miR-33 links SREBP-2 induction to repression of sterol transporters

Tyler J. Marquart[a], Ryan M. Allen[a], Daniel S. Ory[b], and Ángel Baldán[a,c,1]

[a]Edward A. Doisy Department of Biochemistry and Molecular Biology, Saint Louis University, St. Louis, MO 63104; [b]Diabetic Cardiovascular Disease Center, Washington University, St. Louis, MO 63110; and [c]Saint Louis University Center for Cardiovascular Research, St. Louis, MO 63104

Edited by William S. Sly, Saint Louis University School of Medicine, St. Louis, MO, and approved May 27, 2010 (received for review April 15, 2010)

The sterol regulatory element binding protein 2 (SREBP-2) and the liver X receptor (LXR) control antagonistic transcriptional programs that stimulate cellular cholesterol uptake and synthesis, and cholesterol efflux, respectively. The clinical importance of SREBP-2 is revealed in patients with hypercholesterolemia treated with statins, which reduce low-density lipoprotein (LDL) cholesterol levels by increasing hepatic expression of SREBP-2 and its target, the LDL receptor. Here we show that miR-33 is encoded within SREBP-2 and that both mRNAs are coexpressed. We also identify sequences in the 3′ UTR of ABCA1 and ABCG1, sterol transporter genes both previously shown to be regulated by LXR, as targets for miR-33–mediated silencing. Our data show that LXR-dependent cholesterol efflux to both ApoAI and serum is ameliorated by miR-33 overexpression and, conversely, stimulated by miR-33 silencing. Finally, we show that ABCA1 mRNA and protein and plasma HDL levels decline after hepatic overexpression of miR-33, whereas they increase after hepatic miR-33 silencing. These results suggest novel ways to manage hypercholesterolemic patients.

ABCA1 | ABCG1 | cholesterol | high-density lipoprotein | miRNA

Atherosclerosis is a progressive disorder wherein lipid-loaded macrophages initially accumulate in the subendothelial space (1, 2). Subsequently, more advanced plaques develop that contain extracellular lipid, a necrotic core, and inflammatory cells (1, 2). Such plaques can eventually rupture, leading to the formation of thrombi (1, 2). Together, these changes in the artery wall can result in heart attack, stroke, and peripheral artery disease, which collectively accounted for >30% of all deaths in the United States during the last decade (3).

The development of atherosclerosis and the risk of a myocardial infarction are accelerated by a number of factors, including hypercholesterolemia (3, 4). Statins represent the most common pharmacologic treatment for hypercholesterolemic patients (5, 6). Statins inhibit hepatic 3-hydroxy-3-methylglutaryl-CoA reductase, the rate-limiting enzyme in the cholesterol synthesis pathway (6). This decrease in sterol synthesis/levels results in increased nuclear localization of SREBP-2, which then promotes the transcription of the low-density lipoprotein (LDL) receptor (LDLR), ultimately leading to increased clearance of circulating LDL-cholesterol (6–8).

MicroRNAs (miRNAs) are small, noncoding 20- to 24-nt RNAs that promote the down-regulation of their target genes by binding to specific, partially complementary regions in the 3′ UTR of the target mRNA. This results in RNA interference and/or translational repression of the target gene (reviewed in refs. 9 and 10). miRNAs can be transcribed from their own promoter or can be encoded in the introns of other genes (9, 10). It is speculated that in the latter case, these miRNAs might be expressed when the "hosting" mRNA is transcribed. Regardless, the original Pri-miRNA (200–400 nt) is first processed by the exonuclease Drosha, resulting in a Pre-miRNA (100–150 nt), which is then exported to the cytoplasm for further cleavage by Dicer to generate the mature miRNA (20–24 nt) (9, 10). Expression of particular miRNAs has been found to be tissue-, developmental-, and even disease-specific, revolutionizing our understanding of gene expression regulatory networks. Recent studies have shown that miRNAs function as key mediators in multiple normal and disease-related biological processes (11–15). Consequently, novel therapeutical approaches that exploit miRNA-dependent gene silencing offer promising approaches for the management of multiple diseases.

In this report, we identify miR-33 as a miRNA that is cotranscribed with *Srebp-2* and controls critical aspects of cholesterol homeostasis, namely the repression of the LXR target genes ABCA1 and ABCG1, which promote the efflux of sterols from cells and/or are required for high-density lipoprotein (HDL) formation.

Results

miR33 Is Evolutionarily Conserved in Intron 16 of SREBP-2. In an attempt to identify miRNAs that might affect cholesterol homeostasis, we performed an in silico analysis of human genes encoding nuclear receptors and transcription factors known to affect lipid homeostasis. This analysis identified sequences corresponding to miR-33 within intron 16 of human SREBP-2 (Fig. 1*A*). Importantly, these sequences are conserved across multiple animal species (Fig. 1*B*). To test the hypothesis that *Srebp-2* and miR-33 are coexpressed and regulated by the same metabolic/sterol stimuli, we incubated mouse and human primary macrophages in media containing low or high sterols (see *Materials and Methods*), conditions known to regulate *Srebp*-2, SREBP-2 target genes, and LXR target genes (7–16). As expected, excessive exogenous cholesterol resulted in increased expression of the LXR target gene *Abca1*, with concomitant repression of the SREBP-2 target *Ldl-r*, whereas the opposite pattern of gene expression was found in cells incubated in media lacking cholesterol (Fig. 1 *C* and *D*). Importantly, and in agreement with our proposal, miR-33 levels paralleled those of *Srebp-2*; they were repressed in cells incubated in high sterols and induced in cells incubated in media devoid of cholesterol (Fig. 1 *C* and *D*).

