Reduced Mural Cell Coverage and Impaired Vessel Integrity After Angiogenic Stimulation in the Alk1-deficient Brain


Objective—Vessels in brain arteriovenous malformations are prone to rupture. The underlying pathogenesis is not clear. Hereditary hemorrhagic telangiectasia type 2 patients with activin receptor-like kinase 1 (Alk1) mutation have a higher incidence of brain arteriovenous malformation than the general population. We tested the hypothesis that vascular endothelial growth factor impairs vascular integrity in the Alk1-deficient brain through reduction of mural cell coverage.

Methods and Results—Adult Alk1lox/lox mice (loxP sites flanking exons 4–6) and wild-type mice were injected with 2×10^7 PFU adeno-associated-cre recombinase and 2×10^6 genome copies of adeno-associated virus-vascular endothelial growth factor to induce focal homozygous Alk1 deletion (in Alk1lox/lox mice) and angiogenesis. Brain vessels were analyzed 8 weeks later. Compared with wild-type mice, the Alk1-deficient brain had more fibrin (99±30×10^3 pixels/mm^2 versus 40±13×10^3; P=0.001), iron deposition (508±506 pixels/mm^2 versus 6±49; P=0.04), and Iba1+ microglia/macrophage infiltration (888±420 Iba1+ cells/mm^2 versus 240±104 Iba1+; P=0.001) after vascular endothelial growth factor stimulation. In the angiogenic foci, the Alk1-deficient brain had more α-smooth muscle actin negative vessels (52±9% versus 12±7%, P<0.001), fewer vascular-associated pericytes (503±179/mm^2 versus 931±115, P<0.001), and reduced platelet-derived growth factor receptor-β expression.

Conclusion—Reduction of mural cell coverage in response to vascular endothelial growth factor stimulation is a potential mechanism for the impairment of vessel wall integrity in hereditary hemorrhagic telangiectasia type 2-associated brain arteriovenous malformation. (Arterioscler Thromb Vasc Biol. 2013;33:305-310.)

Key Words: activin receptor-like kinase 1 ■ brain arteriovenous malformation ■ iron deposition ■ pericyte ■ platelet-derived growth factor receptor-β

Brain arteriovenous malformations (bAVMs) are tangles of abnormal, dilated vessels that directly shunt blood between arteries and veins. Surrounding the AVM nidus, but extrinsic to the lesion, there may be a network of dilated capillaries. These abnormal vessels are prone to rupture and cause life-threatening intracranial hemorrhage. The pathogenesis of bAVM and the exact vascular defects that cause the rupture are not known. Knowledge of the underlying mechanisms could provide critical insights for the development of novel therapies to reduce the risk of life-threatening spontaneous rupture.

AVMs in various organs, including the brain, are common in patients with hereditary hemorrhagic telangiectasia (HHT), an autosomal dominant disorder. The 2 main subtypes of HHT (HHT1 and HHT2) are caused by loss-of-function mutations in the endoglin \((ENG)\) and activin receptor-like kinase 1 \((ALK1)\), or \(ACVR1L\) genes, both of which participate in transforming growth factor-β signaling. These HHT mutations can be viewed as risk factors for bAVM. Compared with the prevalence of sporadic bAVMs in the general population \((10/100000)\), the prevalence in HHT1 \((ENG)\) is 1000-fold higher, and in HHT2 \((ALK1)\), 100-fold higher. Previously, we created a bAVM model in adult mice through focal Alk1 deletion and vascular endothelial growth factor (VEGF) stimulation. This model mimics both macroscopic and microscopic morphological features of the human lesional phenotype, including large dysplastic, tangled vessels and arteriovenous shunting. Some abnormal vessels with a diameter >15 μm have no α-smooth muscle actin (α-SMA) positive cells on their wall.

Here, we tested the hypothesis that VEGF stimulation in the Alk1-deficient brain impairs vascular integrity through reduction of mural cell coverage. We demonstrated that compared with wild-type (WT) mice, VEGF stimulation in the Alk1-deficient brain not only reduces vascular α-SMA positive cells but also pericytes, which is associated with extravasation of intravascular components. Because the platelet-derived growth factor receptor-β (PDGFR-β)/PDGF-BB signaling pathway has been...
implicated in regulating pericyte recruitment to newly formed blood vessels, we also analyzed the expression of PDGF-β and PDGF-BB in the Alk1-deficient brain after VEGF stimulation.

