Cerebral Cavernous Malformation-1 Protein Controls DLL4-Notch3 Signaling Between the Endothelium and Pericytes

Gerald B. Schulz; Elfriede Wieland, MSc; Joycelyn Wüstemüller, PhD; Gwénoia Boulday, PhD; Iris Moll; Elisabeth Tournier-Lasserve, MD; Andreas Fischer, MD

Background and Purpose—Cerebral cavernous malformation (CCM) is a neurovascular dysplasia characterized by conglomerates of enlarged endothelial channels in the central nervous system, which are almost devoid of pericytes or smooth muscle cells. This disease is caused by loss-of-function mutations in CCM1, CCM2, or CCM3 genes in endothelial cells, making blood vessels highly susceptible to angiogenic stimuli. CCM1- and CCM3-silenced endothelial cells have a reduced expression of the Notch ligand Delta-like 4 (DLL4) resulting in impaired Notch signaling and irregular sprouting angiogenesis. This study aimed to address if DLL4, which is exclusively expressed on endothelial cells, may influence interactions of endothelial cells with pericytes, which express Notch3 as the predominant Notch receptor.

Methods—Genetic manipulation of primary human endothelial cells and brain pericytes. Transgenic mouse models were also used.

Results—Endothelial cell–specific ablation of Ccm1 and Ccm2 in different mouse models led to the formation of CCM-like lesions, which were poorly covered by periendothelial cells. CCM1 silencing in endothelial cells caused decreased Notch3 activity in cocultured pericytes. DLL4 proteins stimulated Notch3 receptors on human brain pericytes. Active Notch3 induced expression of PDGFRB2, N-Cadherin, HBEGF, TGFB1, NG2, and S1P genes. Notch3 signaling in pericytes enhanced the adhesion strength of pericytes to endothelial cells, limited their migratory and invasive behavior, and enhanced their antiangiogenic function. Pericytes silenced for Notch3 expression were more motile and could not efficiently repress angiogenesis.

Conclusions—The data suggest that Notch signaling in pericytes is important to maintain the quiescent vascular phenotype. Deregulated Notch signaling may, therefore, contribute to the pathogenesis of CCM. (Stroke. 2015;46:1337-1343. DOI: 10.1161/STROKEAHA.114.007512.)

Key Words: angiogenesis effect • DLL4 protein • Notch3 protein • pericytes

Cerebral cavernous malformations (CCMs) are low-flow vascular malformations in the brain affecting ≤1 in 250 individuals. CCM lesions consist of a mulberry-like cluster of enlarged and irregular blood vessels surrounded by a thick, multilayered basal membrane. The endothelial channels have poorly developed tight and adherens junctions, and are rarely covered by pericytes, astrocytes, or vascular smooth muscle cells (VSMCs). Growth and bleeding of CCM lesions cause neurological defects, such as pharmacorefractory epilepsy, migraine-like headaches, focal neurological deficits, and hemorrhages.

CCM is caused by loss-of-function mutations in the CCM1 (KRIT1), CCM2 (OSM), and CCM3 (PDCD10) genes. The detailed pathobiology is still not understood. However, many functions of these genes in cultured endothelial cells were recently described. Increased stress fiber formation, cell shape changes, and weakening of adherens junctions were observed after silencing CCM1-3 genes in endothelial cells. A recent landmark study suggested that endothelial cells adopt a more mesenchymal cell-like phenotype (endothelial-to-mesenchymal transition) on loss of the Ccm1 gene. This behavior is, in part, because of decreased Notch signaling. Second, CCM proteins are crucial for endothelial cell interactions with the extracellular matrix. Finally, CCM1 inhibits angiogenesis via induction of Notch signaling.

