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## Brain Arteriovenous Malformation Modeling, Pathogenesis and Novel Therapeutic Targets

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### Abstract

Patients harboring brain arteriovenous malformation (bAVM) are at life-threatening risk of rupture and intracranial hemorrhage (ICH). The pathogenesis of bAVM has not been completely understood. Current treatment options are invasive and  $\approx 20\%$  of patients are not offered interventional therapy because of excessive treatment risk. There are no specific medical therapies to treat bAVMs. The lack of validated animal models has been an obstacle for testing hypotheses of bAVM pathogenesis and testing new therapies. In this review, we summarize bAVM model development; and bAVM pathogenesis and potential therapeutic targets that have been identified during model development.

### Keywords

Activin-like kinase 1; Angiogenesis; Brain arteriovenous malformation; Conditional knockout; Endoglin; Hereditary hemorrhagic telangiectasia; Mouse models

### Introduction

Brain arteriovenous malformations (bAVMs) are complex tangles of abnormal, dilated channels that do not have a typical artery or vein structure. They are important risk factors of intracranial hemorrhage (ICH), especially in children and young adults [1–3]. The etiopathogenesis is currently not well understood. Prevention of new or recurrent ICH is the primary rationale to treat AVMs, with some combination of resection, embolization and/or radiotherapy. All of these therapies are invasive and associated with considerable side effects [4–6]. Other than nonspecific control of symptomatology, e.g., headache and seizures, no specific medical therapy is available to directly treat AVMs or decrease spontaneous rupture risk. About 20% of patients currently are not offered treatment due to

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#### Compliance with Ethics Requirements

This Review Article does not contain any studies with human or animal subjects. All cited studies describe ethical standards in cited manuscripts.

#### Conflict of Interest

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excessive risks associated with the treatment [4]. There is also considerable controversy regarding unruptured bAVMs being treated using invasive modalities, as treatment risk may outweigh the natural history risk of spontaneous rupture [6]. The lack of proper animal models has critically hampered research progress and new therapy development.

Brain AVMs are traditionally regarded as congenital lesions, which are thought to arise in the third week of gestation secondary to disordered embryogenesis. Primordial vascular channels fail to differentiate into mature intervening capillaries and veins, and instead create arteriovenous shunts without intervening capillaries [7, 8]. However, despite frequent use of prenatal ultrasound, there is remarkably little evidence to show that AVMs are congenital lesions arising during embryonic development. In fact, the mean age at presentation (detection) is roughly 40 years of age, with normal distribution. Although it is possible that the lesions uniquely arise prenatally (and a small number do), lacking sufficient data, it would be premature to infer an adequate explanation. There are multiple reports of AVM growing or regressing, and of local AVM regrowth after treatment [9]. AVMs have been shown to occasionally arise *de novo* after a normal angiogram and regrow after resection, either *de novo* from a retained fragment [10–12] or from a lesion treated with radiotherapy [12, 13]. This evidence supports the hypothesis that bAVM can form postnatally.

More than 95% of bAVMs are sporadic [14]. The genesis of bAVMs has been enigmatic. About 5% of bAVM are due to hereditary hemorrhagic telangiectasia (HHT) [15], a familial disease characterized by AVMs in multiple organs and mucocutaneous telangiectasias (small AVMs) [16]. The two main subtypes of HHT (HHT 1 & 2) are caused by mutations in two genes implicated in transforming growth factor- $\beta$  (TGF- $\beta$ )/bone morphogenic protein (BMP) canonical signaling pathways: Endoglin (ENG), and Activin-like kinase 1 (ALK1; ACVLR1) [17]. As a class, the inherited AVMs in HHT have some distinguishing morphological features, but are generally similar to the sporadic lesions and cannot be distinguished individually on the basis of their angioarchitecture [18, 19]. The prevalence of bAVM in HHT1 (ENG-deficient) is 1000-fold higher, and HHT2 (ALK1-deficient) is 100-fold higher than the prevalence in the general population (10/100,000) [20]. Modeling HHT1 and HHT2 bAVMs has been fruitful.

This review discusses the strategies that have been used for bAVM model development, and the pathogenesis as well as potential new therapeutic targets (Fig. 1) that have been identified using available models.

## Modeling Brain AVM in Animals

Historically, “AVM” models have been largely based on extradural arteriovenous (A–V) fistulas to study hemodynamic changes or develop platforms for technology development [21–38]. They can be categorized into 2 subtypes. 1) Hemodynamic models, in which the normal extracranial vasculature is surgically manipulated to create a shunt from the contralateral side through the Circle of Willis and into the ipsilateral jugular vein without an intervening capillary bed. This is most commonly accomplished by anastomosing the common carotid artery to the jugular vein [21, 22, 35]. 2) Angiographic models, which utilize essentially the same anastomotic technique to achieve high-flow, low-resistance

hemodynamics that take advantage of the “AVM-like” angiographic appearance of the rete mirabile normally present in artiodactyl (even-toed ungulates) species [24, 25]. With few exceptions [38], they are extradural in nature; none display the clinical syndrome of recurrent hemorrhage into the brain parenchyma or cerebrospinal fluid (CSF) spaces. No other model has a *parenchymal* nidus. Therefore nidus growth and hemorrhage mimicking the human disease do not occur.

Many different kinds of developmental gene defects result in antenatal hemorrhage, which may or may not be related to brain AVMs. The proteins identified in studies of brain hemorrhage may be related to AVM biology, such as integrin  $\alpha V\beta 8$  [39]. Manipulating the proteins of interest may yield vascular structures reminiscent of the human disease. For example, endothelial expression of constitutively active Notch-4 elicited reversible “AVMs” in adult mice [40], or endothelial overexpression of Notch-4 intracellular domain resulted in brain AVMs in young mice [41]. Knockout integrin  $\alpha V\beta 8$  plus focal vascular endothelial growth factor (VEGF) stimulation induced capillary dysplasia in the brain [42]. In addition, homozygous knockout of matrix Gla protein (Mgp) have also resulted in AVM formation in the brain and multiple organs [43]. However, the story becomes more interesting when such models are focused on genes that are clearly related to the human disorder, i.e., those genes described above which underlie HHT. A logical approach to animal models is to focus on genes that are clearly related to the human disease phenotype.

An important conceptual advance in modeling brain AVMs has been to consider HHT [44] as a familial form of the more common sporadic disorder, or at least posit that HHT possesses a similar enough phenotype to sporadic brain AVM so that knowledge of the inherited gene pathways can shed light on sporadic disease pathogenesis.

Inactivating a single allele of *Eng* or *Alk1* in mice reproduces certain aspects of the human disease in animal models [45, 46], but spontaneous lesions in the brain are rare and subtle, mostly in aged mice [45, 47]. More pronounced forms of cerebral microvascular dysplasia can be induced using VEGF stimulation in *Eng*<sup>+/-</sup> or *Alk1*<sup>+/-</sup> mice [48–50], which can be enhanced by increasing tissue perfusion rates [49]. However, the dysmorphic vessels developed in *Eng*<sup>+/-</sup> or *Alk1*<sup>+/-</sup> mice are at the capillary level. No arteriovenous (A–V) shunt can be detected.

