Review Article

JCBFM

Journal of Cerebral Blood Flow & Metabolism
2021, Vol. 41(12) 3141–3156

DOI: 10.1177/0271678X211026771
journals.sagepub.com/home/jcbfm
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Review of treatment and therapeutic targets in brain arteriovenous malformation

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Abstract

Brain arteriovenous malformations (bAVM) are an important cause of intracranial hemorrhage (ICH), especially in younger patients. The pathogenesis of bAVM are largely unknown. Current understanding of bAVM etiology is based on studying genetic syndromes, animal models, and surgically resected specimens from patients. The identification of activating somatic mutations in the Kirsten rat sarcoma viral oncogene homologue *(KRAS)* gene and other mitogen-activated protein kinase (*MAPK*) pathway genes has opened up new avenues for bAVM study, leading to a paradigm shift to search for somatic, *de novo* mutations in sporadic bAVMs instead of focusing on inherited genetic mutations. Through the development of new models and understanding of pathways involved in maintaining normal vascular structure and functions, promising therapeutic targets have been identified and safety and efficacy studies are underway in animal models and in patients. The goal of this paper is to provide a thorough review or current diagnostic and treatment tools, known genes and key pathways involved in bAVM pathogenesis to summarize current treatment options and potential therapeutic targets uncovered by recent discoveries.

Keywords

Brain arteriovenous malformation, mouse models, somatic mutations, signaling pathways, therapeutic targets

Received 31 March 2021; Revised 27 May 2021; Accepted 28 May 2021

Introduction

Brain arteriovenous malformations (bAVMs) represent a relatively rare but important cause of intracranial hemorrhage (ICH) and neurological morbidity, especially in children and young adults. The population prevalence of bAVM is 10-18 per 100,000 adults, with a new detection rate of ~1.3 per 100,000 person-years. BAVMs are comprised of a complex tangle of blood vessels called the nidus, in which there are direct arterial-venous connections without a normal intervening capillary bed. These high-flow, arteriovenous shunts are prone to rupture with an overall annual ICH rate of 1-3% per year.[1]

The vast majority of bAVMs present as a solitary lesion without known family history (sporadic), while about 5% of bAVMs occur in patients with genetic syndromes, primarily Hereditary Hemorrhagic Telangiectasia (HHT; also called Osler-Weber-Rendu syndrome) and capillary malformation-arteriovenous malformation (CM-AVM, Figure 1). Many early animal models were established by knocking out HHT causative genes. Similarly, many signaling pathways involved in bAVM pathogenesis and therapeutic targets have been identified through establishing and analyzing these models as well as studying HHT and sporadic bAVM patients. However, there are still no specific

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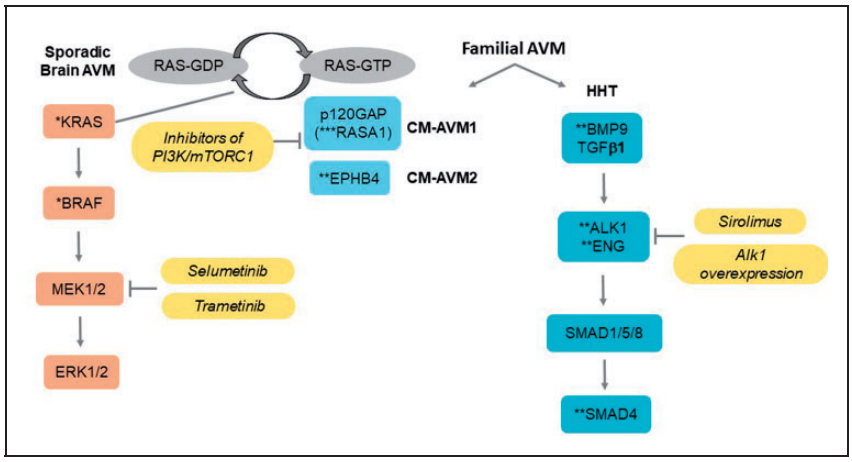

Figure 1. Major pathways for familial vascular diseases with brain AVMs (Hereditary Hemorrhagic Telangiectasis (HHT) and Capillary Malformation-Ateriovenous Malformation (CM-AVM)), sporadic brain AVM, and therapeutic targets. *Somatic mutations, and **germline mutations, and ***germline and somatic mutations identified in vascular malformations.

medical therapies available for the treatment of bAVMs. Treatment modalities are limited to interventional approaches, such as surgery, radiosurgery, or endovascular embolization, and many complex lesions require multi-modal therapy. However, these procedures are associated with risks, including transient or permanent neurological morbidity or death,[2] and not all patients can be offered treatment. There is a compelling need for novel therapies to prevent and/or reduce bAVM bleeding or rupture. In this review, we have summarized current and potential therapeutic targets identified based on clinical and experimental findings highlighting relevant signaling pathways (Figure 2).

Current diagnosis and treatment options for brain arteriovenous malformation

Diagnosis

BAVMs are most commonly diagnosed as part of a work up for a new neurological deficit typically related to a spontaneous ICH. Overall approximately half of all bAVM patients will present initially with ICH, though this fraction is higher for younger (< 20 years) patients given the reduced use of screening imaging for issues like headache or other neurological issues.[3] The annual rate of ICH after diagnosis but before treatment (i.e., natural history) is estimated to be 2.3% (95% CI: 2.0% - 2.7%), and is higher for ruptured (4.8%, 95% CI: 3.9%-5.9%) than unruptured (1.3%, 95% CI: 1.0%-1.7%) cases at presentation.[3]

Computerized tomography (CT) scan is the primary means to screen patients with new focal neurological deficits demonstrating a high degree of sensitivity for hemorrhage. CT angiography also provides excellent accuracy in the detection of bAVMs.[4] In the event of a spontaneous ICH in a patient <40 years or ≥40 years without hypertensive or coagulopathic risk factors, magnetic resonance imaging (MRI) is often performed given its more sensitive soft tissue differentiation.[5] High-resolution post-contrast enhanced imaging is also essential in the identification of small or micro (< 1 cm) bAVMs typical of HHT syndrome. In addition to these more standard MR series, there is growing evidence that quantitative flow methods (e.g., 4D flow) may be used to grade pre- and post-treatment effects on fluid dynamics to and surrounding an AVM.[6]

In the event CT and/or MR reveal findings suggestive of a bAVM or the clinical scenario is concerning for a potential secondary vascular malformation in the setting of ICH regardless the cross-sectional angiographic findings, digital subtraction angiography (DSA) is required. DSA better delineates arterial afferent and venous efferent components, the presence of flow-related aneurysms, venous stenoses, and physiological proliferative angiopathy, all high-risk features implicated in natural history and/or surgical risk assessment of bAVM. Additionally, DSA may be used to quantify flow characteristics – information that may prove helpful in determining management risks.[7] Molecular and mechanical imaging may also prove helpful in grading bAVM characteristics.[8] Such methods are experimental at this time, though have proven useful in other vascular and other hypervascular neurological pathologies.

