Control of Very Low-Density Lipoprotein Secretion by N-Ethylmaleimide-Sensitive Factor and miR-33

Ryan M. Allen, Tyler J. Marquart, Jordan J. Jesse, Ángel Baldán

Rationale: Several reports suggest that antisense oligonucleotides against miR-33 might reduce cardiovascular risk in patients by accelerating the reverse cholesterol transport pathway. However, conflicting reports exist about the impact of anti-miR-33 therapy on the levels of very low-density lipoprotein-triglycerides (VLDL-TAG).

Objective: We test the hypothesis that miR-33 controls hepatic VLDL-TAG secretion.

Methods and Results: Using therapeutic silencing of miR-33 and adenoviral overexpression of miR-33, we show that miR-33 limits hepatic secretion of VLDL-TAG by targeting N-ethylmaleimide-sensitive factor (NSF), both in vivo and in primary hepatocytes. We identify conserved sequences in the 3'UTR of NSF as miR-33-responsive elements and show that Nsf is specifically recruited to the RNA-induced silencing complex following induction of miR-33. In pulse-chase experiments, either miR-33 overexpression or knock-down of Nsf lead to decreased secretion of apolipoproteins and TAG in primary hepatocytes, compared with control cells. Importantly, Nsf rescues miR-33-dependent reduced secretion. Finally, we show that overexpression of Nsf in vivo increases global hepatic secretion and raises plasma VLDL-TAG.

Conclusions: Together, our data reveal key roles for the miR-33–NSF axis during hepatic secretion and suggest that caution should be taken with anti-miR-33-based therapies because they might raise proatherogenic VLDL-TAG levels. (Circ Res. 2014;115:10-22.)

Key Words: lipoproteins, VLDL ■ liver ■ miR-33 ■ N-ethylmaleimide-sensitive factor ■ triglycerides

Very low-density lipoproteins (VLDLs) are triacylglyceride-rich lipoproteins synthesized in the liver. After secretion, the triacylglycerol (TAG) core of VLDL is metabolized in peripheral tissues by lipoprotein lipase, rendering cholesterol ester-rich low-density lipoproteins that can accumulate in the arterial subendothelial spaces and trigger the development of atherosclerosis. The biogenesis of VLDL begins with the microsomal triacylglycerol transfer protein (MTP)-dependent lipidation of apolipoprotein B (apoB) in the endoplasmic reticulum (ER) of the hepatocyte (reviewed by Tiwari and Siddiqi1 and Sundaram and Yao2). Nascent lipoprotein-containing vesicles are then trafficked from the ER to the Golgi, where apoB is glycosylated and phosphorylated, and eventually to the plasma membrane for secretion into the sinusoidal space and release into the circulation. The trafficking of VLDL from ER to Golgi has been particularly well studied, and we now know that APOB-containing vesicles are different from other protein-containing vesicles budding out of the ER, not only in cargo but also in coating proteins. Hence, Siddiqi et al.,3 Siddiqi,4 and Gusarova et al.5 showed that efficient vesicular transport of VLDL out of the ER requires not only the coat complex II machinery but also the presence of the vesicle N-ethylmaleimide-sensitive factor-attachment protein receptor (v-SNARE) SEC22B, which is recognized in the Golgi by the target SNAREs (t-SNAREs) STX5, BET1, and GOSR1. The binding of v-SNARE and t-SNARE to form the so-called cis-SNARE complex is one of the hallmarks of directional vesicular trafficking, allowing vesicle and target membranes to come in close proximity thus facilitating membrane fusion and the release of the cargo into the lumen of the target compartment (reviewed by Zhao et al.6). The cis-SNARE complexes are eventually resolved by the hexameric ATPase N-ethylmaleimide-sensitive factor (NSF), which is recruited to the SNARE complex by the adaptor protein soluble NSF-attachment protein (SNAP).6 Whether NSF activity is also required for membrane fusion is still much debated. Nevertheless, data suggest that disruption of NSF activity alters intracellular vesicular trafficking and prevents normal secretion in a variety of cells.6

MicroRNAs (miRNA) are small, ≈22 nucleotide RNA molecules that associate to the RNA-induced silencing complex (RISC) and modulate the expression of target messenger RNA.
(mRNA) by binding to complementary sequences (generally in the 3′UTR) and subsequently promoting translational suppression or RNA degradation. In the past decade, miRNAs have been recognized as essential regulators of multiple (patho)physiological processes. We and others have reported on miR-33 (also known as miR-33a or miR-33a-5p): an intragenic miRNA encoded within intron 16 of SREBP-2 (Sterol regulatory element-binding protein 2) that controls the expression of several transmembrane sterol transporters that include ABCA1 (ATP-binding cassette, subfamily A, member 1), ABCG1 (ATP-binding cassette, subfamily G, member 1), NPC1 (Niemann-Pick disease, type C1), ABCB11 (ATP-binding cassette, subfamily B, member 11), and ATP8B1 (ATPase, aminophospholipid transporter, class I, type 8B, member 1). Early reports using miR-33−/− mice or mice where miR-33 expression was silenced with antisense oligonucleotides showed increased circulating high-density lipoprotein (HDL) cholesterol and bile secretion. Importantly, non-human primates also exhibited increased HDL cholesterol levels after treatment with anti-miR-33 oligonucleotides. Consistent with these data, silencing of anti-miR-33 led to increased reverse cholesterol transport in vivo in both wild-type and atherosclerosis-prone Ldlr−/− mice, compared with control oligonucleotides. The reverse cholesterol transport pathway mobilizes extrahepatic cholesterol into HDL back to the liver, where it is metabolized to bile acids and subsequently secreted into the bile for final excretion through the feces. Thus, reverse cholesterol transport is regarded as atheroprotective, and strategies that promote and accelerate the flow of cholesterol through this pathway are predicted to be effective in reducing the risk of cardiovascular disease in patients. These reports suggested that silencing of hepatic miR-33 expression (eg, anti-miR-33 oligonucleotides) might reduce cardiovascular risk in patients. However, we and others have reported conflicting results after therapeutic silencing of miR-33 in Ldlr−/− mice.

Herein, we show that prolonged silencing of miR-33 results in a sustained raise in hepatic secretion, leading to increased plasma levels of VLDL-TAG and other liver-secreted proteins in chow-fed C57BL/6 mice, compared with animals treated with saline or control oligonucleotides. Using mouse primary hepatocytes, we demonstrate that NSF is recruited to the RISC after miR-33 overexpression and that NSF mediates miR-33-dependent changes in secretion. Finally, we show that manipulation of hepatic NSF levels in vivo results in changes in hepatic secretion, including changes in plasma VLDL-TAG. Collectively, our data uncover NSF as a key regulator of hepatic secretion and suggest a role for miR-33 on intracellular vesicular trafficking.

Methods

Male 12-week-old C57BL/6 mice (NCI–Charles River Laboratories) were maintained on a 12 hours/12 hours light/dark cycle with unlimited access to food and water. Where indicated, mice were injected intraperitoneally with 200 μL saline, or 5 mg/kg control (5′-TCCTAGAAAGAGTGA) or anti-miR-33 (5′-TAGCAACTACAATAGCA) oligonucleotides (a kind gift from Miragen Therapeutics, Inc) once a week. Other animals were infused via tail vein with empty or NSF adenoviral vectors (2×109 pfu). Mouse cohorts are described in Online Table I. All animal studies were reviewed and approved by the Institutional Animal Use and Care Committee at Saint Louis University.

Detailed Methods are provided in the Online Data Supplement.

