Control of Cholesterol Metabolism and Plasma High-Density Lipoprotein Levels by microRNA-144

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Rationale: Foam cell formation because of excessive accumulation of cholesterol by macrophages is a pathological hallmark of atherosclerosis, the major cause of morbidity and mortality in Western societies. Liver X nuclear receptors (LXRs) regulate the expression of the adenosine triphosphate–binding cassette (ABC) transporters, including adenosine triphosphate–binding cassette transporter A1 (ABCA1) and adenosine triphosphate–binding cassette transporter G1 (ABCG1). ABCA1 and ABCG1 facilitate the efflux of cholesterol from macrophages and regulate high-density lipoprotein (HDL) biogenesis. Increasing evidence supports the role of microRNA (miRNAs) in regulating cholesterol metabolism through ABC transporters.

Objective: We aimed to identify novel miRNAs that regulate cholesterol metabolism in macrophages stimulated with LXR agonists.

Methods and Results: To map the miRNA expression signature of macrophages stimulated with LXR agonists, we performed an miRNA profiling microarray analysis in primary mouse peritoneal macrophages stimulated with LXR ligands. We report that LXR ligands increase miR-144 expression in macrophages and mouse livers. Overexpression of miR-144 reduces ABCA1 expression and attenuates cholesterol efflux to apolipoprotein A1 in macrophages. Delivery of miR-144 oligonucleotides to mice attenuates ABCA1 expression in the liver, reducing HDL levels. Conversely, silencing of miR-144 in mice increases the expression of ABCA1 and plasma HDL levels. Thus, miR-144 seems to regulate both macrophage cholesterol efflux and HDL biogenesis in the liver.

Conclusions: miR-144 regulates cholesterol metabolism via suppressing ABCA1 expression and modulation of miRNAs may represent a potential therapeutical intervention for treating dyslipidemia and atherosclerotic vascular disease. (Circ Res. 2013;112:1592-1601.)

Key Words: ABCA1 ■ cardiovascular research ■ cholesterol efflux ■ cholesterol homeostasis ■ high-density lipoprotein ■ lipids and lipoprotein metabolism ■ microRNAs

Foam cell formation because of excessive accumulation of cholesterol by macrophages is a pathological hallmark of atherosclerosis, the major cause of morbidity and mortality in Western societies.1,2 Macrophages cannot limit the uptake of cholesterol and, therefore, depend on cholesterol efflux pathways for preventing their transformation into foam cells.1,2 Several ABC transporters, including adenosine triphosphate–binding cassette transporter A1 (ABCA1) and adenosine triphosphate–binding cassette transporter G1 (ABCG1), facilitate the efflux of cholesterol from macrophages. ABCA1 and ABCG1 are thought to act in sequence to lipoprotein metabolism and then mature high-density lipoprotein (HDL) to generate larger α-HDL particles destined for clearance by the liver.3-5 Mutations in the Abca1 gene cause Tangier disease, which is characterized by defects in cholesterol efflux and cholesterol ester accumulation in macrophages, and increase the risk of development of atherosclerosis.6-8 In the liver, ABCA1 also plays a critical role in the biogenesis of HDL and its deficiency leads to a dramatic reduction of plasma HDL levels.

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The expression of ABCA1 and of ABCG1 is upregulated in states of cholesterol excess by liver X receptor (LXR).\(^5\) LXR\(s\) are activated by oxysterol metabolites of cholesterol and play key roles in regulating multiple components of the reverse cholesterol transport pathway, cholesterol conversion to bile acid, and intestinal cholesterol absorption.\(^3,10\) Moreover, LXR also regulates cellular cholesterol homeostasis by activating the transcription of the inducible degrader of the low-density lipoprotein (LDL) receptor, an E3 ubiquitin ligase that triggers ubiquitination of the LDL receptor on its cytoplasmic domain, thereby targeting it for degradation.\(^11\)

Over the past decade, it has become progressively more clear that a large class of small noncoding RNAs, known as microRNAs (miRNAs), function as important regulators of a wide range of cellular processes by modulating gene expression.\(^12\) In general, miRNAs regulate gene expression posttranscriptionally by base-pairing to target miRNAs.\(^13\) In animals, most investigated miRNAs form imperfect hybrids with sequences in the 3′-untranslated region (3′ UTR), with the mRNA 5′-proximal seed region (positions 2–8) providing most of the pairing specificity.\(^13,14\) Imperfections in the central portion of miRNA–mRNA duplexes preclude RNAi-like cleavage. Instead, the miRNA association results in translational repression, frequently accompanied by a considerable degradation of the mRNA by a non-RNAi mechanism.\(^13,14\) To date, several miRNAs have been described to regulate lipid metabolism, including miR-122, miR-370, miR-378/378*, miR-758, miR-106, and miR-33.\(^15-23\) Recently, our group and others identified miR-33a/b as intronic miRNAs located within the sterol response element–binding protein (SREBP) genes, Srebp1 and Srebp2.\(^23\) These loci encode for the membrane-bound transcription factors, SREBP1 and SREBP2, which activate the synthesis of fatty acids and the synthesis and uptake of cholesterol. Coincident with the transcription of Srebp1 and Srebp2, the embedded miR-33a and miR-33b are transcribed, and these negative regulators act to repress a number of genes involved in regulating cellular cholesterol export and fatty acid oxidation, including Abca1, Abcg1, Niemann-Pick C1 (Npc1), carnitine palmitoyltransferase 1A (Cpt1a), carnitine O-octanoyl transferase (Cot), hydroxycity-CoA dehydrogenase-3-ketoacyl-CoA thiolase-enoyl-CoA hydratase (trifunctional protein) \(\beta\)-subunit (Hudhbb), and 5′ AMP-activated protein kinase (Ampk).\(^3,24\)

Antagonists of miR-33 in mice increase liver and macrophage ABCA1 expression and promote reverse cholesterol transport and regression of atherosclerosis.\(^25\) Of note, inhibition of miR-33a/b in nonhuman primates raises plasma HDL and lowers very LDL triglycerides (TG).\(^26\) These data suggest that antagonism of endogenous miR-33 may be useful as a therapeutic strategy for treating metabolic syndrome and atherosclerosis.

