

Supplementary information

Supplemental methods

Orthotopic GL261 glioma model

Mouse GL261 glioma cell line was a generous gift from Dr. Karl Plate (Goethe University Medical School, Germany), with which GL261-GFP cell line was created by lentiviral infection of GFP-expressing vector into the GL261 cells. Under anesthesia, 2.0×10^5 of GL261 or GL261-GFP mouse glioma cells were implanted into right hemisphere of 8 to 10-week-old C57BL/6J mice with the aid of stereotaxic apparatus (RWN Life Science).

SPR measurement of 4E2

The binding kinetics of 4E2 was measured with SPR (Bioacore T200, Cytiva). 8 nM of antibody was immobilized with protein-A chip with a 20 $\mu\text{l}/\text{min}$ for 30 sec. For association, human or mouse Tie2 (Sinobiologics) in PBS containing 0.005 % Tween 20 was flowed with a 30 $\mu\text{l}/\text{min}$ for 5 min. The concentration of analyte was 0.781, 1.563, 3.125, 6.25, 12.5, 50, 100, 200 and 400 nM. Dissociation was done with the same buffer with a 30 $\mu\text{l}/\text{min}$ for 10 min. 10 mM Glycine buffer (pH 1.5) was used for the regeneration. The association and dissociation constant were evaluated with BIAevaluation software version 3.0 with a 1:1 stoichiometry.

Permeability

HUVECs were grown as a 3d-old mature monolayer on 0.1% gelatin-coated 0.4 μm -size pore inserts (PIHP01250, Millipore). Cells were treated as required, and incubated with FITC-dextran (60 kDa, 0.5 mg/ml, FD70S, Sigma). Each sample from the bottom chamber was read in triplicate on the Multilabel Microplate Reader (Hidex, Chameleon).

Flow cytometry

For target binding, 1×10^5 cells of human Tie2-overexpressing CHO-K1 cells were washed at room temperature in PBS containing 10% fetal bovine serum (PBS/FBS) and incubated with 4E2 at 4 $\mu\text{g}/\text{ml}$ final concentration for 30 min at 4 °C. Cells were stained with PE-conjugated anti-human FC-antibody in PBS/FBS at 4 °C for 15 min. As mentioned above, mouse Tie2-overexpressed CHO-K1 cells were incubated with 10 $\mu\text{g}/\text{ml}$ of 4E2 for 30 min at 4 °C. For positive controls, 4 $\mu\text{g}/\text{ml}$ of PE-conjugated anti-human and anti-mouse Tie2 antibodies were

bound to human and mouse Tie2-overexpressing CHO-K1 cells. After washing in ice-cold PBS/FBS, the stained cells were resuspended in PBS/FBS and kept on ice for FACS. For competitive binding assay, human Tie2-overexpressing CHO-K1 cells were allowed to bind 10 µg/ml of 4E2 for 30 min, and then bind 0-30 µg/ml His-clustering ANGPT1 (or ANGPT2) for 15 min. 4E2 and ANGPTs are detected by fluorescently labeled-secondary antibodies, respectively. The stained cells were analyzed using a BD FACS Lyric.

Bioluminescence imaging

Prior to imaging, the mice bearing brain tumors were anesthetized with inhalation of isoflurane gas, and injected intravenously with 150 mg/kg of D-luciferin solution (LUCK-100, GoldBio). Luminescence signal emitted from tumors was captured 10 min after injection using an IVIS Spectrum (IVIS Lumina S5, PerkinElmer). *In vivo* bioluminescence imaging was acquired using 10–60 seconds exposure and small or large binning depending on the light produced, 1 f/stop, and an open filter. Living image software (Caliper Life Sciences) was used for all image acquisition and data analysis.

T2-weighted MRI imaging

Mice were initially anesthetized with 4% isoflurane (Piramal Critical Care, Bethlehem) in a 7:1 mixture of air and oxygen. The anesthesia level was reduced to 1.5% during the data acquisition. The head of the animal was fixed with a bite bar to reduce the head motion. The respiration rate of the animal was monitored using an MRI-compatible pressure-sensitive pillow (Model 1025, SA instruments) and maintained within a normal range of 60-90 bpm. The body temperature was monitored using a rectal probe (Model 1025, SA instruments) and maintained at 37 ± 0.5 °C by using a hot air. MRI experiments were performed on a 3-Tesla MRS 3000 preclinical scanner of 17-cm bore size (MR solutions, Surrey, United Kingdom) with a 20-mm birdcage mouse head coil. For MR imaging, a T2-weighted 2D Fast spin echo (FSE) sequence was used, and the scan parameters were as follows: scan direction = axial, TR = 3,000 ms, TE = 68 ms, $TE_{\text{base}}/TE_{\text{eff}} = 17 \text{ ms}/68 \text{ ms}$, echo train length = 8, number of averages = 8, field of view = $22 \times 22 \text{ mm}^2$, matrix size = 256×256 , slice thickness = 0.8 mm (no gap), number of slices = 16, and total scan time = 11 min 4 sec.