Human and Murine ABCA1 and Murine ABCG1 Have Functional miR-33 Responsive Elements in Their 3′ UTR. Because SREBP-2 and LXR control antagonistic aspects of cellular sterol homeostasis, we hypothesized that miR-33 might be involved in repression of LXR target genes, such as ABCA1 and ABCG1, which are known to promote the efflux of cholesterol from cells (17–25). Analysis of the 3′ UTR regions of ABCA1 and ABCG1 identified sequences in both genes that are partially complementary to miR-33 sequences (Fig. 2 *A*–*C*). In the case of human ABCA1, we identified a proximal element (nucleotides 120–172 downstream of the stop codon, which we have termed box 1) that contains three overlapping putative miR-33–responsive elements,

Author contributions: Á.B. designed research; T.J.M., R.M.A., and Á.B. performed research; D.S.O. contributed new reagents/analytic tools; T.J.M., R.M.A., and Á.B. analyzed data; and T.J.M. and Á.B. wrote the paper.

Conflict of interest statement: The authors are pursuing a patent related to this work.

*This Direct Submission article had a prearranged editor.

Freely available online through the PNAS open access option.

[1]To whom correspondence should be addressed. E-mail: abaldan1@slu.edu.

This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1005191107/-/DCSupplemental.

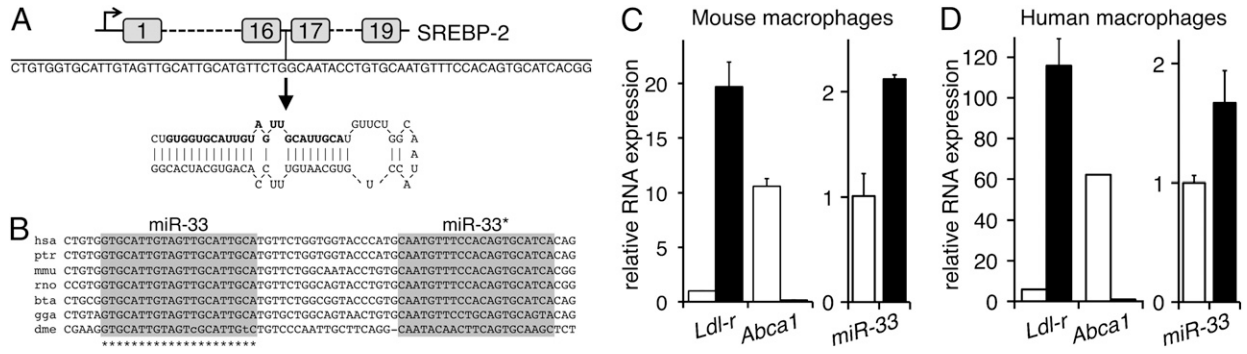

Fig. 1. miR-33 and SREBP-2 are coexpressed. (*A* and *B*) The intragenic miR-33 is encoded within intron 16 of SREBP-2 (*A*), and both its sequence and genomic position are conserved across evolution (hsa, human; ptr, chimpanzee; mmu, mouse; rno, rat; bta, cow; gga, chicken; dme, fruit fly) (*B*). (*C* and *D*) Expression of miR-33 and selected SREBP-2 and LXR target genes in mouse (*C*) and human (*D*) primary macrophages following 48 h incubation in media containing low (closed bars) or high (open bars) levels of sterols (see *Materials and Methods*). $^{**}P < 0.01$. Data are mean ± SD of three (human) or two (mouse) independent experiments in triplicate.

and a distal element (nucleotides 1465–1481 after the stop codon; box 2) (Fig. 2 *A* and *C*). For human ABCG1, a single putative miR-33–responsive element was identified at nucleotides 516–541 after the stop codon (Fig. 2 *B* and *C*). The same putative sequences are found in nucleotides 120–171 and 1426–1442 in the mouse ABCA1 gene, and in nucleotides 715–733 in the mouse ABCG1 gene. Importantly, these sequences are evolutionarily conserved across animal species (Fig. 2 *A* and *B*). To assess the functionality of these sequences, we transfected HEK293 cells with luciferase reporters containing the different miR-33 putative elements inserted after the stop codon. Cotransfection of a plasmid expressing miR-33 resulted in a 50–60% decrease in luciferase activity when the reporter plasmid contained the human/mouse box 1 sequence from ABCA1 or the mouse ABCG1 sequence (Fig. 2*D*, lanes 5, 6, 9, and 10). The specificity of this effect is supported by the finding of no repression when the reporter gene contained either sequences corresponding to box 2 of ABCA1 (Fig. 2*D*), or mutant box 1 or mutant mouse ABCG1 sequences (Fig. S1). Intriguingly, the miR-33–responsive element in the human ABCG1 gene is degenerate compared with the mouse and rat sequences and does not confer miR-33 responsiveness (Fig. 2 *B–D*, lanes 11 and 12). In comparison, a perfect miR-33 complementary sequence resulted in 95% repression of luciferase activity (Fig. 2*D*, lanes 3 and 4).

