MicroRNA-758 Regulates Cholesterol Efflux Through Posttranscriptional Repression of ATP-Binding Cassette Transporter A1

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Objective—The ATP-binding cassette transporter A1 (ABCA1) is a major regulator of macrophage cholesterol efflux and protects cells from excess intracellular cholesterol accumulation; however, the mechanism involved in posttranscriptional regulation of ABCA1 is poorly understood. We previously showed that microRNA-33 (miR-33) is 1 regulator. Here, we investigated the potential contribution of other microRNAs (miRNAs) to posttranscriptional regulation of ABCA1 and macrophage cholesterol efflux.

Methods and Results—We performed a bioinformatic analysis for identifying miRNA target prediction sites in ABCA1 gene and an unbiased genome-wide screen to identify miRNAs modulated by cholesterol excess in mouse peritoneal macrophages. Quantitative real-time reverse transcription–polymerase chain reaction confirmed that miR-758 is repressed in cholesterol-loaded macrophages. Under physiological conditions, high dietary fat excess in mice repressed miR-758 both in peritoneal macrophages and, to a lesser extent, in the liver. In mouse and human cells in vitro, miR-758 repressed the expression of ABCA1, and conversely, the inhibition of this miRNA by using anti-miR-758 increased ABCA1 expression. In mouse cells, miR-758 reduced cellular cholesterol efflux to apolipoprotein A1 (apoA1), and anti-miR-758 increased it. miR-758 directly targets the 3′-untranslated region of Abca1 as assessed by 3′-untranslated region luciferase reporter assays. Interestingly, miR-758 is highly expressed in the brain, where it also targets several genes involved in neurological functions, including Slc38a1, Ntm, Epha7, and Mytl1.

Conclusion—We identified miR-758 as a novel miRNA that posttranscriptionally controls ABCA1 levels in different cells and regulates macrophage cellular cholesterol efflux to apoA1, opening new avenues to increase apoA1 and raise high-density lipoprotein levels. (Arterioscler Thromb Vasc Biol. 2011;31:2707-2714.)

Key Words: ABC transporter • atherosclerosis • lipids • lipoproteins • macrophages

Cellular cholesterol levels are tightly regulated and represent a balance among cholesterol uptake, endogenous synthesis, and efflux. Many diseases result from perturbations in lipid homeostasis, including atherosclerosis, metabolic syndrome, type II diabetes, and Alzheimer disease. In mammals, 2 transcription factors are recognized to control cellular cholesterol homeostasis: the sterol response element–binding proteins and the liver X receptors (LXRs). The sterol response element–binding proteins control both endogenous cholesterol synthesis and uptake by regulating several sterol-dependent genes, including statins1,2 and the low-density lipoprotein receptor (LDLR). The LXRs are members of the nuclear receptor subfamily and are activated in response to cellular cholesterol.3 Under cellular cholesterol excess, LXR targets genes such as the ATP-binding cassette transporter A1 (ABCA1) and G1 (ABCG1), which promote cellular cholesterol efflux and maintain cellular sterol homeostasis.4,5 The removal of excess cholesterol from peripheral tissues, such as macrophage foam cells, has been recognized as a key mechanism to prevent atherogenesis.6 ABCA1 promotes macrophage cholesterol efflux, and deficiency or mutations in this transporter lead to defects in cholesterol efflux and cholesterol ester accumulation in macrophages and increase the risk of developing cardiovascular diseases.7-9

ABCA1 also plays an important role in regulating cholesterol metabolism in the brain. Cholesterol is required for myelination, dendrite differentiation, and synaptic activity. Disturbances in central nervous system cholesterol homeostasis are implicated in neurodegenerative disorders, including Alzheimer and Huntington diseases.10 Several studies have demonstrated that ABCA1 facilitates the cholesterol efflux of central nervous system cholesterol to apolipoprotein E (apoE), as the absence of ABCA1 compromises apoE
secretion from both astrocytes and microglia. Moreover, the apoE that is present in the cerebrospinal fluid of ABCA1-deficient mice is poorly lipidated. Interestingly, in amyloid mouse models of Alzheimer disease, ABCA1 deficiency exacerbates amyloidogenesis, whereas ABCA1 overexpression ameliorates amyloid plaque load, suggesting a role for ABCA1 in Aβ metabolism.

