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MicroRNA 302a is a novel modulator of cholesterol homeostasis and atherosclerosis

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Abstract

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

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

Conclusions—These studies identify miR-302a as a novel modulator of cholesterol efflux and a potential therapeutic target for suppressing atherosclerosis.

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DISCLOSURES

There are no conflicts of interest for any of the authors.

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Keywords

ABCA1; cholesterol homeostasis; macrophages; microRNA; HDL; Atherosclerosis; Macrophages; ATP-binding cassette transporter; Cholesterol efflux

INTRODUCTION

Atherosclerosis is a chronic inflammatory disease of the arterial wall, as well as a disorder of lipid metabolism [1]. Cholesterol is an essential structural component in the cell membrane and a precursor in metabolic pathways, including steroid hormone and bile acid synthesis. The accumulation of cholesterol-loaded macrophages in the arterial wall, termed foam cell formation, is a hallmark feature of early atherosclerotic lesions [2]. Low-density lipoprotein (LDL) particles are internalized by macrophages in the arterial wall via multiple pathways including through the LDL receptor (LDLR) on the cell surface and hydrolyzed to free cholesterol in lysosomes [3]. When excessive cholesterol is taken up in the artery wall, macrophages activate a compensatory pathway to efflux the cholesterol, mediated by the ATP-binding cassette (ABC) transporters ABCA1 and ABCG1 [4]. These transporters promote cellular cholesterol efflux to high-density lipoprotein (HDL) and its associated apolipoprotein (apoA-1), a crucial step in the initiation of reverse cholesterol transport (RCT) to the liver for excretion [5]. During systemic hypercholesterolemia, however, this homeostatic mechanism is overwhelmed, leading to the development of foam cells and fatty streak lesions [6]. Lipid loaded and activated macrophage foam cells can significantly contribute to the maintenance and progression of atherogenesis by producing nitric oxide, reactive oxygen species, inflammatory lipids, growth factors, and pro-inflammatory cytokines (e.g. interleukin (IL)-1, IL-6, interferon (IFN)-γ and tumor necrosis factor (TNF)-α) [7]. Understanding the mechanisms that regulate these responses could therefore be of considerable value in developing new approaches to prevention and treatment of atherosclerosis. The regulation of these lipid metabolism mediators such as ABCA1, however, is complex and likely to involve post-transcriptional mechanisms.

MicroRNAs (miRs) are a recently recognized class of highly conserved, noncoding short RNA molecules that regulate gene expression at the post-transcriptional level. MiRs act as negative regulators of gene expression by inhibiting messenger RNA (mRNA) translation or promoting mRNA degradation [8]. Importantly, several miRs have been recognized very recently to influence cholesterol homeostasis [9–12].

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

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

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MATERIALS AND METHODS

Materials and methods are available in the online-only supplement.

RESULTS

Regulation of miR-302a is inversely correlated with cellular cholesterol levels

It has been shown that specific miRs can be regulated as well as regulate many different functions in macrophages, including cholesterol homeostasis. Therefore, we performed an unbiased genome-wide screen of miRs modulated by cellular cholesterol content in primary macrophages. We identified a subset of 47 miRs differentially regulated in mouse macrophages by cholesterol enrichment (Supplemental Table I), of which 14 were down-regulated and 33 were up-regulated. Confirmation of these miR candidates using real time polymerase chain reaction (PCR) identified miR-302a as one of the most strongly modulated miRs in BMDM after treatment with modified LDL. MiR-302a expression in BMDM was significantly down-regulated by acetylated (Ac)LDL and oxidized (ox)LDL stimulation for 6 hours *in vitro*. This was accompanied by the up-regulated expression of the cholesterol transporter genes *Abca1* and *Abcg1* (Figure 1A). Interestingly, stimulation with native LDL does not seem to affect miR-302a expression but does up-regulate ABC transporter genes. To show that these observations are valid in the human system as well, we performed the same experiment using freshly isolated human peripheral blood mononuclear cells (PBMCs). Real time PCR analysis using primary human macrophages confirmed the results seen in BMDM. MiR-302a was significantly down-regulated after treatment with AcLDL and/or oxLDL for 6 hours with a concomitant up-regulation of *ABCA1* and *ABCG1* gene expression (Figure 1B). Next, we investigated miR-302a expression in the aorta of *Ldlr*$^{-/-}$ mice that had been fed a normal chow and/or a high fat diet (HFD) for 8, 12 and 24 weeks. We found that miR-302a was highly expressed in the aorta of these mice. However, consistent with our *in vitro* findings, after 12 weeks of atherogenic diet feeding, miR-302a was markedly down-regulated, while *Abca1* and *Abcg1* gene expression was up-regulated (Figure 1C). This pattern suggests that miR-302a is regulated by hypercholesterolemia in *Ldlr*$^{-/-}$ mice.