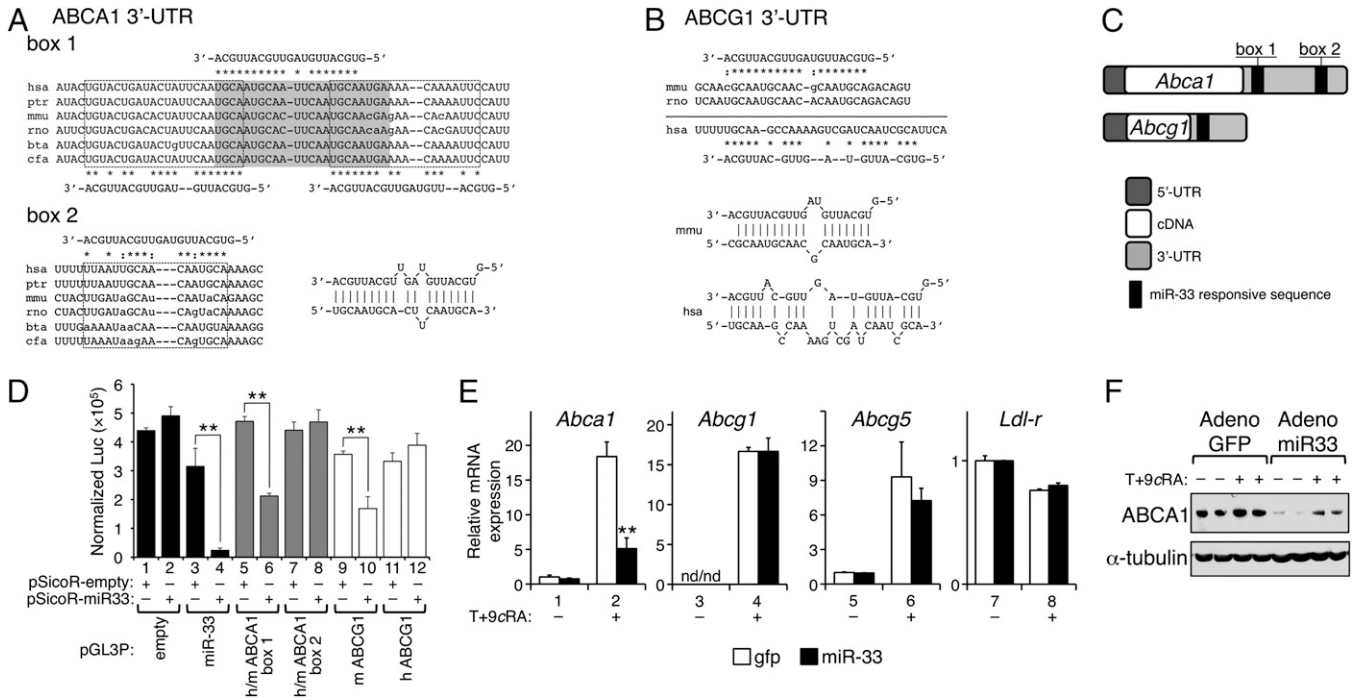

Fig. 2. The expression of human and mouse ABCA1, and of mouse ABCG1 are regulated by miR-33. (*A–C*) Evolutionary conserved sequences in the 3′ UTR of ABCA1 and ABCG1 are partially complementary to miR-33. Annealings of miR-33 to some of the sequences are shown. (*D*) Luciferase activity in HEK293 cells after cotransfection of different constructs containing these putative response elements for miR-33 cloned downstream of the reporter stop codon, cotransfected with or without a miR-33 expression plasmid. Repression of luciferase activity suggests that these sequences are physiological targets for miR-33. Deviation from miR-33 complementarity results in loss of regulation by miR-33 (ABCA1 box 2; human ABCG1 sequence). (*E*) Expression of selected genes in Hep3B human hepatoma cells 48 h after transduction with an empty or a miR-33 adenovirus. (*F*) ABCA1 protein levels also are decreased after transduction of Hep3B cells with the miR-33 adenovirus. Where indicated, cells were incubated for 16 h with LXR:RXR agonists [1 μmol/L T0901317 (T) and 1 μmol/L 9-*cis* retinoid acid (9cRA)]. $^{**}P < 0.01$. Data are mean ± SD of three independent experiments in duplicate.

We next measured the ability of miR-33 to modulate the expression of endogenous ABCA1 and ABCG1 mRNAs. To accomplish this, we transduced Hep3B cells with an empty adenovirus or an adenovirus encoding miR-33, and then incubated them in the absence or presence of agonists for LXR and RXR (T0901317 and 9-cis-retinoic acid, respectively) (Fig. 2*E*). In agreement with the foregoing data, overexpression of miR-33 resulted in reduced expression of ABCA1, but not ABCG1, mRNA (Fig. 2*E*, lanes 1–4), as well as reduced ABCA1 protein expression in these human hepatoma cells (Fig. 2*F*); other lipid metabolism genes remained unaffected by miR-33 expression (Fig. 2*E*, lanes 5–8). Collectively, these results identify human and murine ABCA1 and murine ABCG1 as bona fide targets for miR-33.