In addition to classical transcription regulators, a class of noncoding RNAs termed microRNAs (miRNAs) has emerged as critical regulators of gene expression, acting predominantly at the posttranscriptional level. This large family of short (22 nucleotides), double-stranded regulatory noncoding RNAs is encoded in the genome, and each member is processed from primary transcripts by the sequential actions of Drosha and Dicer enzymes. On incorporation into the cytoplasmic RNA-induced silencing complex, miRNAs bind to partially complementary target sites in mRNA 3′-untranslated regions (3′UTRs), which results in translational repression, mRNA destabilization, or a combination of both. We and others have recently shown that miRNA-33 regulates cholesterol efflux and high-density lipoprotein (HDL) biogenesis by downregulating the expression of ABC transporters ABCA1 and ABCG1. Notably, silencing of miR-33 in vivo increases hepatic ABCA1 expression and plasma HDL levels.

Bioinformatic predictions and experimental approaches indicate that a single miRNA could target more than 100 mRNAs. Similarly, a single miRNA could be regulated by many miRNAs. This may be the case of Abca1, which has a very large 3′UTR (3.3 kb) and more than 100 potential miRNA candidates, according the miRNA target prediction algorithms. In the present study, we aimed to identify new miRNAs that regulate ABCA1 expression. We show that miR-758 posttranscriptionally regulates the expression of ABCA1 in different cell lines, including macrophages, hepatocytes, and astrocytes, thereby attenuating cholesterol efflux to apoA1. Furthermore, the high expression of miR-758 in the brain suggests an important role for this miRNA in regulating cellular cholesterol metabolism in this organ.

Materials and Methods
See the Supplemental Data, available online at http://atvb.ahajournals.org, for an extended description of the methods used in this study.

miRNA Mimics, miRNA Inhibitors, and ABCA1 Small Interfering RNA Transfection
Cells (50%–70% confluence) were transfected with various of 40 nmol/L miRIDIAN miRNA mimics, miRIDIAN miRNA inhibitors (Dharmacon), and small interfering RNA ABCA1 (smart pool, Dharmacon) using Lipofectamine RNAiMAX (Invitrogen) using the manufacturers’ protocols. In all experiments, an equal concentration of a nontargeting control mimic sequence (Con-miR) or inhibitor-negative control sequence was used as a control for nonsequence-specific effects in miRNA experiments. Cells were transfected for 24 hours and treated with or without either T0901317 (3 μmol/L) or acetylated low-density lipoprotein (AcLDL) (120 μg/mL) for an additional 24 hours.

3′UTR Luciferase Reporter Assays
cDNA fragments corresponding to the entire 3′UTR of human Abca1 was amplified by reverse transcription–polymerase chain reaction from total RNA extracted from HepG2 cells with Xhol and NotI linkers. The polymerase chain reaction products were directionally cloned downstream of the Renilla luciferase open reading frame in the psiCHECK2TM vector (Promega) that also contains a constitutively expressed firefly luciferase gene, which is used to normalize transfections. Site-directed mutations in the seed region of predicted miR-758 sites within the 3′UTR of human 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 (Costar) and cotransfected with 1 μg of the indicated 3′UTR luciferase reporter vectors and the miR-758 mimic or negative control mimic (Con-miR) (Dharmacon) using 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 (Con-miR). Experiments were performed in triplicate, and at least 3 independent experiments were performed.

Cholesterol Efflux Assays
J774 macrophages and human H4 neuroglioma cell line were plated in 12-well plates (1×10⁶ cells/well) and transfected with either a Con-miR, a miR-758, an inhibitor-negative control sequence, or an anti-miR-758 (Dharmacon) for 24 hours. Cells were loaded with 0.5μCi/mL 3H-cholesterol (PerkinElmer) for an additional 24 hours. Then, cells were washed twice with PBS and incubated with 2% fatty acid–free BSA (FAFA, Sigma) in medium in the presence of acetyl-coenzyme A acetyltransferase inhibitor (2 μmol/L) for 2 hours before the addition of 50 μg/mL human apoA1 or 50 μg/mL HDL in FAFA medium with or without the indicated treatments. Supernatants were collected after 6 hours, and radioactivity was counted and expressed as a percentage of total cell 3H-cholesterol content (total effluxed 3H-cholesterol + cell-associated 3H-cholesterol).

Statistical Analysis
The results are expressed as a mean±SEM. Statistical comparisons between groups were done by the Student t test or analysis of variance (ANOVA), and post hoc multiple comparisons were done by the Student-Newman-Keuls test, using the Statgraphics Plus v5.0 program (Statistical Graphics, USA).

