The Nuclear Receptor FXR Uncouples the Actions of miR-33 From SREBP-2

Elizabeth J. Tarling, Hannah Ahn, Thomas Q. de Aguiar Vallim

Objective.—To determine whether activation of farnesoid X receptor (FXR) alters cellular and plasma cholesterol homeostasis as a result of regulation of Srebp-2 and miR-33.

Approach and Results.—Chromatin immunoprecipitation sequencing data identified an FXR response element within intron 10 of the Srebp-2 gene. Consistent with this observation, treatment of mice with FXR-specific agonists (GSK2324 or GW4064) rapidly increased hepatic levels of Srebp-2 mRNA, precursor sterol response element binding protein 2 (pSREBP-2) protein, and miR-33. Furthermore, miR-33 targets, that include ABCA1 (ATP binding cassette transporter A1), NSF (N-ethylmaleimide-sensitive factor), and CPT1 (carnitine palmitoyltransferase 1), were all reduced in GSK2324-treated mice. In contrast, neither nuclear SREBP-2 protein (nSREBP-2) nor SREBP-2 target genes were induced after FXR activation. The inability to process pSREBP-2 to nSREBP-2 is likely a consequence of the induction of insulin INSIG-2A (induced gene 2A) by FXR agonists. Finally, we show that FXR-dependent induction of both Srebp-2 and miR-33 is ablated in Scap−/− mice that lack nuclear SREBP-2.

Conclusions.—We demonstrate that the activation of FXR uncouples the expression of nuclear SREBP-2 and miR-33, and the regulation of their respective target genes. Further, we conclude that the FXR agonist-dependent increase in miR-33 requires transcription of the Srebp-2 gene. (Arterioscler Thromb Vasc Biol. 2015;35:787-795. DOI: 10.1161/ATVBAHA.114.304179.)

Key Words: cholesterol ■ FXR ■ miR-33, mouse ■ Nr1h4 protein, mouse ■ sterol regulatory element binding protein 2

Sterol response element binding protein 2 (SREBP-2) is a master regulator of genes involved in both cholesterol synthesis and low-density lipoprotein endocytosis.1,2 The transcriptional regulation of the Srebp-2 gene and subsequent processing of precursor SREBP-2 (pSREBP-2) to generate mature nuclear, transcriptionally active SREBP-2 (nSREBP-2) is controlled at multiple levels by cellular sterols.1 Briefly, the precursor form of SREBP-2 contains 2 transmembrane domains that lead to its localization in the endoplasmic reticulum (ER) where it forms a complex with SREBP cleavage activated protein (SCAP) and insulin induced gene 2 (INSIG-2), a family of resident ER proteins. Decreased sterol content of the ER results in dissociation of INSIG from the pSREBP-2:SCAP complex and translocation of the latter to the Golgi. Two proteases cleave pSREBP-2 in the Golgi to release the soluble amino-terminal fragment of the protein (nSREBP-2). nSREBP-2 translocates to the nucleus, where it binds to sterol response elements (SREs) in target genes, resulting in transcriptional activation of these SRE-containing genes. Target genes include those encoding enzymes of cholesterol biosynthesis, the low-density lipoprotein receptor that facilitates endocytosis of low-density lipoprotein, and Srebp-2 itself.2 Therefore, SREBP-2 protein functions in a feed-forward pathway to maintain sterol homeostasis.1,3 SREBP-2 maturation is repressed when cells accumulate either lanosterol or oxysterols or both. These sterols interact with INSIG and SCAP and prevent the vesicular transport of SCAP:pSREBP-2 to the Golgi, thus attenuating proteolytic cleavage of pSREBP-2.3-5

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MicroRNAs are small (22 nucleotides) RNA molecules that fine-tune gene expression by binding to mRNAs containing complementary sequences to the miRNA seed region resulting in either degradation of the target mRNA or translational arrest.6,7 The net result is a decrease in functional protein.8 Recent studies identified a microRNA, miR-33a, that is localized to intron 16 of the Srebp-2 gene.9-11 Importantly, changes in cellular sterol levels result in similar fold changes in both Srebp-2 and miR-33.9-11 Further, miR-33 was shown to target Abca1 mRNA levels leading to decreases in ATP binding cassette transporter A1 (ABCA1) protein concentrations.9-11 This decrease in ABCA1 protein resulted in decreased efflux of cellular cholesterol to lipid-poor apoproteins and to a corresponding decrease