In addition to miR-33, miR-758 and miR-106b recently have been shown to regulate the expression of ABCA1 at the posttranscriptional level.\(^19,22\) miR-758 is downregulated after cholesterol loading in macrophages and in the liver of mice fed a high-fat diet.\(^22\) Overexpression of miR-758 and miR-106b reduces ABCA1 expression in macrophage, hepatic, and neuronal cell lines.\(^19,22\) Thus, the posttranscriptional regulation of ABCA1 expression by miRNAs seems to be complex and mediated by multiple miRNAs.

In the current study, we present evidence that ABCA1 is posttranscriptionally regulated by miR-144 in vitro and in vivo. miR-144 overexpression inhibits ABCA1 expression in different cell lines, including hepatocytes and monocyte/macrophages, thereby attenuating cholesterol efflux to apolipoprotein A1 (ApoA1). Most importantly, in vivo delivery of miR-144 to mice represses ABCA1 expression in the liver, reducing circulating HDL-cholesterol levels. Conversely, silencing of miR-144 in mice increases the expression of ABCA1 and plasma HDL levels. Thus, miR-144 seems to regulate both macrophage cholesterol efflux and HDL biogenesis in the liver. We also report that LXR ligands increase miR-144 in macrophages and mouse livers and that ABCA1 is a target of LXR-induced miR-144. These data reveal how an inducible miRNA comprises a negative feedback loop to ensure a tight regulation of cholesterol homeostasis.

**Methods**

Because of space limits, a detailed description of the Materials and Methods is presented in the Online Data Supplement.

**Animals**

Male C57BL/6 mice were purchased from Jackson Laboratories (Bar Harbor, ME) and LXR\(\alpha\)-null and LXR\(\beta\)-null mice were kindly provided by David Mangelsdorf. Eight-week-old male C57BL/6 mice were randomized into 4 groups (n=24 mice): nontargeting control negative control sequence (Con-inh; n=6); and miR-144-inhibitors (Inh-miR-144; n=6). mirVana miRNA mimics and inhibitors (Life Technologies) were complexed with Invivofectamine 2.0 reagent (Invitrogen) and injected intravenously twice (every 2 days) at 7 mg/kg dose. All animals were kept under constant temperature and humidity in a 12-hour controlled dark/light cycle. Mice were fed a standard pelleted diet. In another set of experiments, mice were treated with LXR synthetic agonist T0901317 (T090; 10 mg/kg body weight) by oral gavage 2 days after injection with Con-inh or inh-miR-144. After 6 days, mice were fasted for 12 to 14 hours before blood samples were collected by retro-orbital venous plexus puncture. Plasma cholesterol levels and lipoprotein fractionation were analyzed as described. All animal experiments were approved by the Institutional Animal Care Use Committee of New York University Medical Center.

**miRNA Microarray Analysis**

Mouse peritoneal macrophages were stimulated with 3 μmol/L T090 for 24 hours. Total RNA was extracted using TRIzol (Invitrogen), and miRNA was purified from 40 μg of total RNA using the miRNA Isolation Kit (Qiagen). The purity and integrity of both the total RNA sample and the enriched miRNA were verified using the Agilent Bioanalyzer (Agilent Technologies, Santa Clara, CA). Mouse peritoneal macrophage miRNAs were amplified and hybridized to illumina expression profiling microarrays according to the manufacturer’s directions. Quadruplicates
miR-144 Mimic and Anti–miR-144 Transfection

Mouse peritoneal macrophages J774, THP-1, HepG2, Huh-7, Hepa, and EAhy926 cells were transfected with 40 nmol/L mirVANA miRNA mimic (miR-144) or with 60 nmol/L mirVANA miRNA inhibitor (Inh-miR-144; Dharmacon) using RNAiMax (Invitrogen). For cotransfection experiments with miR-33 and miR-144 mimics, we used 2.5 nmol/L each. All experimental control samples were treated with an equal concentration of a nontargeting control mimic (Con-miR) or inhibitor negative control sequence (Con-inh) for use as controls for nonsequence-specific effects in miRNA experiments. Verification of miR-144 overexpression and knockdown was determined using quantitative polymerase chain reaction as described.

miR-144 and Anti–miR-144 Particle Delivery In Vivo

The mirVana miRNA inhibitors and mimics (Life Technologies) were complexed with Invivofectamine 2.0 reagent (Inviogen) to form the nanoparticles suitable for in vivo applications. miRNA oligonucleotides (3 mg/mL in water, 750 μL) were mixed with manufacturer’s complexation buffer (750 μL), and then Invivofectamine 2.0 reagent was added (1500 μL). After 30-minute incubation at 50°C, dialysis was performed in 4 L phosphate-buffered saline (PBS) to remove excessive salts and solvents. The resulting miRNA mimic/inhibitor concentration was 0.7 mg/mL; after a 200-μL injection in the tail vein of a 20-g mouse, it resulted in a 7-mg/kg body weight miRNA dose (∼10 nmol per animal).