Methods

For complete details on Methods and Materials, please refer to the online-only Data Supplement. The experimental protocols involving animal usage were approved by the Institutional Animal Care and Use Committee of the University of California, San Francisco. All studies involving patients were approved by the University of California, San Francisco institutional review board, and patients gave informed consent.

Animal Model

Adult Alk11f/2f mice (exons 4–6 flanked by loxP sites)5 and C57BL/6 mice were used. The 1f allele indicates a null allele derived from the conditional (2f) allele by cre-mediated recombination of the 2 loxP sites flanking exons 4 to 6 in the germ cells. Briefly, the mice were anesthetized with isoflurane and placed in a stereotactic frame with a holder (David Kopf Instruments, Tujunga, CA). A burr hole was drilled in the pericranium 2 mm lateral to the sagittal suture and 1 mm posterior to the coronal suture. Adenovious-cre recombinase (Ad-Cre, 2×107 plaque forming units) and adeno-associated virus (AAV)-VEGF (2×109 genome copies) were stereotactically injected into the basal ganglia of 8-week-old Alk11f/2f mice, about 3 mm under the surface of the cortex. An additional group received the same amount of the vectors in the cortex (Methods and Figure I in the online-only Data Supplement). Ad-green fluorescent protein and AAV-LacZ were used as control for Ad-Cre and AAV-VEGF. Cytomegalovirus promoter was used to drive Cre and VEGF expression in Ad-Cre and AAV-VEGF vectors. Gene expression was mostly detected in endothelial cells, neurons, and astrocytes.6,6 Two control groups with intact Alk1 gene were included: (1) WT mice injected with Ad-Cre and AAV-VEGF; and (2) Alk12f/2f mice injected with Ad-green fluorescent protein and AAV-LacZ. A third control group was Alk1-deficient mice that had not been treated with VEGF. Alk12f/2f mice injected with Ad-Cre and AAV-LacZ.

Statistical Analysis

Data are presented as mean±SD. For quantification of Prussian blue-staining, we used the Kruskall–Wallis test followed by the Wilcoxon rank-sum test. Fisher exact test was used to analyze the associations of α-SMA vessel/Prussian blue staining. We measured the linear relationship between vessel-associated pericyte and fibrin deposition, as well as Iba1+ cells and Prussian blue-positive area using Pearson correlation coefficient. The linear relationship between the numbers of ZIC1+ cells and fibrin deposition, as well as Prussian blue-positive area and Iba1+ cells, was determined using a simple linear regression analysis, which generates the corresponding R² estimate. Because of the skewed nature of Prussian blue area and the number of Iba1+ cell count, the skewed observations were log-transformed before performing this analysis. The other data were analyzed using 1-way ANOVA to compare the means of each group. A probability value <0.05 was considered statistically significant. Sample sizes were n=6 for each group.

Results

Alk1 Deficiency Potentiates Vascular Integrity Impairment After VEGF Stimulation

Focal Alk1 deletion and VEGF stimulation were induced as previously described.4 Injection of Ad-Cre into the brain of Alk12f/2f and Alk11f/2f mice reduced ALK1 expression significantly (Figure V in the online-only Data Supplement). Two-photon imaging demonstrated the dilated dysmorphic cerebrovasculature in the angiogenic focus of Alk11f/2f mice that received Ad-Cre and AAV-VEGF in the cortex (Figures IA and IB, and II in the online-only Data Supplement). The morphology of dysplastic vessels induced in the Alk11f/2f cortex was similar to that of bAVM in HHT patients (Figure IC and ID in the online-only Data Supplement). To investigate the integrity of the abnormal vessels, we analyzed fibrin deposition in the extravascular space. When soluble blood protein fibrinogen extravasates in the CNS, it is converted to insoluble fibrin by thrombin.7 VEGF stimulation resulted in a low level of fibrin deposition on the vessel wall and in the brain parenchyma adjacent to the vessels in the brain of WT mice (40±13×10³ pixels/mm², Figure 1A) and Alk12f/2f mice (45±16×10³ pixels/mm²; P=0.98). Without VEGF stimulation, the Alk1-deficient brain had a low level of fibrin deposition (10±5×10³ pixels/mm²). However, significantly more fibrinogen extravasation was detected in the angiogenic foci of the Alk1-deficient brain (99±30×10³ pixels/mm², P=0.001 versus WT, P=0.003 versus Alk12f/2f, Figure 1C). Perivascular fibrin deposition was mostly around the dysplastic vessels (Figure 1B).