Treatment

Decisions to treat a bAVM should carefully weigh the risks of neurologic morbidity from eventual ICH versus

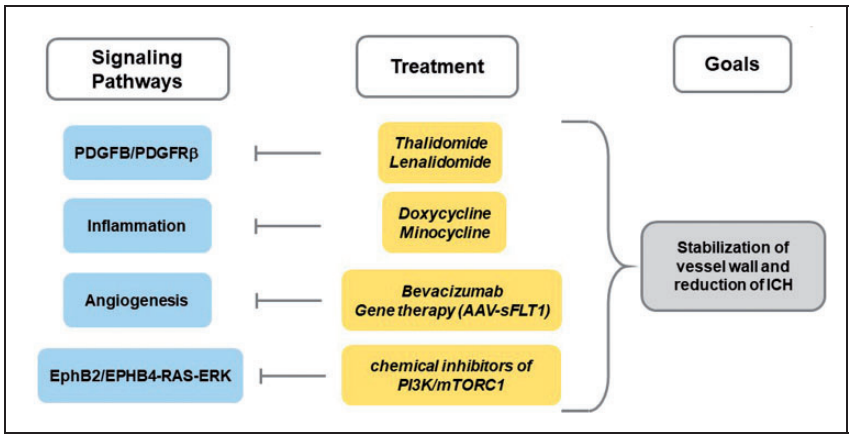

Figure 2. Other pathways dysregulated in brain AVM pathogenesis and implicated therapeutic targets.

those associated with interventional treatment. With high-risk features, hemorrhage rates of bAVM may be up to 34%.[9] Randomized controlled trials and clinical studies have suggested risks of treatment may outweigh risks of rupture for unruptured bAVMs, and treatment remains controversial given the lower risk of ICH in these lesions.[1,10] Like most neurovascular disorders, the timing and modality of bAVM treatment is largely dictated by the acuity and severity of ICH. For those cases with rapid clinical deterioration, surgical decompression with or without hematoma and/or nidal resection based on CT imaging alone is often needed. For the majority of cases, however, treatment considerations are multidisciplinary with input from neurological surgery, neurology, and neurointerventional radiology teams. There is no established natural history scoring system, though a number of series have noted increased ICH risks for deep seated lesions, those with a single or deep draining vein, the presence of nidal or peri-nidal aneurysm, and infratentorial location.[11] For patients with a prior history of hemorrhage, treatment is often recommended given the elevated observed rate of secondary hemorrhage, particularly within the first year of ictus.[12] As a general rule, smaller (< 3 cm), well-circumscribed lesions with fewer arterial afferents and venous efferents centered in non-eloquent locations respond more favorably to any intervention. For larger, more angiographically complex, and/or eloquently located lesions, decisions are more nuanced and a treatment team will often discuss combination strategies designed to address discrete high-risk bAVM features (e.g. flow-related, nidal, or pseudoaneurysms), deep or perforating arterial afferents more difficult to surgically access, and/or high-flow direct AV shunts through embolization.

Higher rates of obliteration are reported with surgical resection and patient outcome heavily driven by patient selection. Patient selection for surgical resection is aided by surgical grading scales which estimate risks of outcomes after surgery – such as the Spetzler-Martin and Lawton-Young grading scales.[13,14] Factors including size, patterns of venous drainage, eloquence of the brain region containing the bAVM, patient age, rupture status, and configuration of the nidus (diffuse vs. compact) have been shown to impact outcomes following surgery. Due to neuroplasticity, surgical outcomes are better in younger patients.[15] With appropriate patient selection, surgery is also a safe, viable treatment option in the elderly.[16]

The intent of bAVM surgery is the complete removal of the nidus and elimination of AV shunting. When these are not possible, surgery can be a useful adjuvant to eliminate blood flow from high risk features – such as intranidal or flow-related aneurysms.[17] Successful surgical resection is defined as no residual arteriovenous shunting on postoperative angiography. However, in certain high risk populations, such as ruptured bAVMs in children, higher rates of recurrence (20%) have been reported and delayed angiography is often recommended between 1 and 5 years after complete resection.[18,19]

For bAVMs in deep, eloquent or surgically in accessible locations, stereotactic radiosurgery (SRS) may be an attractive treatment option. Radiation damages the endothelial cell lining and induces proliferation of smooth-muscle cells which leads to progressive stenosis of bAVM feeding arteries. Eventual occlusion occurs over several years.[20] Radiation induced changes may be seen in the surrounding brain and limits radiation dosage which may be safely applied.[20] With large bAVMs, volume staged radiosurgery may be required.[21] Even if complete obliteration is not achieved, volume staged radiosurgery may decrease the size of the nidus to make it acceptable for surgery.

Embolization has largely been used as an adjunctive aimed to make microsurgical resection safer and more

complete. Embolization carries 1-10% peri-procedural stroke risk depending on the series and is often a function of the number of pedicles treated and embolic agent used.[22] Complications may occur when embolizing neighboring physiological arteries, and obstructing venous outflows causing subarachnoid hemorrhage, ischemia, and ICH, respectively. There is limited evidence as to the impact of embolization on surgical performance,[23] as well as evidence of better clinical outcomes from such a tandem approach.[24] Embolization as an adjunct to radiosurgery has also evolved over time, with evidence suggesting that such practice may lessen the effect of radiation therapy while adding embolic risk.[25] As such, this practice, unless embolization is used to target a high-risk feature (e.g. pseudoaneurysm), is less favorable.

A number of case series describe embolization as a curative approach.[26] There is less evidence in support of this practice, though some have proved successful with follow up intervals up to 3 years without evidence of angiographic recurrence.[26] As with the other modalities, smaller, angiographically simpler lesions prove safer and more amenable to definitive treatment.[27] Within this curative cohort is a group using a combination of transarterial and transvenous techniques. This approach has proven effective in a select number of cases,[28–30] though may carry significantly higher rates of peri-procedural stroke relative to adjunctive embolization when applied to larger, more complex lesions.

