**Bone marrow derived *Alk1* mutant endothelial cells and clonally expanded of somatic *Alk1* mutant endothelial cells contribute to the development of brain arteriovenous malformations in mice**

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## Supplementary Materials and Methods

### BM Transplantation

BM transplantation was performed as previously described [1, 2]. Briefly, BM cells were collected from the tibia and femurs of 8 to 10-week-old *Pdgfb*iCreER;*Alk1*$^{f/f}$;Ai14$^{+/-}$ mice or enhanced green fluorescent protein (EGFP) transgenic mice (the Jackson Laboratory, Bar harbor, ME) by flushing and aspiration with PBS containing 1% fetal bovine serum. Cells were centrifuged at 3000g for 10 minutes and resuspended in PBS at a concentration of 1X10$^7$ cells/ml. 200 µl of cell suspension (2X10$^6$ cells) were injected into lethally irradiated (9.7 Gy, GC3000 Irradiator, MDS-Nordion, Ontario, Canada) wild type (WT) C57BL recipient mice (the Jackson Laboratory) via the tail vein. To facilitate the determination of the reconstitution rate, we transplanted BM cells collected from EGFP transgenic mice to WT control mice. Peripheral blood was collected 4 weeks after BM transplantation. Flow cytometric analysis (LSR II, BD Biosciences, San Jose) was used to determine the nucleated GFP$^+$ donor cells in the recipients' blood.

**Stereotactic injection of AAV-VEGF into the brain**

To induce brain arteriovenous malformation (bAVM), an adeno-associated viral vector expressing vascular endothelial growth factor (AAV-VEGF) [3] was stereotactically injected into the basal ganglia at the beginning of model induction. After induction of anesthesia by 4% isoflurane inhalation, 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. Two μl viral suspension containing $2X10^9$ genome copies (gcs) of AAV-VEGF were stereotactically injected into the right basal ganglia, 3 mm underneath the brain surface, at a rate of 0.2 μl per minute using a Hamilton syringe. The needle was withdrawn after 10 minutes and the wound was closed with a 4-0 suture.

**Bromodeoxyuridine (BrdU) labeling**

To observe EC proliferation, we treated a group of AAV-VEGF injected *Pdgfb*iCreER;*Alk1*$^{2f/2f}$ mice with BrdU (100 mg/kg of body weight, Sigma-Aldrich, St Louis, MO) through i.p. injection on the 5th and 6th days after TM (0.01 mg/kg of body weight) treatment and collected their brains on the 7th days after TM treatment (Fig. 3A).

**Immunohistochemistry**

For detection of Ai14$^+$ BMDECs and EC-clonal expansion, mice were perfused with 4% paraformaldehyde (PFA) intracardially after being anesthetized with isoflurane inhalation. Brains were collected, incubated in 4% paraformaldehyde containing 20% sucrose for 2 days, and then frozen in dry ice, and sectioned into 20-μm-thick sections using a Leica CM1950 Cryostat (Leic Microsystems, Wetzlar, Germany).

For immunostaining, the brains were freshly frozen in dry ice and cut into 20-μm-thick coronal sections using a Leica CM1950 Cryostat (Leica Microsystems). Sections were incubated at 4°C overnight with the following primary antibodies: anti-CD31 (1:100, Santa Cruz, Biotechnology, CA or 1:500, R&D, Systems, Minneapolis, MN), anti-Alk1 (1:50, R&D, Systems, Minneapolis, MN), anti-Ki67 (1:50, eBioscience, San Diego, CA), anti-RFP (1:300 Takara Bio, Japan), anti-

Erg (1:100, Abcam, Burlingame, CA) or anti-BrdU (1:500, Invitrogen, CA, USA). Alexa Fluor 594-conjugated (1:200, Invitrogen, Carlsbad, CA), Alexa Fluor 488-conjugated (1:200, Invitrogen) or Alexa Fluor 647-conjugated (1:100, Invitrogen) antibody was used as secondary antibody to visualize positive signals. Vectashield antifade mounting medium containing 4′, 6-diamidino-2-phenylindole (DAPI, Vector Laboratories, Burlingame, CA) was used to stain the cell nuclei and mount the slides.