In addition to fibrin, we found that the Alk1-deficient brain had hemosiderin deposition after VEGF stimulation (Figure 2). Prussian blue staining showed that after VEGF stimulation, Alk1-deficient mice had 10-fold higher Prussian blue-positive area (508±506 pixels/mm²) compared with WT (61±49 pixels/mm²; P=0.04) and Alk12f/2f mice (51±75 pixels/mm²; P=0.03; Figure 2B). Without VEGF stimulation, the Alk1-deficient brain had a low level of Prussian blue-positive area (8±13 pixels/mm²). Moreover, red blood cells (RBCs) were detected in the brain parenchyma outside the blood vessels in the Alk1-deficient brain, with macrophages in the surrounding region clearing RBCs (Figure 2C). Iron deposition was also detected in the nidus and perinidus areas of nonruptured human brain AVM specimens (Figure 2D), suggesting that vessel leakage could be an important phenotype related to the pathogenesis of the human disease.

Figure 1. The dysplastic vessels in the angiogenic foci of activin receptor-like kinase 1 (Alk1)-deficient brain have increased permeability than vessels in the angiogenic foci of the normal brain. A, Representative images show vessels and fibrin in the viral-injected region. B, High magnification images show the location of fibrin. The fibrin lies outside the dysplastic vessel wall in the Alk1-deficient brain. C, Bar graph shows the quantification of fibrin. In the viral-injected region. There was significantly more fibrin deposition in the vascular endothelial growth factor (VEGF)-treated Alk1-deficient brain than VEGF-treated (wild-type) WT mice (*P=0.001) and Alk12f/2f (P=0.003) groups, and Alk1-deficient brain without VEGF treatment (*P<0.001). Scale bars: 50 μm.
During active angiogenesis, some pericytes are also positive for macrophage/microglial infiltration. 

**Figure 3.** Prussian blue staining positively correlated with macrophage/microglia infiltration. A, Representative images of Iba1 antibody staining (red). Scale bar: 50 μm. B, Bar graph shows quantification of Iba1+ cells. The vascular endothelial growth factor (VEGF)-stimulated activin receptor-like kinase 1 (Alk1)-deficient group had more Iba1+ cells than VEGF-treated wild-type (WT) mice (*P*<0.001) and Alk1+/- (p=0.013) groups, and untreated Alk1-deficient group (*P*>0.002). C, Graph shows Prussian blue-positive area positively correlated with the number of Iba1+ cells (R^2^=0.2, *P*<0.05).

**Correlation of Iron Deposition With Microglia/Macrophages**

To investigate the relationship between inflammation and vascular integrity, we analyzed the number of microglia/macrophages in the angiogenic foci of Alk1-deficient mice, which is significantly more than those in WT (12±7%) and Alk1+/- mice (15±11%) (Figure 4B; *P*=0.001). Without VEGF stimulation, the Alk1-deficient brain had a similar number (17±9%) of α-SMA+ vessels as VEGF-treated WT and Alk1+/- mice (Figure 4B, *P*>0.05). Interestingly, in the Alk1-deficient mice, Prussian blue-positive staining appeared to be mostly located on the wall and the surrounding brain parenchyma of α-SMA+ dysplastic vessels (Figure 4C). Detailed quantification showed that among vessels >15 μm, 53% of α-SMA+ vessels were located within or near a Prussian blue-positive area; only 9% of α-SMA+ vessels were localized near a Prussian blue-positive area (Figure 4D; *P*<0.001). This suggests that lack of α-SMA+ cell coverage is associated with RBC extravasation.

**Iron Deposition in Vessels Without α-SMA+ Cell Coverage**

Smooth muscle cells are α-SMA+ and have an elongated, thin spindle-like shape lining outside the endothelial cells of arteries and veins. In normal conditions, vessels >10 μm usually have 1 or several layers of smooth muscle cells on their wall. During active angiogenesis, some pericytes are also positive for α-SMA staining. Here, we quantified the percentage of α-SMA+ vessels (>15 μm) in the angiogenic foci and found that 52±9% vessels were α-SMA+ in Alk1-deficient mice, which is significantly more than those in WT (12±7%) and Alk1+/- mice (15±11%) (Figure 4B; *P*=0.001). Without VEGF stimulation, the Alk1-deficient brain had a similar number (17±9%) of α-SMA+ vessels as VEGF-treated WT and Alk1+/- mice (Figure 4B, *P*>0.05). Interestingly, in the Alk1-deficient mice, Prussian blue-positive staining appeared to be mostly located on the wall and the surrounding brain parenchyma of α-SMA+ dysplastic vessels (Figure 4C). Detailed quantification showed that among vessels >15 μm, 53% of α-SMA+ vessels were located within or near a Prussian blue-positive area; only 9% of α-SMA+ vessels were localized near a Prussian blue-positive area (Figure 4D; *P*<0.001). This suggests that lack of α-SMA+ cell coverage is associated with RBC extravasation.