Custom-built confocal microscopy for intravital imaging

To visualize *in vivo* vascular remodeling, a custom-built video-rate laser-scanning confocal

microscopy system was used as described¹. The imaging system was equipped with three continuous wave laser modules with output wavelengths at 488 nm (MLD488, Cobolt), 561 nm (Jive, Cobolt), and 640 nm (MLD640, Cobolt), respectively. Combining a rapidly rotating 36 facets polygonal mirror (MC-5, aluminum coated, Lincoln Laser) and galvanometer mirror scanner (6230H, Cambridge Technology), a video-rate Raster-pattern laser-scanning was achieved. The scanning laser beam was then delivered to the deep brain tissue through the tube imaging window by using objective lenses PlanApo λ 10x (Nikon, 10 $\times$, 0.45NA). The multi-color fluorescence signals were split by dichroic beam splitters (FF484-Di01, FF560-Di01, FF649-Di01, Semrock) and detected by photomultiplier tubes (PMT; R9110, Hamamatsu) through bandpass filters (FF02-525/50, FF01-600/37, FF01-685/40, Semrock). Electronic signal outputs from the PMTs were digitized by a frame grabber (Solios, Matrox). The video-rate images with a frame rate of 15 Hz and a size of 1024 $\times$ 1024 pixels were displayed and recorded in real-time by custom-written software using Matrox Imaging Library (MIL10, Matrox).

Window implantation and intravital imaging

To monitor tumor microenvironment in deep brain region, we developed a customized brain-implantable tube imaging window chamber integrated with custom-built laser-scanning confocal microscope. After craniotomy with diameter of 3 mm, the cortical tissue was carefully aspirated until the tumor-initiating area appeared. Then the tube window chamber, consisting of a stainless-steel cannula (height: 1.8mm, OD: 3.2 mm, ID: 2.8mm) and a coverglass (0.1mm thickness, 3mm diameter; Warner Instruments), was gently implanted. The top part of the window chamber and headplate was tightly glued to the skull surface using light-cured dental adhesive (Optibond FL, Kerr) and dental acrylic resin. To prevent the window surface from contamination, the implanted tube imaging window chamber was filled with a low viscosity silicone elastomer (Kwik-Cast sealant, World Precision Instruments) during the recovery period, which was then removed right before the intravital imaging. For intravital imaging, the mouse was anesthetized and placed on a xyz-motorized stage with a customized stereotaxic device. To visualize the blood vessels, Dylight-488 conjugated *Lycopersicon esculentum* lectin (DL-1174, Vector) was intravenously injected before the intravital imaging.

Transmission electron microscopy (TEM)

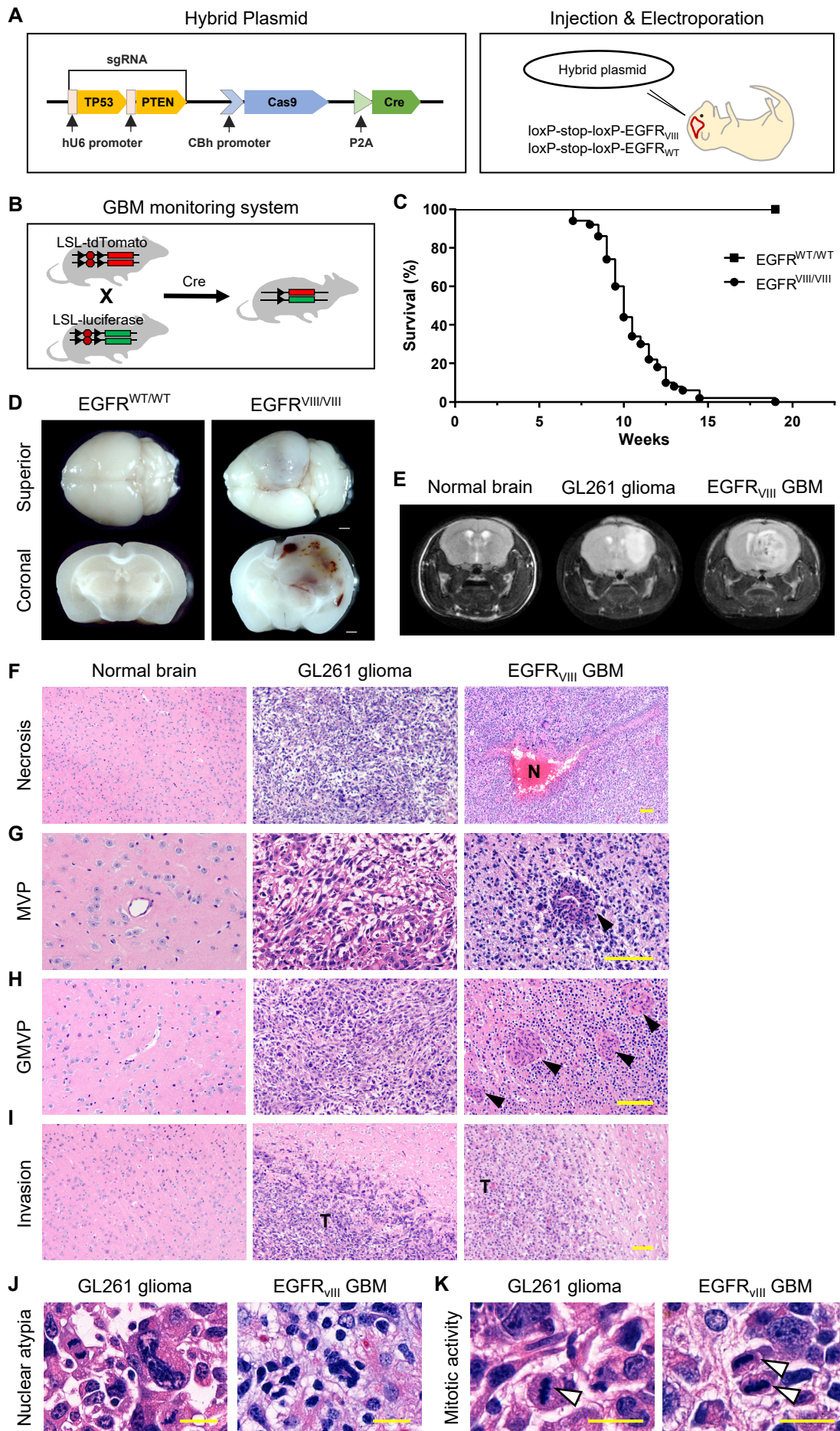
Mice were cardiac perfused with 2% PFA and 2% glutaraldehyde in PBS. Brain was minced into small pieces (less than 1 mm³) and incubated in the same fixative overnight at 4 °C. Fixed brain was post-fixed with 1% osmium tetroxide in PBS at 4 °C for 1 hr. Brain was dehydrated with a series of ethanol gradient, substituted with propylene oxide, and finally embedded in EMbed-812 resin (EMS). Polymerization was performed at 60 °C for 40 hr. Brain resin block was cut at a thickness of 70 nm (Leica EM UC7, Leica). Sections were stained with 2.5% uranyl acetate and Reynold's lead citrate. Images were viewed with Tecnai G2 spirit TWIN (FEI) electron microscope at 120 kV acceleration voltage.