Results
ABCA1 Expression Is Highly Regulated by miRNAs
The 3′UTR of Abca1 is particularly long, and we hypothesized that it is susceptible to being targeted by miRNAs. To determine the potential miRNA candidates that regulate ABCA1 expression, we used a combination of bioinformatic tools for miRNA target prediction (TargetScan [http://www.targetscan.org] and miRanda [http://www.microrna.org]). TargetScan revealed 136 predicted miRNAs that target ABCA1, whereas miRanda predicted 57. To assess whether these predicted miRNAs were modulated by cellular cholesterol content, we undertook an unbiased genome-wide screen. Interestingly, miR-291b-5p, miR-672, miR-673-5p, miR-758, and miR-33 were downregulated (Supplemental Table I), suggesting a physiological role for these miRNAs in regulating cellular cholesterol homeostasis. In our previous work, we studied the role of miR-33 in regulating ABCA1 and cellular cholesterol efflux and were motivated to do so by its intriguing genomic location (intron 16 of Srebf2). To directly examine the effects of the other miRNAs identified in our screening (miR-291b-5p, miR-672, miR-673-5p, and miR-758) on ABCA1 expression, we transfected human macrophages (THP-1) with these miRNAs and treated them with AcLDL or T0901317 to
stimulate ABCA1 expression. Transfection of human macrophages with miR-758 (190-fold increase expression) but not a control miRNA (Con-miR) or other miRNAs strongly decreased the stimulation of ABCA1 protein expression (Figure 1A and 1B). miR-33 was used as a positive control for inhibition of ABCA1 expression (Figure 1A). Interestingly, miR-291b-5p transfection instead inhibited ABCA1 increase its expression. This finding suggests that miR-291b-5p may regulate ABCA1 expression by targeting other genes involved in the regulation of ABCA1 expression. miR-758 also repressed ABCA1 protein in mouse peritoneal macrophages (Figure 1B) and hepatic cell lines (HepG2 and Hepa) (Figure 1C and 1D), indicating that its effect is not cell type specific. Notably, inhibition of endogenous miR-758 by anti-miR-758 (3-fold decrease expression) increased the expression of ABCA1 in macrophages and hepatic cells (Figure 1E and 1F), suggesting a physiological role of miR-758 in regulating the expression of this transporter.

We further assessed whether the cotransfection of miR-33 and miR-758 had an additive inhibitory effect on the ABCA1 protein expression. As seen in Supplemental Figure I, Huh7 cells transfected with miR-33 and miR-758 expressed reduced ABCA1 levels compared with cells transfected with the individual miRNAs, suggesting an additive inhibitory effect of both miRNAs on ABCA1 expression.

miR-758 Directly Targets the 3′UTR of Abca1

The human Abca1 3′UTR has 2 computationally predicted miR-758 binding sites (Figure 2A). Site 2 is highly conserved between species, whereas site 1 is conserved only in human and nonhuman primates (Figure 2B). To assess the effects of miR-758 on the 3′UTR of human Abca1, we used luciferase reporter constructs. miR-758 markedly repressed the activity of the Abca1 3′UTR reporter construct (Figure 2C). Site-directed mutations in site 2, which is widely conserved among several species, abolishes the miR-758 repression of Abca1 3′UTR activity, suggesting that this site is the most important in the posttranscriptional repression of Abca1 by miR-758 (Figure 2D).

miR-758 Regulates Macrophage Cellular Cholesterol Efflux to Apolipoprotein A1

The ability of ABCA1 to stimulate the efflux of cholesterol from cells in the periphery, particularly cholesterol-laden macrophages in atherosclerotic plaques, is an important antiatherosclerotic mechanism.6,22 Transfection of J774 macrophages with miR-758 reduced ABCA1 expression (Figure 3A, top) and attenuated cholesterol efflux to apolipoprotein A1 (apoA1) (Figure 3A, left). miR-758 did not impair cholesterol efflux to HDL in J774 macrophages, consistent with the lack of miR-758 binding sites in the mouse/human Abcg1 3′UTR and other compensating mechanisms (Figure 3A, right). Notably, antagonism of endogenous miR-758 increased ABCA1 protein (Figure 3B, top) and cholesterol efflux to apoA1, but not to HDL (Figure 3B). Similar results were obtained using human THP-1 macrophages (data not shown). Furthermore, to directly assess the role of ABCA1 in
the miR-758 repression of cholesterol efflux, we cotransfected J774 cells with miR-758 and ABCA1 small interfering RNA. As seen in Figure 4, the effect of miR-758 on cholesterol efflux to ApoA1 was significantly attenuated after ABCA1 silencing, suggesting that miR-758 regulates cholesterol efflux via the ABCA1 transporter (Figure 4). Thus, manipulation of cellular miR-758 levels alters macrophage cholesterol efflux, a critical step in the reverse cholesterol transport pathway for the delivery of cholesterol excess to the liver.

