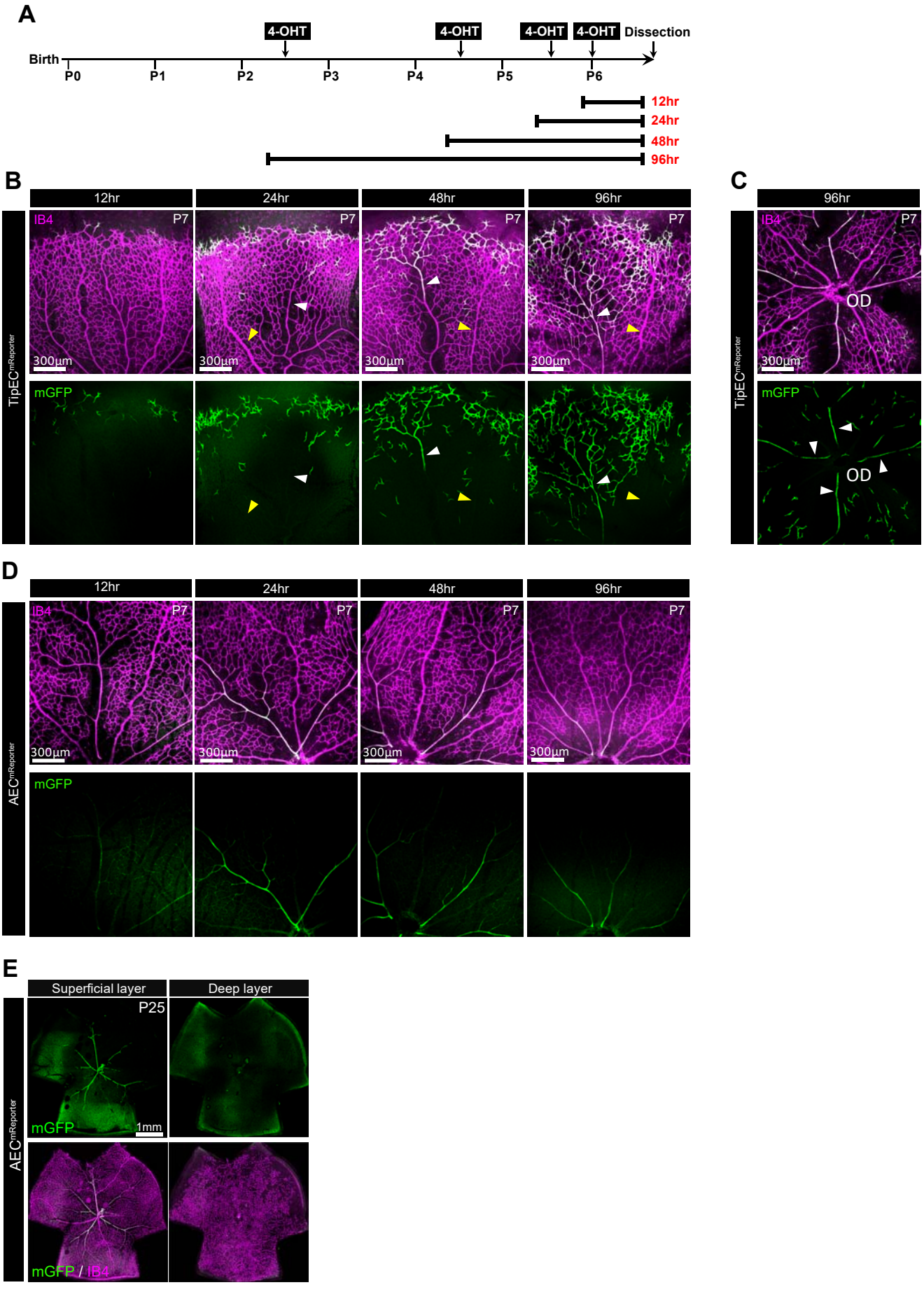
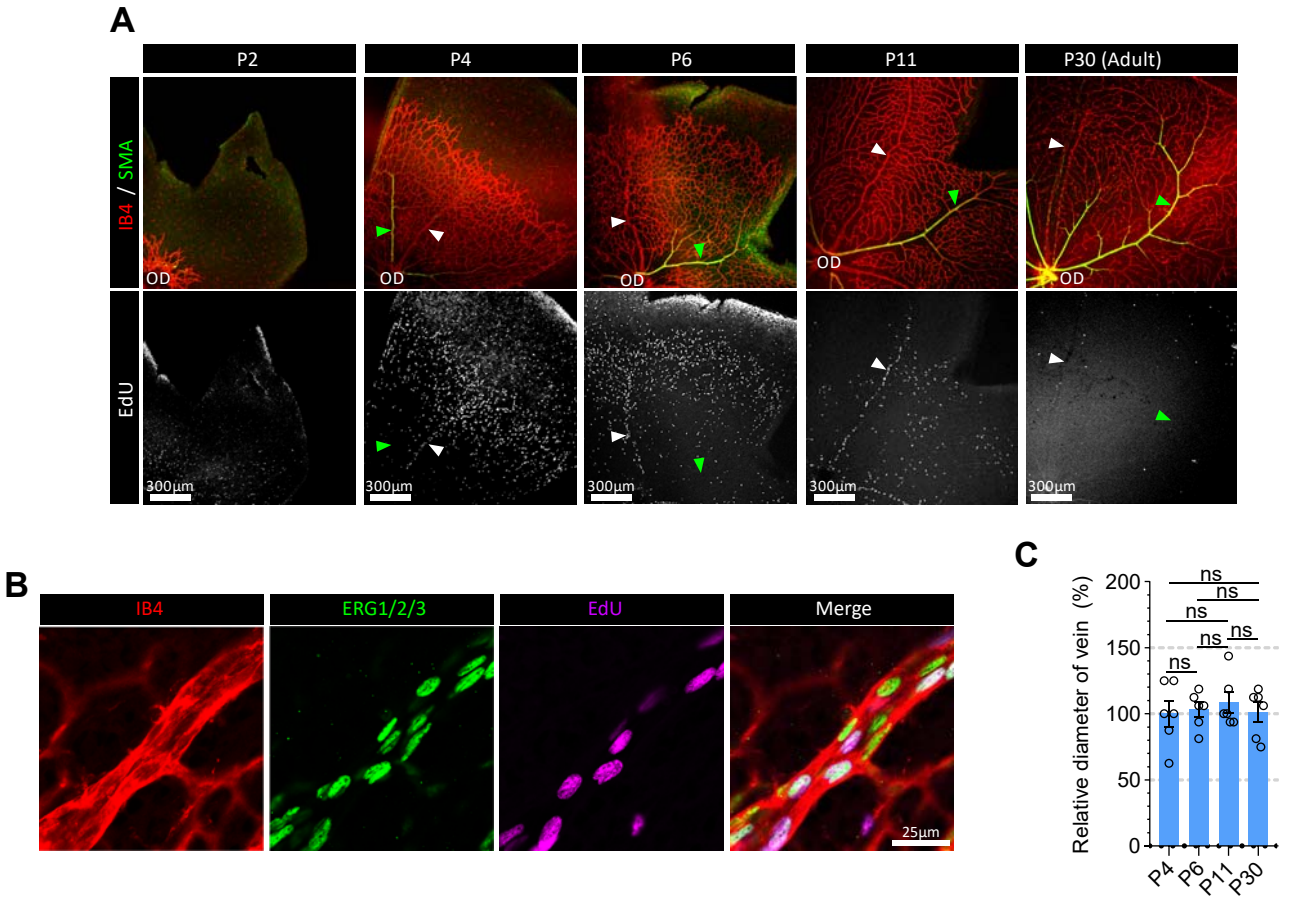
SUPPLEMENTAL MATERIAL