Molecular characteristics of miR-302a

Analysis of sequence alignment revealed that miR-302a is an intrinsic miR localized on chromosome 4 in humans and on chromosome 3 in mice, both within intron 8 of the La ribonucleoprotein domain family member 7 (*LARP7*) gene (Figure 2A). LARP7 belongs to the LARP RNA-binding protein family, and modulates the metabolism and function of a variety of RNA species [13]. Many mammalian miRs are located within introns of protein-coding genes and therefore are typically co-ordinately expressed and processed with the precursor mRNA in which they reside [14, 15]. Accordingly, the mature form of miR-302a appears to be co-expressed with the *Larp7* host gene in several different mouse tissues examined (Figure 2B). Interestingly, miR-302a is highly expressed in the aorta in comparison to *Larp7* whereas in the spleen *Larp7* is abundantly expressed with very little expression of miR-302a. Additionally, we found that in liver, aorta and BMDM, miR-302a and Larp7 expression were coordinately down-regulated by cholesterol loading suggesting

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the regulation of miR-302a by cholesterol (Figure 2C). Moreover, miR-302a is highly conserved in different organisms (Figure 2D), which led us to investigate miR-302a for further validation of its role in cholesterol metabolism.

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

MiR-302a regulates ABCA1 in primary macrophages at the post-transcriptional level

To test the specific effect of miR-302a on ABCA1 expression, we treated BMDM with AcLDL (to load the cells with cholesterol) or the liver X receptor (LXR) ligand T0901317 (to directly stimulate the expression of ABCA1) after transfecting the cells with mimic miR-302a to increase the intracellular levels of miR-302a. Our data show that mimic miR-302a strongly decreased the stimulation of Abca1 mRNA and protein (Figure 3A and C, Supplemental Figure IA) in primary mouse macrophages. MiR-302a up-regulation, however, had no effect on the related *Abcg1* gene expression. To further determine whether miR-302a is specifically involved in regulating Abca1 expression in BMDM, we inhibited endogenous miR-302a by transfecting the BMDM with an anti-miR-302a construct. Introduction of anti-miR-302a indeed resulted in increased levels of Abca1 mRNA and protein (Figure 3B and D, Supplemental Figure IB). As with mimic miR-302a, no effect on *Abcg1* expression was observed. MiR-302a over-expression and/or knockdown were confirmed by real time PCR analysis (data not shown). In summary, these data clearly indicate that Abca1 expression in primary mouse macrophages is regulated by miR-302a. Of note, BMDM transfected with anti-miR-302a showed a down-regulation of inflammatory genes (Figure 3E) indicating a link between Abca1 expression and inflammation.

MiR-302a specifically targets the 3′UTR of mouse and human ABCA1 and regulates cellular cholesterol efflux to apoA-1

To determine the effects of miR-302a on the 3′UTR of human and mouse ABCA1, we used luciferase reporter constructs. As shown in Figure 4A, B and C, co-transfection with mimic miR-302a markedly repressed both mouse and human ABCA1 3′UTR activity compared to control miR, suggesting that miR-302a is able to bind to the 3′UTR of both mouse and human ABCA1. Mutations in the seed base-pairing sequence of the predicted miR-302a target sites in the ABCA1 reporter abrogated the miR-302a repression of mouse and human 3′UTR activity, indicating a direct interaction of miR-302a with these sites (Figure 4B and C). Together, these experiments identify ABCA1 as conserved target of miR-302a.

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 anti-

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atherosclerotic mechanism [5]. Transfection of BMDM with miR-302a diminished cholesterol efflux to apoA-1 compared to control miR after treatment with AcLDL for 24 hours (Figure 4D). Similar results were seen using primary human macrophages (Figure 4F). Conversely, inhibition of endogenous miR-302a with anti-miR-302a increased ABCA1 protein and cholesterol efflux to apoA-1 in primary murine and human macrophages (Figure 4E and G). Thus, manipulation of cellular miR-302a levels alters macrophage cholesterol efflux capacity, a critical step in the reverse cholesterol transport pathway for the delivery of excess cholesterol to the liver [5].

Anti-miR-302a treatment *in vivo* increases ABCA1 in aorta and liver of *Ldlr*$^{-/-}$ mice

In addition to coordinating cellular cholesterol efflux, ABCA1 is also intimately involved in initiating HDL formation in the liver [17]. Therefore, we tested the effect of manipulating miR-302a levels *in vivo* in *Ldlr*$^{-/-}$ mice using 2′ fluoro/methoxyethyl-modified (2′F/MOE-modified) phosphorothioate backbone antisense oligonucleotides provided by our collaborators at Regulus Therapeutics. The delivery of antisense nucleotides has been used successfully in mice to inhibit the function of various miRs and to increase expression of their target genes with no evident toxicity [18]. To assess the effects of inhibiting miR-302a in a model of atherosclerosis, *Ldlr*$^{-/-}$ mice were fed a HFD over 12 weeks while being injected intraperitoneal (i.p.) with anti-miR-302a or control anti-miR oligonucleotides. Consistent with previous studies [19, 20], the treatment did not induce a detectable immune response, as differential blood count in mice treated with either control anti-miR or anti-miR-302a did not differ from those of untreated *Ldlr*$^{-/-}$ mice fed a HFD. Efficient delivery was confirmed by measuring levels of its target gene ABCA1 in the livers and aorta of mice at sacrifice. An increase was seen in *Abca1* expression in liver and aorta of anti-miR-302a treated *Ldlr*$^{-/-}$ mice (Figure 5A) as well as in Abca1 protein levels in the liver (Figure 5B). No change in *Abcg1* mRNA was observed.

