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Supplemental material

Novel experimental model of brain arteriovenous malformations using conditional \textit{Alk1} gene deletion in transgenic mice
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SUPPLEMENTARY METHODS

Free-Floating Brain Sections

Anesthesia was induced with an intraperitoneal injection of a ketamine-xylazine mixture. Mice were transcardially perfused using a GP1000 peristaltic pump (Fisher Scientific) with phosphate-buffered saline (PBS) containing heparin (50 units/mL) followed by 4% paraformaldehyde in 0.1-M phosphate buffer (PB, pH 7.4). Brains were extracted from the cranium, fixed in 4% paraformaldehyde at 4 °C overnight, and then washed with PBS. Tissues were cryoprotected by dehydration in 30% sucrose for 2 days and then sectioned into 40-µm coronal slices using a Leica VT 1000S vibratome. Sections were preserved at -20 °C in a 25% glycerol, 30% ethylene glycol, 45% 0.1-M PB solution.

Immunofluorescence

Brain sections were washed 6 times in 0.1-M PB and blocked and permeabilized with 10% goat serum and 0.4% Triton X-100 in 0.1M PB for 2 hours at room temperature. After washing with PB, tissues were incubated in primary antibodies diluted in 2% goat serum and 0.4% Triton X-100 in 0.1M PB overnight at 4 °C. Tissues were washed in 0.1M PB and incubated with secondary antibodies diluted in 2% goat serum and 0.4% Triton X-100 in 0.1M PB for 2 hours at 4 °C. Nuclear staining was performed using DAPI (Sigma Aldrich, St. Louis, MO). Tissues were mounted on gelatin-coated slides using Prolong Gold Antifade Reagent (ThermoFisher Scientific, Waltham, MA). The following primary antibodies were used in this study: CD68 (Rat, 1:100, Bio-Rad, cat# MCA1957T, Hercules, CA) and CD31 (Rat, 1:50,
Biocare Medical, cat# CM 303A, Pacheco, CA). Alexa fluor 647 (Rat, 1:500, Invitrogen, cat# A-21247, Carlsbad, CA) was used as a secondary antibody.

**Prussian Blue Staining**

Hemorrhage was visualized by staining ferric iron deposits with the iron stain kit (Abcam, cat# ab150674, Cambridge, MA). Brain sections (40-μm thickness) were equilibrated with distilled water, replaced with a mixture (50:50) of potassium ferrocyanide and hydrochloric acid solution, and incubated for 3-10 minutes until the blue color became visible in the tissue. After rinsing with distilled water, the tissues were mounted with prolong gold antifade (ThermoFisher Scientific, cat# P36930, Waltham, MA).

**Isolation of Brain Endothelial Cells**

Mice carrying Tagln-cre(+)::R26mTmG/+ were decapitated, and each head was dipped into 70% ethanol on ice, as previously described. The brain was swiftly removed and stored in Dulbecco’s PBS (ThermoFisher Scientific) on ice. The cerebellum and olfactory bulb were removed from the brain. The meninges were removed by rolling the brain on cellulose chromatography paper. The brain was homogenized in a Dounce tissue grinder (30 strokes) in 5 mL of working medium containing 2 mM L-glutamine in DMEM-F12 (ThermoFisher Scientific) (4 °C). The homogenate was centrifuged at 1350 × g for 5 minutes at 4 °C, and the supernatant was removed. The pellet was resuspended with 15 mL of dextran solution containing 18% dextran, penicillin-streptomycin, 2 mM L-glutamine in Dulbecco’s PBS and vortexed extensively for 2 minutes. The cell suspension was centrifuged at 6080 × g for 10 min at 4 °C, and the supernatant was removed. The fluffy myelin layer (top) and the dextran were removed as
completely as possible. The pellet was resuspended in 10 mL of digestion medium containing 1 mg/mL collagenase (Roche Diagnostics, Indianapolis, IN), 0.147 µg/mL TLCK (Sigma-Aldrich), 4 µg/mL DNase I (Roche Diagnostics) in DMEM (ThermoFisher Scientific) (37 °C) and incubated for 1 hour and 15 minutes in a 37 °C water bath (with frequent shaking every 15 minutes). The cell suspension was centrifuged at 1350 × g for 5 minutes at room temperature. The digestion medium was removed, and the pellet was resuspended in 10 mL warm Dulbecco’s PBS. The cell suspension was centrifuged at 1350 × g for 5 min at room temperature. The collagen was removed from the wells of the 6-well plate, which was coated with the collagen a day before, and the wells were washed with Dulbecco’s PBS twice. The pellet was resuspended in 2.5-mL full medium/well containing 20% plasma-derived bovine serum (First Link), antibiotic-antimycotic (ThermoFisher Scientific), 2 mM L-glutamine (ThermoFisher Scientific), 15 lU/mL heparin (ThermoFisher Scientific), 30 µg/mL ECGS (Sigma-Aldrich), and 8 µg/mL puromycin (Sigma-Aldrich) and plated on collagen-coated wells on the plate. The next day, the medium was replaced with new medium containing puromycin. The medium was then replaced with new medium without puromycin 1–2 times per week. On day 7 after isolation, the cells were stained with the CD31 antibody (Rat, 1:50, Biocare Medical, cat# CM 303A).

**CD31 Staining and Expression of Reporter Genes in Brain Endothelial Cells**

To stain with an endothelial marker in cultured primary brain endothelial cells isolated from a mouse carrying Tagln-Cre(+)R26mTmG/+, cells were fixed with ice-cold methanol for 5 minutes at -20 °C, and after washing with PBS, the cells were incubated with CD31 antibody (Rat, 1:50, Biocare Medical, cat# CM 303A) in 1% bovine serum albumin in PBS-T containing 0.1% Tween 20 (Sigma Aldrich) for 16 hours at 4 °C. The cells were incubated with the
secondary antibody, Alexa fluor 647 (Rat, 1:500, Invitrogen, cat# A-21247), for 1 hour, and nuclear staining was performed using DAPI (Sigma Aldrich). Using the Keyence microscope, the CD31-stained, red fluorescent (Cre-negative), and green fluorescent (Cre-positive) cells were observed.

REFERENCE: