Materials and Methods

Reagents

Chemicals were obtained from Sigma unless otherwise noted. Human lipoproteins (acetylated LDL, HDL) were obtained from Biomedical Technologies Inc (Stoughton, MA). The synthetic LXR ligand T0901317 was from Cayman Chemical. Human ApoAI was obtained from Meridian Life Sciences.

Cell Culture and stimulation

THP-1, HepG2 and HEK293 cells were obtained from American Type Tissue Collection. HEK293 cells stably expressing ABCA1 used to test FLAG-ORP6 functionality were cultured as previously described[1]. HepG2 and HEK293 cells were maintained in Dulbecco's Modified Eagle Medium (DMEM, Sigma) containing 10% fetal bovine serum (FBS) and 2% penicillin/streptomycin in 10 cm^2 dishes at 37°C and 5% CO_2. THP-1 cells were maintained in Roswell Park Memorial Institute (RPMI)-1640 media (Sigma) supplemented with 10% FBS and 2% penicillin/streptomycin in 10 cm^2 dishes at 37°C and 5% CO_2. Peritoneal macrophages from adult female C57BL/6 mice were harvested by peritoneal lavage four days after intraperitoneal injection of thioglycollate. ACK lysing buffer (Lonza) was used to lyse the red blood cells, according to the manufacturer's protocol. The cells were maintained in culture as an adherent monolayer in medium containing DMEM, 10% FBS, and with conditioned medium from L929 mouse fibroblasts (20%) as described[2]. For bone marrow-derived macrophage (BMDM) preparation, bone marrow cells were flushed from the femur and tibia of hind legs from mice, centrifuged and lysed as described above, and the resulting cells were seeded at a density of 1 x 10^6 cells per mL in DMEM containing 10% FBS and supplemented with 15% L929-conditioned medium as described[2]. Cells were allowed to differentiate until day 5, after which they were collected with 10 mM EDTA and were plated at a density of 1 x 10^6 cells per mL in DMEM containing 2% FBS and 7.5% L929-conditioned medium. THP-1 differentiation into macrophages was induced using 100 nM phorbol-12-myristate acetate (PMA) for 72h. Cells were stimulated with 37.5 µg/mL acetylated low density lipoprotein (acLDL) and 10 µM TO901317 at the time points as indicated in figure legends.

Animal Studies

Samples were obtained from mice and monkeys treated with control or miR-33 inhibitors and mimics as we previously reported[3-5]. All mouse experiments were approved by the Institutional Animal Care Use Committee of New York University Medical Center. Briefly, to assess the effects of dietary cholesterol in mice, 6 week old *Ldlr*$^{-/-}$ mice (Jackson Laboratory, n=5-6/group) were placed on chow or western diet (0.3% cholesterol and 21% (wt/wt) fat (from Dyets Inc) diet for 14 weeks[5]. To modulate miR-33 expression in C57BL/6 mice, 6 mice per group were retro-orbitally injected with lentiviral vectors encoding the miR-33 precursor (miR-33), anti-sense to miR-33 (anti-miR-33) or scrambled controls (scr-miR) (System Biosciences Inc) at 2x10^9 pfu/mouse in 100 µL PBS and mice were sacrificed after 6 days, as previously described[5]. For miR-33 inhibition in *Ldlr*$^{-/-}$ mice, 6 week old mice were fed a western diet for 14 weeks and then switched to chow and treated weekly with anti-miR33 or mismatch control oligonucleotides (10 mg/kg subcatenously (s.c.) Regulus Therapeutics) for 4 weeks as described[4]. Hearts were isolated and sectioned through the aortic root and CD68$^+$ plaque macrophages were isolated by laser capture microdissection as described[4]. All monkey studies were approved by Wake Forest University Health Science Institutional Animal Care and Use Committee. Male African green monkeys (n=5/group) were fed diets low (0.002 mg/kcal) or high (0.4 mg/kcal) in cholesterol for 10 weeks, and liver RNA was harvested as described[6]. For miR-33 inhibition, male African green monkeys (n=6/group) were fed a chow diet and treated weekly with anti-miR33 or mismatch control oligonucleotides (5 mg/kg s.c., Regulus Therapeutics) for 4 weeks as described[3]. Liver RNA was harvested and Affymetrix gene expression array analyses were performed as described[3].