Anti-miR-302a treatment increases circulating HDL levels and reduces atherosclerosis progression in *Ldlr*$^{-/-}$ mice

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

In assessing atherosclerotic lesion development, a marked reduction in plaque formation in aorta (Figure 6B) as well as in aortic roots (Figure 6C) was observed in *Ldlr*$^{-/-}$ mice treated with anti-miR-302a in comparison to *Ldlr*$^{-/-}$ mice treated with control miR, as visible after Oil red O staining. Interestingly, quantitative immunostaining revealed that the reduction of plaque area was associated with a significant increase in the relative content of macrophages

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(Figure 6D), whereas the necrotic core size was significantly decreased in anti-miR-302a *Ldlr*$^{-/-}$ mice (Figure 6F). Additionally, the relative content of smooth muscle cells (SMCs) was significantly increased in anti-miR-302a treated *Ldlr*$^{-/-}$ mice (Figure 6E) indicating remodelling of plaques toward a rather early and more stable lesion phenotype with a lesser degree of inflammation (Figure 3E). Together, these results indicate that anti-miR-302a treatment supports the efflux of cholesterol from plaque macrophages leading to an overall reduction in atherosclerotic burden.

DISCUSSION

There is strong evidence that high levels of circulating HDL are associated with positive cardiovascular outcomes, independent of levels of LDL cholesterol, and as such, therapies to raise HDL are actively being pursued [21]. Direct infusion of HDL in apoE-deficient mice [22] or human subjects [23] with established atherosclerosis, reduces plaque size. The ABC transporter ABCA1 is primarily responsible for initiating HDL formation in the liver [24]. The current work reveals that miR-302a exerts post-transcriptional control of the ABCA1 cholesterol transporter in primary macrophages resulting in the regulation of cellular cholesterol efflux. By targeting Abca1 via *in vivo* delivery of anti-miR-302a in *Ldlr*$^{-/-}$ mice, circulating plasma HDL levels were increased leading to less atherosclerosis when compared with *Ldlr*$^{-/-}$ mice treated with a control anti-miR. However, further validation needs to be performed to identify the HDL type generated by anti-miR-302a treatment.

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

There have been several reports of miRs regulating lipid metabolism. MiR-122, for example, was identified as the most highly expressed miR in the adult liver, where it accounts for 70% of all miRs [26]. Using antisense strategies, several groups have reported that inhibition of miR-122 in the liver results in sustained decreases in plasma cholesterol levels in both mice and non-human primates [27, 28]. Most recently, five independent studies demonstrated that miR-33a targets ABCA1 and ABCG1 and limits the efflux of cholesterol to apoA-1 in both macrophages and hepatocytes [29–33]. Conversely, anti-miR-33a treatment upregulates ABCA1 expression both *in vitro* and *in vivo*, promoting the efflux of cholesterol to apoA-1 and increasing circulating HDL levels [29, 32, 33]. In addition to these findings, inhibition of miR-33 expression *in vivo* increases RCT and curbs atherosclerotic plaque regression [20, 34].

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Here we report that a newly identified miR, miR-302a, targets ABCA1 and modulates the cholesterol efflux to apoA-1 in primary macrophages. Additionally, anti-miR-302a treatment increases circulating HDL levels and decreases atherosclerosis development. Aside from ABCA1, VLDLr, Osbpl5, Pmvk and lep are also predicted to be target of miR-302a, all of which can influence lipid metabolism (Supplemental Table 2).

An interesting connection between lipid metabolism and inflammation in macrophages revealed by others suggests that cholesterol loading of macrophages results in a pro-inflammatory phenotype whereas cholesterol efflux dampens this inflammation [35–37]. In our study, real time PCR analysis of anti-miR-302a-transfected macrophages showed that aside from the regulation of Abca1 there was an alteration of the inflammatory state of these cells when compared with miR-302a-transfected cells. This likely led to less inflammation in the lesions of anti-miR-302a-treated *Ldlr*$^{-/-}$ mice, and therefore a slower progression of atherosclerosis.

As the host gene of miR-302a, *LARP7* gene encodes a protein which is found in the 7SK small nuclear ribonucleoprotein (snRNP). Although little is known about the function of this protein what is known is that this snRNP complex inhibits a cyclin-dependent kinase, which is required for paused RNA polymerase II at a promoter to begin transcription elongation. Via its ability to control transcription and elongation, *LARP7* is thought to be involved in general cellular processes like cell growth as well as tumorigenesis. This makes it very likely to be involved in a broad range of different processes including inflammation and lipid metabolism, even though *LARP7* is not a transcription factor regulating gene expression involved in cholesterol metabolism. Interestingly, a recent study showed that loss of function mutation in *LARP7* causes a syndrome of facial dysmorphism, intellectual disability, and primordial dwarfism [38].

The ability of miRs to modulate important biological pathways offers opportunities for the manipulation of miR function using oligonucleotide inhibitors or miR mimics. Antisense oligonucleotides directed against specific miR sequences are efficiently taken up by a variety of tissues [39]. In addition, miR mimics and inhibitors are relatively stable in plasma and can simply be injected to reach their cellular gene targets without apparent toxicity. The challenge to directly target a specific inflamed tissue and/or a specific cell type is still remaining. However, Rayner et al. were able to show that anti-miR treatment indeed is capable of targeting plaque macrophages and directly altering gene expression in these cells [20] supporting our data showing less plaque formation possibly due to a higher cholesterol efflux rate in mice treated with anti-miR-302a.

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

Supplementary Material

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Nonstandard Abbreviations and Acronyms

LDL	Low density lipoprotein
HDL	High density lipoprotein
AcLDL	Acetylated LDL
OxLDL	Oxidized LDL
miR	Micro RNA
ABC	ATP-binding cassette transporter
HFD	High fat diet
BMDM	Bone marrow-derived macrophages
SMC	Smooth muscle cells

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SIGNIFICANCE

Atherosclerosis is a very common disease of the artery in which both cholesterol levels and inflammation play a crucial role in its pathogenesis. Of the inflammatory cells, macrophages play a key role in both initiation and progression of the disease as they can become foam cells in the artery wall and directly contribute to the growth of the atherosclerotic plaque. While studying the lipid metabolism of macrophages we found that a microRNA called 302a (miR302a) may be able to control the metabolism of lipids in macrophages by inhibiting a cholesterol transport molecule called ABCA1. Through many experiments we found that mi indeed binds the 3′UTR region of ABCA1 and therefore inhibits ABCA1 expression. Inhibiting miR302a resulted in less lipid accumulation in macrophages. In a mouse model of atherosclerosis, we used a synthetic miR302a-inhibiting agent to show that lowering miR302a in the body significantly raised HDL levels and attenuated the extent of atherosclerosis compared to control mice.

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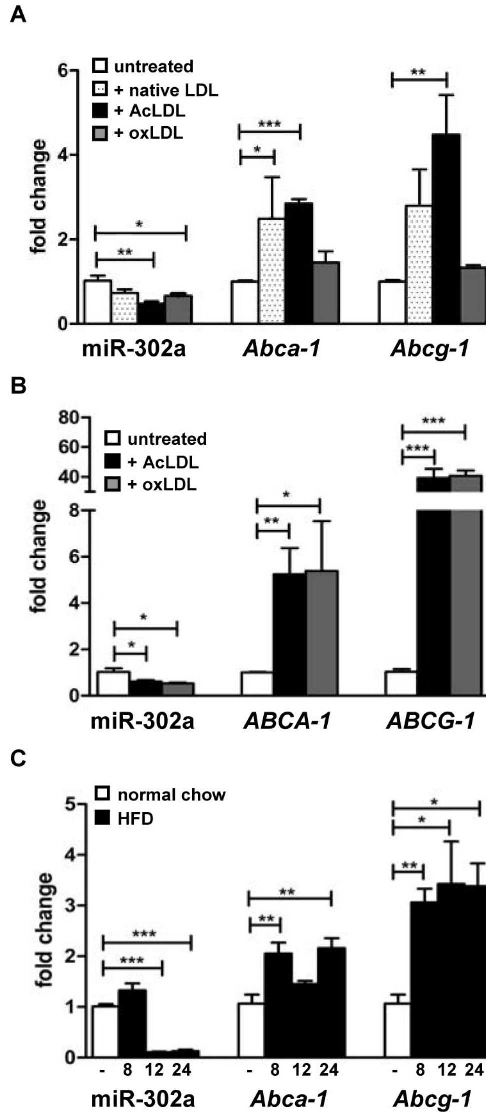

Figure 1. MiR-302a is inversely correlated with ABCA1 and ABCG1

Real time PCR analysis of miR-302a, *Abca1* and *Abcg1* expression in primary mouse macrophages (A) or primary human macrophages (B). Macrophages were either untreated or loaded with cholesterol by either AcLDL (10 μg/ml) or oxLDL (10 μg/ml) treatment for 6 hours (n=4 independent experiments). In addition, mouse macrophages were treated with 10 μg/ml native LDL. *P<0.05, **P<0.005, ***P<0.0005. (C) Real time PCR analysis of miR-302a, *Abca1* and *Abcg1* expression in aorta of $Ldlr^{-/-}$ mice (n=4 per group) fed either a normal chow or an atherogenic diet for 8, 12 and 24 weeks. (A) to (C), data are expressed as mean ± SEM. ANOVA with Bonferroni's multiple comparison test was used.

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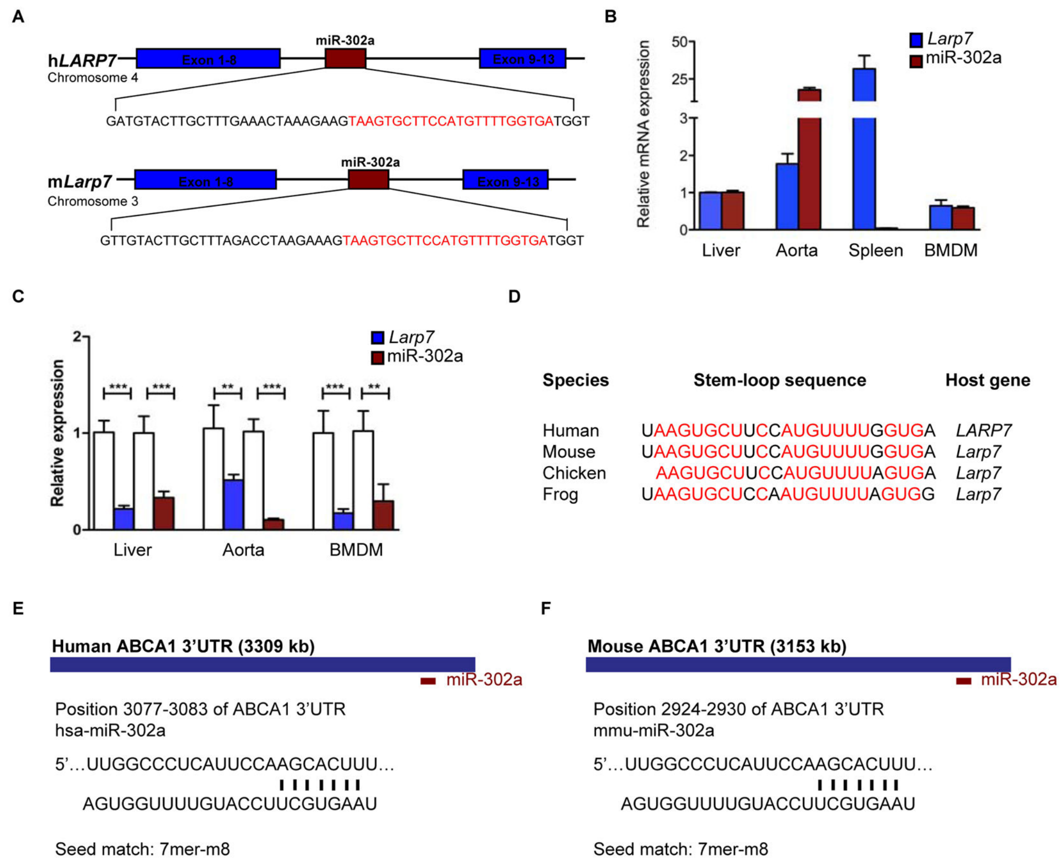