Notch signaling is essential to restrain sprouting angiogenesis and loss ofDll4 or Notch1 in mice leads to a chaotic, tip cell–rich, and excessively branched vessel network that is poorly functional. Notch signaling requires protein interactions between a Notch ligand (Dll1, Dll3, Dll4, Jag1, and Jag2) and the Notch receptor (Notch1, Notch2, and Notch3). The data suggest that Notch signaling in pericytes is important to maintain the quiescent vascular phenotype. Deregulated Notch signaling may, therefore, contribute to the pathogenesis of CCM.
and a receptor (Notch-1-4) located on adjacent cells. This leads to processing by a disintegrin and metalloproteinase domain protease and γ-secretase to release the Notch intracellular domain. Notch intracellular domain enters the nucleus and activates the transcription factor recombination signal-binding protein-Jk to induce gene expression of HES and HEY transcriptional repressors,10 and several cell-type–specific target genes, such as ephrin-B2 in endothelial cells and platelet-derived growth factor (PDGF) receptor β in VSMC.11,12

Notch signaling also occurs between endothelial and mural cells. Endothelial-specific ablation of recombination signal-binding protein-Jk reduces pericyte adhesion leading to hemorrhage.13 The endothelial cell–specific loss of the Notch ligand Jagged-1 dramatically impairs VSMC differentiation.14 Finally, Notch3 expression is transmitted through the vessel wall in an autoregulatory loop that requires the contact of VSMC with endothelial cells.15 In contrast to VSMC, the functions of Notch signaling in pericytes are poorly understood. One would assume that Notch receptors on pericytes can be stimulated by ligands located on endothelial cells because both the cell types form peg-and-socket contacts containing adherens and gap junctions. Pericytes are important to inhibit angiogenesis, to stabilize the vessel wall, to limit vessel permeability,16 to strengthen the blood–brain barrier, 17 and to control cerebral blood flow.18 This study was aimed to determine whether endothelial DLL4 ligands, whose expression is controlled by CCM1, can stimulate Notch3 receptors on pericytes and to evaluate functions of Notch3 signaling in pericytes.

Materials and Methods

Cell Culture

Human umbilical vein endothelial cells (HUVECs) were cultured in Endopan-3 medium with supplements (PAN Biotech) for ≤4 passages. Human brain microvascular endothelial cells (Cell Systems, ACBRI 376) were cultured in Complete Classic Medium (Cell Systems, 420-500). Human brain pericytes (HBPCs) were purchased from ScienCell, California and grown in pericyte medium with 2% fetal bovine serum, and supplements (ScienCell). HBPCs were characterized by immunostaining against smooth muscle actin. The manipulation with siRNA or shRNA, Western blotting, and quantitative polymerase chain reaction are described in the online-only Data Supplement.

Mouse Cavernoma Tissue and Ccm1-Deficient Mice

The endothelial cell–specific Ccm1-deficient mice have been described before.16 Mice were injected with 20 μg/g tamoxifen at postnatal day 1 and euthanized at day 8 to obtain cavernoma tissue from the cerebellum.

In Vivo Spheroid-Based Angiogenesis Assay

This assay was performed as described before.5,20,21 In short, HUVECs were transduced with lentiviral particles expressing shRNA against either CCM1 or CCM1 cDNA. After antibiotic selection spheroids containing 150 cells were generated. Thousand spheroids were embedded in a 1:1 mixture of Endopan-3/methocel/ fibrinogen containing 1 μg/mL vascular endothelial growth factor-A 165 and fibroblast growth factor 2. Immediately before injection, this mixture was diluted 1:1 with growth factor–reduced Matrigel (BD Biosciences) and 1 U/mL thrombin (Calbiochem). This mixture was injected subcutaneously into the flank of 6- to 8-week-old CB17 SCID mice (Charles River). Each mouse received 2 injections with CCM1-mutant and control cells, respectively. Plugs were removed 28 days later, fixed with 4% paraformaldehyde and embedded in paraffin. All animal procedures were performed in accordance with the local committee for animal experiments. Immunohistochemistry methods are further detailed in the online-only Data Supplement.

rhDLL4 Stimulation

Recombinant human DLL4 protein (2.5 μg; R&D) or 2.5 μg IgG as control (Rockland) were diluted in 1 mL 0.1% gelatin in phosphate buffered saline. Six-well dishes were coated with 1 mL of this mixture for 12 hours at 4°C. The solution was removed and 5×10³ HBPC were seeded. RNA and proteins were isolated 24 hours later.