For quantification of $Ai14^+$ BMDECs in the brains of mice transplanted with *Pdgfb*icreER;*Alk1*$^{2f/2f}$;Ai14 BM, for CD31 (ECs) staining, an alkaline phosphatase labeled goat anti-rat antibody were used as the secondary antibody (1:400, abcam). Vector Red Substrated Kit (Vector Laboratories) was used to visualize the positive staining (red). For RFP (Ai14) staining, a biotinylated goat anti-rabbit antibody was used as secondary antibody (1:600. Vector Laboratories). Vectastine ABC-HRP Kit and ImmPact DAB stubstrate, Peroxidase (HRP, Vector Laboratories) were used to visualize the positive staining (brown).

**Quantification of Vessel Density, Dysplasia Index, $Ai14^+$, $BrdU^+$, $Ki67^+$, $Alk1^-$ and $Alk1^+$ cells**

For quantification of vascular density (number of vessels per $mm^2$), dysplasia index (the number of vessels larger than 15 μm per 200 vessels) [4] and $Ai14^+$ cells, a group of mice were injected with a fluorescein labeled lectin (lycopersicon esculentum lectin, Vector Laboratories) through jugular veins, after being anesthetized with isoflurane inhalation. Twenty minutes later, animals were perfused with PBS followed by 4% PFA intracardially. Brains were collected, incubated in 4% PFA containing 20% sucrose for 2 days, and then frozen in dry ice, and sectioned into 20-μm-thick sections using a Leica CM1950 Cryostat (Leica Microsystems). Two sections per brain approximately 100 μm nostril or caudal to the injection site were selected for the quantifications. Brain sections were examined and imaged using a Keyence fluorescence microscopy under a 20X objective lens (Model BZ-9000, Keyence Corporation of America, Itasca, IL). A total of six images were taken from each brain, and three from each brain section (to the right, to the left and below the injection site). The images were coded by a researcher who did not participate in the quantification. All quantifications were performed by at least two researchers who were blinded with the group assignment.

For quantification of $Alk1^-$, $Alk1^+$ ECs and proliferating ECs, two sections per brain approximately 100 μm nostril or caudal to the injection site were selected from the fresh frozen brain samples. $Alk1^-$ and $Alk1^+$ ECs were quantified on sections co-stained with anti-CD31, and anti-Alk1 antibodies. Proliferating ECs were quantified on sections co-stained with anti-Erg, anti-Alk1 and anti-Ki67 or anti-BrdU antibodies.

**Confocal Microscopy**

Confocal images were taken using a Zeiss 780 upright laser scanning confocal microscope (Carl Zeiss AG, Oberkochen, German) with a 34 detector array. Images were obtained with a 20X objective lens, with water immersion. Images were composed of a single 2 μm optical section and channels were overlaid in Image J 1.52.

**Latex Perfusion**

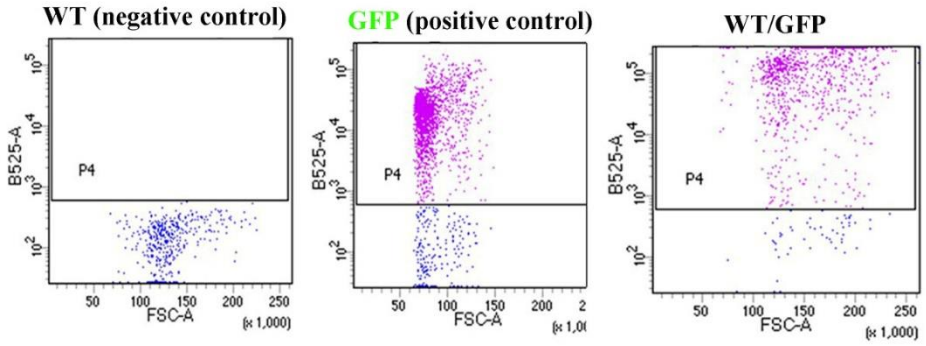
Mice were deeply anesthetized by isoflurane inhalation. The abdominal and thoracic cavities were opened. Both left and right atria were cut off. Blue latex dye (1 ml, Connecticut Valley Biological Supply Co. Burlington, NC) was injected into the left cardiac ventricle using a 21-gauge needle attached to a 5 ml syringe. The brains and intestines were harvested and fixed with 4% PFA overnight. The arteriovenous (AV) shunts in the intestines were examined and imaged directly. The brains were dehydrated with methanol series and clarified with benzyl alcohol/benzyl benzoate (1:1 ratio). After clarification, the brains were cut coronally and imaged.