Figure 2. Molecular characteristics of miR-302a

(A) Schematic overview of the LARP7 gene locus, showing the miR-302a coding sequence within intron 8. (B) Expression profile of miR-302a and *Larp7* host gene in liver, aorta and spleen of C57BL/6 mice (n=4 per group) and primary mouse macrophages.. (C) Real time PCR analysis of miR-302a and *Larp7* in liver and aorta of *Ldlr*$^{-/-}$ mice (n=3 per group) fed an atherogenic diet for 12 weeks and primary mouse macrophages loaded with cholesterol by AcLDL treatment. As controls (white bars), *Ldlr*$^{-/-}$ mice fed a normal chow and primary macrophages without cholesterol loading were used. (B) and (C), data are expressed as mean ± SEM. ***P*<0.005, ****P*<0.0005. (D) Schematic representation of the miR-302a stem-loop sequence and its conservation among species. (E&F) Schematic overview demonstrating the predicted target site of miR-302a in the 3′UTR region of human *ABCA1* (E) and mouse *Abca1* (F). ANOVA with Bonferroni's multiple comparison test was used.

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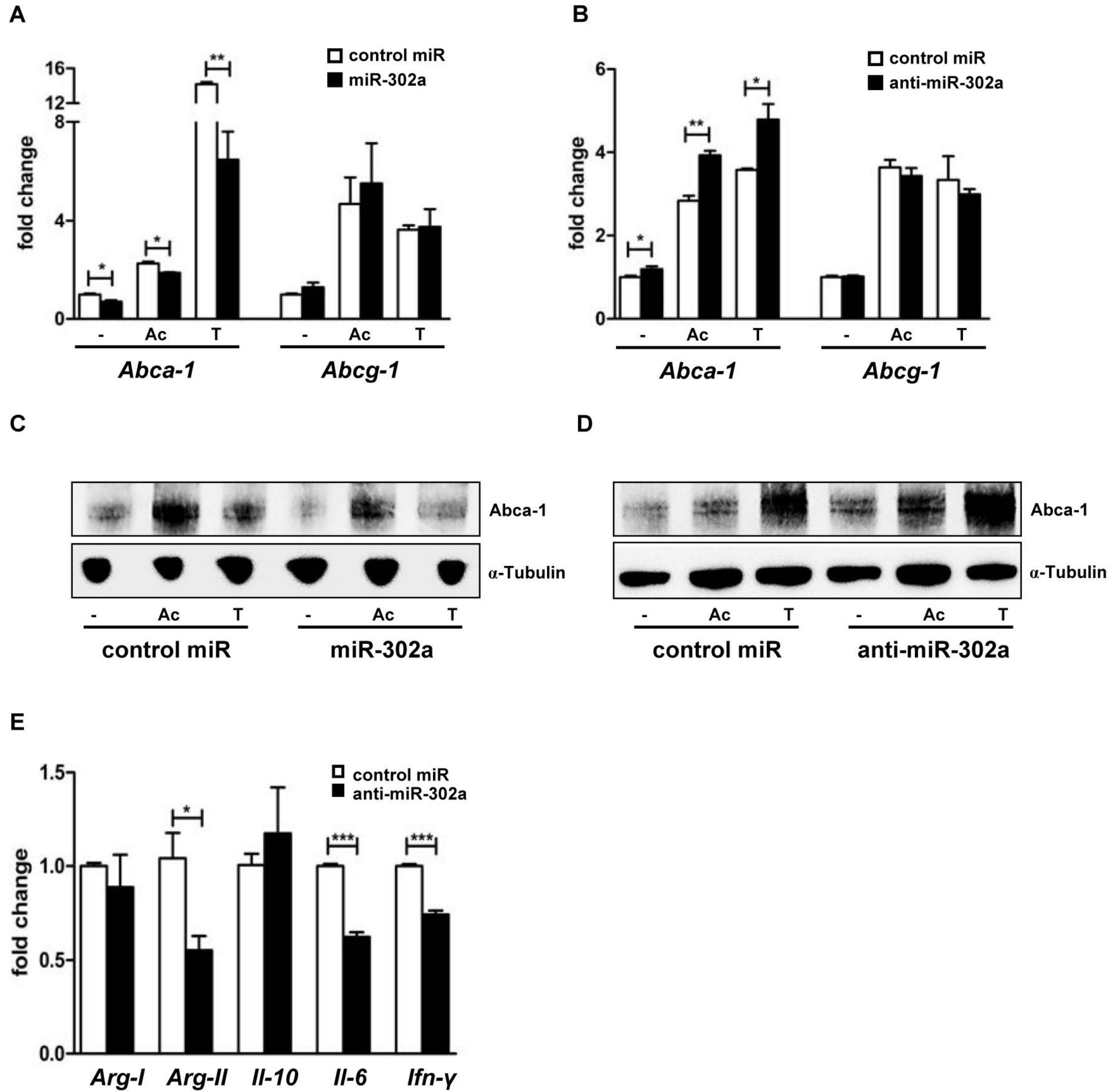