Adhesion, Coculture, Tube Formation, and Spheroid-Based Angiogenesis Assays

HBPC were stained with a red fluorescent dye (CMTPX CellTracker Red, LifeTechnologies), detached by accutase and 1×10⁵ cells were seeded on top of a confluent HUVEC monolayer in a 24-well plate and incubated for 40 minutes. Before fixation with 4% paraformaldehyde in phosphate buffered saline, the cells were washed twice with warm culture medium to remove nonadhesive cells. Quantification was done by automatically counting cells of 5 fields of view per well with cell®P software. For direct coculture 1.5×10⁵ HBPC were seeded on CCM1-silenced HUVEC 1 day after transfection (ratio, 1:1) and incubated for 24 hours.

For tube formation 2.5×10⁴ HBPC and HUVEC were seeded together on top of Matrigel in a 48-well plate. The ratio HBPC/HUVEC was 1:1 (Notch3 overexpressing HBPC) or 1:4 (Notch3-silenced HBPC). The assay was stopped 18 hours later. Bright field microscopy was performed with Zeiss Cell Observer and cumulative length of the capillary network measured with cell®P software. By means of tile scans, the whole well was analyzed and considered as 1 sample. The spheroid-based angiogenesis assay was performed as described.11 The cumulative sprout length of 30 spheroids was measured (Olympus IX 50, cell®P software).

Statistical Analysis

Results are expressed as mean±SD. Comparisons between 2 groups were analyzed with SigmaPlot 12.0 software by a 2-sided t test. P≤0.05 were considered as statistically significant.

Results

Endothelial CCM1 Expression is Prerequisite for Pericyte Coverage

Endothelial channels are poorly covered by mural cells within CCMs.3–5 Our groups have recently developed mouse models for this neurovascular disease.6,8,19 Endothelial cell–specific ablation of Ccm1 in newborn mice by Cre-ERT2/loxP technology leads to the formation of enlarged blood vessels and vascular conglomerates in the brain and the retina.19 Immunohistological analysis revealed that CCM lesions in the cerebellum of 8-day-old iCcm1loxP/loxP-deficient mice contained only few periendothelial cells. The nonaffected blood vessels adjacent to CCM lesions had a normal appearance and were covered by pericytes similar as in control animals (Figure 1A).

Second, we analyzed mural cell coverage of vessels derived from CCM1-silenced human endothelial cells in a xenograft mouse model. In this approach, the HUVECs were stably transduced with shRNA against CCM1 and grafted subcutaneously in immunodeficient mice. HUVEC form a vascular network that anastomoses with the invading murine vessels.20,21 We had shown before that CCM1 gene depletion leads to the formation of a hyperdense vascular network with
and increased vessel lumen size. The abnormal vessels were significantly less covered by desmin-positive periendothelial cells compared with control (16% versus 50%; \(P<0.001\); Figure 1B and 1C). As such, both the animal models recapitulate hallmark symptoms of human CCM lesions.

Previously, we found that forced CCM1 protein expression inhibits sprouting angiogenesis because of elevated DLL4-Notch signaling. Here, we show that HUVEC overexpressing CCM1 formed only few vessels after xenotransplantation into mice (Figure 1A and IB in the online-only Data Supplement). Microvessel density was reduced by \(80\%\) compared with grafted endothelial cells expressing green fluorescent protein. These vessels were, however, covered by desmin-positive mural cells in the same extent as in the control animals (Figure 1C in the online-only Data Supplement). Taken together, a certain threshold dose of CCM gene expression in endothelial cells seems to be necessary to recruit and stably incorporate mural cells into the blood vessel wall.