Palliative embolization may be used in certain cases where conventional therapies cannot safely and effectively treat a bAVM and a patient has progressive signs or symptoms refractory to medical interventions. In the setting of pain or bothersome pulsatile tinnitus, targeted embolization of dural arterial supply to a bAVM may reduce such symptoms. In rare instances where progressive venous stenosis occurs, venous hypertension can cause headaches, seizures, and/or other focal neurological issues. In these instances targeted embolization of arterial afferents to reduce the shunt volume may be effective.

These interventional options focus on removing the bAVM nidus or high-risk features to reduce flow through the lesion. However, not all bAVM lesions can be safely treated with available options, and the ARUBA randomized controlled trial results suggest that unruptured bAVMs (roughly half of all cases) should not be treated.[1] Therefore, many groups have been focused on identifying medical therapies to slow or stabilize lesion progression based on genetic discoveries and signaling pathways identified to date and described below.

Genetic discoveries in bAVM

The genetic contributions to bAVMs are multifactorial, including germline and somatic mutations, epigenetic alterations, and genetic modifiers which can alter the expressivity of disease-causing genes. Different approaches have been used to inform on the key genes and biological pathways that contribute to bAVM development and disease progression. Genetic and epigenetic factors may also have clinical utility as bAVM diagnostic or prognostic markers, or as potential therapeutic targets. To identify new therapeutic targets, we highlight human studies that have identified key genetic discoveries that have resulted in a paradigm shift and suggest areas for future bAVM research strategies.

Germline mutation in bAVM

There are two main subgroups of bAVM patients with known underlying genetic disorders: HHT and CM-AVM (Figure 1). HHT is an autosomal dominant disease characterized by systemic vascular fragility, telangiectasias and AVMs in various organs, including the brain. The majority of HHT is caused by heterozygous, loss-of-function mutations in the following genes: (1) endoglin (*ENG,* HHT1), encoding an ancillary TGFβ receptor; (2) activin receptor-like kinases 1 (*ALK1*, also named *ACVRL1*, HHT2), encoding a type I TGFβ receptor; and (3) mothers against decapentaplegic homology 4 (*SMAD4*, juvenile polyposis-HHT), encoding a mediator of TGFβ signaling. Mutations in bone morphogenetic protein 9 (*BMP9*, also named *GDF2*, HHT5), encoding a secreted ligand of the TGFβ superfamily, have also been reported to cause an HHT-like syndrome.[31] ENG and ALK1 are primarily expressed in endothelial cells to regulate the development of AV network through TGFβ and BMP signaling pathways.

CM-AVM is another autosomal dominant disorder caused by germline, heterozygous loss of function mutations in *RASA1* (CM-AVM1) or *EPHB4* (CM-AVM2), and characterized by multiple cutaneous capillary malformations co-occurring with fast-flow vascular anomalies, such as AVM or AV fistula. *RASA1* encodes RAS p21 protein activator 1, which is a negative regulator of the Ras pathway through its GTPase activating protein. RASA1 interacts with receptor tyrosine kinases including EPH family receptors, amongst which the EPHB4 receptor is involved in regulating AV morphology.[32]

Interestingly, *de novo* damaging heterozygous germline mutations in *EPHB4* have been identified in Vein of Galen malformations (VOGM), another rare congenital AVM sometimes present in newborns.[33,34] The

Ephrin receptor signaling pathway was over-represented in VOGM cases with the following genes in an interactome: *EPHB4, RASA1, EPHA4, EPHA6, ITGB1, ITNS1,* and *NGEF*.[34] In addition, one *RASA1* mutation was identified in 55 VOGM probands, suggesting a potentially shared molecular mechanism between VOGM and CM-AVM.[34] Therefore, the EphrinB2-EphB4-RASA1 signaling axis has a role in human cerebrovascular development and disease, which may provide diagnostic and therapeutic targets for patients with cerebrovascular disorders including bAVM.[33]

Findings in sequencing studies

Whole exome sequencing studies in bAVM have identified several rare germline mutations. For example, a stop-gain mutation (c.C739T:p.R247X) in *SMAD9* was discovered, with reduced levels of vascular SMAD9 protein and phosphorylated SMAD4, a downstream effector of the BMP signaling pathway, in AVM perinidal blood vessels.[35] Whole exome sequencing of 5 bAVM patients identified germline mutations enriched in pathways controlling endothelial homeostasis and 2 novel pathways: cilia morphogenesis and ion homeostasis.[36] In addition, Scimone et al.[37] performed whole exome sequencing to evaluate a young boy with a sporadic bAVM and detected 20 likely gene-disrupting variants affecting many genetic loci, including a *de novo* nonsense variant in the *STK4* gene.

Whole exome sequencing of 100 unrelated bAVM trios led to the discovery of four pathogenic heterozygous variants in four bAVM patients.[38] One variant was in *ENG,* and three others were damaging variants in novel candidate genes: *PITPNM3*, *SARS* and *LEMD3*. These whole exome sequencing data were included in a larger follow-up study of a total of 112 bAVM trios which investigated rare and deleterious compound heterozygous mutations associated with bAVM.[39] A total of 16 genes had compound heterozygous variants that were recurrent in more than one trio. Two genes, *LRP2* and *MUC5B,* were recurrently mutated in three trios. LRP2 is a receptor for lipocalin 2 (LCN2), which is involved in inflammation and may have a role in brain andothelial cell angiogenesis.[40] The *LRP2* mutations identified in bAVM trios were all novel and predicted to be harmful, and all three bAVMs were located in the left parietal lobe. The mutations in the *MUC5B* gene were all missense mutations. However, *MUC5B* is a large gene and sequencing studies often reveal many unexplained variations in this gene; thus *MUC5B* is not considered a strong candidate gene for bAVM. The following genes were recurrently mutated in two trios: *DNAH14, DNAH5, FCGBP, HERC2, HMCN1, MYH1, NHSL1, PLEC, RP1L1* and five genes known to have a role in vascular disease or angiogenesis including *MYLK, HSPG2, PEAK1, PIEZO1,* and *PRUNE2*. This study supports a role for rare recessive compound heterozygous variants in bAVM and future functional studies will be required to assess the impact on bAVM pathology.[39]

Somatic mutation in bAVM

Somatic mutations arise during development or in disease pathogenesis in a somatic cell and are subsequently found only in a subset of cells in each affected individual. Activating somatic mutations in the *Kirsten rat sarcoma viral oncogene homologue (KRAS)* gene and other mitogen-activated protein kinase (MAPK) pathway genes have been detected in bAVM tissue using high-throughput or targeted sequencing technology,[41–44] suggesting a role for the RAS/RAF pathway and MAPK-ERK signaling pathway. Using whole exome sequencing of DNA from sporadic bAVM tissue, Nikolaev et al.[41] identified the presence of 3 recurrent, somatic activating mutations in *KRAS* (c.35G>A [G12D], c.35G>T [G12V], c.183A>T [Q61H]), with low-allelic representation (<5%) and not present in DNA from paired blood samples. Since that report, several groups have confirmed the presence of these rare but recurrent *KRAS* mutations, identified additional *KRAS* and *BRAF* mutations,[42] and found that these mutations are also present in spinal cord AVM.[45] Peripheral AVM lesions also harbor somatic mutations in other members of the RAS/MAPK pathway, including *MAP2K1* and *BRAF*, a proto-oncogene,[46,47] which suggests there may be a potential common signaling pathway for treatment of AVMs located both within or outside the central nervous system (CNS). A recent meta-analysis of 6 studies including 1726 patients with bAVM estimated the frequency of *KRAS* somatic mutations is 55%, while the prevalence of *BRAF* somatic mutation is 7.5%.[44] Gao et al.[48] performed whole exome sequencing in 14 paired bAVM tissue and blood DNA samples, and validated *KRAS* mutations in 56 patients. A total of 24 candidate somatic variants in 11 MAPK pathway genes were identified, including *KRAS* G12V in 15% and *KRAS* G12D in 32% of bAVM lesions,[48] and novel mutations in *PDGFRB*. Only *KRAS* mutations have been reported in multiple patients (recurrent), whereas remaining somatic mutations identified to date are private mutations (*e.g.*, specific and isolated to a single individual or family).

It is unknown whether somatic mutation burden is an inciting event in bAVM formation or appears later in the disease course, as part of endothelial repair, progression to hemorrhage (*e.g.*, as a result of high intranidal blood flow and chronic inflammation triggering

changes in vascular genes such as flow-sensitive genes), or development of high-risk features (*e.g.*, associated aneurysms).[42,47] BAVMs are rarely observed in utero, VOGM being the exception, hence the somatic mutations are likely to occur later in post-natal development. Genotype-phenotype studies have not identified associations with age at presentation, sex, presenting symptom, AVM size, or location, when comparing patients with and without somatic mutations or with mutation burden.[43,48] These findings highlight the need to characterize bAVM tissue for somatic *KRAS* and other RAS/MAPK gene mutations to better understand the relevance in bAVM pathogenesis and phenotypes. Future studies will need to elucidate the precise timing of when these somatic mutations arise in the endothelial cells or other cell types.

Somatic mutations have also been observed in vascular malformations from patients with HHT and CM-AVM. Two somatic mosaic *RASA1* mutations (c.2035C>T and c.1507C>T) were identified in a facial AVM of a patient with CM-AVM who has the germline *RASA1* c.2035C>T mutation.[49] Somatic mutations identified in telangiectasia from HHT patients resulted in bi-allelic loss of *ENG* or *ALK1*.[50] These studies suggest that the focal nature of vascular malformations in these familial diseases follow a two-hit genetic mechanism, where patients inherit a germline mutation followed by a second somatic mutation in the same gene to seed lesion formation, rather than haploinsufficiency of the protein.[50] The mechanism for sporadic lesions likely follows a two-hit mechanism, although the second hit may be genetic or environmental, as also suggested by animal studies. Additional studies are needed to further define the impact of these mutations in bAVM development or progression.

A current limitation of somatic mutation studies is that they rely on availability of AVM tissue. For peripheral AVMs, conventional biopsy methods may be used. However for CNS AVMs, not all can be safely treated by microsurgical resection and open surgical biopsy is not possible due the risks of stroke. As such, our group has demonstrated a method to safely and accurately collect cells using endovascular means for bAVM specific genetic diagnosis.[51] This technique may prove instrumental in determining which cases will most favorably respond to certain therapies, medical or otherwise, in addition to more generally expanding our understanding of the molecular genetics of secondary vascular disorders. Additionally, next generation sequencing liquid biopsy using cell-free DNA may be a useful noninvasive approach to investigate *KRAS* mutations in bAVM patients.[52] Future studies are needed to determine whether blood-based markers can inform on *KRAS* somatic mutation burden and the relevance to bAVM hemorrhage.

Differential microRNA and mRNA expression in bAVM

Expression studies have demonstrated a role for gene regulation in bAVM, including microRNAs, which are non-coding RNA that regulate the expression of target genes.[53] Specific microRNAs (miR-18a, miR-137, and miR-195 all downregulated in bAVM) have been shown to inhibit vasculogenesis or improve endothelial cell function in bAVM.[54] A recent study of patient-derived bAVM endothelial cells demonstrated that miR-18a increases TSP-1 and decreases VEGF by reducing plasminogen activator inhibitor-1/SERPINE1 (PAI-1) levels.[55] In addition, miR-18a decreased the expression of bone morphogenetic protein 4 (BMP4) and hypoxia-inducible factor 1α (HIF-1α), and blocked the BMP4/ALK2/ALK1/ALK5 and Notch signaling pathways.[55] miR-199a-5p, miR-7-5p and miR-200b-3b are upregulated in peripheral blood of bAVM patients, involved in VEGF signaling, and may be useful biomarkers for bAVMs.[56] Chen et al. also observed upregulated let-7b-3p in the blood of bAVM patients, although the function of this miRNA in bAVM is unknown.[56] It remains unknown if miRNAs are involved in AVM development, However in mice, mutations in *Drosha*, a core nuclease that executes the initiation step of miRNA processing in the nucleus, caused vascular abnormalities similar to HHT telangiectasia in mice.[57]

Next-generation RNA sequencing has identified differential expression on a transcriptome-wide level comparing tissue samples of 12 bAVMs to 16 intracranial control arteries.[58] A total of 736 upregulated genes in bAVM are implicated in cytoskeletal machinery, cell-migration, neutrophils and macrophages, and inflammatory cytokines, which is consistent with older transcriptome studies in bAVM tissue.[59–61] In addition, 498 genes are downregulated, including genes involved in the angiopoietin-TIE system and TGF-β signaling. In line with previous studies, *ANGPT1* and its receptor (*TEK*) were downregulated.[60] The study points to involvement of loss of cerebrovascular quiescence, and impaired integrity of the vascular wall in the pathophysiology of bAVMs, and supports a potential role for therapeutics promoting vessel maturation.