Figure 3. MiR-302a regulates ABCA1

(A,B) Real time PCR analysis of *Abca1* and *Abcg1* gene expression in BMDM transfected with (A) control miR and 200 nM miR-302a, or (B) control miR and 200 nM anti-miR-302a. 24 hours after transfection, primary macrophages were either untreated or stimulated with 40 μg/ml AcLDL and/or 10 μM T0901317 for an additional 24 hours (n=4 independent experiments). Data are expressed as mean ± SEM. *$P<0.05$, **$P<0.005$. (C,D) Western blot analysis of Abca1 protein expression in primary mouse cells after transfection with (C) control miR-302a and 200 nM miR-302a, or (D) control miR and 200 nM anti-miR-302a. Macrophages were either untreated or treated with 40 μg/ml AcLDL and/or 10 μM T0901317 for 48 hours 24 hours after transfection. Representative blots are shown. (E) Gene expression analysis of inflammation markers (Arginase-I (*Arg-I*), Arginase-II (*Arg-II*), Interleukin-10 (*Il-10*), Interleukin-6 (*Il-6*) and Interferon gamma (*Ifn-γ*)) in BMDM after transfection with either control miR or 200 nM anti-miR-302a using real time PCR. Data are

expressed as mean ± SEM. *$P<0.05$, ***$P<0.0005$. ANOVA with Bonferroni's multiple comparison test was used.

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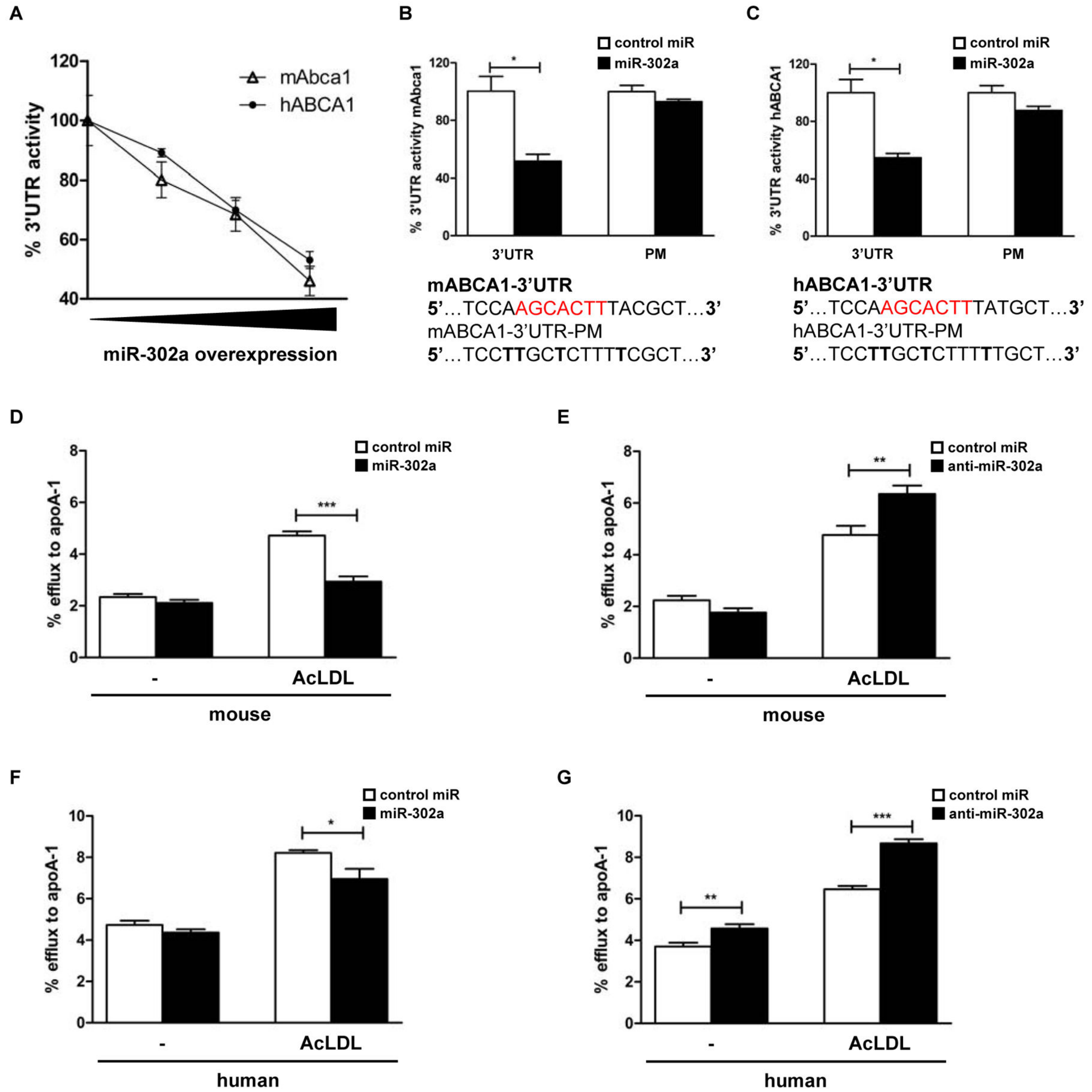