### DLL4 Induces Notch3 Signaling in Pericytes

Depletion of CCM1 or CCM3 in endothelial cells is followed by impaired DLL4-Notch signaling and excessive sprouting. We hypothesized that CCM1-depletion could also affect Notch signaling between endothelial cells and pericytes. It has not yet been formally demonstrated that DLL4 ligands can stimulate Notch receptors on pericytes. An efficient and rather clean method to stimulate Notch receptors is to cultivate cells on immobilized, recombinant ligand proteins. When human brain pericytes (HBPC) were cultured on immobilized DLL4 protein a robust induction of Notch3 protein expression was observed. Importantly, the rate of cleaved, and thus active, Notch3 intracellular domain was significantly increased. The mural cell–specific Notch target gene PDGFR receptor-β (PDGFRB) was also induced (Figure 2A). Consequently, increased mRNA expression of the classical Notch target genes HEY2, HEYL, and PDGFRB was observed on stimulation of pericytes with DLL4 (Figure 1A in the online-only Data Supplement).

Next, we silenced CCM1 expression in HUVEC (knockdown rate \(80\%\)) and cocultured these with HBPC. Silencing of CCM1 significantly reduced endothelial DLL4 expression to \(60\%\) (\(P<0.01\)) but did not affect expression levels of the Notch ligands DLL1 and JAG1 (Figure 2B; Figure IIB in the online-only Data Supplement). Endothelial CCM1 silencing decreased expression of NOTCH3 protein in cocultured HBPC (Figure 2B). Consequently, less NOTCH3 expression was associated with decreased amounts of the active Notch3 intracellular domain (NOTCH3-ICD) and mRNA of its target genes PDGFRB and HEYL (Figure IIC in the online-only Data Supplement). This is cell-type–specific because HUVEC do not express NOTCH3 (Figure IID in the online-only Data Supplement). The same was seen when CCM1 was silenced in human brain microvascular endothelial cells (Figure 2B). In addition, NOTCH3 and PDGFRB levels in pericytes were restored or even increased by DLL4 overexpression in HUVEC after CCM1 silencing (Figure 2C). Taken together, DLL4 is not only important for NOTCH1 receptor stimulation within the endothelium but can also activate NOTCH3 receptors on pericytes.

### Notch3 Signaling in Pericytes Regulates Different Aspects of Pericyte–Endothelial Cell Interactions

To explore the functions of Notch3 signaling in pericytes (HBPC), NOTCH3 gene expression was silenced by 2 independent lentiviral shRNAs. Both the shRNAs significantly reduced NOTCH3 mRNA levels by \(90\%\) and the resulting protein depletion was confirmed by Western blotting. Notch3 gain-of-function experiments were conducted by overexpressing the NOTCH3-ICD with lentiviral vectors.
First, the ability of HBPC to invade into a collagen matrix was assessed. NOTCH3 silencing led to a >2-fold increase in pericyte outgrowth (Figure 3A), whereas overexpression of NOTCH3-ICD significantly reduced the invasion capacity of pericytes (Figure 3B). This was further analyzed by pericyte–endothelial cell coculture assays. The adhesion of pericytes to a dense HUVEC monolayer was strongly impaired after NOTCH3 silencing in pericytes (51% reduction). Consistently, the overexpression of NOTCH3-ICD significantly increased the rate of pericyte adhesion by ≈100% (Figure 3C and 3D).

The adhesion of pericytes to endothelial cells helps to maintain vessels in a quiescent, nonangiogenic state.17,23 A coculture Matrigel assay with HUVEC and HBPC was used to study a potential role of Notch3 signaling in human brain pericytes. When HBPC were added to HUVEC in a 1:1 ratio, this almost prevented tube formation. However, silencing NOTCH3 in pericytes allowed endothelial tube formation (Figure 4A and 4B). Elevated Notch3 signaling in HBPC led to a remarkable reduction of tube formation, even when HBPC were cocultured with HUVEC only in a 1:4 ratio (Figure 4C and 4D). In summary, the experiments imply that the inhibition of Notch signaling in pericytes makes these cells more motile, less adhesive, and impairs their capability to inhibit angiogenesis.