Results

Long-Term Silencing of miR-33 Raises Plasma VLDL-TAG in Chow-Fed Mice

To gain insight into the physiological consequences of long-term silencing of miR-33 expression, we dosed chow-fed C57BL/6 mice IP with saline or control or anti-miR-33 oligonucleotides (5 mg/kg) once per week for 11 weeks. We did not observe significant changes in body weight gain among the different groups (Online Figure IA). Consistent with our reported data in Western diet–fed Ldlr−/− mice, mice receiving anti-miR-33 oligonucleotides showed sustained elevated plasma TAG levels, compared with control animals (Figure 1A). TAG lipoprotein profiles of pooled plasma samples at weeks 3 and 11 (Figure 1B) and Western blot analysis of plasma APOB100 at week 11 (Figure 1C) were consistent with an increase in circulating VLDL-TAG in mice dosed with anti-miR-33, compared with controls. The levels of circulating APOB48, however, remained unchanged between groups. Plasma cholesterol levels increased, as expected, in the anti-miR-33 group for the first 5 weeks, but returned to normal thereafter (Online Figure IB). Analysis of the livers showed a >70% drop in miR-33 levels (Figure 1D) and a concomitant induction of known miR-33 targets (ie, ABCA1, CPT1A) at both the mRNA (Online Figure ID) and protein (Figure 1E) levels in animals receiving anti-miR-33 treatment, compared with controls. As described previously, the anti-miR-33 treatment resulted in a significant decrease in the amounts of hepatic FASN (Figure 1E; Online Figure ID). Data also show that at the mRNA level, both Mttp and Apob, were modestly induced in the anti-miR-33 group, compared with control group (Online Figure ID). However, no significant changes were noted at the protein levels for Mttp and APOB48 between treatments (Figure 1E). The levels of APOB100, in contrast, were modestly increased in the livers of the anti-miR-33 group, compared with controls (Figure 1E). Finally, analysis of hepatic lipid contents revealed a significant increase in the amounts cholesteryl esters in mice receiving anti-miR-33, compared with controls (Online Figure 1E), but no changes in TAG, nonesterified fatty acids or cholesterol, or phosphatidylcholine (Online Figure 1E). Collectively, data in Figure 1 and Online Figure I suggest that prolonged silencing of miR-33
using oligonucleotides in chow-fed mice results in elevated VLDL-TAG and APOB100 in circulation.

**MiR-33 Limits Hepatic VLDL Secretion In Vivo**

We hypothesized that the changes in circulating TAG in the anti-miR-33 treatment group might be the result of accelerated hepatic VLDL secretion. To test this proposal, we measured hepatic TAG secretion in vivo in a second cohort of mice using the tyloxapol method (see the Online Detailed Methods). Briefly, 6 weeks into the treatment with saline or control or anti-miR-33 oligonucleotides, mice were fasted overnight and then injected intravenously with tyloxapol, which inhibits lipoprotein lipase activity and thus prevents the degradation of circulating TAG. Consequently, TAG accumulates in circulation over time in direct proportion to the rate of hepatic secretion. Data in Figure 2A show the kinetics of plasma TAG accumulation in the 3 groups of mice over 3 hours. As expected, no changes in plasma TAG were noted between mice receiving saline or control or anti-miR-33 oligonucleotides, mice were fasted overnight and then injected intravenously with tyloxapol, which inhibits lipoprotein lipase activity and thus prevents the degradation of circulating TAG. Consequently, TAG accumulates in circulation over time in direct proportion to the rate of hepatic secretion. Data in Figure 2A show the kinetics of plasma TAG accumulation in the 3 groups of mice over 3 hours. As expected, no changes in plasma TAG were noted between mice receiving saline or control or anti-miR-33 oligonucleotides; in contrast, circulating TAG accumulated in the anti-miR-33 group at a significantly faster pace (Figure 2A). The actual secretion rates calculated from the slopes of the different curves demonstrated that depletion of miR-33 in the liver results in accelerated VLDL secretion, compared with controls (Figure 2B).

We next used mouse primary hepatocytes to further validate a role for miR-33 on VLDL secretion. Cells were transduced with an empty or miR-33-encoding adenovirus, pulsed with cold- or [14C]-oleate and chased for 24 hours in fresh media. As expected, miR-33 overexpression resulted in decreased mRNA levels of known targets (data not shown). Consistent with a role for miR-33 on VLDL secretion, we noted both reduced amounts of APOB100 and APOB48 (Figure 2C) and reduced radioactivity (Figure 2D) in supernatants recovered from cells overexpressing miR-33, compared with control cells. Intriguingly, the levels of apolipoprotein A1 (APOA1) were significantly decreased in the supernatants from cells overexpressing miR-33, compared with control cells (Figure 2C). Lipids extracted from both the media and the cells were resolved by thin layer chromatography along with standards for different classes of lipids. Data show that the amounts of [14C]-labeled TAG, DAG (diacylglycerides), and FFA (free fatty acids), but not PL (phospholipids), were significantly reduced in the supernatants from cells overexpressing miR-33, compared with control cells (Figure 2E and 2G). Whether the DAG and FFA in the media are actively secreted or the result of the hydrolysis of secreted TAG is unknown. Importantly, these changes in lipid secretion were not the result of decreased incorporation of [14C]-oleate into different classes of lipids in cells overexpressing miR-33 because intracellular-labeled lipids did not differ between control and miR-33 cells (Figure 2F and 2H). Additionally, the changes in lipid and APOB secretion in miR-33-overexpressing hepatocytes were not the result of altered de novo lipogenesis or fatty acid β-oxidation either, as measured by the incorporation of [14C]-acetate into different classes of lipids and the release of labeled water from [3H]-palmitate, respectively. Although no significant changes were noted, both parameters trended down in hepatocytes overexpressing miR-33, compared with control cells (Online Figure IIA–IIC). However, and consistent with a reduction in VLDL secretion, the levels of total intracellular TAG were modestly increased in cells overexpressing miR-33.

**Figure 1.** Silencing of miR-33 results in sustained elevation of plasma very low-density lipoprotein (VLDL)-TAG in mice. Chow-fed C57BL/6 mice were injected intraperitoneally with saline (n=5), control oligonucleotide (n=7; 5 mg/Kg per week), or anti-miR-33 oligonucleotide (n=7; 5 mg/kg/wk) for 11 weeks. Animals were fasted overnight before blood collection or euthanasia. **A**, Plasma triglyceride levels over time. Data are mean±SEM; *P≤0.05. **B**, TAG lipoprotein profiles at weeks 3 and 11, as determined by fast protein liquid chromatography. **C**, Immunoblots for apolipoprotein B (APOB) and apolipoprotein A1 (APOA1) in individual plasma samples from week 11. **D**, Relative hepatic miR-33 expression at week 11. Data are mean±SEM; **P≤0.01. E**, Immunoblots for selected proteins in the same livers. ABCA1 indicates ATP-binding cassette, subfamily A, member 1; CPT1A, carnitine palmitoyltransferase 1A; DGAT2, diacylglycerol O-acyltransferase 2; FASN, fatty acid synthase; HDL, high-density lipoprotein; LDL, low-density lipoprotein; and MTTP, microsomal triglyceride transfer protein.
compared with control cells (Online Figure IID); likewise, total intracellular cholesterol levels were elevated in the same cells (Online Figure IID), likely the result of reduced ABCA1 expression. Taken together, data from Figures 1 and 2 suggest that hepatic miR-33 modulates the secretion of VLDL-TAG both in vitro and in vivo.

APOB and MTTP Are Not Direct miR-33 Targets

Data in Figure 1 and Online Figure ID showing elevated APOB100 in circulation and moderately increased levels of hepatic Apob and Mttp following silencing of miR-33 are compatible with one or both of these genes being a direct target of the miRNA. That proposal would be consistent with the well-known role of these genes during VLDL biogenesis. Indeed, analysis of APOB and MTTP revealed the presence of conserved sequences that were partially complementary to miR-33, compared with controls. In contrast, the levels of Atp8b1, Abcc11, and Cpt1a, which encode the secreted lipoproteins apoB100, apoA1, and apoA4, respectively, remained unchanged between treatments. These data suggested that NSF could be a direct target of miR-33. To validate this proposal, we then tested whether the Nsf is actively recruited to the RISC after miR-33 overexpression.