Manipulation of miR-33 Expression Alters Cholesterol Metabolism Both in Vitro and in Vivo. We tested the hypothesis that miR-33–dependent repression of ABCA1 and/or ABCG1 could affect cellular sterol homeostasis in vitro and in vivo. To test the proposal that miR-33 modulates the efflux of cellular cholesterol, we transfected HEK293 cells with an empty plasmid or a plasmid encoding miR-33 and then incubated the cells with [^{3}H]-cholesterol in the presence or absence of ligands for LXR and RXR to induce ABCA1 and ABCG1. Importantly, altering miR-33 levels produced no differences in total cellular cholesterol content or [^{3}H]-cholesterol uptake (Fig. S2). The ability of the cells to efflux the radiolabeled sterol to BSA, ApoAI, and FBS was analyzed 6 h later (see *Materials and Methods* for details). The data shown in Fig. 3*A* demonstrate that after activation of LXR:RXR, miR-33 overexpression resulted in reduced cholesterol efflux to ApoAI or FBS (compare lanes 5 and 6 to lanes 7 and 8, and lanes 9 and 10 to lanes 11 and 12). Thus, miR-33 overexpression decreases the ability of cells to efflux cholesterol. Conversely, silencing miR-33 using antisense oligonucleotides resulted in enhanced LXR agonist–dependent cholesterol efflux in the same HEK293 cells (Fig. 3*B*; compare lanes 5 and 6 to lanes 7 and 8, and lanes 9 and 10 to lanes 11 and 12). These data offer a molecular explanation for previously reported results showing that treatment with statins, which induce *Srebp-2,* resulted in decreased expression of both

