streptavidin (GE Healthcare). NIS-Elements imaging software (Nikon) was used to quantify vascular coverage at the venous leading edge of the retinal vasculature (vascular area relative to the total retinal area analyzed) in control and iCCM2 retinas ($n = 4$ animals with similar weight in each group, 6–8 fields per retina).

Immunofluorescence was analyzed using a Nikon Eclipse 80i microscope or by confocal microscopy (TCS-SP2-AOBS, Leica).

Statistics. Student’s two-tailed nonpaired t test was used to determine statistical significance for in vitro analysis. The significance level was set at $P < 0.05$. Kaplan-Meier test was used to determine survival curve of the iCCM2 animals versus controls.

Online supplemental material. Fig. S1 shows the analysis of tamoxifen-induced recombination. Fig. S2 shows additional analysis of CCM2 lesions in the cerebellum. Fig. S3 shows the cerebellar and retinal CCM lesions obtained after Cam1 and Cam3 ablation at P1. Fig. S4 shows the analysis of AJ and TJ junctions in vitro in CCM2 KO ECs. Fig. S5 shows the analysis of molecular regulators of claudin-5 expression in vitro in CCM2 KO ECs. Fig. S6 shows the analysis of impact of the postnatal timing for Cam2 ablation on the vascular phenotype severity. Online supplemental material is available at http://www.jem.org/cgi/content/full/jem.20110571/DC1.

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