SUPPLEMENTAL MATERIAL

Thalidomide Reduces Hemorrhage of Brain Arteriovenous Malformations in a Mouse Model

Supplementary Methods

Animals

Animal husbandry was provided by the staff of the IACUC, under the guidance of supervisors who are certified Animal Technologists, and by the staff of the Animal Core Facility. Veterinary care was provided by IACUC faculties and veterinary residents located on the San Francisco General Hospital campus. All mice were housed in a pathogen-free area in 421×316 cm^2 cages, maximus 5 mice per cage, with 12 hours light/dark cycle.

Immunohistochemistry

For mice treated with thalidomide starting 2-weeks after model induction, animals were transcardially perfused with PBS followed by 4% paraformaldehyde (PFA). Brain samples were collected and fixed in PFA for 48 hours in 4^{o}C, and then submerged in 20% sucrose-PFA solution until they sunk to the bottom of the solution. Brain samples were then snap-frozen in dry ice and cut into 20-μm-thick coronal sections using a Leica RM2155 Microtome (Leica Microsystems, Wetzlar, Germany). Two sections per brain approximately 500 μm rostral or caudal to the injection site were chosen for staining.

For mice treated with thalidomide or lenalidomide starting 8-weeks after model induction, animals were injected with a fluorescein labeled lectin (lycopersicon esculentum lectin, VectorLabs #FL-1171, Burlingame, CA) through jugular veins, after being anesthetized with isoflurane inhalation. Twenty minutes later, animals were perfused with PBS followed by 4% paraformaldehyde (PFA) transcardially. Brain samples were collected and treated as aforementioned.

Sections were incubated at 4°C overnight with anti-α-smooth muscle actin antibody (Thermo Scientific Cat #MA11547, Waltham, MA) to stain smooth muscles; fluorescein lycopersicon esculentum -lectin (Vector Laboratory, Burlingame, CA), anti-CD13 (R&D systems, AF2335, Minneapolis, MN), anti-platelet endothelial cell adhesion molecular 1 (CD31, Santa Cruz Biotechnology, sc-18916, Santa Cruz, CA; and R&D systems, AF3628) to stain endothelial cells or anti-PDGFB antibody to detect PDGFB expression (abcam, Cambridge, MA, ab23914,) Alexa Fluor 594-conjugated or Alexa Fluor 488-conjugated (Invitrogen, Carlsbad, CA) was used as secondary antibody. Vascular density (number of vessels per mm^2), dysplasia index (the number of vessels larger than 15 μm per 200 vessels), and vSMC-coverage [1] were quantified by at least two researchers who were blended with the group assignment. The pericyte coverage was quantified as described in previous paper [2]. Briefly, the CD13-positive and CD31-positive surface area was quantified utilizing NIH ImageJ software, and pericyte coverage is expressed as the percentage of the CD31-positive endothelial area covered by CD13-positive pericytes' processes.

Knockdown of *ALK1* Expression by shRNA in Human Brain Microvascular Endothelial Cells (HBMECs)

HBMECs were purchased from ScienCell (Carlsbad, CA) and cultured in Endothelial Cell Medium (ECM, cat#1001, ScienCell). To knockdown ALK1 expression, HBMECs were infected with lentivirus vector expressing *ALK1* shRNA (lenti-sh*ALK1*, SHCLNV-NM_000020, Sigma-Aldrich). Cells infected with a lentivirus expressing green fluorescent protein (lenti-GFP, SHC002V, Sigma-Aldrich) were used as control. These lentiviral vectors also carry a puromycin-resistant gene. Lentivirus was added to HBMEC cultures when the cells were 80% confluent, together with 8 μg/ml polybrene (hexadimethrine bromide, Sigma-Aldrich), and were incubated with the cells at 37^{o}C overnight. Successfully infected cells were enriched by puromycine selection (1ug/ml, Clontech laboratories Inc., Mountain View, CA). Puromycin resistant clones were pooled 2-3 weeks later. ALK1 expression was analyzed using qRT-PCR. HBMECs with more than 70% down-regulation of ALK1 expression were treated with 10 ng/ml VEGF in RPMI1640 + 2% of bovine serum albumin (BSA) for 16 hours and used for further analyses.