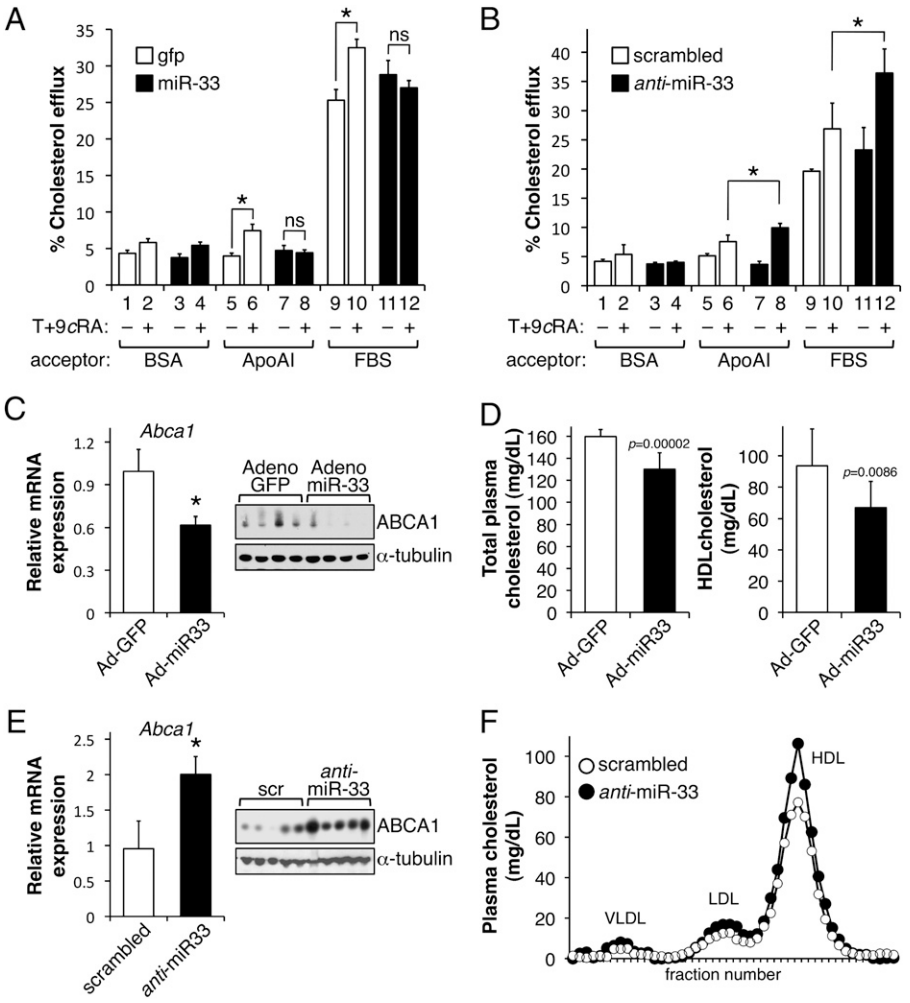

Fig. 3. Manipulation of miR-33 expression alters HDL lipidation. (*A* and *B*) Cholesterol efflux assay in HEK293 cells transfected with an empty plasmid or a plasmid encoding miR-33 (*A*), or with scrambled or miR33-specific hairpin inhibitors (*B*) (see *Materials and Methods* for details). After 36 h, the cells were washed and incubated for 16 h in media supplemented with [^{3}H]-cholesterol (1 μCi/mL) in the presence or absence of LXR:RXR ligands, as described in Fig. 2. After 16 h, fresh media supplemented with BSA (0.2%), ApoAI (15 μg/mL), or FBS (20%) was added to the cells. Radioactivity in the media and in cell lysates was measured 6 h later. The percentage of efflux is expressed as dpm in the media versus total dpm (media + cells). *$P < 0.01$. Data are mean ± SD of eight wells (two independent experiments in quadruplicate). (*C*–*F*) For in vivo experiments, 8- to 10-wk-old male C57BL/6 mice were infused i.v. with 2×10^9 empty or miR-33 adenovirus (*C* and *D*) or with 5 mg/kg/d scrambled or anti–miR-33 antisense oligonucleotides for 3 consecutive d (*E* and *F*) (n = 6–8 for Ad-GFP/Ad-miR33; n = 5/group for antisense oligonucleotides). The levels of hepatic ABCA1 mRNA and protein were measured 5 d (adenovirus) or 12 d (antisense oligonucleotides) after the infusion (*C* and *E*). Total cholesterol and HDL-cholesterol were measured in plasma of these same mice, using colorimetric enzymatic kits (*D*), and cholesterol distribution across plasma fractions were analyzed by FPLC (*F*). *$P < 0.01$. Data are mean ± SD.

ABCA1 and/or ABCG1 mRNAs and attenuated cholesterol efflux in macrophages and endothelial cells (26, 27).

To put these latter results in a more physiological context, we injected empty or miR-33–expressing adenovirus, or scrambled or anti–miR-33 antisense oligonucleotides, into the tail veins of C57BL/6 mice and measured hepatic ABCA1 mRNA and protein expression and plasma lipoprotein and cholesterol levels 5 d or 12 d after the infusion of the adenovirus or the antisense oligonucleotides, respectively. The data show that miR-33 adenoviral transduction resulted in reduced ABCA1 mRNA and protein (Fig. 3*C*), along with a 19% reduction in total plasma cholesterol (130.0 ± 15.0 vs. 159.8 ± 6.4 mg/dL, $P = 0.00002$) and a 29% reduction in HDL-cholesterol (66.8 ± 16.8 vs. 93.6 ± 23.6 mg/dL, $P = 0.0086$) , compared with mice transduced with the empty adenovirus ($n = 6$ for Ad-GFP; $n = 8$ for Ad-miR33) (Fig. 3*D*). Conversely, silencing miR-33 with antisense oligonucleotides resulted in a substantial increase in both *Abca1* expression (Fig. 3*E*) and HDL-cholesterol levels as measured by FPLC (Fig. 3*F*). Importantly, these effects were specific for *Abca1*, because the expression of other lipid-related genes did not change following manipulation of hepatic miR-33 expression (Fig. S3). Collectively, these in vitro and in vivo studies strongly suggest that miR-33 modulates intracellular cholesterol homeostasis and lipoprotein metabolism, presumably by silencing the expression of ABCA1. Whether other miR-33 targets are also essential for this effect remains to be determined.

Discussion

The experiments shown herein both challenge and expand our current understanding of how cholesterol homeostasis is regulated in the cell and in the whole body. Although SREBP-2 and LXR are known to respond to changes in cellular sterols (28–30), these two pathways had been thought to operate independently. However, it is now clear that the two pathways are intimately connected through the inducible degrader of LDLR (Idol-1) (31) and miR-33 (this report) (Fig. 4). We speculate that miR-33–dependent silencing of the sterol transporters ABCA1 and/or ABCG1 evolved to ensure minimal loss of sterols during conditions of very low intracellular cholesterol that could compromise cell viability. Nevertheless, this paradigm that links the SREBP-2 and LXR pathways (Fig. 4) may provide new opportunities to evaluate and treat dyslipidemias and other cholesterol-related disorders. For example, the current findings suggest that statins not only induce hepatic SREBP-2 and LDLR expression, thus reducing plasma LDL-cholesterol levels and decreasing the risk of myocardial infarction (3, 5), but also lead to an increase in miR-33 that results in partial repression of ABCA1. This model is in apparent contradiction with data from large clinical trials that show a modest, but significant, increase in plasma HDL-cholesterol levels in patients treated with statins (recently reviewed in ref. 32). Interestingly, these latter studies show great variability between individuals, and the effects of some statins on HDL-cholesterol are not always dose-dependent. The exact molecular basis for such statin-dependent increases in HDL-cholesterol remains obscure, but some authors have suggested that it might be not the result of increased HDL secretion by hepatocytes, but rather a secondary consequence of decreased sterol exchange between HDL and VLDL/LDL, due to reduced plasma cholesterol ester transfer protein (CETP) levels and/or activity (reviewed in ref. 33). It is safe to conclude that the specific effects of statins on HDL secretion, metabolism, and clearance remain to be fully elucidated. We propose that the new miR-33 pathway impacts the initial, ABCA1-dependent steps of ApoAI lipidation and subsequent secretion of HDL.

Nevertheless, we hypothesize that induction of miR-33 levels after treatment with statins also might contribute to some of the pleiotropic, LDL-independent effects of these drugs, such as modulation of inflammatory cascades or cell proliferation. Additional experiments to find new physiological targets of miR-33 are needed to confirm this hypothesis. Finally, because hepatic ABCA1 is critical to the generation of plasma HDL (34, 35), it seems likely that a combination therapy that includes statins and miR-33 antagomirs might result in both decreased LDL levels and increased HDL levels, thus improving the prognosis for patients with hypercholesterolemia and cardiovascular disease.

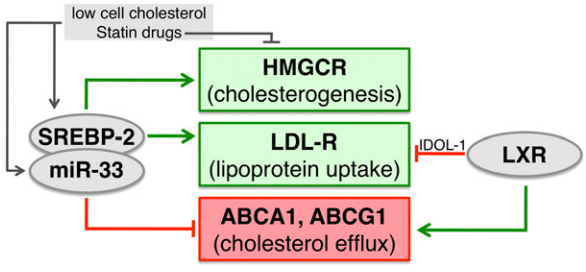

Fig. 4. A unified paradigm for cholesterol homeostasis. SREBP-2 and LXR coordinately regulate the positive (green) and negative (red) balances of intracellular cholesterol metabolism. Both pathways are not independent, but intersect through IDOL-1 and miR-33. Statin drugs, or conditions of low intracellular cholesterol, induce both SREBP-2 and the intragenic miR-33, leading to increased synthesis and uptake of sterols, as well as minimizing sterol loss through repression of ABC transporters.

Materials and Methods

Plasmid Constructs and Production of Adenovirus. A fragment containing miR-33 flanked by 150 bp upstream and 150 bp downstream of genomic sequence was amplified by PCR using Platinum Pfx enzyme (Invitrogen) from mouse genomic DNA obtained from tail biopsy specimens from C57BL/6 mice. The reverse primer used in the PCR contained the appropriate terminator sequence for RNA-pol-III (TTTTTCT). This fragment was cloned into the HpaI-XhoI sites of pSicoR-GFP (Addgene), which provides a U6 promoter to control the expression of the transgene (and a GFP cassette under control of a cytomegalovirus promoter), thus generating pSicoR-miR33. The integrity of the clones was analyzed by sequencing. Adenoviruses were produced using the AdEasy Adenoviral System (Stratagene) by cloning a XbaI-XhoI fragment containing the U6 promoter with or without the miR-33 sequences from the pSicoR vectors into pAdTrack (which also contains a GFP cassette). The pAdTrack and pAdTrack-miR33 vectors were electroporated into pAdeasy-1 cells to generate the final adenoviral vectors. Recombinant vectors were identified by the presence of a 4.5-kb PacI restriction fragment. These final vectors were transfected individually into AD293 cells (Stratagene) for the production and amplification of the adenoviral particles. The adenoviruses were purified by CsCl gradient ultracentrifugation, and the titers were calculated to be 1×10^{12} plaque-forming units (pfu)/μL for Ad-GFP and 1×10^{11} pfu/μL for Ad-miR33. Experiments that used the empty pSicoR or the empty adenoviral vector are referred to as "gfp."

Cells. HEK293 cells (American Type Culture Collection) and Hep3B cells (a kind gift from Dr. Richard Lee, Isis Pharmaceuticals) were maintained in DMEM plus 10% FBS. Human monocyte-derived macrophages from three healthy donors and mouse thioglycollate–elicited primary peritoneal macrophages were obtained and maintained as described previously (36). Murine macrophages were incubated for 48 h in media A [DMEM supplemented with 10% lipoprotein-deficient serum (LPDS) (Intracel), 100 μmol/L mevalonic acid (Sigma-Aldrich), and 50 μmol/L pravastatin (Cayman Chemicals)] in the presence or absence of 10 μg/mL of cholesterol (Sigma-Aldrich) and 1 μg/mL of 25-hydroxycholesterol (Sigma-Aldrich). Human macrophages were incubated in media A with or without supplementation with 40 μg/mL of oxidized LDL (Biomedical Technologies). Where indicated, cells were transfected with pSicoR-GFP or pSicor-miR33, or with miRIDIAN-scrambled or miR33-specific hairpin inhibitors (Dharmacon), using Lipofectamine 2000 (Invitrogen) or Dharmafect 1 (Dharmacon) reagents, respectively, or were transduced with Ad-GFP or Ad-miR-33 adenovirus. Where indicated, cells were incubated for 16 h in the absence or presence of agonists for LXR (1 μmol/L T0901317; Cayman Chemicals) and RXR (1 μmol/L 9-*cis*-retinouic acid; Sigma-Aldrich).

Cholesterol Efflux Assays. HEK293 cells were seeded in 24-well plates (0.25×10^6 cells per well) and transfected with pSicoR-GFP or pSicoR-miR33, or with

scrambled or miR33-specific hairpin inhibitors. After 36 h in complete media, the cells were washed and incubated for an additional 16 h in DMEM supplemented with 10% LPDS and [^{3}H]-cholesterol (1 μCi/mL). After 16 h, the cells were washed with PBS and then incubated in DMEM supplemented with 0.2% BSA for a 2-h equilibration period. When indicated, the cells were incubated with T0901317 (1 μmol/L) and 9-*cis* retinoic acid (1 μmol/L) during radiolabeling and equilibration. To determine cholesterol efflux, the cells were rinsed three times with PBS and then incubated for 6 h in DMEM supplemented with 0.2% BSA and, where indicated, either ApoAI (15 μg/mL) or FBS (10%). Subsequently, the media was removed, the cells washed with PBS, and the radioactive content of the media and cells was determined by scintillation. Cholesterol efflux was determined by dividing the radioactive content of the media by the sum of the radioactivity in the cells and media.

