Supplementary Data

Materials and Methods

Isolation and characterization of endothelial progenitor cells

The mice were sacrificed as described in the main text. Bone marrow (BM) was harvested by flushing the femurs and tibias. BM-derived mononuclear cells (BMNCs) were isolated using density gradient centrifugation (Lymphoprep 1.083, Sigma-Aldrich). The BMNCs were washed thrice in phosphate-buffered saline and then resuspended in EGM-2 MV BulletKit medium (Lonza) containing endothelial basal medium (EBM-2) and 5% fetal bovine serum and supplemented with recombinant human (rh) EGF, rhFGF-B, rhVEGF, rhIGF-1, ascorbic acid, and heparin. Next, the cells were seeded in cell culture flasks or plates (precoated with fibronectin) at a density of $4 \times 10^6/cm^2$ and were cultured at 37°C in 5% CO_2 in a humidified incubator. After 2 days of culture, the medium was changed for the first time; thereafter, the medium was changed every 3 days. To confirm the phenotype of the endothelial progenitor cells (EPCs), the cells were processed for immunofluorescent staining to evaluate the expression of VEGF receptor 2, CD34, and CD133 (Santa Cruz Technology). In addition, the cells were incubated with acLDL-Dil (10 mg/mL) and fluorescein isothiocyanate-labeled lectin (UEA-1, 10 mg/mL); cells positive for acLDL-DiI and UEA-1 were identified as EPCs.

Sequences of the oligonucleotides used to produce shRNA targeting Stim1 mRNA

5′-GATCCGCGACTTCTGAAGAGTCTACCTTCAAG
AGAGGTAGACTCTTCAGAAGTCGCTTTTTTG-3′
3′-AATTCAAAAAAGCGACTTCTGAAGAGTCTACC
TCTCTTGAAGGTAGACTCTTCAGAAGTCGCG-5′

Primer sequences

Stim1: 5′-CTTGGCCTGGGATCTCAGAG-3′ (fw)
5′-TCAGCCATTGCCTTCTTGCC-3′ (rev)
Orai1: 5′-CCATCGCTACCACCTTCA-3′ (fw)
5′-ACGTCCACAACCTCAACTC-3′ (rev)
TRPC1: 5′-ATTCGGGTGGACATTCTT-3′ (fw)
5′-GTGGATTATTGGGATGATTTGG-3′ (rev)

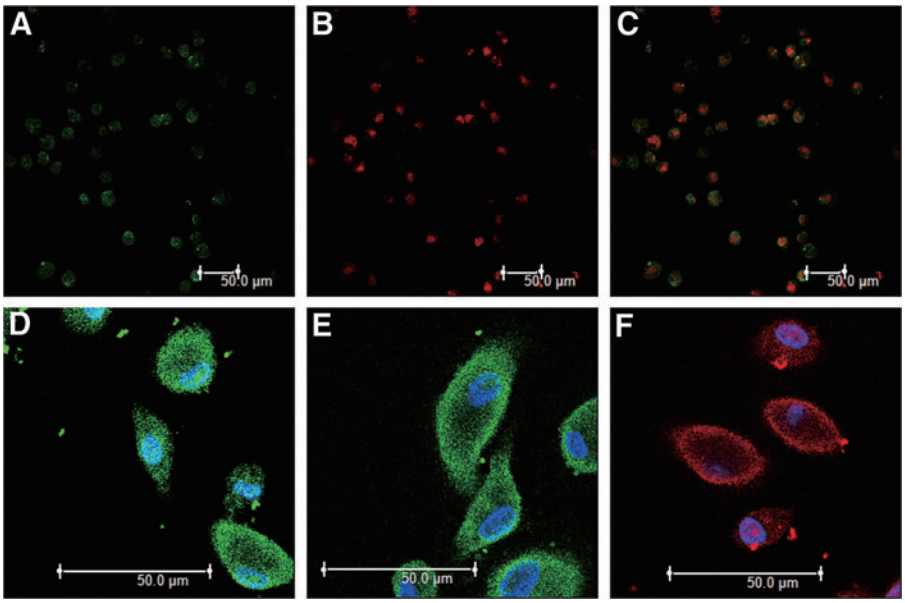
Results

Identification of EPCs

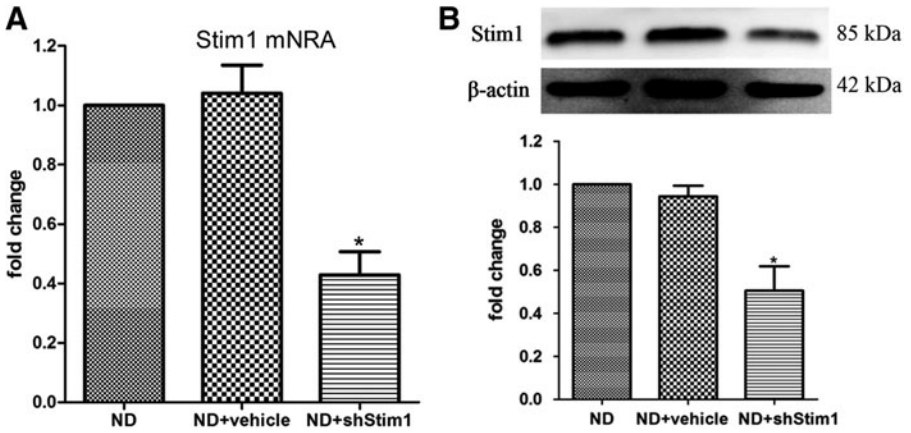
EPCs isolated from BM were cultured in EGM-2 MV BulletKit medium. After 6–7 days in culture, attached EPCs showed the ability to uptake DiI-LDL and bind UEA-1 (Supplementary Fig. S1A–C). Furthermore, immunofluorescence assays showed that the attached EPCs stained positive for CD34, CD133, and VEGFR2 (Supplementary Fig. S1D–F).