Prussian blue staining

Accustain Iron Stain Kit (Sigma-Aldrich, St. Louis, MO) was used to detect iron deposition. Two sections per brain that were adjacent to the sections used for vessel quantification were incubated in freshly prepared working iron stain solution for 15 minutes, washed in distilled water, and counterstained with pararosaniline solution for 3 minutes. Prussian blue-positive staining were quantified by image J software and data are presented as total pixels in a given basal ganglia divided by the total area of that basal ganglia (mm^2). Because of the skewed nature of Prussian blue area, the data were 10 log-conversed before the analysis.

RNA Extraction and Quantitative Real-Time RT-PCR (qRT-PCR) Analyses

Total RNA was extracted from cells or tissues using RNAzol RT (Molecular Research Center, Cincinnati, OH) and reverse-transcribed into cDNA using SuperScript III First-Strand Synthesis System (Invitrogen, Carlsbad, CA). Real-time PCR was performed using TaqMan Fast Advanced Master Mix (Applied Biosystems, Foster City, CA). Gene-specific primers and probes purchased from Applied Biosystems were used: *Alk1* (Mm00437432_m1), *Pdgfb* (Mm00440677_m1), *Cxcr4* (Mm01996749), *Il1b* (Mm00434228), *Tnf* (Mm00443260), *Gapdh* (Mm99999915_g1), *ALK1* (Hs00953798_m1), *PDGFB* (Hs00966522_m1), and *GAPDH* (Hs02758991_g1). All samples were run in triplicate, and relative gene expression was calculated using the comparative threshold cycle (CT) and normalized to Gapdh for mouse samples or GAPDH for human samples (ΔCT).

Western blot

Mice were anesthetized with isoflurane. Brain tissues around vector-injection sites were collected and protein was extracted using a cell lysis buffer (R&D Systems) supplemented with Protease inhibitor cocktail (Sigma-Aldrich #8849) and 1mM PMSF (Cell Signaling, Danvers, MA, #8553). Protein quantification was performed via the Bradford method (Bio-Rad, 500-0006, Hercules, CA) using a microplate reader (Emax, Molecular Devices, Sunnyvale, CA). Protein samples were loaded into 4–20% Tris-Glycine gels (Bio-Rad) and transferred onto nitrocellular membranes (Bio-Rad). Immunoblotting were performed using primary antibodies specific to

Pdgfrβ (Abcam, Cat#ab32570, Cambridge, UK), Tek (Tie-2, Santa Cruz Biotechnologies, C-20, Catalog number: sc-324) and Tuba4A (α-tubulin, Thermo Fisher, Cat#: PA5-19489, Waltham, MA) Blots were then labeled with a goat anti-rabbit secondary antibody: (Li-Cor, Cat #IRDye680LT, Lincolin, NB). Pdgfrβ and Tuba4A bands were detected by LI-COR Quantitative western blot scanner and quantified using LI-COR imaging software.

Supplemental Tables

Stroke Online Supplement

Table I. Checklist of Methodological and Reporting Aspects for Articles Submitted to *Stroke* Involving Preclinical Experimentation

Methodological and Reporting Aspects	Description of Procedures
Experimental groups and study timeline	☒ The experimental group(s) have been clearly defined in the article, including number of animals in each experimental arm of the study. ☒ An account of the control group is provided, and number of animals in the control group has been reported. If no controls were used, the rationale has been stated. ☒ An overall study timeline is provided.
Inclusion and exclusion criteria	☒ A priori inclusion and exclusion criteria for tested animals were defined and have been reported in the article.
Randomization	☒ Animals were randomly assigned to the experimental groups. If the work being submitted does not contain multiple experimental groups, or if random assignment was not used, adequate explanations have been provided. ☒ Type and methods of randomization have been described. ☐ Methods used for allocation concealment have been reported.
Blinding	☐ Blinding procedures have been described with regard to masking of group/treatment assignment from the experimenter. The rationale for nonblinding of the experimenter has been provided, if such was not feasible. ☒ Blinding procedures have been described with regard to masking of group assignment during outcome assessment.
Sample size and power calculations	☒ Formal sample size and power calculations were conducted based on a priori determined outcome(s) and treatment effect, and the data have been reported. A formal size assessment was not conducted and a rationale has been provided.
Data reporting and statistical methods	☒ Number of animals in each group: randomized, tested, lost to follow-up, or died have been reported. If the experimentation involves repeated measurements, the number of animals assessed at each time point is provided, for all experimental groups. ☒ Baseline data on assessed outcome(s) for all experimental groups have been reported. ☒ Details on important adverse events and death of animals during the course of experimentation have been provided, for all experimental arms. ☒ Statistical methods used have been reported. ☒ Numeric data on outcomes have been provided in text, or in a tabular format with the main article or as supplementary tables, in addition to the figures.
Experimental details, ethics, and funding statements	☒ Details on experimentation including stroke model, formulation and dosage of therapeutic agent, site and route of administration, use of anesthesia and analgesia, temperature control during experimentation, and postprocedural monitoring have been described. ☒ Different sex animals have been used. If not, the reason/justification is provided. ☒ Statements on approval by ethics boards and ethical conduct of studies have been provided. ☒ Statements on funding and conflicts of interests have been provided.

