# SUPPLEMENTAL MATERIALS

## Supplemental Methods

*Bone-marrow transplantation (BMT)*

Donor mice were strain- and age-matched to recipient mice aged 6-8 weeks. The BMT procedure was performed as described previously[1,2] with minor modifications. Briefly, the BM in recipient mice was ablated with lethal irradiation (12 Gy), then mice were transplanted with $5\times10^5$ donor BM cells, and the transplanted BM was allowed to regenerate for 4-6 weeks before subsequent experimental procedures were performed. $ApoE^{-/-}$ mice were fed a 1% cholesterol-containing Western diet after transplantation to maintain elevated serum cholesterol levels.

*Incorporation of infused EPCs*

The incorporation of systemically infused $ApoE^{-/-}$ and WT EPCs was evaluated in both $ApoE^{-/-}$ mice and WT mice. The infused EPCs were cultured from mouse BM,[1,2] and EPC transfusion was performed as described previously.[3] Briefly, mice were splenectomized to prevent homing of the transfused EPCs to the spleen. Seven days later, the carotid denudation procedure was performed, and $1\times10^6$ DiI-acLDL-labeled EPCs were injected into the tail vein. Mice were sacrificed 14 days after carotid denudation, and carotid arteries were harvested for *en face* histological assessments. Transfused EPCs were visualized with fluorescent microscopy as described previously.[3]

*Re-endothelialization*

Re-endothelialization was evaluated as described previously[4,5]; assessments were performed in 1) ApoE$^{-/-}$ and WT mice, 2) ApoE$^{-/-}$ mice transplanted with BM from ApoE$^{-/-}$ or WT donor mice, 3) WT mice transplanted with BM from ApoE$^{-/-}$ or WT donor mice, and 4) WT mice transplanted with BM from Tie2-Cre Notch1$^{+/-}$ or Tie2-Cre donor mice. Briefly, Evans blue dye (Sigma-Aldrich Co., St. Louis, MO, USA) diluted with saline (5%) was injected into the heart, the heart was perfused with saline to wash out the whole blood and excess dye, then the stain was fixed via perfusion of 4% paraformaldehyde (PFA) in phosphate-buffered saline (PBS). Ten minutes after PFA perfusion, mice were sacrificed, and carotid arteries were harvested for *en face* histological assessments; regions of damaged endothelium incorporate the stain and appear blue whereas re-endothelialized tissue is resistant to the dye. The areas stained blue and the unstained areas were outlined and measured with Image J software.

*Cellular contributions to atherosclerotic lesion formation*

The formation of atherosclerotic lesions was studied in ApoE$^{-/-}$ and WT mice transplanted with BM from Tie2-LacZ donor mice. Mice were sacrificed 2, 4, and 6 weeks after carotid artery denudation, then carotid arteries were harvested, paraffin embedded, and sectioned. Lesion formation was visualized with Masson's trichrome staining, then the intima/media ratio was calculated and reported as a percentage. Evidence of apoptosis and β-galactosidase (β-gal) expression (signaling the presence of BM-derived endothelial cells) or apoptosis and CD68 expression (signaling the presence of monocytes/macrophages) was evaluated via double immunofluorescent

staining. Sections were stained for apoptosis by using a fluorescein *in situ* cell-death detection kit (F. Hoffmann-La Roche Ltd, Basel, Switzerland) according to the manufacturer's instructions. β-gal expression was visualized by overnight incubation with rabbit polyclonal anti-β-gal antibody (1:1000, Cappel Laboratories Inc., Cochranville, PA, USA) at 4˚C followed by a 30-minute incubation with Cy3-goat anti-rabbit IgG antibody (1:2000, Jackson ImmunoResearch Laboratories Inc., West Grove, PA, USA). CD68 expression was visualized by overnight incubation with anti-CD68 antibody (1:100) (AbD Serotec, Oxford, UK) at 4˚C followed by a 30-minute incubation with Cy3-rabbit anti-rat IgG antibody (1:500, Jackson ImmunoResearch Laboratories Inc.). Nuclei were counterstained with DAPI (1:5000, Sigma-Aldrich, Co.), then sections were mounted in aqueous mounting medium, and images were examined under a fluorescent microscope (Nikon ECLIPSE TE200, Nikon Inc., Melville, NY, USA).

*EPC function (proliferation, adhesion, and migration) and apoptosis*

EPC functional assays were performed with EPCs isolated from the BM of ApoE$^{-/-}$ mice, WT mice, Tie2-Cre mice, and Tie2-Cre Notch1$^{+/-}$ mice. The effect of cholesterol on EPC function was determined by repeating the assays in the presence of 0, 50, 150, 300, and/or 500 mg/dL cholesterol (Sigma-Aldrich Co.).