Supplemental Figure I. Lineage tracing of TipE C and AEC in vivo.



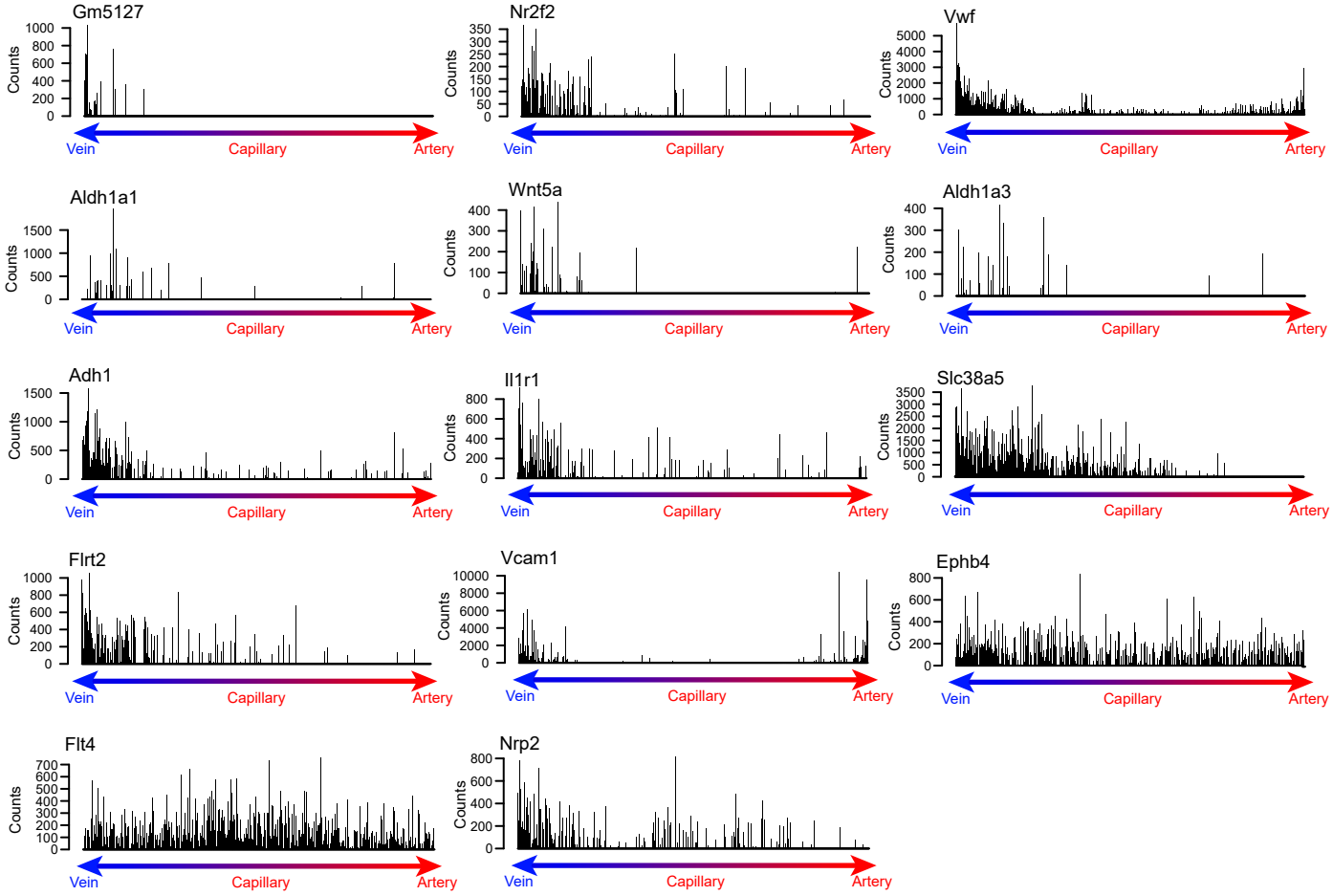
A. Timeline of the experimental procedure for 4 -hydroxytamoxifen (4 -OHT) administration and dissection. 4-OHT was administrated at indicated day and dissected at P7 (postnatal day 7). **B.** Tracing of mGFP-expressing TipECs (green) in the P7 retinal vasculature (Isolectin B4, pu rple) of $TipEC^{mReporter}$ mice at indicated time points after 4 -OHT administration. mGFP expressing TipECs at the vascular front were observed at 12hr after 4-OHT administration. After 48hr, mGFP expressing TipECs were incorporated into arteries (white arrowheads), but not veins (yellow arrowhead). **C.** mGFP expression in the main artery of $TipEC^{mReporter}$ mice indicates the incorporation of TipECs into arteries (white arrowheads), but not veins (96hr after 4-OHT administration). (OD : optic disc) **D.** Tracing of mGFP-expressing AECs (green) in retinal vasculature (Isolectin B4 , purple) of $AEC^{mReporter}$ mice at indicated time points after 4 -OHT administration. mGFP expressing arterial ECs were observed at 12hr after 4 -OHT administration. GFP expressing AECs remained arteries over time until 96hr. **E.** P25 retinal whole mount Immunostaining of $AEC^{mReporter}$ mice showing vasculature (Isolectin B4 , purple) and $mGFP^{+}$ ECs (green) on superficial and deep layer after 4 -OHT administration at P2.5.

Supplemental Figure II. VECs show strong proliferation activity.



