Tissue-specific conditional CCM2 knockout mice establish the essential role of endothelial CCM2 in angiogenesis: implications for human cerebral cavernous malformations

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SUMMARY
Cerebral cavernous malformations (CCM) are vascular malformations of the brain that lead to cerebral hemorrhages. In 20% of CCM patients, this results from an autosomal dominant condition caused by loss-of-function mutations in one of the three CCM genes. High expression levels of the CCM genes in the neuroepithelium indicate that CCM lesions might be caused by a loss of function of these genes in neural cells rather than in vascular cells. However, their in vivo function, particularly during cerebral angiogenesis, is totally unknown. We developed mice with constitutive and tissue-specific CCM2 deletions to investigate CCM2 function in vivo. Constitutive deletion of CCM2 leads to early embryonic death. Deletion of CCM2 from endothelial cells severely affects angiogenesis, leading to morphogenic defects in the major arterial and venous blood vessels and in the heart, and results in embryonic lethality at mid-gestation. These findings establish the essential role of endothelial CCM2 in proper vascular development and strongly suggest that the endothelial cell is the primary target in the cascade of events leading from CCM2 mutations to CCM cerebrovascular lesions.

INTRODUCTION
Cerebral cavernous malformations (CCM) are slow-flow vascular anomalies characterized by densely packed vascular sinusoids embedded in a collagen matrix without intervening neural tissue (Russell and Rubinstein, 1989). These clusters of vascular sinusoids (also called caverns) are lined by a thin endothelium and by rare subendothelial cells (Clatterbuck et al., 2001). An absence of tight junctions between endothelial cells (ECs) has been reported in CCM lesions upon ultrastructural examination. Most of these lesions are located within the central nervous system (CNS) but they may also affect the retina (for a review, see Labauge et al., 2007). Clinical onset is generally around 20-30 years of age but symptoms can start in early infancy and in old age. The most common manifestations include headaches, seizures and focal neurological deficits caused by cerebral hemorrhages.

From large series studies, the prevalence of CCM in the general population has been estimated to be close to 0.1-0.5% and CCM bleeding is involved in more than 10% of young patients who show intracerebral hemorrhage (Otten et al., 1989; Moussa et al., 2006). Both sporadic and familial autosomal dominant forms of the disorder have been identified (Rigamonti et al., 1998; Labauge et al., 2007). Familial CCM (FCCM) cases are characterized by the presence of multiple lesions upon cerebral magnetic resonance imaging (MRI) and the major risk endured by FCCM patients is the recurrence of cerebral hemorrhages.

Our group and others have identified three CCM genes namely CCM1/KRIT1, CCM2/MGC4607 (also called malcavernin) 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). The mutations detected in CCM patients are loss-of-function mutations. It has been suggested that a Knudson two-hit mechanism is likely to be involved in CCM pathophysiology, as reported previously in some other vascular conditions (Brouillard et al., 2002). This is based on the observed autosomal dominant pattern of CCM inheritance and the presence of multiple lesions in FCCM, contrasting with the detection of a single lesion in nonhereditary cavernous angiomas. In addition, recent data showing biallelic mutations in CCM genes (germline and somatic) have been reported (Gault et al., 2005; Akers et al., 2008; Pagenstecher et al., 2008).

The CCM1 gene encodes KRIT1, a 736-amino acid protein containing three ankyrin domains and a FERM (protein 4.1, ezrin, radixin, moesin) domain. CCM2/MGC4607 encodes a protein containing a phosphotyrosine-binding domain (PTB) and CCM3/PDCD10 encodes a protein, without a known conserved functional domain, which was shown recently to interact with STK serine threonine kinases (Ma et al., 2007; Voss et al., 2007). Ccm genes have highly similar patterns of expression in both the embryo and adult mouse. Within the CNS, Ccm transcripts are detected mostly in neuronal cell layers (Petit et al., 2006; Plummer et al.,