**Western blot**

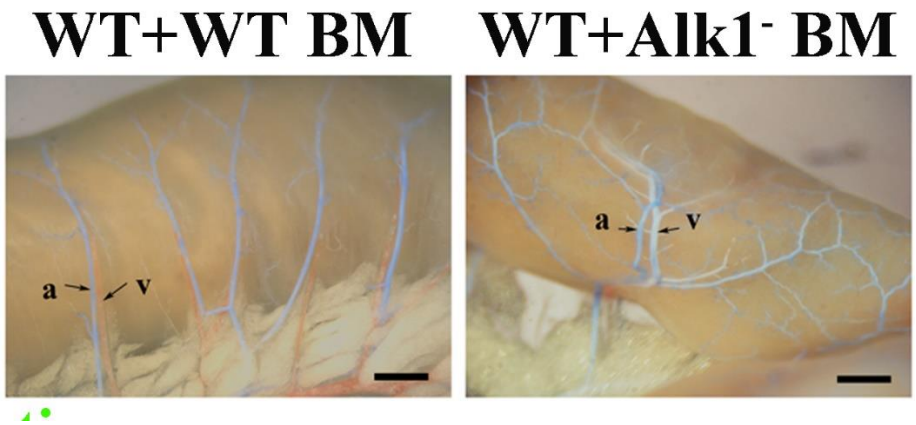
Brain tissues (1 $mm^3$) containing the vector-injection site were collected. Protein was extracted using a cell lysis buffer (Cell Signaling, Danvers, MA) supplemented with protease inhibitor cocktail (Sigma-Aldrich), 1mM PMSF (Cell Signaling) and quantified by the Bradford method (Bio-Rad, Hercules, CA). The protein concentration was determined using a microplate reader (Emax, Molecular Devices, Sunnyvale, CA). Protein samples 150 μg were loaded into 4–20% Tris-Glycine gels (Bio-Rad, Berkeley, CA) and transferred onto nitrocellulose membranes (Bio-Rad). Immunoblottings were performed using primary antibodies specific to Alk1 (1:1000,

Abcam, Cambridge, MA) and Gapdh (1:1000, Abcam, Cambridge, MA). A goat anti-rabbit IgG antibody or donkey anti-goat antibody (Li-Cor, Lincoln, NE) was used as the secondary antibody. Alk1 and Gapdh bands were detected by Li-Cor Quantitative western blot scanner and quantified using Li-Cor imaging software.

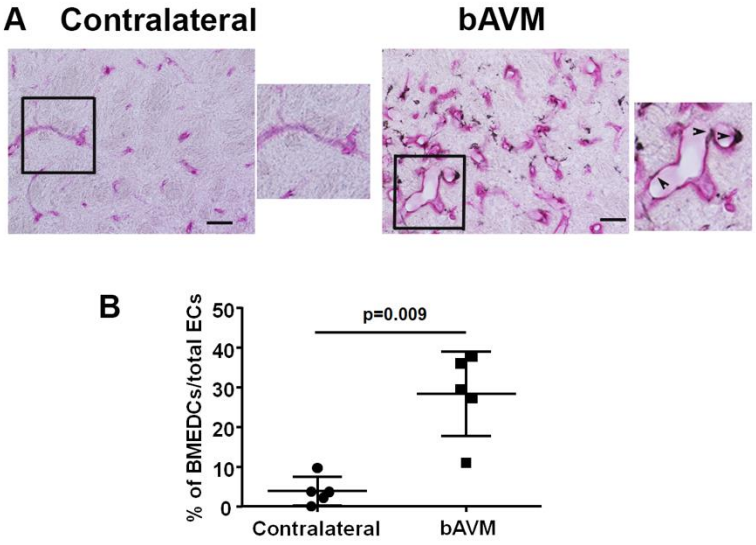
## Supplementary Figures.