Cholesterol Efflux Assays

J774 macrophages were transfected with a control mimic (Con-miR), an miR-144 mimic, a control inhibitor (Con-inh), or an anti–miR-144 inhibitor (Dharmacon) at 40 nmol/L and seeded at a density of 1×10⁶ cells per well 1 day before loading with 0.5-μCi/mL 3H-cholesterol. For 24 hours, cells were washed twice with PBS and incubated in RPMI supplemented with 2-mg/mL fatty acid–free bovine serum albumin (BSA) bovine serum albumin (BSA) media in the presence of acetyl-coenzyme A acetyltransferase inhibitor (2 μmol/L) for 2 hours before addition of 50 μg/mL human ApoAI in fatty acid–free BSA media with or without the indicated treatments. Supernatants were collected after 6 hours and expressed as a percentage of total cell 3H-cholesterol content (total effluxed 3H-cholesterol plus cell-associated 3H-cholesterol). In another set of experiments, we transfected Huh-7 cells with control mimic (Con-miR), miR-144 mimic, miR-33 mimic, or a combination of miR-144/miR-33 mimics at 5 nmol/L final concentration and performed the cholesterol efflux assays as described.

Lipid Analysis and Lipoprotein Profile Measurement

Mice were fasted for 12 to 14 hours before blood samples were collected by retro-orbital venous plexus puncture. Plasma was separated by centrifugation and stored at −80°C. Total plasma cholesterol and HDL cholesterol were enzymatically measured with the Amplex red cholesterol assay kit (Molecular Probes, Invitrogen) according to the manufacturer’s instructions. The lipid distribution in plasma lipoprotein fractions was assessed by fast-performance liquid chromatography gel filtration with 2 Superose 6-hour 10/30 columns (Pharmacia).
the upregulation of miR-144 and pri-miR-144/451 (primary miRNA transcript) by LXR agonists was impaired in macrophages isolated from LXRα-null and LXRβ-null mice (Figure 2C and 2D), suggesting a transcriptional regulation of miR-144 and miR-451 by LXR. Finally, we also found that the miR-144/451 promoter activity was induced in Huh-7 cells treated with T090 (Figure 2E). Collectively, these data demonstrated that treatment of macrophages and hepatic cells with LXR agonist resulted in higher expression of miR-144.

Next, we examined the in vivo expression of miR-144 in mice. miR-144 was widely expressed in mouse tissues and is particularly abundant in the liver, spleen, and aorta (Online Figure IIIA). To determine the cell type that expressed a higher amount of miR-144 in the liver, we isolated primary hepatocytes and Kupffer cells by isopycnic centrifugation. As shown in Online Figure IIIB, hepatocytes express significantly higher levels of miR-144 compared with Kupffer cells. We further analyzed whether miR-144 was regulated by dietary cholesterol. To this end, we measured its expression in mice fed with either a chow diet or a high-fat diet for 5 weeks. As expected, treatment of C57BL6 mice with a high-fat diet increased body weight and plasma cholesterol and TG levels (Online Figure IIIC). Interestingly, hepatic miR-144 levels are regulated by high-fat diet in vivo (Online Figure IIIC, right).

miR-144 Regulates ABCA1 Expression in Macrophages and Hepatic Cells

We next determined the effect of miR-144 overexpression and inhibition on ABCA1 mRNA and protein expression. Transfection of mouse peritoneal macrophages with miR-144 increased its expression 350-fold (not shown) and significantly inhibited ABCA1 mRNA levels (Figure 3A, left). Moreover ABCG1 also was downregulated at the mRNA level in macrophages transfected with miR-144 (Figure 3A, right). Even though ABCG1 is not a direct target of miR-144, the inhibition of retinoid X receptor β (data not shown), which is a predicted target for miR-144, by this miRNA may influence ABCG1 expression. To further assess the effect of miR-144 on ABCA1 and ABCG1 protein expression, we treated mouse peritoneal macrophages with acetylated LDL (to enrich cholesterol) or T090 (to directly stimulate expression of the 2 genes). Transfection of mouse peritoneal macrophages with miR-144 mimics but not a control miRNA (Con-miR) strongly decreased the stimulation of ABCA1 (Figure 3B, left). In contrast to ABCA1, ABCG1 expression was slightly reduced in miR-144 overexpressing macrophages. Similar effects were observed in human THP-1 cells (Figure 3B, right). miR-144 also repressed ABCA1 mRNA and protein expression in hepatic and endothelial cells, indicating that its effects are not
Abca1 both miRNAs on miR-33 or miR-144 alone. We also directly tested the effect of the expression of ABCA1 compared with cells transfected in Figure 4A, cotransfection of both miRNAs slightly reduced 33 and miR-144 mimics at a very low dose (5 nmol/L). As seen on ABCA1 expression, we cotransfected Huh-7 cells with miR-33 and miR-144 resulted in a 60% decrease in luciferase activity, consistent with a direct interaction of miR-144 with these sites (Figure 3D). Sites 2 and 4 seem to be the most important sites for miR-144 repression because their mutations are required for the significant derepression of Abca1 3′ UTR activity by miR-144. We further confirmed these results using the mouse Abca1 3′ UTR construct, which has 2 highly conserved predicted miR-144 binding sites. As seen in Online Figure V, overexpression of miR-144 significantly reduced the Abca1 3′ UTR activity and specific point mutations in the miR-144–binding sites abolish its inhibitory effect.

miR-33 and miR-144 Have an Additive Effect on ABCA1 Protein Expression

We have previously reported that miR-33, an intronic miRNA encoded in the Srebp genes, regulates ABCA1 expression. To determine whether miR-33 and miR-33 have an additive effect on ABCA1 expression, we cotransfected Huh-7 cells with miR-33 and miR-144 mimics at a very low dose (5 nmol/L). As seen in Figure 4A, cotransfection of both miRNAs slightly reduced the expression of ABCA1 compared with cells transfected with miR-33 or miR-144 alone. We also directly tested the effect of both miRNAs on Abca1 3′ UTR activity. Cotransfection of miR-33 and miR-144 resulted in a 60% decrease in luciferase activity, whereas miR-144 or miR-33 alone suppressed the 3′ UTR activity 30% to 40% (Figure 4B). To determine the effect of both miRNAs on cellular cholesterol efflux, we transfected Huh-7 cells with miR-33, miR-144, or with a combination of both. miR-33 and miR-144 inhibited cholesterol efflux, but the combination of both miRNAs do not further reduce the cholesterol export in this human hepatic cell line (Figure 4C). This could be explained because we used a very low dose of miRNA mimics (5 nmol/L) in this experiment and because T090 induces the endogenous expression of miR-144 in hepatic cells and not in COS-7 cells, where the 3′ UTR assays were performed.