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By embryonic day E14.5, moderate labeling of all three transcripts can be observed in the large arterial and venous blood vessels and in the heart. Ccm1 mRNA is not detected in small cerebral vessels at any stage of development. Ccm2 and Ccm3 mRNAs are weakly, and only transiently, detected within meningeal and parenchymal cortical vessels at P8. Regarding CCM protein expression in tissue sections, controversial results have been published that are most likely explained by a lack of antibody specificity. Inter-relations between neural and vascular cells are crucial for proper cerebrovascular development and, therefore, the specificity. Inter-relations between neural and vascular cells are published that are most likely explained by a lack of antibody mRNAs are weakly, and only transiently, detected within meningeal vessels and in the heart. Transcripts can be observed in the large arterial and venous blood vessels and in the heart. Ccm1 mRNA is not detected in small cerebral vessels at any stage of development. Ccm2 and Ccm3 mRNAs are weakly, and only transiently, detected within meningeal and parenchymal cortical vessels at P8. Regarding CCM protein expression in tissue sections, controversial results have been published that are most likely explained by a lack of antibody specificity. Inter-relations between neural and vascular cells are crucial for proper cerebrovascular development and, therefore, the specificity. Inter-relations between neural and vascular cells are published that are most likely explained by a lack of antibody mRNAs are weakly, and only transiently, detected within meningeal

Recent in vitro data suggest strongly that CCM proteins are scaffold proteins that exist as a complex in cells (Zhang et al., 2001; Zawistowski et al., 2002; Zawistowski et al., 2005; Hilder et al., 2007). CCM1 has been shown to interact with (1) the PTB domain of CCM2 protein, (2) RAP1A, a small Ras-like GTPase (Serebriiski et al., 1997; Glading et al., 2007), and (3) ICAP1, a protein that binds to the cytoplasmic tail of integrin β1 (Zhang et al., 2001; Zawistowski et al., 2002). The yeast ortholog of CCM2, OSM (osmosensing scaffold for MEKK3), has been identified as a scaffold protein that interacts with kinases involved in the p38 mitogen-activated kinase pathway in response to osmolarity stress (Uhlik et al., 2003). It also interacts with CCM1 and CCM3, as well as with MEKK3, RAC1, RIN2 (a protein shown to regulate E-cadherin internalization), actin and tubulins. In addition, CCM2 and CCM3 bind to phosphatidylinositol phospholipids (Hilder et al., 2007). Altogether, these data suggest that the three CCM proteins are members of a larger signaling complex that is involved in cell-cell junction homeostasis and cytoskeleton remodeling.

These biochemical data have provided useful insights into CCM protein function. However, their in vivo function, particularly during cerebral angiogenesis, remains obscure. In the zebrafish embryo, Ccm1 and Ccm2 are expressed mostly in the brain, the notochord and the posterior cardinal vein. Their inactivation leads to an early death with massive dilatation of both the heart and large venous vessels (Mably et al., 2006; Hogan et al., 2008). Constitutive deletion of CCM1 in the mouse leads to abnormal arterial morphogenesis including both enlargement and narrowing of large trunk arteries and ultimately to mid-gestation death (Whitehead et al., 2004).

Here, we used constitutive and tissue-specific inactivation of CCM2 to investigate its role in mouse vascular development. We showed that (1) neuroepithelial expression of CCM2 is dispensable for proper vascular development and (2) endothelium-specific inactivation of CCM2 results in embryonic lethality at mid-gestation, abnormal angiogenesis and massive heart and blood vessel defects.

**RESULTS**

**Targeting Ccm2 in mice**

We targeted the Ccm2 locus in embryonic stem (ES) cells by using a linearized construct containing exons 3 and 4 of the Ccm2 gene, which encode most of the PTB (Fig. 1A and Methods). Mutations leading to transcripts with these two exons deleted have been detected in several CCM patients and result in a frameshift and a premature stop codon, leading to partial mRNA decay (Denier et al., 2004) (supplementary material Fig. S1). Ccm2 gene targeting was confirmed by Southern blot on ES cells and mice, and by northern blot and western blot analysis (Fig. 1B-D; supplementary material Fig. S1). When Ccm2+/floxed (Ccm2-/+floxed) mice were interbred, we did not obtain any Ccm2-null pups (from a total of 96 offspring) (Table 1), strongly suggesting in utero lethality. During embryogenesis, all of the different genotypes were recovered at a Mendelian ratio (Table 1). The absence of the CCM2 protein in null embryos was confirmed by western blot analysis (Fig. 1D). No phenotypic difference between embryos was detected upon dissection at E8.5 (supplementary material Fig. S3). At E9.5, mutant mice were recognized because their very pale
yolk sacs (YSs) showed a wrinkled appearance (supplementary material Fig. S3). Homozygous null embryos showed a developmental delay with failure to complete turning and signs of resorption. Most Ccm2-null embryos had a massive pericardial edema at E9.5 and their hearts, which were sometimes still beating, were showing a retarded S-shape. No Ccm2-null embryos were found alive at E10.5; all of them were already in resorption (supplementary material Fig. S3). Altogether, our results show that constitutive deletion of Ccm2 leads to cardiovascular failure and mid-gestation embryonic death.