Table II. ALK1 expression in HBMECs with ALK1 knockdown (KD)

	*WT		ALK1 KD	
	#Control	VEGF	#Control	VEGF
Means	1.05	0.89	0.32	0.29
SDs	0.22	0.20	0.08	0.09

Note: ALK1 expression was measured by qRT-PCR. The data are fold changes compared to that of WT Controls

*WT: cells treated with Lenti-GFP, a control vector for Lenti-shALK1.

#Control: cells that have not been treated with VEGF.

Table III. PDGFB expression in ALK1 knockdown (KD) HBMECs

	*WT		ALK1 KD	
	#Control	VEGF	#Control	VEGF
Means	1.02	1.52	0.45	0.64
SDs	0.28	0.68	0.20	0.18

Note: PDGFB expression was measured by qRT-PCR. The data are fold changes compared to that of WT Controls

*WT: cells treated with Lenti-GFP, a control vector for Lenti-shALK1.

#Control: cells that have not been treated with VEGF.

Table IV. Mortality in each treatment group

Group	**DMSO (T)**	**THA**	**DMSO (L)**	**LEN**
#'s of death/total #'s	0/31	3/37	0/8	1/9

Note: DMSO (T): control for thalidomide treatment; DMSO (L): control for lenalidomide treatment;

THA: Thalidomide, LEN: Lenalidomide.

Supplemental Figures

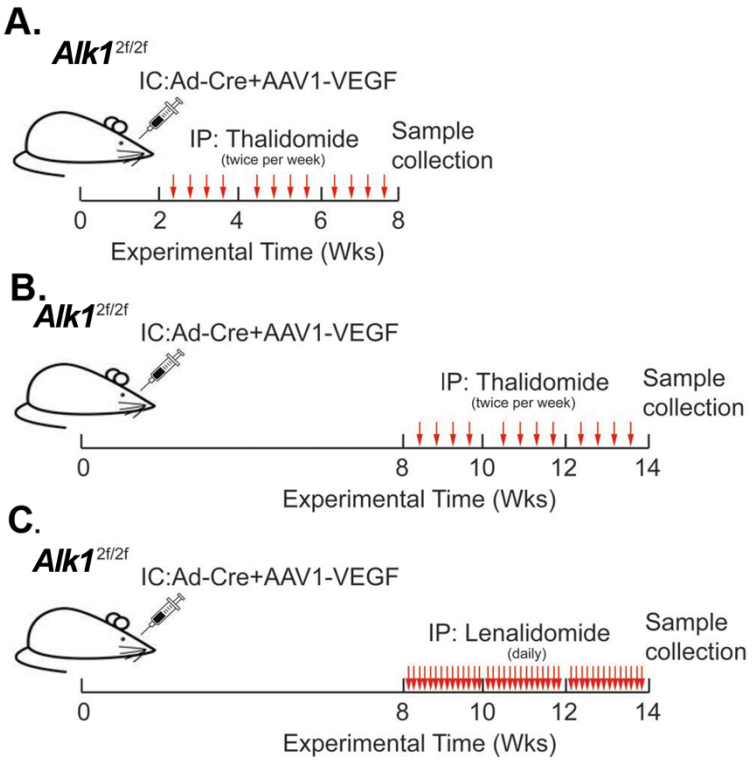

Figure I. Experimental Design. A. Design for thalidomide treatment starting 2-weeks after model induction. Brain AVM was induced in *Alk1*$^{2f/2f}$ mouse through intra-basal ganglia injection of AAV1-VEGF (an AAV-VEGF vector packaged in AAV serotype 1 capsid) and Ad-Cre. Two weeks later, thalidomide was injected intraperitoneally twice per week (red arrows) for 6 weeks. **B.** Design for thalidomide treatment starting 8 weeks after model induction. **C.** Design for lenalidomide treatment starting 8 weeks after model induction. Lenalidomide was intraperitoneally injected daily (red arrows) for 6 weeks.

