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Arteriovenous Malformation in the Adult Mouse Brain Resembling the Human Disease

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Abstract

Objective—Brain arteriovenous malformations (bAVM) are an important cause of hemorrhagic stroke. The underlying mechanisms are not clear. No animal model for adult bAVM is available for mechanistic exploration. Patients with Hereditary Hemorrhagic Telangiectasia Type2 (HHT2) with activin receptor-like kinase 1 (*ALK1*; *ACVRL1*) mutations have a higher incidence of bAVM than the general population. We tested the hypothesis that VEGF stimulation with regional homozygous deletion of *Alk1* induces severe dysplasia in the adult mouse brain, akin to human bAVM.

Methods—*Alk1*$^{2f/2f}$ (exons 4–6 flanked by loxP sites) and wild-type (WT) mice (8–10 weeks old) were injected with Ad-Cre (2×10^7 PFU, adenoviral vector expressing Cre recombinase) and AAV-VEGF (2×10^9 genome copies, adeno-associated viral vectors expressing VEGF) into the basal ganglia. At 8 weeks, blood vessels were analyzed.

Results—Gross vascular irregularities were seen in *Alk1* 2f/2f mouse brain injected with Ad-Cre and AAV-VEGF. The vessels were markedly enlarged with abnormal patterning resembling aspects of the human bAVM phenotype, displayed altered expression of the arterial and venous markers (EphB4 and Jagged-1), and showed evidence of arteriovenous shunting. Vascular irregularities were not seen in similarly treated WT mice.

Interpretation—Our data indicate that post-natal, adult formation of the human disease bAVM is possible, and that both genetic mutation and angiogenic stimulation are necessary for lesion development. Our work not only provides a testable adult mouse bAVM model for the first time, but also suggests that specific medical therapy can be developed to slow bAVM growth and potentially stabilize the rupture-prone abnormal vasculature.

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Introduction

Brain arteriovenous malformations (bAVMs) are an abnormal tangle of blood vessels that shunt blood directly from the arterial to venous circulation.1 An important cause of hemorrhagic stroke, especially in children and young adults, there is little known about how bAVMs develop and progress. Only surgical, endovascular or radiation-induced obliteration is available for treatment, which has associated risks of neurological injury. There are no specific medical therapies to treat the disease.

The longstanding assumption that bAVMs are congenital lesions2 colors approach to both treatment and research into novel medical therapies. Considering the high utilization of prenatal ultrasound, there is little evidence for this common belief that bAVMs arise during embryonic development.3 The mean age at presentation (detection) is roughly 40 years of age, with a normal distribution.4, 5 Some bAVMs do arise prenatally,6 but invoking congenital formation for all lesions may not be the best explanation.7 The usual clinical behavior, e.g, natural history pattern for hemorrhage, begins near puberty.8 There have been multiple reports of de novo growth and local bAVM recurrence after treatment.9–11 Although rare (≈1–5%), such events support the notion that bAVMs are not static congenital defects, but rather undergo active vascular change with potential for post-natal growth.12

An underlying genetic predisposition may also play a role in bAVM formation. A familial form of bAVM is seen in Hereditary Hemorrhagic Telangiectasia (HHT). This autosomal dominant disease is caused by mutations in primarily two genes—Endoglin (OMIM: 131195) in HHT1 and *ALK1* (*ACVRL1*; OMIM: 601284) in HHT2— that result in functional haploinsufficiency; both genes are involved in TGFβ superfamily signaling. Brain AVMs are seen in HHT1 and HHT2 patients with an approximate penetrance of 20% and 2%, respectively.13 Both sporadic and familial bAVMs, despite some differences, have remarkably similar vascular phenotype, unique among brain vascular malformations.14

Recently a concept has emerged that a response to a perturbation or injury appears to be a necessary component to initiate vascular dysplasia,15–17 which is hypothesized to be an early stage of bAVM development based on the following observations: Deletion of *Alk1* during development resulted in severe vascular dysplasia and arteriovenous shunting, whereas a systemic deletion of *Alk1* in the adult mouse did not provoke vascular malformation in the skin and brain.16 Induction of a wound in the skin of *Alk1*-deficient adult mice led to vascular dysplasia and arteriovenous shunting around the wound. These results suggest that formation of an AVM phenotype in the adult skin requires both a genetic predisposition (e.g., *Alk1*-deficiency) and environmental factors (e.g., wounding).