Cerebrovascular development does not require expression of CCM2 in neuroepithelial precursor cells

To assess whether CCM2 expression in neuroepithelial cells is required for proper vascular development in the mouse brain, we specifically ablated CCM2 in these cells by using a nestin-Cre line that expresses Cre under the control of the neural enhancer element of the nestin promoter (Tronche et al., 1999). This transgene drives Cre-mediated recombination in neural precursor cells giving rise to both neurons and glia, but does not drive recombination in ECs (Graus-Porta et al., 2001). Briefly, we sequentially crossed the transgenic nestin-Cre line with Ccm2\textsuperscript{-/-} and Ccm2 floxed mice to generate NPKO mice (nestin-Cre; Ccm2\textsuperscript{-/-flox}). We obtained NPKO mice with the expected Mendelian ratio at weaning (27% from a total of 176 offspring). NPKO mice were fertile and indistinguishable from their control littermates at up to 1 year of age. They did not have any significant weight difference or detectable neurological defect.

The neural-specific recombination of the floxed allele and the absence of the CCM2 protein within the brain of NPKO mice were confirmed at P8 (Fig. 2A,B). In addition, X-Gal staining performed on E12.5 embryos, obtained from crosses between nestin-Cre; Ccm2\textsuperscript{+/–} and Ccm2\textsuperscript{-/–flox} mice, demonstrated an absence of recombination in the ECs. We also performed immunoblotting for α-tubulin and CCM2 with protein lysates from NPKO and control littermates at P8. No CCM2 protein was detected in NPKO brain lysates. A Ccm2\textsuperscript{-/-flox} brain from a littermate was used as a control for the absence of recombination (second lane from the left). The DNA fragment in the first lane is slightly lower than the floxed fragment and corresponds to the 8.13 kb wild-type allele.

Fig. 2. Conditional deletion of CCM2 from neuroglial precursors does not lead to major cerebrovascular defects. (A-C) Analysis of the specific inactivation of CCM2 in the neuralglial compartment. (A) Southern blot analysis using 12 μg of genomic DNA extracted from tissues at P8. DNA was digested by HindIII and hybridized with the external radiolabeled 3’ probe P2. The floxed allele (8.2 kb) in NPKO was recombined specifically within the brain of NPKO mice. A Ccm2\textsuperscript{-/-flox} brain from a littermate was used as a control for the absence of recombination (second lane from the left). The 6.3 kb and the 8.2 kb DNA fragments represent the Ccm2-deleted and floxed alleles, respectively. Note that the DNA fragment in the first lane is slightly lower than the floxed fragment and corresponds to the 8.13 kb wild-type allele. (B) Western blot analysis of CCM2 protein expression within the brain (100 μg protein lysates) and the lung (70 μg protein lysates) from NPKO and control littermates at P8. CCM2 protein was not detected in NPKO brain lysates. Protein lysate from Ccm2\textsuperscript{-/-flox} HEK cells was used as a positive control (200 ng). Note that the second lane of the blot is free of sample. Immunoblotting for α-tubulin on the same blot was performed as a loading control. (C) β-galactosidase expression analysis on a E12.5 embryo obtained after crossing a nestin-Cre; Ccm2\textsuperscript{-/-flox} animal with a Rosa26R reporter line (genotype of the embryo: nestin-Cre; Rosa26R; Ccm2\textsuperscript{-/-flox}). Note that the blood vessels (red arrow) are not blue, demonstrating an absence of recombination in the ECs. (D,E) Analysis of the brains from NPKO and control mice. (D) Analysis of 2 mm-thick brain coronal sections from 2-month-old NPKO and control animals under a dissecting microscope, showing slices of the cerebrum (left panels) and the cerebellum (right panels). (E) Hematoxylin and eosin (H&E) staining on 10 μm paraffin-embedded brain sections from NPKO or control mice at P19. B, brain; C, control; cc, corpus callosum; co, cortex; cpu, caudate putamen; Fb, forebrain; fd, fascia dentata; H, heart; Hb, hindbrain; h, hippocampus; K, kidney; L, liver; Lu, lung; Mb, midbrain; Nt, neural tube; ob, olfactory bulb; S, spleen; T, toe; v, ventricle.
Endothelial-specific ablation of CCM2 leads to mid-gestation embryonic death

