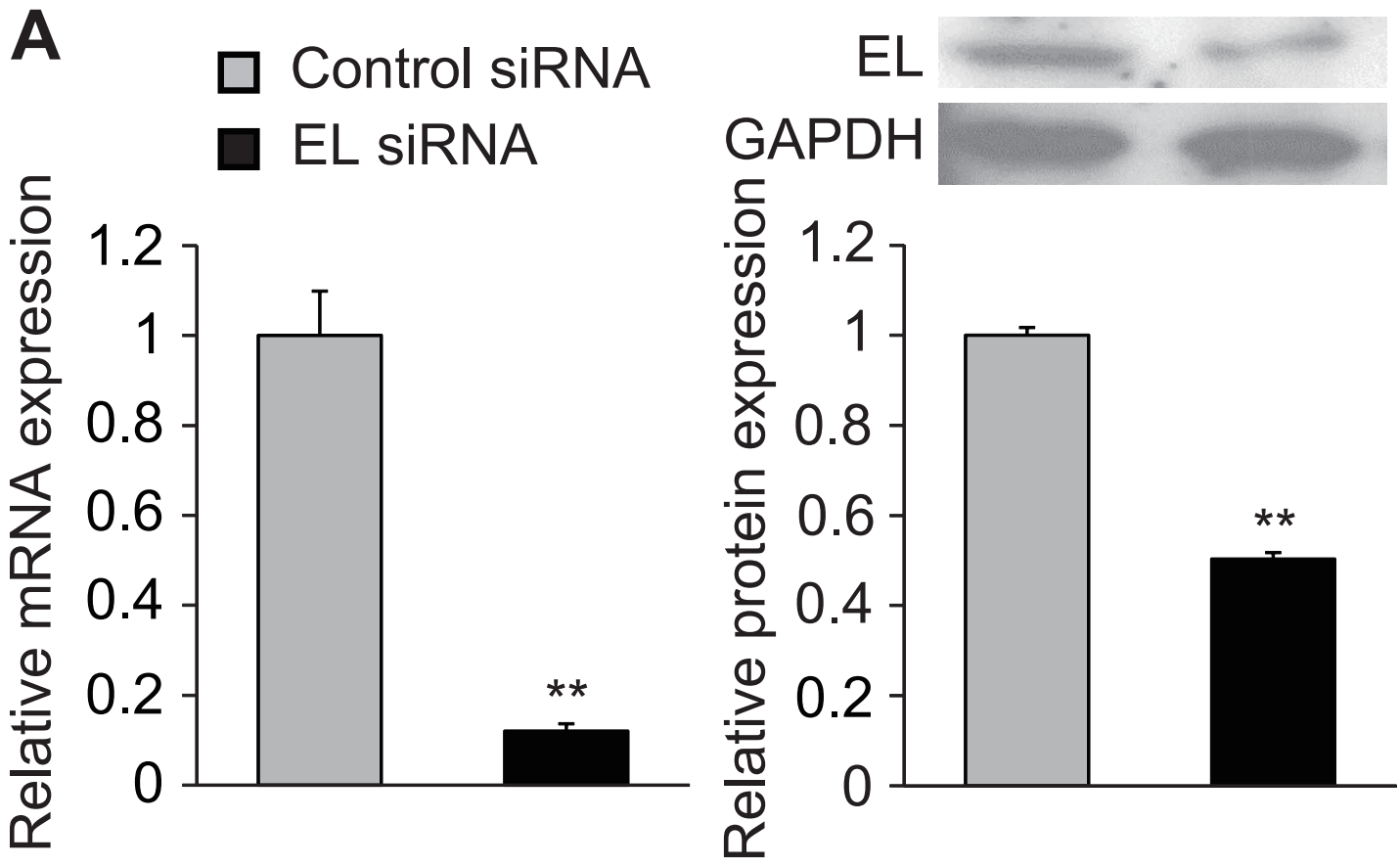
Supplement Material

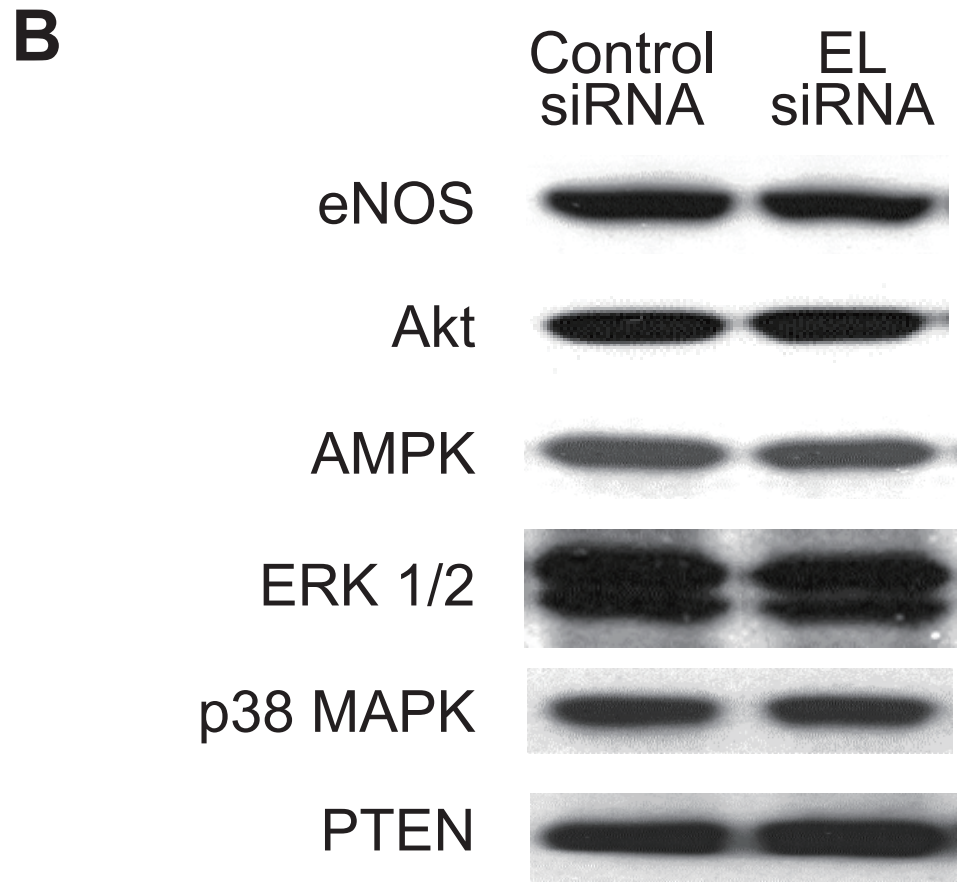
Supplement Figure Legends

Supplement Figure I. siRNA to Endothelial Lipase (EL) Decreased Expression and Protein levels of EL But Not Other Relevant EC Targets. A. EL siRNA treatment as outlined in Methods significantly decreased EL mRNA (90%, left panel) and protein (50%, right panel) levels as compared to control siRNA, as seen on RT-PCR and Western blotting. B. Similar EL and control siRNA EC treatment protocols as employed in 1A and throughout the manuscript did not alter mRNA or protein levels of eNOS, Akt, AMPK, ERK1/2, p38 MAPK, or PTEN (abbreviations as per manuscript).

Supplement Figure II. S1P Chromatograms. A. Endogenous and Exogenous, C17-labeled S1P Detection In Murine Plasma. HPLC tandem MS was performed on murine plasma from wildtype mice to which C17-labelled S1P had been added, yielding a precursor ion m/z 364 (C17 standard) and the product ion m/z 79 (phosphate head group). Comparison to wildtype plasma without the addition of exogenous S1P demonstrated a precursor ion m/z 378.2 with the expected product ion (m/z 79). B. S1P chromatograms on plasma from wildtype mice (n = 4). C. S1P chromatograms on plasma from EL-deficient mice (n = 6). The upper number on the S1P peak represents quantification of the S1P detected.