Loss of both alleles of *Eng* or *Alk1* in mice is embryonically lethal [51, 52]. Oh and colleagues have created A–V fistulas in the neonatal brain through knockout of *Alk1* from *Alk1*-expressing cells [53]. Brain and spinal cord AVMs have also developed in mice with *SM22* $\alpha$ -Cre-mediated *Alk1* deletion during the embryonic developmental stage [54]. However, most of the mice died shortly after birth.

Our group has developed the first adult onset brain AVM model using a combination of focal *Alk1* homozygous deletion and VEGF stimulation (Table 1, Figs. 2 & 3) [55]. This model mimics many aspects of the human bAVM lesion, such as A–V shunting, microhemorrhage and macrophage infiltration [55–57]. Since an adenoviral vector is used to mediate cre expression (Ad-Cre) in this model, the inflammation caused by the adenoviral

vector complicates the mechanism analysis (Table 1). In addition, Ad-Cre could not mediate significant *Eng* deletion in *Eng<sup>fl/fl</sup>* mice [58].

Using Cre transgenic mouse lines, we have now developed two other adult onset models (Figure 2) [59, 60] and one developmental onset bAVM model that have low mortality [60] (Table 1). Fully-developed bAVMs were detected in adult R26CreER;*Eng<sup>fl/fl</sup>* mice eight weeks after induction of global *Eng* gene deletion and brain angiogenesis (Figs. 2 & 4), and in *Pdgfb-iCre;Alk1<sup>fl/fl</sup>*; mice four weeks after induction of brain angiogenesis and two weeks after induction of endothelial *Alk1* deletion (Fig. 2). The bAVM that developed in *Pdgfb-iCre;Alk1<sup>fl/fl</sup>* mice occurred in a relatively shorter time. The mice died 10–14 days after tamoxifen-induced *Alk1* deletion. The bAVM in R26CreER;*Eng<sup>fl/fl</sup>* mice developed more slowly. The mice survived for an extended period, more than eight weeks after *Eng* deletion (Table 1, Fig. 2). Thus, this model is more suited for testing new therapies.

The developmental onset bAVM model was developed by using *SM22 $\alpha$ -Cre* transgenic line to delete *Eng* during the embryonic developmental stage. Unlike conventional *Eng*-homozygous knockout mice (*Eng<sup>-/-</sup>*) that are embryonically lethal [61–63], *SM22 $\alpha$ -Cre;Eng<sup>fl/fl</sup>* mice were born with, and in life developed, various degrees of AVMs in the central nervous system, with more than 95% penetrance at five weeks of age (Table 1) [60]. They showed important clinical aspects of human lesions, including A–V shunting and spontaneous hemorrhages (Fig. 5). Further, AVM phenotypes were similar to those previously observed in *SM22 $\alpha$ -Cre;Alk1<sup>fl/fl</sup>* mice [54]. These mice, however, had less lethality. Since bAVMs in this model were developed spontaneously without local manipulation, their lesion progression more closely mimics human disease, and thus is a better model than others for bAVM mechanistic study and for new drug testing (Table 1).

## Brain AVM Pathogenesis

Molecular and histological analysis of human bAVM specimens shows that the level of angiogenic factors and inflammatory cytokines are higher in bAVMs than in the normal brain. bAVMs are also infiltrated with inflammatory cells [16, 56, 57, 64–68]. However, the pathogenesis of bAVMs is not completely understood. By modeling HHT bAVMs in animals and analyzing the phenotype of these models, we have identified the following factors playing a role in bAVM pathogenesis (Fig. 1).

### (1) Homozygous causative gene deletion in endothelial cells

The prevailing view is that HHT is caused by haploinsufficiency of one of its causative genes in somatic endothelial cells. However, inactivation of the remaining wild-type allele appears to have a powerful effect, irrespective of the mechanism by which it is inactivated, e.g., loss of heterozygosity or loss of protein during inflammation [69]. As mentioned above, the loss of a single allele of one of the causative genes for HHT is ineffective for bAVM formation in mice [45, 46]. In contrast, loss of both alleles of any HHT-causative gene is embryonically lethal [51, 52], and conditional (tissue/time-specific) homozygous deletion of *Eng* [69] or *Alk1* [53, 54] results in striking vascular malformations resembling the AVMs found in HHT [53, 54]. We showed that homozygous knockout of *Eng* in just ~1% endothelial cells in mice resulted in a more severe cerebrovascular dysplasia after VEGF

stimulation than in *Eng*<sup>+/-</sup> mice [58]. *Eng* null endothelial cells were present in vessels in bAVM lesions in *SM22α;Eng*<sup>ff</sup> mice and *RosaCreER;Eng*<sup>ff</sup> mice [60]. Moreover, analysis of human brain and lung AVMs in HHT indicates that haploinsufficiency of *ENG* is not sufficient to cause lesion development [70]. Interestingly, bAVMs developed only in *Mgp*<sup>-/-</sup> mice, not in *Mgp*<sup>+/-</sup> mice [43]. In addition, there is compelling proof-of-principle evidence that loss of function of the wild-type allele is relevant to vascular malformations, demonstrated in two related disorders: somatic mucocutaneous venous malformations [71], and cerebral cavernous malformations [72].

Although *Alk1* has been reported to be predominantly expressed in endothelial cells [73], it has also been shown in smooth muscle cells and splenic macrophages [74]. Milton et al recently reported that *Alk1* deletion driven by *SM22α*-Cre resulted in AVMs in the brain and spinal cord [54]. We have explored the cellular loci of endogenous *Alk1* in bAVM pathogenesis, by cross breeding *Alk1*<sup>ff</sup> mice with *Pdgfb*-iCreER (endothelial-specific promoter), *NG2*-iCreER (pericyte-specific promoter), or *LysM*-Cre (macrophage-specific promoter) mice. Neither macrophage-nor pericyte-*Alk1* gene deletion caused mortality in embryonic or neonatal mice [59]. Postnatal VEGF stimulation did not lead to an AVM phenotype in any organ, including the brain. However, endothelial-*Alk1* deletion led to spontaneous AVM development in the intestine and lung, and in the brain after angiogenic stimulation [59]. AVMs also developed around ear wounds (Fig. 6) [59]. Similarly, deletion of *Eng* in all cell-types in adult mice by Rose-CreER transgene led to AVM formation in the brain after angiogenic stimulation, and around the ear wounds [60]. No AVM developed in any organs in *LysM*-Cre;*Eng*<sup>ff</sup> mice, including the brain after angiogenic stimulation [60]. This suggests that homozygous causative gene deletion in endothelial cells is a requirement for AVM formation.