Figure 4. MiR-302a targets the 3′UTR of ABCA1 and regulates cellular cholesterol efflux in vitro

(A) Luciferase reporter activity in COS-7 cells co-transfected with the indicated 3′UTR luciferase reporter vectors and increasing concentrations (0, 50, 100 and 200 nM) of control miR or miR-302a of mouse Abca1 (mAbca1) and human ABCA1 (hABCA1). (B,C) Activity of luciferase reporter constructs fused to the 3′UTR of mAbca1 (B) and hABCA1 (C) in COS-7 cells transfected with 200 nM control miR or miR-302a. As control, mAbca1 3′UTR and hABCA1 3′UTR containing the indicated point mutations (PM)(bold) in the miR-302a target site (represented in red) were used. Data are expressed as mean ± SEM and are representative of 4 independent experiments. **P*<0.05. (D–G) Cholesterol efflux to apoA-1 in primary mouse (D) and primary human (F) macrophages stimulated with 50 μg/ml AcLDL and transfected with control miR or 100 nM miR-302a (n=4 independent experiments). Cholesterol efflux to apoA-1 in primary mouse (E) and primary human (G) macrophages stimulated with 50 μg/ml AcLDL and transfected with control miR or 200 nM

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anti-miR-302a (n=4 independent experiments). Data are expressed as mean ± SEM. **P*<0.05, ***P*<0.005, ****P*<0.0005. ANOVA with Bonferroni's multiple comparison test was used.

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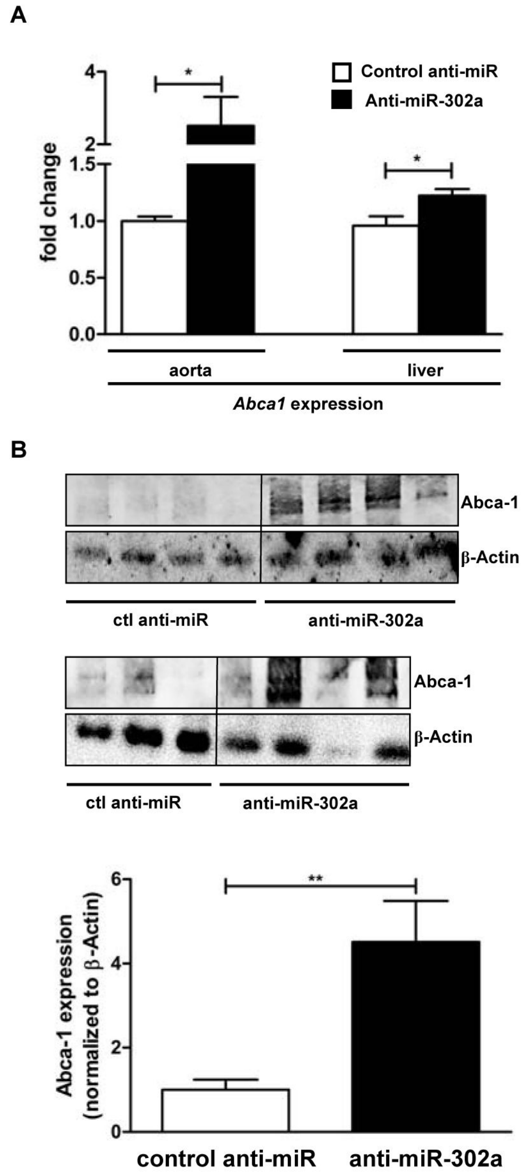

Figure 5. Anti-miR-302a treatment in vivo increases Abca1 expression in aorta and liver of Ldlr$^{-/-}$ mice

(A) Real time PCR analysis of *Abca1* gene expression in liver and aorta of *Ldlr*$^{-/-}$ mice after *in vivo* delivery of control anti-miR and anti-miR-302a (i.p. injections of 10 mg/kg per week over a period of 8 weeks). (B) Western blot analysis of Abca-1 protein expression in liver lysates of *Ldlr*$^{-/-}$ mice after *in vivo* delivery of control anti-miR and anti-miR-302a (i.p. injections of 10 mg/kg per week over a period of 8 weeks). Data are expressed as mean ± SEM. **P*<0.05, ***P*<0.005. 2-tailed Student's t test was used.