NSF Is a Direct, Functional miR-33 Target

We next turned our attention to genes encoding proteins involved in VLDL vesicular trafficking (see the introductory text). First, we tested whether overexpression of miR-33 in either mouse primary hepatocytes or human HuH7 hepatoma cells could reduce the expression of any of such genes. Data in Figure 4A and 4B and Online Figure IIIA and IIIB show that both the mRNA and protein levels of NSF are decreased in cells overexpressing miR-33, compared with controls. In contrast, the levels of NAPA (which encodes α-SNAP, the main SNAP expressed in the liver), SEC22B, GOSR1, STX5, or BET1 remained unchanged between treatments. These data strongly suggest that NSF is a direct target of miR-33. Based on these results, we next sought to characterize the miR-33 responsive elements in NSF. The miRNA target prediction algorithm Targetscan identified 3 conserved sequences in the 3’UTR of mouse and human NSF that are partially complementary to miR-33 (sites 1–3 in Figure 4D). Interestingly, elements 2 and 3 are partially overlapping, similar to the miR-33 elements in Abca1.8–12 These sequences, or the entire 3’UTR of mouse or human NSF, were cloned downstream of a luciferase reporter and transiently transfected into HEK293...
cells in the presence or absence of a plasmid encoding miR-33. Data from these experiments show that the 3′ UTR fragments and the cluster containing sites 2 and 3, but not site 1, were able to confer responsiveness to miR-33 (Figure 4E). Importantly, specific point mutations in element 2 or 3 that prevent interaction with the seed sequence of miR-33 abrogated the response to the miRNA (Figure 4E). Together, these data identify sequences 2 and 3 as functional miR-33 responsive elements.

Finally, we checked the hepatic expression of NSF, NAPA, and selected v- and t-SNARE in the livers of mice dosed with saline or control or anti-miR-33 oligonucleotides (Figure 1). Consistent with the data shown above, NSF was increased both at the mRNA and protein levels in the livers of the anti-miR-33 group, compared with controls (Figures 4F; Online Figure IIIC). No significant changes were noted for the other sequences studied, with the exception of a modest increase of Stx5 at the mRNA but not protein level in anti-miR-33 treated livers (Figures 4F; Online Figure IIIC). Taken together, the in vivo and in vitro data shown in Figure 4 identify NSF as a physiological target of miR-33.

NSF Controls TAG and APOB Secretion in Primary Hepatocytes

Based on the well-known role of NSF on intracellular vesicular trafficking, we hypothesized that miR-33-dependent changes in NSF expression may account for the altered secretion of VLDL-TAG in mice dosed with anti-miR-33 oligonucleotides (Figures 1A and 1B and 2A and 2B). We therefore tested whether changes in NSF abundance modulate the secretion of TAG and APOB using antisense oligonucleotides to knock-down Nsf in mouse primary hepatocytes. By titrating the amounts of small interfering RNA used, we aimed at mimicking the levels of NSF repression obtained when miR-33 was overexpressed (compare Figure 5A and 5B versus Figure 4A and 4B). Although this approach was successful in dose-dependently decreasing the protein levels of NSF, it did not affect the levels of intracellular MTTP or APOB (Figure 5B). At the mRNA level, however, we noted an increase in Mttp and a decrease in both β-oxidation genes (Cpt1a and Acox) and lipogenic genes (Fasn, Acc, and Scd1) in samples transfected with anti-Nsf oligonucleotides, compared with controls (Online Figure IVA). Of critical significance for our hypothesis, the decrease in intracellular NSF in cells transfected with the anti-Nsf small interfering RNA was paralleled by a dose-dependent reduction in APOB100, APOB48, and APOA1 recovered from the supernatants of the same cells, compared with controls (Figure 5C). Remarkably, we also observed a dose-dependent increase in intracellular APOA1 in the same cells (Figure 5B). Taken together, these data suggest that the changes in the amounts of apolipoproteins recovered in the media of cells with reduced NSF expression are not due to reduced protein synthesis but rather to decreased secretion. The fact that APOB did not also accumulate in the low-NSF media of cells with reduced NSF expression are not due to associated protein degradation or autophagy of excess nonsecreted APOB (reviewed by Olofsson and Borén20).
In parallel experiments, we tested whether reduced NSF expression would also alter lipid secretion. Hence, following transfection with nontargeting or anti-Nsf small interfering RNA, primary hepatocytes were pulsed with [14C]-oleate and subsequently chased for 24 hours in fresh medium. Data show that partial knock-down of NSF did not alter the ability of cells to incorporate the labeled fatty acid into different classes of lipids (Figure 5D and 5E) but led to decreased recovery of radiolabeled lipid species (TAG, DAG, and FFA, but not PL) from the supernatants (Figure 5F and 5G), compared with control cells. These data are strikingly reminiscent of the results obtained after miR-33 overexpression in primary hepatocytes (Figure 2E–2F) and suggest both that NSF activity is rate limiting for constitutive hepatic lipoprotein secretion and that NSF likely mediates miR-33-dependent changes in VLDL secretion.

NSF Overexpression Enhances TAG and APOB Secretion and Rescues the Effect of miR-33

To complement the knock-down studies shown above, we next performed overexpression experiments in which primary hepatocytes were transduced with either an empty adenovirus or an adenovirus encoding murine NSF. The next day, cells were washed and incubated in fresh media for an additional 24 hours without supplementation of fatty acids. Our data show that MTTP protein levels did not change in cells overexpressing NSF, compared with control cells (Figure 6A), despite the significant increase in mRNA levels (Online Figure IVB). Meanwhile, the levels of APOB protein, but not mRNA, were slightly increased in the same cells, compared with controls (Figure 6A; Online Figure IVB). On the other hand, supernatants from cells transduced with the empty adenovirus contained little APOB48/100 as determined by Western blot (Figure 6B), as expected. In contrast, supernatants from cells transduced with the NSF adenovirus contained increased amounts of APOB100/48 and APOA1. These results are consistent with those from knock-down experiments above and strongly suggest that NSF activity enhances lipoprotein secretion. Importantly, manipulation of NSF levels with either small interfering RNA or adenovirus did not result in significant cell toxicity or death, as measured...
by lactate dehydrogenase activity released to the supernatant, ability to cleave 3(4,5-dimethylthiazol-2-yl)2,5-diphenyltetrazolium bromide (MTT), activation of caspases 3 and 7, or Tnf-α expression (Online Figure IVC).

Next, to validate the role of NSF on hepatic VLDL secretion in vivo, we transduced male C57BL/6 mice (n = 5) with empty or NSF adenoviral vectors, via tail vein injection. After 7 days, the animals were fasted overnight, euthanized, and liver and plasma samples collected. Using this approach, NSF was efficiently overexpressed in mice receiving Adeno-Nsf, compared with controls (Figure 6C and 6D). Similar to data from primary hepatocytes, increased NSF levels were not paralleled by significant changes in the abundance of hepatic MTTP and APOB (Figure 6D), although Mttp levels were slightly increased in the same livers (Figure 6C). Importantly, plasma TAG levels, but not cholesterol, were significantly elevated in mice overexpressing NSF, compared with controls (Figure 6E), which translated into a ≈30% increased hepatic secretion rate (Figure 6I). An additional experiment in a third cohort of mice (n = 6) transduced with adenoviral vectors and used for tyloxapol experiments resulted in virtually identical results (Online Figure VA and VB). Taken together, our studies both in primary hepatocytes and in vivo strongly suggest that changes in hepatic NSF expression alter VLDL-TAG secretion.