Proliferation was evaluated by using a colorimetric MTS assay kit (Promega Corporation, Madison, WI, USA) according to the manufacturer's instructions. Cells ($1\times10^4$ cells/well) were seeded on 96-well plates and cultured in 0.5% fetal bovine serum (FBS) for 24 hours, then the culture medium was replaced with 5% FBS/EBM-2

medium supplemented with cholesterol. After 72 hours in culture, proliferation was visualized with MTS dye, and the optical density (OD) at 490-nm wavelength was measured in 8 wells with a plate reader. Proliferation was reported as the mean relative OD (i.e., normalized to the mean OD of WT or Tie2-Cre EPCs in the absence of cholesterol).

Adhesion was evaluated as described previously.[6] Briefly, EPCs ($2.5\times10^4$ cells/well) in 5% FBS/EBM-2 medium supplemented with cholesterol were seeded on 96-well plates precoated with vitronectin, collagen type I, fibronectin, and laminin (Sigma-Aldrich, Co.) and incubated for 1 hour at 37˚C under 5% carbon dioxide, then washed 3 times with PBS. The attached cells were fixed and stained with DAPI, then visualized under a fluorescent microscope (10× magnification). The adhered cells were counted in 8 wells, and adhesion activity was reported as the mean number of attached cells.

Migration was evaluated with a modified Boyden's chamber assay as described previously.[2] Briefly, cells suspended in EBM-2 medium supplemented with cholesterol were placed in the upper chamber ($5\times10^4$ cells/chamber), and the lower chamber was filled with medium containing 50 ng/mL recombinant mouse VEGF protein (R&D Systems, Inc., Minneapolis, MN, USA). The chamber was incubated for 16 hours at 37˚C under 5% carbon dioxide, then the EPCs that had migrated into the lower chamber were fixed with 2% PFA/PBS for 10 minutes and stained with DAPI. Migrated cells were viewed under a fluorescent microscope (40× magnification) and counted in 4 chambers,

5 HPFs per chamber. Migration activity was reported as the mean number of migrated cells.

Apoptosis was evaluated by the TUNEL method with an *in situ* cell-death detection kit (F. Hoffmann-La Roche Ltd) according to the manufacturer's instructions. Briefly, cells ($1\times10^5$ cells/well) were seeded on 4-well glass slides and cultured in 0.5% FBS for 24 hours, then the culture medium was replaced with 5% FBS/EBM-2 medium supplemented with cholesterol. After 48 hours in culture, cells were fixed with 2% PFA/PBS, TUNEL stained, and viewed under a fluorescent microscope (20× magnification). The number of TUNEL positive cells and the total number of cells were determined in 5 HPFs per well. Apoptosis was reported as the mean percentage of TUNEL-positive cells per HPF.

*Quantitative real-time RT-PCR*

EPCs were isolated from the BM of ApoE$^{-/-}$ and WT mice and lysed with RNA-Stat (Tel-Test, Inc., Friendswood, TX, USA), then RNA was extracted and reverse transcribed by using an iScript cDNA Synthesis Kit (Bio-Rad Laboratories, Hercules, CA, USA). Amplification was performed with a Taqman 7300 (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's instructions. Primer and probe sequences are indicated below. Relative mRNA expression was calculated with the comparative $C_T$ method (relative expression=$2^{\Delta CT}$) and normalized to the expression of the endogenous 18S gene.

VEGF primers: forward, CATCTTCAAGCCGTCCTGTGT; reverse, CAGGGCTTCATCGTTACAGCA. VEGF probe: CCGCTGATGCGCTGTGCAGG. Endothelial nitric oxide synthase (eNOS) primers: forward, TCTGCGGCGATGTCACTATG; reverse, CATGCCGCCCTCTGTTG. eNOS probe: AACCAGCGTCCTGCAAACCGTGC. Notch1 primers: forward, CGTGGTCTTCAAGCGTGATG; reverse, GCTCTTCCTCGTGGCCATAG. Notch1 probe: CAAGGCCAGCAGATGATCTTCCCG.

**Supplemental Table.** *Serum Cholesterol Levels (mg/dL)*[*]

| Mouse strain | Before Injury | Time After Arterial Injury (weeks) | | | |
|---|---|---|---|---|---|
| | | 1 | 2 | 4 | 6 |
| WT | 51.4±4.4 | 52.8±6.8 | 60.2±4.7 | 61.0±3.2 | 56.0±6.7 |
| ApoE$^{-/-}$ | 458.0±38.6 | 445.9±30.7 | 460.9±31.3 | 471.4±23.9 | 431.3±34.4 |

*Within each mouse strain, cholesterol levels before injury did not differ significantly from the levels observed at subsequent time points.

**Supplemental References**


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