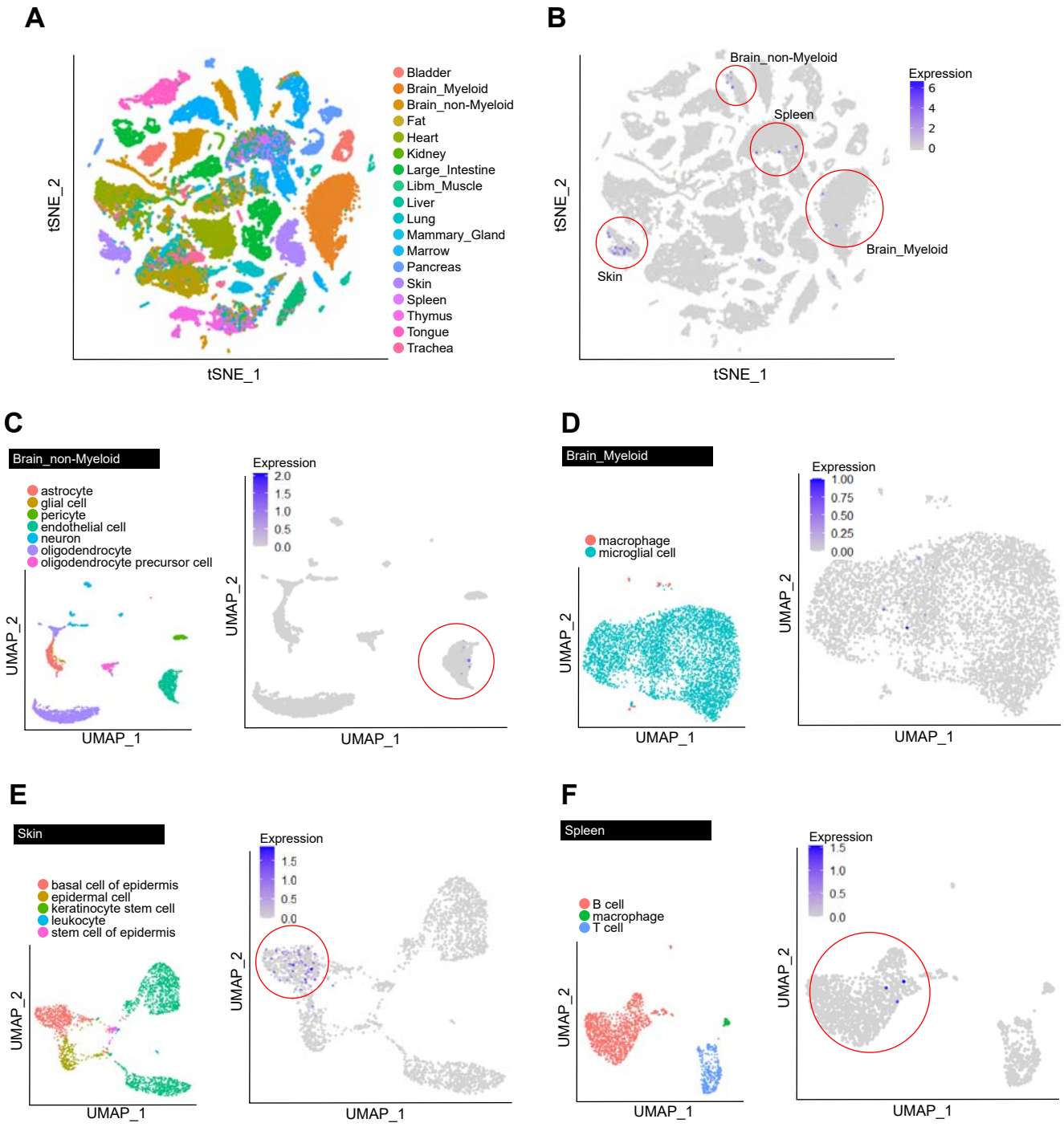
A. Confocal images showing proliferation (EdU) in retinal whole mounts at indicated stages. Upper panels show Isolectin B4 (red) and SMA staining (green); bottom panels show EdU staining (white) only. From left to right, retinal whole mount stainings at P2, P4, P6, P11, and P30 are shown. Note that proliferation is absent in arteries (green arrowheads) already at P4 but persists in veins (white arrowheads) until P11. At P30, no EdU-positive ECs were detected in the retinal vasculature. (OD : optic disc) (Scal e bars: 300μm) **B.** Confocal images showing IB4 (red), ERG1/2/3 (green) and EdU (cyan) staining in a vein of retinal vasculature. **C.** Quantification of relative diameter of veins in the retinal vasculature at indicated stages. (*n*=6, ns: not significant, one-way ANOVA followed by a multiple comparison procedure for pairwise comparisons).

Supplemental Figure III. Expression of VEC marker candidates across endothelial arteriovenous zonation



