ONLINE SUPPLEMENT

Endoglin Deficiency in Bone Marrow Is Sufficient to Cause Cerebrovascular Dysplasia in the Adult Mouse after VEGF Stimulation

Supplemental Methods

Bone Marrow Transplantation

BM transplantation was performed as previously described.[1] Briefly, BM cells were collected from the tibia and femurs of 8 to 10-week-old male donor mice by flushing and aspiration with PBS containing 1% fetal bovine serum (FBS). Then, cells were centrifuged at 1,200 rpm for 10 minutes and resuspended in PBS at a concentration of 1×10^7/ml. Two hundred µl of cell suspension (2×10^6 cells) were immediately injected into lethally irradiated (9.7 Gy, GC3000 Irradiator, MDS-Nordion) recipient mice via the tail vein. To facilitate detection of BMDCs in the angiogenic foci, WT and *Eng*$^{+/-}$ [2] mice expressing enhanced green fluorescent protein (EGFP) were used as donors in the BM homing study.

AAV-VEGF Stereotactic Injection

AAV-VEGF viral vector was described previously.[3] CMV promoter was used to drive human $VEGF_{165}$ cDNA expression in the vector. The vector was packaged in an AAV serotype 1 capsid. This viral vector mediates VEGF expression in brain endothelial cells, neurons, and astrocytes.[3] AAV-VEGF was injected into the brain 4 weeks after BM transplantation.[1] After induction of anesthesia by isoflurane inhalation, mice were placed in a stereotactic frame with a holder (David Kopf Instruments), 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 2×10^9 genome copies (gcs) of AAV-VEGF were stereotactically injected into the right basal ganglia, 3 mm under the cortex, at a rate of 0.2 µl per minute using a Hamilton syringe. The needle was withdrawn after 10 min and the wound was closed with a suture.

Vascular Density and Dysplasia Index

Six weeks later, vascular density (the mean vessels obtained from six images) and dysplasia index (number of vessels >15 µm in diameter per 100 vessels) were assessed on two lectin-stained (1:200; Vector Laboratories) coronal sections per animal, 0.5 mm rostral and 0.5 mm caudal to the virus injection site 20 µm in thickness, by three blinded investigators using NIH Image 1.63 software.[1] Images for quantification were taken from three areas (to the right and left of and below the injection site) of each section under the 20X microscopic objective lens.

Immunohistochemistry

Mice were anesthetized with isoflurane inhalation and perfused with 4% paraformaldehyde (PFA). Brain samples were collected, frozen in dry ice, and sectioned at 20 µm in thickness (CM1900 Cryostat, Leica). Two coronal sections per mouse, 0.5 mm rostral and 0.5 mm caudal to the virus injection site, were chosen and stained with primary antibodies against CD68 (1:50, AbD Serotec) and CD31 (1:50, Abcam). Expression was subsequently detected by fluorescent secondary antibodies Alexa Fluor 594 goat anti-rat IgG (1:500, Invitrogen) for CD68 and goat anti-rabbit IgG (1:500, Invitrogen) for CD31. Images for quantification were taken as detailed above.

Monocyte/macrophage Culture

BM was harvested from tibias and femurs of 8-week-old WT and *Eng*$^{+/-}$ mice. BMDCs were grown in the medium containing mouse macrophage-colony stimulating factor (M-CSF; 7.5 ng/ml, Akron Biotech) and 10% FBS for 7 days.[4] The monocyte/macrophage-enriched culture was serum-starved in the macrophage serum-free medium (Invitrogen) for 6 hours. Then, the culture was treated with various concentrations of VEGF (0, 10, 50, and 100 ng/ml, R & D Systems) diluted in the macrophage serum-free medium for 18 hours.

Quantitative Real-time qRT-PCR

Total RNAs were isolated from WT and *Eng*$^{+/-}$ monocytes/macrophages using TRIzol reagent (Invitrogen) according to the manufacturer's protocol. cDNA was synthesized by reverse transcription using SuperScript III First-Strand Synthesis System kit (Invitrogen). TaqMan Gene Expression Assays (Applied Biosystems) was used for qPCR to quantify relative expression of each gene using Mx3000P QPCR System (Agilent Technologies). Predesigned qPCR primers are listed in Supplemental Table S1.