We further explored the cooperativity of miR-33 and miR-144 in regulating ABCA1 expression by analyzing the miR-33 levels in the setting where miR-144 is inhibited and vice versa. As seen in Online Figure VIA, inhibition of miR-144 does not alter miR-33 levels and miR-33 inhibition does not change miR-144 expression. Moreover, the inhibitory effect of miR-33 and miR-144 on ABCA1 expression is independent of the endogenous expression of both miRNAs (Online Figure VIB). Altogether, these results suggest that miR-144 and miR-33 may cooperate to regulate the expression of ABCA1 in vitro.

miR-144 Expression Regulates Cellular Cholesterol Efflux and HDL Levels In Vivo

ABCA1 plays a critical role in regulating cellular cholesterol efflux to ApoA1. To determine whether miR-144 modulates the efflux of cellular cholesterol, we transfected J774 murine macrophages with miR-144 and then incubated the cells with 3H-cholesterol in the presence of acetylated LDL and T090 to induce ABCA1. As expected, miR-144 overexpression inhibited ABCA1 expression (Figure 5A, upper) and attenuated cholesterol efflux to ApoA1 (Figure 5A, bottom). Importantantly, antagonism of endogenous miR-144 increased ABCA1 expression (Figure 5B, upper) and cellular cholesterol efflux to ApoA1 (Figure 5B, bottom). Thus,
manipulation of cellular miR-144 levels alters macrophage cholesterol efflux, a critical step in the reverse cholesterol transport pathway for the delivery of excess cholesterol to the liver.

In addition to regulating cellular cholesterol efflux, ABCA1 plays a key role in regulating HDL biogenesis in the liver. Thus, we studied the effects of miR-144 levels in vivo by injecting mice with miR-144 mimic particles. Efficient overexpression with 250-fold increase (not shown) was confirmed using quantitative real-time polymerase chain reaction in the liver. Consistent with our in vitro results, miR-144 significantly reduced ABCA1 mRNA and protein expression in the liver (Figure 6A and 6B). We also found a decrease of ABCG1 mRNA and protein expression (Figure 6A and 6B). Other lipid-related genes, including SR-BI, a cognate receptor for HDL in the liver, CD36, and Niemann-Pick C1, were not affected in mice treated with miR-144 particles (Figure 6B).

The data show that miR-144 inhibition in basal conditions resulted in a significant reduction of miR-144 levels (Figure 7A) and an increase of liver ABCA1 protein expression without changes at the mRNA level (Figure 7B and 7C, upper). In another group of mice, we induced the endogenous expression of miR-144 by oral gavage with T0901317. Under this condition, miR-144 was induced 2.3-fold and inhibited 2.1-fold after the injection of anti–miR-144 oligonucleotides (Figure 7A). miR-144 inhibition significantly increased ABCA1 protein and mRNA expression (Figure 7B and 7C, bottom). We further analyzed the effects of anti–miR-144 treatment on total cholesterol, HDL cholesterol, and TG plasma levels. In vivo delivery of anti–miR-144 particles resulted in an increase of plasma HDL cholesterol levels, suggesting that endogenous expression of miR-144 is important in regulating lipoprotein metabolism (Figure 7D). The lipoprotein fractionation analysis also demonstrated that the increased plasma HDL cholesterol was independent of the cholesterol distribution in other lipoproteins (Figure 7E). As expected, plasma TG levels were slightly increased after T090 treatment, but no differences were not affected by anti–miR-144 treatment (Figure 7D, right). Altogether, these results establish that miR-144 overexpression or inhibition reduces and increases circulating HDL, respectively.
Discussion

Since their discovery in *Caenorhabditis elegans*, miRNAs have emerged as critical fine-tuners of many biological processes. Recent advances in the understanding of lipid metabolism have revealed that miRNAs, particularly miR-122 and miR-33, play major roles in regulating cholesterol and fatty acid metabolism. We and others provided identification of a highly conserved miRNA family, miR-33a/b, within the intronic sequences of the *Srebp* genes in organisms ranging from *Drosophila* to humans. The 3′ UTR of *Abca1* contains 3 highly conserved binding sites for miR-33a/b, and the expression of ABCA1 mRNA and protein is strongly repressed by miR-33a/b overexpression in a variety of cell types. In addition to miR-33, other miRNAs, including miR-758, miR-106b, and miR-26, have been shown to regulate the expression of ABCA1 at the posttranscriptional level. In the present study, we report a novel miRNA, miR-144, that regulates the expression of ABCA1 in macrophages, hepatocytes, and endothelial cells. Overexpression of miR-144 inhibits ABCA1 expression and reduces cellular cholesterol efflux in macrophages. Importantly, in vivo manipulation of miR-144 levels in the liver regulates plasma HDL levels. Because HDL levels correlate inversely with coronary artery disease, anti–miR-144 treatment may be useful to prevent atherosclerosis.