RNA Analyses

RNA was extracted using Trizol and Direct-zol columns (Zymo Research). 1 µg RNA was reverse-transcribed using iScript cDNA Synthesis Kit (Bio-Rad), and RT-PCR analysis was conducted using

KAPA SYBR green Supermix and a Mastercycler Realplex (Eppendorf) and the following species-specific primers (m=murine, h=human, monk=monkey, F=forward, R=reverse): mGAPDH_F TGTGAGGGAGATGCTCAGTG; mGAPDH_R TGTTCCTACCCCCAATGTGT; hGAPDH_F GAAGGTGAAGGTCGGAGTC; hGAPDH_R GAAGATGGTGATGGGATTTC; MonkOSBPL6_F TACTGGGAGCTTCGAAAGGA; MonkOSBPL6_R AGTGGTTAGGGGCCTTGTCT; hOSBPL6_F TGTCTCAGGCACTCAACCAG; hOSBPL6_R GGCTCTCATTGGCTACTTGC; mOSBPL6_F CTGGAGACCAGGTTCTCTGC; mOSBPL6_R ATGTAGCGTCTTCGCGATCT; MonkOSBPL1a_F GATTGGAAGCAGTGACAGCA; MonkOSBPL1a_R TGGCATCGTGATCTTGGATA; hOSBPL1a_F GATTGGAAGCAGTGACAGCA; hOSBPL1a_R TGGCATCGTGATCTTGGATA; mOSBPL1a_F CTGAAGTTCTGGGGCAAGAG; mOSBPL1a_R CCTTGCCAAAAAGACCACAT; hABCA1_F GGTTTGGAGATGGTTATACAATAGTTGT; hABCA1_R CCCGGAAACGCAAGTCC; mABCA1_F AAAACCGCAGACATCCTTCAG; mABCA1_R CATACCGAAACTCGTTCACCC. Fold change in mRNA expression was calculated using the comparative cycle method ($2^{-\Delta\Delta Ct}$). For miRNA detection, 1 µg RNA was reverse-transcribed using Qiagen miScript Kit (Quiagen), and RT-PCR analysis was conducted using miScript SYBR Green PCR Kit with miScript Primer Assays.

siRNA, plasmid, miRNA and anti-miR transfections

THP-1 or HepG2 were transfected 50 nM miRIDIAN miRNA mimics (miR-33 or miR-27b) or with 60 nM miRIDIAN miRNA inhibitors (anti-miR-33) (Dharmacon) utilizing Lipofectamine RNAiMAX (Invitrogen). For OSBPL6 silencing in THP-1 macrophages, cells were transfected with ON-TARGETplus SMARTpool siRNA (Human OSBPL6, L-009437-01-005, ORF or Non-targeting Control Pool D-001810-10-20) using Lipofectamine RNAiMAX. All experiment control samples were treated with an equal concentration of a non-targeting control mimics sequence (Con miR) or inhibitor negative control sequence (Con Inh), for use as controls for non-sequence-specific effects in miRNA experiments.

Luciferase Reporter Experiments

hABCA1, hOSBPL6 3'UTR luciferase/renilla constructs were obtained from GeneCopoeia. Point mutations in the seed region of predicted miR-33 or miR-27b sites within the 3'UTR of hOSBPL6 were generated using Quickchange XL site-directed mutagenesis kit (Stratagene) according to the manufacturer's protocol. All constructs were confirmed by sequencing. HEK293 cells were cotransfected with a total of 1 µg of the indicated 3'UTR luciferase reporter vector and 50 nM miRNA mimic or control mimic using Lipofectamine 2000 (Invitrogen). Luciferase activity was measured using the Dual Glo Luciferase Assay System (Promega). Firefly luciferase activity was normalized to the corresponding renilla luciferase activity and plotted as a percentage of the control (cells co-transfected with the corresponding concentration of control mimic). Experiments were performed in triplicate wells of a 96-well plate and repeated at least three times. The hOSBPL6 promoter construct was obtained from GeneCopoeia. Deletion of the LXR response element (LXRE) was conducted using Quickchange XL Site directed mutagenesis kit (Stratagene) and verified by sequencing. HEK293 cells were cotransfected with a total of 1 µg of the promoter and Secreted Alkaline Phosphatase (SEAP) control plasmid using Lipofectamine 2000 (Invitrogen). The Secrete-Pair™ Dual Luminescence Assay Kit (GeneCopoeia) was used to analyze the activities of *Gaussia* Luciferase (GLuc) and SEAP of the dual-reporter system.