PI 3-kinase inhibitor (LY-294002) inhibited eNOS and Akt phosphorylation

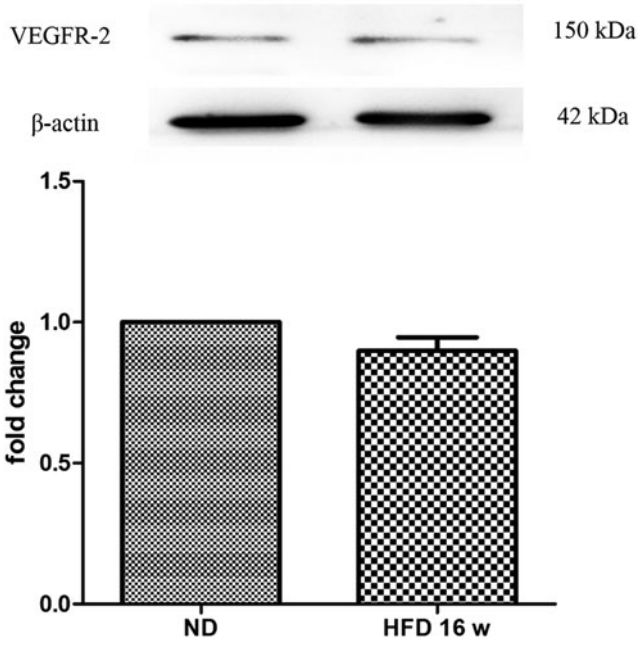
After exposure of EPCs to VEGF, the phosphorylation at Ser^{1177} of eNOS was markedly increased (Supplementary Fig. S4A, left). This effect was partly abolished by PI3K inhibition (Supplementary Fig. S4A, right). In addition, although total Akt levels were unaffected by VEGF, Ser^{473} Akt phosphorylation increased after exposure to VEGF (Supplementary Fig. S4A, left), and LY294002 attenuated VEGF-stimulated Ser473 Akt phosphorylation (Supplementary Fig. S4A, right). These data suggested that VEGF-induced eNOS phosphorylation occurs (at least partly) through the PI3K/Akt pathway.



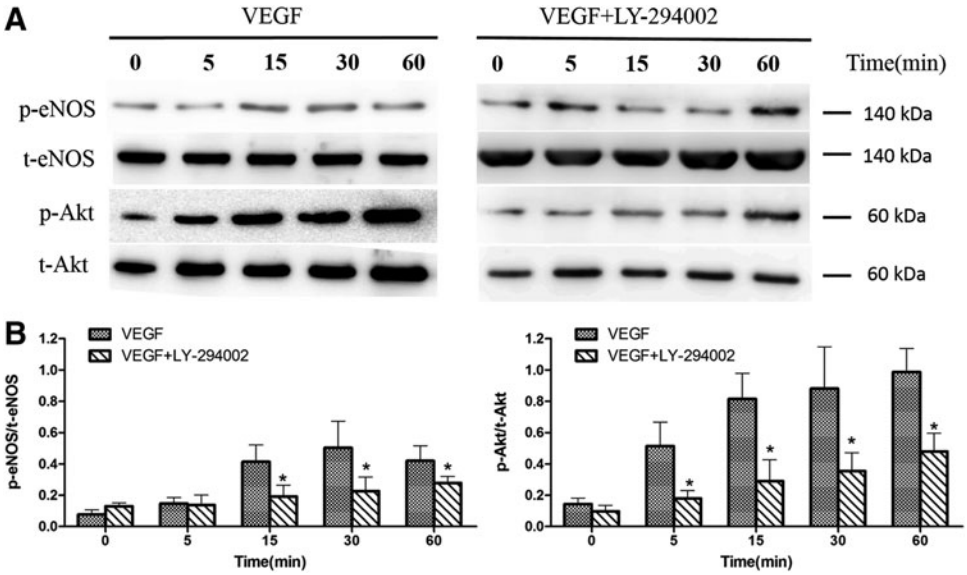
SUPPLEMENTARY FIG. S1. Characteristic endothelial phenotypes of the endothelial progenitor cells (EPCs). **(A)** EPCs stained with fluorescein isothiocyanate UEA-1 (lectin) (*green*). **(B)** Endocytosis of DiI-acLDL (*red*) by EPCs. **(C)** EPCs showed double-positive staining for DiI-acLDL and UEA-1. Immunofluorescence detection of CD34 (*green* **D**), CD133 (*green* **E**), and VEGFR2 (*red* **F**) in EPCs. The cells were counterstained with DAPI to label the nuclei (*blue*). Representative of three experiments.



SUPPLEMENTARY FIG. S2. Stim1 gene silencing in EPCs. Transfection of EPCs with shRNA targeting Stim1 markedly decreased Stim1 mRNA **(A)** and protein **(B)** levels after 72 h of transfection. $n = 3$, $P < 0.05$ veruss ND-EPCs.



SUPPLEMENTARY FIG. S3. Western blotting analysis indicated no significant differences in the levels of VEGFR expression in ND-EPCs and HFD-EPCs. $n = 3$.



SUPPLEMENTARY FIG. S4. LY-294002 inhibited VEGF-induced eNOS and Akt phosphorylation. **(A)** Representative western blots for the detection of phospho (p)-eNOS and p-Akt after treatment with VEGF alone or in the presence of LY-294002. **(B)** Quantitative temporal analyses of eNOS (*left*) and Akt (*right*) phosphorylation. $n = 3$, $^*P < 0.05$ versus VEGF treatment.