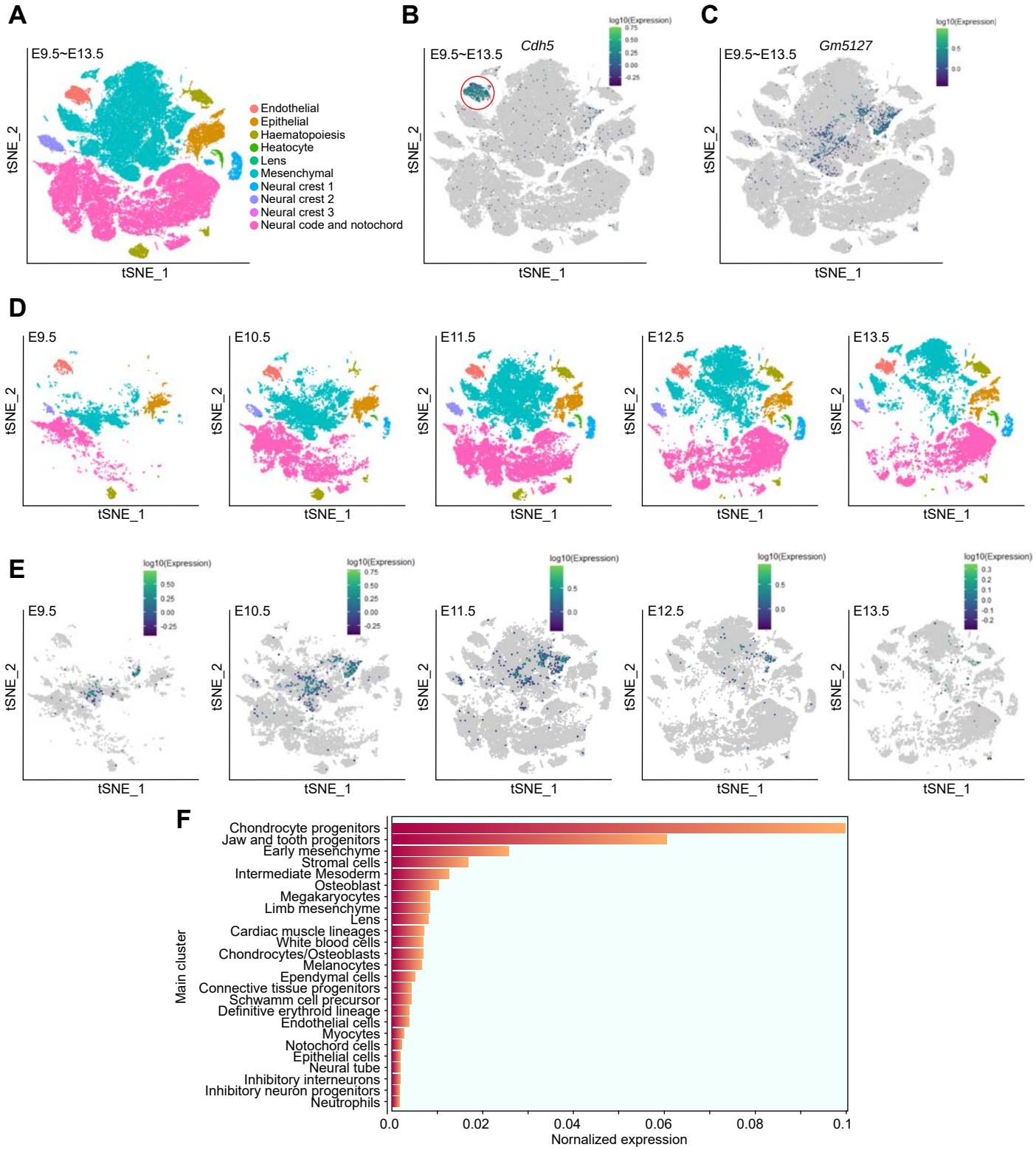
Bar plots showing endothelial zonal expression of various VEC mar ker candidates from scRNAseq dataset (Brain atlas) including Gm5127, Nr2f2, VwF. Aldh1a1, Wnt5A. Aldh1a3, Adh1, Il1r1, Alc38a5, Flrt2 Vcam1, Ephb4, Flt4 and Nrp2.

Supplemental Figure IV. Characterization of Gm5127 expression using Tamula Muris atlas



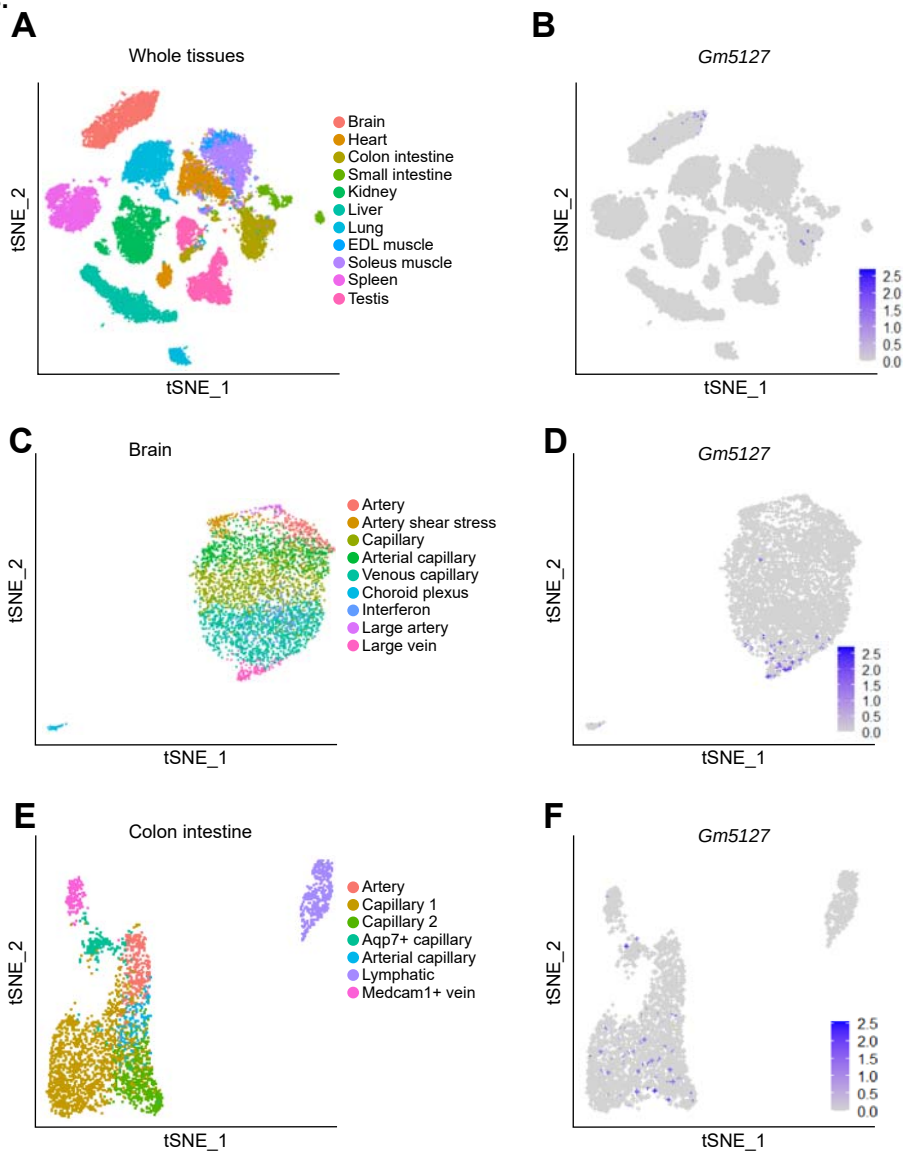
A. *t*-SNE visualization of scRNAseq atlas from 20 organs (colored by organ) (Tamula Muris atlas; 44,949 mouse cells). **B.** *t*-SNE visualization of cells colored by expression level of Gm5127. Four major organs (Brain non-MyeloiD. skin, spleen and brain Myeloid) expressing Gm5127 are indicated by red circle **C.** *t*-SNE visualization of brain non -myeloid cells. left: colored by cell ontology class, right: colored by expression level of Gm5127. Red circle indicates endothelial cluste r showing Gm5127 expression. **D.** *t*-SNE visualization of brain myeloid cells. left: colored by cell ontology class, right: colored by expression level of Gm5127. **E.** *t*-SNE visualization of skin cells. left: colored by cell ontology class, right: colored by expression level of Gm5127. Red circle indicates basal cell of epidermis cluster showing Gm5127 expression. **F.** t-SNE visualization of spleen cells. left: colored by cell ontology class, right: colored by expression level of Gm5127. Red circle indicates B cell cluster showing Gm5127 expression.

