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 homeostatis ■ 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 lipidate nascent 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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Nonstandard Abbreviations and Acronyms

3′UTR	3′-untranslated region
ABCA1	adenosine triphosphate–binding cassette transporter A1
ABCG1	adenosine triphosphate–binding cassette transporter G1
ApoA1	apolipoprotein A1
HDL	high-density lipoprotein
LXR	liver X nuclear receptor
miRNA	microRNA
SREBP	sterol response element–binding protein
T090	T0901317
TG	triglycerides

The expression of ABCA1 and of ABCG1 is upregulated in states of cholesterol excess by liver X nuclear receptor (LXR).[9] LXRs 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.[5,10] 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 cytoplasmatic 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 mRNAs.[13] In animals, most investigated miRNAs form imperfect hybrids with sequences in the 3′-untranslated region (3′UTR), with the miRNA 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-octanyl transferase (*Crot*), hydroxyacyl-CoA dehydrogenase-3-ketoacyl-CoA thiolase-enoyl-CoA hydratase (trifunctional protein) β-subunit (*Hadhb*), and 5′ AMP-activated protein kinase (*Ampk*).[23,24] 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 LXRα-null and LXRβ-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 mimic (Con-mir; n=6); miR-144-mimic (miR-144; n=6); inhibitor negative control sequence (Con-inh; n=6); and miR-144-inhibitors (Inh-miR-144). 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 pellet 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

for each condition were used for microarray analysis. Raw data were normalized and analyzed by GeneSpring GX software version 11.5 (Agilent Technologies). miRNAs showing an altered expression in T090 treatment compared with control were identified using a *t* test unpaired with $P<0.2$ and fold change ≥1.5, asymptotic *P* value computation, and no multiple testing correction. The 1.5-fold–change threshold was chosen on the basis of its use in previously published articles using these particular types of microarrays. Hierarchical cluster analysis of average value per condition was performed using average linkage and Pearson uncentered correlation as a measure of similarity.

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 mi*RIDIAN* miRNA mimic (miR-144) or with 60 nmol/L mi*RIDIAN* 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 sequence (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 (Invitrogen) 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 1×10^6 cells per well 1 day before loading with 0.5-μCi/mL ^{3}H-cholesterol for 24 hours. Then, 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 ApoA1 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 ^{3}H-cholesterol content (total effluxed ^{3}H-cholesterol plus cell-associated ^{3}H-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 −80ºC. Total plasma cholesterol and HDL cholesterol were enzymatically measured with the Amplex red cholesterol assay kit (Molecular Probes, Invitrogen) according to the manufacture'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).

Primary Hepatocyte and Kupffer Cells Isolation

Primary hepatocyte and Kupffer cells were isolated using standard protocols.[27]

Statistical Analysis

All data are expressed as ±SEM. Statistical differences were measured by either a Student *t* test or a 2-way ANOVA with Bonferroni correction for multiple comparisons when appropriate. A value of $P\leq0.05$ was considered statistically significant. Data analysis was performed using GraphPad Prism 5.0a software (GraphPad, San Diego, CA).

An expanded Materials and Methods section is available in the Online Data Supplement.

Results

miR-144 is Upregulated in LXR-activated Macrophages and in the Liver of Mice Fed a High-fat Diet

To determine whether LXR-induced miRNAs can regulate cholesterol metabolism at posttranscriptional levels, we undertook an unbiased genome-wide screen of miRNAs modulated by LXR ligands. We identified 32 miRNAs differentially regulated in mouse peritoneal macrophages treated with T090, a synthetic LXR ligand (Figure 1A). Interestingly some of the miRNAs induced by T090, including miR-370 and miR-122, were previously identified as important regulators of lipid metabolism.[15,16,18,28] To determine the potential candidates involved in regulating cholesterol metabolism, we analyzed the predicted target genes using a combination of bioinformatic tools for miRNA target predictions (TargetScan [http://www.targetscan.org] and miRanda [http://www.microrna.org]), gene ontology (Panther [http://www.pantherdb.org/]), and protein–protein interactions ([String (http://string-db.org/]). We found miR-144 as a strong predicted candidate to regulate genes involved in metabolic processes, including lipid metabolism (Online Figure IB and ID). Protein–protein interaction analysis showed that miR-144–predicted targets might play an important role in regulating lipid homeostasis (Figure 1E and 1F). Interestingly, ABCA1 was a strong predicted target with 7 potential binding sites in the 3′UTR (Online Figure IA), with some of them conserved in most vertebrates (Online Figure IB).

