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Integrin $\beta 8$ Deletion Enhances Vascular Dysplasia and Hemorrhage in the Brain of Adult *Alk1* Heterozygous Mice

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Abstract Brain arteriovenous malformation (bAVM), characterized by tangled dysplastic vessels, is an important cause of intracranial hemorrhage in young adults, and its pathogenesis and progression are not fully understood. Patients with haploinsufficiency of transforming growth factor- β (TGF- β) receptors, activin receptor-like kinase 1 (ALK1) or endoglin (ENG) have a higher incidence of bAVM than the general population. However, bAVM does not develop effectively in mice with the same haploinsufficiency. The expression of integrin $\beta 8$ subunit (ITGB8), another member in the TGF- β superfamily, is reduced in sporadic human bAVM. Brain angiogenic stimulation results at the capillary level of vascular malformation in adult *Alk1* haploinsufficient (*Alk1*^{+/-}) mice. We hypothesized that deletion of *Itgb8* enhances bAVM development in adult *Alk1*^{+/-} mice. An adenoviral vector expressing Cre recombinase (Ad-Cre) was co-injected with an adeno-associated viral vector expressing vascular endothelial growth factor (AAV-VEGF) into

the brain of *Alk1*^{+/-};*Itgb8*-floxed mice to induce focal *Itgb8* gene deletion and angiogenesis. We showed that compared with *Alk1*^{+/-} mice ($4.75 \pm 1.38/\text{mm}^2$), the *Alk1*^{+/-};*Itgb8*-deficient mice had more dysplastic vessels in the angiogenic foci ($7.14 \pm 0.68/\text{mm}^2$, $P = 0.003$). More severe hemorrhage was associated with dysplastic vessels in the brain of *Itgb8*-deleted *Alk1*^{+/-}, as evidenced by larger Prussian blue-positive areas (1278 ± 373 pixels/ mm^2 vs. *Alk1*^{+/-} : 320 ± 104 pixels/ mm^2 ; $P = 0.028$). These data indicate that both *Itgb8* and *Alk1* are important in maintaining normal cerebral angiogenesis in response to VEGF. *Itgb8* deficiency enhances the formation of dysplastic vessels and hemorrhage in *Alk1*^{+/-} mice.

Keywords Activin receptor-like kinase 1 · Brain arteriovenous malformations · Brain hemorrhage · Integrin $\beta 8$ · Mouse model

Li Ma and Fanxia Shen contributed equally to this work.

Dr. Young passed away 3 years ago.

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Introduction

Brain arteriovenous malformation (bAVM) is a vascular disease with tangles of dysplastic, dilated vessels that shunt blood directly from arteries to veins [1, 2]. These abnormal vessels are prone to rupture, causing life-threatening intracranial hemorrhage. The annual rupture rate is 2–4 % [3]. While most bAVMs occur sporadically, a few are associated with genetic disorders, such as Osler-Weber-Rendu syndrome, also called hereditary hemorrhagic telangiectasia (HHT). HHT is the most common familial disorder associated with bAVMs [4]. It is an autosomal dominant vascular disease mainly caused by haploinsufficiency of the genes in the transforming growth factor- β (TGF- β)/bone morphogenic protein (BMP) superfamily: endoglin (*ENG*, type 1 HHT), activin receptor-like kinase 1 (*ALK1*, type 2 HHT) [5, 6], and *SMAD4* (JPHT, HHT and juvenile polyposis). Common polymorphisms in *ALK1* are associated with sporadic

bAVM [7–9]; however, few spontaneous bAVMs develop in *Alkl*^{+/-} (similar to HHT2 genotype) mice [10]. Dysmorphic vessels at the capillary level developed in the brain only after focal angiogenic stimulation [11, 12], suggesting that other factors are required for bAVM development.

We showed previously that compared with normal brain tissue, there is a reduced level of integrin $\beta 8$ (ITGB8) in human bAVM tissue [13]. ITGB8 is expressed primarily in neuroepithelial cells, serving as a major pathway for transforming growth factor- β (TGF- β) activation and signaling in vivo [14, 15]. ITGB8 in perivascular astrocytes plays a central role in regulating brain vessel homeostasis through modulation of TGF- β activation and expression of TGF- β -responsive genes that promote vessel differentiation and stabilization [16]. Focal deletion of *Itgb8* in the mouse brain reduces the activation of TGF- β and induces microscopic vascular dysplasia in the angiogenic foci of the vascular endothelial growth factor (VEGF)-stimulated adult mouse brain [13]. Similar to *Alkl*^{+/-} brain, the dysplasia is found primarily in capillaries.