Supplemental Figure V. Characterization of Gm5127 expression in embryonic stages using MOCA atlas.



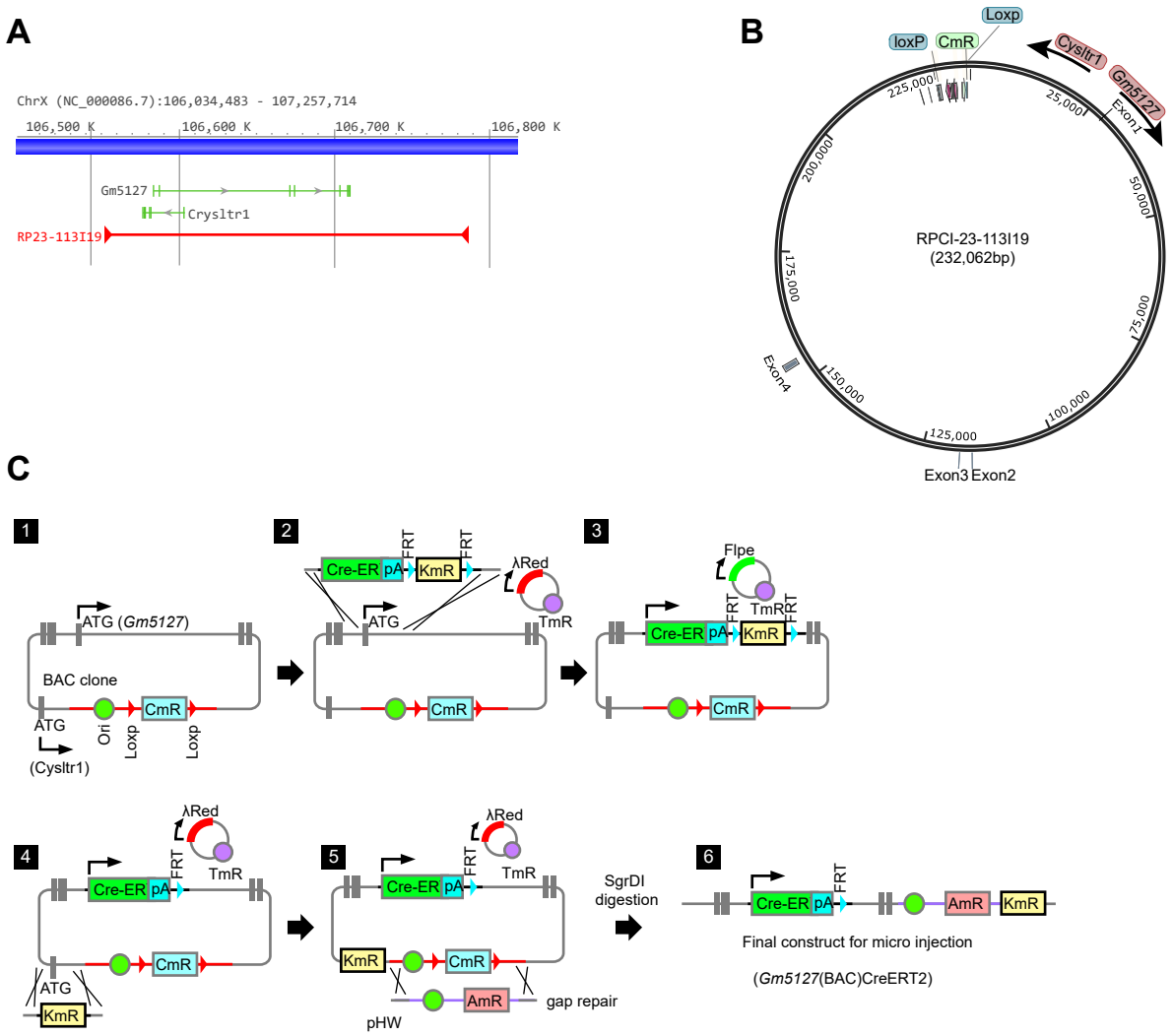
A. *t*-SNE visualization of 100,000 mouse cells from E9.5~13.5 (colored by main trajectory) (MOCA atlas). **B.** *t*-SNE visualization of cells colored by expression level of Cdh5 showing endothelial cluster (red circle). **C.** *t*-SNE visualization of cells colored by exp ression level of Gm5127. Note that most cells in the endothelial cluster do not express Gm5127 and Gm5127 -expressing cells are mainly located in the mesenchymal cluster. **D.** *t*-SNE visualization showing cells from each embryonic stage from E9.5 to E13.5 (colored by main trajectory) (cell numbers: n = 8,221 for E9.5; 19,331 for E10.5; 33,802 for E11.5; 26,183 for E12.5; 18,252 for E13.5) **E.** *t*-SNE visualization colored by expression level of Gm5127 for each embryonic stage (E9.5~E13.5). **F.** Bar plot showing the normalized expression of Gm5127 for top 25 cell types in MOCA altas. Note that endothelial cell is the 18 th cell type expressing Gm5127 across 38 major cell types.

Supplemental Figure VI . Characterization of Gm5127 expression in endothelial cells across various tissues.