Whole blood transcriptome (mRNA) profiling in 40 bAVM patients (20 ruptured vs. 20 unruptured) identified molecular signatures of ICH, including increased levels of *FAS*, *TLR10*, *TNFAIP6*, *IL1R1*, and *IL18R1*, and suggests involvement of the MAPK, VEGF, Wnt and several inflammatory pathways.[53] Future studies will be needed to further define the role of microRNAs and mRNAs in bAVM and determine whether they can be useful noninvasive clinical biomarkers for bAVM.

Epigenetic factors in cerebrovascular disease and bAVM

Epigenetic mechanisms provide tight control at the transcriptional level that differentially modifies gene expression and protein activity. Targeted candidate gene methylation studies in bAVM and intracranial aneurysm patients suggest that methylation of *CDKN2A* is associated with bAVM and methylation of *PDGFD* is associated with increased risk of both bAVM and intracranial aneurysm.[62] Genetic variants in these or related genes have been associated with bAVM or bAVM-associated aneurysms.[48,63] In addition, key components of the m6A methyltransferase complex, Wilms' tumour 1-associating protein (WTAP) and Methyltransferase-like 3 (METTL3), which is an important epigenetic regulator, are down-regulated in bAVM lesions and inhibits angiogenesis,[64] with METTL3 downregulation leading to continuous activation of the Notch signaling pathway.[64] DNA methylation of key candidate genes involved in pathways that contribute to bAVM progression, such as flow-sensitive genes, may disrupt the regulation of transcription in immune cells or supporting vascular cells that stabilize the bAVM lesion. Future studies are needed to investigate the role of gene regulation, e.g., through mechanisms such as DNA methylation, in bAVM pathogenesis. These new avenues of bAVM research may also lead to the identification of new therapeutic targets for bAVM, as epigenetic regulators may be targeted to correct gene expression perturbations in disease.

Other genetic factors associated with bAVM and bAVM hemorrhage

Common genetic variants may influence bAVM disease course and increase risk of ICH. We have identified inflammatory genes associated with risk of bAVM hemorrhage in three settings: presentation with ICH,[65] new ICH after diagnosis,[66] and ICH after treatment.[67] In particular, our group and others have identified several pro-inflammatory cytokine variants that increase risk of ICH by 2-4 fold in bAVM patients, including interleukins and tumor necrosis factor alpha (*TNFA*).[68] In addition, two *EPHB4* variants (rs314313 and rs314308) were found to be associated with risk of ICH in Caucasian subjects with sporadic bAVM.[65] *EPHB4*, which is expressed by venous endothelial cells, is involved in kinase dependent forward signaling, which regulates diverse endothelial cell functions and angiogenesis along with concomitant activation of ERK1/2.[69] Thus, it is plausible that common *RASA1* and *EPHB4* variants could influence disease severity in vascular diseases with involvement in the complex RAS-ERK and EPHRINB2-EPHB4-RASA1 signaling pathways.

In summary, we do not yet know the cause of sporadic bAVM, however there are several genetic factors that may influence sporadic bAVM disease including, e.g., gain or loss of function genetic mutations, epigenetic changes, or genetic modifiers. Figure 1 illustrates several major pathways (and key genes) in both hereditary diseases with bAVM and sporadic bAVM. Although these are not the same genes, the pathways interact, suggesting a spectrum of vascular diseases displaying vascular malformations as part of the phenotype. Genetic associations are not necessarily causal for bAVM. However, mechanistic/functional studies in *in vitro* and *in vivo* animal models as described in detail in the next section below reveal a strong role for these genes in bAVM development and/or hemorrhage.

Signaling pathways, current AVM animal models, and potential therapeutic targets

Tgf-β signaling and HHT animal models

Mutations in the TGF-β pathway impairs vascular morphogenesis and angiogenesis. Mice deficient in the components of the TGF-β pathway exhibited embryonic lethality due to vascular defects. Five type I receptors and seven type II receptors have been identified thus far. Accessory receptors have also been identified to be involved in the formation of receptor complexes, including ENG, beta-glycan, BMP, and activin membrane-bound inhibitor homology (BAMBI). In the canonical Smad pathway, TGFβ/BMP dimers induce the heteromeric complex formation of TβRI/TβRII. The TβRII then phosphorylates and activates the TβRI, which in turn propagates the signal to the nucleus through the Smad family of co-activators. Alternatively, the phosphorylated TβRI receptor can activate non-Smad pathways.

Mutations of TGF-β signaling pathway genes, including *ENG, ALK1, SMAD4* and *BMP9* cause HHT. The endothelial cell TGF-β signaling is characterized by the balanced signaling through the TGF-β type I receptors: the endothelial cell dominant ALK1 and the uniquitously expressed ALK5. TGF-β fine tunes the intricate equilibrium between ALK1 and ALK5. Low doses of TGF-β stimulates endothelial cell proliferation and migration via ALK1 to activate angiogenesis, while high doses of TGF-β increases the production of extracellular matrix (ECM) components, leading to a quiescent endothelium. ALK5 is required for efficient ALK1 signaling and the ratio of those two receptors as well as accessory receptor ENG determines the relative response to TGF-β.[70] Interestingly, ENG can be shed off from the endothelial cell membrane as a soluble form (solENG) affecting the delicate balance of TGF-β signaling required for angiogenesis by

scavenging TGF-β ligands. Overexpression of solENG caused bAVMs in mice.[71] solENG was also found to specifically bind to BMP9 and BMP 10, leading to the inhibition of blood vessel formation.[72] BMPs have also been implicated in endothelial cell function and angiogenesis. Blocking both BMP9 and BMP10 induced AVM development in the retinal vasculature, but it remains elusive whether BMP9 and BMP10 are both required for ENG-ALK1 signaling.

Mice carrying mutations on both alleles of *Eng* or *Alk1* genes were embryonic lethal and showed obvious defects in angiogenesis and cardiac development.[73,74] However, mice with heterozygous mutations in either of these genes can survive to adult stage and recapitulate relatively mild phenotypes seen in HHT patients,[74,75] suggesting that additional factors, such as mutation on the other allele (second hit) and environmental mediators are required for bAVM development. Moreover, morpholino-induced knockdown of *Eng* or *Alk1* in zebrafish models recapitulate the morphologic, functional, and molecular defects seen in human AVMs, allowing visualization of precise spatiotemporal patterns during vascular development.[76]

Tamoxifen-inducible conditional knockout (iKO) mouse models have been developed to allow temporal control of *Eng* or *Alk1* deletion in specific cells and at specific developmental stages. Brain focal angiogenic stimulation (VEGF administration) with either *Eng* or *Alk1* iKO globally or specifically in endothelial cells induced a robust and reproducible bAVM phenotype in adult mice, including vascular dysplasia, arteriovenous shunt, and microhemorrhage.[77–79] In the skin, arteriovenous shunts only developed around skin wounds in *Alk1* or *Eng* iKO mice.[80,81] In addition, bAVM can develop spontaneously in mice that have *Alk1* or *Eng* deleted at the perinatal stage.[79,82] These data indicate that in addition to *Eng* or *Alk1* mutation, response to injury/angiogenic stimulation is necessary to cause AVM development in the brain and other organs.