## (2) Response-to-injury and angiogenic stimulation

The bAVM phenotype includes an active angiogenic and inflammatory component that is inconsistent with a static congenital anomaly [75]. Although HHT patients have genome-wide haploinsufficiency of one of the causative genes, AVMs did not form at random locations. In addition, only a few vascular dysplasia developed in the brain of *Eng*<sup>+/-</sup> [76] and *Alk1*<sup>+/-</sup> [45] adult mice, and was only seen in older mice [45, 47]. There were more pronounced forms of cerebral microvascular dysplasia in the VEGF-stimulated brain at the angiogenic foci in *Eng*<sup>+/-</sup> or *Alk1*<sup>+/-</sup> mice [48–50].

We hypothesize that the environmental stimulus (injury) is required for triggering bAVM formation. Induction of *Alk1* gene deletion in adult mice resulted in AVM and hemorrhage in the lung and gastrointestinal tract, but not in the skin or brain. Upon wounding, *Alk1*-deleted mice developed vascular dysplasia and A–V shunts around the skin wound [53]. A macroscopic level of vascular dysplasia that mimics many phenotypes of human bAVM was induced by injecting AAV-VEGF (an adeno-associated viral vector expressing VEGF) into the basal ganglia of *Alk1*-deleted brain [55].

Taken together, both genetic manipulation and angiogenic stimulation are required for AVM development. The angiogenic stimulus can be a minor injury, exogenous growth factor

delivery, or high endogenous angiogenic factors in the brain of young and perinatal individuals.

### (3) Impaired mural cell recruitment

Brain AVM can cause ICH and serious neurological disability or death. ICH is the first clinical symptom in about 50% of bAVM patients. The malformed vessels are fragile and prone to rupture, causing bleeding into the brain. We showed that 30% of unruptured and non-hemorrhagic bAVMs demonstrated microscopic evidence of hemosiderin in the vascular wall [56]. The presence of silent intralesional microhemorrhages may be a biomarker for the risk of ICH. However, the underlying mechanisms for bAVM rupture and micro-hemorrhage are not fully understood.

Analyzing our established bAVM model, we found that vascular mural cell coverage is reduced in the AVM lesion and accompanied by vascular leakage and microhemorrhage [55, 57]. Many dysplastic vessels do not have a smooth muscle cell layer. Iron-deposition (Prussian blue positive staining) is present around the dysplastic vessels, and the vascular pericytes are reduced in the bAVM lesions as well [65]. Knockdown of *ALK1* attenuates the increase of PDGFB in human brain microvascular endothelial cells (HBMECs) following VEGF stimulation, and reduces the ability of HBMEC to recruit pericytes [65]. Our data suggest that PDGFB signaling could be one of the underlying mechanisms for vascular destabilization and microhemorrhage (Fig. 1).

### (4) Bone marrow-derived cells

VEGF stimulation resulted in dysplasia at the capillary level in the brain of *Eng*<sup>+/-</sup> mice [50]. Similar degrees of cerebrovascular dysplasia developed in the brain of wild-type (WT) mice transplanted with bone marrow (BM)-derived from *Eng*<sup>+/-</sup> mice following VEGF stimulation. In addition, the dysplasia in *Eng*<sup>+/-</sup> mice could be partially rescued by transplantation of WT BM [77]. This suggests that *Eng* haploinsufficiency in BM-derived cells is sufficient to cause cerebrovascular dysplasia in the adult mouse after angiogenic stimulation.

So far, the cell type(s) in the BM underlying AVM formation are unknown. There is evidence for two primary—probably complementary—cell types that serve as a locus for the phenomena: (1) BM-derived endothelial cells that incorporate into the angiogenic neovasculature [77, 78]; and (2) BM-derived monocytes/macrophages that may provide critical repair functions in response to injury [79–82] and/or provide guidance involving Notch signaling during angiogenesis [83, 84]. However, deletion of *Alk1* or *Eng* in macrophages alone did not cause AVM formation, suggesting that gene-deficiency in macrophages is not an initiating factor. The involvement of macrophages might be associated with pathological vascular remodeling and vascular destabilization.

The involvement of BM-derived endothelial cells in focal angiogenesis has been shown in several conditions, such as tumor formation. BM-derived endothelial cells seed tumor vascular beds, regulating tumor angiogenesis [85, 86], and can incorporate into vessels in

the brain angiogenic foci in mouse models [77, 78, 87]. In addition, endothelial cell progenitor cells have been identified in vessels in adult human sporadic bAVMs [88].

### (5) Inflammatory cells and cytokines

Supporting evidence for myeloid cells playing a critical role in AVM progression include: (1) most of the BM- derived cells that home to the brain angiogenic foci are CD68<sup>+</sup> or CD45<sup>+</sup> [77, 87]; and (2) intra-peritoneal administration of neutrophil neutralizing antibody reduces AAV-VEGF-mediated angiogenesis and MMP-9 activity [89]. Other experiments suggest that both neutrophils and macrophages are relevant to large vessel remodeling as well [90].

Normal human monocytes rescue the impairment of *Eng*<sup>+/-</sup> mice in repairing myocardial injury, whereas monocytes from HHT patients fail to improve the myocardial repair [79, 81]. HHT1 monocytes migrate to SDF1 $\alpha$  less effectively than normal monocytes, which is associated with an increase of CD26 expression [79, 81]. These data suggest that the function of monocytes in vascular repair or remodeling is defective in HHT patients, which could result in abnormal vascular remodeling and thus promote AVM progression.

Abnormal expression patterns of inflammatory mediators and cytokines, as well as an influx of inflammatory cells into AVMs have been observed by a number of investigators [91–95]. Inflammatory markers are overexpressed in human AVMs, including myeloperoxidase (MPO) and IL-6, both of which highly correlate with matrix metalloproteinase-9 (MMP-9) expression.

Remodeling of the vascular network in AVMs is facilitated by a number of proteases that can enlarge the vascular elements in the nidus. This remodeling is partially mediated through VEGF activity and modulated by pro-angiogenic signals such as MMP expression. MMPs maintain and remodel the extracellular matrix [96]. MMPs, including MMP-9, are major components of neutrophil tertiary granules and are also synthesized by monocytes and lymphocytes. MMP-9 is expressed at significantly higher levels in bAVMs than in control tissue [92, 97], but the source of this expression is unclear. MMP-9 expression and activity during inflammation are stimulated by the cytokines IL-8, IL-1  $\beta$ , and IL-6. MMP-9 degrades key components of the cerebrovascular matrix including laminin, denatured collagen, and tight junction proteins such as ZO-1 leading to blood-brain barrier leakage and hemorrhage [98, 99]. MMP-9 is expressed in the endothelial cell/peri-endothelial cell layer of AVMs. Along with endothelial and smooth muscle cells, inflammatory cells seem to be a major contributor to the abnormally high levels of MMP-9 in AVM tissue [92]. The MMP-9 signal co-localizes with MPO, and expression correlates with both MPO and IL-6 levels, which suggests that the source of MMP-9 levels may be the inflammatory cells in the environment [92]. In addition, BM-derived cells that home to bAVMs might also be an MMP-9 source [78].