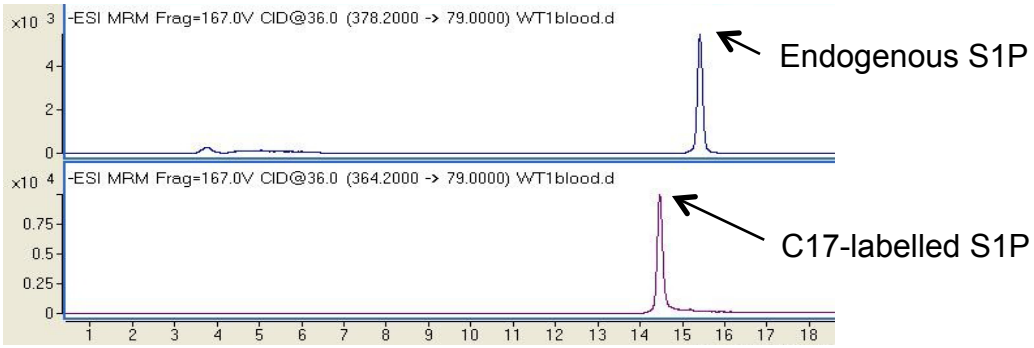
Supplement Figure I



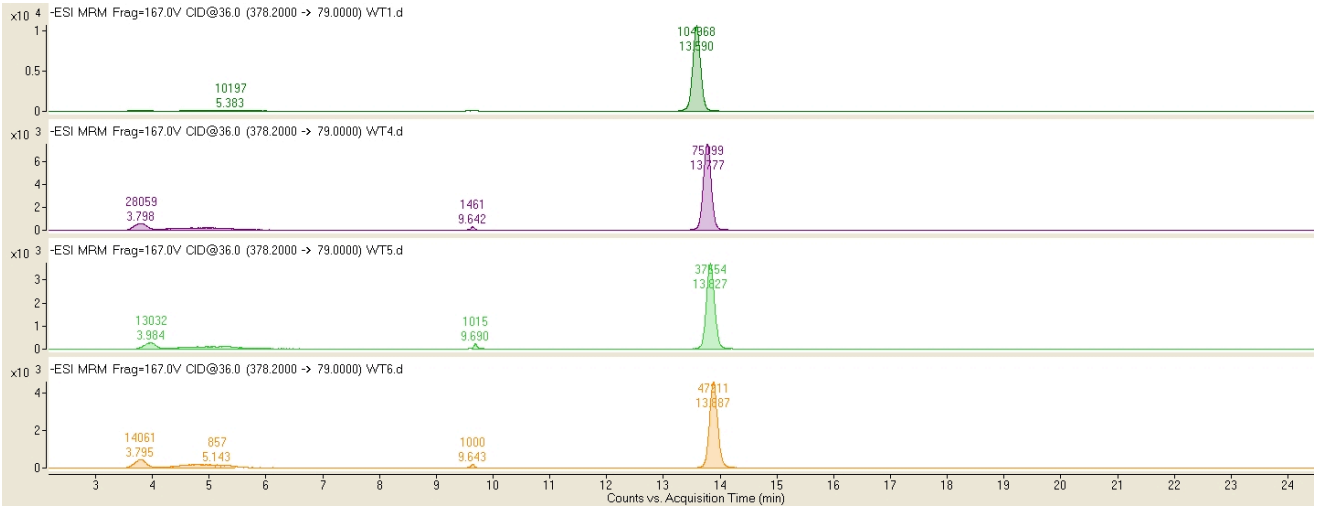


Supplement Figure II

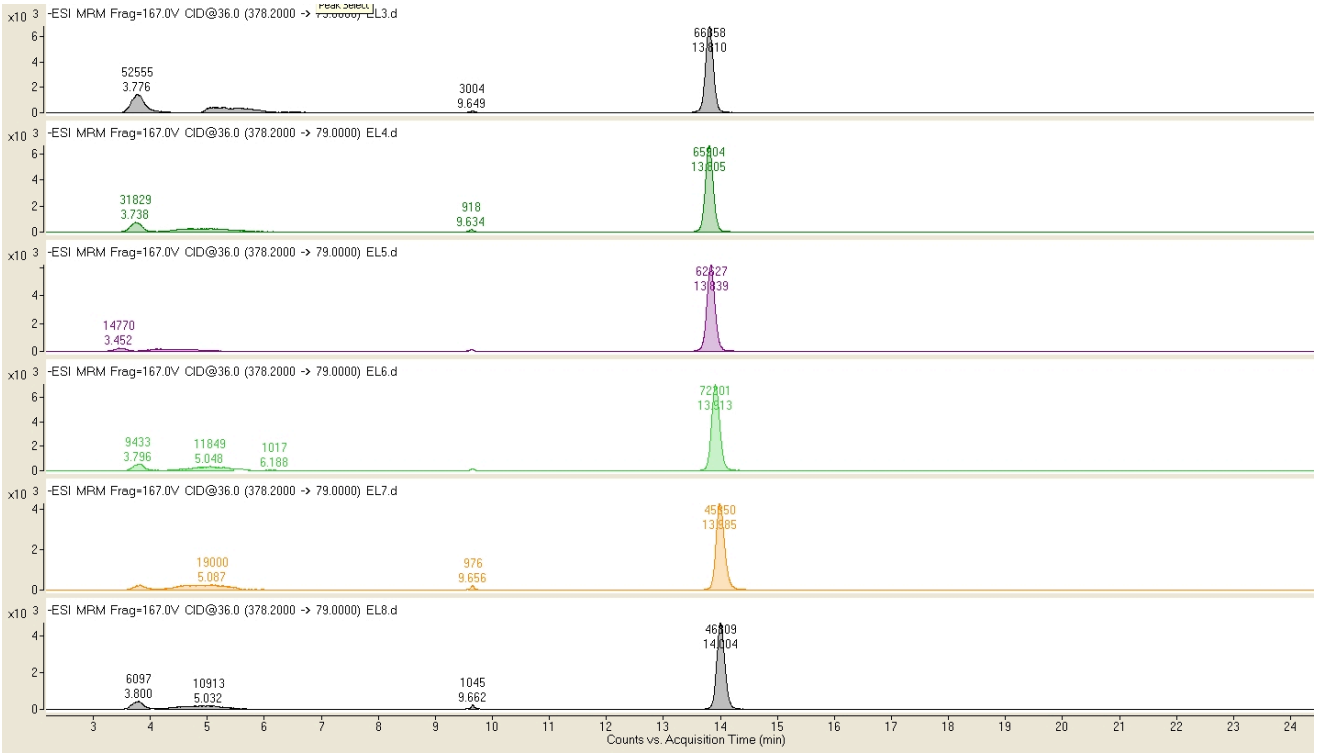
A. Chromatograms of wildtype murine plasma with and without addition of C17-labelled S1P