In order to determine whether the disruption of CCM2 in ECs leads to vascular defects, we generated mice with an endothelium-restricted deletion of CCM2, by using a previously well-characterized Tie2-Cre transgenic mouse (Kisanuki et al., 2001). Intercrosses between Tie2-Cre; Ccm2<sup>+/−</sup> and Ccm2 floxed mice did not produce any Tie2-Cre; Ccm2<sup>−/−</sup> mice (ECKO) at weaning (from a total of 77 offspring) (Fig. 3A). During embryogenesis, all the genotypes were obtained at the expected Mendelian ratios at the different stages tested.

EC-specific Cre expression was confirmed in the YS and embryo vasculature at E9.5, and Cre-mediated recombination of the Ccm2 floxed allele was observed in the endothelium (supplementary material Fig. S4 and S5).

At E8.5, ECKO embryos could not be distinguished from controls (data not shown). At E9.5, most of the ECKO embryos were comparable in size to the control embryos and did not show any obvious anomaly. However, 10% of them were retarded and failed to complete turning, showing a phenotype similar to the one described in the constitutive null embryos at this stage (supplementary material Fig. S3 and data not shown). At E10.5, ECKO embryos exhibited phenotypes of variable severity, which we assigned to three classes (Fig. 3B-E). Class I included 19% of ECKO embryos dissected at this stage and corresponded to ECKO embryos showing no significant size difference compared with controls (Fig. 3C). A slight developmental delay restricted to the craniofacial part of the embryos was noticed in several ECKO embryos of this group. Most ECKO embryos were assigned to class II (58%); they were characterized by a marked general developmental delay (Fig. 3D). Class III included the remaining 23% of ECKO embryos, which showed general growth arrest, a failure to complete turning and/or signs of resorption; the heart was still beating in most of these embryos (Fig. 3E). At E10.5, most ECKO embryos had a pericardial edema that was more severe in class II and III embryos. Half of the mutants had signs of hemorrhage in the pericardial cavity and in the trunk. Interestingly, we never observed any hemorrhage within the upper part of the embryo and the head. No ECKO embryos were found alive at E11.5 and E12.5.

Abnormal remodeling of the extra-embryonic vasculature in ECKO embryos

The blood islands of the YS are the first sites of vasculogenesis and their fusion gives rise to the primitive vascular plexus. In wild-type embryos at around E9, the initial honeycomb-like pattern of the YS vasculature is remodeled into a more mature and branched pattern of vitelline vessels (Risau, 1997). YSs of ECKO embryos were wrinkled, similar to those of Ccm2-null embryos, allowing the identification of mutant embryos during dissection. YS vessels remained in the honeycomb pattern and failed to develop into large vitelline vessels (Fig. 4B; supplementary material Fig. S4B). Endodermal and mesodermal layers were less frequently connected compared with in control embryos and almost no blood cells were detected in the immature vessels (Fig. 4C,D). These data suggest that vasculogenesis occurred normally in ECKO YSs but that angiogenesis was impaired, with severe remodeling defects.

Vascular defects were also observed in the placental labyrinth. At E10.5, the different layers of the placenta were difficult to define and appeared poorly organized in ECKO embryos (Fig. 4F). Further, embryonic blood vessels did not invade the labyrinthine layer to the same extent as seen in control placenta.

### Table 1

<table>
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<tr>
<th>Age</th>
<th>Total Number</th>
<th>Viable ECKO&lt;sup&gt;+&lt;/sup&gt;/ECKO total</th>
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<td>74</td>
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<tr>
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<td>32/38</td>
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<td>P21</td>
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* Heart is still beating
Disease Models & Mechanisms

Embryonic vasculature in ECKO embryos is abnormal and shows impaired recruitment of vascular smooth muscle cells

Visualization of the embryonic vasculature at E9.5, using whole-mount platelet endothelial cell adhesion molecule (PECAM) staining, revealed the presence of all major blood vessels, paired dorsal aortae (DA), branchial arch arteries, cardinal veins and intersomitic vessels in ECKO embryos, suggesting that vasculogenesis occurred normally.