Recently, Kim et. al. demonstrated that overexpression of Alk1 can rescue the AVM phenotypes in both *Alk1*- and *Eng*-iKO mice through normalizing the expression of Smad and Notch target genes and restoring the effect of Bmp9 on suppression of pAkt in Eng-deficient endothelial cells.[81] However, overexpression of Eng failed to inhibit the AVM manifestations in *Alk1*-iKO mice. Therefore, Eng is signaling upstream of Alk1. Increasing Alk1 expression could be a therapeutic option.

KRAS-MAPK signaling and sporadic bAVM models

Emerging evidence suggests the RAS-MAPK signaling cascade is important in sporadic bAVMs and non-CNS AVMs.[41,45–47] The somatic, *de novo* activation mutations in *KRAS*/*BRAF* and *MAP2K1*/*MEK* were shown to activate the MAPK-ERK signaling pathway in AVM endothelial cells, leading to increased angiogenesis, cell migration and proliferation.[41,45–47] Robust MAPK/ERK activity were detected in all bAVM tissues, including those without detectable *KRAS* mutations, suggesting that this pathway plays a key role in AVM pathogenesis.[41]

Endothelial *KRAS* activating mutations cause conformational changes in KRAS and render it constitutively active by preventing GTP hydrolysis.[83] BRAF is the downstream effector of KRAS, which is recruited to the cell membrane following KRAS activation. As a serine/threonine kinase, RAF activation phosphorylates MAPK (a.k.a. MEK, mitogen-activated protein kinase kinase 1), which phosphorylates and activates downstream ERK1 and ERK2. ERK1 and ERK2 further activate and phosphorylate a variety of nuclear transcription factors and kinases, resulting in a large number of KRAS-induced cellular responses. Expression of $KRAS^{G12V}$ in endothelial cells *in vitro* stimulated ERK activity, and activated specific genes involved in angiogenesis and Notch signaling. These effects of $KRAS^{G12V}$ were reversed by inhibition of MAPK-ERK signaling using MEK inhibitor (U0126).[41]

Mouse and zebrafish models that mimic sporadic bAVM features have been recently generated through somatic endothelial cell-specific gain of function mutation in *KRAS*.[84,85] Using both postnatal and adult mice, Fish et al. demonstrated that endothelial cell-specific gain of function mutations in *KRAS* (G12D or G12V) are sufficient to induce bAVMs,[84] even in the setting of uninjured adult vasculature. Using a brain endothelial cell-specific AAV vector, AAV-BR1, mediated brain endothelial cell gene transfer, Park et al. confirmed that KRAS mutations promote bAVM development via the MEK/ERK pathway.[85] In addition, using the embryonic zebrafish model, Fish et al. demonstrated that activation of MEK but not PI3K signaling is required for KRAS-mediated AVM progression.[84] Similarly, Park et al showed that inhibition of MEK/ERK by trametinib treatment attenuated $KRAS^{G12V}$-induced bAVM growth in mice.[85]

There are two case reports of off-label use of the MEK-inhibitor trametinib in patients with KRAS-positive chest wall AVMs, one of which demonstrated significant reduction in the cardiac output fraction to the lesion after 6 months of treatment.[86,87] Together, these animal and human studies indicate that MEK inhibition is a promising therapy for the treatment of bAVMs and should be evaluated in future studies.

Notch signaling and bAVM models

Aberrant activation of Notch signaling is involved in the etiology of bAVMs.[88,89] Canonical Notch signaling

controls cell fate decisions in various developmental processes. This pathway is an intercellular signaling pathway, where both receptor and ligand are membrane-bound on adjacent juxtaposed cells. There are four transmembrane Notch receptors (1-4) and five membrane-bound ligands (Jagged 1, 2, D-like ligand 1, 3, and 4). Notch ligand-receptor interaction is followed by proteolytic cleavage to release the intracellular domain of the receptor, which is subsequently trafficked to the nucleus to mediate the transcription of Notch target genes.

Endothelial cell expression of a constitutively active Notch-4 allele in adult mice caused vascular defects in the liver, uterus, and skin, but not in brain.[88] The defective vessels were reversed upon repression of Notch-4 expression. Similarly, endothelial cell expression of a constitutively active Notch-1 resulted in similar hepatic vascular lesions. These findings provide the first evidence that Notch signaling in adult endothelium is sufficient to render the development of AVMs.[88] Expression of constitutive Notch-4 or Notch-1 in neonatal mice recapitulated the phenotypes of human bAVMs.[88] Blockage of Notch signaling through deletion of *Rbpj* in endothelial cells of postnatal mice also caused features of bAVMs.[90] Arteriovenous shunts showed decreased Efnb2 (arterial marker) and increased Ephb4 (venous marker) expression.

Previous studies have shown that arteriovenous shunts were observed in both mouse and zebrafish models carrying mutants of genes in the Notch pathway, which prompted the investigation of the Notch pathway in AVM pathogenesis.[81,91] In *Alk1* KO mouse models, decreased Notch signaling was found in AVMs.[91] Alk1 signaling inhibits angiogenesis by cooperating with the Notch pathway. In addition, combined blockade of Alk1 and Notch signaling exacerbated hypervascularization, and activation of Alk1 by its high-affinity ligand Bmp9 rescued the hyper-sprouting induced by Notch inhibition.[91] These findings demonstrate a direct crosstalk between ALK1 and NOTCH pathways during vascular morphogenesis that may be relevant to the pathogenesis of HHT.

Notch signaling also plays a very important role in regulating mural cell differentiation and function. There are 2 types of mural cells: pericytes and vascular smooth muscle cells (vSMCs). Pericytes and vSMCs both express Notch 1, 2, and 3. Notch signaling has been found to modulate vSMC differentiation, survival, and vasculature. *In vitro* studies demonstrated that Notch signaling is essential for pericyte survival and adhesion to endothelial cells. *In vivo* studies using mouse and zebrafish models found that Notch-3 signaling promoted pericyte proliferation and limited vascular permeability.[92] Deletion of *Rbpj* in pericytes resulted in reduced pericyte coverage and induced AVM development.[92] Moreover, the loss of Notch signaling in pericytes downregulated *Pdgfrb* levels and increased pericyte apoptosis, indicating a critical role for Notch in pericyte survival.[93]

Proteins involved in Notch signaling, including the receptor, its ligands, and downstream signals, are expressed in bAVM tissue.[89,94] Therefore, the role of Notch in bAVM pathogenesis merits further exploration.