B. S1P Chromatograms of plasma samples from wildtype mice



C. S1P Chromatograms of plasma samples from EL-deficient mice



Methods

Reagents

Tetrahydrolipstatin was obtained from Roche Laboratories (Nutley, NJ). Pooled normal human HDL and LDL were from Sigma-Aldrich and Biomedical Technologies respectively (Stoughton, MA). Mouse anti-endothelial nitric oxide synthase (eNOS) and mouse anti-phospho-eNOS (Ser^{1177}) antibodies were from BD Biosciences (San Diego, CA). Rabbit polyclonal antibodies against Akt and phospho-Akt (Ser^{473}) were from Cell Signaling Technology (Danvers, MA). W146 and S1P were from Avanti Polar Lipids (Alabaster, AL). CAY10444 was from Cayman Chemical (Ann Arbor, MI). All other reagents were from Sigma (St. Louis, MO).

Cell culture

Bovine aortic endothelial cells (BAEC) were obtained from Cambrex (Walkersville, MD), grown in Dulbecco modified Eagle medium (DMEM) containing 10% fetal bovine serum (FBS), 1% glutamine, penicillin, streptomycin, and fungizone, and studied between passages 6 and 8 as before.[1, 2]

siRNA transfection

Small interfering RNA (siRNA, Ambion, Austin, TX) against endothelial lipase was 5'-ACGUGACAGCCAAAACCUUtt-3' and the negative control sequence was: 5'-AUUGUAUGCGAUCGCAGACtt-3'. BAEC were transfected with siRNA when cells were ~50–70% confluence. Transfected cells were maintained in antibiotic-free DMEM/10% FBS. Transfection with siRNA (30 nM) was done using Lipofectamine 2000 (0.15% vol/vol) following the manufacturer's protocol (Invitrogen, Carlsbad, CA) in media supplemented with 10% FBS 5 h after transfection; experiments were conducted 48 h after transfection.

Endothelial cell migration and proliferation assay

To assess the effect of HDL and EL on cell migration, co-culture inserts from Culture-Inserts (Ibidi, Madison, WI) were used. BAEC (5 x 10^5 cells/ml, 70 μl) were seeded in two cell culture reservoirs separated by a silicon insert. HDL concentrations chosen were based on prior published reports [3] and concentration-ranging studies performed here. After 18 h, the cells were washed with phosphate buffered solution (PBS) prior to serum starvation in serum-free DMEM containing .1% bovine serum albumin and no other mitogens. After 6 h, the inserts were removed, the cells were rinsed twice with PBS, and the culture medium replaced by fresh medium with vehicle, HDL (75 μg/ml), W146 (10 μM), CAY10444 (10 μM) or S1P (100 nM). Cell images were obtained using an inverted microscope (ECLIPSE TE 2000-U, Nikon, Tokyo, Japan) 12 h after insert removal and migration quantified using Image-Pro Plus software. The cells were incubated for 24 h, then trypsinized and counted using a hemocytometer to assess cell proliferation.