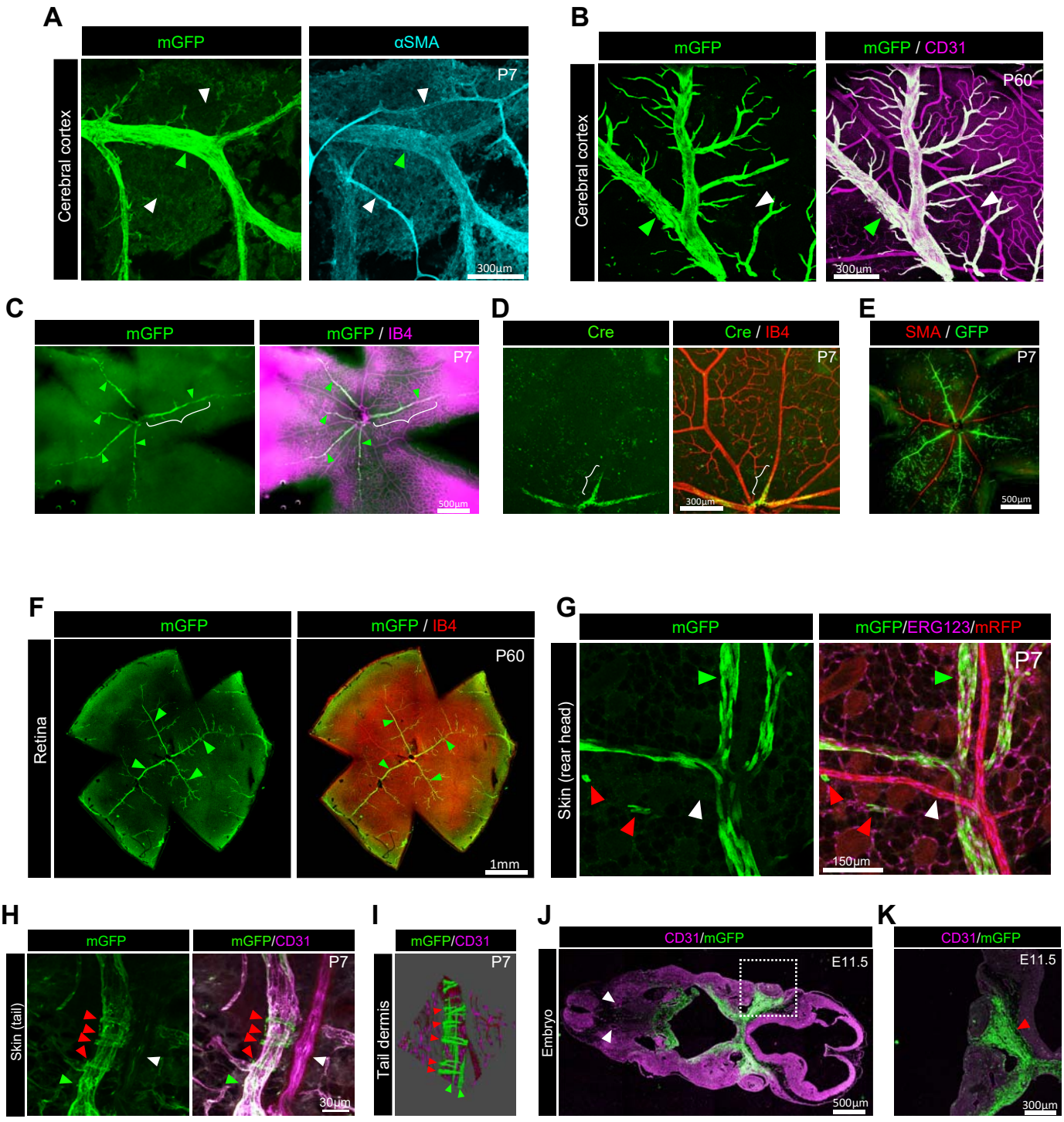
A. *t*-SNE Visualization of endothelial cells from 11 different organs (cell number = 32,567)(colored by tissue)(EC atlas). **B.** *t*-SNE visualization of endothelial cells colored by expression level of *Gm5127*. Note that only ECs fro m brain and colon intestine express *Gm5127*. **C.** *t*-SNE visualization of endothelial cells from brain (EC atlas) (cell number = 3,482)(colored by cell type). **D.** *t*-SNE visualization of endothelial cells from brain colored by expression level of *Gm5127*. Note th at the location of *Gm5127* expressing ECs is limited to large vein cluster. **E.** *t*-SNE visualization of endothelial cells from colon intestine (EC atlas) (cell number = 2,832)(colored by cell type). **F.** *t*-SNE visualization of endothelial cells from colon intestine colored by expression level of *Gm5127*. Note that the Gm5127 -expressing ECs are in capillary clusters, but not vein.

Supplemental Figure VII. A strategy for generation of *Gm5127* promoter-driven inducible Cre mice (*Gm5127*(BAC)CreER^{T2}) using BAC clone modification.



A. Display of genomic sequence for *Gm5127* in the NCBI genome browser (NCBI accession: NC_000086.7). *Gm5127* gene is located on the positive strand of XqD. while Crysltr1 gene is on the negative strand. Genes and their dire ction of transcription are indicated by green arrows and gray arrowheads, respectively. The genomic sequence inserted in the BAC clone (RP23-113I19) is indicated by red line. **B.** Original BAC clone containing genomic sequence of *Gm5127*. Exons for *Gm5127* are indicated as gray box and the start codons (*Gm5127* and *Crysltr1*) included in the clone are indicated by black arrows **C.** 1. Vector map showing schematic structure of BAC clone (RP23-113I19). 2. Cells with *λRed* recombinase expression plasmid and BAC clone were electroporated with PCR fragments containing CreER^{T2}/kanamycin resistant (*KmR*) cassette. 3. Cells were electroporated with a plasmid containing inducible *Flpe* proteins to remove *KmR* sequence flanked by two FRT sites. 4. Cells were electroporated with PCR fragments containing kanamycin resistant cassette to remove start codon of *Crsltr1*. 5. Modified genomic sequence for *Gm5127* was retrieved into pHW vector using gap repair recombineering. 6. After SgrDI digestion, linearized vector was used for microinjection.