Soluble ENG (extracellular domain) has been shown to contribute to another vascular disease: preeclampsia [100]. Soluble ENG (sENG) is distinct from long (L) and short (S) form ENG, which have cytoplasmic tails of 47 and 14 amino acids, respectively [101]. sENG also increases in bAVMs [102]. It is not clear how sENG is formed. A related Type



III TGF- $\beta$  receptor, betaglycan, appears to be shed through a process that is mediated by MMP-1 [103]. Several different MMPs are also found in AVM nidus tissue [92, 104, 105], suggesting that similar mechanisms may contribute to the formation of sENG or soluble ALK1 (sALK1) [100]. TNF- $\alpha$  can induce the release of sENG from normal placental villous explants [106]. Thus, inflammatory proteins and cytokines in AVMs could cause shedding of sENG and promote bAVM instability. Another interesting observation is the increased levels of immunoglobulins within bAVMs when compared to the control brain [93].

Vascular inflammation is central to the pathogenesis of several vascular diseases [107, 108], including intracranial aneurysm growth [109, 110] and abdominal aortic aneurysm formation [111, 112]. In addition, associations between single nucleotide polymorphisms (SNPs) in cytokines such as TNF- $\alpha$  and increased bAVM intracerebral hemorrhage risk have been described [113]. SNPs in IL-6 were also associated with a hemorrhagic clinical presentation in bAVM patients [91], and the highest risk genotype IL-6 (GG) was associated with the highest IL-6 expression levels in bAVM tissue [105].

## (6) Hemodynamic changes

Vessels in an AVM are subjected to higher-than-normal flow rates. High vascular flow rates in *ALK1*<sup>+/-</sup> mice using vasodilators (nicardipine or hydralazine) after focal VEGF stimulation increased the number of dysplastic vessels in the brain angiogenic foci [49].

Cerebral venous hypertension is a common symptom in bAVMs [114]. Cerebral venous hypertension causes at least one kind of fistula (dural arteriovenous fistula) to form [27, 30] through a mechanism involved in the induction of angiogenesis [27]. In rats, non-ischemic levels (15–23 mmHg) of cerebral venous hypertension cause expression of HIF-1 $\alpha$ , and its downstream signal, VEGF [115]. Further, HIF-1 $\alpha$ , VEGF, SDF-1 $\alpha$  expression, neutrophils, macrophage and MMP-9 activity increase in the brain of the mouse cerebral venous hypertension model. Capillary density in the parasagittal cortex also increases in the mouse VH model. These findings suggest that mild nonischemic cerebral venous hypertension results in a pro-angiogenic state [116]. Thus, cerebral venous hypertension could represent a kind of injury that triggers bAVM development in subjects carrying mutant genes.

Hemodynamic stress can also trigger vascular inflammation that initiates vascular remodeling and angiogenesis. High shear stress activates endothelial cells and upregulates leukocyte adhesion molecules, including intercellular adhesion molecule-1 (ICAM-1) and monocyte chemoattractant protein-1 (MCP-1) [117–120]. Shear stress activates endothelial and smooth muscle cells and promotes their production and release of angiogenic factors and other cytokines critical for vascular remodeling [121, 122].

## New Therapeutic Targets

Current treatment options for bAVMs are invasive and associated with excessive risk [4, 123]. There are no specific medical therapies to treat bAVMs. Through analysis of surgically resected bAVM specimens and our novel mouse models, we have identified the following targets that might be used to develop new therapies (Fig. 1).

## (1) Anti-angiogenesis

Excessive VEGF expression appears to be a fundamental part of bAVM pathophysiology for both sporadic bAVM [50, 55, 124] and bAVM in HHT [125–127]. Compelling evidence supports interruption of VEGF signaling as a therapeutic strategy. Bevacizumab (Avastin) normalized cardiac output in HHT patients harboring liver AVMs [128], and was effective in the treatment of severe epistaxis caused by hemorrhage from small mucosal AVMs (telangiectasias) [129–136]. Importantly, we demonstrated that after establishment of the bAVM phenotype in a conditional *Alk1* deletion mouse model [55], intra-peritoneal bevacizumab treatment reduced the number of abnormal vessels, suggesting that maintenance of the bAVM phenotype is dependent on tonic VEGF signaling [137]. However, antibody therapy has many drawbacks, including concerns regarding hemorrhage [138] and the need for prolonged periods of intermittent intravenous (i.v.) infusions.

A promising alternative is the use of AAV-mediated expression of *soluble* FMS-related tyrosine kinase 1 (sFLT1), also called VEGF receptor (VEGFR)-1. sFLT1 contains the extracellular domain of VEGFR-1, which binds to VEGF in the tissue, thus reducing downstream signaling through membrane-bound VEGFRs. Injection of sFLT1 in an AAV construct packaged in AAV serotype 2 capsid (AAV2) into the vitreous humor of nonhuman primates effectively inhibited laser-induced choroidal neovascularization [139]. The AAV2-sFLT1 was well tolerated and capable of mediating long-term sFLT1 expression [140]. We showed that co-injection of AAV2-sFLT with AAV1-VEGF into the brain or intravenous injection of AAV9-sFLT at the time of intra-brain injection of AAV1-VEGF completely blocked VEGF-induced brain angiogenesis [141].

## (2) Vascular integrity

Unlike cancer-related chemotherapy that aims to shrink abnormal tumor tissue as cytotoxic therapy, the concept for the treatment of bAVMs would be to stabilize vascular tissue and thereby decrease the risk of spontaneous ICH [142].

Microhemorrhage is present in unruptured human bAVMs [56] and in our bAVM mouse model lesions [57]. The vessels in bAVM mouse models have less mural cell coverage, which could be due to decreased expression of platelet-derived growth factor B (PDGFB) [55, 57]. Lebrin et al [143] demonstrated that thalidomide treatment increased PDGFB expression in endothelial cells and stimulated mural cell coverage.

Thalidomide belongs to a class termed immunomodulatory drugs (IMiDs). Because of thalidomide's well-known adverse effects that limit patient tolerance, e.g., peripheral neuropathy and drowsiness [144], the search for related molecules has yielded a second generation of IMiDs. Lenalidomide, the most widely used in the group, is effective in treating multiple myeloma and myelodysplastic syndrome [145].

