ONLINE SUPPLEMENT

De novo cerebrovascular malformation in the adult mouse after endothelial *Alk1* deletion and angiogenic stimulation

Supplemental Methods

Animal Breeding and Modeling

For induction of endothelial *Alk1* deletion, we crossbred *Alk1*$^{2f/2f}$ (exons 4-6 flanked by loxP sites) mice[1] with mice expressing a tamoxifen (TM)-inducible Cre recombinase (iCreER) driven by *Pdgfb* promoter (*Pdgfb*-iCreER).[2] *Pdgfb* is predominately expressed in endothelial cells; therefore, tamoxifen (TM) treatment will induce endothelial-*Alk1* deletion in *Pdgfb*-iCreER;*Alk1*$^{2f/2f}$ mice.

Eight-week-old *Pdgfb*-iCreER;*Alk1*$^{2f/2f}$ mice were anesthetized with isoflurane inhalation and placed in a stereotactic frame with a holder (David Kopf Instruments, Tujunga, CA). A burr hole was drilled in the pericranium 2mm lateral to the sagittal suture and 1mm posterior to the coronal suture. Adeno-associated viral vector (AAV)-expressing **CMV promoter driving** VEGF (AAV-VEGF)[3] was packaged in AAV serotype 1 capside and was stereotactically injected into the basal ganglia of the brain (2X10^9 genome copies). AAV-LacZ was used as control for AAV-VEGF. Two weeks later, a single dose of TM (2.5 mg/25 g body weight) was injected intra-peritoneally (i. p.) and brain samples were collected 10 days later.

For pericyte *Alk1* deletion, *Alk1*$^{2f/2f}$ mice were crossbred with mice expressing iCreER under the transcriptional control of the *NG2* promoter (*NG2*-iCreER).[4] NG2 is predominately expressed by pericytes, and thus, TM administration will induce pericyte-*Alk1* deletion in *NG2*-iCreER;*Alk1*$^{2f/2f}$ mice. TM (4 mg/25g body weight, i.p.) was injected for three consecutive days into 8-week-old *NG2*-iCreER;*Alk1*$^{2f/2f}$ mice. AAV-VEGF (2X10^9 genome copies) was stereotactically injected into the basal ganglia of the brain eight weeks after the TM treatment. Samples were collected eight weeks later.

ROSA26tm14 (CAG-tdTomato) (Ai14) reporter mice that have a loxP flanking stopper before Ai14 coding sequence were used to monitor cre activity.

Latex Perfusion

Mice were deep-anesthetized with isoflurane inhalation. The abdominal and thoracic cavities were opened. Both left and right atria were cut off. Blue latex dye (1 ml, Connecticut Valley Biological Supply Co.) was injected slowly into the left cardiac ventricle using a 25-gauge 5 ml syringe. The brain, ear and intestine were harvested and fixed with 4% paraformaldehyde overnight. The brain and ear were dehydrated with methanol series and clarified with benzyl alcohol/benzyl benzoate (1:1 ratio). After clarification, the brain was cut coronally and images were taken.

Immunohistochemistry

Ten days after TM injection, brain samples were collected, sectioned and stained as previously described.[5]

Briefly, brain tissue was frozen in dry ice and cut into 20-μm-thick coronal sections using a Leica CM1950 Cryostat (Leica Microsystems, Wetzlar, Germany). Sections were incubated at

4°C overnight with the following primary antibodies: anti-CD31 (1:100, Santa Cruz Biotechnology, CA), anti-ki67 (1:100, abcam, MA), anti-CD68 (1:100, abcam, MA), anti-αSMA (1:1000, Sigma-Aldrich, MO). Alexa Fluor 594-conjugated (1:500), Alexa Fluor 488-conjugated IgG (1:500), and Cy5-conjugated goat anti-rat (1:500, Invitrogen, Carlsbad, CA) were used as secondary antibodies.