In this study, we investigated whether this concept is applicable to formation of a bAVM phenotype in adult mice and we demonstrate for the first time that a pathway known to be associated with the Mendelian form of bAVM, HHT2, can be manipulated by an environmental stimulus in the post-natal adult mouse brain to result in a phenotype consistent with the clinical manifestations of bAVM in humans. This proof-of-concept has important consequences for the approach to pre-clinical therapy development: if bAVMs are a post-natally acquired disease and form in response to an injury or angiogenic stimulus, medical therapy to slow or arrest their growth may be developed as an important step towards improving care of these patients.

Materials and Methods

Animals

Experimental procedures for using laboratory animals were approved by the Institutional Animal Care and Use Committee of the University of California, San Francisco. Adult (8 to 10-week old) *Alk1*-floxed mice (*Alk1*$^{2f/2f}$) with loxP sites flanking Exons 4–618 and C57BL/6 mice (Jackson Laboratory, Bar Harbor, ME) were used. We also included additional groups of *Alk1*$^{2f/2f}$ / ROSA26 (+/creER)16 and Rosa26-lacZ cre reporter (R26R, Jackson Laboratory) as controls.

Viral Vectors

Ad-Cre (adenoviral vector with CMV promoter driving Cre recombinase expression) and control Ad-GFP vectors were purchased from Vector Biolabs (Philadelphia, PA). AAV-VEGF and AAV-LacZ have been previously described.19, 20 We chose to use an adenoviral vector to deliver Cre gene since protein production following transduction peaks earlier than with AAV.21 Therefore, we co-injected Ad-Cre with AAV-VEGF to favor Cre-mediated *Alk1* deletion before the peak of VEGF stimulation.19

Viral Vector Transduction in the Mouse Brain

Following induction of anesthesia with isoflurane, the mice were placed in a stereotactic frame with a holder (David Kopf Instruments, Tujunga, CA), and a burr hole was drilled in the pericranium 2 mm lateral to the sagittal suture and 1 mm posterior to the coronal suture. Three μl viral suspension containing 2×10^7 plaque forming unit (PFU) adenoviral vectors and 2×10^9 genome copies (gcs) of AAV viral vectors were stereotactically injected into the right basal ganglia at a rate of 0.2 μl per minute using a Hamilton syringe. The needle was withdrawn after 10 min and the wound was closed with a suture.22

Statistical Analysis

The effects of mouse genotype (WT versus *Alk1*-floxed), AAV vectors (AAV-VEGF versus AAV-LacZ) and adenoviral vectors (Ad-Cre versus Ad-LacZ) on capillary density and dysplasia index were analyzed using three-way ANOVA, followed by a student's t test to compare the means between groups. Data are presented as mean ± SEM. A p value < 0.05 was considered statistically significant.

The Supplemental Methods section describes additional methods.

Results

Injection of Ad-Cre Leads to Regional Gene Deletion in the Adult Mouse Brain

To regionally and conditionally delete *Alk1* in the brain of adult mice, we used a strategy of injecting Ad-Cre into the basal ganglia of *Alk1*-floxed mice.18 The effect of Ad-Cre-mediated gene deletion has been evaluated in R26R reporter mice.23 After injection of Ad-Cre (2×10^7 PFU) into the basal ganglia of R26R mice, we detected LacZ gene expression in 51% of endothelial cells, 85% of neurons, and 74% of astrocytes; expression was restricted to the local injection site, without evidence of distant infection, e.g., LacZ expression in other organs (Supplemental Figures 1 and 2). The success of *Alk1* gene deletion around the injection site was demonstrated by the presence of the *Alk1* 1f allele in the genomic DNA (Supplemental Figure 3). Ad-GFP did not affect the integrity of the *Alk1* gene.

Induction of Localized Cerebrovascular Malformations in the Adult Mouse Brain

To investigate whether angiogenic stimulation induces the bAVM phenotype in *Alk1*-deficient adult mice, we co-injected Ad-Cre (2×10^7 PFU) and AAV-VEGF (2×10^9 gcs)[19, 24] into the basal ganglia of *Alk1*-floxed mice. Gene expression mediated by AAV vectors was detected around the injection sites (Supplemental Figure 4). Gross cerebrovascular irregularities were observed around the injection site eight weeks following vector injection (Figure 1A, 1B and Supplemental Figure 5). The vessels were enlarged and dilated with grossly abnormal patterning resembling diffuse bAVM seen in humans (Figure 1C and 1D). Injection of Ad-GFP with AAV-VEGF into the brain of *Alk1*-floxed mice or injection of Ad-Cre with AAV-VEGF into the brain of WT mice resulted in angiogenesis with normal vascular structure. Injection of Ad-Cre with AAV-LacZ to *Alk1*-floxed mice did not change the vascular structure of the brain (Figure 1A). Thus, VEGF stimulation as an environmental insult is sufficient to induce the development of severe cerebrovascular dysplasia in *Alk1*-deficient adult mice.