We next confirmed the induction of miR-144 by LXR ligands using quantitative real-time polymerase chain reaction analysis. As seen in Figure 2A, mouse peritoneal macrophages, human monocytes (THP-1), and human hepatic (Huh-7) cells treated with T090 showed an increased expression of miR-144. Similar results were obtained using another LXR ligand (GW3965) in THP-1 and Huh-7 cells (Online Figure IIA) and after cholesterol loading with acetylated LDLs in mouse peritoneal macrophages (Online Figure IIB). The maximum stimulation of miR-144 expression was observed at 0.1 μmol/L of T090 and 3 μmol/L of GW3965 in human THP-1 and Huh-7 cells (Online Figure IIC and IID). In addition to miR-144, miR-451, which is encoded in the same genomic cluster, also was induced on stimulation with T090 in mouse primary macrophages, THP-1 and Huh-7 (Online Figure IIE). To examine whether LXR ligands regulated the expression of these miRNAs at the transcriptional level, we examined the expression of primary transcripts containing the stem loop of miR-144 and miR-451. As shown in Figure 2B, LXR ligands increased the expression of pri-miR-144/451. Furthermore,

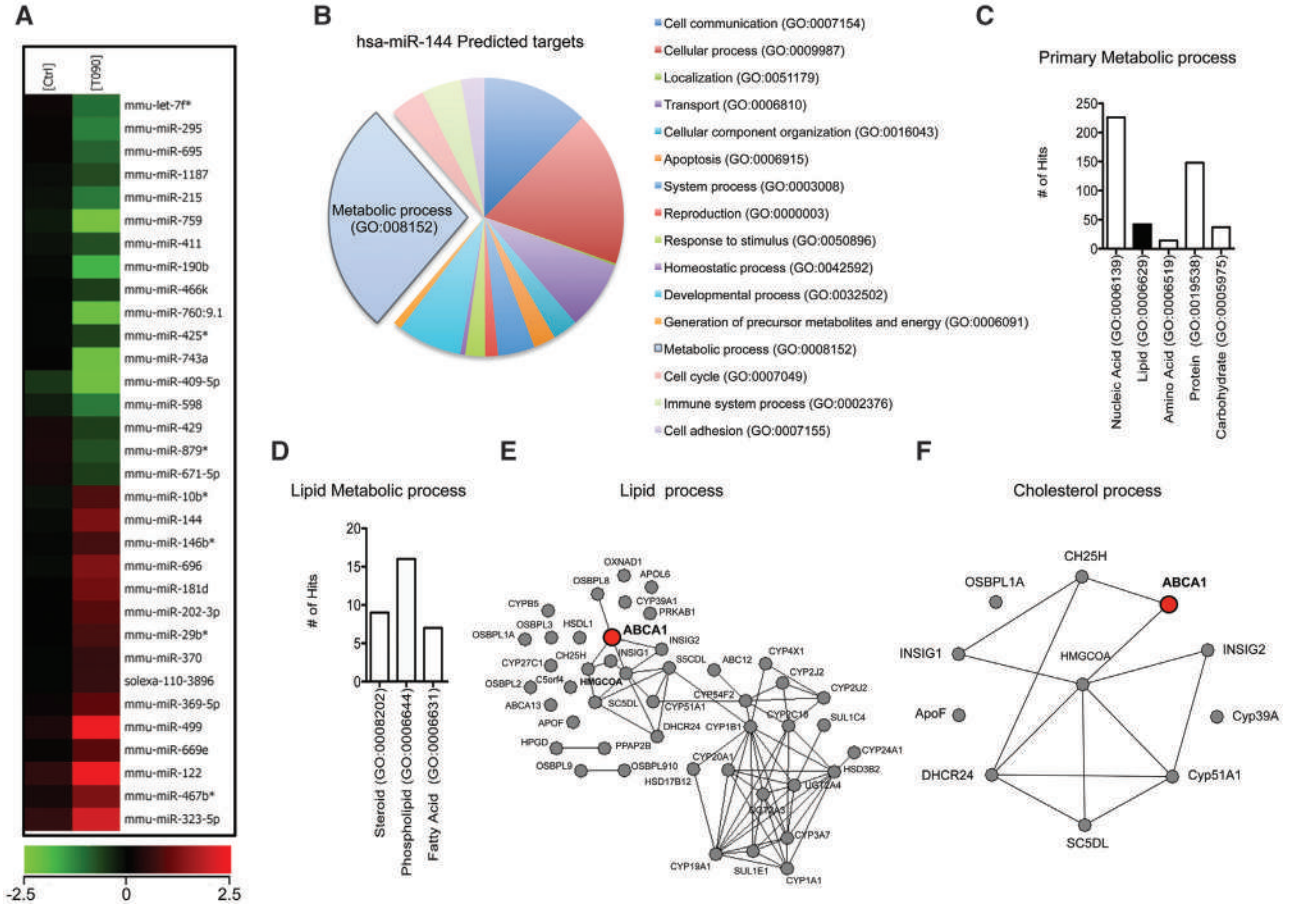

Figure 1. miR-144 expression is upregulated in liver X nuclear receptor–treated macrophages and has multiple lipid-related predicted target genes. A, microRNA (miRNA) array analysis of miRNAs upregulated (red) or downregulated (green) from peritoneal macrophages treated with T0901317 (n=4). **B** to **D**, Gene ontology analysis of the miR-144–predicted target genes using Panther software. **E** and **F**, Protein–protein interaction analysis of the miR-144–predicted targets using String software and Navigator 2.2. ABCA1 indicates adenosine triphosphate–binding cassette transporter A1.