IMiDs possess a number of immunomodulatory, anti-inflammatory and anti-angiogenic properties that are pertinent to bAVM therapy [144, 146, 147]. The IMiDs do not inhibit endothelial cell proliferation, but rather, migration [148, 149]. There is a remarkable overlap in the pathways targeted by IMiDs in neoplastic disorders [144] with those dysregulated in bAVM, e.g., NF $\kappa$ B activation [94], VEGF overexpression [66], HIF-1 $\alpha$  [150],  $\alpha$ v $\beta$ 3 [67,

151], and cytokine elaboration [105], including TNF- $\alpha$  [152] and IL-1 $\beta$  [153]. Both thalidomide and lenalidomide improved outcomes and reduced TNF- $\alpha$  and IL-1 $\beta$  expression in an amyotrophic lateral sclerosis mouse model [154, 155], suggesting that the drug can cross the blood-brain barrier in a setting with some impairment of barrier integrity like our bAVM model [65]. In early-phase human studies, thalidomide showed promise for decreasing hemorrhage from gastrointestinal or nasal telangiectasias (epistaxis) [143, 156–163]. Only a single case of lenalidomide used to treat gastrointestinal telangiectasias has been reported [164].

The anti-angiogenic mechanism of thalidomide is poorly understood but it has shown some clinical benefit in the treatment of gastrointestinal hemorrhage and epistaxis in patients with HHT [143, 159]. We have tested thalidomide in our brain AVM mouse model. Serial injections of thalidomide over a 6-week period attenuated dysplastic vessel formation. Furthermore, it decreased hemorrhage and improved vascular smooth muscle cell coverage in the bAVM lesion [65].

### (3) Anti-inflammation and BM/monocyte transfusion

As discussed above, AVMs in humans and in animal models have been associated with an increased inflammatory response [16, 56, 57, 64, 65]. Inflammatory markers, including MPO, IL-6 and MMP-9, are overexpressed in human AVMs. MMPs and proinflammatory cytokines can interact with each other to carry out both physiological and pathological vascular remodeling. We have shown that MMP-9 plays an important role in VEGF-induced brain angiogenesis [78]. Inhibition of MMP-9 might lead to a reduction of angiogenesis.

Tetracycline class drugs are emerging as clinically applicable nonspecific MMP inhibitors that have the potential to enhance vascular stability, thus reducing the risk of spontaneous hemorrhage. They also possess neuroprotective properties. Settings investigated to date include cerebral ischemia [165], ICH [166, 167], neurodegenerative disorders [168, 169], traumatic brain injury [170], and atherosclerotic disease [171]. In clinical trials, tetracyclines decreased MMP in abdominal aortic aneurysms and carotid plaques [171–173]. Doxycycline can reduce MMP levels in bAVM [174]. Further, animal studies showed that doxycycline reduces MMP-9 activity in VEGF-stimulated brain angiogenic foci and reduces parenchymal angiogenesis [175]. Our Phase I study assessing the feasibility of using minocycline and doxycycline as potential long-term vasculostatic therapy for brain vascular malformations showed that it is feasible to propose a long-term trial to assess the potential benefit of tetracycline therapy to decrease hemorrhagic risk in bAVM [142].

BM-derived cells participate in VEGF-stimulated brain angiogenesis [78, 87] and the formation of vascular dysplasia in the brain of *Eng*<sup>+/-</sup> mice [77]. Moreover, normal human monocytes rescue the impairment of *Eng*<sup>+/-</sup> mice in repairing myocardial injury [79, 81]. These data suggest that correction of gene mutations in BM or monocytes through BM transplantation or monocyte transfusion could also be therapies for bAVMs.

## Summary

The pathogenesis and pathophysiology of bAVMs are complex and currently unclear. Evidence obtained from modeling HHT bAVMs suggests that the initiation and progression of AVMs require interplay among several factors (Fig. 1), including: (1) homozygous loss-of-function of causative genes in somatic endothelial cells; (2) angiogenic stimulation (response-to-injury); (3) participation of BM-derived cells; (4) inflammation; and (5) hemodynamic changes. Animal studies have also identified some potential therapeutic targets (Fig. 1).

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## Abbreviations

<b>bAVM</b>	Brain arteriovenous malformation
<b>ICH</b>	Intracranial hemorrhage
<b>HHT</b>	Hereditary hemorrhagic telangiectasia
<b>ENG</b>	Endoglin
<b>ALK1</b>	Activin-like kinase 1
<b>VEGF</b>	Vascular endothelial growth factor
<b>Pdgfb</b>	Platelet derived growth factor-b
<b>A-V shunt</b>	Arteriovenous shunt
<b>f</b>	Allele with 2 loxp site flanking the target sequence