Cell viability assay

Cell viability was determined by a colorimetric assay using 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT, Sigma-Aldrich, final concentration 0.5 mg/ml). BAEC were plated in a 96-well plate at a density of 3×10^4 cells per well in 10% FBS media for 24 h. Three duplicate wells were set up in each sample. At least three independent experiments were carried out. The cells were incubated with MTT (4 h, 37ºC). The plate was gently agitated until the color reaction was uniform; OD_{570} nm was determined by microplate reader (SpectraMax Plus384, Molecular Devices, Sunnyvale, CA). Results shown are an average fold-change of cell viability relative to the control (control siRNA in vehicle).

Tube formation assay

24-well plates were coated with Matrigel (BD Biosciences, San Diego, CA, 250 μl/well) and incubated (30 min, 37ºC) for gel polymerization. Forty-eight hours after siRNA transfection, BAEC were trypsinized and re-plated on Matrigel-treated wells at a density of 3 X 10^4 cells/well in serum-free DMEM (250 μl) with vehicle or HDL (75 μg/ml). The cells were incubated (9 h, 37ºC, 5% CO_2 incubator) and imaged using phase-contrast microscopy (ECLIPSE TE 2000-U, Nikon). Four random fields of view (FOV)/well were examined and photographed by a blinded observer. For quantification, a node was defined as an aggregation of cells from which three or more tube-like structures originated; a tube was defined as a continuous stretch of at least two cells containing no more than two nodes. The total tube length was measured using Image-Pro Plus software. Each experimental arm was repeated in five wells.

Mice

Male homozygous EL-/- mice, which were backcrossed with the C57BL6/J strain for at least 12 generations, and age-matched C57BL6/J wild-type (WT) mice were used in this study as described previously, including 12-h light/dark cycle housing with food and water *ad libitum*.[4] All experimental procedures were conducted according to Harvard Medical School Institutional Animal Care and Use Committee protocols.

Aortic ring angiogenesis assay

Aortic ring angiogenesis assays were performed using thoracic aortas removed from 1–2 month old mice (WT and $EL^{-/-}$), following established methods.[5, 6] Aortas were transferred into a dish containing sterilized cold PBS. Peri-aortic fibro-adipose tissues were carefully removed under surgical microscopy, the aorta cut into rings (1 mm wide), and washed 5 times (Medium 199, Lonza, Allendale, NJ). A 96-well plate was coated with Matrigel (50 μl, BD Biosciences, San Diego, CA). After polymerization (37ºC), the aortic rings were placed in the wells and

covered with Matrigel (50 μl). After polymerization (30 min, 37ºC), 100 μl RPMI 1640 (Lonza, Walkersville, MD) was added to each well, either with or without HDL (75 μg/ml). After 4-5 days of culture, the aortas were photographed with a phase contrast microscope by a blinded observer. The area of EC outgrowth was analyzed using Image-Pro Plus software.

Western blot analysis

Western blotting was performed as before.[7] For cell extraction, cells were lysed in immunoprecipitation buffer (Boston BioProducts, Ashland, MA) containing protease and phosphatase inhibitor cocktails (both Roche Diagnostics, Indianapolis, IN). Proteins from whole cell lysates of BAEC were separated on 4-12% Bis-Tris gel (Invitrogen) and transferred to a polyvinylidene fluoride membrane (Perkin Elmer, Waltham, MA). After transfer, the membrane was blocked in 5% nonfat milk in Tris-buffered saline (TBS) plus 0.1% Tween 20 (1 h), probed with primary antibodies and washed (TBS-Tween20, 15 min, three times). The membranes were probed with secondary antibodies for 1 h and washed three times. The signal was detected using enhanced chemiluminescence (Perkin Elmer, Waltham, MA) using manufacturer's protocols.

mRNA isolation and real-time quantitative PCR (RT-qPCR)

RNA was isolated from BAEC using RNeasy (Qiagen, Valencia, CA) and quantified using NanoDrop spectrophotometry (Thermo Scientific, Wilmington, DE). cDNA was prepared using High Capacity cDNA Reverse Transcription Kit (Applied Biosystems). RT-qPCR was performed using iQ SYBR Green Supermix and iCycler Real-Time PCR Detection System (Bio-Rad). The sequences used for PCR were as follows: *LIPG*, 5'-CAGAGGATGAAGGATGCGATC-3' for forward primer and 5'-TGACACCAGTTTGTACAGCC-3' for reverse primer; *ACTB*, 5'-GGCACCCAGCACAATGAAGATCAA-3' for forward primer and 5'-

ATCGTACTCCTGCTTGCTGATCCA-3' for reverse primer. mRNA levels were normalized to β-actin as internal control.