**Alk1 Deficiency Leads to Reduced Number of Vessel-associated Pericytes After VEGF Stimulation**

To determine whether the impaired vascular integrity in the Alk1-deficient brain is also related to the number of pericytes on the vessel wall, we used anti-ZIC1 and anti-Ng2 antibodies to identify pericytes. Both Ng2 and ZIC1 staining showed that vessels in the Alk1-deficient brain have less pericyte coverage after VEGF stimulation (Figure 5A). Because other cell types in the adult brain, (ie, oligodendrocyte progenitor cells), also express Ng2, and Ng2 positive staining is located on the cytoplasm membrane, it is difficult to quantify vascular-associated Ng2 positive cells. ZIC1 has been reported to be a specific marker for pericyte-nuclei in the mouse embryo brain. To test if ZIC1 is pericyte-specific in the adult mouse brain, we performed double immunostaining using the anti-ZIC1 antibody with anti-NeuN, anti-glial fibrillary acidic protein, and anti-desmin (Figure III in the online-only Data Supplement). We did not find ZIC1 positive astrocytes; however, some NeuN positive cells in the basal ganglia were ZIC1 positive, suggesting that ZIC1 is expressed by cells other than pericytes in the adult mouse brain. To quantify the vascular-associated...
pericytes, we only counted the ZIC1+ nuclei located on the vessel wall (Figure 5B).

The number of vascular-associated pericytes in the angiogenic foci of Alk1-deficient brain (503±179 ZIC1+ nuclei/mm² vessel area, Figure 5C) was 45% less than that in the angiogenic foci of the WT (931±115; P<0.001) and Alk1<sup>2f/2f</sup> (936±145; P<0.001) mice. Alk1-deficient-only did not reduce the number of vascular-associated pericytes (888±108, P=0.95). More importantly, the number of pericytes on the vessel wall inversely correlated with the level of fibrin deposition (R²=0.45, P=0.0003; Figure 5D). This indicates that the reduction of vascular pericytes correlated with impairment of vascular integrity in the lesion.

**PDGFR-β Expression is Reduced in the Alk1-deficient Brain**

To determine whether reduced vascular mural cell coverage in the Alk1-deficient brain was associated with the reduction of PDGFR-β and PDGF-BB, we analyzed the levels of PDGFR-β and PDGF-BB protein expression. We found that PDGFR-β expression in the angiogenic foci of Alk1-deficient brain was reduced as compared with that in the angiogenic foci of WT and Alk1<sup>2f/2f</sup> mice (P<0.001; Figure VIB in the online-only Data Supplement). Alk1-deficient brain had a similar level of PDGFR-β with or without VEGF stimulation (P=0.99). There was no difference in PDGFR-BB expression among all the groups (P=0.28; Figure VIC in the online-only Data Supplement).

**Discussion**

Compared with the mice that have normal Alk1 gene, we found that the vascular integrity was impaired in the brain of Alk1-deficient mice after VEGF stimulation, as evidenced by increased fibrin and iron deposition; small pockets of extravasated RBCs outside the vessels; and macrophage/microglia infiltration on the vessel wall and in the brain parenchyma near the dysplastic vessels. The dysplastic vessels in the angiogenic foci of Alk1-deficient brain displayed less mural cell coverage than the normal cerebrovascular. Consistent with the reduction of vascular pericytes, PDGFR-β expression in the angiogenic foci of Alk1-deficient mice was reduced. Iron deposition was found near the α-SMA- vessels, as well as in the nonruptured human bAVM specimen. A key finding from our study is that Alk1 plays a role in maintaining the integrity of vessels in the adult brain during angiogenesis. Patients with mutations in ALK1 and HHT2-associated bAVM may be prone to the same loss of vascular integrity because plasma and tissue levels of VEGF seem to be elevated in these patients.10

Roughly half of all bAVM patients come to clinical attention with an intracranial hemorrhage, and providing protection against the risk of spontaneous ICH after diagnosis is the
Hemoglobin breakdown products are reabsorbed or cleared by macrophages, and hemosiderin often remains the only sign of a previous hemorrhage. Even with relatively iron-insensitive magnetic resonance sequences, it is intriguing that $\approx 14\%$ to $20\%$ of bAVM patients with nonhemorrhagic history exhibit signs of prior hemorrhage events. We recently described that in patients with unruptured bAVMs and with no history of hemorrhage, $30\%$ of resected surgical specimens contain microscopic evidence of hemosiderin in the vascular wall or intervening stromal tissue. The underlying mechanisms for bAVM rupture and microbleeding in unruptured bAVM patients are not fully understood and might be involved in reduced mural cell coverage. However, the dysplastic vessels in the VEGF-stimulated Alk1-deficient brain probably and more closely mimic an early developmental stage of HHT2-associated bAVM and perinidal capillaries, rather than the fully formed and mature lesion seen in symptomatic human disease. Further study is needed to analyze pericyte and mural cell coverage in human bAVM. Interestingly, abnormal structural integrity of the perinidal vessels has been suggested. At the microscopic level, there is evidence of abnormal BBB structure, increased vessel permeability, RBC diapedesis, and capillary bleeding in human perinidal bAVM, similar to our findings here.

Inflammatory cell types, for example, macrophages and neutrophils, have been detected in human bAVM surgical specimens, even in those without a history of hemorrhage or previous treatment with embolization or radiosurgery. Polymorphic genetic variations in several inflammatory cytokines are associated with bAVM hemorrhage. We found that in the angiogenic foci of Alk1-deficient mice, the number of macrophage/microglia increased in the lesion and positively correlated with the degree of iron deposition (Prussian blue-positive area). Moreover, the distribution of macrophages/microglia resembled the pattern of CD68 positive cells in human bAVM, present in and around the dysplastic vessel wall. The number of $\beta 1^+ \alpha$ cells positively correlated with the degree of iron deposition. Thus, inflammation might also be involved in HHT2-associated bAVM pathogenesis and rupture of dilated dysplastic vessels. Further experiments are needed to determine the roles of ALK1 in inflammatory pathways.

It has been suggested that ALK1 regulates vascular smooth muscle cell (vSMC) differentiation and recruitment to the perivascular region during the embryonic stage. In E10.5 Alk1–/– mouse embryos, vSMCs were reduced in the dorsal aorta region. In human bAVMs, SMC vessels (>15 μm) have also been reported. Taken together with our findings, these data suggest that loss of vSMC coverage is present in Alk1-deficient embryos, as well as in the Alk1-deficient adult mouse brain and human bAVM. How this commonality in phenotypes contributes to the pathogenesis or clinical sequelae remains to be elucidated.

During vessel assembly, sprouting endothelial tubes recruit mesenchymal progenitors that differentiate into vSMC or pericytes. The recruitment of pericytes to vessels indicates the maturation of a vascular system, as well as the establishment and maintenance of blood–brain barrier integrity. The importance of pericytes in maintaining cerebrovascular integrity in adults has been described by Bell et al. Tu et al analyzed the ultrastructure of a specimen from an unruptured bAVM and found that pericytes were less common in perinidal capillaries than in control vessels. The number of lysosomes and pinocytotic vesicles in the residual pericytes increased, indicating BBB opening. Caution is needed in interpreting these findings, as these changes may be epiphenomenal and are because of some process unrelated to the original pathogenesis of the lesion, for example, some consequence of long-standing high flow rates in the vascular structures. Nonetheless, if these changes in human specimens are related to the pathogenesis, they are consistent with our findings. Here, we showed that the dysplastic vessels in the angiogenic foci of Alk1-deficient brain have a reduced number of pericytes associated with increased fibrinogen extravasation, suggesting that Alk1 is involved in mural cell recruitment during angiogenesis in the adult mouse brain. How Alk1 regulates this process needs to be clarified and is a potentially important subject of future research.