However, in E9.5 ECKO embryos, the DA already had an irregular appearance and were thinner than in controls (Fig. 5B). At E10.5, the vascular phenotype was even more severe; the DA were now highly irregular in appearance and had narrow lumens (Fig. 5E,J). In the caudal region, DA failed to fuse in most ECKO embryos, suggesting that mural cell recruitment could be detected at E9.5 and DA sections were clearly SMA positive at E10.5 in both the trunk and the caudal region (Fig. 5O and data not shown). In contrast, mural cells were hardly detectable in the DA of ECKO embryos although SMA staining was detected in the somites and in the heart (Fig. 5P; Fig. 6l and data not shown). Only the class I ECKO embryos showed some rare mural cell recruitment in the DA (data not shown).

Heart defects in ECKO embryos

Although most ECKO embryo hearts were still beating at E10.5 (the hearts of a few class III embryos were not), they were enlarged and showed edematous pericardial swelling and bleeding, a hallmark of cardiac failure (Fig. 3C-E). In class I and some class II ECKO embryos, the size of the heart, assessed on an atrioventricular axis, relative to the size of the embryo from the hindbrain/midbrain boundary to the rostral part of the limbbud, was about 17% larger than in control embryos. Chamber specification appeared normal and, at this stage, ECKO embryos showed both a common atrial and a common ventricular chamber (Fig. 6F).

Several embryos had an enlarged outflow tract and almost all of them had an enlarged atrium (Fig. 6A-D). In most severe cases, the massively enlarged atrium and sinus venosus led to distortion of the embryo. However, the atrial wall of ECKO embryos was comparable to control embryos, with a single layer of endocardial cells surrounded by a few layers of cardiomyocytes, including a monolayer of SMA-positive cells (Fig. 6F,L). Interestingly, the dilations that were seen in the atrial chamber of the heart, as well as in the venous system, could not be correlated to an increase in the proliferative rate of ECs, as determined by double labeling with antibodies for PECAM and phosphorylated histone 3 (data not shown).

Ventricular trabeculations were strongly reduced with, in some extreme cases, detachment of the endocardial cells from the myocardium (Fig. 6) and data not shown). This much thinner...
ventricular wall could be responsible for blood cells leaking into the pericardial cavity, which was observed in some ECKO embryos. In addition, there was a paucity of cells in the cushions in the atrioventricular canal (Fig. 6H), suggesting a reduction in the ability of endocardial cells to invade the cardiac jelly and undergo endocardial-mesenchymal transformation (Conway et al., 2003).

DISCUSSION

Loss-of-function mutations in the CCM2 gene in humans lead to cerebrovascular malformations, causing recurrent brain hemorrhages. Here, we demonstrate that, in spite of CCM2 being predominantly expressed in the neuronal layers within the CNS, deletion of CCM2 from neuroglial precursor cells in mice does not have a major phenotypic effect. In contrast, deletion of CCM2 from ECs leads to a very severe vascular phenotype. Vasculogenesis appears normal in ECKO embryos, contrasting with marked angiogenesis remodeling abnormalities in the extra-embryonic and embryonic vasculature that lead to major heart defects and both arterial and venous defects. These data establish the role of endothelial CCM2 in angiogenesis.

In constitutive null embryos, lethality occurred one embryonic day earlier than in ECKO embryos. Further, whilst all null embryos are already in resorption at E10.5, only 15% of ECKO embryos are dead at this stage. This delayed lethality might be explained by the incomplete and progressive endothelial-specific recombination induced by the Tie2-Cre cell line. Kisanuki et al. showed that EC-specific recombination induced by this Tie2-Cre line starts at E7.5 in some blood islands but is still not complete at E9.5. However,
we cannot exclude a role for CCM2 in other cell types, such as in vascular smooth muscle cells, which might also explain the more severe phenotype of constitutive null embryos.