Confocal Microscopy

Confocal images were taken with a Spectral Confocal Microscope (Nikon) using three laser lines (405, 488, and 561 nm). Z-stacks were rendered into a three-dimensional (3D) image using the NIS-Elements AR 3.0 software (Nikon).

Statistical Analysis

Data are shown as mean ± SD. Two-way analysis of variance (ANOVA) was used to determine a statistical significance among groups, followed by pair-wise multiple comparisons using the post-hoc Bonferroni test. Student's t-test was performed when two groups were compared. A p value of <0.05 was considered statistically significant.

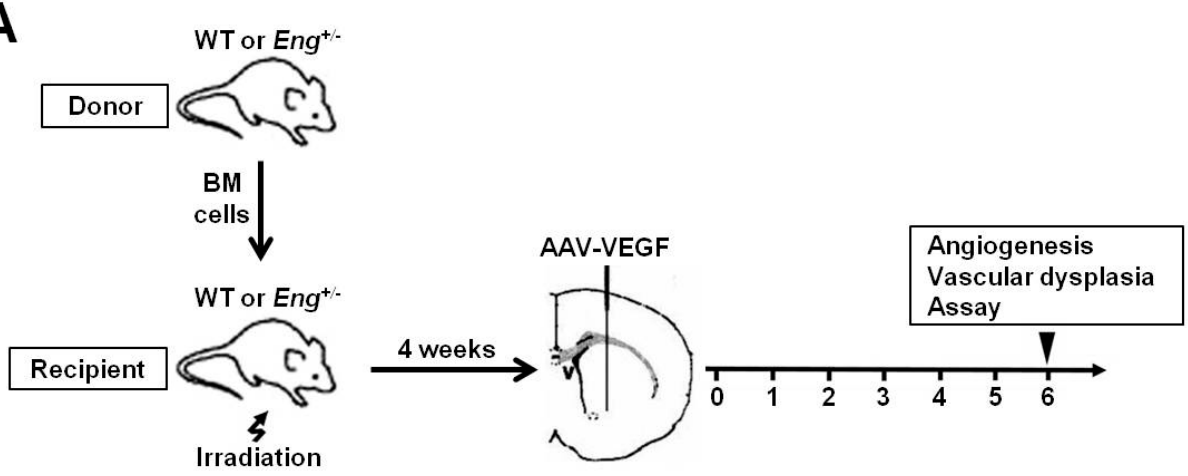
Supplemental Table

Gene	Name	Taqman Assay ID
Mmp9	matrix metallopeptidase 9	Mm00442991_m1
Notch1	Notch gene homolog 1 (Drosophila)	Mm00435249_m1
Gapdh	glyceraldehyde-3-phosphate dehydrogenase	Mm99999915_g1

Supplemental Table S1. TaqMan Gene Expression Assays primers used for qPCR.