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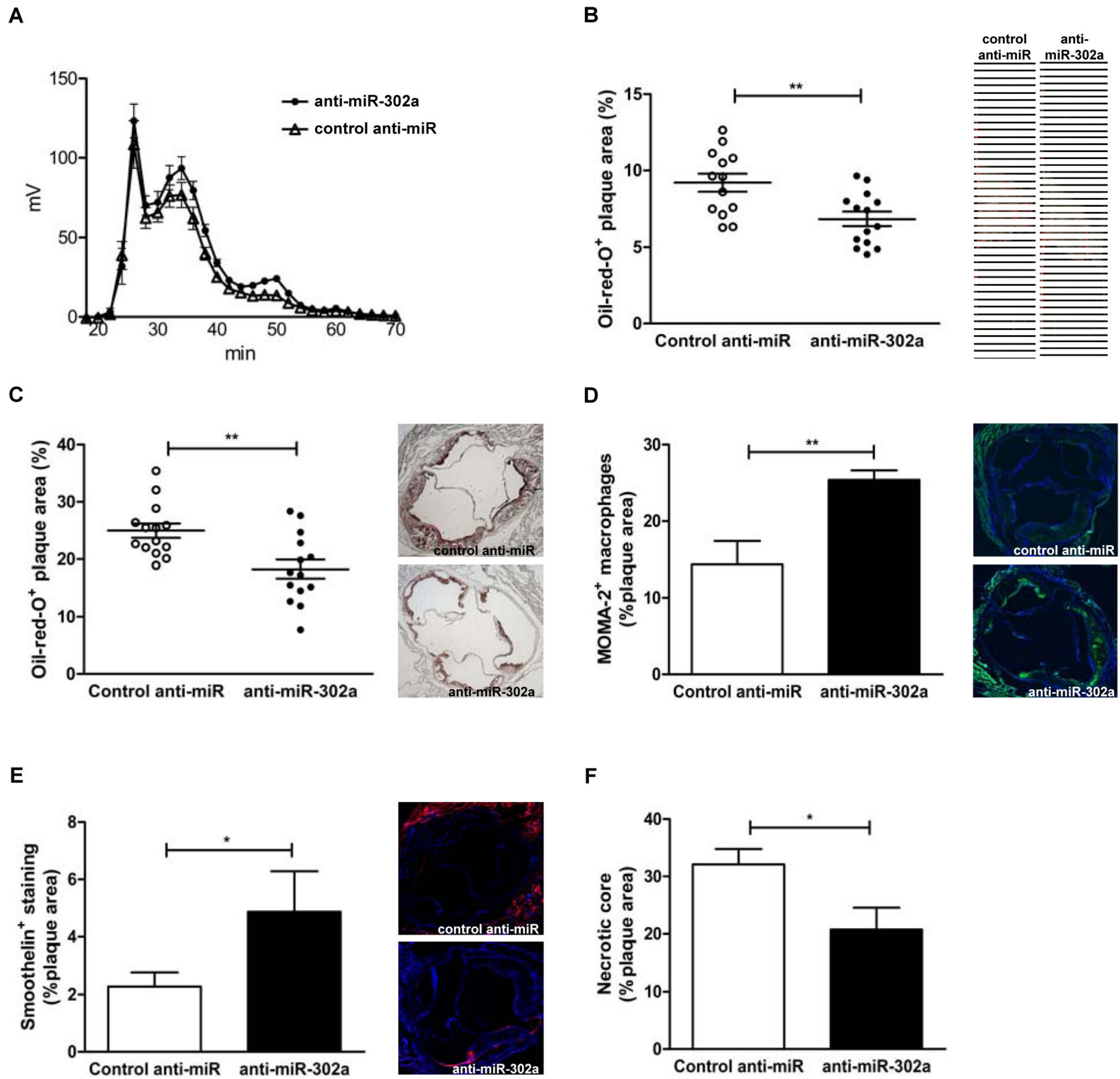

Figure 6. Anti-miR-302a treatment increases circulating HDL levels and reduces athersclerosis progression in Ldlr$^{-/-}$ mice inducing a stable plaque phenotype with less degree of inflammation

(A) HPLC lipoprotein profiles from plasma of Ldlr$^{-/-}$ mice treated with control anti-miR or anti-miR-302a (i.p. injections of 10 mg/kg per week over a period of 8 weeks) (n=7 mice per group). (B,C) Quantification of Oil Red O$^+$ lipid depositions in the aorta (B) and aortic root (C) of Ldlr$^{-/-}$ mice treated with either control anti-miR or anti-miR-302a. Fluorescence microscopy was used to analyze levels of MOMA-2-positive macrophages (D) and smoothelin-positive smooth muscle cells (SMC) (E) in the aortic root of Ldlr$^{-/-}$ mice treated with either control anti-miR or anti-miR-302a. Representative images are shown. (F) Quantification of necrotic cores within aortic root lesion of Ldlr$^{-/-}$ mice with either control

anti-miR or anti-miR-302a. Data are expressed as mean ± SEM. $^{*}P<0.05$, $^{**}P<0.005$. 2-tailed Student's t test was used.

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Table 1

Lipid profiles in *Ldlr*$^{-/-}$ mice treated with control anti-miR or anti-miR-302a

	Triglyceride [mg/dL] n=14/group	Total cholesterol [mg/dL] n=14/group	VLDL/LDL [mg/dL] n=7/group	HDL [mg/dL] n=7/group
12 week atherogenic diet in *Ldlr*$^{-/-}$ mice				
+ control anti-miR	133.2 ± 1.6	442.0 ± 39.7	394.5 ± 36.3	48.04 ± 4.9
+ anti-miR-302a	133.0 ± 1.1	563.2 ± 53.3	498.1 ± 47.2	64.92 ± 7.6*

*$P<0.05$