Human brain tumor specimens

After receiving informed consent, tumor specimens with corresponding clinical records were obtained from patients undergoing surgery at the Samsung Medical Center. This study was approved by the local institutional review board (IRB file number 201004004) and conducted in accordance with all relevant ethical regulations for research using human specimens. To obtain non-tumor brain samples, the local institutional review board (IRB file number 906-210118-BR-004-01) of Chonnam National University Medical School and Hospital approved all autopsies as research activity involving non-living, post-mortem subjects in accordance with ethical regulations and waived the requirement to obtain informed consent owing to the inaccessibility to and unavailability of personal identification information and testing for heritable traits of the deceased. Accordingly, all specimens and data, with the exception of age, gender, and medical histories, were anonymized prior to all analyses.

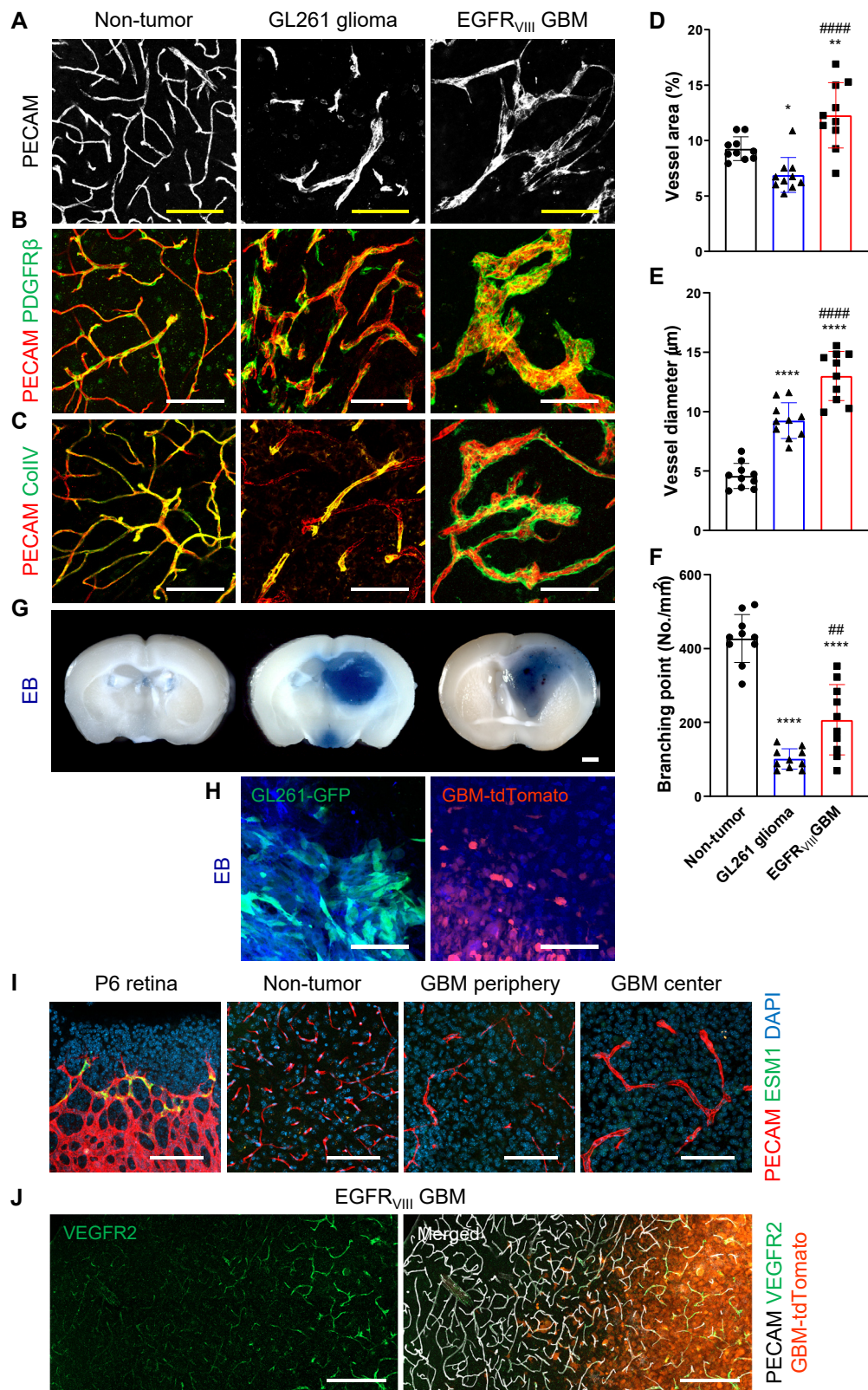
Supplemental reference

- 1 Lee, J., Kong, E., Hong, S., Moon, J. & Kim, P. In vivo longitudinal visualization of the brain neuroinflammatory response at the cellular level in LysM-GFP mice induced by 3-nitropropionic acid. *Biomed Opt Express* **11**, 4835-4847, doi:10.1364/BOE.393690 (2020).



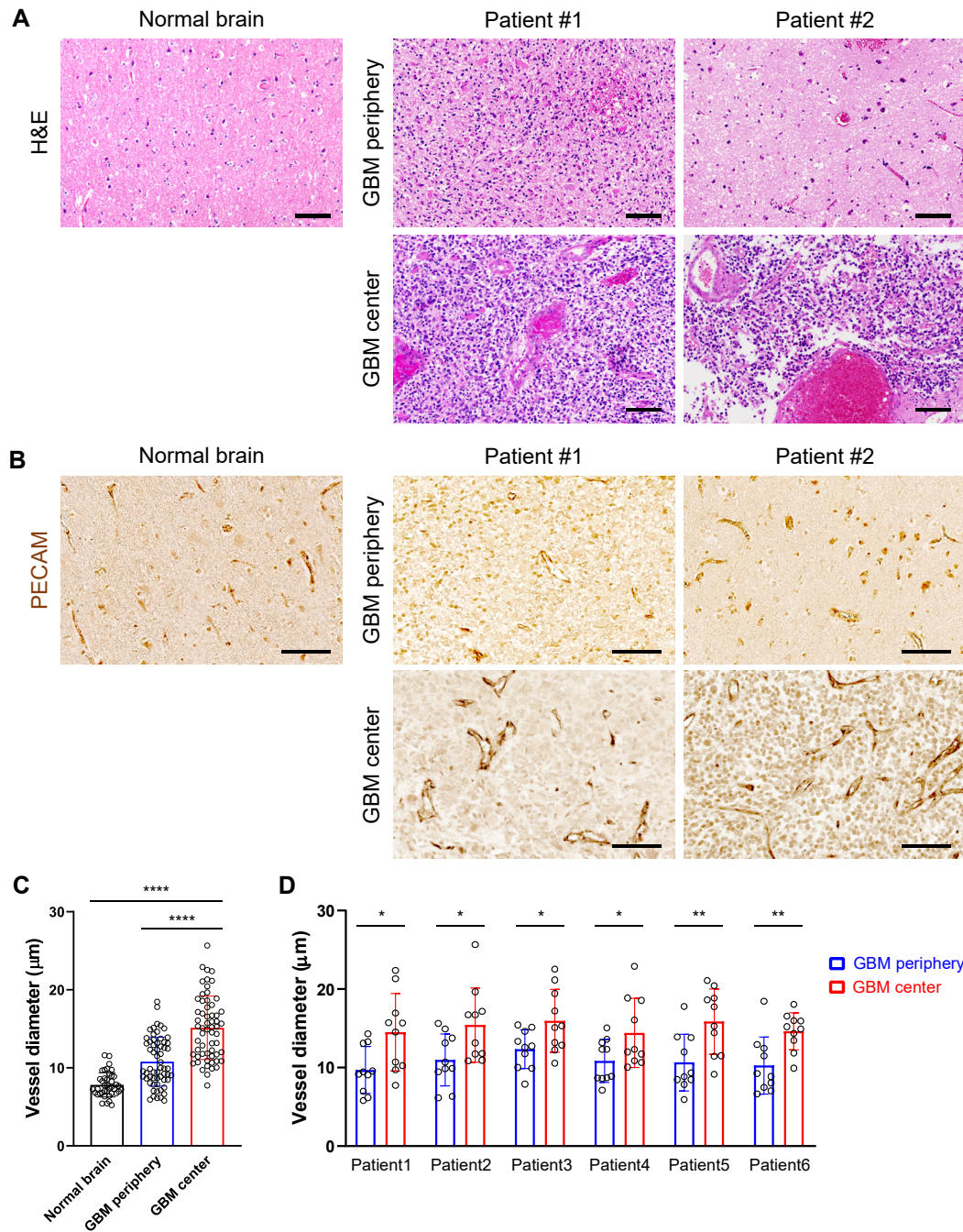
Supplementary Fig. 1. EGFR_{VIII} GBM mouse model represents histopathologic features of grade IV glioma