Notch3-Regulated Genes are Key Players in Pericyte Physiology

To gain first insights into the transcriptome of Notch3 signaling in human brain pericytes, we performed quantitative reverse transcription polymerase chain reaction with selected genes that are known to guide critical pericyte–endothelial cell interactions. NOTCH3 silencing in HBPC significantly reduced both mRNA and protein level of PDGFRB2 and the adhesion molecule N-Cadherin, as well as the expression of the heparin-binding epidermal growth factor (HBEGF) and transforming growth factor β-1 (TGFB1; Figure 5A and 5C). Consistent to this NOTCH3-ICD, overexpression significantly increased expression of PDGFRB2, N-Cadherin, HBEGF, TGFB1, and sphingosine-1-phosphate S1P (Figure 5B and 5C). These changes seem to be specific because expression of several additional genes were not changed (Figure III in the online-only Data Supplement). Taken together, Notch3 signaling regulates complex genetic programs that are essential for

**Figure 2.** DLL4 induces Notch3 signaling in pericytes. A, Western blot analysis 24 hours after seeding human brain pericytes (HBPCs) on 2.5 μg/mL recombinant human DLL4 (rhDLL4) or IgG-Fc–coated plates showed DLL4-induced increase of NOTCH3, as well as Notch3 intracellular domain (NOTCH3-ICD) and platelet-derived growth factor receptor-β (PDGFRB) in pericytes. B, Western blot analysis of human umbilical vein endothelial cells (HUVECs) only, HUVEC and HBPC, or human brain microvascular endothelial cells (HBMECs) and HBPC cocultured for 24 hours. Endothelial CCM1-depletion with siRNA (siCCM1) regulates DLL4 expression level in endothelial cells, as well as NOTCH3 and PDGFRB protein level in pericytes. Nonsilencing siRNA duplexes served as control (siNS). n=3, results are shown as mean±SD, **P<0.01, *P<0.05.

C, HUVEC were transduced with lentivirus for DLL4 overexpression or green fluorescent protein (GFP) control. Direct coculture of HBPC with CCM1-silenced HUVEC for 24 hours reduced NOTCH3 and PDGFRB mRNA level in pericytes. Endothelial DLL4 overexpression restored decreased Notch signaling in pericytes. GFP overexpression in endothelial cells served as control. n=3, results are shown as mean±SD, ***P<0.001, **P<0.01. n.s. indicates not significant.
interplay of pericytes with endothelial cells. Impaired stimulation of Notch3 receptors by DLL4-depleted endothelial cells would create a situation where pericytes attach more loosely to endothelial cells and are more migratory and invasive. Second, such pericytes are severely impaired to inhibit angiogenesis.

Discussion

Many functions of the CCM proteins were deciphered in the past years. It is obvious that these proteins execute critical roles to regulate cytoskeletal remodeling, adherens junctions, cell polarity, lumen formation, endothelial-to-mesenchymal transition, and angiogenesis.1,2,6,9,19,22,24–26 Nevertheless, it is not yet clear which of these functions is predominantly responsible for the initiation of lesion formation. In this regard, it is noteworthy that endothelial cells silenced for CCM gene expression are more susceptible toward angiogenic stimuli,8,9 implicating that minor injuries or hypoxic episodes may trigger CCM development.

Impaired Notch signaling was observed in CCM1- or CCM3-silenced endothelial cells,8,9 and endothelial cell–specific Ccm1 expression was shown to restore cell–cell interactions in CCM1/2 or CCM3-deficient endothelium.8,9 This suggests that Notch signaling is critical for a proper adhesion of pericytes to the endothelium, which is essential to maintain the integrity of the vessel wall. Notch signaling has been shown to be essential for the maintenance and differentiation of pericytes, with a role in the regulation of cell–cell adhesion.27 These findings are consistent with our observations that Notch3 signaling reduces invasion and migration of pericytes but promotes adhesion to endothelial cells. A and B. Pericyte spheroids were embedded in a collagen matrix for 24 hours. Silencing of NOTCH3 (A) increased cell invasion into the extracellular matrix, whereas expression of NOTCH3 intracellular domain (NOTCH3-ICD); B) severely impaired this function. n=4 experiments with 10 spheroids analyzed per group. Scale bar, 400 μm. C and D, human brain pericytes were stained with a fluorescent dye and added on top of an endothelial cell monolayer. After 40 minutes, adherent pericytes were detected with a fluorescent microscope (Olympus IX50) and cell^P software. n=6 experiments for shRNA and n=4 for lentiviral overexpression experiments. Results are presented as mean±SD, ***P<0.001, **P<0.01. HUVEC indicates human umbilical vein endothelial cell.