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

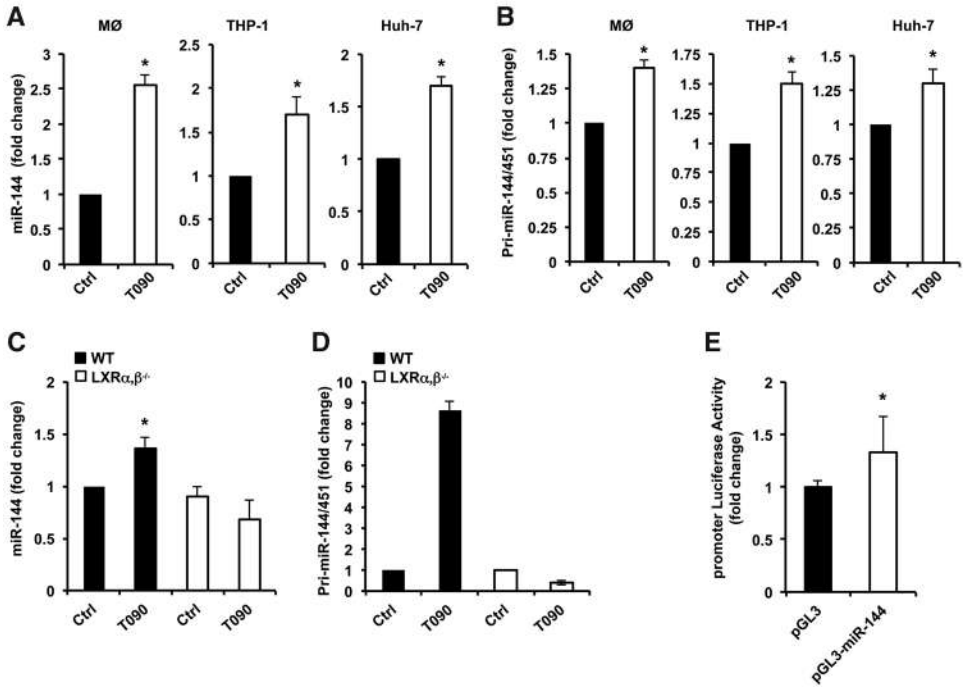

Figure 2. Liver X nuclear receptor (LXR) induces miR-144 transcriptional expression. A, Quantitative real-time polymerase chain reaction (qRT-PCR) analysis of miR-144 expression in mouse peritoneal macrophages (MØ, **left**), THP-1 cells (**center**), and Huh-7 cells (**right**) treated with T0901317 (T090). **B**, qRT-PCR analysis of pri-miR-144/451 expression in mouse peritoneal macrophages (**left**), THP-1 cells (**center**), and Huh-7 cells (**right**) treated with T090. **C** and **D**, qRT-PCR analysis of miR-144 (**C**) and pri-miR-144/451 (**D**) expression from wild-type (WT) and LXR $\alpha,\beta^{-/-}$ (LXR$\alpha,\beta^{-/-}$) macrophages treated with T090. **E**, miR-144/451 promoter-luciferase reporter activity in Huh-7 cells treated with T090. Data are expressed as relative expression and correspond to mean±SEM of 3 independent experiments. *Significantly different from cells without treatment (Ctrl), $P \leq 0.05$.

specific to cell type (Online Figure IVA and IVB). We further determined the role of endogenous miR-144 on ABCA1 expression in mouse peritoneal macrophages and human THP-1 cells. Importantly, inhibition of miR-144 by anti–miR-144 antisense oligonucleotides (Inh-miR-144) increased the expression of ABCA1 in both cell types (Figure 3C).

To assess the effects of miR-144 on the 3′UTR of human *Abca1*, we used a luciferase reporter construct. miR-144 (40 nmol/L) markedly repressed *Abca1* 3′UTR activity (Figure 3D). Mutation of the miR-144 target sites relieved miR-144 repression of the *Abca1* 3′UTR activity, consistent with a direct interaction of miR-144 with these sites (Figure 3D and 3E). 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-144 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 ^{3}H-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). Importantly, antagonism of endogenous miR-144 increased ABCA1 expression (Figure 5B, upper) and cellular cholesterol efflux to ApoA1 (Figure 5B, bottom). Thus,

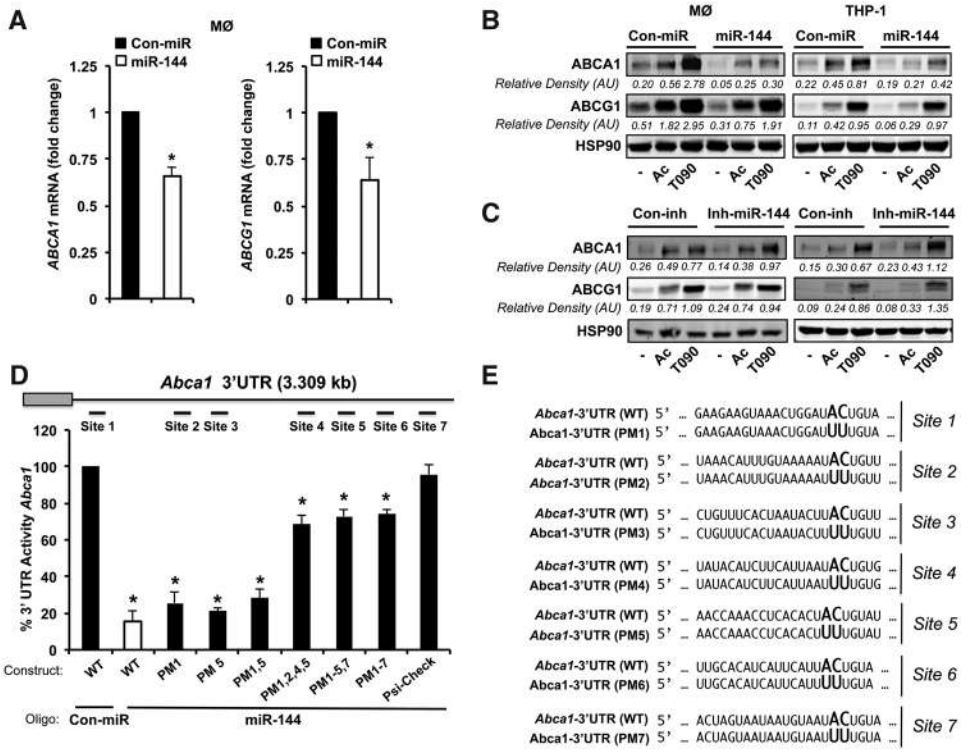

Figure 3. miR-144 levels regulate adenosine triphosphate–binding cassette transporter A1 (ABCA1) expression. **A**, Quantitative real-time polymerase chain reaction analysis of *ABCA1*, and *ABCG1* expression in mouse peritoneal macrophages transfected with miR-144 mimics. Data are expressed as relative expression and correspond to the mean±SEM from 3 independent experiments. *Significantly different from cells transfected with control mimic (Con-miR), *$P \leq 0.05$. **B** and **C**, Western blot analysis of ABCA1, adenosine triphosphate–binding cassette transporter G1 (ABCG1), and HSP90 in mouse peritoneal macrophages and THP-1 cells transfected with (**B**) Con-miR or miR-144, or (**C**) control inhibitor (Con-inh) or anti–miR-144 in the presence or in the absence of acetylated low-density lipoprotein (Ac-LDL) or T0901317 (T090). Data correspond to a representative experiment among 3 that gave similar results. Values of the band densitometry analysis are shown. **D**, Luciferase reporter activity in COS-7 cells transfected with Con-miR or miR-144 (40 nmol/L) mimic and human *Abca1* 3′-untranslated region (UTR) containing the indicated point mutations (PM) in the miR-144 target sites. Data are expressed as mean percent 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. $P \leq 0.05$. **E**, Human *Abca1* 3′UTR containing the indicated PM in the miR-144 target sites.

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 inhibition of ABCA1 expression after 6 days of treatment with miR-144 particles leads to a significant reduction in total cholesterol and HDL cholesterol levels without changes in TG or cholesterol distribution in other lipoproteins (Figure 6C and 6D). To put these latter results in a more physiological context, we inhibited the expression of miR-144 using particles conjugated with miR-144 antisense oligonucleotides. 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 T090. 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.

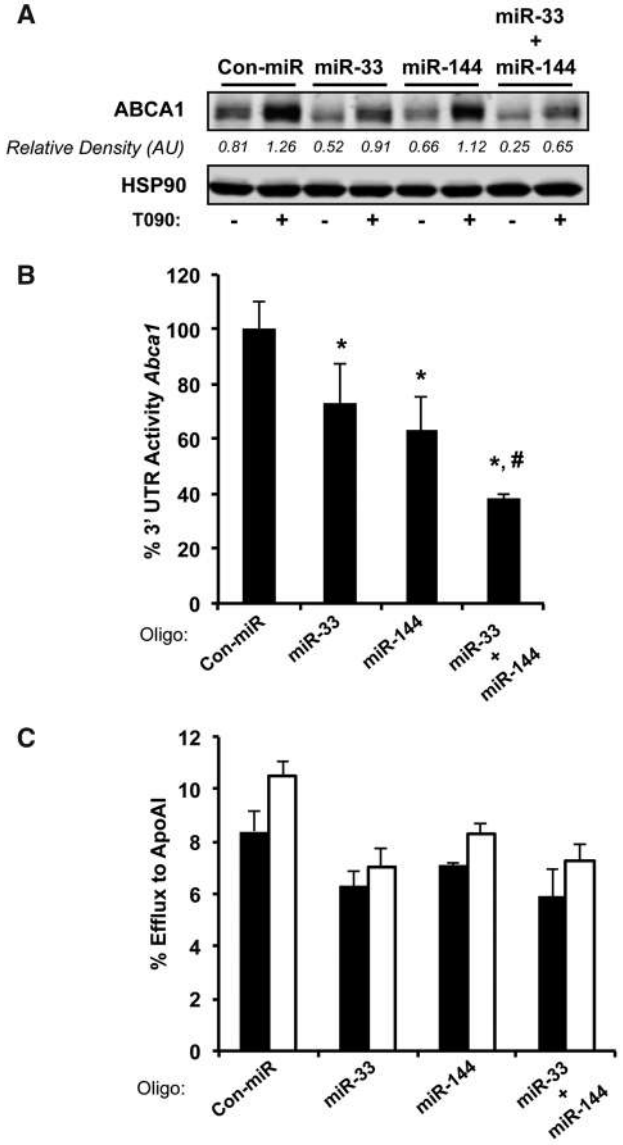

Figure 4. miR-33 and miR-144 cooperate to regulate adenosine triphosphate–binding cassette transporter A1 (ABCA1) expression. **A**, Western blot analysis of Huh-7 cells transfected with 5 nmol/L nontargeting control mimic (Con-miR), miR-33, miR-144, and miR-33/miR-144 (**upper**). Data correspond to a representative experiment among 3 that gave similar results. Quantification of specific bands was conducted by densitometric analysis and is shown at the **bottom** of each band. **B**, Activity of the luciferase reporter construct fused to the 3′-untranslated region (UTR) of *Abca1* in COS-7 cells. Cells were transfected with Con-miR or miR-33, or miR-144, or miR-33 and miR-144 together. All microRNA constructs were transfected at a final concentration of 5 nmol/L. Relative luciferase activity is presented and data are the mean±SEM of 3 independent experiments in triplicate. *Significantly different from cells cotransfected with Con-miR and *Abca1* 3′UTR. $P \leq 0.05$. #Significantly different from cells cotransfected with miR-33 or miR-144 and *Abca1* 3′UTR. $P \leq 0.05$. **C**, Total cholesterol efflux to apolipoprotein A1 (ApoA1) in Huh-7 cells stimulated with T0901317 and expressing control miR (Con-miR), miR-33, miR-144, or miR-33 and miR-144. Data are the mean±SEM of 2 independent experiments performed in triplicate.

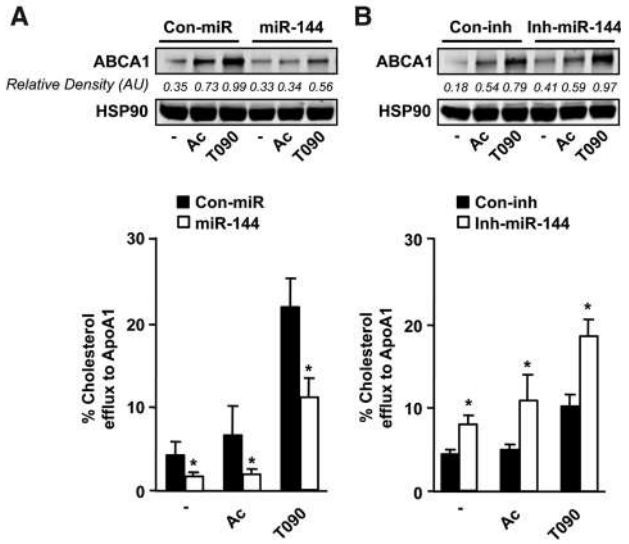

Figure 5. Modulation of miR-144 regulates macrophage cholesterol efflux. A, Total cholesterol efflux to apolipoprotein A1 (ApoA1) in J774 mouse macrophages stimulated with acetylated low-density lipoprotein (Ac-LDL) or T0901317 (T090) and expressing control miR (Con-miR) or miR-144. **Upper**, Adenosine triphosphate–binding cassette transporter A1 (ABCA1) protein expression assessed by Western blotting in the same conditions. **B**, Total cholesterol efflux to ApoA1 in J774 mouse macrophages stimulated with Ac-LDL (Ac) or T090 and expressing control inhibitor (Con-inh) or Inh-miR-144. **Upper**, ABCA1 protein expression assessed by Western blotting in the same conditions. Data are the mean±SEM of 3 independent experiments performed in triplicate. *Significantly different from cells transfected with Con-miR (**A**) or Con-inh (**B**). $P \leq 0.05$.

Discussion

Since their discovery in *Caenorhabditis elegans*, miRNAs have emerged as critical fine-tuners of many biological processes.[29,30] 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.[15,16,20,21,23] 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.[20,21,23] 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.[19,22,31] 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

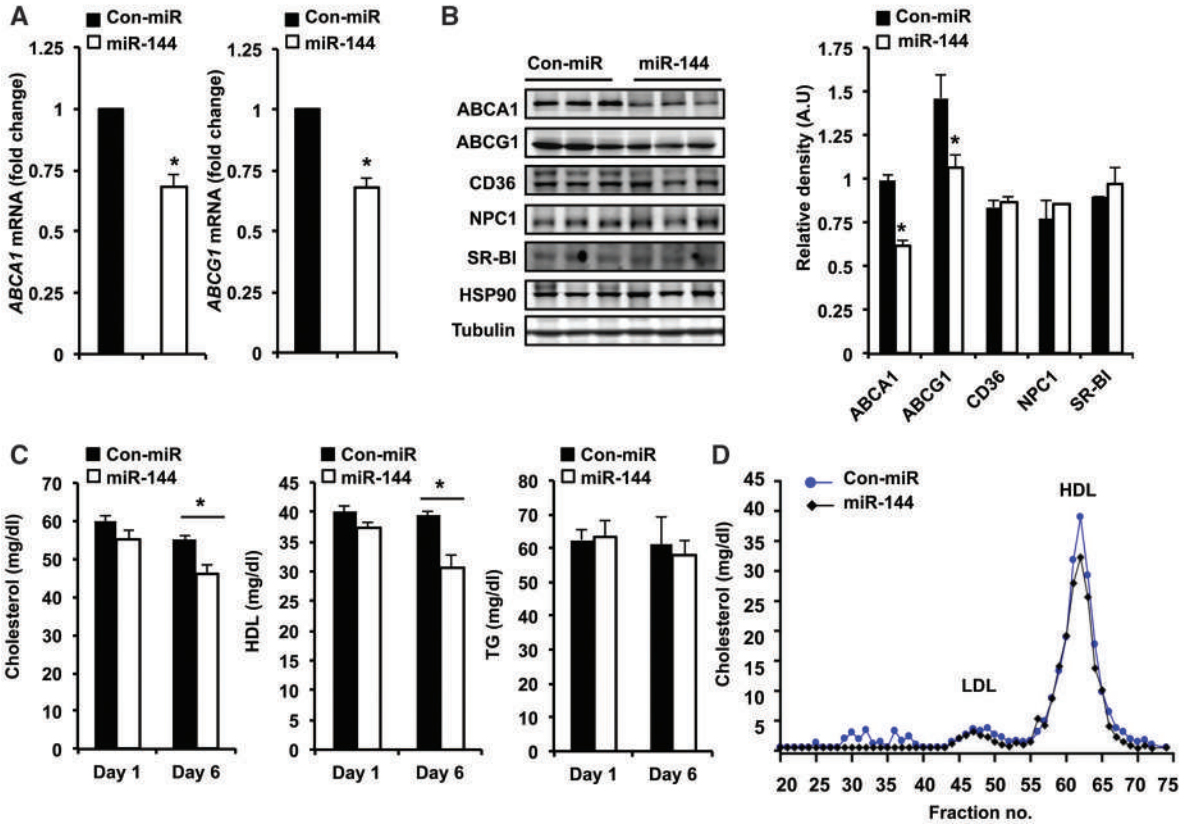

Figure 6. Delivery of miR-144 oligonucleotides significantly reduces liver adenosine triphosphate–binding cassette transporter A1 (ABCA1) expression and high-density lipoprotein (HDL) levels in mice. A, Quantitative real-time polymerase chain reaction analysis of *ABCA1* and *ABCG1* expression from mouse livers treated with miR-144 mimics. Data are the mean±SEM from 6 mice per group. *Significantly different from mice injected with nontargeting control mimic (Con-miR) conjugated particles. $P \leq 0.05$. **B**, Analysis of hepatic gene expression 6 days after injection of Con-miR or miR-144. Western blot analysis of liver tissue from 3 representative mice per group. Quantification analysis is shown in the **right**. Data are the mean±SEM from 6 mice per group. *Significantly different from mice injected with Con-miR conjugated particles. $P \leq 0.05$. **C** Total cholesterol (**left**), HDL cholesterol (**center**), and triglyceride (TG; **right**) levels in mice treated with Con-miR or miR-144 mimics (n=6). *Significantly different from mice injected with nontargeting Con-miR conjugated particles. $P \leq 0.05$. **D** Lipoprotein profile from mice treated with Con-miR or miR-144 mimics (n=6). Cholesterol distribution across plasma fractions was analyzed by fast protein liquid chromatography. ABCG1 indicates adenosine triphosphate–binding cassette transporter G1.

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[32] recently have demonstrated that miR-613, a LXR-induced miRNA, targets LXRα and played an important role in the autoregulation of the human *Lxrα* gene.[32] Interestingly, miR-613 transcriptional expression is activated by SREBP1c, a LXR direct target. In a similar manner, it also has been shown that miRNAs regulate the expression of adhesion molecules (SELE and ICAM-1) in response to tumor necrosis factor through a negative feedback loop to control inflammatory responses.[33] This kind of regulation, in which miRNA-directed target repression acts to oppose the overall outcome of an induced biological response, 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.[13] 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.[13,14] 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.[19,22,23] 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

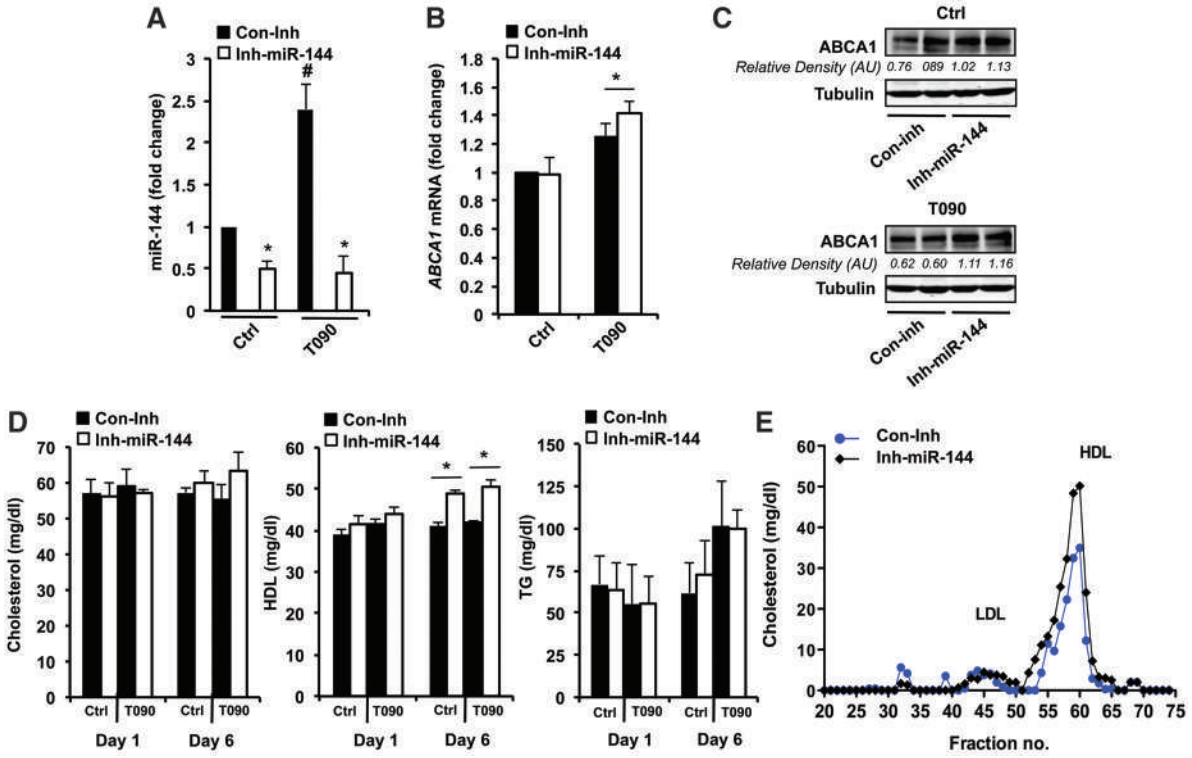

Figure 7. Inhibition of miR-144 increases hepatic adenosine triphosphate–binding cassette transporter A1 (ABCA1) expression and raises high-density lipoprotein (HDL) plasma levels. A, Quantitative real-time polymerase chain reaction (qRT-PCR) analysis of miR-144 levels from mouse livers treated with miR-144 inhibitor (Inh-miR-144) conjugated particles. Data are expressed as relative expression and correspond to the mean±SEM from 6 mice per group. *Significantly different from mice injected with control inhibitor (Con-inh). #Significantly different from mice untreated and injected with Con-Inh or treated with T0901317 (T090) and injected with Inh-miR-144 compared with mice treated with T090 and injected with Con-Inh. $P \leq 0.05$. **B**, qRT-PCR analysis of hepatic ABCA1 gene expression mouse liver treated or untreated with T090 and Con-inh or Inh-miR-144. Significantly different from mice injected with Con-inh. $P \leq 0.05$. **C**, Western blot analysis of ABCA1 expression from liver tissue of mice injected with Con-inh or Inh-miR-144 and treated with vehicle (**upper**) or T090 (**bottom**). Quantification of specific bands was conducted by densitometric analysis and is shown at the **bottom** of each band. **D**, Total cholesterol, HDL cholesterol and triglyceride (TG) plasma levels in mice injected with Con-miR or miR-144 mimics and treated or not with T090 (n=6). Data are the mean±SEM from 6 mice per group. Significantly different from mice injected with Con-inh, *$P \leq 0.05$. **E**, Lipoprotein profile analysis from mice treated with Con-inh or Inh-miR-144 (n=6). Cholesterol distribution across plasma fractions was analyzed by fast protein liquid chromatography.

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 the binding in 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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Novelty and Significance

What Is Known?

- Adenosine triphosphate–binding cassette transporter A1 (ABCA1) plays a critical role in regulating cellular cholesterol efflux in macrophages, and liver and hepatic ABCA1 expression is required for plasma high-density lipoprotein (HDL) formation.
- Treatment of mice and nonhuman primates with miRNA antisense oligonucleotides, such as miR-33 antisense oligonucleotides, can increase HDL cholesterol levels and promote atherosclerosis regression.

What New Information Does This Article Contribute?

- 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.