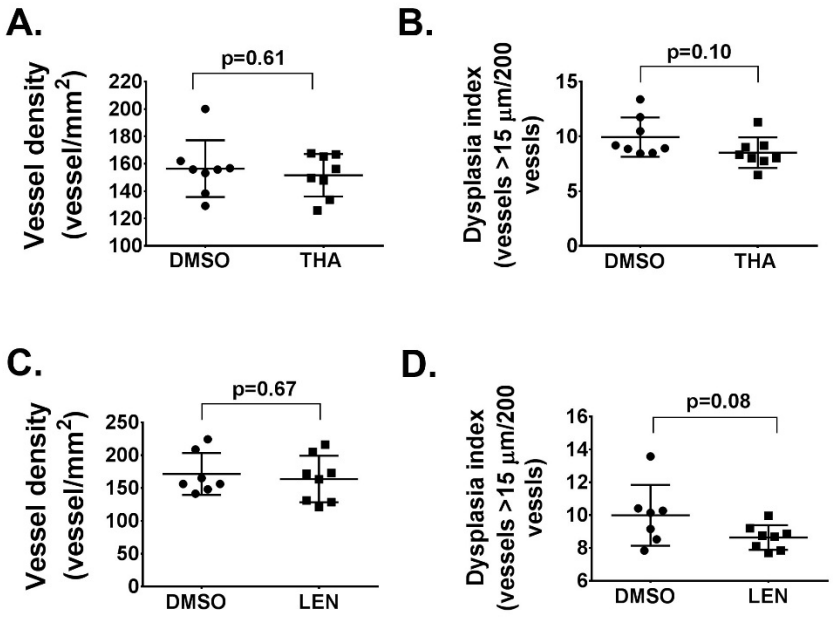

Figure II. Thalidomide and lenalidomide treatments did not change vessel densities or dysplasia index in pre-existing bAVM. A & B. Quantification of vessel densities and dysplasia index of thalidomide-treated mice (THA). N=8. **C & D** Quantification of vessel densities and dysplasia index of lenaledomide-treated mice (LEN). N=7 in DMSO group and N=8 in lenalidomide group. P values were determined by t-test analysis.

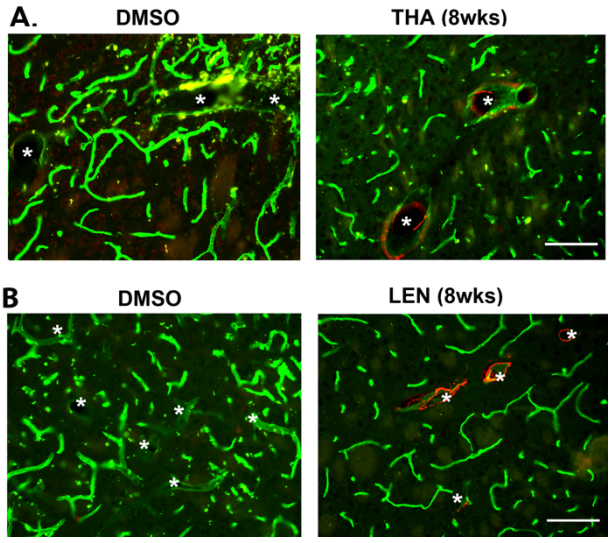

Figure III. Thalidomide and lenalidomide increase mural cell-coverage in established bAVMs. A & B. Representative images of brain sections. Vessels were stained by lectin (green); vSMCs were stained by antibody specific to smooth muscle actin-α (red). * indicates dysplasia vessels. THA (8wks): thalidomide treatment started 8 weeks after model induction. LEN (8wks): lenalidomide treatment started 8 weeks after model induction. Scale bars: 100 µm.