Histological analysis of lectin-perfused brain demonstrated that the dysplastic vessels were irregular with larger lumens than normal capillaries. Some were covered with smooth muscle (αSMA positive) and others were not. The arterial marker, Jagged-1, and the venous marker, EphB4, were detected in the endothelial cells lining the vessels. The specific staining of Jagged-1 and EphB4 antibodies were confirmed on the arteries and veins of the small intestine, basilar artery and jugular vein (Supplemental Figure 6). Some dysplastic vessels had endothelial cells that expressed both Jagged-1 and EphB4 (Figure 2A and 2B), suggesting that aberrant arterial-venous determination may be involved in the altered phenotype. Neuronal tissue was observed in between the dysplastic vessels as indicated by NeuN positive neurons scattered among the vessels and on the dysplastic vessel walls (Figure 2C). This is also similar to the clinical phenotype of diffuse bAVMs, where areas of brain parenchyma are seen intervening between vascular structures in the nidus (Figure 2D).[25, 26]

To quantify the angiogenic response and the degree of dysplasia, we analyzed the capillary density and dysplasia index. Overall, mean capillary density differed among groups, with a trend towards increased capillary density in all AAV-VEGF-injected groups as compared to the LacZ-injected group ($p= 0.07$, Figures 3A and 3B). The mean capillary density of *Alk1*-floxed mice treated with Ad-Cre and AAV-VEGF (353 ± 55) was higher than that of *Alk1*-floxed mice treated with Ad-Cre and AAV-LacZ (177 ± 16, $p=0.02$). The mean capillary densities of other groups were: 248 ± 36 (WT/Ad-Cre and AAV-VEGF), and 327 ± 75 (*Alk1*-floxed mice /Ad-GFP and AAV-VEGF).

The dysplasia index was significantly higher in the brain of *Alk1*-floxed mice treated with Ad-Cre and AAV-VEGF as compared to all other groups ($p<0.01$, Figure 3B). Dysplasia index values were: 0.40 ± 0.19 (WT/Ad-Cre and AAV-VEGF), 2.9 ± 0.23 (*Alk1*-floxed mice/Ad-Cre and AAV-VEGF), 0.24 ± 0.24 (*Alk1*-floxed mice/Ad-Cre and AAV-LacZ), and 0.68 ± 0.31 (*Alk1*-floxed mice/Ad-GFP and AAV-VEGF). Thus, the combination of *Alk1* deletion and VEGF stimulation was necessary to induce vascular dysplasia in this model.

Alk1 Deletion with VEGF Stimulation Leads to Arteriovenous (A–V) Shunting in the Brain

To assess if there was A–V shunting in the dysplastic vessels that we induced by combining *Alk1* deletion and VEGF stimulation, we used two methods. First, we injected 20 μm fluorescent microspheres into the common carotid artery. In the normal brain, beads will lodge in the capillary bed; in the presence of A–V shunting, beads will pass through the cerebrovascular bed and lodge in the lungs. Beads were found in the brain of all groups.

However, beads were found in the lung only in the *Alk1*-floxed mice treated with Ad-Cre and AAV-VEGF, suggestive of A–V shunting in this group (Figure 4A). There were a few fluorescent beads in the lung of AAV-VEGF-injected control mice, consistent with vascular dilation present in VEGF-treated tissues.27

Second, we perfused the animals with latex through the left ventricle of the heart. The latex particles we used are too large to pass through the capillary bed and only cast arterial structures. However, in the presence of an A–V shunt, latex also casts veins.16 In the brain of control mice, the latex only perfused arteries. Latex injection cast tortuous dysplastic vessels and veins in the brain of the *Alk1*-floxed mice treated with Ad-Cre and AAV-VEGF (Figure 4B); it did not enter capillaries and veins in the brain of *Alk1*-floxed/ ROSA26(+/ CreER) mice that received tamoxifen treatment to globally delete *Alk1* (Figure 4B). Thus, deletion of the *Alk1* gene is not sufficient to trigger the development of cerebrovascular dysplasia with A–V shunting; additional stimulation, such as angiogenic signaling, is necessary.