(A) Hybrid plasmid for both CRISPR/Cas9-mediated gene inactivation and the ectopic *Cre* expression (left). Its delivery into the brain cells of neonatal mice harboring the *EGFR_{VIII}* gene cassette (right). (B) Breeding of mice carrying *tdTomato* and *luciferase* genes for tracking tumor cells *ex vivo* and *in vivo*. (C) Survival curves for *EGFR^{VIII/VIII}* ($n = 50$) and *EGFR^{WT/WT}* ($n = 30$) mice after genetic manipulation. $P = 0.0303$. (D) Macroscopic images showing normal brain of *EGFR^{WT/WT}* and tumor-bearing brain of *EGFR^{VIII/VIII}*. (E) Coronal T2-weighted magnetic resonance images highlighting EGFR_{VIII} GBM with heterogeneous high-signal-intensity mass. (F-I) H&E staining images for histopathologic features of grade IV glioma: (F) Necrosis with pseudo-palisades, (G) microvascular proliferation (MVP, arrowheads), (H) glomeruloid microvascular proliferation (GMVP, arrowheads), and (I) invasion. Note common features of grade IV glioma in EGFR_{VIII} GBM. N, necrosis; T, tumor region. (J-K) H&E staining images for nuclear atypia (J) and mitotic activity (K). Arrowheads indicating mitotic chromatin condensation. Scale bars: 1 mm (C), 100 μ m (F-I), 20 μ m (J-K).



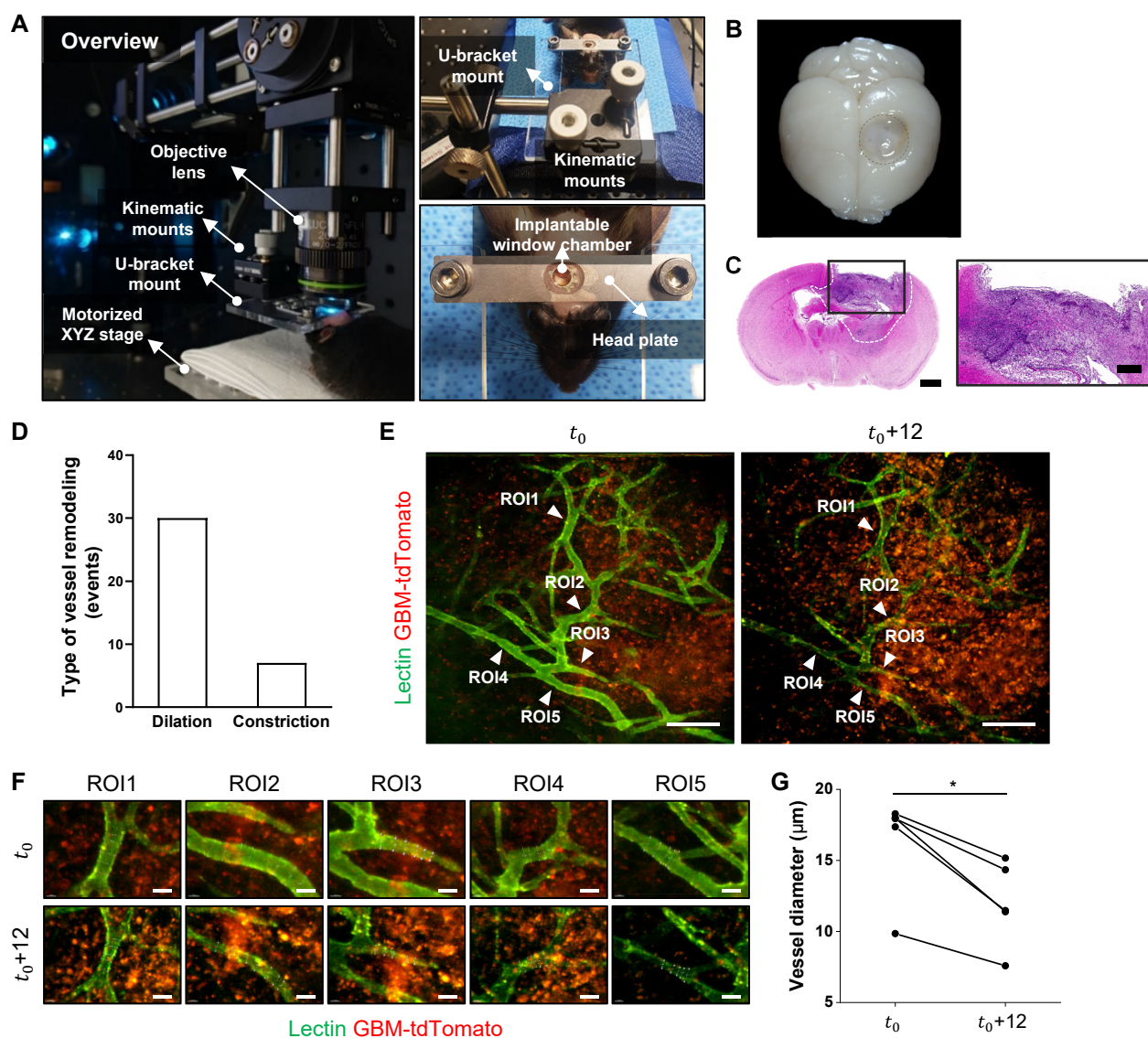
Supplementary Fig. 2. Increased vascular heterogeneity without sprouting activity in a spontaneous GBM model

(A) Immunostaining for PECAM-positive vessels in non-tumor brain, GL261 glioma, and EGFR_{VIII} GBM and quantification of (D) vessel area, (E) vessel diameter, and (F) vessel branching point. Note the biggest deviations in EGFR_{VIII} GBM. (B) Immunostaining for PDGFR β -positive pericytes. (C) Immunostaining of collagen type IV (ColIV)-positive basement membrane. (G-H) Gross image of Extravasated Evans blue (EB, G) and immunostaining of Evans blue at the tumor periphery (H). (I) Immunostaining of ESM1 in P6 retina, non-tumor brain, and the periphery and center of EGFR_{VIII} GBM. (J) Panorama view showing a gradual increase in VEGFR2 levels in vessels from the non-tumor area toward the GBM center. Scale bars: 100 μ m (A-C, H, I), 1 mm (G), 200 μ m (J). *, $P < 0.05$; **, $P < 0.01$; ****, $P < 0.0001$ vs Non-tumor; ##, $P < 0.01$; #####, $P < 0.0001$ vs GL261 glioma.