Compared with *miR-33a/b*, which are located within introns of *Srebp* genes and regulated by host genes, *miR-144* is an intergenic miRNA located in the same locus as *miR-451*. Here, we show that the primary transcript (pri-miR-144/451) expression is regulated by LXR ligands; however, whether LXR regulates pri-miR-144/451 expression by direct interaction with its promoter remains to be answered. Preliminary
data from our laboratory show that the miR-144/451 promoter has 2 predicted binding sites for SREBP transcription factors. Because SREBP1c expression is activated by LXR, it is plausible that LXR activates SREBP1c and, subsequently, miR-144/451 expression. Another possibility is that the increase in ABCA1 and ABCG1 expression by LXR agonists causes a depletion of cellular cholesterol, leading to an increase in SREBP2 and SREBP1a activity. Further experiments are warranted to understand the molecular mechanism that regulates the expression of miR-144/451 at the transcriptional level. Similarly, Ou et al recently have shown that SREBP1c, a LXR direct target, is likely to be important for fine-tuning cellular processes.

One of the most interesting aspects of miRNA biology is that 1 miRNA often regulates multiple genes that are involved in a specific signaling cascade or cellular mechanism, making miRNAs potent biological regulators. However, defining the gene targets through which an miRNA functions is probably also the most tedious aspect of miRNA research. A given miRNA can be predicted to target several hundred genes, and 60% of mRNAs have predicted binding sites for 1 or multiple miRNAs in their 3'UTR. Under baseline conditions, miRNAs seem to act as moderate regulators that act as a rheostat to fine-tune gene expression, but under conditions of stress or disease, they seem to exert more pronounced functions. To date, it has been reported that at least 4 miRNAs are able to regulate ABCA1 expression in several cell lines and tissues. The contribution of each in modulating ABCA1 expression will be determined by the abundance of each miRNA in different tissues and the biological stimuli that regulate their expression. For instance, miR-33 and miR-758 are downregulated under cholesterol loading conditions...
to increase the expression of ABCA1 and to promote cholesterol export. By contrast, LXR stimulation increases miR-144 and ABCA1 expression to fine-tune cellular cholesterol efflux in macrophages. The regulation of ABCA1 by miR-33, miR-758, miR-106b, and miR-144 also could be influenced by the relative expression of other miR-33, miR-758, miR-106b, and miR-144 mRNA targets that can compete for their 3′UTRs. This could be even more complex with the recent identification of competing endogenous RNAs.34,35 These RNA transcripts share the miRNA response element with the target genes and can regulate each other by competing for miRNA binding. Nevertheless, our data using miR-144 antisense oligonucleotides suggest that the endogenous levels of miR-144 in macrophages and hepatic cell lines are important in regulating ABCA1 expression and cellular cholesterol efflux. Finally, because hepatic ABCA1 is critical for the generation of plasma HDL, it seems likely that a combination therapy that includes both miR-144 and miR-33 antisense oligonucleotides might result in increased HDL levels, thus improving the prognosis for patients with cardiovascular disease.

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Disclosures

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References

We identified a new microRNA (miR-144) that regulates cholesterol metabolism in macrophages and human hepatic cell lines. Inhibition of miR-144 in macrophages increases ABCA1 expression and cholesterol efflux to apolipoprotein A1. Inhibition of miR-144 in vivo increases hepatic ABCA1 expression and plasma HDL levels.

miRNAs recently have emerged as having an important role in regulating macrophage cholesterol efflux and reverse cholesterol transport. Using an unbiased genome-wide screen, we identified miR-144 as a novel regulator of cholesterol metabolism in vitro and in vivo. We show that overexpression of miR-144 reduces ABCA1 expression and attenuates cholesterol efflux to apolipoprotein A1 in macrophages. In contrast, endogenous inhibition of miR-144 expression increases ABCA1 expression and cholesterol efflux to apolipoprotein A1. Most importantly, delivery of miR-144 oligonucleotides to mice attenuates ABCA1 expression in the liver, reducing HDL levels. Conversely, silencing of miR-144 in mice increases the expression of ABCA1 and plasma HDL levels. Thus, genetic manipulation of this pathway could represent a novel therapeutic intervention to increase reverse cholesterol transport and to ameliorate atherosclerotic vascular disease.
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SUPPLEMENTAL MATERIAL

Cristina M. Ramírez, PhD; Noemi Rotllan, PhD; Alexander V. Vlassov, PhD; Alberto Dávalos, PhD, Mu Li, BS; Leigh Goedeke, MS; Daniel Cirera-Salinas, MS; Juan F. Aranda, PhD; Elisa Araldi, MS; Alessandro Salerno, PhD; Amarylis Wanschel, PhD; Jiri Zavadil, PhD; Antonio Castrillo, PhD; Jungsu Kim, PhD; Yajaira Suárez, PhD and Carlos Fernández-Hernando, PhD

ONLINE DETAILED MATERIALS AND METHODS

Materials

Chemicals were obtained from Sigma unless otherwise noted. Human lipoproteins (acetylated LDL) were obtained from Biomedical Technologies Inc. The synthetic LXR ligand T090 is from Cayman Chemical. A mouse monoclonal antibody against ABCA1 was purchased from Abcam and a mouse monoclonal HSP90 antibody was purchased from BD Bioscience. Rabbit polyclonal antibodies against ABCG1 and SR-B1 were obtained from Novus. Secondary fluorescently labeled antibodies were from Molecular Probes (Invitrogen).

Cell Culture

Human monocytic (THP-1), human hepatic (HepG2 and Huh-7), mouse macrophages (J774), mouse hepatic (HEPA), human endothelial (EAhy296) and monkey kidney fibroblast (COS-7) cells were obtained from American Type Tissue Collection (ATTC). THP-1 and J774 cells were maintained in RPMI 1640 media (Sigma) supplemented with 10% fetal bovine serum (FBS) and 2% penicillin-streptomycin in 10 cm² dishes at 37°C and 5% CO₂. THP-1 differentiation into macrophages was induced using 100 nM phorbol-12-myristate acetate (PMA) for 72 h. HepG2, HEPA and COS-7 cells were maintained in Dulbecco’s Modified Eagle Medium (DMEM) containing 10% FBS and 2% penicillin-streptomycin. EAhy296 cells were grown in DMEM containing 10% FBS and penicillin-streptomycin, L-glutamine and HAT (Sigma). Peritoneal macrophages from adult male C57BL/6J mice were harvested by peritoneal lavage four days after intraperitoneal injection of thioglycollate (3% w/v). Bone marrow derived macrophages were isolated from WT and LXR-deficient mice. 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 Ac-LDL or 3 µmol/L T090 for 48 h.