RNA Analysis. RNA was isolated from cells with TRIzol reagent, using a slight modification of the manufacturer's protocol to preserve miRNAs. In brief, after homogenization in 2 mL of TRIzol, 400 μL of chloroform and 200 μL of 2 mol/L sodium acetate (pH 4.2) were added before separation of the organic and aqueous phases. The latter was combined with 500 μL of acid phenol:chloroform: isoamylic acid and then centrifuged again. RNAs in the aqueous phase were then precipitated with 3 mL of ethanol overnight at 4 °C. After centrifugation, the RNA pellet was resuspended in water and stored at −80 °C until use. Alternatively, RNAs were isolated using the mirVana miRNA Isolation Kit (Ambion). cDNAs were generated from 5 μg of DNase1-treated RNA using SuperScript III and Random hexamers (Invitrogen). Real-time PCR was done with SYBR Green reagent (Roche), using a LightCycler 480 real-time PCR detection system (Roche). Primer sets are available from the authors on request. Values were normalized to GAPDH and calculated using the comparative C_T method. The expression of miR-33 was normalized to U6 and analyzed using Taqman miRNA assays (Applied Biosystems).

Protein Analysis. Protein extracts from cells or livers were obtained and analyzed as described previously (31). ABCA1 levels were determined using rabbit polyclonal anti-ABCA1 (Novus Biologicals, 1:1,000 dilution in TBS + 1% Tween 20) and normalized to α-tubulin (Santa Cruz Biotechnologies, 1:5,000 dilution in TBS + 1% Tween 20).

Murine Studies. Male C57BL/6J mice were obtained from Jackson Laboratories and maintained in a 12-h/12-h light/dark cycle with unlimited access to chow and water. The mice (n = 6–8 per group) were infused via tail vein injection with adenoviral vectors (2×10^9 pfu) or with control or anti–miR-33 antisense oligonucleotides (5 mg/kg/d for 3 consecutive d; miRagen Therapeutics). Blood and livers were collected 5 d (for adenovirus) or 12 d (for antisense oligonucleotides) postinfusion. Total cholesterol and HDL-cholesterol were assayed enzymatically with Waco Chemicals Cholesterol E and HDL-Cholesterol E Kits. Plasma lipoprotein profiles for cholesterol were determined in pooled plasma samples from five mice by FPLC as described previously (37).

Note Added in Proof. While this manuscript was under revision, two independent laboratories reported similar findings (38, 39).

ACKNOWLEDGMENTS. We thank Paul Loewenstein and Erin Touchette for help with adenovirus production and i.v. infusion into mice, respectively. We thank Eva van Rooij (miRagen Therapeutics) for help with anti-miR in vivo experiments. We also thank our friends and colleagues at the Doisy Research Center for helpful discussions. This work was supported by startup funding from the Doisy Department of Biochemistry and Molecular Biology and from the Saint Louis University Center for Cardiovascular Research (to Á.B.) and National Institutes of Health Grants P50 HL083762 and P60 DK020579 (to D.S.O.).