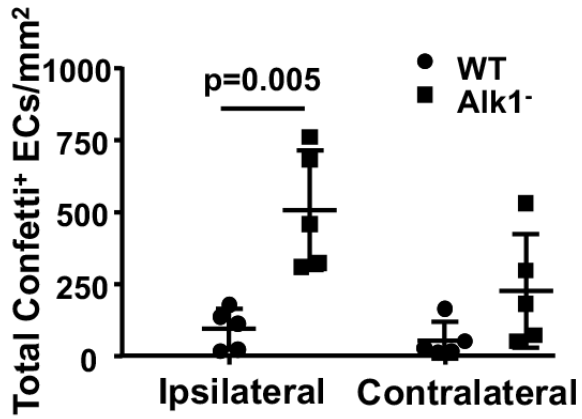

**Supplementary Fig. 1. Recipients' BM reconstituted by donor BM.** Representative images of FACS analysis of $GFP^+$ nucleated cells in the peripheral blood of WT mice transplanted with BM of EGFP transgenic mice (WT/GFP) 4 weeks after the BM transplantation. WT or EGFP transgenic mice (GFP) were used as the control.



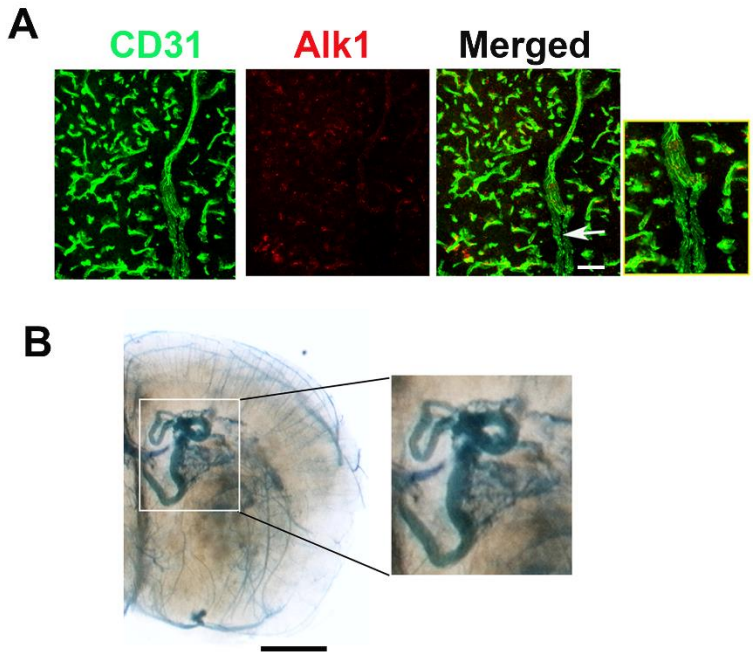
**Supplementary Fig. 2: Deletion of *Alk1* in BM derived ECs (BMDECs) caused AV shunts in the intestines.** Latex casted intestinal vessels. Left image shows normal mesenteric vessels. Mesenteric veins (red) go parallel to the mesenteric arteries (blue latex perfused) and no latex dye was detected in the veins. Right image shows latex in the mesenteric veins, which indicates the presence of AV shunts in the intestine. a, artery; v, vein. Scale bars: 1 mm.