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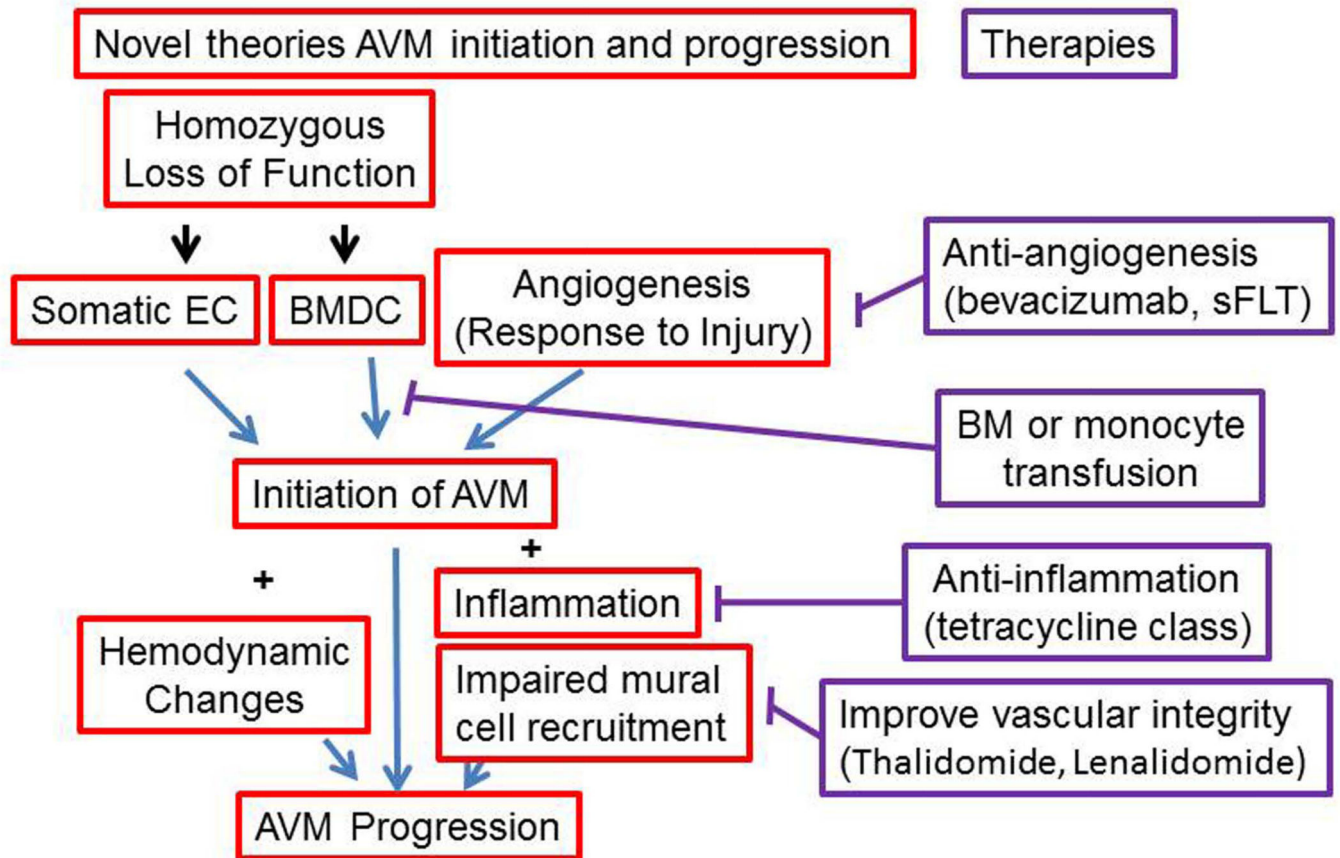
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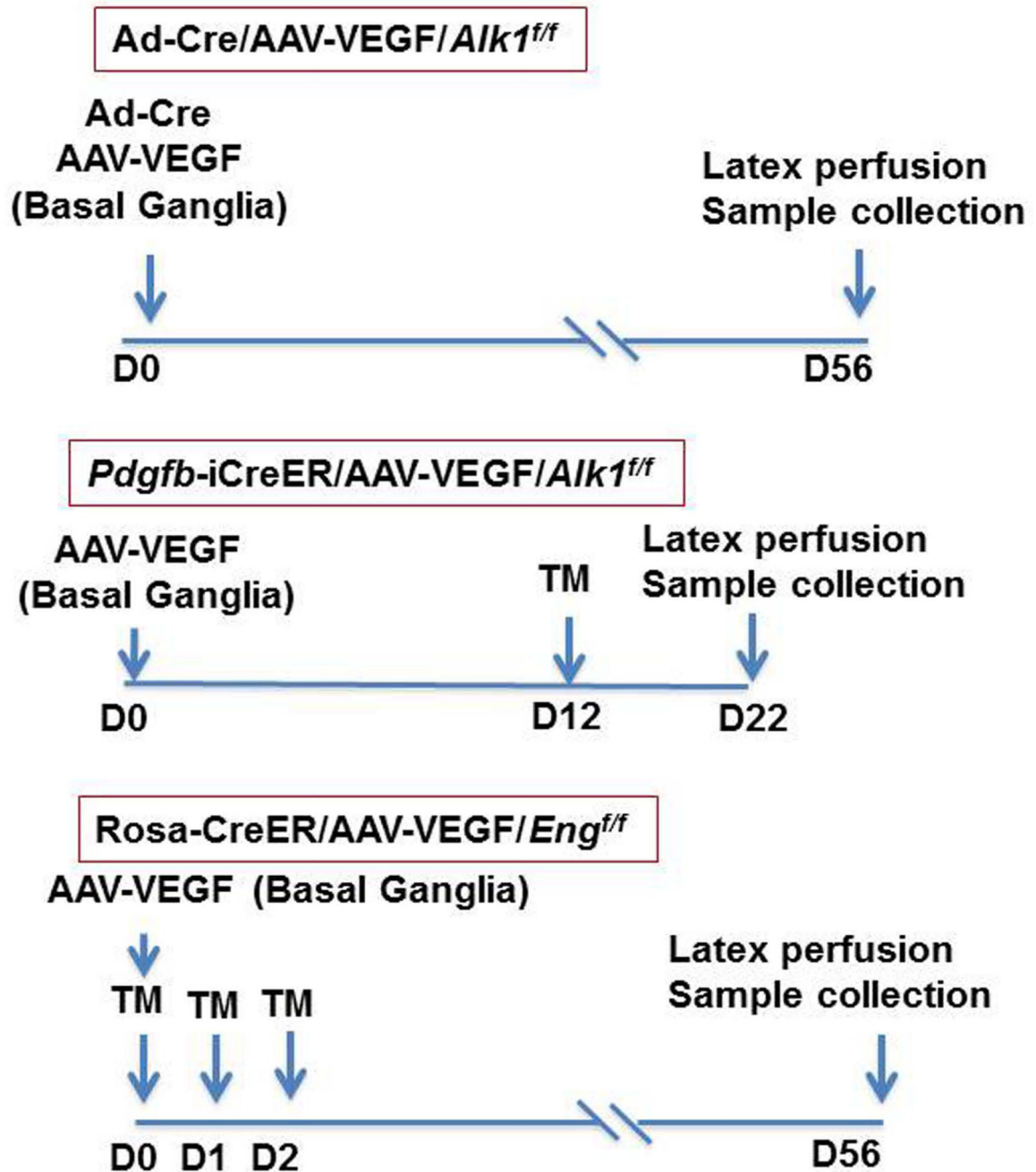
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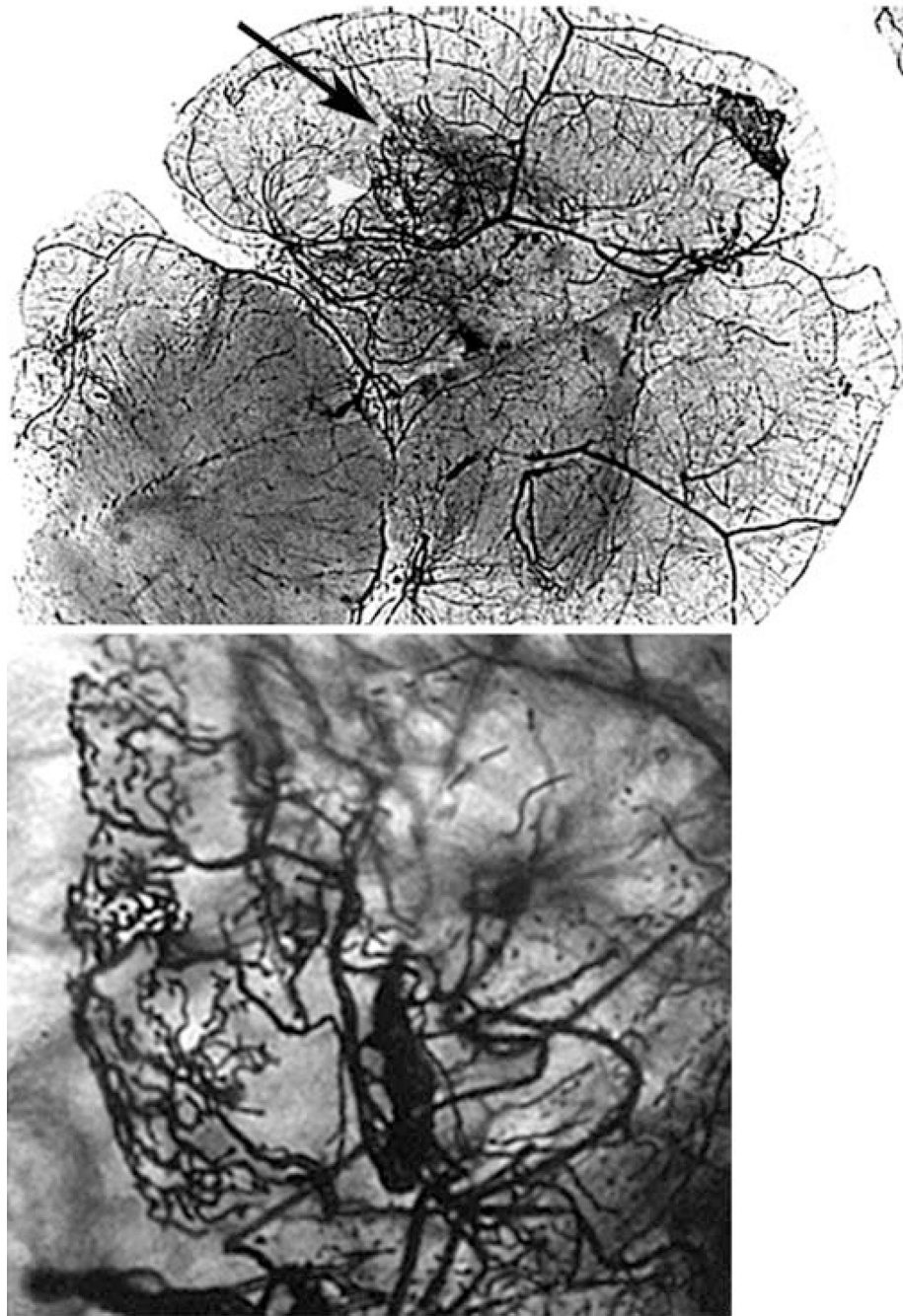
**Fig. 1. Novel theories of AVM initiation and progression, and new therapeutic targets**  
 EC: endothelial cell; BMDC: bone marrow-derived cells; sFLT: *soluble* FMS-related tyrosine kinase 1 (sFLT1), also called VEGF receptor-1