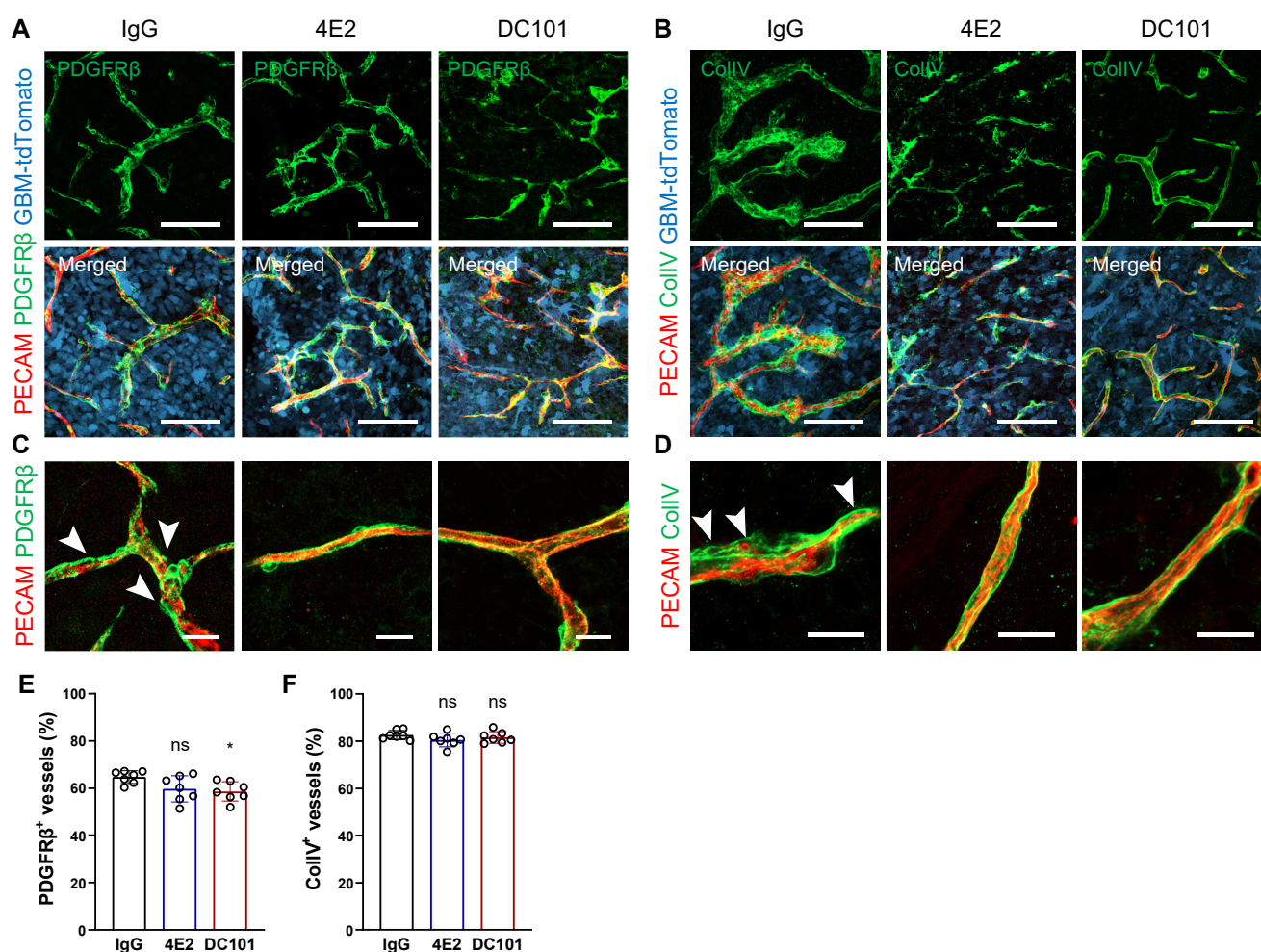
Supplementary Fig. 3. Tumor vessels are dilated in human GBM.

Vascular comparison between the periphery and the center of human GBMs. **(A)** H&E staining of brain tissues from control human subjects and paired specimens from patients with GBM. Peripheral GBM region (upper) and central GBM region (lower). **(B)** Immunostaining for PECAM-positive blood vessels and **(C)** quantification of vessel diameter in normal brain ($n = 40$), GBM periphery and GBM center ($n = 60$ per group). **(D)** Comparison of vessel diameter in paired tissues from each patient ($n = 10$ per group). Scale bars: 100 μ m (**A-B**). *, $P < 0.05$; **, $P < 0.01$; ****, $P < 0.0001$.