Chromatin Immunoprecipitation Assay

2 x 10^7 THP1 cells were differentiated with PMA for 3-5 days and treated overnight with 10 µM TO901317. Cells were treated with 1% formaldehyde to cross-link and the reaction was stopped with 0.125 M glycine, followed by washing in ice cold PBS. To collect nuclei, cells were pelleted and lysed using Farnham lysis buffer, and nuclei were then lysed using RIPA buffer with 1% SDS. Lysates were disrupted by passage through a 25g needle and sonicated until chromatin was sheared to 500-2000 bp fragments. Cellular debris was cleared by centrifugation and supernatants were collected to test sonication fragment size. For immunoprecipitation 30 µg of chromatin was incubated with a cocktail of 3 µg LXRa antibody and 3 µg LXRb antibody or isotype matched IgG as a control. Bound chromatin was

immunoprecipitated using protein G Dynabeads (Life Technologies). After immunoprecipitation beads/chromatin complexes were collected, washed and dissociated at 65°C and supernatant containing chromatin was incubated at 65°C overnight to reverse crosslinks. Finally, DNA was purified using PrepEase DNA Clean-Up Kit (Affymetrix, Santa Clara, CA) and 1 µL of DNA was used for quantitative PCR, as described above.

Fluorescence microscopy

For OSBPL6 intracellular localization, HEK293 cells were plated into 8-well Labtek chamber slides (ThermoScientific) coated with poly-D-lysine, and transfected with 500 ng of FLAG-ORP6 plasmid DNA alone or along with pEGFP-N1-VAPA(1-242) DNA (Addgene). FLAG-ORP6 was generated by cloning a N-terminal Flag-tagged human *OSBPL6* (isoform E, amplified by PCR from HEK293 cDNA) into the MluI/XmaI sites of the pWXLd plasmid (Addgene) in place of GFP. 48 h post-transfection, cells were fixed in 4% paraformaldehyde (PFA), blocked/permeabilized in 2.5% BSA/0.1% Triton X-100, and stained for 1-2h at room temperature with a monoclonal anti-FLAG antibody (F3165, Sigma) to visualize FLAG-OSBPL6 along with the indicated antibodies as markers of the endocytic pathway (EEA1 SC6414 Santa Cruz, Rab7 #50533 Abcam, LAMP1 #24170 Abcam) and the ER (Calnexin #2679P Cell Signaling). For Golgi staining, CellLight Golgi-RFP (C10593, Life Technologies) or an antibody against Giantin (Abcam ab24586) was used, according to the manufacturer's protocol. Co-localization of FLAG-OSBPL6 with subcellular organelle markers was assessed using a Leica SP5 confocal microscope. For neutral lipid and free cholesterol visualization, THP-1 macrophages were transfected with control or OSBPL6 siRNAs for 48h and subsequently treated with acLDL (50 µg/mL) in the presence or absence of ACATi (10 µM) for 24h, fixed in 4% PFA, and neutral lipids were stained using Bodipy 493/503 (Life Technologies, 10 µg/mL) in PBS for 1h or free cholesterol was stained using Filipin III (25 µg/mL, Fisher Scientific). For immunostaining of atherosclerotic plaques, frozen sections were fixed in cold acetone and permeabilized in 0.1% Triton X-100. Sections were incubated with anti-OSBPL6 (1:500, ab96286; Abcam) and anti-MOMA-2 (1:500, MCA519G AbD Serotec) to detect macrophages, followed by fluorescent goat anti-rabbit or goat anti-rat secondary antibodies (1:400 A-11008 or A-11081, Life Technologies). Sections were mounted and visualized using a Nikon Eclipse microscope. Where indicated, a short permeabilization with saponin was used prior to fixation to release cytosol and facilitate visualization of membrane-bound proteins; for this, cells were incubated for 1min with a cold solution of with 0.05% saponin in 80 mM K-PIPES buffer, pH = 6.8, 5 mM EGTA, 1 mM MgCl2 at 4°C, as previously described[7].

Electron microscopy

THP-1 macrophages treated with control siRNA or OSBPL6 siRNA were incubated for 24h with acLDL (50µg/mL) for 24h and fixed in glutaraldehyde prior to cryosectioning and EM imaging at the OCS Microscopy Core of the NYU Medical Center.