**Fig. 2. Development of adult onset brain AVM models**

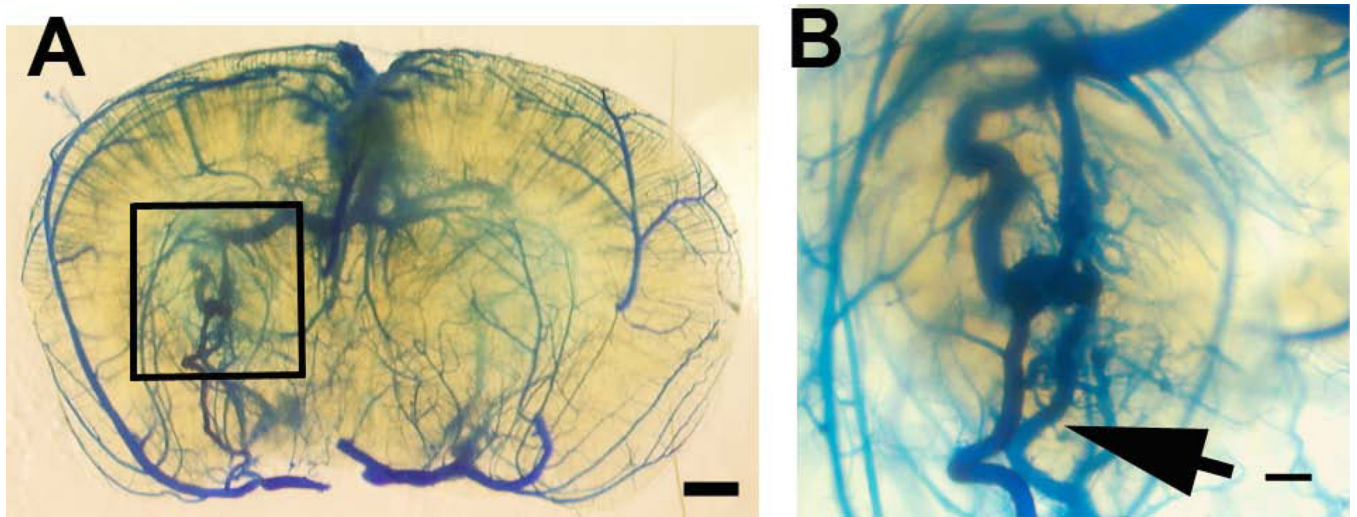
AAV1-VEGF [ $2 \times 10^9$  viral genome (vg)] is used to stimulate brain focal angiogenesis. Ad-Cre (Ad-Cre/AAV-VEGF/*Alk1<sup>f/f</sup>* model), *Pdgfb-iCreER* (*pdgfb-iCreER*/AAV-VEGF/*Alk1<sup>f/f</sup>*) and Rosa-CreER (*Rosa-CreER*/AAV-VEGF/*Eng<sup>f/f</sup>*) are used to delete *Alk1* or *Eng* in *Alk1<sup>f/f</sup>* or *Eng<sup>f/f</sup>* mice.