PDGFR-β/PDGFB signaling regulates pericyte recruitment and differentiation to nascent capillaries. The differentiation of mesenchymal cells into the pericyte/vSMCs lineage is dependent on PDGFR-β expression in the mouse. Pdgfb or Pdgfrβ null mice have cerebral hemorrhage with an absence of microvascular pericytes in the brain vessels and endothelial hyperplasia. Here, we demonstrated that the expression of PDGFR-β was reduced in the angiogenic foci of Alk1-deficient mice, suggesting a possible link between Alk1 and PDGFR-β/PDGFB-BB signal pathways. However, it is not clear whether the reduced expression of PDGFR-β is caused by the reduced number of pericytes in the tissue. We found that PDGFR-β protein was reduced in the brain of untreated Alk1-deficient mice even though the number of vascular-associated pericytes was not reduced, possibly because we used homogenous brain tissues. Gene expression by other cell types may influence the analysis. Therefore, the specific effect of pericytes may not be accurately reflected in the protein analysis. The exact mechanism that links PDGFR-β/PDGFB-BB signaling and Alk1 will require further investigation.

We did not detect any changes in PDGFB-BB expression in the Alk1-deficient brain. Other than endothelial cells, PDGF-BB expression has been detected in the neurons of the adult rat brain. Endothelial-specific Pdgfb deletion causes vSMC/pericyte deficiency, whereas its deletion in hematopoietic cells or neurons has no obvious effect on the vascular structure. Although our data suggest that Alk1 deletion does not affect the level of PDGF-BB expression, it is possible that the neuronal expression of PDGF-BB in the adult brain masks the changes of endothelial cell-derived PDGF-BB. Lebrin et al. showed that thalidomide upregulated PDGF-BB expression, increased mural cell coverage, and rescued vessel wall defects in ENG–/– mice. These findings suggest that the alteration of PDGFR-β/PDGFB-BB signaling could be one of the underlying mechanisms for HHT2-associated bAVM pathogenesis and provide an impetus to develop new therapies.

This study has 2 limitations. (1) Because adenoviral vector itself could cause local inflammation, we could not determine in our current model whether vascular leakage leads to local inflammation or vice versa during the pathogenesis of the cerebrovascular dysplasia. We have now developed new models without local viral vector injection. We would like to address
those questions in the future using different models. (2) We do not know whether loss of mural cell coverage is a primary or secondary effect of Alk1 deficiency. Additional experiments are needed to identify the primary cell type that causes this phenotype.

In conclusion, in a mouse model that simulates aspects of human bAVM, especially those associated with HHT2, we described and quantified a series of abnormalities consistent with compromised vascular integrity of the affected vasculature. A common theme was extravascular localization of intravascular components. The extravasation of intravascular components correlated with the reduction of vascular mural cells and increase of macrophage burden. Reduced expression of PDGF-R-β in the angiogenic foci of Alk1-deficient brain suggests that PDGF-R-β/PDGFB signaling could be involved in regulating mural cell recruitment in HHT2-associated bAVM. Modulation of PDGF-R-β/PDGFB signaling shows promise in the development of novel therapies to stabilize the abnormal vasculature and reduce the risk of HHT2-associated bAVM rupture.

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Disclosures
None.

References
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Supplemental Methods

Immunohistochemistry

Eight weeks after viral injection, brain sections were collected and immunostaining was performed as previously described.

One set of animals was perfused under deep anesthesia with PBS followed by 4% paraformaldehyde (PFA). The brain samples were embedded in paraffin and cut into 5-µm-thick coronal sections using a Leica RM2155 Microtome (Leica Microsystems, Wetzlar, Germany). The sections were used for α-SMA, Prussian blue, and Iba1 staining. Another set of brain samples was frozen in dry ice and cut into 20-µm-thick coronal sections using a Leica CM1950 Cryostat (Leica Microsystems, Wetzlar, Germany). The frozen sections were used for ZIC1 and fibrin staining.

Two sections per brain within the injection site were chosen for staining. Three images were taken from each section (right and left of, and below the injection site) under a 20X objective lens (Leica MZFL III microscope, Leica Microsystem, Bannockburn, IL). Vessels with diameter larger than 15 µm, either with or without α-SMA positive cells, were quantified on lectin/α-SMA antibody double-stained sections. Pericytes associated on the vessel wall were counted on ZIC1 antibody-stained sections and were normalized with the total vessel area.

Sections were incubated at 4°C overnight with the following primary antibodies: anti-α-SMA (1:1000, Sigma-Aldrich, St. Louis, MO); anti-Iba-1 (1:200, Wako Chemicals USA, Richmond, VA), anti-fibrinogen (1:300, United States Biological, Swampscott, MA), anti-ZIC1 (1:100, Novus Biologicals, CO), anti-NG2 (1:50, R&D Systems, MN), anti-CD31 (1:100, Santa Cruz Biotechnology, CA), or fluorescein lycopersicin esculentum-lectin (1:200, Vector Laboratory, Burlingame, CA).