The neural expression of Ccm2 and other Ccm genes suggested that CCM vascular lesions could be secondary to neural defects. However, NPKO mice did not show neurological symptoms and we did not observe any vascular lesion within the brain of NPKO mice, up to 6 months of age. Interestingly, constitutive ablation of CCM1 did not lead to neural lesions in Ccm1<sup>−/−</sup> embryos (Whitehead et al., 2004). Further analysis of NPKO mice would be required to search for subtle neuropathological lesions that might affect the cerebral cortex and to investigate the functional consequences of a complete loss of CCM2 in neurons.

Some of the vascular defects described here in ECKO embryos are reminiscent of those reported in embryos with constitutive CCM1 inactivation (Whitehead et al., 2004). In Ccm1<sup>−/−</sup> embryos, vasculogenesis seems intact but angiogenesis is severely compromised. Ccm1<sup>−/−</sup> embryos die at E10.5 and show multiple arterial morphogenesis defects including a variable narrowing of both branchial arch arteries and the proximal dorsal aorta, as well as heart defects including atrial enlargement and signs of cardiovascular failure. In addition, Ccm1<sup>−/−</sup> embryos show a defect in mural cell recruitment, similar to that observed in our ECKO embryos. However, the main phenotype reported in Ccm1<sup>−/−</sup> embryos is an extensive vascular dilation of the dorsal and caudal aortae, and of the cranial and intersomitic vessels, which was never observed in our ECKO embryos. These defects have been associated with an increase in the proliferative rate of ECs of the dilated dorsal aortae and a downregulation of arterial-specific markers including Efnb2, neither of which was observed in ECKO embryos. Interestingly, no dilation of the venous compartment was reported in Ccm1<sup>−/−</sup> embryos, contrasting with the dilation of the common cardinal vein and sinus venosus in CCM2 ECKO embryos and dilation of the major large venous vessels in ccm1<sup>−/−</sup> zebrafish embryos (Hogan et al., 2008).

Recessive loss-of-function mutations in <i>santa</i> and <i>valentine</i>, the respective orthologs of CCM1 and CCM2 in zebrafish, lead to similar and major cardiac and vascular defects (Mably et al., 2006; Hogan et al., 2008). The cardiac phenotype observed in these mutants is also highly similar to that observed in <i>heart of glass</i> mutants (Mably et al., 2003) and is characterized by a massive dilation of the heart chambers, which show only one layer of cardiomyocytes instead of the two or three layers present in wild-type embryos. Enlargement of the atrium is one of the main phenotypic features in ECKO embryos; however, we did not observe any anomaly in the number of cell layers composing the atrial wall of these embryos. The vascular phenotype of Ccm1<sup>−/−</sup> embryos, and to a lesser degree ccm2<sup>−/−</sup> zebrafish embryos, is characterized by a progressive and massive dilation of venous vessels including the caudal vein and posterior cardinal vein, contrasting with the normal development of dorsal aorta and intersomitic vessels (Hogan et al., 2008). This venous defect is associated with a progressive thinning and spreading of ECs, which is EC cell-autonomous, strongly suggesting that CCM1 is involved in the control of EC shape. Interestingly, the authors did not observe an increase in EC proliferation rate or any modification in the expression of the arterial specification markers.

Blood vessel growth and differentiation involves a broad spectrum of genetic and physical signals, such as blood flow; one of the main challenges in the analysis of these various animal models is to differentiate the primary effects of mutations on the vasculature from the secondary effects of altered blood flow (Carmeliet, 2000; Lucitti et al., 2007). Indeed, most of the genes expressed in ECs are expressed in endocardial cells and when mutated, many of them, such as Ccm2, lead to cardiac defects. Altered blood flow may in turn influence extra-embryonic and embryonic vessel morphogenesis and remodeling (le Noble et al., 2004; Jones et al., 2008). Altered expression of arterial-specific markers such as Efnb2 can also be a secondary effect of abnormal blood flow (le Noble et al., 2004). Even very subtle differences in flow patterns may result in remodeling abnormalities (Lucitti et