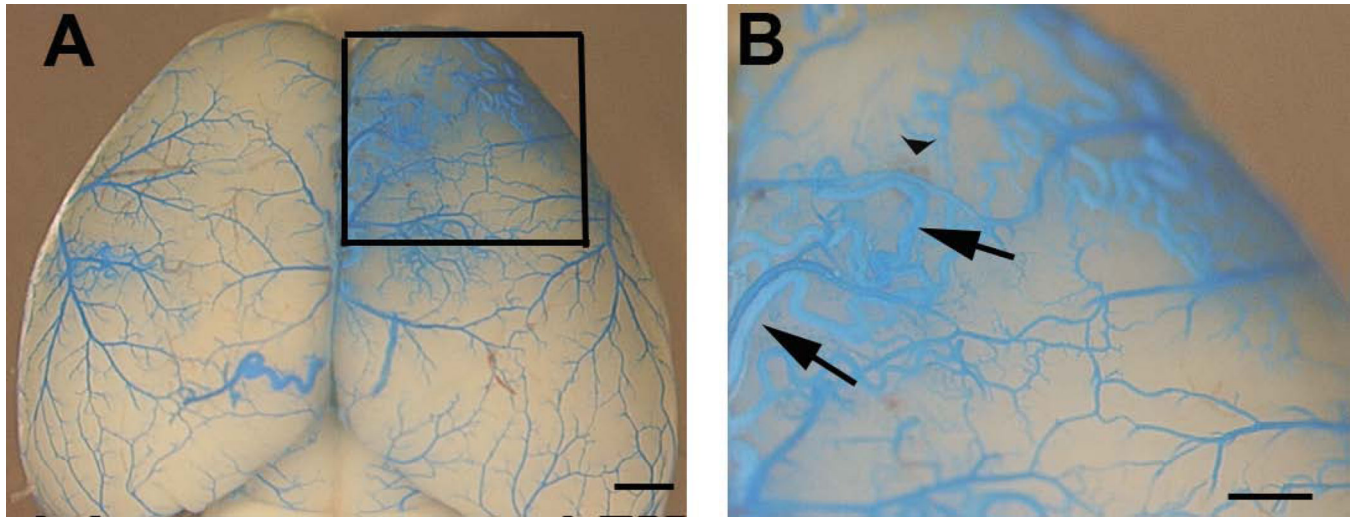


**Fig. 3. Vessel casting showing AVM in the brain angiogenic region**

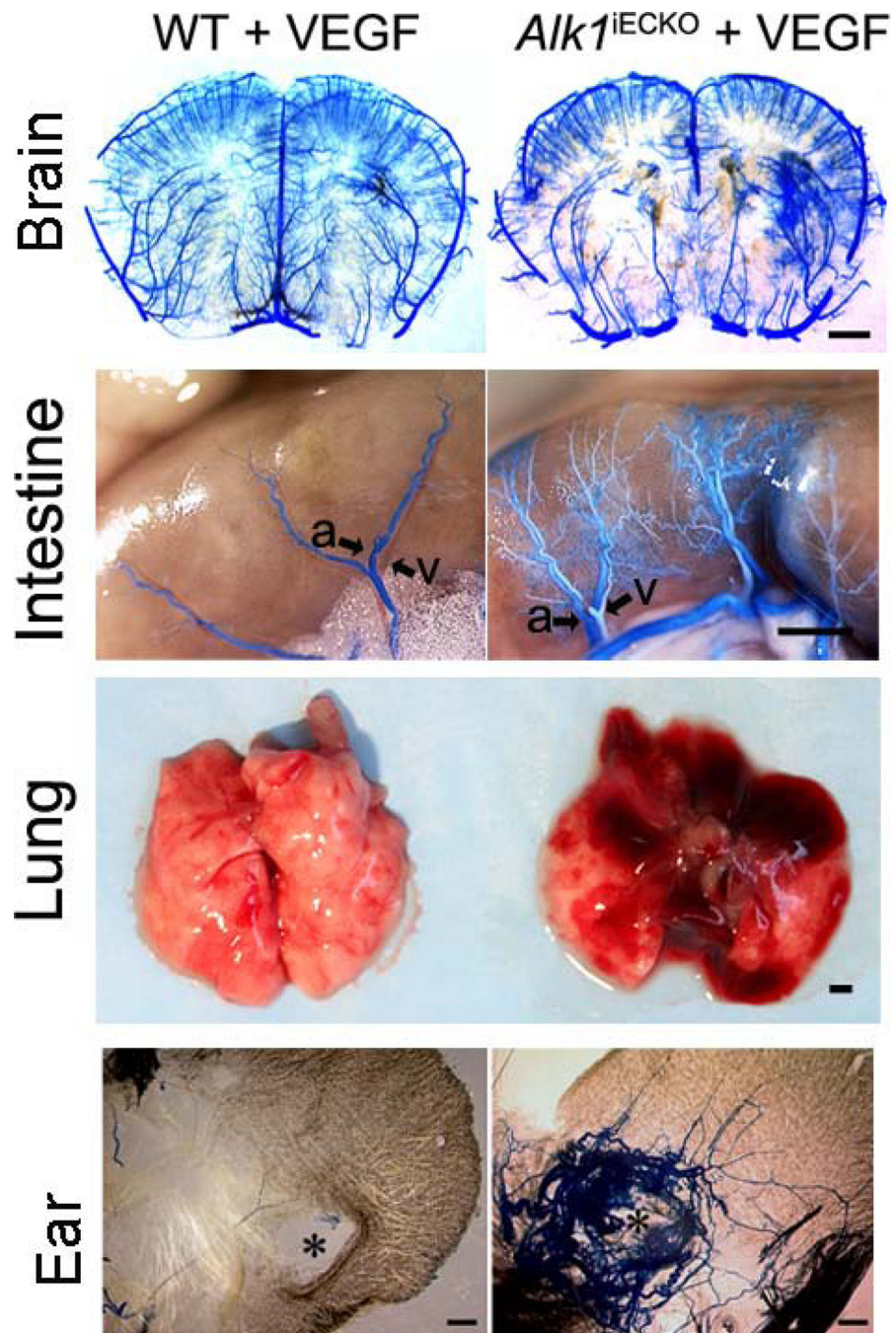
Large tangled vessels resembling bAVM were detected at the injection site of Ad-Cre and AAV-VEGF in the brain of *Alk1*-floxed mice (black arrow). Bottom images show the enlarged angiogenic foci of the images at the top. Scale bar = 100 $\mu$ m.



**Fig. 4. Adult onset bAVM of  $Eng^{2f/2f};R26CreER$  mice after TM and VEGF treatment**  
(A) AVM (squared region) in the brain of  $Eng^{2f/2f};R26CreER$  mice 8 weeks after intra-brain injection of AAV-VEGF and intraperitoneal injection of TM. (B) Enlarged image of the AVM lesion. Latex-perfused veins are clearly shown (arrows). Scale bars: 1 mm in (A) and 200  $\mu$ m in (B).



**Fig. 5. Developmental onset AVMs in the postnatal brain of *Eng<sup>2ff/2ff</sup>;SM22 $\alpha$ -Cre* mice**  
(A) Representative images of latex dye casting show the AVM vessels (squared region) in the brain of 5-week-old *Eng<sup>2ff/2ff</sup>;SM22 $\alpha$ -Cre* (B) Enlarged images of dotted boxes shown in (A). Arrows indicate latex-casted veins. Arrow head indicates hemorrhage. Due to the particle size in the latex, the dye enters the vein after intra-left cardiac perfusion only when the A–V shunts are present. Scale bars: 1 mm in (A) and 500  $\mu$ m in (B).



**Fig. 6. Brain AVM in mice with endothelial-Alk1 deletion and focal VEGF stimulation ( $Alk1^{iECKO}+VEGF$ )**

AVMs also developed in the intestine, lung and around the ear-tag wound. a: artery; v: vein. Scale bars: 1 mm.

**Table 1**

## Brain AVM mouse models

Models	Onset	Advantages	Disadvantages
Ad-Cre/AAVVEGF/ <i>Alk1<sup>ff</sup></i>	Adult	Low mortality. No AVM in other organs.	Inflammation caused by adenoviral vector complicates mechanistic analysis.
<i>Pdgfb-iCreER/AAVVEGF/Alk1<sup>ff</sup></i>	Adult	Brain AVM develops in a relatively shorter time.	AVM develops in multiple organs and mice die 10–14 days after tamoxifen-
Rosa-CreER/AAV-VEGF/ <i>Eng<sup>ff</sup></i>	Adult	Low mortality.	AVM develops in multiple organs.
SM22 $\alpha$ - <i>Cre/Eng<sup>ff</sup></i>	Embryonic	AVM develops spontaneously. A good model for mechanistic study and for new drug testing.	Embryonic onset. 50% mice die before 6 weeks of age.