BrdU Labeling and Quantification

5-Bromodeoxyuridine (BrdU; Sigma) was injected i.p. daily (100 mg/kg) for 10 days following TM administration. Two sections, 0.5mm apart, per brain within the injection site were chosen for double staining with anti-ERG and anti-BrdU antibodies. Sections were fixed with 4% paraformaldehyde for 5 min, and incubated with anti-ERG (1:100, abcam, MA). Alexa Fluor 488-conjugated IgG (1:500) was used as secondary antibody. After completion of anti-ERG staining, the sections were then treated with 2 mol/L HCl at 37°C for 30 minutes and rinsed in 0.1 mol/L boric acid (pH 8.5) at room temperature for 10 minutes. Sections were incubated overnight with anti-BrdU (1:500, Sigma, MO) primary antibody at 4°C. Fluor 594-conjugated (1:500) was used as secondary antibody. Three images were taken from each section (right and left of, and below the injection site) under a 40X objective (Leica MZFL III microscope, Leica Microsystem, Bannockburn, IL). Both ERG^+ and $BrdU^+$ cells were blind-counted.

Prussian Blue Staining

Accustain Iron Stain Kit (Sigma-Aldrich, MO) was used to detect iron deposition. Slides were incubated in freshly prepared working iron stain solution for 15 minutes, washed in distilled water, and counterstained with pararosaniline solution for 3 minutes.

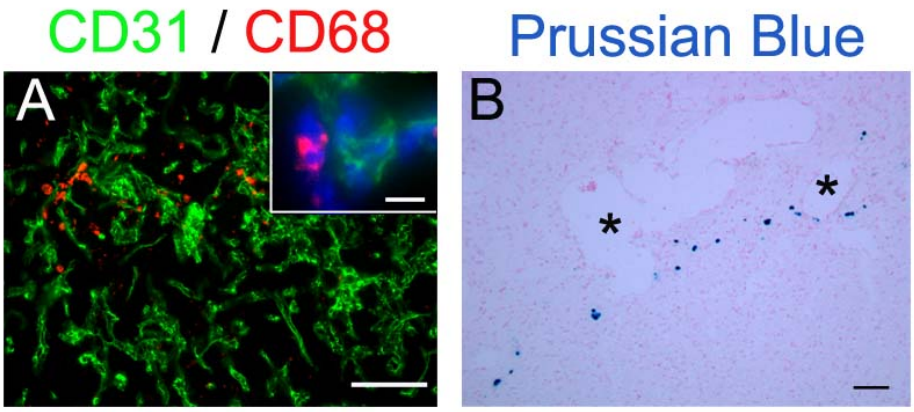
Vessel Density and Dysplasia Index Quantification

Dysplasia Index was quantified as previously described.[6] Briefly, two sections, 0.5 mm apart, per brain within the injection site were stained for vessels using anti-CD31 antibody. Three images were taken from each section (right and left of, and below the injection site) under a 20X objective. Total vessel area and the number of vessels with a diameter larger than 15 μm in each picture were counted using NIH Image 1.63 software. The vessel density was expressed as the number of pixels pre mm^2. The Dysplasia Index was calculated as the number of vessels with a diameter > 15 μm per 200 vessels examined.

