MicroRNA 302a Is a Novel Modulator of Cholesterol Homeostasis and Atherosclerosis

Svenja Meiler, Yvonne Baumer, Emma Toulmin, Kosal Seng, William A. Boisvert

Objective—Macrophage foam cell formation is a key feature of atherosclerosis. Recent studies have shown that specific microRNAs (miRs) are regulated in modified low-density lipoprotein–treated macrophages, which can affect the cellular cholesterol homeostasis. Undertaking a genome-wide screen of miRs regulated in primary macrophages by modified low-density lipoprotein, miR-302a emerged as a potential candidate that may play a key role in macrophage cholesterol homeostasis.

Approach and Results—The objective of this study was to assess the involvement of miR-302a in macrophage lipid homeostasis and if it can influence circulating lipid levels and atherosclerotic development when it is inhibited in a murine atherosclerosis model. We found that transfection of primary macrophages with either miR-302a or anti–miR-302a regulated the expression of ATP-binding cassette (ABC) transporter ABCA1 mRNA and protein. Luciferase reporter assays showed that miR-302a repressed the 3′ untranslated regions (UTR) activity of mouse Abca1 by 48% and human ABCA1 by 45%. In addition, transfection of murine macrophages with miR-302a attenuated cholesterol efflux to apolipoprotein A-1 (apoA-1) by 38%. Long-term in vivo administration of anti–miR-302a to mice with low-density lipoprotein receptor deficiency (Ldlr<sup>−/−</sup>) fed an atherogenic diet led to an increase in ABCA1 in the liver and aorta as well as an increase in circulating plasma high-density lipoprotein levels by 35% compared with that of control mice. The anti–miR-302a–treated mice also displayed reduced atherosclerotic plaque size by ≈25% and a more stable plaque morphology with reduced signs of inflammation.

Conclusions—These studies identify miR-302a as a novel modulator of cholesterol efflux and a potential therapeutic target for suppressing atherosclerosis. (Arterioscler Thromb Vasc Biol. 2015;35:323-331. DOI: 10.1161/ATVBAHA.114.304878.)

Key Words: ABCA1 protein ◼ atherosclerosis ◼ cholesterol-efflux regulatory protein ◼ HDL cholesterol ◼ macrophages ◼ microRNA

Atherosclerosis is a chronic inflammatory disease of the arterial wall, as well as a disorder of lipid metabolism. Cholesterol is an essential structural component in the cell membrane and a precursor in metabolic pathways, including steroid hormone and bile acid synthesis. The accumulation of cholesterol-loaded macrophages in the arterial wall, termed foam cell formation, is a hallmark feature of early atherosclerotic lesions. Low-density lipoprotein (LDL) particles are internalized by macrophages in the arterial wall via multiple pathways including through the LDL receptor (LDLR) on the cell surface and hydrolyzed to free cholesterol in lysosomes. When excessive cholesterol is taken up in the artery wall, macrophages activate a compensatory pathway to efflux the cholesterol, mediated by the ATP-binding cassette (ABC) transporters, such as ABCA1 and ABCG1. These transporters promote cellular cholesterol efflux to high-density lipoprotein (HDL) and its associated apolipoprotein A-1 (apoA-1), a crucial step in the initiation of reverse cholesterol transport to the liver for excretion. During systemic hypercholesterolemia, however, this homeostatic mechanism is overwhelmed, leading to the development of foam cells and fatty streak lesions. Lipid loaded and activated macrophage foam cells can significantly contribute to the maintenance and progression of atherogenesis by producing nitric oxide, reactive oxygen species, inflammatory lipids, growth factors, and proinflammatory cytokines (eg, interleukin-1, interleukin-6, interferon-γ, and tumor necrosis factor-α). Understanding the mechanisms that regulate these responses could, therefore, be of considerable value in developing new approaches to the prevention and treatment of atherosclerosis. The regulation of these lipid...
metabolism mediators such as ABCA1, however, is complex and likely to involve post-transcriptional mechanisms.

MicroRNAs (miRs) are a recently recognized class of highly conserved, noncoding short RNA molecules that regulate gene expression at the post-transcriptional level. MiRs act as negative regulators of gene expression by inhibiting messenger RNA (mRNA) translation or promoting mRNA degradation. Importantly, several miRs have been recognized recently to influence cholesterol homeostasis.