To further demonstrate potential direct A–V connections, we developed a novel technique to label the arteries and veins individually with different colored latex dye. We injected green latex dye into the jugular vein to label intracerebral veins, and blue latex dye into the left ventricle of the heart to label cerebral arteries. The direct connections of arteries and veins were detected in the brain of *Alk1*-floxed mice treated with Ad-Cre and AAV-VEGF (Figure 4C). Thus, by regional deletion of the *Alk1* gene plus VEGF stimulation, we created cerebrovascular dysplasia with direct A–V connections.

Discussion

We demonstrate here for the first time the induction of a mouse brain phenotype that mimics adult-onset human bAVM formation. The novel demonstration is that the mouse phenotype was induced by an “environmental” stimulus—VEGF—in genetically modified adult animals. The mouse model developed by *Alk1* deletion, in combination with VEGF stimulation, mimics macroscopic morphological features of the human disease phenotype, such as large dysplastic, tangled vessels and A–V shunting. Histological analysis demonstrates similarities in microvascular morphology between the mouse model and human bAVMs. Induced vascular dysplasia resulted from disruption of *Alk1* signaling, making the most direct comparison of the lesions we produced to those lesions found in the context of HHT2. However, given the morphological similarities between sporadic and germline bAVMs, the induction of this mouse phenotype provides the first proof-of-principle that the human disease can be induced by a combination of genetic predisposition and a response to local environmental changes. This demonstration has important implications for developing novel medical interventions for a potentially highly morbid clinical disorder, widely held to be “congenital.”

Attempts to model bAVM disease have historically focused on simulating altered cerebral hemodynamics to study perioperative neurological complications,28 or developing therapeutic materials such as embolic agents.29 The modeled lesions are largely extradural in nature and do not display the clinical syndrome of recurrent hemorrhage into the brain parenchyma or cerebral-spinal fluid spaces.30 Therefore, a *parenchymal* nidus is not formed, and nidus growth and hemorrhage mimicking the human disease do not occur.5 More recently, disease formation studies have addressed prenatal induction of the bAVM phenotype,16, 18, 31 but these studies have not addressed how the disease might develop in the adult.

The deletion of *Alk1* alone does not appear sufficient to induce significant cerebrovascular abnormalities. In 7-to-21 month old *Alk1*+/− mice, spontaneous vascular lesions were seen in skin, extremities, oral cavity and in many internal organs. However, brain abnormalities occurred only in one out of 47 animals, which showed a dilated cerebellar blood vessel.32 Post-natal conditional homozygous deletion of *Alk1* in adult mice systematically caused vascular malformations to form in the GI track, lung and uterus, but brain and skin lesions were not evident.16 However, wounding induced the formation of A–V fistulas in the skin of these mice.16

Following a wound, there is a release of various growth factors and cytokines that may depend on functional *Alk1* signaling to properly mount an angiogenic response. Park et al have further shown that VEGF delivered by pellet in the skin also induces a similar phenotype to the wounding experiment (personal communication). We have previously shown that VEGF stimulation in adult mice heterozygous for *Alk1* leads to modest abnormalities in vascular morphology, which we termed *vascular dysplasia*.33 This led us to propose the term “response-to-injury” to describe a two-step process for the development of severe dysplasia resulting in the formation of a bAVM, a term that differentiates it from a “two-hit” genetic model, i.e., biallelic somatic and germline mutations, which has been demonstrated, for example, in cerebral cavernous malformations.34, 35 The “response-to-injury” concept is supported by current experiments and previous reports that have examined abnormal vascular responses in both $Alk1^{+/-}$ 16, 36 and $Endoglin^{+/-}$ mice.15, 33

Since our genetic manipulation involved *Alk1*, our study is directly related to bAVM in the context of HHT2. Nonetheless, the induction of an abnormal vascular phenotype in the *Alk1*-deleted mice has relevance to the sporadic disease. As a class, the inherited bAVMs in HHT have distinguishing morphological characteristics such as smaller size, higher incidence of single-hole fistulas, higher incidence of cortical location and lesion multiplicity. Otherwise, bAVMs in HHT patients have a morphology generally similar to sporadic lesions and cannot be distinguished individually on the basis of their angioarchitecture.14, 37

Compared to the prevalence of sporadic lesions in the normal population, the presence of an *ENG* or *ALK1* mutation results in approximately a 1,000- and 100-fold increased risk, respectively, of developing a bAVM.1 The greatly elevated risk of bAVM development in the Mendelian disorders raises the possibility that germline *sequence variants* of these and other genes in shared pathways may likewise pose a significant risk for *sporadi*c bAVM development.