Figure 3. Notch3 signaling reduces invasion and migration of pericytes but promotes adhesion to endothelial cells. A and B. Pericyte spheroids were embedded in a collagen matrix for 24 hours. Silencing of NOTCH3 (A) increased cell invasion into the extracellular matrix, whereas expression of NOTCH3 intracellular domain (NOTCH3-ICD); B) severely impaired this function. n=4 experiments with 10 spheroids analyzed per group. Scale bar, 400 μm. C and D, human brain pericytes were stained with a fluorescent dye and added on top of an endothelial cell monolayer. After 40 minutes, adherent pericytes were detected with a fluorescent microscope (Olympus IX50) and cell^P software. n=6 experiments for shRNA and n=4 for lentiviral overexpression experiments. Results are presented as mean±SD, ***P<0.001, **P<0.01. HUVEC indicates human umbilical vein endothelial cell.
knockout mice. This may significantly contribute to uncontrolled angiogenesis during lesion formation because loss of Notch signaling sensitizes endothelial cells for the growth factor vascular endothelial growth factor. The question why Notch signaling sensitizes endothelial cells for the growth factor vascular endothelial growth factor has, however, not yet been systematically addressed.

The presence of pericytes at the blood–brain barrier is essential for limiting transendothelial transport, and the control of blood perfusion. The blood–brain barrier is impaired in cerebral cavernomas. Our approaches to silence CCM gene expression exclusively in endothelial cells resulted in formation of enlarged and irregular branched endothelial channels. These malformations were additionally only poorly covered by pericytes. We still detected some remaining pericytes in cavernoma-like lesions of iCcm1ΔΔ mice. Because the progression of the number and size of these lesions occurs rapidly, we intended to analyze an early state after gene ablation. Even 7 days after tamoxifen injection the vascular channels were strongly enlarged, irregular, and had either lost or failed to recruit pericytes. Moreover, the data suggest that signaling from the endothelium toward pericytes is severely affected on impaired CCM gene expression in endothelial cells.

This study showed that DLL4 is important for the cross-talk of endothelial cells with pericytes in CCM. Other groups provided evidence of DLL4 enhancing pericyte coverage of tumor vessels, and defective vessel maturation and perfusion is a result of DLL4 ablation. Defective recruitment of pericytes, hyperproliferative angiogenesis, enhanced permeability, insufficient maturation and perfusion are all well-known aspects in CCM pathology and underline the relevance of DLL4-Notch3 signaling in this neurological entity.

To date, it is known that the Notch ligand Jagged-1 on endothelial cells is crucial to stimulate Notch3 signaling in the adjacent layer of VSMCs. However, Jagged-1 expression is not altered in CCM1 or CCM3-silenced endothelial cells, whereas only DLL4 protein levels are decreased. DLL4 is expressed exclusively on endothelial cells in the vasculature. It has not yet been formally tested whether DLL4 can stimulate Notch3 receptors in pericytes, which are abundantly expressed on these cells. Our data revealed that recombinant DLL4 protein can efficiently stimulate Notch3 signaling in pericytes. This may have several implications. We found that Notch3 signaling in human brain pericytes enhanced the adhesion to endothelial cells and limited the migratory and invasive behavior of mural cells. DLL4-Notch3 signaling would help to maintain stable endothelial cell–pericyte interactions within the vessel wall and, therefore, may play a substantial role in blood–brain barrier homeostasis. The role of Notch3 in pericytes for maintaining blood–brain barrier function as well as positively controlling pericyte coverage was recently shown in zebrafish. These findings are in accordance with our work pointing out Notch3 as functionally relevant for the pericyte–endothelial cell interplay.