Supplemental Figures

A



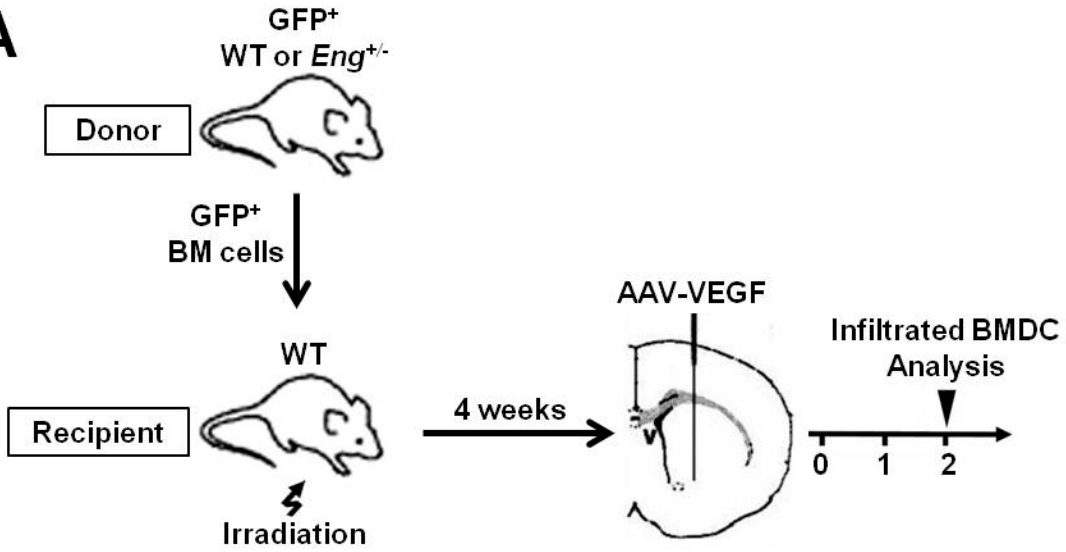
B

Groups	BM Recipient	BM Donor	VEGF	n
WT + WT BM	WT	WT	+	6
Eng + WT BM	*Eng*$^{+/-}$	WT	+	6
WT + Eng BM	WT	*Eng*$^{+/-}$	+	6
Eng + Eng BM	*Eng*$^{+/-}$	*Eng*$^{+/-}$	+	6

Supplemental Figure S1. Experimental design and groups for angiogenesis and vascular dysplasia analyses in the adult mouse brain.

(A) Experimental design for Figure 1. (B) Experimental groups for Figure 1.

A

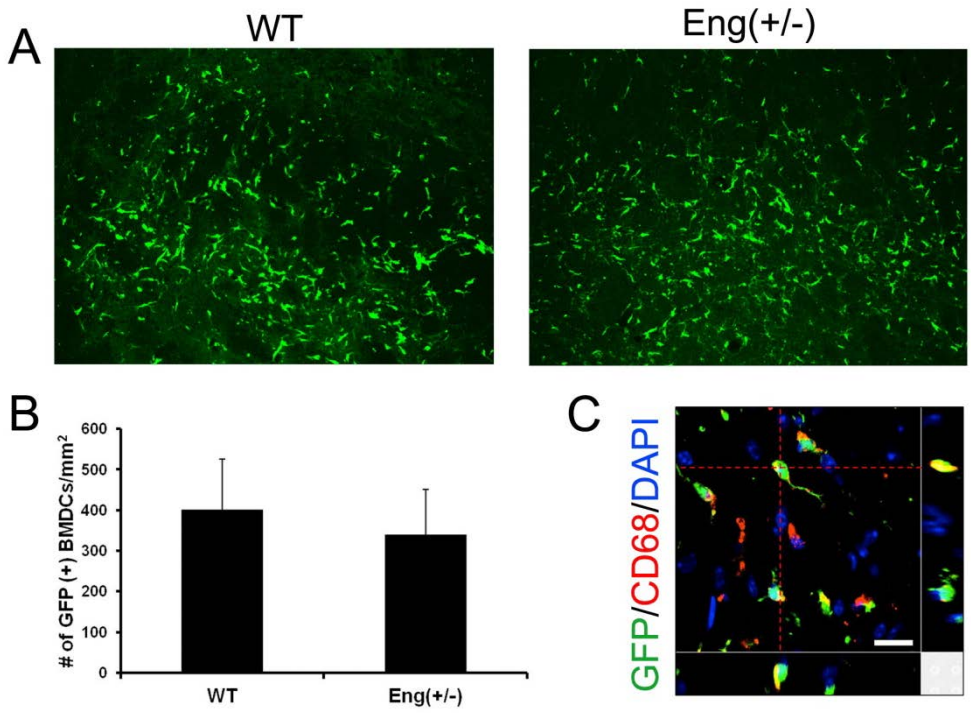


B

Groups	BM Recipient	BM Donor	VEGF	n
WT + WT BM	WT	GFP+ WT	+	6
WT + Eng BM	WT	GFP+ *Eng*+/-	+	6