RNA isolation and quantitative real-time PCR

Total RNA was isolated using TRIzol reagent (Invitrogen) according to the manufacturer’s protocol. For mRNA quantification, cDNA was synthesized using iScript RT Supermix (Bio-Rad), following the manufacturer’s protocol. Quantitative real-time PCR was performed in triplicate using iQ SYBR green Supermix (BioRad) on Real-Time Detection System (Eppendorf). The mRNA level was normalized to GAPDH as a house keeping gene. The human primer sequences used were: ABCA1, 5'-GGTTTGGAGATGGTTATACATAGTTG-3' and 5'-CCCGGAACGCAAATCC-3'; ABCG1, 5'-TCACCAGTTCTGCTCATCTT-3' and 5'-GCAGATGTCAGGACCAGG-3'; LXRβ, 5'-TTCGCTAAGCAAGTGCCTGGTTCTC-3' and 5'-AGTCGTCCTTTGCTAGTGAAG-3'; GAPDH, 5'-AACCTTTGCATTGGAAG-3' and 5'-ACACATTTGGGGTGAACA-3'; RXRβ, 5'-GCTCGGCTCGCAGGGC-3' and 5'-TGGCAAAACCGCACCTTG-3'; PPARα, 5'-AAGAGTAGCTTGGAGCCTCGGC-3' and 5'-GCTCGTGAAGCGTCTCGG-3'. The mouse primers sequences used were: ABCA1, 5'-GGTTTGGAGATGGTTATACATAGTTG-3' and 5'-CCCGGAACGCAAATCC-3'; ABCG1, 5'-TCACCAGTTCTGCTCATCTT-3' and 5'-GCAGATGTCAGGACCAGG-3'; RXRβ, 5'-
TCTCAGGGGATCCGTCCGTC-3' and 5'-CGACACTGGAGTTGATCTGAG-3'; LXRβ, 5'-CAGCTGCAGTGCAACAAACGATCT-3' and 5'-ACCTGCTTGGCAAAGTCCACAATC-3'; CYP7A1, 5'-AGCAACTAAACAACCTGCCAGTACTA-3' and 5'-GTCCGGATATTCAAGGATGCA-3'; Actin, 5'-CAGCTGCAGTGCAACAAACGATCT-3' and 5'-ACCTGCTTGGCAAAGTCCACAATC-3'; CYP7A1 5'-AGCAACTAAACAACCTGCCAGTACTA-3’ and 5'-GTCCGGATATTCAAGGATGCA-3'; Actin 5'-TGAGAGGGAAATCGTGCGTGAC-3' and 5'-ACCTGCTTGGCAAAGTCCACAATC-3'; CYP7A1 5'-AGCAACTAAACAACCTGCCAGTACTA-3’ and 5'-GTCCGGATATTCAAGGATGCA-3'; Actin 5'-TGAGAGGGAAATCGTGCGTGAC-3' and 5'-ACCTGCTTGGCAAAGTCCACAATC-3'; CYP7A1 5'-AGCAACTAAACAACCTGCCAGTACTA-3’ and 5'-GTCCGGATATTCAAGGATGCA-3'; Actin 5'-TGAGAGGGAAATCGTGCGTGAC-3' and 5'-ACCTGCTTGGCAAAGTCCACAATC-3'; CYP7A1 5'-AGCAACTAAACAACCTGCCAGTACTA-3’ and 5'-GTCCGGATATTCAAGGATGCA-3'; Actin 5'-TGAGAGGGAAATCGTGCGTGAC-3' and 5'-ACCTGCTTGGCAAAGTCCACAATC-3'; CYP7A1 5'-AGCAACTAAACAACCTGCCAGTACTA-3’ and 5'-GTCCGGATATTCAAGGATGCA-3'; Actin 5'-TGAGAGGGAAATCGTGCGTGAC-3' and 5'-ACCTGCTTGGCAAAGTCCACAATC-3'; CYP7A1 5'-AGCAACTAAACAACCTGCCAGTACTA-3’ and 5'-GTCCGGATATTCAAGGATGCA-3'; Actin 5'-TGAGAGGGAAATCGTGCGTGAC-3' and 5'-ACCTGCTTGGCAAAGTCCACAATC-3'; CYP7A1 5'-AGCAACTAAACAACCTGCCAGTACTA-3’ and 5'-GTCCGGATATTCAAGGATGCA-3'; Actin 5'-TGAGAGGGAAATCGTGCGTGAC-3' and 5'-ACCTGCTTGGCAAAGTCCACAATC-3'; CYP7A1 5'-AGCAACTAAACAACCTGCCAGTACTA-3’ and 5'-GTCCGGATATTCAAGGATGCA-3'; Actin 5'-TGAGAGGGAAATCGTGCGTGAC-3' and 5'-ACCTGCTTGGCAAAGTCCACAATC-3'; CYP7A1 5'-AGCAACTAAACAACCTGCCAGTACTA-3’ and 5'-GTCCGGATATTCAAGGATGCA-3'; Actin 5'-TGAGAGGGAAATCGTGCGTGAC-3' and 5'-ACCTGCTTGGCAAAGTCCACAATC-3'; CYP7A1 5'-AGCAACTAAACAACCTGCCAGTACTA-3’ and 5'-GTCCGGATATTCAAGGATGCA-3'; Actin 5'-TGAGAGGGAAATCGTGCGTGAC-3' and 5'-ACCTGCTTGGCAAAGTCCACAATC-3'; CYP7A1 5'-AGCAACTAAACAACCTGCCAGTACTA-3’ and 5'-GTCCGGATATTCAAGGATGCA-3'; Actin 5'-TGAGAGGGAAATCGTGCGTGAC-3' and 5'-ACCTGCTTGGCAAAGTCCACAATC-3'; CYP7A1 5'-AGCAACTAAACAACCTGCCAGTACTA-3’ and 5'-GTCCGGATATTCAAGGATGCA-3'; Actin 5'-TGAGAGGGAAATCGTGCGTGAC-3' and 5'-ACCTGCTTGGCAAAGTCCACAATC-3'; CYP7A1 5'-AGCAACTAAACAACCTGCCAGTACTA-3’ and 5'-GTCCGGATATTCAAGGATGCA-3'; Actin 5'-TGAGAGGGAAATCGTGCGTGAC-3' and 5'-ACCTGCTTGGCAAAGTCCACAATC-3'; CYP7A1 5'-AGCAACTAAACAACCTGCCAGTACTA-3’ and 5'-GTCCGGATATTCAAGGATGCA-3'; Actin 5'-TGAGAGGGAAATCGTGCGTGAC-3' and 5'-ACCTGCTTGGCAAAGTCCACAATC-3'; CYP7A1 5'-AGCAACTAAACAACCTGCCAGTACTA-3’ and 5'-GTCCGGATATTCAAGGATGCA-3'; Actin 5'-TGAGAGGGAAATCGTGCGTGAC-3' and 5'-ACCTGCTTGGCAAAGTCCACAATC-3'; CYP7A1 5'-AGCAACTAAACAACCTGCCAGTACTA-3’ and 5'-GTCCGGATATTCAAGGATGCA-3'; Actin 5'-TGAGAGGGAAATCGTGCGTGAC-3' and 5'-ACCTGCTTGGCAAAGTCCACAATC-3'; CYP7A1 5'-AGCAACTAAACAACCTGCCAGTACTA-3’ and 5'-GTCCGGATATTCAAGGATGCA-3'; Actin 5'-TGAGAGGGAAATCGTGCGTGAC-3' and 5'-ACCTGCTTGGCAAAGTCCACAATC-3'; CYP7A1 5'-AGCAACTAAACAACCTGCCAGTACTA-3’ and 5'-GTCCGGATATTCAAGGATGCA-3'; Actin 5'-TGAGAGGGAAATCGTGCGTGAC-3' and 5'-ACCTGCTTGGCAAAGTCCACAATC-3'; CYP7A1 5'-AGCAACTAAACAACCTGCCAGTACTA-3’ and 5'-GTCCGGATATTCAAGGATGCA-3'; Actin 5'-TGAGAGGGAAATCGTGCGTGAC-3' and 5'-ACCTGCTTGGCAAAGTCCACAATC-3';

For miRNA quantification, total RNA was reverse transcribed using the RT² miRNA First Strand kit (SABiosciences). Primers specific for human and mouse miR-144 (SABiosciences) were used and values normalized to SNORD38B as a housekeeping gene. For pri-miRNA quantification, cDNA was synthesized using TaqMan® reverse transcription reagents (Applied Biosystems), following the manufacturer's protocol. Quantitative real-time PCR was performed in triplicate using TaqMan Universal Master Mix (Applied Biosystems) on a Real-Time PCR System (Applied Biosystems). The pri-miRNA level was normalized to 18S as a housekeeping gene.

For mouse tissues, total RNA from liver, spleen, lung, kidney, brain, heart, aorta and peritoneal macrophages from C57BL6 mice was isolated using the Bullet Blender Homogenizer (Next Advance) in TRIzol. 1 µg of total RNA was reverse transcribed using the RT² miRNA First Strand kit (SABiosciences) for miR-144 quantitation and normalized to SNORD66 using quantitative PCR as described above.

Western blot Analysis
Cells 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 NaP, 1 mM orthovanadate and 1 mg/ml of protease inhibitor cocktail (Roche) and 0.25 mg/ml AEBSF (Roche). Cell lysates were rotated at 4°C for 1 h before the insoluble material was removed by centrifugation at 12000 x g for 10 min. After normalizing for equal protein concentration, cell lysates were resuspended in SDS sample buffer before separation by SDS-PAGE. Following overnight transfer of the proteins onto nitrocellulose membranes, the membranes were probed with the indicated antibodies, and protein bands were visualized using the Odyssey Infrared Imaging System (LI-COR Biotechnology). Densitometry analysis of the gels was carried out using ImageJ software from the NIH (http://rsbweb.nih.gov/ij/).

miR-144/451 Promoter- Luciferase Reporter Assay
The pGL3-miR-CRE1 promoter-luciferase vector was kindly provided by M. Weiss. Huh-7 cells were plated into 12-well plates and cotransfected using Lipofectamine® LTX & Plus Reagent (Invitrogen), following manufacturer's instructions. Cells were transfected with 1µg of pGL3-miR-CRE1 promoter vector or the pGL3-promoter vector (Promega) as a control, and also cotransfected with 1µg of Renilla plasmid, which was used to normalize transfections. At 24h post transfection, cells were treated with 3 µmol/L T090 or vehicle. After 6h of treatment, cells were lysed with Passive Lysis Buffer (Promega) and 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 pGL3-promoter. Experiments were performed in triplicate, and at least 3 independent experiments were performed.