Finally, we tested whether NSF could rescue the miR-33-dependent decrease in TAG and APOB secretion. Primary hepatocytes were transduced with or without adenovirus encoding miR-33 and Nsf in media supplemented with 0.8 mmol/L oleate and in the absence (A–C) or presence (D–G) of 1 μCi/mL [14C]-oleate, and then chased in fresh media for 24 hours. A, Relative mRNA levels of Nsf. Data are mean±SEM; **P ≤ 0.01, ***P ≤ 0.001. B, Immunoblots for selected intracellular proteins. C, Immunoblots for apolipoprotein B and apolipoprotein A1 in supernatants from the same cells. D, Intracellular lipids were extracted from pulsed/chased cells and resolved by thin layer chromatography. Labeled lipids visualized with a PhosphorImager. E, Spots above were scraped from the silica plate, and radioactivity determined by scintillation and normalized to intracellular protein. Data are mean±SEM. **P ≤ 0.01. F, Lipids were extracted from the supernatants of the same cells and resolved by thin layer chromatography as above. G, Spots were scraped from the silica plate, and radioactivity determined by scintillation and normalized to intracellular protein. Data are mean±SEM. APOA1 indicates apolipoprotein A1; APOB, apolipoprotein B; and MTTP, microsomal triglyceride transfer protein.
oleate, then pulsed with [14C]-oleate, and chased for 24 hours in fresh medium. As expected, the secretion of APOB was higher in these cells, compared with those cultured in media without oleate supplementation (Figure 7B versus Figure 6B). Nevertheless, data in Figure 7A–7D show that the secretion of both APOB and labeled TAG was decreased by miR-33 and increased by NSF, respectively. Importantly, NSF overexpression reverted the effects of miR-33 on both APOB and TAG secretion (Figure 7B and 7C) without altering the incorporation of [14C] into intracellular lipid pools (Online Figure VI), suggesting that NSF indeed mediates miR-33-dependent changes in VLDL secretion.

The miR-33–NSF Axis Controls the Overall Secretory Pathway in Primary Hepatocytes

Because NSF is a universal ATPase required for the resolution of all cis-SNARE complexes, we next hypothesized that changes in the levels of miR-33 or NSF should also alter the global pattern of secretion from the hepatocyte, which may account for the consistent changes in APOA1 secretion noted in previous figures. To test this proposal, we performed a [35S]-Met/Cys pulse-chase experiment in primary mouse hepatocytes transduced with empty, miR-33, or NSF adenoviral vectors. Similar specific activities (ie, dpm/μg protein) were recorded for intraacellular protein extracts (Figure 7E), consistent with no changes in protein synthesis among the different treatments. However, the amounts of labeled proteins recovered from the supernatants were significantly decreased in cells overexpressing miR-33, and increased in cells overexpressing Nsf, compared with control cells (Figure 7F). Together, these data are consistent with an overall effect of miR-33 and NSF on secretion.

We next tested whether NSF could rescue the miR-33-dependent decrease in global secretion. Figure 7G provides a proof-of-concept experiment in human HuH7 hepatoma cells that were cotransfected with both intracellular (LUC) and constitutively secreted (GLUC) luciferase vectors in the presence or absence of expression vectors for miR-33 and NSF. The GLUC and LUC activities were recorded in supernatants and cell extracts, respectively, over time (see Online Detailed Methods). Data show that miR-33 significantly decreased the secretion of GLUC, compared with basal conditions. Not only did NSF raise basal GLUC secretion but it also reversed the effect of miR-33 (Figure 7G). Importantly, the changes in normalized media GLUC activity cannot be explained by changes in Gluc expression because similar mRNA levels were noted among the different treatments (Figure 7G, insert). These in vitro results provide decisive evidence for our hypothesis that the miR-33–NSF axis regulates the overall secretory pathway in hepatocytes from both mice and humans.

Based on these data, we reasoned that global changes in hepatic secretion should also be observed in vivo after manipulation of miR-33 or NSF. Hence, we checked the levels of several proteins associated (APOB, APOA1, APOE, LCAT) or not (ALBUMIN, TRANSFERRIN, C3A) with lipoprotein metabolism in plasma samples from mice treated with saline, scrambled, or miR-33 oligonucleotides (Figure 8A) or mice transduced with empty or NSF adeno viral vectors (Figure 8B). In agreement with our hypothesis, samples from both anti-miR-33- and Adeno-Nsf-treated mice showed higher titers for most, but not all, of the proteins studied. On the contrary, immunoglobulin G, which is not secreted from the liver, remained unchanged among groups. A potential limitation of these in
vivo data is that circulating levels of proteins are likely affected by a multiplicity of factors besides secretion, which include uptake in peripheral tissues, degradation, and renal clearance, and thus, plasma levels may not directly reflect changes in hepatic output. To avoid this limitation, we performed new tyloxapol experiments in mice simultaneously injected with [35S]-Met/Cys, thus allowing us to study the appearance in circulation of newly synthesized hepatic proteins. Data in Figure 8C and 8D and 8H and 8I show that either anti-miR-33 treatment or adenoviral-mediated NSF overexpression resulted, again, in accelerated TAG accumulation in plasma, compared with control animals. Such rise in VLDL secretion was paralleled by the increased abundance of plasma [35S]-labeled APOB, APOA1, and ALBUMIN in the same mice, compared with controls (Figure 8E–8G and 8J–8L). Taken together, data in Figure 8 support our proposal that miR-33 regulates global secretion in the hepatocyte by targeting NSF.

Discussion

Impaired lipid homeostasis is generally recognized as a risk factor for the development of atherosclerosis, the primary cause of heart attack and stroke, which collectively account for ≈32% of all deaths in the United States.22 The vast majority of dyslipidemic patients are prescribed statins, which act as competitive inhibitors of 3-hydroxy-3-methylglutaryl-CoA reductase, the rate-limiting enzyme in cholesterologenesis. The therapeutic benefit of statins is due, at least in part, to the increased maturation of SREBP-2, which then promotes the expression of the low-density lipoprotein receptor and thus the accelerated clearance of proatherogenic lipoproteins from circulation. Importantly, SREBP-2 levels are also induced by statins because SREBP-2 transactivates its own promoter. We and others showed that conditions that promote SREBP-2 maturation (such as reduced intracellular contents or treatment with statins) also raise the levels of its intragenic miR-33.8–10 Therefore, from a clinical perspective, it may be important that statins induce the expression of miR-33. Our data suggest that miR-33/NSF-dependent changes in VLDL secretion, together with the accelerated uptake of APOB-containing lipoproteins via the low-density lipoprotein receptor, may help explain the significant drop in plasma TAG noted in patients and animal models taking statins.23,24 Studies in patients using stable isotopes and radiolabeled lipoproteins to measure hepatic APOB secretion in response to statins have been mixed, though: some authors reported decreased APOB secretion25–29 while others found no changes.30–33

However, caution must be taken when translating the results from anti-miR-33 studies from mice to humans. Primates, but...
not rodents, express a second copy of miR-33 from within an intron of SREBP-1. MiR-33a (cotranscribed with SREBP-2) and miR-33b (cotranscribed with SREBP-1) share the same seed sequence but differ in 2 nucleotides in the 3′ region. Whether these 2 nucleotides confer target specificity remains to be determined. Nonetheless, Rayner et al. showed that an oligonucleotide that is 100% complementary to miR-33a effectively reduces the functional levels of both miR-33a and miR-33b.

Importantly, the transcription of SREBP-2 and SREBP-1 is controlled by different nutritional and hormonal stimuli, and only SREBP-2 responds to statins. Consequently, it is likely that murine studies are missing certain regulatory circuits controlled by miR-33b in the primate liver, particularly under lipogenic conditions where SREBP-1 is induced. A role for miR-33 on FFA and TAG metabolism was first proposed by Gerin et al., and later confirmed by Dávalos et al., when...
they showed that genes encoding enzymes involved in fatty acid β-oxidation (CPT1A, HADHD, and CROT) and insulin signaling (IRS2, PRKAA1, and SIRT6) are functional targets of miR-33 in primates, rodents, and flies. From these studies, it was inferred that miR-33 might function to limit fatty acid synthesis and utilization in the hepatocyte. More recently, the gluconeogenic genes phosphoenolpyruvate carboxykinase 1 and glucose-6-phosphatase, catalytic subunit have also been reported as functional targets of miR-33a/b.37 Collectively, these studies show miR-33 as a regulatory hub for multiple intracellular metabolic processes, coordinating the expression of genes involved in sterol, fatty acid, and glucose homeostasis.