A prime importance of this study is that it demonstrates that the bAVM phenotype formation is not necessarily congenital. Other than Vein of Galen malformations, true congenital bAVMs are relatively rare.3 The scarce data available on longitudinal assessment of bAVM growth after detection suggest that approximately 50% of cases display some degree of interval growth,12 but the relationship of such growth with natural history remains unknown. If bAVMs form and grow during post-natal life, this opens up the possibility that medical therapy can be developed to slow growth and stabilize abnormal vessels that are prone to rupture. The present model system will be useful in screening a variety of strategies to achieve these ends. Proof-of-principle for pharmacological therapy for another abnormal vascular phenotype in the setting of HHT has recently been shown: use of thalidomide to stabilize the abnormal vessels in nasal mucosa that are prone to repeated bouts of severe epistaxis.38

Our study had several limitations. Injection of the adenovirus, Ad-Cre, may lead to local injury and inflammation39 that may affect vascular integrity. In addition, VEGF stimulation has been associated with breakdown of the blood-brain barrier,27, 40 which may lead to

cytokine release and a vascular response. However, our data show that neither *Alk1* deletion nor VEGF stimulation alone causes the phenotype. The deletion of *Alk1* was a necessary component to induce the observed vascular phenotype, but further work remains to be done to confirm if in fact there is a defect in canonical or non-canonical pathways involved in the generation of the phenotype, as has been suggested for endoglin.41

Another limitation is the lack of definitive documentation of A–V shunting. A lack of oxygen extraction by the surrounding tissue would be needed to confirm a true A–V shunt. However, the microscopic and macroscopic findings in our model, coupled with the demonstration of bead passage into the lung, support the existence of the shunt.

In addition, we found that *Alk1* deletion increased brain angiogenic response as shown in Figure 3A and B which is consistent with our previous finding in the cortex of $Alk1^{+/-}$ mice.17 The effect of *Alk1* in endothelial cells *in vitro* is controversial. Constitutively active *Alk1* increased the migration and proliferation of embryonic mouse cells.42, 43 However, in cultured human endothelial cells, constitutive overexpression of *Alk1* inhibited cell migration and proliferation,44 and downregulation of *Alk1* accelerated cell migration.45 Our data show that in the adult mouse brain, *Alk1* deletion increased neovascular formation in response to VEGF stimulation. The underlying mechanisms need to be dissected in the future.

In summary, we have experimentally induced a distinct vascular phenotype that mimics pertinent aspects of human brain AVMs. The “response-to-injury” after VEGF stimulation against an *Alk1* deficient background is a plausible scenario for post-natal growth or recurrence of bAVM. Importantly, identifying the factors involved in the progression of the human disease, regardless of underlying genetic defect, may allow for development of a medical therapy to lessen the risk of spontaneous rupture.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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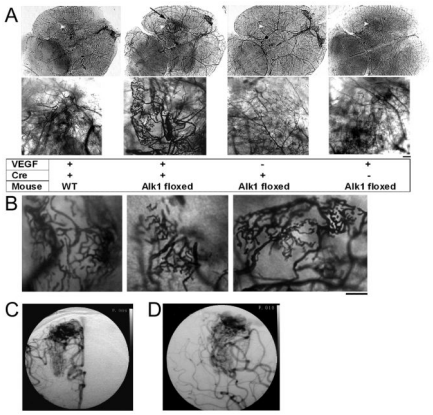

Figure 1. Vessel casting showing that VEGF stimulation induced distinct cerebrovascular abnormalities at the *Alk1*-deleted region

(A) Large tangled vessels resembling a bAVM were detected at the injection site of Ad-Cre and AAV-VEGF in the brain of *Alk1*-floxed mice (black arrow). *Alk1* deletion (Ad-Cre injection) without VEGF had no effect on the cerebrovascular structure. Overexpression of VEGF in the brain without *Alk1* deletion (WT mice or *Alk1* mice injected with control adenoviral vector) induced normal angiogenesis. The bottom images show the enlarged angiogenic foci of the images on top. Scale bar = 100 μm. Injection sites are indicated by white arrow heads.