In this study, we tested the hypothesis that deletion of *Itgb8* in the brain of *Alkl*^{+/-} mice enhances bAVM development. We found that focal deletion of *Itgb8* in the adult *Alkl*^{+/-} brain enhanced the formation of dysplastic vessels and hemorrhage.

Methods

Animals and Experimental Groups

The experimental protocols involving animal usage were approved by the Institutional Animal Care and Use Committee (IACUC) of the University of California San Francisco (UCSF) and conformed to National Institutes of Health (NIH) guidelines. The staff of the Animal Core Facility and the IACUC of UCSF provided animal husbandry under the guidance of supervisors who are certified animal technologists, and IACUC faculty members and veterinary residents located on the San Francisco General Hospital campus provided veterinary care.

Adult male and female mice (8 to 10 weeks old) that have one allele of the *Alkl* gene deleted (*Alkl*^{+/-}) and have the exon 4 of *Itgb8* gene flanked by loxP sides (*Itgb8*-floxed) [13] were used (Table 1). Mouse genotypes were determined through PCR genomic DNA isolated from tails using the following primer sets. For *Alkl*^{+/-}: 5'-CCACTCATTCTCCTGGGTA-3' (wild-type allele), 5'-ACTTCCTGACTAGGGGAGGAGTAG-3' (common), and 5'-GTGTTTCATGGTAGTGGGTGAT-3' (knockout allele). For *Itgb8*-floxed locus: 5'-GTGGTTAAGAGC ACCGATTG-3' and 5'-CACTTTAGTATGCTAATGATGG-3'.

The following vectors were injected into the brain to induce vascular dysplasia: (1) adenoviral vector expressing Cre recombinase (Ad-Cre), adenoviral vector with CMV promoter

driving Cre recombinase expression, to mediate deletion of floxed sequence in *Itgb8*; (2) Ad-GFP, adenoviral vector with green fluorescent protein (GFP) gene, as vector control for Ad-Cre; (3) adeno-associated viral vector (AAV)-VEGF, adeno-associated viral vector expressing vascular endothelial growth factor (VEGF), to induce brain angiogenesis; and (4) AAV-LacZ, adeno-associated viral vector expressing β -galactosidase (LacZ), as vector control for AAV-VEGF. Brain focal angiogenesis was induced because our previous study showed that cerebral vascular dysplasia occurs more robustly in the *Alkl*^{+/-} brain after angiogenic stimulation [12]. We chose an adenoviral vector to deliver the Cre gene since protein production following transduction peaks earlier than with AAV [17]. Therefore, we co-injected Ad-Cre with AAV-VEGF to favor Cre-mediated *Itgb8* deletion before the peak of VEGF stimulation [18]. AAV-VEGF and AAV-LacZ were prepared as described previously [18, 19]. Ad-Cre and Ad-GFP were purchased from Vector Biolabs (Philadelphia, PA).

Mice were randomly assigned to the following groups (Table 2) and were injected with: (A) Ad-Cre + AAV-VEGF, (B) Ad-GFP + AAV-VEGF, (C) Ad-Cre + AAV-LacZ, and (D) Ad-GFP + AAV-LacZ.

Stereotactic Injection of Viral Vectors

After being anesthetized with 2 % isoflurane inhalation, the mice were placed in a stereotactic frame with a holder (David Kopf Instruments, Tujunga, CA), and a burr hole was drilled in the pericranium 2 mm lateral to the sagittal suture and 1 mm posterior to the coronal suture. Three-microliter viral suspension containing 2×10^7 plaque forming unit (PFU) of adenoviral vectors and 2×10^9 genome copies (gcs) of AAV viral vectors were stereotactically injected into the right basal ganglia at a rate of 0.2 μ l/min; the needle was withdrawn after 10 min and the wound was closed with a suture [20].