1. Lusis AJ (2000) Atherosclerosis. *Nature* 407:233–241.
2. Glass CK, Witztum JL (2001) Atherosclerosis: The road ahead. *Cell* 104:503–516.
3. Rosamond W, et al.; American Heart Association Statistics Committee and Stroke Statistics Subcommittee (2007) Heart disease and stroke statistics—2007 update: A report from the American Heart Association Statistics Committee and Stroke Statistics Subcommittee. *Circulation* 115:e69–e171.
4. Kannel WB, Castelli WP, Gordon T (1979) Cholesterol in the prediction of atherosclerotic disease: New perspectives based on the Framingham Study. *Ann Intern Med* 90:85–91.
5. Baigent C, et al.; Cholesterol Treatment Trialists' (CTT) Collaborators (2005) Efficacy and safety of cholesterol-lowering treatment: Prospective meta-analysis of data from 90,056 participants in 14 randomised trials of statins. *Lancet* 366:1267–1278.
6. Steinberg D (2006) Thematic review series. The pathogenesis of atherosclerosis: An interpretive history of the cholesterol controversy. Part V: The discovery of the statins and the end of the controversy. *J Lipid Res* 47:1339–1351.
7. Brown MS, Goldstein JL (1997) The SREBP pathway: Regulation of cholesterol metabolism by proteolysis of a membrane-bound transcription factor. *Cell* 89:331–340.
8. Goldstein JL, Brown MS (2009) The LDL receptor. *Arterioscler Thromb Vasc Biol* 29: 431–438.
9. Bartel DP (2009) MicroRNAs: Target recognition and regulatory functions. *Cell* 136: 215–233.
10. Olena AF, Patton JG (2010) Genomic organization of microRNAs. *J Cell Physiol* 222: 540–545.
11. Trang P, Weidhaas JB, Slack FJ (2008) MicroRNAs as potential cancer therapeutics. *Oncogene* 27(Suppl 2):S52–S57.
12. Coolen M, Bally-Cuif L (2009) MicroRNAs in brain development and physiology. *Curr Opin Neurobiol* 19:461–470.
13. van Rooij E, et al. (2008) Dysregulation of microRNAs after myocardial infarction reveals a role of miR-29 in cardiac fibrosis. *Proc Natl Acad Sci USA* 105:13027–13032.
14. Ortega FJ, et al. (2010) MiRNA expression profile of human subcutaneous adipose and during adipocyte differentiation. *PLoS ONE* 5:e9022.
15. Asirvatham AJ, Gregorie CJ, Hu Z, Magner WJ, Tomasi TB (2008) MicroRNA targets in immune genes and the Dicer/Argonaute and ARE machinery components. *Mol Immunol* 45:1995–2006.
16. Venkateswaran A, et al. (2000) Control of cellular cholesterol efflux by the nuclear oxysterol receptor LXR alpha. *Proc Natl Acad Sci USA* 97:12097–12102.
17. Kennedy MA, et al. (2005) ABCG1 has a critical role in mediating cholesterol efflux to HDL and preventing cellular lipid accumulation. *Cell Metab* 1:121–131.
18. Baldán A, Bojanic DD, Edwards PA (2009) The ABCs of sterol transport. *J Lipid Res* 50(Suppl):S80–S85.
19. Cavelier C, Lorenzi I, Rohrer L, von Eckardstein A (2006) Lipid efflux by the ATP-binding cassette transporters ABCA1 and ABCG1. *Biochim Biophys Acta* 1761:655–666.
20. Gelissen IC, et al. (2006) ABCA1 and ABCG1 synergize to mediate cholesterol export to apoA-I. *Arterioscler Thromb Vasc Biol* 26:534–540.
21. Kobayashi A, et al. (2006) Efflux of sphingomyelin, cholesterol, and phosphatidylcholine by ABCG1. *J Lipid Res* 47:1791–1802.
22. Out R, et al. (2008) Coexistence of foam cells and hypocholesterolemia in mice lacking the ABC transporters A1 and G1. *Circ Res* 102:113–120.
23. Tall AR, Yvan-Charvet L, Terasaka N, Pagler T, Wang N (2008) HDL, ABC transporters, and cholesterol efflux: Implications for the treatment of atherosclerosis. *Cell Metab* 7: 365–375.
24. Vaughan AM, Oram JF (2006) ABCA1 and ABCG1 or ABCG4 act sequentially to remove cellular cholesterol and generate cholesterol-rich HDL. *J Lipid Res* 47:2433–2443.
25. Wang X, et al. (2007) Macrophage ABCA1 and ABCG1, but not SR-BI, promote macrophage reverse cholesterol transport in vivo. *J Clin Invest* 117:2216–2224.
26. Wong J, Quinn CM, Gelissen IC, Jessup W, Brown AJ (2008) The effect of statins on ABCA1 and ABCG1 expression in human macrophages is influenced by cellular cholesterol levels and extent of differentiation. *Atherosclerosis* 196:180–189.
27. Zeng L, et al. (2004) Sterol-responsive element-binding protein (SREBP) 2 down-regulates ATP-binding cassette transporter A1 in vascular endothelial cells: A novel role of SREBP in regulating cholesterol metabolism. *J Biol Chem* 279:48801–48807.
28. Radhakrishnan A, Ikeda Y, Kwon HJ, Brown MS, Goldstein JL (2007) Sterol-regulated transport of SREBPs from endoplasmic reticulum to Golgi: Oxysterols block transport by binding to Insig. *Proc Natl Acad Sci USA* 104:6511–6518.
29. Repa JJ, Mangelsdorf DJ (2002) The liver X receptor gene team: Potential new players in atherosclerosis. *Nat Med* 8:1243–1248.
30. Edwards PA, Kennedy MA, Mak PA (2002) LXRs: Oxysterol-activated nuclear receptors that regulate genes controlling lipid homeostasis. *Vascul Pharmacol* 38:249–256.
31. Zelcer N, Hong C, Boyadjian R, Tontonoz P (2009) LXR regulates cholesterol uptake through Idol-dependent ubiquitination of the LDL receptor. *Science* 325:100–104.
32. Natarajan P, Ray KK, Cannon CP (2010) High-density lipoprotein and coronary heart disease: Current and future therapies. *J Am Coll Cardiol* 55:1283–1299.
33. Chapman MJ, Le Goff W, Guerin M, Kontush A (2010) Cholesteryl ester transfer protein: At the heart of the action of lipid-modulating therapy with statins, fibrates, niacin, and cholesteryl ester transfer protein inhibitors. *Eur Heart J* 31:149–164.
34. Singaraja RR, et al. (2006) Hepatic ATP-binding cassette transporter A1 is a key molecule in high-density lipoprotein cholesteryl ester metabolism in mice. *Arterioscler Thromb Vasc Biol* 26:1821–1827.
35. Singaraja RR, et al. (2001) Human ABCA1 BAC transgenic mice show increased high density lipoprotein cholesterol and ApoAI-dependent efflux stimulated by an internal promoter containing liver X receptor response elements in intron 1. *J Biol Chem* 276: 33969–33979.
36. Venkateswaran A, et al. (2000) Human white/murine ABC8 mRNA levels are highly induced in lipid-loaded macrophages: A transcriptional role for specific oxysterols. *J Biol Chem* 275:14700–14707.
37. Zhang JR, et al. (2008) Niemann-Pick C1 protects against atherosclerosis in mice via regulation of macrophage intracellular cholesterol trafficking. *J Clin Invest* 118: 2281–2290.
38. Najafi-Shoushtari SH, et al. (2010) MicroRNA-33 and the SREBP host genes cooperate to control cholesterol homeostasis. *Science*, 10.1126/science.1189123.
39. Rayner KJ, et al. (2010) miR-33 contributes to the regulation of cholesterol homeostasis. *Science*, 10.1126/science.1189862.