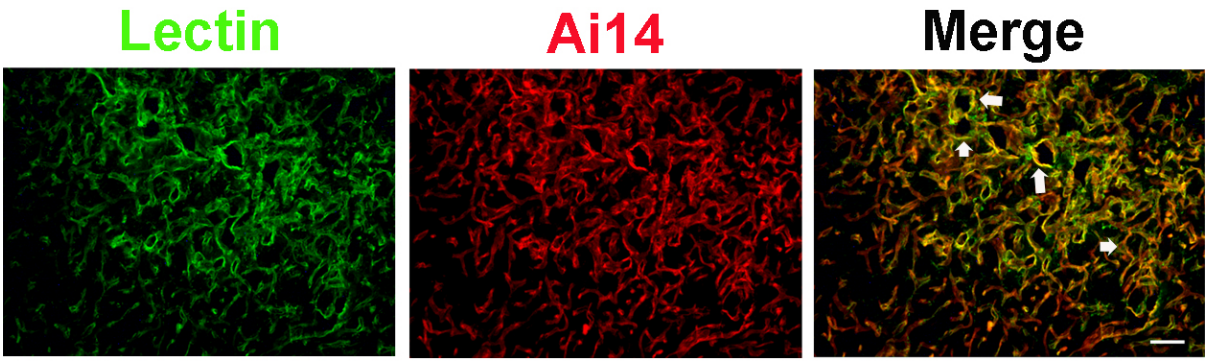
Statistical Analysis

Data are presented as mean ± standard deviation (SD). The data were analyzed using one-way ANOVA to compare the means of each group. A p value < 0.05 was considered statistically significant. Sample sizes were n=6 for each group.

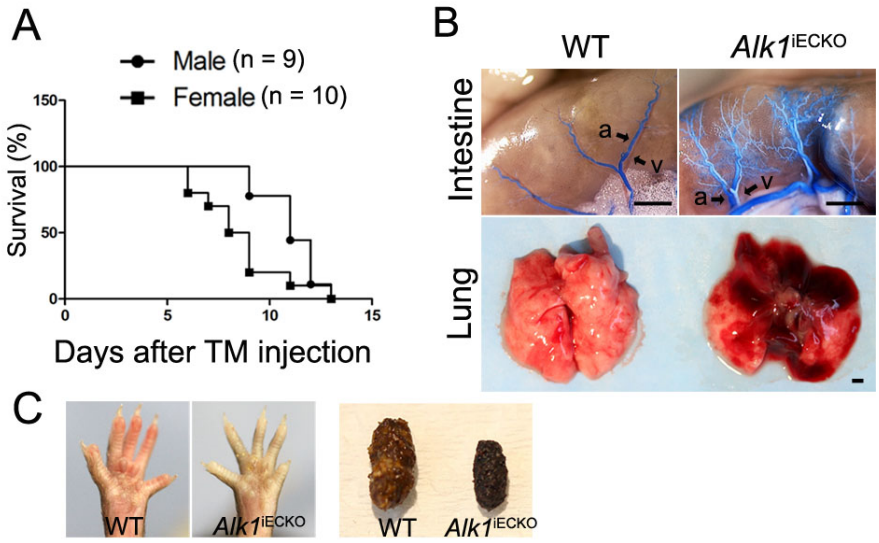
Supplemental Figures



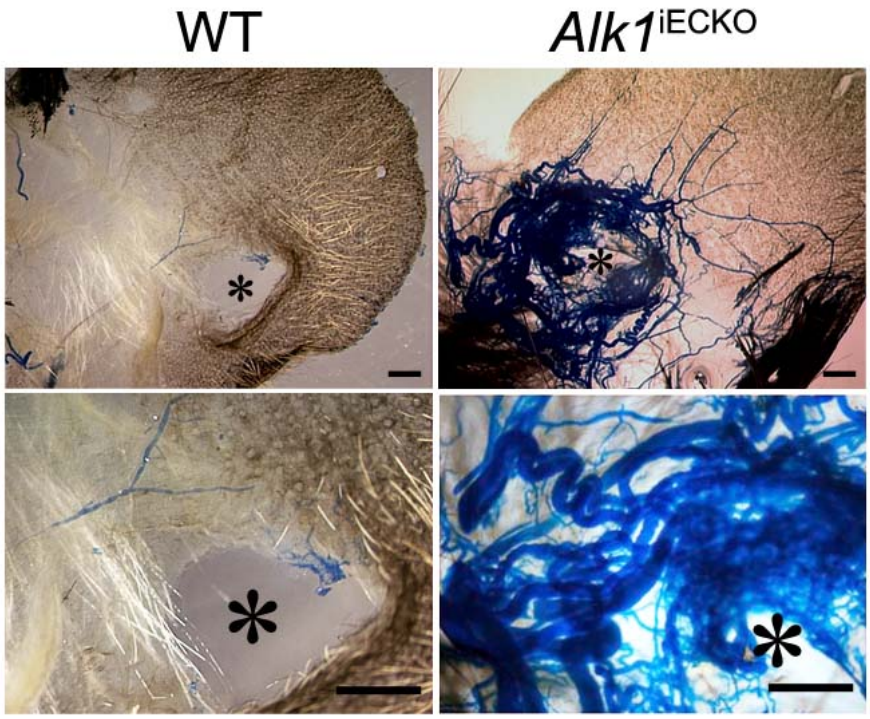
Supplementary Figure I. Macrophage infiltration and microhemorrhage. (A) Microphage infiltration in bAVM lesion. Microphages were detected by immunostaining using an anti-CD68 specific antibody (red). Blood vessels were visualized by immunostaining using an anti-CD31-specific antibody (Green) (B) Prussian blue staining. Iron deposition caused by microhemorrhage was detected around the dysplastic vessels (indicated by *). Scale bar: 100 µm. Insert scale bar: 10 µm.