Other pathways and AVM models

Matrix GLA protein (MGP). MGP, an antagonist of BMPs, is expressed in endothelial cells and plays an essential role in endothelial cell function by affecting BMP, TGFβ and VEGF signaling.[95] BMP-SMAD signaling increases the expression of ALK1, which in turn induces the expression of MGP and further sequesters BMP, thereby forming a negative feedback loop.[96] Deletion of *Mgp* ($Mgp^{-/-}$) in mice induces Notch signaling by enhancing expression of Notch ligands, Jagged 1 and Jagged 2, dysregulates endothelial cell differentiation, and causes bAVM development.[97] Crossing $Mgp^{-/-}$ mice with Jagged deficient mice diminished Notch activity, normalized endothelial cell differentiation, and prevented bAVMs, but not pulmonary or renal AVMs.[97] These findings suggest that endothelial cell-Rbpj is required at postnatal stage for maintaining of vascular integrity and preventing arteriovenous shunt and AVM development.

PDGFB/PDGFRβ signaling. Accumulating data demonstrate that the abnormal vascular remodeling and vascular instability are associated with bAVM development and progression, including dilated perinidal capillaries,[98] intranidal or feeding artery aneurysms,[99] and microhemorrhage and rupture.[100] However, the exact mechanisms underlying bAVM hemorrhage remain unclear. Abnormal expression of PDGFB and PDGFRβ has been described in bAVMs in humans and rodents.[101–103] Pdgfrβ expression was reduced in the bAVM lesions of *Alk1* iKO mice, which was associated with a reduction of mural cell coverage, suggesting a possible crosstalk between ALK1 and PDGFB/PDGFRβ signaling pathways.[103] Both pericyte number and coverage are reduced in resected tissue from sporadic bAVM patients.[101] Importantly, pericyte reductions are greatest in bAVMs with clinical hemorrhage and are associated with a higher microhemorrhage burden in unruptured cases, suggesting that reduction of pericytes contributes to bAVM hemorrhage.[101]

PDGFRβ is expressed in multiple cell types, including pericytes, vSMCs, and neurons.[104] Its ligand, PDGFB is secreted from the endothelial cells of

angiogenic sprouts where it works as an attractant for recruiting pericytes. PDGFB and PDGFRβ are key elements in regulating pericyte recruitment and maintaining vascular integrity and stabilization.[105]

Thalidomide treatment was shown to increase PDGFB expression in endothelial cells and induce vessel maturation by increasing mural cell coverage.[106] Thalidomide belongs to a group of drugs known as immunomodulatory drugs (IMIDs), which works to modulate the immune system. Neurotoxic adverse effects of thalidomide promoted the discovery of newer derivatives, e.g. lenalidomide, which demonstrates effectiveness in treating multiple myeloma and myelodysplastic syndrome.[107] The IMIDs have been shown to inhibit endothelial cell proliferation and migration.[108] The anti-angiogenic mechanism of thalidomide remains elusive, but it has demonstrated clinical benefits in treating gastrointestinal hemorrhage and epistaxis in HHT patients.[106,109] Thalidomide or lenalidomide treatment also reduced hemorrhage, attenuated dysplastic vessel formation, and improved vascular smooth muscle cell coverage in mouse bAVM lesions.[110] In addition, lentiviral vector mediated overexpression of Pdgfb in mouse brain has also reduced bAVM severity in *Alk1* iKO bAVM mouse model.[110] These data demonstrate that PDGFB/PDGFRβ pathway can be a target for developing new therapies to reduce AVM hemorrhage.

Ephrinb2/EphB4 signaling. Elevated expression of EphB4 and ephrinB2 were detected in patients with AVMs.[111,112] Among the 14 Eph receptors and 8 Ephrin, EphrinB2/EphB4 are the first ones discovered to be differentially expressed in arterial and venous endothelial cells. EphrinB2/EphB4 signaling has been implicated in the regulation of multiple vascular events, including sprouting angiogenesis, vascular morphogenesis, arteriovenous differentiation and vascular homeostasis.[113]

EphrinB2 is expressed in endothelial cells and their surrounding mesenchymal and mural cells, while EphB4 is specifically expressed in endothelial cells.[114] EphrinB2 and EphB4 have been deemed as the primary molecular markers for endothelial cell arteriovenous specification. Accumulating evidence suggest that EphrinB2-EphB4 signaling plays an very important role in AVMs and other cerebrovascular disorders.[111] Embryos harboring homozygous mutations in *Efnb2* and *Ephb4* exhibited vascular defects and AVMs.[115] An *in vitro* model of HHT2 showed that loss of *Alk1* gene blocked Bmp9 signaling, resulting in reduced EphrinB2 expression, enhanced Vegfr2 expression, and dysregulated endothelial cell sprouting and anastomosis.[116] In addition, EphrinB2 is a crucial regulator of Pdgfrβ expression in vSMCs, and thereby acts as a molecular switch controlling the downstream signaling activity induced by PDGFB/PDGFRβ. mTORC1 overactivition was observed in both morpholino-treated zebrafish and cultured HEK293T cells with *EphB4* knocked in.[117] The zebrafish phenotype could be rescued by inhibiting mTOR or RAS-MAPK signaling. *EphrinB2* ablation enhanced Pdgfb-induced Mapk and Jnk activation and diminished Tiam1/Rac1 signaling, a pathway critical for cell migration, proliferation, and spreading.[118]

RASA1 is a direct downstream effector of EPHB4. Knockdown of *Ephb4* and *Rasa1* in zebrafish shared a similar phenotype of vascular deformities and caudal vascular plexus malformation.[32] *Rasa1* KO mice are embryonic lethal and exhibited several blood vessel abnormalities, suggesting that RASA1 is essential in vasculogenesis.[119] Knock-in *Rasa1* lacking the arginine finger, which is required for its interaction with Ras, resulted in embryonic lethality and several vascular abnormalities similar to *Rasa1* KO mice. These findings suggest that dysfunction of Ras-Mapk and EphrinB2/EphB4 pathways work synergistically in the context of vascular development. Phenotypes induced by knockdown of *Ephb4a* or *Rasa1* can be rescued by chemical inhibitors of PI3K/mTORC1.