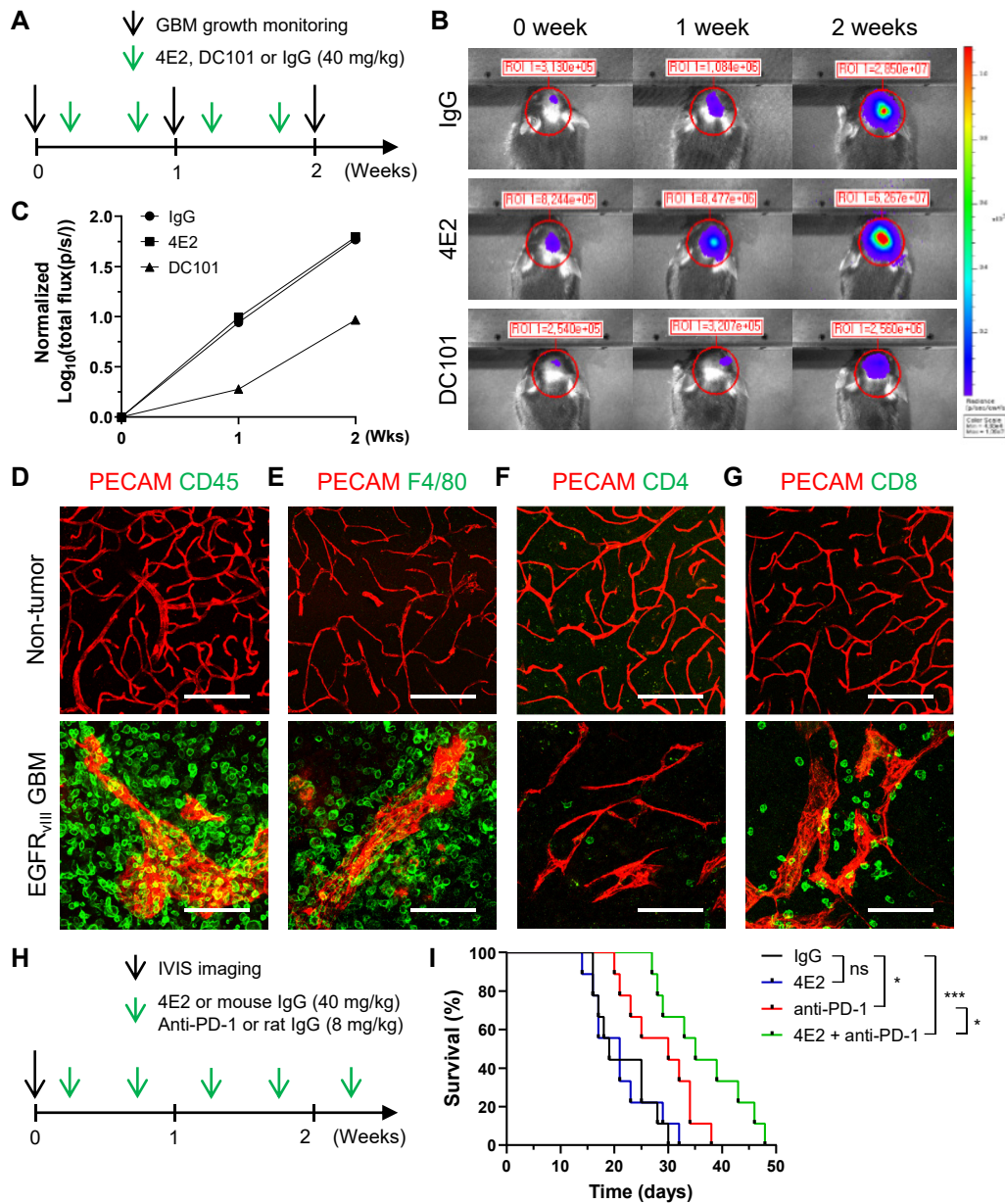
Supplementary Fig. 4. Dynamics of tumor vessel remodeling in the GBM periphery

(A) Photograph of imaging setup and anesthetized GBM mouse implanted with the tube imaging for intravital imaging (B) Macroscopic bright field image of mouse brain. (C) H&E staining of coronal section of mouse brain bearing EGFR_{VIII} GBM (broken white lines) after the removal of window chamber (left). Magnification of boxed area in (B) showing the depth of window chamber inserted below the skull surface (right). (D) Quantification of vessel dilation and constriction in GBM. (E) Vasoconstriction in control GBM and (F) high magnification of region of interests (ROIs) with time interval of 12 hours ($t_0 \sim t_0+12$). (G) Quantification of vessel diameter. Scale bars: 1 mm (C, left), 500 μm (C, right), 200 μm (E), 20 μm (F). *, $P < 0.05$.