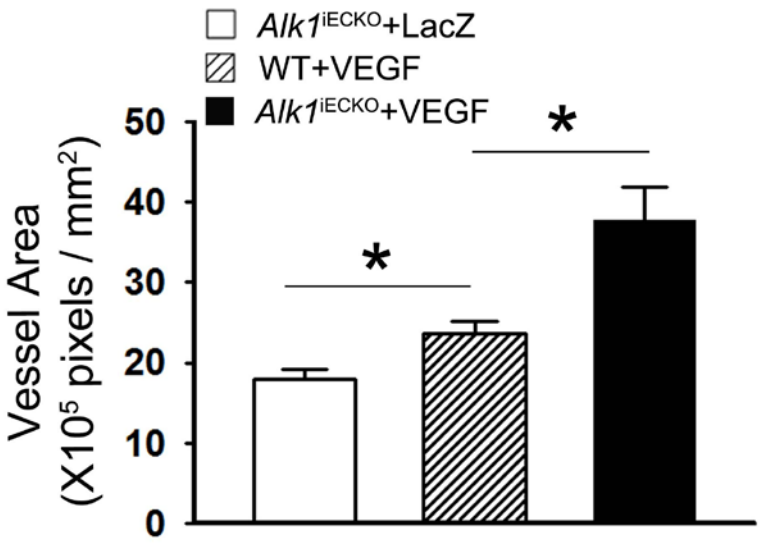
Supplementary Figure II. Ai14 reporter (red) in the endothelial cells (green) at the angiogenic foci of the brain. AAV-VEGF was injected into *Pdgfb*-iCreER;Ai14$^{+/-}$;*Alk1*$^{2f/2f}$ brain. Cerebrovascular endothelial cells were stained with fluorescent labeled Lectin (green). Arrows indicate dilated dysplasia vessels. Scale bar: 100 µm.



Supplementary Figure III. Endothelial-*Alk1* deletion (both *Alk1*^{iECKO}+VEGF or *Alk1*^{iECKO}+LacZ) in adult mice results in lethality, with AVMs in the intestine and hemorrhages in the intestine and lung. (A) Survival curve of adult mice following TM administration. There is a significant difference between male and female mice. *p = 0.011. (B) Representative images show AVMs in the intestine and hemorrhage in the lung. Latex is present in the vein. Due to the particle size, intra-left cardiac ventricle-perfused latex will present in the vein only when there is an arteriovenous shunt. AVM phenotype was detected only in the brain of *Alk1*^{iECKO}+VEGF mice (Figure 2). a: artery. v: vein. Scale bar: 1 mm. (C) Both *Alk1*^{iECKO}+VEGF or *Alk1*^{iECKO}+LacZ mice displayed pale paws and darkened feces, indicating internal bleeding and anemia.