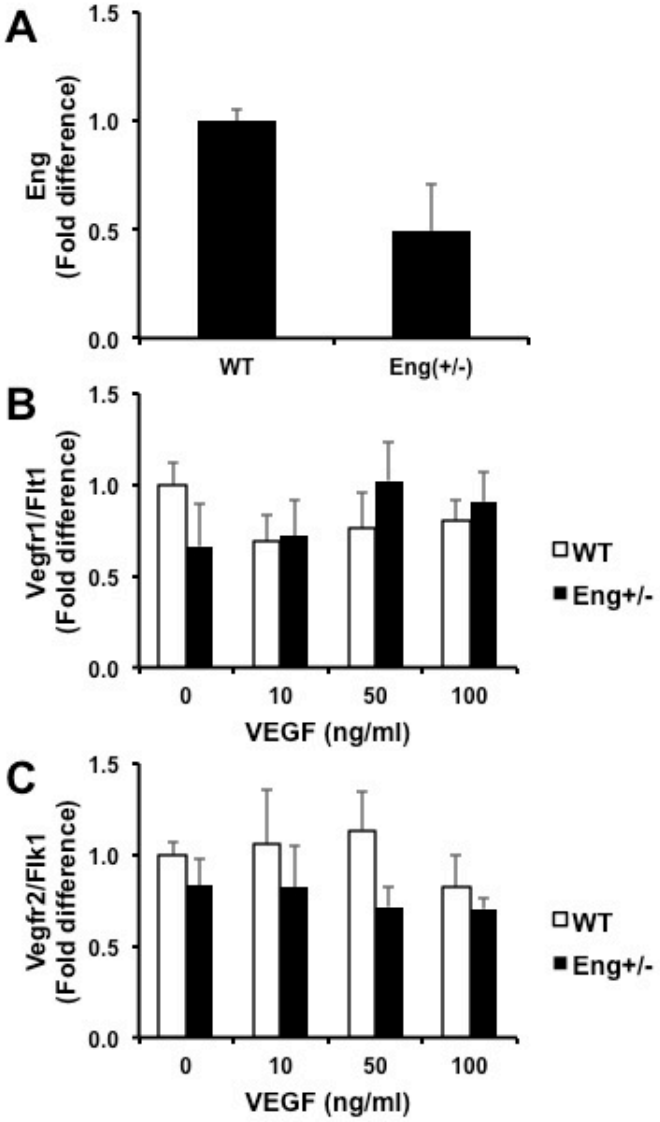
Supplemental Figure S2. Experimental design and groups for BMDC homing assay.

(A) Experimental design for Figure 2 and Supplemental Figure S3. (B) Experimental groups for Figure 2 and Supplemental Figure S3.



Supplemental Figure S3. WT and *Eng*$^{+/-}$ BMDCs had equivalent homing ability and most of BMDCs in the angiogenic foci were CD68^{+} monocytes/macrophages.

(A) Representative images of GFP^{+} WT and *Eng*$^{+/-}$ BMDCs in the brain angiogenic foci. (B) Bar graph shows quantification of recruited GFP^{+} BMDCs. Data: mean±SD. n=6 per group. (C) A representative confocal image shows co-localization of GFP/CD68/DAPI. Scale bar is 10 μm.



Supplemental Figure S4. *Eng*$^{+/-}$ monocytes/macrophages expressed a half level of *Eng* compared to WT and both *Vegfr1/Flt1* and *Vegfr2/Flk1/Kdr*.

(A) Basal *Eng* expression. Quantification of (B) *Vegfr1/Flt1* and (C) *Vegfr2/Flk1/Kdr* expression. Expression levels are relative to that of WT-untreated cells. Data: mean±SD from three independent experiments (n=3 per group).

Supplemental References

1. Hao Q, Liu J, Pappu R, Su H, Rola R, Gabriel RA, et al. Contribution of bone marrow-derived cells associated with brain angiogenesis is primarily through leucocytes and macrophages. Arterioscler Thromb Vasc Biol. 2008;28:2151-2157.
2. Arthur HM, Ure J, Smith AJ, Renforth G, Wilson DI, Torsney E, et al. Endoglin, an ancillary TGFbeta receptor, is required for extraembryonic angiogenesis and plays a key role in heart development. Dev Biol. 2000;217:42-53.
3. Shen F, Su H, Liu W, Kan YW, Young WL, Yang GY. Recombinant adeno-associated viral vector encoding human VEGF165 induces neomicrovessel formation in the adult mouse brain. Front Biosci. 2006;11:3190-3198.
4. Stanley ER. The macrophage colony-stimulating factor, CSF-1. Methods Enzymol. 1985;116:564-587.