**miR-758 Tissue and Cellular Expression and Its Regulation by Plasma Lipid Levels In Vivo**

Next, we examined the in vivo expression of miR-758 in mice. miR-758 was widely expressed in mouse tissues and is particularly abundant in the brain, heart, and aorta (Figure 5A). Interestingly, whole brain tissue expressed very high levels of miR-758 compared with other tissues. We also evaluated the miR-758 expression in different cell lines from mouse (Figure 5B) and human (Figure 5C) origin. In accordance with the high expression of miR-758 in the brain, neurogliomas, particularly the astrocyte cell line CCF-STTG1, expressed very high levels of miR-758 (≈75-fold) compared with the other human cell lines analyzed, suggesting an important role for this miRNA in the brain physiology.

To determine whether miR-758 is regulated under different physiological conditions, we measured its expression in mice fed either a chow or high-fat diet for 5 weeks. As expected, treatment of C57BL/6 mice with a high-fat diet increased body weight and plasma cholesterol and triglyceride levels (Figure 5D). Consistent with our in vitro observations,
To determine the potential role of miR-758 in regulating neuronal functions, we transfected glioblastoma H4 cells with miR-758 mimics. As seen in Figure 6A, miR-758 overexpression significantly decreased ABCA1 protein expression. Conversely, endogenous inhibition of miR-758 resulted in an increase in ABCA1 protein levels (Figure 6B), confirming that ABCA1 is regulated by miR-758 in neural cell lines. Next, we examined the effects of miR-758 manipulation on cholesterol efflux in H4 cells. As expected, miR-758 overexpression led to a reduction in cholesterol efflux to ApoA1 (Figure 6A), whereas the endogenous inhibition of miR-758 increased cellular cholesterol efflux (Figure 6B). Thus, these findings demonstrate that miR-758 effects on ABCA1 result in the retention of cellular cholesterol and that manipulating cellular miR-758 levels can influence neuronal cholesterol homeostasis. Interestingly, several genes involved in amino acid synthesis (sodium-coupled neutral amino acid transporter 1 [SLC38A1], neurite outgrowth (neurotrophin [NTM]), and the development of nervous system (ephrin type-A receptor 7 [EPHA7], and myelin transcription factor 1-like [Mytl1]) are predicted targets for miR-758.

To assess whether miR-758 regulates the expression of these targets, we transfected H4 cells with miR-758. As seen in Figure 6C, miR-758 overexpression reduced significantly the mRNA expression of SLC38A1, IGF1, NTM, STXBP1, and EPHA7, suggesting an important role of miR-758 in regulating neurological functions. Whether other miR-758 targets can contribute to this effect and which physiological or pathological conditions underlie the regulation of miR-758 gene expression remain to be determined.

Discussion

HDL and its principal apolipoprotein, apoA1, play a critical role in the removal of cellular cholesterol excess and its transport to the liver, a process called reverse cholesterol transport. When the excess cholesterol originates from foam cells in arterial plaques, reverse cholesterol transport is thought to underlie the atheroprotective effect of HDL.6,22
ABCA1 and ABCG1 transporters promote cellular cholesterol efflux to HDL and its associated apolipoprotein, apo-A1, in peripheral cells, including macrophages. ABCA1 is also primarily responsible for initiating HDL formation in the liver, a finding brought to light when mutation in Tangier disease, a condition characterized by low plasma HDL, was mapped to the ABCA1 gene.\textsuperscript{23,24} Transcriptionally, ABCA1 expression is regulated primarily by the heterodimeric complex of LXR with the retinoid X receptor (RXR) and also by other agonists in a LXR-dependent and -independent manner in peripheral cells.\textsuperscript{25} In the liver, evidence suggests that ABCA1 is also regulated by sterol response element–binding protein-2.\textsuperscript{26} Posttranscriptional regulation of ABCA1, by calpain-mediated proteolytic degradation, has also been described.\textsuperscript{27,28}