Because complex metabolic pathways, such as lipid metabolism, are often coordinately regulated by a variety of homeostatic mechanisms and 1 gene can be under the repressive mechanism of multiple miRs, we performed an unbiased genome-wide screening of miRs regulated in mouse bone marrow–derived macrophages (BMDM) with or without treatment with modified LDL. We identified miR-302a as a potential candidate targeting ABCA1 leading us to hypothesize that miR-302a is involved in cholesterol transport and efflux.

On the basis of our findings that miR-302a regulates ABCA1 at the post-transcriptional level and anti–miR-302a treatment prevents atherosclerosis progression, miR-302a may be a promising therapeutic target to treat atherosclerosis.

**Materials and Methods**

Materials and Methods are available in the online-only Data Supplement.

**Results**

**Regulation of miR-302a Is Inversely Correlated With Cellular Cholesterol Levels**

It has been shown that specific miRs can be regulated as well as regulate many different functions in macrophages, including cholesterol homeostasis. Therefore, we performed an unbiased genome-wide screen of miRs modulated by cellular cholesterol content in primary macrophages. We identified a subset of 47 miRs differentially regulated in mouse macrophages by cholesterol enrichment (Table I in the online-only Data Supplement), of which 14 were downregulated and 33 were upregulated. Confirmation of these miR candidates using real-time polymerase chain reaction identified miR-302a as one of the most strongly modulated miRs in BMDM after treatment with modified LDL. MiR-302a expression in BMDM was significantly downregulated by acetylated LDL (AcLDL) and oxidized LDL stimulation for 6 hours in vitro. This was accompanied by the upregulated expression of the cholesterol transporter genes Abca1 and Abcg1 (Figure 1A).

Interestingly, stimulation with native LDL does not seem to affect miR-302a expression but does upregulate ABC transporter genes. To show that these observations are valid in the human system as well, we performed the same experiment using freshly isolated human peripheral blood mononuclear cells. Real-time polymerase chain reaction analysis using primary human macrophages confirmed the results seen in BMDM. MiR-302a was significantly downregulated after treatment with AcLDL or oxidized LDL for 6 hours with a concomitant upregulation of ABCA1 and ABCG1 gene expression (Figure 1B). Next, we investigated miR-302a expression in the aorta of Ldlr <sup>−/−</sup> mice that had been fed a normal chow or a high-fat diet for 8, 12, and 24 weeks. We found that miR-302a was highly expressed in the aorta of these mice. However, consistent with our in vitro findings, after 12 weeks of atherogenic diet feeding, miR-302a was markedly downregulated, whereas Abca1 and Abcg1 gene expression was upregulated (Figure 1C). This pattern suggests that miR-302a is regulated by hypercholesterolemia in Ldlr <sup>−/−</sup> mice.
Molecular Characteristics of miR-302a

Analysis of sequence alignment revealed that miR-302a is an intrinsic miR localized on chromosome 4 in humans and on chromosome 3 in mice, both within intron 8 of the La ribonucleoprotein domain family member 7 (LARP7) gene (Figure 2A). LARP7 belongs to the LARP RNA-binding protein family, and modulates the metabolism and function of a variety of RNA species.11 Many mammalian miRs are located within introns of protein-coding genes and, therefore, are typically coordinately expressed and processed with the precursor mRNA in which they reside.14,15 Accordingly, the mature form of miR-302a seems to be coexpressed with the Larp7 host gene in several different mouse tissues examined (Figure 2B). Interestingly, miR-302a is highly expressed in the aorta in comparison with Larp7, whereas in the spleen Larp7 is abundantly expressed with little expression of miR-302a. In addition, we found that in liver, aorta, and BMDM, miR-302a and Larp7 expression were coordinately down-regulated by cholesterol loading suggesting the regulation of miR-302a by cholesterol (Figure 2C). Moreover, miR-302a is highly conserved in different organisms (Figure 2D), which led us to investigate miR-302a for further validation of its role in cholesterol metabolism.

MiRs have been shown to target miRNAs for post-transcriptional repression by base-pairing with mRNA sequences typically located in the 3’UTRs and causing translational inhibition or mRNA cleavage.16 To gain insight into the function of miR-302a, we analyzed its potential gene targets using 4 different prediction programs, such as miRanda, miRwalk, Pictar, and TargetScan, which predict binding sites on miRNAs for a particular candidate miR (Table II in the online-only Data Supplement). We identified a potential binding site for miR-302a in the 3’UTR of the human and mouse ABCA1 gene (Figure 2E and 2F, respectively), a strong indication that miR-302a indeed plays a role in cholesterol metabolism via ABCA1 regulation.

MiR-302a Regulates ABCA1 in Primary Macrophages at the Post-Transcriptional Level

To test the specific effect of miR-302a on ABCA1 expression, we treated BMDM with AcLDL (to load the cells with...
MicroRNAs (MiR)-302a regulates ABCA1. Real-time polymerase chain reaction (PCR) analysis of Abca1 and Abcg1 gene expression in bone marrow–derived macrophages (BMDM) transfected with (A) control miR and 200 nmol/L miR-302a, or (B) control miR and 200 nmol/L anti–miR-302a. Twenty-four hours after transfection, primary macrophages were either untreated or stimulated with 40 μg/mL acetylated LDL (AcLDL) or 10 μmol/L T0901317 for an additional 24 hours (n=4 independent experiments). Data are expressed as mean±SEM. *P<0.05, **P<0.005. Western blot analysis of Abca1 protein expression in primary mouse cells after transfection with (C) control miR and 200 nmol/L miR-302a and 200 nmol/L anti–miR-302a, or (D) control miR and 200 nmol/L anti–miR-302a. Macrophages were either untreated or treated with 40 μg/mL AcLDL or 10 μmol/L T0901317 for 48 hours 24 hours after transfection. Representative blots are shown. E, Gene expression analysis of inflammation markers (Arginase-I [Arg-I], Arginase-II [Arg-II], Interleukin-10 [IL-10], Interleukin-6 [IL-6], and Interferon gamma [IFN-γ]) in BMDM after transfection with either control miR or 200 nmol/L anti–miR-302a using real-time PCR. Data are expressed as mean±SEM. *P<0.05, **P<0.005. ANOVA with Bonferroni multiple comparison test was used.
Therapeutics. The delivery of antisense nucleotides has been used successfully in mice to inhibit the function of various miRs and to increase expression of their target genes with no evident toxicity. To assess the effects of inhibiting miR-302a in a model of atherosclerosis, Ldlr−/− mice were fed a high-fat diet >12 weeks while being injected intraperitoneally with anti–miR-302a or control anti–miR oligonucleotides. Consistent with previous studies, the treatment did not induce a detectable immune response, because differential blood count in mice treated with either control anti–miR or anti–miR-302a did not differ from those of untreated Ldlr−/− mice fed a high-fat diet. Efficient delivery was confirmed by measuring levels of its target gene ABCA1 in the livers and aorta of mice during euthanization. An increase was seen in...
Abca1 expression in liver and aorta of anti–miR-302a–treated Ldlr−/− mice (Figure 5A) as well as in Abca1 protein levels in the liver (Figure 5B). No change in Abcg1 mRNA was observed.

**Anti–miR-302a Treatment Increases Circulating HDL Levels and Reduces Atherosclerosis Progression in Ldlr−/− Mice**

To determine if increased ABCA1 expression in the liver augments HDL biogenesis in anti–miR-302a–treated Ldlr−/− mice, we measured circulating plasma HDL as well as other lipid levels in anti–miR-302a and control anti–miR–treated mice. There was a significant increase in total circulating cholesterol in mice treated with anti–miR-302a (Table). Moreover, analysis of lipoproteins by high-performance liquid chromatography showed an increase in cholesterol content of the HDL fractions (45–58 minutes) of the anti–miR-302a–treated mice compared with that of control anti–miR–treated mice (Figure 6A). In fact, treatment of Ldlr−/− mice with anti–miR-302a raised HDL by 35% compared with that of control mice. Very low-density lipoprotein (VLDL)/LDL cholesterol was also raised in the anti–miR-302a–treated mice, but this was not statistically significant (Table).

In assessing atherosclerotic lesion development, a marked reduction in plaque formation in aorta (Figure 6B) and in aortic roots (Figure 6C) was observed in Ldlr−/− mice treated with anti–miR-302a in comparison with Ldlr−/− mice treated with control miR, as visible after Oil red O staining. Interestingly, quantitative immunostaining revealed that the reduction of plaque area was associated with a significant increase in the relative content of macrophages (Figure 6D), whereas the necrotic core size was significantly decreased in anti–miR-302a–treated Ldlr−/− mice (Figure 6F). In addition, the relative content of smooth muscle cells was significantly increased in anti–miR-302a–treated Ldlr−/− mice (Figure 6E) indicating remodelling of plaques toward a rather early and more stable lesion phenotype with a lesser degree of inflammation (Figure 3E). Together, these results indicate that anti–miR-302a treatment supports the efflux of cholesterol from plaque macrophages leading to an overall reduction in atherosclerotic burden.

### Discussion

There is a strong evidence that high levels of circulating HDL are associated with positive cardiovascular outcomes, independent of levels of LDL cholesterol, and as such, therapies to raise HDL are actively being pursued.31 Direct infusion of HDL in apoE-deficient mice22 or human subjects23 with established atherosclerosis, reduces plaque size. The ABC transporter ABCA1 is primarily responsible for initiating HDL formation in the liver.24 This work reveals that miR-302a exerts post-transcriptional control of the ABCA1 cholesterol transporter in primary macrophages resulting in the regulation of cellular cholesterol efflux. By targeting Abca1 via in vivo delivery of anti–miR-302a in Ldlr−/− mice, circulating plasma HDL levels were increased leading to less atherosclerosis when compared with Ldlr−/− mice treated with a control anti–miR. However, further validation needs to be performed to identify the HDL type generated by anti–miR-302a treatment.

Although we demonstrated that miR-302a modulates Abca1 in primary macrophages, hepatocytes represent the primary metabolic cell type within the liver. Hoekstra et al25 profiled murine hepatocyte miR expression during the development of nonalcoholic fatty liver and showed a decrease in miR-302a levels in response to western-type diet feeding, which coincided with a marked increase in the expression of the miR-302a target gene, Abca1. These findings support our results showing miR-302a is regulating Abca1 on a post-transcriptional level resulting in increased HDL levels in anti–miR-302a–treated Ldlr−/− mice. However, albeit not statistically significant, we also observed an ≈25% increase in VLDL/LDL levels in those same mice. Although the reason for this increase is under active investigation, it is noteworthy that
despite the raised VLDL/LDL levels there was a significant reduction in atherosclerosis in anti–miR-302a–treated mice. This is a strong indication that the antiatherogenic property exerted by a 35% increase in HDL far outweighed the proatherogenic property exhibited by a 25% rise in VLDL/LDL.

There have been several reports of miRs regulating lipid metabolism. MiR-122, for example, was identified as the most highly expressed miR in the adult liver, where it accounts for 70% of all miRs.26 Using antisense strategies, several groups have reported that inhibition of miR-122 in the liver results in sustained decreases in plasma cholesterol levels in both mice and nonhuman primates.27,28 Most recently, 5 independent studies demonstrated that miR-33a targets ABCA1 and ABCG1 and limits the efflux of cholesterol to apoA-1 in both

| Table. Lipid Profiles in Ldlr−/− Mice on Atherogenic Diet Treated With Control Anti–miR or Anti–miR-302a |
|-------------------------------------------------|-------------------------------------------------|-------------------------------------------------|-------------------------------------------------|
| Triglyceride (mg/dL) n=14 per group | Total Cholesterol (mg/dL) n=14 per group | VLDL/LDL (mg/dL) n=7 per group | HDL (mg/dL) n=7 per group |
| Control anti-miR | 133.2±1.6 | 442.0±39.7 | 394.5±36.3 | 48.04±4.9 |
| Anti–miR-302a | 133.0±1.1 | 563.2±53.3 | 498.1±47.2 | 64.92±7.6* |

HDL indicates high-density lipoprotein; LDL, low-density lipoprotein; miR, microRNAs; and VLDL, very low-density lipoprotein.*P<0.05.

Figure 6. Anti–miR-302a treatment increases circulating high-density lipoprotein levels and reduces atherosclerosis progression in Ldlr−/− mice inducing a stable plaque phenotype with less degree of inflammation. A, High-performance liquid chromatography lipoprotein profiles from plasma of Ldlr−/− mice treated with control anti–miR or anti–miR-302a (intraperitoneal injections of 10 mg/kg per week over a period of 8 weeks; n=7 mice per group). Quantification of Oil Red O+ lipid depositions in the aorta (B) and aortic root (C) of Ldlr−/− mice treated with either control anti–miR or anti–miR-302a. Fluorescence microscopy was used to analyze levels of MOMA-2-positive macrophages (D) and smoothelin-positive smooth muscle cells (E) in the aortic root of Ldlr−/− mice treated with either control anti–miR or anti–miR-302a. Representative images are shown. F, Quantification of necrotic cores within aortic root lesion of Ldlr−/− mice with either control anti–miR or anti–miR-302a. Data are expressed as mean±SEM. *P<0.05; **P<0.005. 2-tailed Student t test was used. miR indicates microRNAs.
macrophages and hepatocytes. Conversely, antimiR-33a treatment upregulates ABCA1 expression both in vitro and in vivo, promoting the efflux of cholesterol to apoA-1 and increasing circulating HDL levels. In addition to these findings, inhibition of miR-33 expression in vivo increases reverse cholesterol transport and curbs atherosclerotic plaque regression. Here, we report that a newly identified miR, miR-302a, targets ABCA1 and modulates the cholesterol efflux to apoA-1 in primary macrophages. In addition, anti–miR-302a treatment increases circulating HDL levels and decreases atherosclerosis development. Aside from ABCA1, VLDLR, Osbp5, Pmvk, and lep are also predicted to be target of miR-302a, all of which can influence lipid metabolism (Table II in the online-only Data Supplement).

An interesting connection between lipid metabolism and inflammation in macrophages revealed by others suggests that cholesterol loading of macrophages results in a proinflammatory phenotype, whereas cholesterol efflux dampens this inflammation. Our study, real-time polymerase chain reaction analysis of anti–miR-302a–transfected macrophages showed that aside from the regulation of Abca1 there was an alteration of the inflammatory state of these cells when compared with miR-302a–transfected cells. This likely led to less inflammation in the lesions of anti–miR-302a–treated Ldlr−/− mice, and, therefore, a slower progression of atherosclerosis.

As the host gene of miR-302a, LARP7 gene encodes a protein that is found in the 7SK small nuclear ribonucleoprotein. Although little is known about the function of this protein what is known is that this small nuclear ribonucleoprotein complex inhibits a cyclin-dependent kinase, which is required for paused RNA polymerase II at a promoter to begin transcription elongation. Via its ability to control transcription and elongation, LARP7 is thought to be involved in general cellular processes, such as cell growth and tumorigenesis. This makes it likely to be involved in a broad range of different processes, including inflammation and lipid metabolism, even though LARP7 is not a transcription factor regulating gene expression involved in cholesterol metabolism. Interestingly, a recent study showed that loss of function mutation in LARP7 causes a syndrome of facial dysmorphism, intellectual disability, and primordial dwarfism.

The ability of miRs to modulate important biological pathways offers opportunities for the manipulation of miR function using oligonucleotide inhibitors or miR mimics. Antisense oligonucleotides directed against specific miR sequences are efficiently taken up by a variety of tissues. In addition, miR mimics and inhibitors are relatively stable in plasma and can simply be injected to reach their cellular gene targets without apparent toxicity. The challenge to directly target a specific inflamed tissue or a specific cell type is still remaining. However, Rayner et al were able to show that anti-miR treatment indeed is capable of targeting plaque macrophages and directly altering gene expression in these cells supporting our data showing less plaque formation possibly because of a higher cholesterol-efflux rate in mice treated with anti–miR-302a.

Taken together, our data identify for the first time miR-302a as a modulator of cholesterol efflux and clearly highlights miR-302a to be an attractive therapeutic target for the prevention/treatment of atherosclerosis.

Acknowledgments

We thank Regulus Therapeutics for their generous gift of anti–miR302a and control oligonucleotides. We are grateful to Monica Montgomery and Eric Collier for excellent technical assistance and to Sara McCurdy for editorial assistance. This work was performed within the Russian Government Program of Competitive Growth of Kazan Federal University.

Sources of Funding

This work was supported by National Institutes of Health (NIH) grants R01HL075677 and R01HL81863 as well as Hawaii Community Foundation grant 10ADVC-47037 to W.A. Boisvert. Core facilities were supported by NIH grants P20GM103516, P20RR016453, G12RR03061, and G12MD007601. S. Meiler is recipient of a postdoctoral grant from the Deutsche Forschungsgemeinschaft (ME3898/2-1).

Disclosures

None.

References

MicroRNA 302a Modulates Atherosclerosis

Atherosclerosis is a common disease of the artery in which both cholesterol levels and inflammation play a crucial role in its pathogenesis. Of the inflammatory cells, macrophages play a key role in both initiation and progression of the disease as they can become foam cells in the artery wall and directly contribute to the growth of the atherosclerotic plaque. Although studying the lipid metabolism of macrophages, we found that a microRNA called 302a (miR-302a) may be able to control the metabolism of lipids in macrophages by inhibiting a cholesterol transport molecule called ABCA1. Through many experiments, we found that miR-302a indeed binds the 3′ untranslated region of ABCA1 and, therefore, inhibits ABCA1 expression. Inhibiting miR-302a resulted in less lipid accumulation in macrophages. In a mouse model of atherosclerosis, we used a synthetic miR-302a-inhibiting agent to show that lowering miR-302a in the body significantly raised high-density lipoprotein levels and attenuated the extent of atherosclerosis compared with control mice.
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Arterioscler Thromb Vasc Biol. 2015;35:323-331; originally published online December 18, 2014;
doi: 10.1161/ATVBAHA.114.304878
Arteriosclerosis, Thrombosis, and Vascular Biology is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 1079-5642. Online ISSN: 1524-4636

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://atvb.ahajournals.org/content/35/2/323

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MATERIALS AND METHODS

Reagents and antibodies
Chemicals were obtained from Sigma unless otherwise noted. Human lipoproteins (LDL, AcLDL, oxLDL) and human apoA-1 were obtained from Biomedical Technologies Inc (Stoughton, MA). The synthetic LXR ligand T0901317 was from Cayman Chemical. Antibodies were obtained from Abcam unless stated otherwise.

Cell culture
COS-7 cells were obtained from American Type Tissue Collection (ATTC). Cells were maintained in Dulbecco’s Modified Eagle Medium (DMEM) (Cellgro) containing 10% FBS and 2% penicillin-streptomycin.

For generation of BMDM, BM-cell suspensions were prepared from C57BL/6J mice. Femurs and tibias were removed aseptically from donor mice, marrow cavities were flushed and BM-cell suspensions seeded at 4 x 10^5 cells/ml in DMEM/F12 (Gibco) containing 10% FBS, 1% penicillin-streptomycin, 10 mM L-Glutamine and 20% L929 conditioned medium for 6 days. Differentiated macrophages were counted and replated in DMEM/F12 with 10% FBS and 1% penicillin-streptomycin for various experiments.

Primary human monocytes/macrophages were collected from healthy donors. To obtain monocytes/macrophages, peripheral blood mononuclear cells (PBMCs) were isolated from blood by centrifugation over a density gradient (Ficoll-Hypaque, Pharmacia). PBMCs were removed from the upper band located at the plasma and resolving medium interface and washed with Mg2+- and Ca2+-free PBS. To separate monocytes/macrophages from other cells, PBMCs were resuspended in XVivo 15 medium containing 1% human serum and incubated in plastic six-well culture dishes (Costar). After incubation at 37°C for 1 hour, non-adhering cells were removed by vigorous washing for four times with Mg2+- and Ca2+-free PBS while adhering cells were maintained in XVivo 15 medium containing 1% human serum. Primary human macrophages were counted and used for various experiments.

Mice
All animals were handled in accordance with good animal practice as defined by the relevant animal welfare bodies. All animal work was approved by the University of Hawaii Institutional Animal Care and Use Committee. C57BL/6J and Ldlr^-/- mice were obtained from Jackson Laboratory. Ldlr^-/- mice were placed on either a chow diet or a high fat diet (HFD) containing 15.8% (wt/wt) fat and 1.25% cholesterol (diet 94059; Harlan Teklad) for 8, 12 and 24 weeks. At sacrifice, liver samples, spleen samples and aorta tissue were collected and total RNA was harvested for miR and gene expression analysis. Some 8-week-old Ldlr^-/- mice were placed on a HFD for 12 weeks. Simultaneously, mice were treated with either 2`F/MOE control anti-miR oligonucleotide or 2`F/MOE anti-miR-302a oligonucleotide (a generous gift from Regulus Therapeutics). Mice received 2 intraperitoneal (i.p.) injections of 10 mg/kg anti-miR in the first week, spaced 3 days apart, and weekly injections of 10 mg/kg then for 8 weeks. Mice were sacrificed 4 weeks later.

Quantification and immunohistochemical analysis of atherosclerosis
The extent of atherosclerosis was assessed in aortas by staining for lipid depositions with Oil Red O and quantified using ImageJ software as described 1. Briefly, aortas were opened longitudinally and the percentage of aortic surface covered by lipid deposition was calculated by dividing the stained area over the total aortic surface. The aortic sinus was sectioned to use for various immunohistochemical staining as described below.

The presence of macrophages and smooth muscle cells (SMCs) in the aortic sinus plaque were determined by mAb staining for MOMA-2 (AbCam, USA, ab33451) and SM22alpha
(Proteintech, USA, 10493-1-AP), respectively, and detection with Cy2- and Cy3-conjugated secondary antibodies (AbCam). For all assays, nuclei were labeled using DAPI and images were recorded using Axiovert microscope (Carl Zeiss, Germany).

**miR microarray analysis**

BMDM were stimulated with either medium alone, 10µg/ml AcLDL or 10µg/ml oxLDL for 6 h. Total RNA was extracted using TRIzol (Invitrogen) and miR was purified using the miRNeasy Mini Kit (QIAGEN). A qPCR based miRNA system (mouse miRNome assay) (System Biosciences) was used to screen a total of 709 miRs.

**Quantitative real-time polymerase chain reaction (PCR)**

Total RNA was isolated from BMDM, liver samples, spleen samples and aorta by TRIzol (Invitrogen). cDNA was reverse transcribed from 1µg of DNase-treated total RNA (Promega) for gene expression. Real-time-PCR analysis was performed using SYBR Green RT-PCR system (Clontech) on 7900HT Fast Real-Time System with Power SYBR Green chemistry (Applied Biosystems) with specific primer pairs (Integrated DNA Technologies). The mRNA level was normalized to GAPDH as the housekeeping gene.

<table>
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<tr>
<th>Gene</th>
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For miR quantification, total RNA was reverse transcribed using the miScript Reverse Transcription Kit (QIAGEN) and real time-PCR analysis was performed using miScript SYBR Green PCR Kit (QIAGEN). Primers (QIAGEN) specific for human and mouse miR-302a were used and values were normalized to U6 as the housekeeping gene.
miR-302a and anti-miR-302a transfection

BMDM were transfected with 200nM miRIDIAN miR mimics (miR-302a) or with 200 nM miRIDIAN miR inhibitors (anti-miR-302a) (Dharmacon) utilizing x-tremeGENE siRNA Transfection Reagent (Roche). All experimental control samples were treated with an equal concentration of non-targeting control mimics and/or inhibitor sequence (control miR) for use as controls for non-sequence-specific effects in miR experiments. MiR-302a over-expression and knockdown were verified using qPCR, as described above. For posttranscriptional expression analysis, cells were either unstimulated or stimulated with 10 µM T0901317 and/or 40 µg/ml AcLDL for 48h after transfection.

Immunofluorescence Analysis

48h after transfection with either control miR, miR-302a, or anti-miR-302a (Dharmacon), BMDM were stained for ABCA1. BMDM were carefully washed with PBS and fixed using 2% PFA in PBS (pH 7.4) for 5 min at room temperature (RT) and blocked for 30 min at (RT) using 10% goat serum in 2% BSA/PBS. Primary anti-ABCA1 antibody (1:200) in 2% BSA/PBS was added and incubated overnight at 4°C. After thorough washing secondary goat anti-mouse cy3 antibody (1:300) was added and incubated at (RT) in the dark for 1h. Cell nuclei were counter stained with DAPI 5 min prior to washing and mounting with fluorescence mounting media (DAKO). Immunofluorescence was analyzed using Axiovert microscope (Carl Zeiss, Germany). Five independent pictures were taken using the 40x objective per treatment condition. To determine the fluorescence intensity of the images ImageJ software was used. Briefly, the fluorescence intensity of 50 individual cells was measured using the average cell size per picture (250 cells per individual sample). Afterwards, average fluorescence per sample was normalized to untreated control conditions.