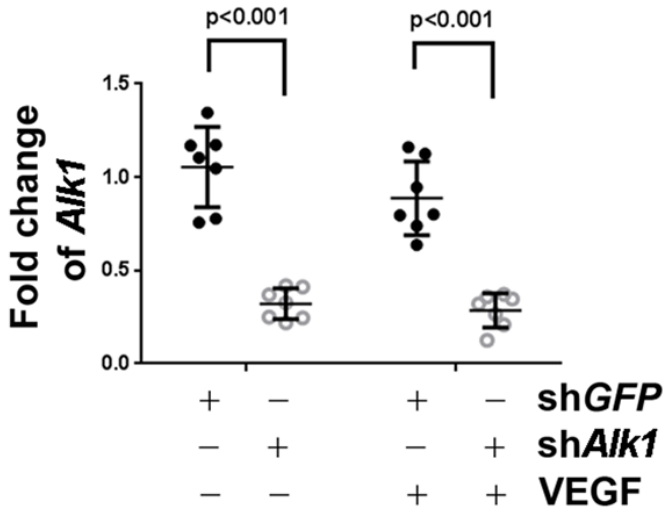

Figure IV. Lenti-shALK1 reduced ALK1 expression in HBMECs. The Y axis is fold changes compared to the mean of Lenti-GFP (shGFP) infected controls without VEGF treatment. N=7. P values were determined by one-way ANOVA analysis followed by Sidak's multiple comparisons.

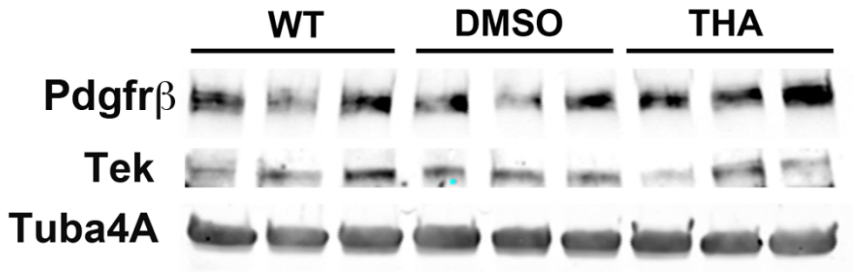

Figure V. Thalidomide increased Pdgfrβ expression. Representative western blot for quantification of Pdgfrβ and Tek expression. Tuba4A is used as loading control.

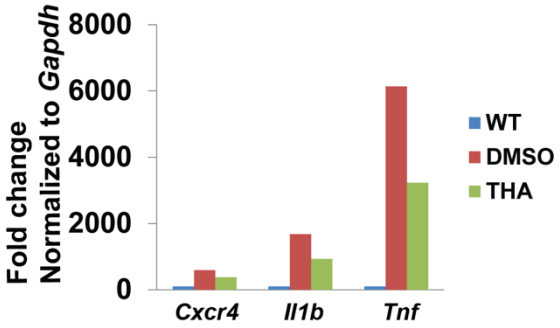

Figure VI. Inflammatory cytokines and macrophages in bAVMs. A. Quantification of expression of Cxcr4, Il1b and Tnfα (Tnf) in microvessels extracted from viral vection injection sites**.** In order to obtain enough RNA for the analysis, microvessels from mice in same group

were pooled: 4 wild type (WT) mice, 7 DMSO treated mice and 9 thalidomide (THA) treated mice.