Cerebrovascular Casting

Using casting technique, we assessed whether dysplastic vessels in mice resemble the AVM nidus in humans. We explored the cerebrovasculature with Microfil cast (Flow Tech, Inc. Carver, MA) 8 weeks after vector injection, as previously described [21]. Briefly, mice were anesthetized using isoflurane inhalation, perfused with 37 °C PBS plus heparin (5 units/ml) through the left ventricle of the heart to wash out blood from the circulation using a Masterflex Pump Controller (Cole Parmer Instrument, Chicago, IL) at 4 ml/min, followed by perfusion with Microfil. This flow rate generated a pressure approximate to murine physiological blood pressure [22]. Brain samples were collected and fixed overnight in 4 % paraformaldehyde (PFA) at 4 °C, then clarified using an alcohol-methyl salicylate clearing sequence according to the manufacturer's instructions. Vasculature was

Table 1 Sample size and mice gender

	Viral vector-injected		Male (<i>n</i> = 6)	Female (<i>n</i> = 12)	Total (<i>n</i> = 17)
	Ad-Cre	AAV-VEGF			
Group A	+	+	1 (20.0)	4 (80.0)	5 (100)
Group B	–	+	1 (25.0)	3 (75.0)	4 (100)
Group C	+	–	3 (50.0)	3 (50.0)	6 (100)
Group D	–	–	1 (33.3)	2 (66.7)	3 (100)

Table entries are No. (%)

imaged under a dissecting microscope (Leica MZFL III microscope, Leica Microsystems, Bannockburn, IL).

Quantitative Assessment of Vessel Morphology

The Microfil cast brain samples were embedded in paraffin. Coronal sections (7- μ m thick) were cut using a microtome (Leica, RM2155, Germany). Two brain coronal sections from each mouse were chosen for vessel quantification, 0.5 mm anterior and 0.5 mm posterior to the needle track. Sections were incubated overnight with fluorescein *Lycopersicon esculentum* lectin (Vector Laboratories Burlingame, CA), 2 g/ml at 4 °C, and then coverslipped with Vectashield mounting medium with 4'-6-diamidino-2-phenylindole (Vector Laboratories, Burlingame, CA) to label cell nuclei. Three areas (to the right and left of and below the injection site) within the angiogenic region of each section were imaged under the $\times 20$ microscopic objective lens for quantification. Vessel density was quantified using NIH Image 1.63 software and reported as mean vessel counts per mm². The dysplasia index (number of vessels larger than 15 μ m in diameter per mm²) was calculated as previously described [12, 23–25]. All quantification was performed by three blinded investigators.

Prussian Blue Staining

Two sections per brain, selected as described earlier, were used for detecting iron deposition with an Iron Stain Kit

(Sigma-Aldrich, St. Louis, MO). Slides were incubated in a freshly prepared working iron stain solution for 15 min, washed in distilled water, and then counterstained with pararosaniline solution for 3 min. The positive stain area was quantified using NIH Image 1.63 software. Data are presented as total Prussian blue-positive pixel numbers (pixels) in the entire area of the hemisphere (mm²).

Statistical Analyses

Data are presented as mean $\pm$ standard deviation (SD). One-way analysis of variance was used to determine statistical significance among multiple groups, followed by pairwise multiple comparisons using the post hoc Tukey test. We measured the linear relationship among vascular density, vascular dysplasia and Prussian blue-positive area using Pearson correlation coefficient. The linear relationship was determined using a simple linear regression analysis, which generates the corresponding R^2 estimate. A *P* value of <0.05 was considered statistically significant. Sample sizes are shown in Table 1.

Results

Itgb8 Deletion Enhances Vascular Dysplasia in the Angiogenic Foci of Adult *Alk1*^{+/-} Brain

Deletion of *Itgb8* plus angiogenic stimulation in the *Alk1*^{+/-} brain did not induce a similar level of cerebrovascular dysplasia as we have observed in the angiogenic-stimulated *Alk1* homozygous deleted brain [23] (Fig. 1). Injection of AAV-VEGF induced a similar level of angiogenesis in the *Alk1*^{+/-} brain with or without *Itgb8* deletion (Fig. 2a, b). However, deletion of *Itgb8* increased the number of dysplastic capillaries in the brain angiogenic foci (7.14 $\pm$ 0.68/mm²), compared with *Alk1*^{+/-} mice (4.75 $\pm$ 1.38/mm², *P* = 0.003) (Fig. 2a, b). Compared with AAV-VEGF-injected *Alk1*^{+/-} brain with *Itgb8* deletion, there were few dysplasia vessels in the AAV-LacZ-injected *Alk1*^{+/-} brain (1.83 $\pm$ 0.23/mm²) and *Itgb8*-deleted *Alk1*^{+/-} brain (2.52 $\pm$ 0.43/mm², *P* < 0.0001) (Fig. 2a, b).