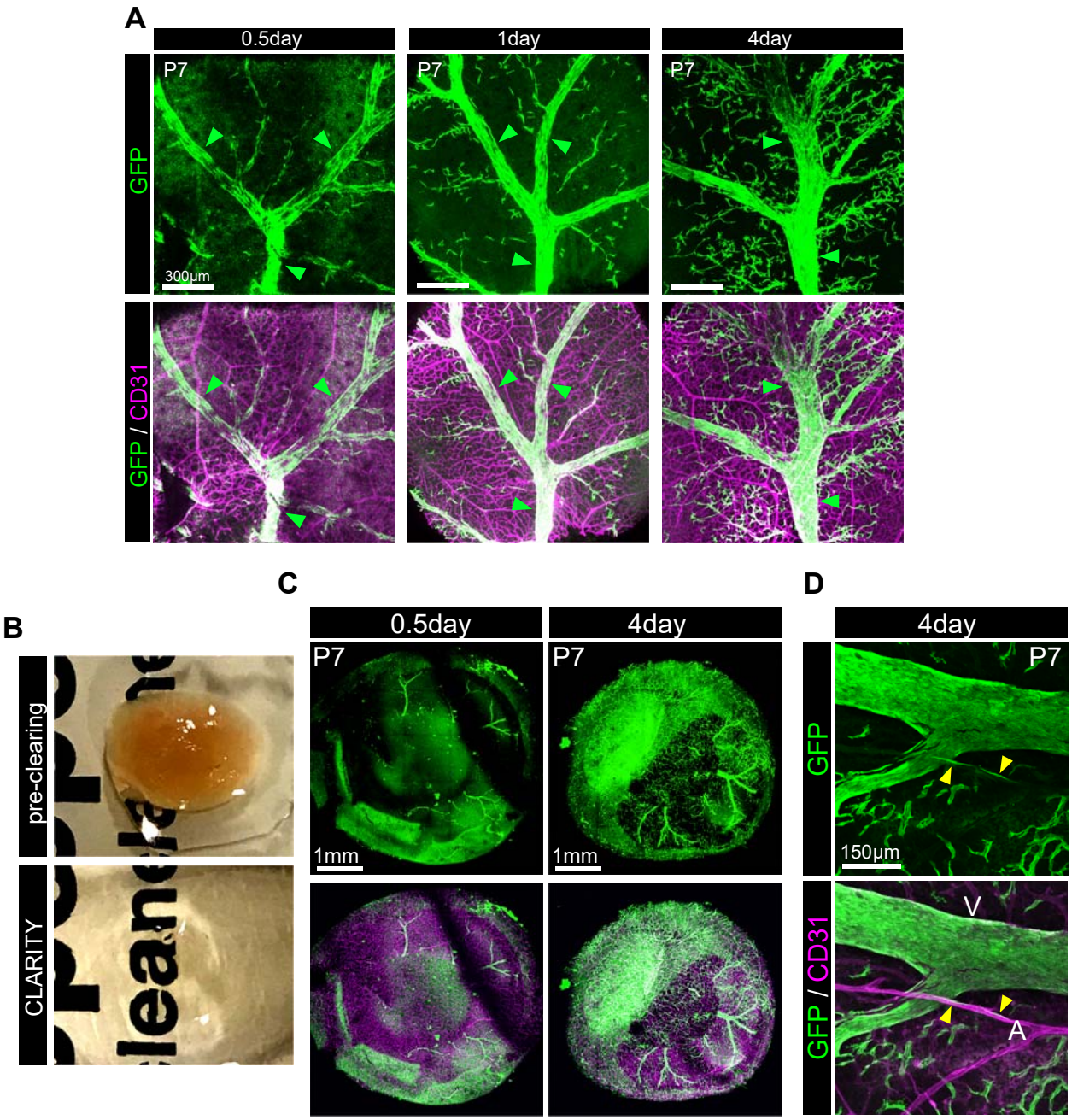
Supplemental Figure VIII. Characterization of Cre activity in *Gm5127*(BAC)CreER^{T2} mice.



A. Immunofluorescent staining of postnatal days 7 (P7) cerebral cortex of VEC^{mReporter} mice showing membrane-localizing GFP (mGFP) expression in veins (green arrowhead), but not arteries (white arrowheads) (αSMA: cyan). αSMA staining shows veins and arteries. **B.** Immunofluorescent staining of postnatal days 60 (P60) cerebral cortex of VEC^{mReporter} mice showing membrane-localizing GFP (mGFP) expression in veins (green arrowhead), but not arteries (white arrowheads). **C.** Immunofluorescent staining of postnatal days 7 (P7) retinal vasculature ofVEC^{mReporter} mice showing membrane-localizing GFP (mGFP) expression in veins (green arrowhead). White brace indicates the proximal vein. **D.** Isolectin B4 (IB4, red) and Cre Ab (green) staining showing the expression of Cre recombinase in the retinal vasculature of *Gm5127*(BAC)CreER^{T2} mice. White brace indicates th e proximal vein. **E.** Immunofluorescent staining of postnatal days 7 (P7) retinal vasculature of VEC^{mReporter} mice showing mGFP (green) in veins and αSMA in arteries (red).

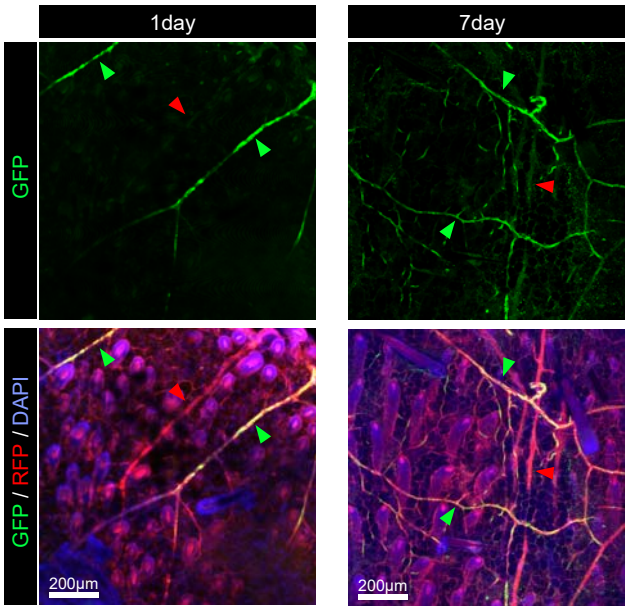
F. Whole mount adulthood (P60) retinal immunohistochemistry with Isolectin B4 (IB4, red) showing vein specific mGFP expression (green) in $VEC^{mReporter}$ mice. **G.** Whole mount postnatal days 7 (P7) skin (rear head) immunohistochemistry for ERG1/2/3 (purple) sho wing mGFP (green) and mRFP (red) expression. mGFP is expressed in venous ECs (green arrowhead) and basal cell of epidermis (red arrowheads), but not arterial ECs (white arrowhead). Note that mGFP showed discontinuous expression in vein. **H.** Whole mount pos tnatal days 7 (P7) skin (tail) immunohistochemistry with CD31 (purple) showing mGFP expression in veins (green arrowhead) and vSMCs (red arrowheads), but not arteries (white arrowhead). **I.** 3D reconstruction of confocal images from skin (tail) showing mGFP expression in veins (green arrowheads and vSMCs (red arrowheads). **J.** Transversal section of E11.5 embryo of $VEC^{mReporter}$ mice with CD31 staining (purple) showing mGFP expression (green). 4-OHT was administrated at 24hr before dissection. The area with white rectangle is shown at higher magnification in the panel g. White arrowheads indicates vena cava. Note that mGFP is not expressed in vena cava of $VEC^{mReporter}$ mice. **K.** Higher magnification images from transversal section of E11.5 embryo of $VEC^{mReporter}$ mice with CD31 staining (purple) showing mGFP expression (green) on head mesenchyme (red arrowhead).