Alexa Fluor 594-conjugated (1:500), Alexa Fluor 488-conjugated IgG (1:500, Invitrogen, Carlsbad, CA), or Cy3-conjugated donkey anti-sheep (1:400, Jackson ImmunoResearch Laboratories, West Grove, PA) were used as secondary antibodies.

Prussian Blue Staining

Accustain Iron Stain Kit (Sigma-Aldrich, St. Louis, MO) was used to detect iron deposition. Paraffin-sections were deparaffinized and rehydrated through graded alcohols. Slides were then incubated in freshly prepared working iron stain solution for 15 minutes, washed in distilled water, and counterstained with pararosaniline solution for 3 minutes. Two sections per brain within the injection site were chosen for staining. Data presented as total Prussian blue positive pixel numbers (pixels) divided by total brain section area (mm²).

In vivo imaging of the vasculature using two-photon microscopy and image analysis
Eight week-old $\text{Alk}^{162f}$ mice were stereotactically injected with Ad-Cre ($2 \times 10^7$ plaque forming units) and AAV-VEGF ($2 \times 10^9$ genome copies) into the cortex, as previously described.$^1$ Briefly, the needle was inserted 1 mm into the cortex and advanced 2 mm parallel to the surface, and the vector was then injected (Supplemental Figure S1). Mice were imaged in vivo 8 weeks after the viral injections. Untreated WT mice were used as controls. In vivo imaging was performed transcranially through a thinned area of the skull over the somatosensory cortex, as previously described.$^2$ Mice were anesthetized intraperitoneally with ketamine (200 mg/kg body weight) and xylazine (30 mg/kg body weight) in 0.9% NaCl solution. A small region (~1 mm in diameter) of the skull was first thinned with a high-speed drill under a dissecting microscope, and then scraped with a microsurgical blade to a final thickness of ~20 µm. A drop (~200 µl) of artificial mouse cerebrospinal fluid (ACSF) was applied over the thinned region for the duration of the experiment. The skull surrounding the thinned region was attached to a custom-made steel plate to reduce respiratory-induced movement. To minimize leakage of the dextran from the dysplasia, rhodamine dextran (2MD, Invitrogen) was used. A single injection of 100µl of a 3% w/v solution of rhodamine dextran (2MD, Invitrogen) diluted in PBS was given intravenously as described$^3$ to label the vasculature immediately before in vivo imaging started. The animal was placed inside a preheated light-tight chamber under a Prairie Technologies multi-photon microscope (Ultima IV In Vivo Imaging System). A Ti-sapphire laser (Mai Tai DeepSee eHP, Spectra Physics) was tuned to 890 nm for two-photon excitation of rhodamine dextran. Stacks of images with a step size of 2 µm were acquired using a water-immersion objective (Olympus 40x, 0.8 N.A.). The 2-photon images were taken from projections of z stacks covering a depth of 130-150µm from pial surface. Images were acquired using low laser power (<30 mW at the sample) and a low-pass emission filter (<700 nm). The acquired image stacks were projected along the z-axis, corrected for brightness and contrast and pseudo-colored using the ImageJ software (NIH).

**Western Blot**

Western blot analysis was performed as previously described with some modifications. The level of PDGFR-β and PDGF-BB expression was measured eight weeks after the viral injection. The level of ALK1 expression was measured two weeks after the viral injection. Animals were anesthetized and the ipsilateral hemisphere at the location of 1.0 mm posterior and 1.0 mm lateral to the injection site was collected. The brain tissue was then lysed in RIPA buffer (Santa Cruz Biotechnology, CA), and an equal amount of protein was loaded to SDS-PAGE (NuPAGE® Novex 4-12% Bis-Tris gel, Invitrogen). After electrophoresis, the protein was electrotransferred onto a PVDF membrane (Bio-Rad, Hercules, CA). After blocking with Odyssey blocking buffer for 1 hour at room temperature, the membranes were incubated overnight with primary antibodies ALK1 (Santa Cruz Biotechnology, CA), PDGFR-β (R&D Systems, MN), and PDGF-BB (Santa Cruz Biotechnology, CA) at 4°C. Subsequently, the membrane was incubated with fluorescent secondary IRDye 800CW- or 680-conjugated immunoglobulin (LI-COR Biotechnology, Lincoln, NE) in 0.1% Tween-20 blocking solution. Images were acquired using the Odyssey infrared imaging system (LI-COR Biotechnology, Lincoln, NE) and analyzed using the software program as specified in the Odyssey software manual. β-actin (Sigma-Aldrich, St. Louis, MO) was used as a loading control. To test whether there was a difference among the four groups, we ran a one-way ANOVA followed by Tukey-corrected pairwise comparisons. For ALK1 data, we log transformed (ln X) the response to better adhere to the ANOVA model’s assumption of normally distributed residuals.
Supplemental References


Supplemental Figure I.