**Fig. 6. Cardiac defects in ECKO embryos.** (A-D) Hearts from control (A) and ECKO embryos (B-D) after whole-mount PECAM staining at E10.5. (E-J) Heart sections from PECAM-stained control (E) and ECKO (F) embryos, counterstained with eosin, showing the common atrial chamber (a) and the common ventricular chamber (v). The black and red boxes in (E,F) are shown enlarged in (G,H) and (I,J), respectively. (G,H) A reduction of cells was observed in the atrioventricular canal in the ECKO heart. (I,J) Ventricular trabeculations are strongly reduced in the ECKO heart (J). (K,L) Double staining for PECAM (red) and SMA (green) showing the atrial wall from a control (K) and an ECKO (L) embryo. Sections were counterstained with DAPI. Bars, 100 µm (E,F); 25 µm (G-J); 20 µm (K,L).
The current generation of mouse models, with their temporally controlled CCM2 deletion from blood vessels that begins at later stages, should help to elucidate this issue and perhaps help to obtain models that more closely recapitulate the human CCM phenotype and thus allow dissection of the relevant cellular and biochemical pathways involved in this condition.

**METHODS**

**Targeting the Ccm2 gene and generation of CCM2 mouse mutants**

ES cells (129 background) were electroporated with a linearized 10.1 kb construct, containing three loxP sites flanking a GFP-hygrozymic resistance cassette (2.7 kb) and exons 3 and 4 of the Ccm2 gene (Fig. 1A). Hygromycin was used to select a positive ES clone, which was then used to inject C57BL/6 blastocysts. The chimeric tri-lox mice were bred with MeuCre40 mice (Leneuve et al., 2003) to remove the cassette and to obtain mosaic animals; these were then backcrossed with wild-type C57BL/6 mice. Ccm2+/− and Ccm2+/lox mutants, selected to be free of the Cre transgene, were backcrossed with C57BL/6 mice at least seven times to establish a C57BL/6 genetic background.

Southern blot analysis was developed to confirm transmission of the targeted Ccm2 locus. Two different DNA fragments that were external to the targeting construct, one at the 5’ end (245 bp) and one at the 3’ end (282 bp), were radiolabeled before being probed with Xbal- or HindIII-digested genomic DNA.

The absence of the Ccm2 protein in mutants was confirmed by western blot. Total protein lysates were prepared from embryos or mouse tissues and lyzed in RIPA buffer that had been supplemented with protease inhibitors. The Ccm2 protein (48.8 kDa) was detected using a purified polyclonal antibody, which was raised against a 15-amino acid peptide located at the C-terminal end of the Ccm2 protein (peptide sequence: NH2-DDRSAPSEGDEWDRM-COOH; Ab made by Eurogentec). Lysates from full-length-CCM2-transfected HEK 293T cells were used as a positive control (200 ng). Western blots for α-tubulin (clone DM 1A, Sigma), performed on the same blots, were used as a control for the amount of protein loaded.

Offspring genotyping was analyzed by PCR on genomic DNA using the following primers (5’ to 3’): wild-type (177 bp) and floxed allele (263 bp), ATGGCACCTTGTGTTTCCAC and TG-GCATCGAGAAATCTTTCA; deleted allele (285 bp), ATG-GACCTTTGCTTTTCCAC and ACCCTGCTGTCTGAAAGG; Cre (370 bp), TCAATTATTGAGGCTAGACC and CGTTTTCTTTCGGATCC.

**Mouse lines**

The nestin-Cre mice (Tronche et al., 1999), Tie2-Cre mice (Kisanuki et al., 2003), Rosa26R reporter line (Soriano, 1999) and Efnb2-tau-lacZ reporter transgenic mouse (Wang et al., 1998) have been described previously. Mice were all bred with a C57BL/6 background. The procedure followed in the care and euthanasia of study animals was in accordance with European Community standards on the care and use of laboratory animals (Ministère de l' Agriculture, France).

**Histology**

Whole brains were fixed by immersion overnight in 4% PFA, before being sectioned in 2 mm coronal slices using an acrylic brain matrix (electron microscopy services) for examination under a dissecting microscope. H&E staining was performed on paraffin-embedded sections. Placentas and YSs were fixed in 4% PFA and embedded in paraffin. Histological analysis was performed on 7 μm sections using H&E staining.