(B) Abnormal vasculature from 3 different *Alk1*-floxed mice injected with Ad-Cre and AAV-VEGF. Scale bar = 100 μm.

(C) & (D) Right internal carotid artery anterior-posterior (C) and lateral (D) projections of an angiogram from an 18-year-old male who underwent microsurgical bAVM resection. The bAVM, supplied by the middle and anterior cerebral arteries, has a diffuse angioarchitecture, similar to the phenotype in the mouse model.

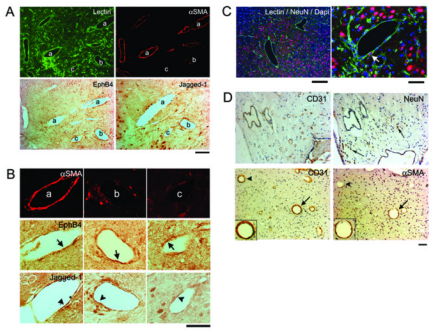

Figure 2. Dysplastic vessels have irregular smooth muscle coverage and express inconsistent arterial and venous markers

(A) A group of dilated irregular vessels labeled with lectin (green). Some of these vessels have smooth muscle coverage (αSMA, red), some do not. Arterial (Jagged-1) and venous (EphB4) markers were expressed by the endothelial cells of vessels with or without smooth muscle. Scale Bar: 50 μm.

(B) Enlarged image of the vessel (a), (b), (c) indicated in (A). Vessel (a) is covered with a complete layer of smooth muscle and (b) has a few αSMA positive cells. Their endothelial cells express EphB4 and Jagged-1 (arrows and arrowheads). Vessel (c) has almost no αSMA positive cells. Most of its endothelial cells express EphB4, a few express Jagged-1. Scale Bar: 50 μm.

(C) Neuronal tissue is present between enlarged irregular vessels. Arrow indicates NeuN positive cell on the dysplastic vessel wall. Scale Bars: 200 μm (left) and 50 μm (right). Corresponding colors for Lectin, NeuN, and Dapi are green, red, and blue, respectively.

(D) Paraffin sections of surgical specimen from patient shown in Figure 1, stained with antibodies specific to CD31, αSMA or NeuN. Top panel shows NeuN positive cells (arrows) detected between the dysplastic vessels. Lower panel shows enlarged vessels with (arrows) or without (arrowheads) smooth muscle layers. Inserts in the lower panel show enlarged images of the vessels indicated by arrows with endothelial cells stained by CD31 antibody and a thin layer of smooth muscle (αSMA) surrounding the endothelial layer. Scale Bar: 50 μm.

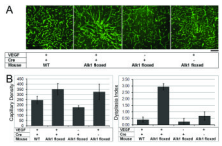

Figure 3. VEGF induced focal angiogenesis in the normal brain and dysplastic vessel formation in the *Alk1*-deleted brain

(A) Representative images show lectin-perfused vessels in the brain. Capillary density is high in all AAV-VEGF-treated groups as compared to the AAV-LacZ control group. Dilated irregular vessels were only observed in the brain with *Alk1* deletion and VEGF stimulation. Scale Bar=50 μm.

(B) Bar graphs show capillary density (left) and dysplasia index (right). Capillary density increased in all groups with VEGF stimulation as compared to the AAV-lacZ control group ($p=0.07$). Dysplasia index increased significantly in *Alk1*-floxed mice treated with Ad-Cre and AAV-VEGF as compared to all other groups ($p<0.01$).