Illustration of cortical injection site. A. Needle path Blue line represents the needle. The blue spot is the needle entry site. B. A small craniotomy (solid circle) was performed to expose the underlying pial vasculature, showing large veins as well as arteries and capillaries at the surface of the somatosensory cortex relative to the midline, bregma and lambda. For this study, in vivo imaging was performed through a thinned skull window that was prepared in the area marked by the broken circle. C. Dysplastic vasculature shown by latex perfusion eight weeks after viral cortical injection. D. Examples of small cortical bAVMs in patient with HHT (genotype unknown). Patient had three bAVMs, two of which are shown. These small lesions are roughly comparable to the results of the mouse lesion shown in (left) lateral projection of internal carotid angiogram showing ≈1 cm frontal bAVM nidus (arrow) fed by branches of the middle cerebral artery and a prominent draining vein (arrowhead). Anterior-posterior projection of a vertebrobasilar injection (right) shows a ≈1 cm temporal bAVM fed by branches of the posterior cerebral artery.
Supplemental Figure II.

Two-photon imaging shows that injection of Ad-Cre and AAV-VEGF induced distinct dysplastic vessels in the cortex of $\text{Alk1}^{1f/2f}$ mouse (right). Left panel is a picture taken from the same brain region of a normal wild-type mouse.
Supplemental Figure III.

ZIC1 expression in the adult mouse brain. Double-staining of NeuN/ZIC1 (A and B), GFAP/ZIC1 (C) and desmin/ZIC1 (D) on the brain sections. There are small (A) and large (B) ZIC1+ nuclei in the adult mouse brain. The small ZIC1+ nuclei did not co-localize with NeuN; however, the large ZIC1+ nuclei did co-localize with NeuN positive nuclei, indicating that other than pericytes, some, but not all, neurons express ZIC1. ZIC1 positive staining was not detected in GFAP+ cells (C). The small ZIC1+ nuclei co-localized with desmin (D), suggesting that they are pericyte nuclei. The inserts on the left corner of each panel show nuclei-stained ZIC1 (DAPI, blue). Scale bar: 20 µm.
Supplemental Figure IV.

Confocal image of NG2 and ZIC1 double staining. A. Confocal image shows that ZIC1 (nuclei) and NG2 (cytoplasm) stain the same cell. Scale bar: 10 µm. B. 3D image shows that ZIC1 and NG2 stain the same cell (arrows).
ALK1 protein levels in $Alk1^{2f/2f}$ and $Alk1^{1f/2f}$ mice. A. Representative images of Western blot. B. Bar graph shows the quantification. Compared to $Alk1^{2f/2f}$ group, ALK1 protein was reduced in the brain of $Alk1^{1f/2f}$ mice ($P=0.016$), Ad-Cre-injected $Alk1^{2f/2f}$ ($P=0.003$), and Ad-Cre-injected $Alk1^{1f/2f}$ ($P<0.0001$) mice. $Alk1^{1f/2f}$ mice injected with Ad-Cre demonstrated further reduced ALK1 protein ($#P = 0.029$). The ALK1 protein level of Ad-Cre-treated $Alk1^{2f/2f}$ and $Alk1^{1f/2f}$ mice was not significant ($P=0.143$). * indicates $P < 0.05$ when compared to all the other three groups.
Supplemental Figure VI.

PDGFR-β expression was reduced in the viral injection sites of Alk1-deficient brain. (A) Representative images of Western blot for PDGFR-β and PDGF-BB. Bar graphs show quantification of PDGFR-β (B) and PDGF-BB (C) expression. The protein level was presented as relative to the WT group. PDGFR-β expression was reduced in the viral-injection sites of Alk1-deficient brain as compared to that in the WT and Alk1^{11/2f} groups (*p<0.001). PDGF-BB expression did not differ among groups (p=0.32).