Altogether, these data demonstrate that the ephrinB2-ephB4-RASA1 signaling axis is essential for development of the vascular system. Inhibition of PI3K/mTORC1 could be a therapeutic target for the treatment of vascular malformation induced by *RASA1* or *EPHB4* mutation.

Additional therapeutic targets not related to specific genetic alteration

Anti-angiogenesis. Excessive expression of VEGF was detected in both HHT and sporadic bAVMs, and angiogenesis is necessary to induce bAVM development in adult mice.[77,120] Compelling evidence supports that inhibitors of VEGF signaling can block angiogenesis and reduce AVM severity in HHT mouse models. Intraperitoneal bevacizumab injection reduced the number of malformed vessels in bAVM model of an *Alk1* iKO mouse.[121] Several VEGF inhibitors have demonstrated clinical efficacy in patients with cancer or ocular vascular disease.[122,123] Of these, a humanized anti-VEGF monoclonal antibody (bevacizumab; Avastin), approved by the FDA for treatment of several cancers, showed promise in treating HHT patients. It normalized cardiac output and improved anemia in HHT patients with severe liver failure and/or refractory anemia.[124] It also demonstrated clinical efficacy and safety in the treatment of severe epistaxis caused by hemorrhage from small mucosal AVMs (telangiectasias).[125] There have been several case reports of off

label use of bevacizumab in treating sporadic bAVMs.[126,127] No serious adverse events have been noted, though bAVMs did not change in size during the study interval.[126,127] However, two reports using bevacizumab to treat adverse radiation effects demonstrated a reduction in perilesional edema by imaging and marked improvement in clinical symptoms.[126,127]

Despite the clinical benefits of bevacizumab, this antibody-based therapy has several drawbacks, including hemorrhage[128] and frequent dosing over an extended period of time. The use of AAV-mediated expression of *soluble* FMS-related tyrosine kinase 1 (sFLT1), the extracellular domain of VEGFR-1, is a promising alternative to the bevacizumab for the treatment of bAVM. sFLT1 is capable of binding VEGF in tissues, preventing its binding to VEGFRs and thus inhibits VEGF-mediated angiogenesis. Intravenous injection of AAV9-sFlt1 reduced bAVM development and bAVM severity in two *Eng* iKO mouse models.[129]

Anti-inflammation. AVMs in humans and animal models are associated with increased inflammation and overexpressed inflammatory markers, e.g., MPO, IL-6, and MMP-9.[103,130] Tetracyclines are clinically available non-specific MMP inhibitors. They can potentially increase vascular stability and reduce the risk of spontanous hemorrhage in a variety of human diseases, including ICH[131] and traumatic brain injury.[132] Doxycycline has been shown to reduce MMP level in bAVMs.[133] Animal studies show that doxycycline is also effective in reducing bleeding risk in bAVMs[133,134] via MMP-9 inhibition.[135] Doxycycline or minocycline has been used in a small pilot study of bAVM patients, with no serious adverse effects noted for up to 2 years.[136] However, there is also no significant evidence of clinical efficacy or hemorrhagic risk reduction in patients.

Bone marrow (BM)/monocyte transfusion. BM-derived cells participate in VEGF-stimulated brain angiogenesis.[137] BM-derived MMP-9 plays an important role in bone marrow cell mobilization and VEGF-induced brain angiogenesis.[137] Transplatation of $Eng^{+/-}$ mouse bone marrow to WT mice resulted in similar degree of capillary dysplasia in the brain angiogenesis in the brain angiogenic region of $Eng^{+/-}$ mice. Transplatation of WT bone marrow to $Eng^{+/-}$ mice reduced the severity of vascular dysplasia.[138] Another study found that injection of normal human monocytes can rescue the defective vessel formation and heart function in $Eng^{+/-}$ mice.[139] Together, these data suggest that bone marrow has a crucial role in blood cell-mediated vascular repair and bone marrow/monocyte transfusion could be used as an potential therapy to reduce bAVM severity.

In summary, the most devastating symptom of bAVM patients is ICH. The treatment for bAVM should aim to stabilize vascular tissue thereby decreasing the risk of spontaneous ICH. Risk factors for hemorrhage of bAVM include elevation of VEGF, loss of vessel wall integrity, and alteration in hemodynamics.[105] Several therapeutic options identified through pathway studies can be explored to strengthen vessel wall and reduce the possibility of ICH, such as increase PDGFB level through thalidomide or lenalidomide treatment or overexpression of PDGFB and anti-angiogenesis through Bevacizumab treatment or overexpression of sFLT1. In addition, anti-inflammation can also stabilize vessel wall and reduce ICH of bAVM patients (Figure 2).

Future prospects

In summary, much progress has been made in understanding sporadic bAVM pathogenesis. The recent discovery of somatic gene mutations in the RAS/MAPK/ERK signaling pathway in bAVMs suggest a common pathway with peripheral AVMs, and have added to the growing number of relevant signaling pathways involved in bAVM. Novel animal models have been developed to elucidate the molecular mechanisms involved and have identified several potential therapeutic targets. Due to the size limitations, AVM models in rodents cannot be used for many preclinical tests, such as for development of novel endovascular treatment. Large animal AVM models generated by creating carotid-jugular fistula, using species exhibiting rete mirabile or autologous implants feature various conceptual advantages in translational research.[140] However, the carotid-jugular fistula, rete mirabile or autologous implants are not true brain AVM. With the advantage of molecular tools, it is possible to induce AVM development in the brain parenchymal in large animals in the future.

Current therapeutic strategies for bAVM are to remove or reduce the risk of hemorrhage with interventional treatment. Likewise potential medical therapies targeting relevant signaling pathways highlighted in this review also aim to stabilize vascular tissue thereby decreasing the risk of spontaneous ICH. Future studies should focus on validating existing targets in animal models, and moving towards clinical trials in AVM patients.

Funding

The author(s) disclosed receipt of the following financial support for the research, authorship, and/or publication of this article: This study was supported by grants from the National Institutes of Health to H.S. (R01 HL122774, NS027713 and NS112819), to H.K. (R01 NS034949, NS099268), and from

the Michael Ryan Zodda Foundation and the Leslie Munzer Neurovascular Research Fund.

Declaration of conflicting interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

Authors' contributions

HK and HS: participated in conception and design of the review; PP, SW, DC, AA and EW participated in writing, HK and HS finalized the manuscript.

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