The potential therapeutic use of anti-miR-33 oligonucleotides in patients at risk of developing cardiovascular disease was proposed following murine studies that showed increased circulating HDL cholesterol,8–10 bile secretion,13 and mobilization of sterols through the reverse cholesterol transport pathway,13,14 compared with control oligonucleotides. The impact of ablation and reduction of miR-33 levels on atherogenesis has been evaluated in several studies using atherosclerosis-prone mouse models. Rayner et al15 showed that a 4-week treatment with 2′-fluoro/methoxyethyl anti-miR-33 oligonucleotides after 14 weeks of Western diet feeding increased plasma HDL cholesterol and accelerated the regression of atheromatous in Ldlr+/− mice. In contrast, using locked nucleic acid oligonucleotides, we16 showed that anti-miR-33 therapy failed to sustain elevated plasma HDL cholesterol levels for the course of 12 weeks, raised the levels of plasma VLDL-TAG, and did not prevent the progression of atherosclerosis in Western diet–fed Ldlr+/− mice. Finally, Rotllan et al18 also reported a progression study in which 2′-fluoro/methoxyethyl anti-miR-33 oligonucleotides did not sustain elevated plasma HDL cholesterol, yet they led to a significant reduction in atheroma without changes in plasma TAG in the same mouse model. The reasons behind the different outcomes among these studies remain to be elucidated, but it is possible that different bioavailability and potency of 2′-fluoro/methoxyethyl and locked nucleic acid oligonucleotides, length of treatment, or interactions with dietary components might explain the discrepancies in HDL, VLDL, and atherogenesis. Finally, ApoE+/−/miR-33+/− mice, but not ApoE−/− mice transplanted with miR-33−/− bone marrow, had decreased atherosclerotic lesions compared with ApoE−/− mice.38 These latter studies imply that increased hepatic HDL biogenesis (or perhaps bile secretion), rather than accelerated macrophage cholesterol efflux, could mediate atheroprotection following whole-body loss of miR-33. However, and paradoxically, the 2 progression studies that used antisense oligonucleotides in Ldlr+/− mice reported no changes in plasma HDL cholesterol among the different treatments, despite elevated hepatic ABCA1 expression in anti-miR-33-treated mice.18,19 Furthermore, while this article was under revision, Horie et al39 reported that miR-33−/− mice developed hepatosteatosis when fed a high-fat diet, but not regular chow, compared with wild-type animals. These authors reasoned that de novo lipogenesis was induced in the knock-out mice via direct derepression of Srebp-1, which was presented as a direct miR-33 target. However, the expression of key lipogenic genes (ie, Fasn, Acc1) was identical in chow-fed wild-type and miR-33−/− mice, complicating the interpretation of the data. In contrast to this latter study, herein we report no changes in the lipogenic ability of mouse primary hepatocytes after miR-33 overexpression. We had also reported that the potential miR-33 sequences present in the 3′-UTR of the mouse Srebp-1 did not confer responsiveness to the miRNA in our hands,13 and Srebp1c and Srebp1a were not enriched in our RISC pull-downs after miR-33 overexpression (data not shown). Incidentally, Horie et al30 did not note changes in plasma TAG in miR-33−/−, compared with wild-type animals, although the values reported were abnormally low and, surprisingly, decreased in high-fat diet versus chow (=20 versus =35 mg/dL, respectively). The reasons behind the discrepancies between the sustained miR-33 silencing (via oligonucleotides) and the miR-33−/− models regarding plasma TAG and HDL cholesterol remain obscure. Besides potential differences in the genetic backgrounds between these animals, a critical distinction is that treatment with antisense oligonucleotides results in only the partial derepression of hepatic miR-33 targets, whereas the expression of miR-33 and miR-33 targets remains unchanged in metabolically relevant extrahepatic tissues (ie, skeletal muscle, heart, adipose, and intestine); conversely, the knock-out model leads to the abolishment of miR-33 and the complete derepression of miR-33 targets in all tissues. The relative contribution of extrahepatic miR-33 to whole-body lipid homeostasis remains to be determined, but it is conceivable that the anti-miR-33 treatment can only partially recapitulate the effects of genetic loss of miR-33. In any case, whether anti-miR-33 therapy will be effective to manage dyslipidemic patients remains an open question that will need further validation.

Rayner et al15 and Rottiers et al16 reported 2 independent studies in African green monkeys that showed the expected raise in HDL cholesterol following anti-miR-33 therapy with either no change in plasma TAG16 or lowered VLDL-TAG,19 compared with vehicle or control oligonucleotides, respectively. The analysis of VLDL metabolism in these and other non-human primates, however, is complicated by the fact that the levels of plasma TAG are only 15% to 30% of those in humans,34,40–42 likely because of the efficient clearance from circulation by lipoprotein lipase.40,41 Indeed, the efficiency of clearance is so high in these animals that interventions that alter hepatic VLDL secretion are difficult to detect by simply measuring plasma concentrations of TAG.41 In the study by Rayner et al,15 VLDL-TAG represented ≈25% of the total TAG in chow-fed monkeys, independent of treatment, consistent with a rapid VLDL turnover in these animals. Over time, VLDL-TAG levels increased in their control oligonucleotide group (both on chow and after switching to a moderate-cholesterol, high-carbohydrate diet), whereas they remained flat in the anti-miR-33 group. This was interpreted as a lowering effect of the anti-miR, compared with the control oligonucleotides. An alternative interpretation is that the control oligonucleotides raised VLDL-TAG, compared with the anti-miR treatment. The absence of a third group of animals injected with saline precludes distinguishing between those 2 possibilities. However, whether the Rayner control oligonucleotide alters the hepatic or peripheral expression of genes involved in VLDL-TAG metabolism or clearance is unknown. On the contrary, the study by Rottiers et al16 presents data from animals fed a high-fat diet and then switched to a high-carbohydrate diet, but no TAG lipoprotein distribution is
shown. In any case, additional studies will be necessary to establish a role for miR-33 on hepatic VLDL-TAG secretion and clearance in primates.

The assembly of secretion-competent VLDL particles was originally regarded as a 2-step process ignited by the MTTP-dependent cotranslational lipidation of APOB as it is translocated across the ER membrane, then followed by addition of lipid droplet-derived bulk TAG within the ER and Golgi lumen. Recent studies in patients with inherited dyslipidemias and in mouse models have identified several additional factors involved in VLDL assembly, maturation, trafficking, degradation, and secretion.\(^\text{1,2,44-46}\) The exact molecular mechanisms that control the ultimate fate of nascent VLDL-containing vesicles (secretion versus degradation) in response to different nutritional and hormonal stimuli are not completely understood, but it is likely that these vesicles are decorated with distinct proteins that define their proper intracellular trafficking.\(^\text{47,48}\) As mentioned in the introductory text, specific v-SNARE (SEC22B) and t-SNAREs (STX5, BET1, and GOSR1) are required for the mobilization of APOB-containing cargo from ER to Golgi but not for other ER-derived secretory vesicles.\(^\text{3,4}\) Interestingly, SEC22B has been reported to bind directly to APOB100.\(^\text{49}\) NSF activity is required for the resolution of the SNARE complexes formed before and or during vesicular membrane fusion, thus allowing the recycling of v- and t-SNAREs.\(^\text{6}\) It is then perhaps not surprising that altered NSF expression results in changes in APOB and TAG secretion, as described in this report. It is unclear which specific steps during VLDL trafficking are directly impacted by NSF: ER to Golgi, fusion with lipid droplets, mobilization through the Golgi, or fusion with plasma membrane and release of the cargo. Because all these steps are SNARE dependent, it is tempting to speculate that NSF likely modulates all trafficking aspects of intracellular VLDL mobilization. Additional studies should provide clues into the consequences of deregulated NSF expression on the intracellular fate of nascent VLDL and whether small molecules that alter NSF activity might be useful to control VLDL secretion and manage dyslipidemic patients. In a broader sense, the data presented herein suggest that long-term therapeutic silencing of miR-33 may result in the profound deregulation of hepatic secretion. Nevertheless, such an increase in not only proatherogenic VLDL but also other liver-derived circulating proteins (eg, coagulation factors) might result in unanticipated side effects. Hence, caution should be taken with the implementation of potential anti-miR-33-based therapies.