Mass Spectroscopy for S1P Quantification

LC/MS/MS analysis was performed using an Agilent 6410 triple quadrupole mass spectrometer in negative ionization mode with an electrospray source ionization interface, adapting prior methods[8]. For LC analysis a Gemini (Phenomenex) C18 column (50 mm x 2.1 mm, 3 μm particle size, 100 angstrom pore) was used with a 50 μm steel mesh filter. Mobile phase A consisted of 95/5 water/methanol, mobile phase B 65/35/5 isopropanol/methanol/water, both supplemented with 0.1% ammonium hydroxide. The flow rate was 0.3 μl/min. The gradient started at 20% B and linearly increased to 100% B (45 minutes), was maintained at 100% B (10 minutes) before equilibrating at 20% B (5 minutes). MS was operated in multiple reaction monitoring (MRM) mode, monitoring for S1P using the precursor ion m/z 378.2 to 79 (phosphate head group) transition $[M - H]^+$. For the C17-S1P standard, the m/z transition was 364.2 to 79. Capillary voltage was set to 3.0 kV, the fragmentor voltage to 200 V with a collision energy of 35 V, with drying gas temperature 350 ºC, drying gas flow 10 L/min and nebulizer pressure was 45 psi. Plasma (80 μl) from wildtype (n = 3) and EL-deficient mice (n = 6) was extracted (modified Folch method) and aliquots injected and compared to control samples spiked with a C17-labelled S1P standard. Sensitivity for S1P measurement was in the high femtomole range. The integrated S1P peak area was normalized to the C17 standard peak. Results presented as mean ± SEM.

Statistical Analysis

Data are expressed as mean ± SEM. Results were analyzed by unpaired Student's *t* test or 1-way ANOVA, as appropriate, followed by Newman-Keuls post hoc test. A p value < 0.05 was considered statistically significant.

References for Material/Methods

1. Ziouzenkova O, Perrey S, Asatryan L, Hwang J, MacNaul KL, Moller DE, Rader DJ, Sevanian A, Zechner R, Hoefler G, Plutzky J. Lipolysis of triglyceride-rich lipoproteins generates PPAR ligands: evidence for an antiinflammatory role for lipoprotein lipase. *Proc Natl Acad Sci U S A.* 2003;100:2730-2735.
2. Ahmed W, Orasanu G, Nehra V, Asatryan L, Rader DJ, Ziouzenkova O, Plutzky J. High-density lipoprotein hydrolysis by endothelial lipase activates PPARalpha: a candidate mechanism for high-density lipoprotein-mediated repression of leukocyte adhesion. *Circ Res.* 2006;98:490-498.
3. Miura S-i, Fujino M, Matsuo Y, Kawamura A, Tanigawa H, Nishikawa H, Saku K. High density lipoprotein-induced angiogenesis requires the activation of Ras/MAP kinase in human coronary artery endothelial cells. *Arterioscler Thromb Vasc Biol.* 2003;23:802-808.
4. Ishida T, Choi S, Kundu RK, Hirata K-I, Rubin EM, Cooper AD, Quertermous T. Endothelial lipase is a major determinant of HDL level. *J Clin Invest.* 2003;111:347-355.
5. Reed MJ, Karres N, Eyman D, Vernon RB. Culture of murine aortic explants in 3-dimensional extracellular matrix: a novel, miniaturized assay of angiogenesis in vitro. *Microvasc Res.* 2007;73:248-252.
6. Sun J, Sukhova GK, Yang M, Wolters PJ, MacFarlane LA, Libby P, Sun C, Zhang Y, Liu J, Ennis TL, Knispel R, Xiong W, Thompson RW, Baxter BT, Shi GP. Mast cells modulate the pathogenesis of elastase-induced abdominal aortic aneurysms in mice. *J Clin Invest.* 2007;117:3359-3368.
7. Kanda T, Brown JD, Orasanu G, Vogel S, Gonzalez FJ, Sartoretto J, Michel T, Plutzky J. PPARgamma in the endothelium regulates metabolic responses to high-fat diet in mice. *J Clin Invest.* 2009;119:110-124.
8. Brown JD, Oligino E, Rader DJ, Saghatelian A, Plutzky J. VLDL hydrolysis by hepatic lipase regulates PPARdelta transcriptional responses. *PLoS One.* 2011;6:e21209.