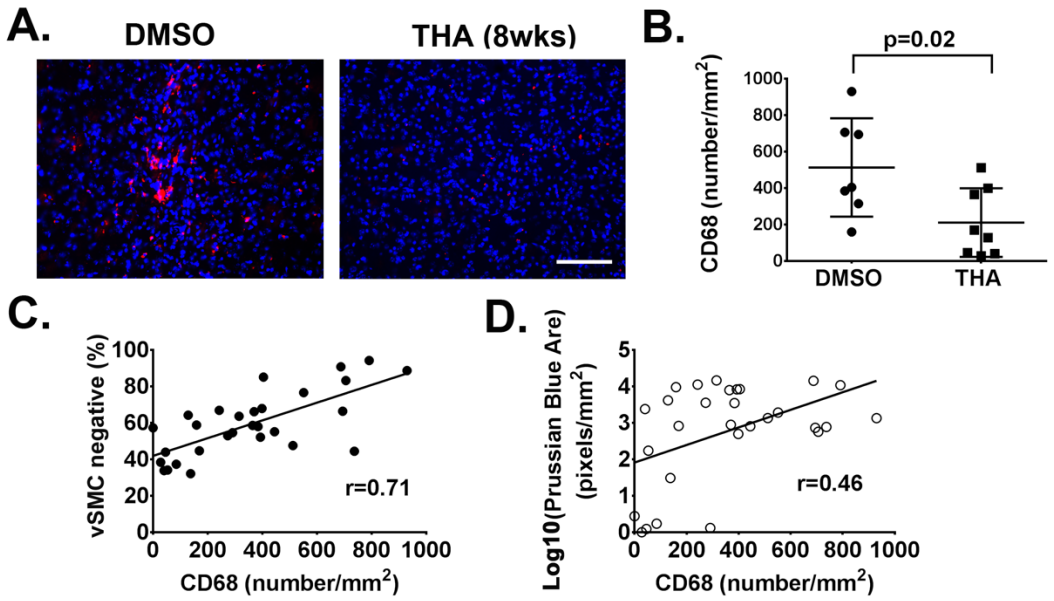

Figure VII. Thalidomide reduced the number of CD68$^+$ cells in bAVM lesions. A. Representative images of CD68 antibody stained sections. CD68$^+$ cells are in red. The nuclei were counterstained with DAPI (blue). Scale bars: 100 µm. THA (8wks): mice received thalidomide treatment starting 8 weeks after model induction. **B**. Quantification of CD68$^+$ cells. N=7 for DMSO group and N=8 for thalidomide (THA) treated group. P=0.02 determined by t-test analysis. **C**. Correlation between the number of CD68$^+$ cells and vSMC negative vessels. P<0.0001 determined by Spearman nonparametric correlation. (**D**) Correlation between CD68$^+$ positive cells and Prussian blue positive area. P=0.012 determined Spearman nonparametric correlation. 10 log conversed (Log 10).

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Human_PDGFB      MNRCWALFLSLCCYLRLVSAEGDPIPEELYEMLSDHSIRSFDDLQRLLHGDPGEEDGAEL
Mouse_PDGFB      MNRCWALFLPLCCYLRLVSAEGDPIPEELYEMLSDHSIRSFDDLQRLLHRDSVDEDGAEL
                 ********* ************************************** *  :******

Human_PDGFB      DLNMTRSHSGGELESLARGRRSLGSLTIAEPAMIAECKTRTEVFEISRRLIDRTNANFLV
Mouse_PDGFB      DLNMTRAHSGVELESSSRGRRSLGSLAAAEPAVIAECKTRTEVFQISRNLIDRTNANFLV
                 ******:*** **** :*********: ****:***********:***.**********

Human_PDGFB      WPPCVEVQRCSGCCNNRNVQCRPTQVQLRPVQVRKIEIVRKKPIFKKATVTLEDHLACKC
Mouse_PDGFB      WPPCVEVQRCSGCCNNRNVQCRASQVQMRPVQVRKIEIVRKKPIFKKATVTLEDHLACKC
                 ********************** :***:********************************

Human_PDGFB      ETVAAARPVTRSPGGSQEQRAKTPQTRVTIRTVRVRRPPKGKHRKFKHTHDKTALKETLG
Mouse_PDGFB      ETVVTPRPVTRSPGTSREQRAKTPQARVTIRTVRIRRPPKGKHRKFKHTHDKAALKETLG
                 ***.: ******** *:********:*********:*****************:*******

Human_PDGFB      A
Mouse_PDGFB      A
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Figure VIII. Sequence alignment of human and mouse PDGFB protein by Clustal Omega (http://www.clustal.org/). Human and mouse PDGFB protein sequences are 96% homologous (231/241 amino acids). Asterisks (*) indicate fully conserved residue; A colon (:) indicates conservation between groups of strongly similar properties; A period (.) indicates conservation between groups of weakly similar properties.