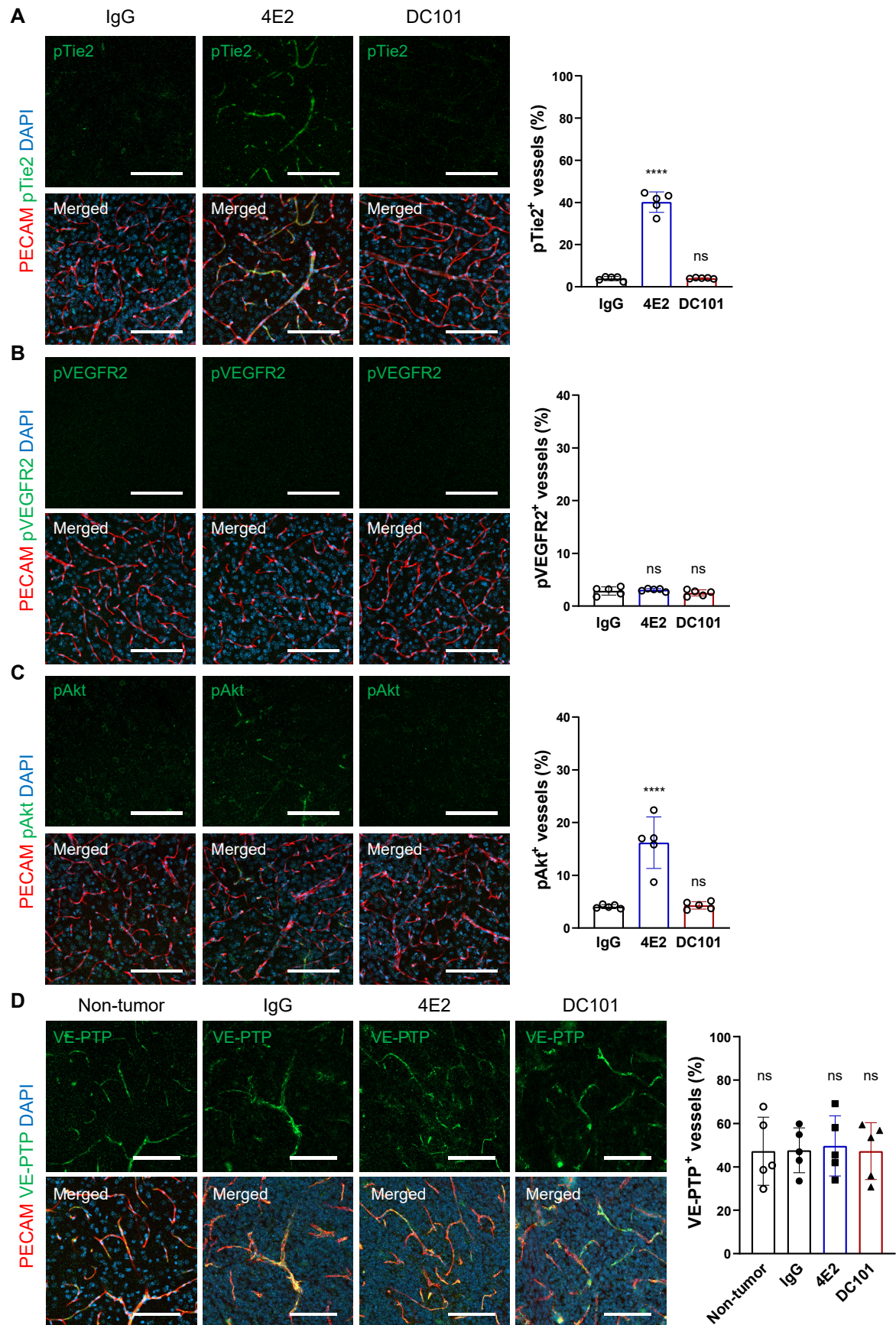


Supplementary Fig. 5. 4E2 and DC101 suppress tumor vascular abnormalities

(A-D) Vascular coverage in EGFR_{VIII} GBM 2 weeks after IgG, 4E2, and DC101 treatment. (A) Coverage of PDGFR β -positive pericytes and (B) ColIV-positive basement membrane in the tumor center regions. (C) High-magnification images of pericytes and (D) basement membrane. Arrowheads indicate their loose contact on GBM vessels. (E) Quantification of the coverage of PDGFR β -positive pericytes ($n = 7$ per group) and (F) ColIV-positive basement membrane ($n = 7$ per group). Scale bars: 100 μ m (A, B), 20 μ m (C, D). ns, not significant; *, $P < 0.05$ vs IgG.



Supplementary Fig. 6. 4E2 combined with anti-PD-1 antibody extended survival of GBM-bearing mice
(A) Experimental schedule of GBM growth monitoring and administration of antibodies. (B) *In vivo* bioluminescence imaging and (C) quantification of luminescent intensity ($n = 6$ per group). (D-G) Immunostaining for CD45 (D), F4/80 (E), CD4 (F), and CD8 (G) in non-tumor brain and EGFR_{vIII} GBM. (H) Experimental schedule of antibody administration for survival tests. (I) Survival curves of GBM-bearing mice treated with IgG, 4E2, anti-PD-1 antibody, or both 4E2 and anti-PD-1 antibody ($n = 9$ per group). Scale bars: 100 μm (D-G). ns, not significant; *, $P < 0.05$; ***, $P < 0.001$.



Supplementary Fig. 7. 4E2 activates Tie2-Akt signaling in non-tumor brain tissues

(A) Increased phospho-Tie2 immunoreactivity in non-tumor brain vessels by 4E2 treatment and quantification ($n = 5$ per group). (B) No detectable phospho-VEGFR2 signal and quantification ($n = 5$ per group). (C) Increased phospho-Akt by 4E2 treatment and quantification ($n = 5$ per group). (D) Immunostaining and quantification of VE-PTP in non-tumor brain and EGFR_{III} GBM treated with IgG, 4E2, and DC101 ($n = 5$ per group). Scale bars: 100 μm (A-D). ns, not significant; ***, $P < 0.0001$ vs IgG.