Lipoprotein Endocytosis

For lipoprotein uptake experiments, macrophages were incubated for 6h with LDL or acLDL (50 µg/mL) that was pre-incubated with ^{3}H-cholesterol (PerkinElmer, Waltham, MA) for 30min at 37°C to incorporate the radiolabel within the lipoprotein core (for a final concentration of 0.5 µCi/mL of ^{3}H-cholesterol). After 6h of uptake, unbound ^{3}H-cholesterol-LDL or ^{3}H-cholesterol-AcLDL was washed 3X with 1X PBS, cells were lysed in 0.5N NaOH and cell lysate radioactivity was quantified. Alternatively, DiI-LDL or DiI-acLDL (AlfaAesar) was used at 10 µg/mL for 4h in 2 mg/mL BSA medium for fluorescence microscopy visualization of lipoprotein endocytosis.

Gene expression in arteries from the Biobank of Karolinska Endarterectomies (BiKE)

Human healthy arterial and plaque tissues were part of the Biobank of Karolinska Endarterectomies (BiKE) at the Centre for Molecular Medicine, Karolinska Institute. Atherosclerotic plaques and clinical data were obtained from patients undergoing surgery for stable or unstable carotid stenosis at the Department of Vascular Surgery, Karolinska University Hospital, Stockholm, Sweden. Control normal

arteries (undiseased macroscopically atherosclerosis free-arteries, iliac and one aorta) were obtained from organ donors without any current or history of cardiovascular disease. All samples are collected with informed consent from patients, organ donors or their guardians. The study is approved by the Ethical Committee of Northern Stockholm. The BiKE study cohort demographics, details of sample collection, processing and analyses have been previously described[8]. Gene expression profiles were analyzed by Affymetrix HG-U133 plus 2.0 Genechip microarrays in n=127 patient plaque tissues and n=10 normal arteries. Robust multiarray average (RMA) normalization and correction for batch effect was performed and processed gene expression data was returned in log2-scale. The microarray dataset is available from Gene Expression Omnibus (GSE21545). miR-27b levels were analyzed by Taqman MicroRNA assays (Life Technologies) on n=19 atherosclerotic plaque samples and n=5 control arteries. Statistical analyses were done with the GraphPad Prism 6 software.

Cholesterol Efflux Assays

THP-1 cells or HepG2 cells were seeded in 24-well plates at a density of 1 x 10^6 cells per well. THP-1 cells were differentiated with PMA for 72h prior to transfection. Cells were transfected with siRNA or plasmid DNA using Lipofectamine2000 for 24h and labeled with 0.5 µCi/mL of ^{3}H-cholesterol (PerkinElmer, Waltham, MA) for 24h. Cells were washed twice with PBS and incubated with 2 mg/mL fatty-acid free BSA (FAFA, Sigma) in media for 24h prior to addition of 50 µg/mL human apoAI in FAFA media with or without the indicated treatments. Supernatants were collected after 24h and efflux was expressed as a percentage of total cell ^{3}H-cholesterol content (total effluxed ^{3}H-cholesterol+cell associated ^{3}H-cholesterol).

Human liver sample preparation

Liver tissue samples (n=200) and corresponding serum samples (n=200) from non-fasting patients were obtained and experimental procedures were performed within the framework of the non-profit foundation HTCR, including the informed patient's consent[9]. RNA from liver samples was isolated with TRIzol according to the manufacturer's instructions and 2 µg RNA was reverse transcribed as we described[10]. Absolute copy numbers of *OSBPL6* were determined in quadruplicate measurements per sample using plasmid standard curves and normalized to *GAPDH* gene expression. Serum levels of apoA1 were determined using an immunoturbidimetric assay (Beckman Coulter) and HDL cholesterol was quantified using an enzymatic assay (Beckman Coulter). Both assays were run on an AU5800 automated analyzer (Beckman Coulter) according to the manufacturers' instructions.

Statistics

Data are presented as mean ± the standard error of the mean (SEM). Statistical significance of differences was evaluated with the Student's t-test or one-way analysis of variance (ANOVA) for multiple group comparisons. For human liver samples, gene expression data were quantile normalized using the statistical software package "R 2.8.0" (www.r-project.org)[11]. Normality of distribution was tested using the Kolmogorov-Smirnov test implemented in the PRISM statistical software (GraphPad). No significant effects of age and sex on gene expression levels were detected. Spearman correlation coefficients between gene expression data and serum apoA1 and HDL cholesterol levels and levels of significance were calculated using the SAS statistic software (SAS Version 9.3, SAS Institute, Inc., Cary, NC, USA).

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