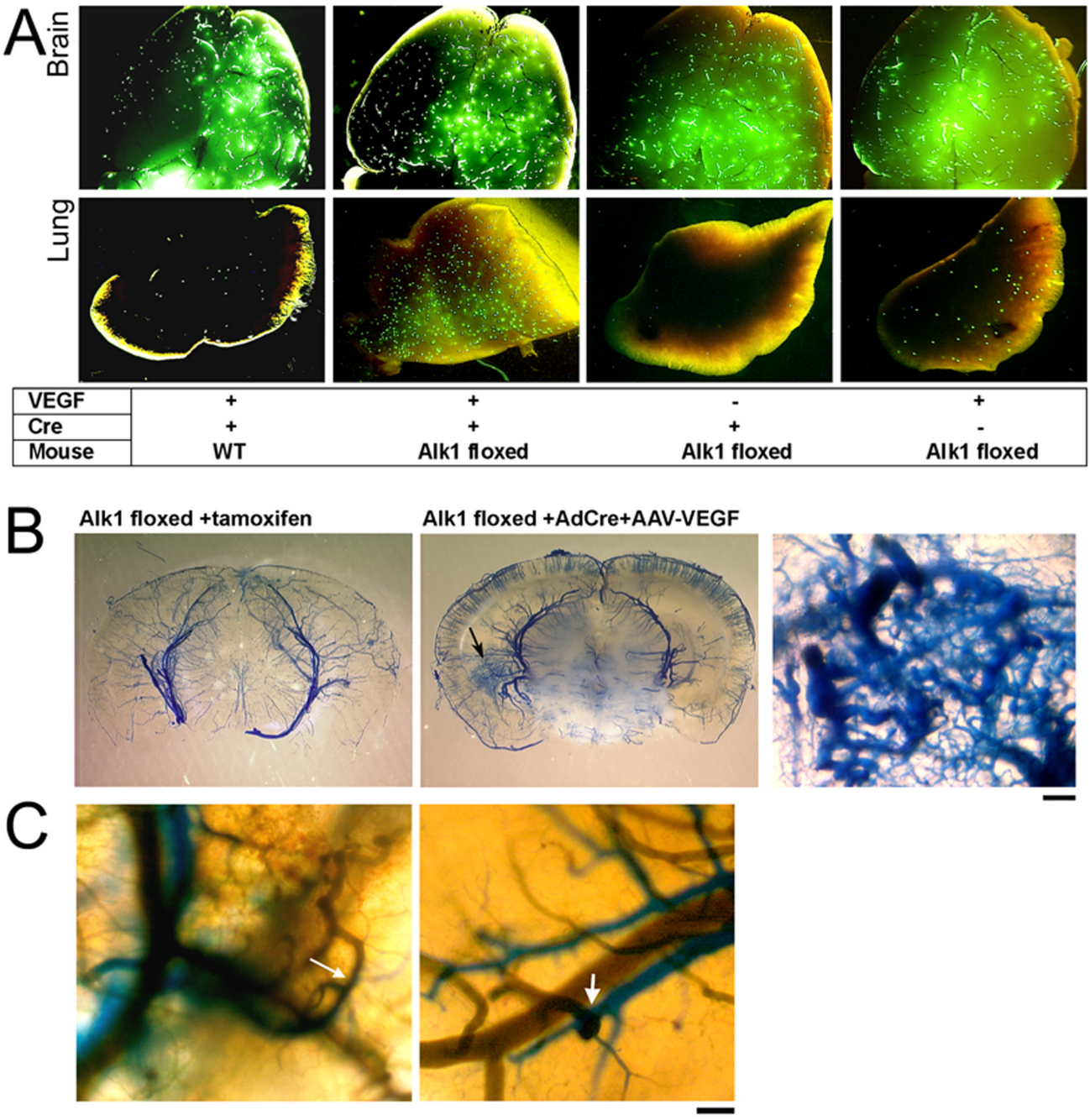

Figure 4. Arteriovenous (A–V) shunting was detected in the brain with regional *Alk1* deletion and VEGF stimulation

(A) Brain and lung samples collected from animals perfused with 20 μm fluorescent beads through the carotid artery. Beads were detected in all brain samples, but only *Alk1*-floxed mice injected with Ad-Cre and AAV-VEGF had a significant amount of beads in the lung. A few beads were detected in the lungs of control mice injected with AAV-VEGF.

(B) Latex-perfused brain. Latex (blue) presented only in cerebral arteries of *Alk1*$^{2f/2f}$/ROSA26(+/creER) mice treated with tamoxifen that had *Alk1* gene deleted globally (left). *Alk1*-floxed mice that received intracerebral injection of Ad-Cre and AAV-VEGF had an increase of vascular density at the injection site (arrow, middle image). At higher

magnification (right), vessels are enlarged and tortuous with a fistula between arteries and veins. Scale Bar = 100 μm.
(C) Two color latex perfusion shows direct connections of cerebral arteries (blue) and veins (green) (arrows). Scale Bar = 100 μm.