Our experiments showed that Notch3 controlled the prototypic signaling mode between the endothelium and mural cells, the PDGFB/PDGFRB signaling system. This is similar to the situation in VSMCs. PDGFB is essential for the recruitment of pericytes in the central nervous system and the establishment of a tight blood–brain barrier. Histopathologic findings provided evidence of elevated levels of PDGF in CCM lesions. Considering that the PDGFβ receptor is downregulated on decreased Notch3 activity in pericytes, this can be seen as a compensatory mechanism.

Finally, we found that the antiangiogenic capability of pericytes is, in part, dependent on Notch3 signaling. Therefore, we hypothesize that CCM gene deletion makes endothelial cells susceptible for growth stimuli and that impaired notch activity aggravates this. It allows excessive vessel branching and facilitates endothelial-to-mesenchymal transition. The resulting endothelial channels are impaired to recruit pericytes, a situation that additionally weakens the blood–brain barrier, vessel integrity, and facilitates further vessel growth.

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Disclosures

None.

References


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SUPPLEMENTAL MATERIAL

Supplemental Methods

Plasmids, virus production and transduction
Lentiviral and adenoviral particles were produced according to ViraPower™ Lentiviral or Adenoviral Expression Systems (Invitrogen). Transduction was done with MOI of 10 for lentivirus and MOI of 50 for adenovirus. 48 hours after transduction cells were cultured in puromycin or blasticidine-containing selection media. Silencing of NOTCH3 was carried out by lentiviral short hairpin RNA transduction (RHS4430-98896002, RHS4430-100995536, Open Biosystems) including a non-silencing control. NOTCH3-ICD cDNA (provided by Urban Lendahl, Karolinska Institute, Sweden) was subcloned into lentiviral (pLenti-V5_DEST) and adenoviral (pAd-V5_DEST) expression plasmids. pLenti-CCM1-IRES-eGFP was used for CCM1 overexpression in HUVEC. Silencing of CCM1 was achieved through short hairpin RNA (RHS4430-98820292, -98913140) and a non-silencing control from Open Biosystems. pLenti-DLL4-IRES-eGFP was used for DLL4 overexpression in HUVEC. All gene manipulation experiments were controlled by qRT-PCR and Western blot.

Immunohistochemistry
Serial paraffin sections were pretreated by steam boiling with a Tris buffer (10 mM Tris pH 9.0, 1 mM EDTA, 0.05 % Tween) for 25 minutes. Sections were deparaffinized, rehydrated and blocked with 10% milk powder in PBS for 1 hour. A 1:100 dilution of anti-mouse Desmin antibodies (Thermo Scientific) in PBS was added over night at 4°C. Alexa Fluor488 donkey anti-rabbit (Invitrogen) 1:200 in PBS was used as secondary antibody. For staining against human CD34 a monoclonal anti-CD34 antibody (QBEND10, Menarini) was added in a 1:50 dilution in PBS for 2 hours at room temperature. After washing with PBST donkey anti-mouse-Cy3 (Dianova) was added in a 1:200 dilution in PBS. Nuclei were stained with DAPI. Vessels were divided into two groups: pericyte covered and uncovered group, respectively. Pericyte coverage was defined by direct colocalization of desmin positive cells with CD34 positive vessels, whereas the vessel had to be covered by at least 50%.Cavernoma tissue was stained with FITC-lectin (Sigma) in a 1:100 dilution in Pblec buffer (PBS, 1% Triton X-100, 0.1mM CaCl2, 0.1mM MgCl2, 0.1mM MnCl2, pH6.8), anti-PDGFRß, anti-Desmin (both Abcam) or anti-SMA-Cy3 (Sigma) antibodies. Fluorescence microscopy was done with Zeiss Imager.Z1 and AxioVision software or Zeiss confocal scanning microscope LSM700 and Zen2011 software.