Table 2 Experimental groups

	Viral vector-injected		<i>Itgb8</i> gene deletion	Angiogenesis
	Ad-Cre	AAV-VEGF		
Group A	+	+	Yes	Yes
Group B	–	+	No	Yes
Group C	+	–	Yes	No
Group D	–	–	No	No

Mice in all groups are *Alk1*^{+/-} (loss of a single allele of *Alk1*)

Ad-Cre adenoviral vector expressing Cre recombinase gene, *AAV-VEGF* adeno-associated viral vectors expressing vascular endothelial growth factor

Fig. 1 Representative images of Microfil cast brain. **a** *Itgb8*-deleted *Alkl*^{+/-} brain with AAV-VEGF injection. **b** *Alkl*^{+/-} brain with AAV-VEGF injection. **c** *Itgb8*-deleted *Alkl*^{+/-} brain with AAV-LacZ injection. **d** *Alkl*^{+/-} brain with AAV-LacZ injection. No tangled abnormal vessel was detected. Increased vascular density (white arrow) was observed around the AAV-VEGF injected sites (**a**, **b**) *Itgb8*-deleted *Alkl*^{+/-} brain showed significant hemorrhage (red arrow) around the AAV-VEGF injection site. Scale bar = 500 μ m

Itgb8 Deletion Increases Hemorrhage in the Brain Angiogenic Foci of *Alkl*^{+/-} Mice

Alkl^{+/-} mice had microhemorrhage in the brain angiogenic foci. Deletion of *Itgb8* enhanced the hemorrhage (Fig. 1). No hemorrhage was observed in AAV-LacZ-injected brain (Fig. 1). Prussian blue-positive areas were ≈ 4 -fold larger in the angiogenic region of *Itgb8*-deleted *Alkl*^{+/-} brain (1278 ± 373 pixels/mm²) than in the *Alkl*^{+/-} brain (320 ± 104 pixels/mm²; $P = 0.028$) (Fig. 3a, b). The Prussian blue-positive area positively

correlated with vascular density ($R^2 = 0.56$, $P = 0.0006$, Fig. 4a) and vascular dysplasia ($R^2 = 0.53$, $P = 0.001$, Fig. 4b). No Prussian blue-positive area was detected in the brain injected with AAV-LacZ (Fig. 3a, b).

Discussion

Using mice with focal *Itgb8* deletion combined with haploinsufficiency of *Alkl*, we demonstrated a role for *Itgb8* in vascular dysplasia in the angiogenic foci. The vascular abnormalities were in the capillaries, as we have observed in the *Alkl*^{+/-} brain [12]. However, deletion of *Itgb8* in the *Alkl*^{+/-} brain caused a significant increase of microhemorrhage in the angiogenic foci (Fig. 5a). The degree of microhemorrhage positively correlated with vessel density and the number of abnormal vessels.

It has been observed that HHT patients with the same mutated gene have different phenotypes. For example, brain [26, 27] and pulmonary [26–29] AVMs are more common among patients with *ENG* mutation, while AVM prevalence and hemorrhage risk in the same organ vary among these patients [30]. The variability of phenotypes cannot be explained by global heterozygous gene deletion. Haploinsufficiency of the HHT causative gene is not sufficient to cause spontaneous lesion development in mice [31]. Inactivation of the remaining WT 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 [32]. For example, the loss of a single allele of one of the HHT causative genes, such as *Eng* or *Alkl*, reproduces

Fig. 2 Deletion of *Itgb8* increases vascular dysplasia in the angiogenic foci of *Alkl*^{+/-} brain. **a** Representative lectin-stained sections. Vessels are labeled by green fluorescence. White arrows indicate dysplastic vessels. The insets show enlarged images of dysplastic vessels. Scale bar = 50 μ m. **b** Quantification of vessel density. **c** Quantification of

dysplasia index. Error bars indicate SD. VEGF AAV-VEGF treatment. Cre Ad-Cre treatment. * $P < 0.0001$ versus brain injected with AAV-LacZ and Ad-Cre; § $P = 0.003$ versus brain injected with AAV-VEGF and Ad-GFP; # $P < 0.05$ versus brain injected with AAV-LacZ and Ad-GFP