Very recently, 4 independent groups have shown that miRNAs play an important role in posttranscriptional regulation of ABCA1\textsuperscript{18–21} by targeting the 3’UTR of Abca1. The 3’UTR of Abca1 is particularly long, and we hypothesized that it might be targeted by miRNAs besides miR-33. By conducting a whole-genome miRNA microarray in cholesterol-loaded macrophages, here we show that other miRNAs can regulate cholesterol homeostasis by targeting ABCA1, expanding our current knowledge of how cholesterol is modulated and the relevance of cholesterol and lipoprotein metabolism by miRNAs.

Two other miRNAs have been shown to have a direct role in regulating cholesterol homeostasis.\textsuperscript{18,29} The liver-restricted miR-122 regulates cholesterol and triglyceride metabolism, and its silencing in mice and nonhuman primates reduces cholesterol synthesis, hepatic fatty acids, and plasma cholesterol levels and increases liver fatty acid \(\beta\)-oxidation.\textsuperscript{30–32} In contrast to miR-122, miR-758 is widely expressed in different tissues and cell types, and its effect on ABCA1 has a more global effect on cellular cholesterol homeostasis. Similarly, miR-33 has also been reported to be expressed in different tissues and cell lines.\textsuperscript{18} Interestingly, here we have shown that miR-758 is highly expressed in the brain, particularly in astrocytes. The brain accounts for almost 25% of total body cholesterol.\textsuperscript{33} In the developed brain, cholesterol is synthesized by astrocytes and oligodendrocytes, and in contrast to oligodendrocytes, the astrocytes supply cholesterol to other neurons by ABC proteins, ABCA1, ABCG1, and ABCG4.\textsuperscript{10,34} ApoE is an important mediator of cholesterol efflux in the brain, and certain neurodegenerative disorders are associated with disturbances in cholesterol metabolism and the presence of the apoE4 isoform.\textsuperscript{10,35} Here we show that manipulating miR-758 levels in neuronal cells can modify both ABCA1 expression and cholesterol efflux to ApoA1. However, the in vivo physiological and pathological changes of miR-758 levels in the brain are not known and need to be evaluated.

Compared with miR-33\textsubscript{a/b}, which are localized within introns of Srebf genes and regulated primarily by host genes,\textsuperscript{18,19} the highly conserved miR-758 is localized in an intergenic region within chromosome 14 and its direct regulation is not known. Here, we confirm that miR-758 is downregulated on cholesterol loading in macrophages both in culture and in a mouse model of hypercholesterolemia (\(\text{Ldlr}^{-/-}\)) induced by high-fat diet, indicating that in vivo, miR-758 in macrophages is regulated by dietary cholesterol.

So far, only 1 specific miRNA, miR-33, has been described to target ABCA1. Here we describe and confirm a second miRNA that controls ABCA1 levels and therefore regulates cellular cholesterol homeostasis. Our screening of cholesterol-loaded macrophages revealed other miRNAs that, according to different miRNA prediction algorithms, also target ABCA1. Why cells expressed several miRNAs (miR-33 and miR-758) regulating this protein and what drives the speci-
ficity of these miRNAs in different cell types under physiological and pathological conditions need to be determined. Through evolution, cholesterol has played a crucial role in the development of both the vertebrate brain and special membrane domains, and its level in our organism is tightly controlled by plasma lipoprotein levels and transcription factors. Imbalances in cholesterol metabolism are recognized as the major risk factor for developing cardiometabolic disease. Therefore, control of cellular cholesterol homeostasis through miRNAs can be an alternative for cells to regulate cholesterol homeostasis in cholesterol-related pathologies.

Additional studies are needed to explore the in vivo effects of miR-758 silencing in HDL biogenesis to decipher the antagonism of endogenous miR-758 as a potentially useful therapeutic strategy to enhance plasma levels of HDL. Understanding the complex network of miRNAs that target ABCA1 may provide new tools to develop therapeutic strategies to combat the burden of cardiovascular disease and atherosclerosis. Our findings increase the knowledge of the complex regulation of ABCA1 and therefore the maintenance of cellular cholesterol homeostasis through miRNAs can be an alternative for cells to regulate cholesterol homeostasis in cholesterol-related pathologies.

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Disclosures

None.

References


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