Supplemental Figure IX. Lineage tracing of VECs in the brain vasculature.



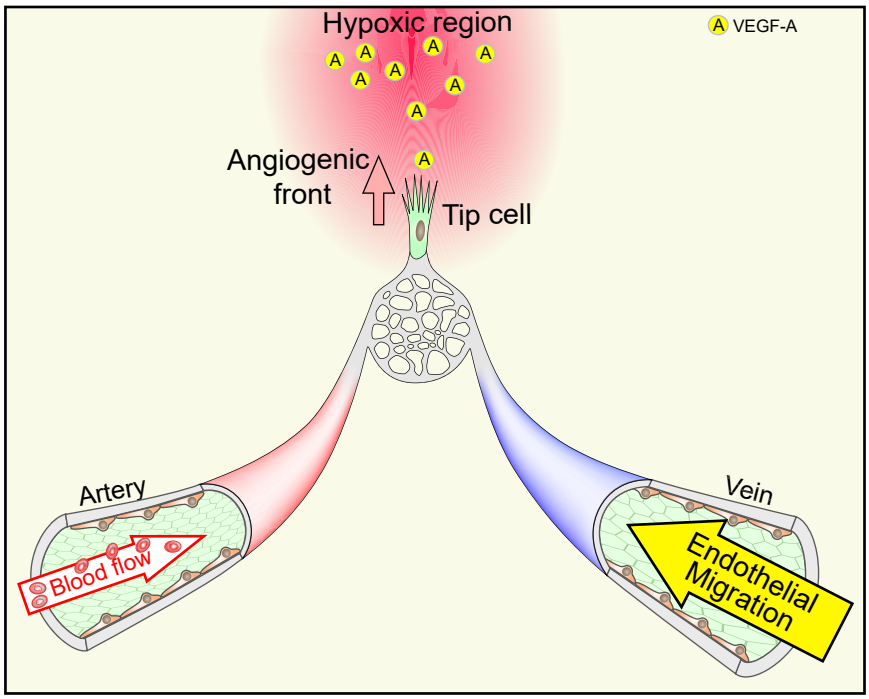
A. Tracing of mGFP-expressing VECs (green) in the P7 brain vasculature (Isolectin B4 , purple) of $VEC^{mReporter}$ mice over time (at 0.5 , 1 and 4 day after 4-OHT administration). Green arrowheads indicate veins. **B.** Images showing a non-treated (upper panel) versus CLARITY-cleared (lower panel) P7 mouse brain tissues. **C.** Extended view by XY projection of confocal images from CLARITY-cleared brain of $VEC^{mReporter}$ mice showing vasculature (CD31, purple) and $mGFP^{+}$ ECs (green). (left: 0.5 days after induction, right : 4 days after induction) **D.** Confocal images showing vasculature (CD31, purple) and GFP^{+} ECs (green) in the brain of $VEC^{mReporter}$ mice. Note that the presence of $mGFP^{+}$ AECs (yellow arrowheads).

Supplemental Figure X. Lineage tracing of VECs in the skin vasculature.



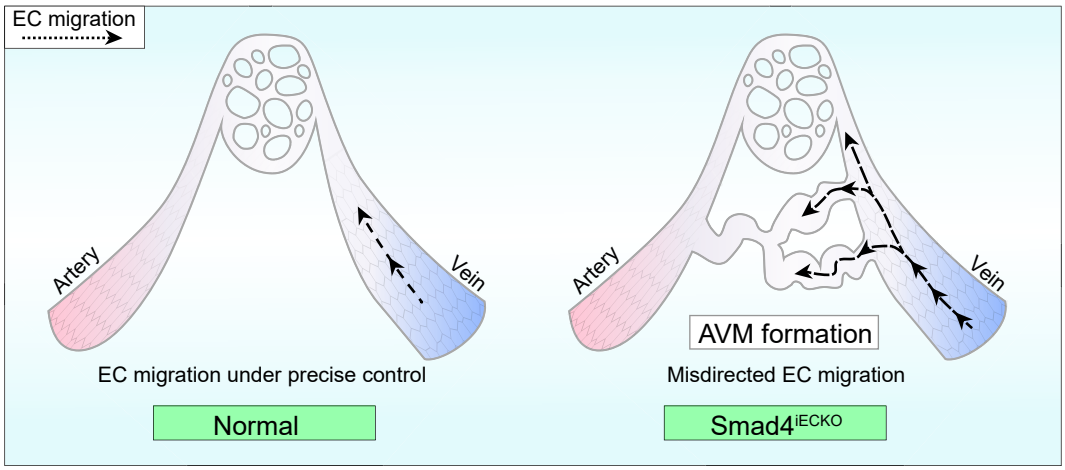
Representative images showing GFP+ ECs in the skin (rear head) of $VEC^{mReporter}$ mice (GFP: green, RFP: red. DAPI: blue, P10) at 1day (left) and 7 day (right) after 4 -OHT administration. Red and green arrowheads indicate arteries and veins, respectively.

Supplemental Figure XI. A working model for angiogenesis with endothelial migration and differentiation.



Cells in the avascular area exposed to hypoxia release growth factors including VEGF to initiate angiogenesis. Among 3 different endothelial subtypes (AEC. VEC and capiEC), VEC acts as primary EC subtype responding to VEGF signal. VEGF stimulate VECs and then VECs migrate into vascular front via reverse migration and differentiated into TipECs. Finally VEC-derived TipECs migrate against blood flow and differentiated into AECs.

Supplemental Figure XII. A working model for AVM formation.



In a normal vasculature, VECs sense blood flow and migrate against blood flow. In Smad4^{iECKO} mice, the migration of VEC is disturbed and the speed of VEC migration is accelerated compared to normal VECs. Finally, the misdirected VEC migration initiates AVM formation and results in abnormal connection between artery and vein (AV shunt).

Supplemental Movie I. Intravital imaging of endothelial cells in cerebral cortex vasculature.

Left panel shows structure of the vasculature in the cerebral cortex of P2 *Cdh5*(BAC)*Rosa26*^{nTnG} mice before 2-photon observation. Endothelial cells were labeled wi th nuclear localizing GFP (nGFP, green). White dotted-rectangle indicates the site of observation. Middle (nGFP) and right (nGFP/nTomato) panels show the cells with nuclear localizing-fluorescent proteins. Endothelial cells were labeled with nGFP (green) and non-endothelial cells were labeled with nTomato (red). White dotted-lines indicate vein and white arrow indicates the direction of blood flow. Cells in white-dotted circles indicate immobile non-endothelial cells showing the stage is remained in a same position over 5hr observation.