Fig. 3 Deletion of *Itgb8* increases hemorrhage in the angiogenic foci in *Alk1*^{+/-} brain. **a** Representative images of Prussian blue-stained sections. The iron depositions are stained blue. Scale bar = 100 μ m. **b** Quantification of Prussian blue-positive area. VEGF AAV-VEGF

treatment. Cre Ad-Cre treatment. * $P = 0.008$ and ** $P = 0.003$ versus brain injected with AAV-LacZ and Ad-Cre or Ad-GFP; # $P = 0.028$ versus brain injected with AAV-VEGF and Ad-GFP

certain aspects of the human disease in animal models, primarily in older animals [10, 33]. In contrast, the loss of both alleles of any HHT causative gene is embryonically lethal in mice [34, 35], and conditional (tissue/time-specific) homozygous deletion of *Eng* [32] or *Alk1* [36, 37] results in striking vascular malformations resembling the AVMs found in HHT. This has also been demonstrated in our previous studies in modeling bAVM in mice [23, 38–40]. AVM development in an HHT gene-deficient mouse model is influenced by mouse strains, which have various levels of plasma TGF- β 1 [41]. Therefore, modifier genes involved in the regulation of TGF- β 1 expression may act in combination with a single functional copy of *Eng* or *Alk1*. Our data suggest that *Itgb8* could be one of the modifiers that influence the AVM progression.

ITGB8 is expressed in the perivascular cell process surrounding cerebral blood vessels, serving as a major pathway for TGF- β activation and signaling in vivo [16, 42, 43]. Perivascular astrocytes interact with vascular endothelial cells to regulate brain vessel differentiation and stabilization through integrin β 8-mediated activation of TGF- β 1 [16]. *Itgb8* deletion

reduces activated TGF- β 1 in both the developmental and adult brain [13, 42]. Excessive vascular sprouting and vascular dysplasia are obvious in the embryonic brain with *Itgb8* deletion [42]. Increased cerebral blood vessel densities that appear to be the remnants of abnormal vessels occurring during development are also observed in *Itgb8* homozygous knockout mice that survive to adulthood [43]. In the adult mouse brain, conditional deletion of *Itgb8* does not increase vascular density or cause vascular dysplasia [44]; however, triggering angiogenesis in the *Itgb8*-deleted adult brain induces vascular dysplasia [13]. Therefore, integrins containing the β 8 subunit appear to be important in regulating vascular development.

We showed previously that VEGF stimulation induced vascular dysplasia in the capillaries in the brain with focal *Itgb8* deletion and in the brain of *Alk1*^{+/-} mice (Fig. 5a) [12, 13]. To test if mutation of both *Itgb8* and *Alk1* could enhance bAVM formation, we crossbred *Itgb8*-floxed mice with *Alk1*^{+/-} mice. Although deletion of *Itgb8* increased the number of abnormal vessels in the angiogenic foci of the *Alk1*^{+/-} brain, the abnormality was still at capillary level, and no macroscopic level of

Fig. 4 Correlation between Prussian blue-positive area and vascular features. **a** Positive correlation of Prussian blue-positive area with vessel density ($R^2 = 0.56$, $P < 0.001$). **b** Positive correlation of Prussian blue-positive area with vascular dysplasia ($R^2 = 0.53$, $P = 0.001$)

a

Genotype	<i>Alk1</i> 2f/2f	<i>Alk1</i> +/-	<i>Alk1</i> +/-; <i>Itgb8</i> 2f/2f	<i>Itgb8</i> 2f/2f
Gene knock-out	Cre		Cre	Cre

	Homozygous <i>Alk1</i> KO	Heterozygous <i>Alk1</i> KO	Heterozygous <i>Alk1</i> Homozygous <i>Itgb8</i> KO	Homozygous <i>Itgb8</i> KO
Microscopic Vascular Dysplasia	Yes	Yes	Yes	Yes, glomeruloid dysplasia
Macroscopic Tangled Vessels with A-V shunt	Yes	No	No	No
Hemorrhage	Yes	No	Yes	No

Fig. 5 Summary of vascular phenotypes in the *Alkl*-deficient brain and depiction of underlying mechanisms. **a** Cerebrovascular phenotype of *Alkl* and/or *Itgb8* knockout (KO) mice in the brain angiogenic foci. Homozygous *Alkl* KO in the brain causes microscopic vascular dysplasia and macroscopic tangled vessels with arteriovenous (A-V) shunt [23]; heterozygous *Alkl* KO or homozygous *Itgb8* KO results in microscopic level of vascular dysplasia only. Heterozygous *Alkl* KO plus *Itgb8* homozygous deletion cause microscopic vascular dysplasia and hemorrhage. *Alkl*^{2f/2f}: exons 4–6 of both alleles of *Alkl* gene are flanked by loxP sides and are deleted when Cre is expressed [23, 47]; *Alkl*^{+/-}: one allele of *Alkl* is deleted [12]; *Itgb8*^{2f/2f}: exon 4 of both alleles of *Itgb8* gene is flanked by loxP sides and is deleted when Cre is expressed [13]. **b** Depiction of the mechanisms of *Alkl* and *Itgb8* deletion in the vascular malformation and hemorrhage. Normal cerebrovasculature is covered with mural cells (including pericytes and smooth muscle cells, *top panel*). *Alkl* deletion in endothelial cells (reduced color intensity, *bottom panel*) reduces vascular mural cell coverage [46], and *Itgb8* deletion in astrocytes (*white color*) results in abnormal sprouting [42]. Therefore, deletion of *Itgb8* in the *Alkl*^{+/-} brain enhances vascular dysplasia and hemorrhage