Acknowledgment

We thank Erin Touche for outstanding technical support with in vivo experiments.

Disclosures

R.M. Allen received support from American Heart Association Predoctoral Fellowship (11PRE7240026). Á. Baldán received support from National Institutes of Health (grant HL107794). R.M. Allen, T.J. Marquart, and Á. Baldán are pursuing a patent related to miR-33. The other author reports no conflicts.

References

4. Siddiqui SA. VLDL exits from the endoplasmic reticulum in a specialized vesicle, the VLDL transport vesicle, in rat primary hepatocytes. Biochem J. 2008;413:333–342.
5. Gusarova V, Brodsky JL, Fisher EA. Apolipoprotein B100 exit from the endoplasmic reticulum (ER) is COPII-dependent, and its lipidation to very low density lipoprotein occurs post-ER. J Biol Chem. 2003;278:48051–48058.
The effects of anti-miR-33 therapy on atherogenesis in mice are
• Short-term anti-miR-33 therapy increases plasma high-density lipo-
  atheroprotective. However, conflicting data have been reported
Several studies suggested that anti-miR-33 therapy might be
atheroprotective. However, conflicting data have been reported
on the impact of anti-miR-33 oligonucleotides on plasma triglyc-
erides (TAG). Here, we show that miR-33 controls the hepatic
secretory pathway via N-ethylmaleimide-sensitive factor. Thus,
manipulation of either miR-33 or N-ethylmaleimide-sensitive
factor levels in both primary hepatocytes and in vivo results in
profound changes in the secretion of not only very low-density
lipoprotein-TAG but also a plethora of liver-derived circulating
proteins. In a general sense, our results reveal the risk of unde-
sired effects after miRNA therapeutic antagonism and highlight
the need for comprehensive characterization and understanding
of physiological miRNA targets. In the case of miR-33, in vivo
antagonism results in the derepression of both antithromogenic
pathways (previous reports) and proatherogenic pathways (this
report). Additional studies will be necessary to establish whether
miR-33 is a safe therapeutic target to manage cardiovascular
disease in patients.
Control of Very Low-Density Lipoprotein Secretion by N-Ethylmaleimide-Sensitive Factor and miR-33
Ryan M. Allen, Tyler J. Marquart, Jordan J. Jesse and Ángel Baldán

_Circ Res._ 2014;115:10-22; originally published online April 21, 2014; doi: 10.1161/CIRCRESAHA.115.303100

_Circulation Research_ is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2014 American Heart Association, Inc. All rights reserved.
Print ISSN: 0009-7330. Online ISSN: 1524-4571

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circres.ahajournals.org/content/115/1/10

Data Supplement (unedited) at:
http://circres.ahajournals.org/content/suppl/2014/04/21/CIRCRESAHA.115.303100.DC1

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in _Circulation Research_ can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to _Circulation Research_ is online at:
http://circres.ahajournals.org//subscriptions/
SUPPLEMENTAL MATERIAL

Allen et al.
Control of very-low density lipoprotein secretion
by N-ethylmaleimide-sensitive factor and miR-33

Detailed Methods

Cells were isolated using Perfusion and Digest buffers from Invitrogen. Cells were resuspended in William’s E Medium (Invitrogen) supplemented with Plating Supplements (Invitrogen), seeded in 24-, 12- or 6-well BioCoat Collagen I plates (BD), and incubated at 37°C and 5% CO$_2$. After 6 h, the media was switched to William’s E supplemented with Maintenance Supplements (Invitrogen). Where indicated, cells were transduced in Maintenance Medium with Adeno-empty, Adeno-miR-33, or Adeno-NSF adenovirus (MOI=3), or transfected with non-targeting (D-001810-10-05) or anti-Nsf (L-058797-00-0005) siRNA oligonucleotides (Dharmacon) at the indicated doses using Dharmafect 1 reagent (Dharmacon). Where indicated, cells were incubated overnight in maintenance media containing 0.8 mM oleate (conjugated to BSA) with or without 1 $\mu$Ci/mL $[^{14}C]$-oleate. The following morning cells were washed three times with warm PBS, and incubated in fresh maintenance medium for up to 24 h. Lipid extracts were obtained from both the supernatants and cells by the Bligh & Dyer method$^1$, and the different classes of lipids were resolved by thin layer chromatography$^2$; specific bands were scraped from the silica glass and the amount of radioactivity determined by scintillation, and normalized to intracellular protein contents. Metabolic labeling of proteins was performed by incubating the cells in Met/Cys-deficient DMEM for 1 h, pulsing in media supplemented with 200 $\mu$Ci/mL $[^{35}S]$-Met/Cys for 30 min, and chasing in maintenance media for 3 h. Protein extracts were concentrated with centrifugal columns (Amicon), resolved by SDS-PAGE, dried, and analyzed with a PhosphorImager (GE Healthcare). De novo lipogenesis was assessed by pulsing cells 6 hours in media supplemented with 2 $\mu$Ci/mL $[^{14}C]$-acetate. The incorporation of $[^{14}C]$ label into different classes of lipids was measured in whole cell lipid extracts as above, and normalized to cell protein contents. Fatty acid $\beta$-oxidation was assayed in cells pulsed 2 h in media containing 125 $\mu$mol/L palmitate (conjugated to 2.5 mg/mL BSA) and 10 $\mu$Ci/mL $[^{3}H]$-palmitate and 1 mmol/L L-carnitine, as described$^3$. Media protein was then precipitated with 10% trichloroacetic acid and $[^{3}H]$-water was purified by ion exchange chromatography and counted by scintillation.
**Plasma Lipid Analysis**

Plasma samples were collected after an overnight fast by superficial temporal vein bleeds prior to the first oligonucleotide injection, one week after the first injection, and every two weeks until animals were sacrificed. Total cholesterol and triglyceride contents were assayed enzymatically using Cholesterol E and Triacylglyceride kits (Wako Chemicals). FPLC lipoprotein profiles from plasma samples were determined by a modified Column Lipoprotein Profile (CLiP) method. Briefly, plasma samples were pooled, diluted in saline (1:5 for cholesterol, 1:2 for triglycerides), and auto-injected (10 μL for cholesterol, 40 μL for triglycerides) into a Superose-6 column (GE Healthcare) using elution buffer (saline/2mmol/L EDTA/0.01% sodium azide [pH = 7.4]) at a flow rate of 0.6 mL/min at 40°C. The FPLC eluate was immediately mixed with cholesterol or triglycerides reagent (Thermo Scientific), and incubated at 40°C in a 5 m KOT coiled reactor. The final mixture entered a capillary spectrophotometric detector at 0.3 mL/min set at 500 nm, and the profiles were collected in real time using LC Solution software (Shimadzu).

**Hepatic and Primary Hepatocyte Lipid Contents Analysis**

Tissue lipids were extracted into chloroform by a modified Folch method and resolubilized in water, as described. Specific lipid classes were quantitated using enzymatic kits for total and free cholesterol, triglyceride, phosphatidylcholine, and non-esterified fatty acids (Wako Chemicals). Primary hepatocytes were scraped in 0.5 mL of cold saline and transferred to a glass tube before lipid extraction as above. Hepatocyte or supernatant lipid extracts were resuspended in 100 μL chloroform, and 20 μL were spotted on thin layer chromatography (TLC) plates (Whatman 4865-821), together with true standards for each class of lipids (Sigma), and resolved as described. Radioactive lipids were visualized with a PhosphorImager. Radioactive spots were scrapped out of the glass with a razor blade and quantified by scintillation.