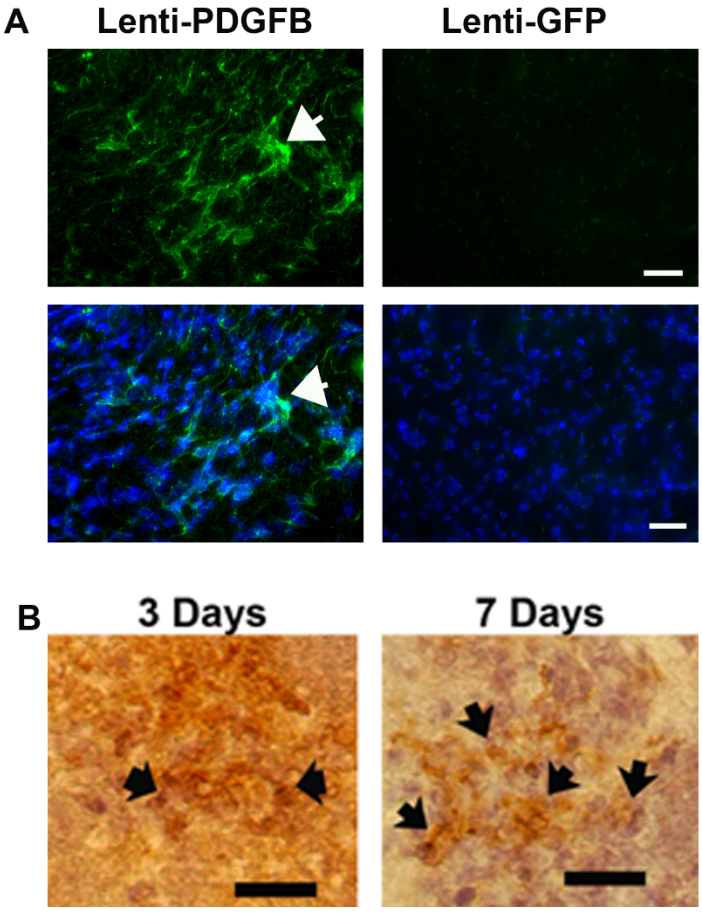

Figure IX. PDGFB and GFP expression in the vector-injection sites. A. PDGFB expression (green, arrows) was detected in the brain of that received Lenti-PDGFB injection (left) by an antibody specific to PDGGB, but not in the brain of that received Lenti-GFP injection (right). The bottom pictures show DAPI counterstained images. B. GFP expression was detected by immunohistochemistry staining (brown, arrows) using an antibody specific to GFG. Scale bar: 25 μm**.**

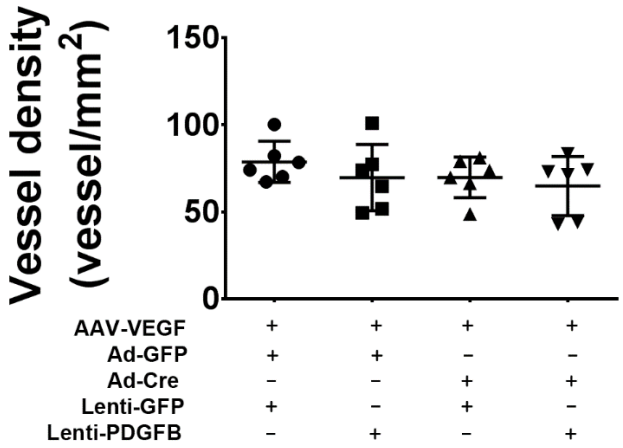

Figure X. Overexpression of PDGFB during bAVM development have no effect on vessel density. N=6. p=0.48 determined by One-way ANOVA analysis.