Quantitative Real-Time PCR (qRT-PCR)
RNA was purified with the RNeasy Kit from Qiagen. 2 µg of RNA was transcribed into cDNA (High Capacity cDNA kit; Applied Biosystems). 20 ng cDNA were used in a 25 µl reaction setup containing POWER SYBR Green Master Mix on an ABI StepOnePlus cycler (Applied Biosystems). Two different housekeeping genes were used for normalization: OAZ1 and RPS29 for pericytes and HPRT1 and OAZ1 for endothelial cells. LinReg software was used for determination of Ct-values and the calculation of ∆∆Ct values. Primer sequences are given in suppl. Table I.

Western Blotting
Cells were lysed (200 mM NaCl, 75 mM Tris, 15 mM NaF, 1.5 mM Na3VO4, 7.5 mM EDTA, 7.5 mM EGTA, 1.5% Triton X-100, 0.75% Igepal CA-630, 1x Roche Complement Protease
Inhibitor) and denatured protein lysates were separated by electrophoresis on a 10% polyacrylamide/SDS gel. After blotting proteins to a nitrocellulose membrane, membranes were blocked with 5% milk powder in TBST for 6 hours at 4°C. Primary antibodies against NOTCH3-ICD (Santa Cruz), DLL4 (Cell Signaling), PDGFRB (Abcam) and N-CADHERIN (BD Biosciences) were incubated over night at 4°C in 5% BSA/TBST. After washing with TBST the membrane was incubated with an HRP-conjugated, goat anti-rabbit secondary antibody (DAKO) for 1 hour at room temperature and detection of chemoluminescence was done using Pierce ECL Western Blotting Substrate (Thermo Scientific). Quantification was done with ImageLab 3.0 software (BioRad).

**Supplemental Tables**

**Table I**

**Oligonucleotides**

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Supplemental Figures

Figure 1. Overexpression of CCM1 reduces vessel density in a xenotransplant model. HUVEC overexpressing CCM1 were injected into immunocompromised mice. 28 days after xenotransplantation plugs were removed and paraffin sections were stained against human CD34 (HUVEC, red). (A, B) Mean vessel density (MVD) was reduced by approximately 80% in CCM1 overexpressing HUVEC compared to the GFP control group. Scale bars, 100 µm. (C) Representative paraffin sections of plugs stained with human-specific anti-CD34 (HUVEC, red) and anti-desmin (pericytes, green). Nuclei were stained with DAPI (blue). Scale bars, 50 µm. n=3 animals for both groups. Error bars are means ± SD, ***p<0.001.
Figure II. DLL4 induces Notch3 signaling in pericytes. (A) qRT-PCR analysis 24 hours after seeding human brain-derived pericytes (HBPC) on 2.5 µg/ml recombinant human DLL4 or IgG-Fc-coated plates showed DLL4-induced increase of Notch3 signaling in pericytes. qRT-PCR of NOTCH3 and Notch target genes HEY2, HEYL, and PDGFRB. n=3, ***p<0.001, **p<0.01. (B) qRT-PCR 48h after silencing CCM1 expression in HUVEC. CCM1-depletion specifically reduced expression level of the Notch ligand DLL4. (C) Direct co-culture of HBPC and CCM1 silenced HUVEC for 24h reduced pericyte NOTCH3, HEYL and PDGFRB mRNA level. n=4, results are shown as means ± SD, ***p<0.001, **p<0.01, *p<0.05. (D) Western blot analysis of endogenous NOTCH3 and PDGFRB level in endothelial cells (HUVEC/ HBMVEC) and human brain pericytes (HBPC).
Figure III. Manipulation of *Notch3* expression in pericytes. qRT-PCR analysis of HUVEC after lentivirus-mediated *NOTCH3* silencing (A) as well as overexpression of *Notch3-ICD* (B). n=3;
Supplemental References