**In vivo VLDL-TAG Secretion Assays**

After an overnight fast, mice were pre-bled via temporal vein puncture and injected i.v. with 500 mg/Kg Tyloxapol (Sigma) in 100 μL saline. Sequential bleedings (< 100 μL each) were performed 60, 120, and 180 min after tyloxapol injection. VLDL secretion rate was calculated as mg of TAG secreted per Kg of body weight per hour, as described. Identical TAG secretion rates to those shown in Figs. 2A and 6H were obtained in mice fasted only for 4 h, from 8 am until noon (data not shown). Where indicated, mice were fasted 4 h (8 am to noon) and then injected with 500 mg/Kg tyloxapol and 500 μCi [35S]-Met/Cys, in 100 μL saline. The relative plasma amounts of specific newly synthesized hepatic proteins was assessed by immunoprecipitating 10 μL of plasma with specific antibodies followed by autoradiography, as described.
RNA Isolation and Quantitative Real Time PCR Analysis
RNA was isolated from cells and livers with Trizol (Invitrogen). Complimentary DNAs (cDNAs) were generated from 1 μg of DNase1-treated RNA using Multi-Scribe Reverse Transcriptase (Applied Biosystems) and random hexamers. Real-time quantitative PCR was done using Power SYBR Green reagent (Applied Biosystems) in a LightCycler-480 (Roche). Primer sets are available upon request. Values were normalized to 36B4 and calculated using the comparative ΔΔCt method.

Protein Extraction and Immunoblotting
Protein contents from liver or cells were extracted in 150 mmol/L NaCl, 1% NP-40, 0.1% SDS, 100 mmol/L Tris-HCl, pH 7.4, supplemented with protease inhibitors (Roche) and cleared by centrifugation at 4°C for 10 min at 10,000×g. Thirty micrograms of protein or 10 μL of 1:100 diluted plasma were resolved in either 4–12% Bis-Tris or 3–8% Tris-Acetate gels (Invitrogen), transferred to PVDF membranes, and probed with different primary and secondary antibodies (Online Table II) in TBS-Tween20 containing 4% non-fat dry milk. Immune complexes were detected with SuperSignal West Pico chemiluminescent substrate (Pierce).

NSF Plasmids and Production of Adenovirus
pCMV-Sport6-mNSF plasmid was purchased from OpenBiosystems (MM1013-202763641). This plasmid was then digested with NheI, to remove the distal section of the NSF 3'UTR containing miR-33 response elements and re-ligated. The resulting NSF transcript containing a truncated 3'UTR was then sub-cloned into pAdTrack-CMV (Stratagene). The pAdTrack-NSF vector was then electroporated into pAdEasy-1 cells to generate the final adenoviral vectors, as described. The final vector was transfected into HEK293ad cells (Stratagene) to produce and amplify adenoviral particles. Replication-deficient adenovirus vectors were then purified by CsCl gradient ultracentrifugation.

Immunoprecipitation of RISC-complexes from primary mouse hepatocytes
RISC-complexes were immunoprecipitated from primary mouse hepatocytes transduced with Adeno-empty or Adeno-miR-33 using an AGO2 antibody, as described. Briefly, 48h after transduction cell proteins were extracted in 150 mmol/L NaCl, 1% NP-40, 0.1% SDS, 100 mmol/L Tris-HCl, pH 7.4, supplemented with protease inhibitors (Roche), 1 mg/mL yeast tRNA (Invitrogen), and 1 U/μL SUPERnase-IN (Ambion), and cleared by centrifugation at 4°C for 10 min at 10,000×g. Extracts were then added to 50 μL of protein G-coupled Dynabeads (Invitrogen) that had been pre-bound to 5 μg of anti-AGO2 antibody (Wako 2D4). After 1 hour of rotation, beads were pelleted and washed 4 times. AGO2-bound RNA was extracted in 1 mL of TRIzol (Invitrogen) and used for RT-qPCR.
Firefly Luciferase Reporter Assays
Transient transfection of HEK293 cells was performed in triplicate in 24-well plates using the calcium phosphate method. Luciferase activity was measured 48 h later using the Luciferase Assay System (Promega) and normalized to β-galactosidase activity to correct for changes in transfection efficiency.

Secreted Gaussia Luciferase Assays
HuH7 hepatoma cells were seeded in 24-well plates and transiently transfected with expression vectors for miR-33, Nsf, firefly luciferase (LUC), and Gaussia luciferase (GLUC; a kind gift of Dr. Bakhos Tannous at Harvard Medical School) using Lipofectamine LTX (Invitrogen). Forty-eight hours after transfection, cells were washed and incubated in 2 mL of fresh media (DMEM + 10% FBS). At indicated times, 20 μL of media was taken for detection of GLUC activity using the Renilla Luciferase Assay System (Promega) and normalized to intracellular LUC activity.

Cell Viability Assays
Mouse primary hepatocytes were seeded in collagen-coated 48-well plates and treated as described for 48 h. Cytotoxicity was measured by the release of intracellular lactate dehydrogenase (LDH) into the culture medium using the LDH–Cytotoxicity Assay kit (Cayman). To estimate viability, cells were incubated for 3 h in the presence of MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; Cayman); healthy cells cleave MTT to produce dark blue formazan crystals that are then solubilized with isopropanol and spectrophotometrically quantified. Apoptosis was estimated using the luminescent Caspase-Glo 3/7 assay (Promega).

Statistical Analysis
Data are shown as mean ± SEM. Differences between groups were analyzed by Student’s t-test, or one-way ANOVA and post-hoc Tukey’s test, as appropriate. Statistical significance was set at *P ≤ 0.05 and **P ≤ 0.01.
Online Figure I. Effects of miR-33 antisense treatment on plasma cholesterol, hepatic gene expression, and hepatic lipid contents. Data shown are from the same mice used in Figure 1. (A) Body weight gain among the different groups. (B) Plasma cholesterol levels over time. Data are mean ± SEM. *P ≤ 0.05. (C) Cholesterol lipoprotein profiles at weeks 3 and 11, as determined by FPLC. (D) Relative expression of selected hepatic transcripts in anti-miR-33-treated mice vs. controls, at week 11. Data are mean ± SEM. *P ≤ 0.05. (E) Hepatic lipid contents at week 11. PC, phosphatidylcholine; UC, unesterified cholesterol; EC esterified cholesterol; NEFA, non-esterified cholesterol. Data are mean ± SEM. *P ≤ 0.05.
Online Figure II. Effect of miR-33 overexpression on intracellular lipid utilization. Mouse primary hepatocytes were transduced with an empty adenovirus or an adenovirus encoding miR-33, and incubated in media supplemented with 0.8 mmol/L oleate. (A) Cells were pulsed 6 h with 2 µCi/mL $[^{14}C]$-acetate and the incorporation of the label to specific classes of lipids measured by thin layer chromatography using total lipid extracts. (B) Specific bands were scraped form the silica glass and the radioactive contents determined by scintillation, and normalized to intracellular protein contents. Data are mean ± SEM of 2 independent experiments in triplicate. (C) A separate group of cells were pulsed 2 h with 125 mmol/L palmitate and 10 µCi/mL $[^{3}H]$-palmitate. The oxidation rates for the labeled fatty acid were estimated by determining the abundance of $[^{3}H]$-water in each sample (see Detailed Methods). A set of non-transduced cells were treated with the $\beta$-oxidation inhibitor etomoxir. Data are mean ± SEM of 2 independent experiments in triplicate. (D) The total intracellular contents of TAG and cholesterol were
determined in a separate set of cells. Data are mean ± SEM of 2 independent experiments in triplicate. 