Western blot Analysis

BMDM and liver cells were lysed in ice-cold buffer containing 1% NP40, 150 mM NaCl, 50 mM Tris and protease inhibitor cocktail (Roche) at 4°C for 30 min while vortexing every 10 min. Insoluble material was removed by centrifugation at maximum speed for 5 min and total protein in the supernatant was determined using a Bradford Assay reagent (Bio-Rad). After adjusting to equal protein concentration, 10 – 30 µg cell lysates were separated by SDS-PAGE followed by transfer of the proteins onto nitrocellulose membranes. Blots were incubated with primary anti-Abca1 (1:500; Novus), anti-α-Tubulin (1:5000; Sigma), or anti-β-actin (1:3000; Sigma) over night, washed, incubated with appropriate HRP-conjugated secondary antibody (1:5000), and visualized. Densitometric analysis was performed with the ImageJ software package (NIH).

3'UTR Luciferase Reporter Assays

cDNA fragments corresponding to the entire 3'UTR of human and/or mouse ABCA1 were amplified by conventional PCR from total RNA extracted from either THP-1 cells or BMDMs with XhoI and Pmel linkers. The PCR products were directionally cloned downstream of the Renilla luciferase open reading frame into the psiCHECK2 vector (Promega) that also contains a constitutively expressed firefly luciferase gene, required for normalizing transfections. Point mutations in the seed region of predicted miR-302a sites within the 3'UTR of hABCA1 and mABCA1 were generated using QuickChange II site-directed Mutagenesis Kit (Agilent Technologies) according to the manufacturer’s protocol. All constructs were confirmed by sequencing. COS-7 cells were plated into 96-well plates (BD) and co-transfected with the indicated 3'UTR luciferase reporter vectors and control mimics or miR-302a (Dharmacon) utilizing X-tremeGENE siRNA Transfection Reagent (Roche). Luciferase activity was measured using the Dual-Glo Luciferase Assay System (Promega). Renilla luciferase activity was
normalized to the corresponding firefly luciferase activity and plotted as a percentage of the control.

**Cholesterol Efflux Assays**

BMDM were transfected with either control mimic, miR-302a mimic, control inhibitor or anti-miR-302a (Dharmacon) and apoA-1-dependent cholesterol efflux assays were performed. Briefly, BMDM were seeded at a density of 0.5 x 10⁶ cells per well one day prior to loading with 0.5µ Ci/ml ^3^H-cholesterol with and without 50 µg/ml AcLDL for 24 h. The cells were washed with PBS, and DMEM containing 10% lipoprotein deficient serum (LPDS) (Intracel) was added into each well and incubated for 1-2 h. For apoA-1-dependent cholesterol efflux, efflux medium (DMEM/0.2% BSA ± 20 µg/ml apoA-1) was added into each well. Supernatants were collected after 6h and expressed as a percentage of total cell ^3^H-cholesterol content (total effluxed ^3^H-cholesterol+cell-associated ^3^H-cholesterol).

**Lipid analysis and Lipoprotein profile measurement**

Mice were fasted for 12-14 h before blood samples were collected. Plasma was separated by centrifugation and stored at -80°C. Triglyceride and cholesterol levels were measured with their respective assay kits (Cayman), according to the manufacturer’s protocol. The lipid distribution in plasma lipoprotein fractions was assessed by high performance liquid chromatography (HPLC).

**Statistical Analysis**

Statistical comparison between groups was performed with PRISM software (GraphPad). Data represent mean ± the standard error of the mean (SEM), and were analysed by 2-tailed Student’s t test and ANOVA with Bonferroni’s multiple comparison test. Significance was accepted at the level of P<0.05.
REFERENCES

Supplemental Table I

Relative miR expression in primary murine macrophages treated with AcLDL and oxLDL vs. untreated control.

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Primary macrophages were treated with AcLDL or oxLDL for 6 h and relative miR expression was analyzed by a qPCR based miR system. Data are represented as mean; n=3.
Supplemental Table II

Predicted target genes of miR-302a related to atherosclerosis, lipoprotein signaling and cholesterol metabolism using four separate computational algorithms: miRanda, miRwalk, Pictar and TargetScan.

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0: not predicted  
1: predicted
**Supplemental Figure I. MiR-302a regulates ABCA1.** Immunofluorescence microscopy of Abca1 protein expression in primary mouse macrophages after transfection with (A) control miR and miR-302a, or (B) control miR and anti-miR-302a. Macrophages were either untreated or treated with AcLDL and/or T0901317 for 48 hours after transfection (n=5 independent experiments). Data are expressed as mean ± SEM. *P<0.05.