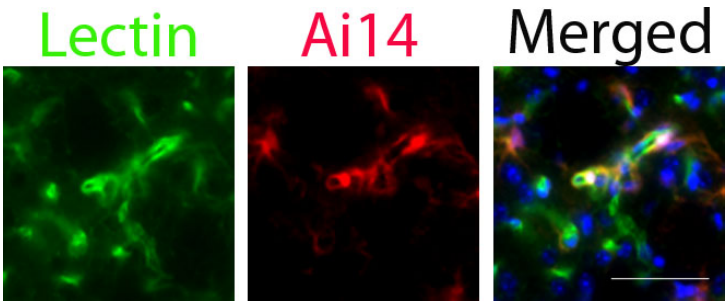


Supplementary Figure IV. Wounding induced vascular malformation in the ear of *Alk1*^{iECKO}+VEGF and *Alk1*^{iECKO}+LacZ mice. Latex blue dye was perfused from the left cardiac ventricle. The image was taken after the ear was dehydrated and clarified. Wound is indicated by *. Scale bar: 1 mm.



Supplementary Figure V. Quantification of vessel density. Brain sections were stained with anti-CD31 antibody. The area covered by the vessel (Vessel Area) was quantified in the viral-injected region. *Alk1*^{iECKO}+VEGF mice had more Vessel Area than WT+VEGF mice (37.7±4.2 X10^5 vs. 23.7±1.42 X10^5 pixels/mm2, P<0.001. Data: mean±SD. *P<0.001.

A



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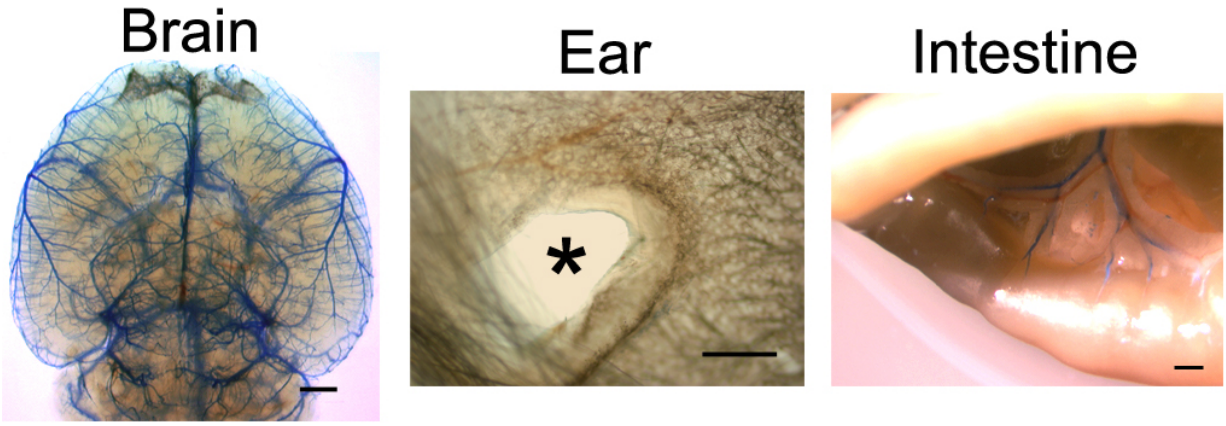

Supplementary Figure VI. Induction of *Alk1* deletion in pericytes of adult mice did not cause AVM development in internal organs. Vessels were labeled with Lectin (green). Scale bar: 50 μm. (A) Cre was activated in the brain pericytes (Ai14, red) of TM-treated NG2-iCreER;Ai14;*Alk1*$^{2f/2f}$ mice. (B) Latex casts. Latex blue dye was perfused from the left heart ventricle of NG2-iCreER;*Alk1*$^{2f/2f}$ mice 8 weeks after AAV-VEGF injection. Scale bar: 1 mm. Ear wound is indicated by *.

Supplemental References

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4. Armulik A, Genove G, Betsholtz C. Pericytes: developmental, physiological, and pathological perspectives, problems, and promises. *Dev Cell*. 2011;21:193-215.
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