3'UTR Luciferase Reporter Assays
cDNA fragments corresponding to the entire 3'UTR of human and mouse Abca1 were amplified by RT-PCR from total RNA extracted from HepG2 or Hepa cells with XhoI and NotI linkers. The PCR product was directionally cloned downstream of the Renilla luciferase open reading frame of the psiCHECK2™ vector (Promega) that also contains a constitutively expressed firefly luciferase gene, which is used to normalize transfections. Point mutations in the seed region of
the predicted miR-144 sites within the 3'UTR of Abca1 were generated using Multisite-Quickchange (Stratagene) according to the manufacturer's protocol. All constructs were confirmed by sequencing. COS-7 cells were plated into 12-well plates and co-transfected with 1 µg of the indicated 3'UTR luciferase reporter vectors and the miR-144 mimic or control mimic (Con-miR) (Dharmacon) utilizing Lipofectamine 2000 (Invitrogen). 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 (cells co-transfected with the corresponding concentration of control mimic). Experiments were performed in triplicate wells of a 12-well plate and repeated at least three times.

**ONLINE FIGURE LEGENDS**

Online Figure I. Sequence and conservation of the miR-144 binding sites in the human Abca1 3'UTR. (A) Sequence alignment of the human Abca1 3'UTR. Relative position of the binding sites is indicated. (B) Sequence alignment of the seven human (Hsa) miR-144 binding sites and the indicated species [Pan troglodytes (Ptr), Mus musculus (Mmu), Rattus norvegicus (Rno), and Oryctolagus cuniculus (Ocu)].

Online Figure II. LXR ligands induce miR-144 and miR-451 expression in macrophages and hepatic cell lines. (A) qRT-PCR analysis of miR-144 expression in THP-1 (left panel) and Huh-7 cells (right panel) treated with GW3965. Data are the mean ± SEM of 3 independent experiments in triplicate. *P< 0.05. (B) qRT-PCR analysis of miR-144 expression in mouse peritoneal macrophages treated with Ac-LDL. Data are the mean ± SEM of 3 independent experiments in triplicate. *P< 0.05. (C and D) qRT-PCR analysis of miR-144 expression in THP-1 (left panel) and Huh-7 cells (right panel) treated with different doses of T090 (C) or GW3965 (D). Data are the mean ± SEM of 2 independent experiments in duplicate. (E) qRT-PCR analysis of miR-451 expression in mouse peritoneal macrophages (left panel), THP-1 cells (middle panel) and Huh-7 cells (right panel) treated with T090. Data are the mean ± SEM of 3 independent experiments in triplicate. *P< 0.05.

Online Figure III. Distribution and dietary regulation of miR-144. (A) qRT-PCR analysis of miR-144 expression in selected mouse tissues. (B) Representative pictures of hepatocyte (Hep) and Kupffer (Kupf) cells isolated by isopycnic centrifugation (upper left panels). The purity of each cell population was confirmed by Western blot (bottom left panel). miR-144 relative expression assessed by qRT-PCR is represented in the right panel. Data are the mean ± SEM of three independent cellular isolations. qRT-PCR analysis of miR-144 expression in the liver of C57BL6 mice (=5 per group) fed in a chow diet or high-fat diet (HFD) for 5 weeks. Graphs (left to right) represent body weight, cholesterol plasma levels, triglycerides plasma levels and miR-144 expression. Data are expressed as relative expression and correspond to mean±SEM of three independent experiments. *Significantly different from mice fed with chow diet, P≤0.05.

Online Figure IV. miR-144 over-expression reduces ABCA1 expression in HepG2, Hepa and EAhy296 cells. (A) qRT-PCR analysis of ABCA1 in HepG2 transfected with miR-144 mimics. Data are the mean ± SEM of 3 independent experiments in triplicate. *P≤ 0.05. (B) Western blot analysis of ABCA1 and HSP90 expression in HepG2, Hepa and EAhy296 cells transfected with miR-144 mimics and treated or untreated with T0901317. Data correspond to a representative blot among two that gave similar results.

Online Figure V. miR-144 levels regulates mouse Abca1 3'UTR activity. Luciferase-reporter activity in COS-7 cells transfected with Con-miR or miR-144 mimic and mouse Abca1 3'UTR.
containing or not the point mutations (PM) in the miR-144 target sites. Data are expressed as mean % of 3'UTR activity of Con-miR ±SEM and are representative of ≥3 experiments. *Significantly different from cells cotransfected with Con-miR and wild-type (WT) 3'UTR. P≤ 0.05.

**Online Figure VI. miR-33 and miR-144 inhibit ABCA1 expression in Huh-7 cells independently of the endogenous levels of each miRNA.** (A) qRT-PCR analysis of miR-33 (left panel) and miR-144 (right panel) expression levels in Huh-7 cells transfected with miR-33 inhibitor (Inh-miR-33) or miR-144 inhibitor (Inh-miR-144 panel) compared with cells transfected with control inhibitor (Con-inh). Data are the mean ± SEM of 2 independent experiments in duplicate. (B) Western blot analysis of ABCA1 expression in Huh-7 cells transfected with miR-144, miR-33, Inh-miR-33 and Inh-miR-144 and treated or not with T0901317 (T090). Data correspond to a representative blot among two that gave similar results. HSP-90 was used as a loading control. Values of the band densitometry analysis are shown.
Online Figure I

**A**

*Abca1* 3'UTR (3.309 kb)

**B**

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Online Figure II
Online Figure IV

A

HepG2

Con-miR
miR-144

ABCA1 mRNA (fold change)

B

HepG2
Hepa
EAhy.926

ABCA1

HSP90

Relative Density (AU)

0.15 1.23 1.17 0.43
0.29 0.48 0.57 0.34
0.14 2.07 1.78 0.53

Treatment
Con-miR
miR-144

- - + -
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- - - +

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*
mouse Abca1 3′UTR (3.150 kb)

Site 1  Site 2  Site 3

% 3′ UTR Activity Abca1

Con-miR  miR-144

WT  WT  PM1  PM2  PM1,2