**Supplementary Fig. 3: More BMDECs were present in bAVM lesion than contralateral brain. A.** Representative images of brain sections co-stained with anti-CD31 (red) and anti-RFP (Ai14 reporter, brown) antibodies. The enlarged images of the rectangle regions in the images on their left show details of normal capillaries (left) and AVM vessels (right). Arrowheads indicated $Ai14^{+}$ BMDECs. Scale bar: 50 μm. Contralateral: contralateral brain corresponding of bAVM lession . bAVM: bAVM lesion. **B. Quantification of BMDECs in total ECs. N=5.**

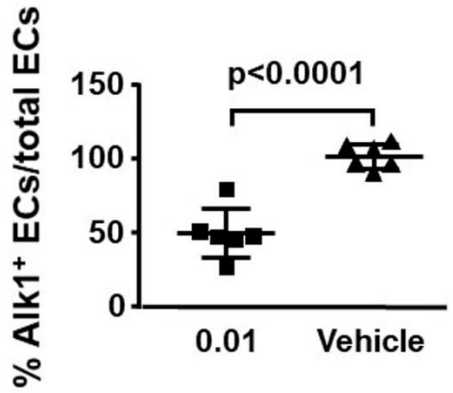



**Supplementary Fig. 4. Brains with Alk1$^-$ ECs have more confetti$^+$ ECs around AAV-VEGF injected sites (bAVM) than WT mice.** Alk1$^-$: brain with Alk1$^-$ ECs. WT: brain of TM treated *Pdfgb*iCre;confetti$^{+/-}$ mice in which confetti locus was activated without *Alk1* gene deletion. Ipsilateral: AAV-VEGF injection side. Contralateral: corresponding brain regions on the contralateral sides. N=5.

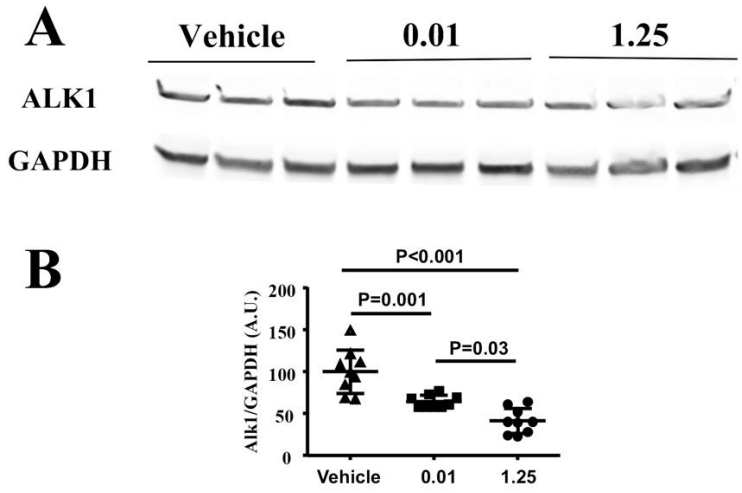



**Supplementary Fig. 5**: **1.25 mg/25g TM treatment mediated bAVM formation and *Alk1* deletion in nearly 100% ECs. A.** Representative images of brain sections co-stained with anti-CD31 (green) and anti-Alk1 (red) antibodies. Arrow indicates an abnormal vessel. The picture on the right shows an enlarged image of an abnormal vessel indicated by an arrow in the picture

on its left. Scale bars: 50 μm. **B.** Latex casted bAVM. Right: an enlarged image of AVM vessels. Scale bar: 500 μm.




**Supplementary Fig. 6. 0.01 mg/25g TM treatment created a mosaic of Alk1$^{+}$ and Alk1$^{-}$ ECs ECs in bAVM lesion.** 0.01: mice treated 0.01 mg/25g of body weight TM. Vehicle: mice treated with corn oil. N=6.




**Supplementary Fig. 7. Increase TM dose reduced Alk1 protein levels in the brain. A**. Representative western blot images. B. Quantification of Alk1 protein. Vehicle: corn oil treated mice; 0.01 or 1.25: mice treated with TM (0.01 mg/25g or 1.25mg/25g of body weight). N=9. A.U.: arbitrary unit.

**References:**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