**$P \leq 0.01$.**
Online Figure III. Changes in mRNA expression following manipulation of miR-33. (A) Relative expression of selected transcripts in HuH7 hepatoma cells transduced with empty or miR-33 adenovirus. Data are mean ± SEM of 3 experiments in triplicate. *$P \leq 0.05$; **$P \leq 0.01$. (B) Immunoblots for proteins of interest in cell extracts from HuH7 cells processed in parallel. (C) Relative expression of selected transcripts in the livers of mice dosed weekly with saline, or control or anti-miR-33 oligonucleotides for 11 weeks, as described in Figure 1. Data are mean ± SEM. *$P \leq 0.05$; **$P \leq 0.01$. 
Online Figure IV. Changes in mRNA expression and cell viability in mouse primary hepatocytes following manipulation of NSF. (A) Relative expression of selected transcripts in mouse primary hepatocytes transfected with non-targeting (siNT) or anti-Nsf (siNsf) oligonucleotides, as described for Figure 5. (B) Relative expression of selected transcripts in mouse primary hepatocytes transduced with empty or Nsf adenoviral vectors, as described for Figure 5. (C, D) Manipulation of NSF levels did not result in cytotoxicity nor compromise the viability of the cells, as measured by LDH activity released to the supernatant, ability to cleave MTT, Caspase-3/7 activation, and Tnfα expression. The different assays were performed 60 h after transduction. Where indicated, cells were incubated in the presence of 2 µg/mL tunicamycin for 12 h. (E) Silencing of NSF did not change intracellular levels of triglycerides or cholesterol. Data are mean ± SEM. *P ≤ 0.05
Online Figure V. Accelerated hepatic VLDL-TAG secretion in mice overexpressing NSF. (A) Kinetics of plasma TAG accumulation in a cohort of C57BL/6 mice (n=6/group) different from that shown in Figure 6H, 7 days after transduction with empty of Nsf adenovirus, and injection i.v. with tyloxapol as described for Figures 2 and 6. Data are mean ± SEM. *P ≤ 0.05. (B) Hepatic TAG secretion rates calculated from the data above. Data are mean ± SEM. *P ≤ 0.05
Online Figure VI. NSF Rescues miR-33–dependent Changes in Secretion without Altering Intracellular Lipid Pools. Incorporation of \(^{14}\text{C}\)-oleate into different classes of intracellular lipids in mouse primary hepatocytes transduced with empty, Nsf, miR-33, or miR-33 plus Nsf adenoviral vectors. These data complement those shown in Figure 7C, D. (A) Lipid extracts were resolved by thin layer chromatography, and exposed to a PhosphorImager screen. (B) Specific bands were scraped out of the silica glass and the radioactive contents determined by scintillation. Data are mean and SEM.
### Online Table I. Animal cohorts used in this study.

<table>
<thead>
<tr>
<th>Cohort</th>
<th>n</th>
<th>Duration</th>
<th>Figures</th>
</tr>
</thead>
<tbody>
<tr>
<td>saline vs. control vs. anti-miR-33, #1</td>
<td>5, 7, 7</td>
<td>11 w</td>
<td>1, 4F, S1</td>
</tr>
<tr>
<td>saline vs. control vs. anti-miR-33, #2</td>
<td>6, 6, 6</td>
<td>6 w*, 24 w**</td>
<td>2A*, 2B*, 8A**</td>
</tr>
<tr>
<td>saline vs. control vs. anti-miR-33, #3</td>
<td>6, 6, 6</td>
<td>6 w</td>
<td>8C–G</td>
</tr>
<tr>
<td>Adeno-empty vs. Adeno-Nsf, #1</td>
<td>5, 5</td>
<td>7 d</td>
<td>6C–G</td>
</tr>
<tr>
<td>Adeno-empty vs. Adeno-Nsf, #2</td>
<td>6, 6</td>
<td>7 d</td>
<td>6H, 6I, 8B</td>
</tr>
<tr>
<td>Adeno-empty vs. Adeno-Nsf, #3</td>
<td>6, 6</td>
<td>7 d</td>
<td>S5</td>
</tr>
<tr>
<td>Adeno-empty vs. Adeno-Nsf, #4</td>
<td>6, 6</td>
<td>7 d</td>
<td>8H–L</td>
</tr>
</tbody>
</table>

### Online Table II. Antibodies used in this study.

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Provider</th>
<th>Catalog #</th>
<th>Dilution</th>
</tr>
</thead>
<tbody>
<tr>
<td>ABCA1</td>
<td>Novus</td>
<td>NB400-105</td>
<td>1:1,000</td>
</tr>
<tr>
<td>β-Actin</td>
<td>SCBT</td>
<td>sc-130656</td>
<td>1:3,000</td>
</tr>
<tr>
<td>APOA1</td>
<td>Meridian</td>
<td>K23500R</td>
<td>1:5,000</td>
</tr>
<tr>
<td>APOB</td>
<td>Meridian</td>
<td>K23300R</td>
<td>1:2,000</td>
</tr>
<tr>
<td>APOE</td>
<td>Meridian</td>
<td>K23100R</td>
<td>1:2,000</td>
</tr>
<tr>
<td>ALBUMIN</td>
<td>Bethyl</td>
<td>A90-134A</td>
<td>1:30,000</td>
</tr>
<tr>
<td>CASPASE 3</td>
<td>Pierce</td>
<td>PA5-16335</td>
<td>1:500</td>
</tr>
<tr>
<td>Complement C3</td>
<td>MP Biomed</td>
<td>55444</td>
<td>1:500</td>
</tr>
<tr>
<td>CPT1A</td>
<td>Proteintech</td>
<td>15184-1-AP</td>
<td>1:2,000</td>
</tr>
<tr>
<td>FASN</td>
<td>Thermo</td>
<td>MA5-14887</td>
<td>1:1,000</td>
</tr>
<tr>
<td>GOSR1</td>
<td>Pierce</td>
<td>MA1-91008</td>
<td>1:1,000</td>
</tr>
<tr>
<td>LCAT</td>
<td>Abcam</td>
<td>ab109417</td>
<td>1:500</td>
</tr>
<tr>
<td>MTTP (human)</td>
<td>SCBT</td>
<td>sc-33116</td>
<td>1:1,000</td>
</tr>
<tr>
<td>MTTP (mouse)</td>
<td>BD Biosci</td>
<td>612022</td>
<td>1:5,000</td>
</tr>
<tr>
<td>NSF</td>
<td>BD Biosci</td>
<td>612272</td>
<td>1:10,000</td>
</tr>
<tr>
<td>PLIN2</td>
<td>Abcam</td>
<td>ab37516</td>
<td>1:1,000</td>
</tr>
<tr>
<td>SEC22B</td>
<td>Pierce</td>
<td>OSS00040W</td>
<td>1:500</td>
</tr>
<tr>
<td>α-SNAP</td>
<td>Pierce</td>
<td>PA5-21782</td>
<td>1:500</td>
</tr>
<tr>
<td>STX5</td>
<td>Pierce</td>
<td>PA5-22359</td>
<td>1:1,000</td>
</tr>
<tr>
<td>TRANSFERRIN</td>
<td>Bethyl</td>
<td>A90-129A</td>
<td>1:5,000</td>
</tr>
<tr>
<td>α-TUBULIN</td>
<td>Sigma</td>
<td>T5168</td>
<td>1:1,000</td>
</tr>
<tr>
<td>[goat IgG]-HRP</td>
<td>SCBT</td>
<td>sc-2352</td>
<td>1:5,000</td>
</tr>
<tr>
<td>[mouse IgG]-HRP</td>
<td>SCBT</td>
<td>sc-2318</td>
<td>1:10,000</td>
</tr>
<tr>
<td>[rabbit IgG]-HRP</td>
<td>SCBT</td>
<td>sc-2749</td>
<td>1:20,000</td>
</tr>
</tbody>
</table>