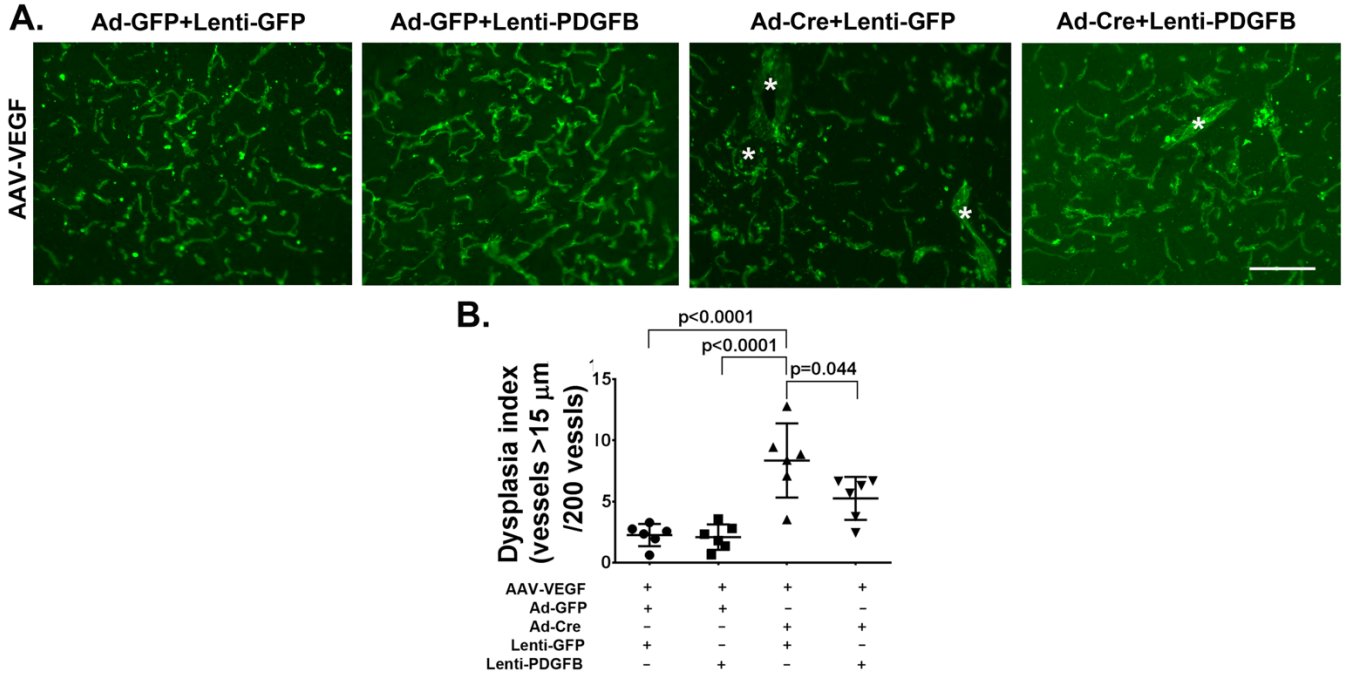

Figure XI. Overexpression of PDGFB inhibited bAVM vessel formation. A. Representative images of brain sections stained with an antibody specific to CD31 (an endothelial cell-marker, green). Ad-GFP+Lenti-GFP: Controls for normal angiogenesis; Ad-GFP+Lenti-PDGFB: Overexpressing PDGFB in angiogenic brain; Ad-Cre+Lenti-GFP; untreated bAVM; Ad-Cre+Lenti-PDGFB; Overexpression of PDGFB in bAVM. Scale bar: 100 μm. **B.** Quantification dysplasia index (the numbers of vessels larger than 15 μm/200 vessels). N=6. P values were determined by one-way ANOVA followed by Sidak's multiple comparisons.

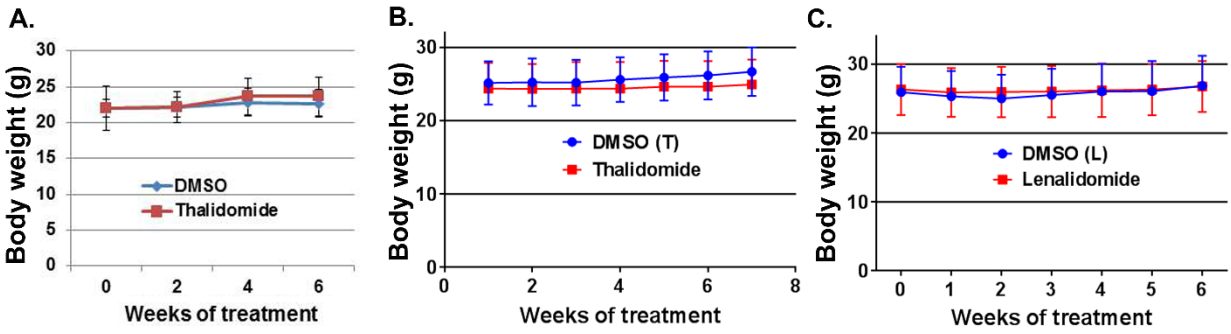

Figure XII. Thalidomide and lenalidomide treatments did not alter mouse body weight. A. Body weight of mice received thalidomide started 2 weeks after model induction. **B.** Body weight of mice received thalidomide started 8 weeks after model induction. **C.** Body weight mice received lenalidomide started 8 weeks after model induction.

References

1. Chen W, Guo Y, Walker EJ, Shen F, Jun K, Oh SP, et al. Reduced mural cell coverage and impaired vessel integrity after angiogenic stimulation in the *alk1*-deficient brain. *Arterioscler Thromb Vasc Biol*. 2013;33:305-310
2. Bell RD, Winkler EA, Sagare AP, Singh I, LaRue B, Deane R, et al. Pericytes control key neurovascular functions and neuronal phenotype in the adult brain and during brain aging. *Neuron*. 2010;68:409-427