dysplasia was formed (Fig. 5a). These data suggest that *Itgb8* is not a key component for bAVM development.

Brain vessels in the *Itgb8*-deficient embryo show defective anastomotic connections and increased endothelial cell proliferation resulting in “glomeruloid” vascular malformations, which are associated with extensive cerebral hemorrhage [45]. The primary ultrastructural defect in the *Itgb8*-null developmental mouse brain appears to be abnormal astrocyte end-feet; the perivascular cells fail to associate with endothelial cells [45]. However, selective ablation of *Itgb8* in endothelial cells or neurons does not cause cerebral hemorrhage [44]. Deletion of *Itgb8* in astrocytes causes cerebral hemorrhage in the embryonic brain, although the phenotype is less severe than in mice with *Itgb8*-deleted ubiquitously. Cerebral hemorrhage in the embryonic brain with targeted deletion of *Itgb8* in neuroepithelium correlates with decreased active TGF- β [42]. However, deletion of *Itgb8* in the adult brain does not cause significant vascular dysplasia and cerebral hemorrhage [44]. In homozygous *Itgb8*-null mice with outbred background that survive to adulthood, cerebral hemorrhage is obvious in newborn mice but surprisingly absent in the adults [43]. These data indicate that the expression of *Itgb8* in neuroepithelial cells and correlated TGF- β activation are essential for maintaining normal cerebrovascular structure in the setting of active angiogenesis, thereby preventing cerebral hemorrhage.

We have shown in this study that *Itgb8* deletion induces severe hemorrhage in the angiogenic foci of *Alkl*^{+/-} adult mouse brain. The degree of hemorrhage as quantified by Prussian blue staining positively correlates with both vascular density and vascular dysplasia. Therefore, integrins containing the $\beta 8$ subunit appear to be important for maintaining cerebrovascular stability and preventing hemorrhage in the angiogenic foci of *Alkl*^{+/-} mice.

Our previous study indicated that ALK1 could play a role in maintaining the integrity of vessels in the adult brain during

angiogenesis [46]. Cerebral hemorrhage in *Alkl* homozygous mutant mice has been found to be associated with impaired vascular integrity and blood-brain barrier (BBB) dysfunction. In the angiogenic foci of *Alkl*-null brain, the dysplastic vessels have fewer pericytes and smooth muscle cells than normal cerebrovasculature (Fig. 5b), positively correlating with the degree of BBB leakage [46]. However, there is no evidence of BBB leakage in the *Itgb8*-deficient embryo before cerebral hemorrhage [42]. The numbers of perivascular smooth muscle cells and pericytes are normal in the brain of *Itgb8*-null embryo [44]. A strong spatial-temporal association of sprouting abnormal angiogenesis and cerebral hemorrhage in *Itgb8*-deficient embryo indicates that abnormal angiogenesis, not BBB leakage, causes hemorrhage in the *Itgb8*-deficient brain [42]. Therefore, the brain hemorrhage we have observed in the angiogenic foci of *Itgb8*-deleted and *Alkl*^{+/-} brain might be due to both abnormal angiogenic sprouting and loss of vascular mural cells (Fig. 5b). Future studies should investigate the interaction of ITGB8 and ALK1 in maintaining vascular integrity.

In summary, our data suggest that both *Itgb8* and *Alkl* play important roles in maintaining normal vascular structure during brain angiogenesis in adult mice, and that *Itgb8* deficiency could enhance vascular dysplasia and hemorrhage in brain vascular malformations in *Alkl*^{+/-} mice, which, in turn, might be associated with bAVM hemorrhage.

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Compliance with Ethical Standards

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Conflict of Interest The authors declare that they have no conflict of interest.

Ethical Approval All procedures performed in studies involving animals were approved by and were in accordance with the ethical standards of the Institutional Animal Care and Use Committee of the University of California San Francisco.

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