**Immunohistochemistry and immunofluorescence**

The following antibodies were used for immunohistochemistry and/or immunofluorescence: anti-PECAM (1:100; MEC13.3, BD Pharmingen); FITC-conjugated anti-αSM A (1:100; Clone 1A4, Sigma); anti-phosphohistone H3 (1:200; Abcam); peroxidase-conjugated anti-rat IgG (H+L) (1:500; Jackson ImmunoResearch Laboratories); Alexa Fluor 594-conjugated anti-rat IgG (H+L) (1:200; Molecular Probe); FITC-conjugated anti-rabbit IgG (H+L) (1:100; Jackson ImmunoResearch Laboratories).

Immunohistochemistry on whole embryos was performed as described previously (Nagy et al., 2003) after neutralization of free aldehyde residues in PFA-fixed embryos by incubation with 100 mM glycine for 20 minutes.

X-gal staining on whole embryos was performed as described previously (Moessler et al., 1996). Sections (7 μm) of the paraffin-embedded embryos were counterstained with 1% eosin.

**Clinical issue**

Bleeding owing to cerebral cavernous malformations (CCM) is involved in 10 percent of young patients who show cerebral hemorrhage. These cerebral vascular malformations can result from either sporadic or familial autosomal dominant (FCCM) conditions. Recurrent bleeding is the major complication in patients affected by FCCM, which is characterized by the presence of multiple CCM lesions. FCCM is caused by loss-of-function mutations in any of the three CCM genes identified so far. High expression levels of the CCM genes in the neuroepithelium indicate that CCM lesions may be caused by a loss of function of CCM genes in neural cells rather than in vascular cells. In vitro data suggest that CCM proteins are members of a large signaling complex involved in cell-cell junction homeostasis and cytoskeleton remodeling; however, their in vivo function, particularly during cerebral angiogenesis, is totally unknown and the mechanisms leading to the formation of CCM lesions are obscure.

**Results**

Here, we used constitutive and tissue-specific inactivation of CCM2 to investigate its role in vascular development in the mouse. Constitutive deletion of CCM2 leads to an early embryonic death. Deletion of CCM2 from neuroglial precursor cells by using nestin-Cre transgenic mice results in mice with a normal phenotype, whereas deletion of CCM2 from endothelial cells by using Tie2-Cre transgenic mice severely hampers angiogenesis, leading to morphogenic defects in the major arterial and venous blood vessels and in the heart, and results in embryonic lethality at mid-gestation.

**Implications and future directions**

This study provides the first definitive demonstration that, despite the strong neuroepithelial expression of CCM2, it is the endothelial expression of CCM2 that is crucial for proper angiogenesis. It also strongly suggests that the endothelial cell is the primary target in the cascade of events leading from CCM2 mutations to CCM cerebrovascular lesions. These findings have important implications for the understanding of the mechanisms surrounding CCM lesion development. They indicate that development of these vascular lesions, which are found almost exclusively within the brain, may require additional events to take place within the cerebral environment, which have yet to be identified.

**doi:** 10.1242/dmm.002584

**Translational impact**

**Clinical issue**

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Immunohistochemistry on frozen sections was performed after fixation in aceton and permeabilization of the tissue. Sections were counterstained with DAPI and mounted in a fluorescent mounting medium (DakoCytomation).

ACKNOWLEDGEMENTS
This work was supported by the Agence Nationale pour la recherche grant ANR-07-MIRAR-002-01 (to E.-L.), the Leducq grant 07 CVD 02 hemorraghic stroke (to E.-L.), INSERM. G.B. has been partly supported by the PHRC grant AOR0301. N.P. was sequentially supported by Lefoulon Delalande and Fédération pour les Maladies Orphelines FMO and ‘Association Cavernomes France’ fellowships. We thank deeply A. Joutel from UMR-S 740, A. Eichmann from INSERM U833 and S.M. Meilhac from CNRS URA 2578 for very helpful discussions, and M. Arnoult for excellent technical help.

COMPETING INTERESTS
The authors declare no competing financial interests.

AUTHOR CONTRIBUTIONS
G.B. was the principal person for the experiments involving mice, and wrote the first draft of the paper; A.B. contributed to breeding, genotyping and phenotyping.

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The authors declare no competing financial interests.

AUTHOR CONTRIBUTIONS

SUPPLEMENTARY MATERIAL
Supplementary material for this article is available at http://dmm.biologists.org/content/1/16/759/suppl/DC1

Received 6 August 2008; Accepted 10 December 2008.

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