SUPPLEMENT MATERIAL

miR-758 regulates cholesterol efflux through post-transcriptional repression of ABCA1

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Materials

Human lipoproteins (acetylated LDL) were a kindly gift from Prof. M.A. Lasunción (Madrid, Spain). The LXR agonist TO901317 was purchased from Cayman Chemical. Human apoAI and HDL were isolated by standard ultracentrifugation protocols from human plasma.

miRNA Microarray Analysis

Mouse peritoneal macrophages were incubated in 0.5% FBS media and treated or not treated 120μg/ml AcLDL for 24 hours. Total RNA was extracted using Trizol (Invitrogen) and microRNA was purified from 40μg of total RNA using the RT^2 qPCR-grade miRNA Isolation Kit (SABiosciences). The purity and integrity of both the total RNA sample and the enriched miRNA was verified using the Agilent Bioanalyzer (Agilent Technologies, Santa Clara, CA). A total of 400 ng of miRNA was reverse transcribed with the RT^2 miRNA First Strand kit (SABiosciences) and used for each set of Human Whole Genome miRNA Array (SABiosciences). 96-well plates were analyzed on a BioRad iCycler (BioRad Laboratories) and results were analyzed was done using SABiosciences software. Each array was performed in triplicate from three independent experiments.

RNA isolation and quantitative miRNA and mRNA real-time PCR

Total RNA from cell lines and mouse tissues were isolated using TRIzol reagent (Invitrogen) according to the manufacturer's protocol. For miRNA quantification, total RNA was reverse transcribed using the RT2 miRNA First Strand kit (SABiosciences). Primers specific for human or mouse miR-758 (SABiosciences) were used and values were normalized to the human housekeeping genes SNORNA38B and mouse SNORNA142. Quantitative real-time PCR was performed in triplicate using iQ SYBR green Supermix (BioRad) on iCycler Real-Time Detection System (BioRad).

Primer sequences

ABCA1, 5'-GGTGATGTTTCTGACCAATGTGA-3' and 5'-TGTCCTCATACCAGTTGAGAGAC-3; SLC38A1, 5'-TGCAGTCCGACCTCCTTCACAAAT-3' and 5'-ATCACAGCAACAATGACAGCCAGC-3'; IGF1, 5'-TTGGGCAAGTCACTTCACCTCTCT-3' and 5'-ACCCTTACAGCAACCCAGGGTAAA-3'; NTM, 5'-AACGCAGTACAGCATCGAGATCCA-3' and 5'-ACTTGCACAATGAGGTGGACCCTA-3'; GLTSCRI1, 5'-ATGAGAGCAAACTGAGTGGCCTGA-3' and 5'-TGTGCAAATGCTCCAGGAAACAGG-3'; STXBP1 5'-TCCCAGATGCTGAAGAAGATGCCT-3' and 5'-AGAGTTTGTCTACGGTGCCTTGGT-3'; SPTLC2 5'-CGAGACCGGTCCTCTGCGGA-3' and 5'-AGGCTCTGTAGGCGGTGGCA-3'; EPHA7 5'-AAGCGGAAAACGCCCCCAGG-3' and 5'-AGAACCCACGGCCACAGGGT-3'.

Cell Culture

THP-1 (human acute monocytic leukemia cell line), HepG2 (human liver hepatocellular cell line), Huh7 (human hepatoma cell line), H4 (human neuroglioma cell line), CCF-STTG1 (human astrocytoma cell line), J774 (murine macrophage cell line), Raw (mouse leukaemic monocyte macrophage cell line), HEPA (mouse hepatoma cel line) and COS-7 (monkey kidney fibroblast cell line) cells were obtained from American Type Culture Collection. Bovine aortic endothelial cells (BAEC), human vascular smooth muscle cells (hVSMC) and mouse vascular smooth

muscle cells (mVSMC) were isolated with standard protocols. THP-1 and J774 cells were maintained in RPMI 1640 media (Sigma) supplemented with 10% fetal bovine serum (FBS) and antibiotics at 37°C and 5% CO2. THP1 was differentiate into macrophages using 100nM phorbol-12-myristate acetate (PMA) for 72h. BAEC, hVSMC, mVSMC, HepG2, HEPA and COS-7 were maintained in Dulbecco's Modified Eagle Medium (DMEM) containing 10% FBS and antibiotics. EAhy296 cells were grown in DMEM containing 10% FBS, antibiotics and supplemented with HAT (Sigma). Peritoneal macrophages from adult male C57BL/6J mice were harvested by peritoneal lavage four days after intraperitoneal injection of thioglycollate. The cells were maintained in culture as an adherent monolayer in medium containing DMEM, 10% FBS, and 20% L-cell-conditioned medium. Cells were stimulated with 120 μg/ml acLDL or 3 μM TO901317.

Mice

All animal experiments were approved by the Institutional Animal Care Use Committee of New York University Medical Center. Six-week old C57BL6, LDLr$^{-/-}$ mice were obtained from Jackson Laboratory. C57BL6 were placed on either a chow diet or high fat diet (HFD) for 5 weeks. A second group of LDL receptor (LDLR) knockout mice were placed on either chow or HFD for 12 weeks. Mice were fasted for 12-14 h and blood samples were collected by retro-orbital venous plexus puncture and sacrifice. Liver samples and peritoneal macrophages were collected and stored at -80ºC. Total RNA was isolated for miR-758 expression was analysis.

Western blotting

Cell were lysed in ice-cold buffer containing 50 mM Tris-HCl, pH 7.5, 125 mM NaCl, 1% NP-40, 5.3 mM NaF, 1.5 mM $Na_4O_7P_2$ and 1mM orthovanadate, 1 mg/ml of protease inhibitor cocktail (Roche) and 0.25 mg/ml AEBSF (Roche). Standard SDS-PAGE western blot analysis was conducted using mouse monoclonal antibody against ABCA1 (Abcam), rabbit polyclonal

antibodie against ABCG1 (Novus), and mouse monoclonal antibody against HSP90 (BD Bioscience). Secondary fluorescently labeled antibodies were used (Invitrogen) and protein bands were visualized using the Odyssey Infrared Imaging System (LI-COR Biotechnology).

Lipid analysis

Mice were fasted for 12-14 h before blood samples were collected by retro-orbital venous plexus puncture. Plasma was separated by centrifugation and stored at -80ºC. Total plasma cholesterol was enzymatically measured with the Amplex red cholesterol assay kit (Molecular Probes), according to the manufacture's instructions. Triglycerides were analyzed by an enzymatic assay kit according to the manufacturer protocol (Wako Chemicals).

SUPPLEMENTAL FIGURE LEGENDS

Supplemental Figure I. Western blot analysis of HepG2 transfected with Con-miR, miR-758, miR-33 and miR-758/miR-33

Supplemental Figure II. *Pri-miR-758* and *miR-758* expression is reduced in cholesterol-loaded macrophages. A, qRT-PCR analysis of *pri-miR-758* and *miR-758* from mouse peritoneal macrophages treated with AcLDL (Ac) or Chol:MCB (methyl-beta-cyclodextrin).

Supplemental Table I. miRNAs regulated by cholesterol loading in mouse peritoneal macrophages. Predicted miRNAs that target ABCA1 are represented in bold. The cut-ooff for fold change were extablished at ± 1.3 fold.

ILMN_GENE Data are the mean ± SEM of three independent experiments in triplicate. *, *P*<0.05 from control.	***p*-value**	**Fold Change**
mmu-miR-696	0.0045	2.65
mmu-miR-291b-3p	0.0537	1.91
mmu-miR-200a*	0.0271	1.73
mmu-miR-129-3p	0.0035	1.54
mmu-miR-296-5p	0.0928	1.36
mmu-miR-291b-5p	0.0353	-4.61
mmu-miR-672	0.1815	-3.05
mmu-miR-297b-5p	0.0417	-2.78
mmu-miR-673-5p	0.2038	-2.22
mmu-miR-880	0.0901	-2.21
mmu-miR-539	0.0567	-2.12
mmu-miR-543	0.0739	-2.03
mmu-miR-758	0.0808	-1.84
mmu-miR-33	0.0485	-1.65
mmu-miR-122	0.3893	-1.54

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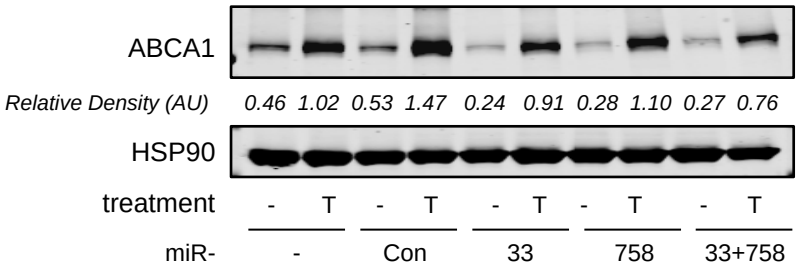
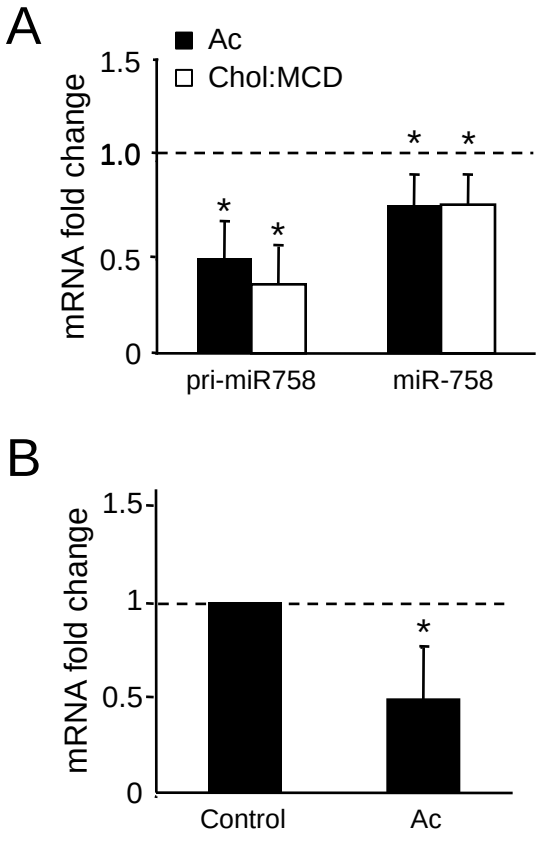


Supplemental Figure I



Supplental Figure II