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plasma, mediated by SREBPs, as well as cholesterol catabolism. Cholesterol catabolism occurs via conversion to bile acids, a pathway that is regulated by the nuclear receptor FXR. A link between these 2 pathways, which work together to maintain cholesterol and bile acid homeostasis, has never been shown at a physiological level. To investigate potential cross talk between FXR and Srebp-2, we investigated whether an FXR response element (FXRE) in the Srebp-2 locus might represent a link between FXR and SREBP-2. Analysis of 2 independent chromatin immunoprecipitation sequencing studies that used antibodies to FXR and mice treated with either vehicle or the FXR agonist GW4064, identified an FXRE in intron 10 of the Srebp-2 gene (Figure 1A). The Srebp-2 locus also contains a microRNA, miR-33, located within intron 16 of the Srebp-2 gene. Importantly, changes in the levels of cellular sterols were shown to result in coordinate regulation of Srebp-2, SREBP-2 target genes and miR-33. To determine whether the FXRE in the Srebp-2 locus is functional and regulates both Srebp-2 and miR-33 expression, we treated wild-type (C57BL/6) mice with a single dose of GSK2324, a potent and specific FXR agonist. Srebp-2 mRNA levels were increased significantly after 2 and 4 hours of GSK2324 treatment (Figure 1B), consistent with direct regulation by FXR. To demonstrate the specificity of the response to FXR agonists, we treated wild-type, Fxr−/− mice or mice that lack hepatic FXR (Fxr L–/– mice) with GSK2324. In contrast, Srebp-2 mRNA levels were similar (1.6-fold) in wild-type treated with GSK2324 or GW4064 and floxed mice treated with GSK2324. In contrast, Srebp-2 induction was not observed in agonist treated whole body, or liver-specific Fxr−/− mice (Figure 1C through 1E).

Based on the previous studies showing coordinate regulation of Srebp-2 and miR-33, we hypothesized that treatment of mice with FXR agonists would also increase the hepatic levels of miR-33 in parallel with the changes in Srebp-2 mRNA. Consistent with this hypothesis, we show that hepatic miR-33 levels were induced within 2 hours of treatment of wild-type mice with GSK2324 (Figure 1F). Hepatic miR-33 levels were further increased after 4 hours (Figure 1F), or after 3 days of treatment of wild-type mice with either GSK2324 or GW4064 (Figure 1G and 1H). In contrast, hepatic miR-33 levels were unchanged in Fxr−/− mice or after treatment of either Fxr−/− mice or mice that lack hepatic FXR (FxrL–/–) with either GSK2324 or GW4064 (Figure 1F through 1H). These data are consistent with a specific requirement for hepatic FXR. Taken together, the data in Figure 1 demonstrate that FXR activation with ≥2 different agonists specifically increases both Srebp-2 and miR-33 expression.

In a recent study, we used microRNA expression profiling followed by high stringency analysis to identify hepatic microRNAs that were regulated in response to FXR activation. This approach identified 5 microRNAs, including a microRNA cluster that encoded both miR-144 and miR-451, which we showed were regulated in response to FXR agonists in vivo. This initial and strict approach failed to identify miR-33. Based on the data of Figure 1, we reanalyzed our original microRNA expression data using less stringent
analysis parameters (removing a fold-change cut off). Under the latter conditions, we identified additional microRNAs, including miR-33, whose levels were altered after FXR activation (Figure 1 in the online-only Data Supplement).

To further evaluate the function of the FXRE within the Srebp-2 locus, we generated a luciferase reporter gene, in which the FXRE was inserted 3′ of the luciferase coding sequence. In addition, we inserted the Srebp-2 proximal promoter, which contains ≥1 SRE, upstream of luciferase (Figure 1J). Plasmids containing this reporter gene and an expression plasmid for FXR were transfected into Hep3B cells, and the cells were then treated for 24 hours with either the FXR agonist GSK2324 or vehicle. Figure 1J shows that GSK2324 treatment resulted in a significant increase in luciferase activity. Taken together, these results suggest that the FXRE in intron 10 of the Srebp-2 gene is functional and that activation of hepatic FXR leads to increased expression of Srebp-2.
to induction of both Srebp-2 mRNA and miR-33. However, it remains unclear from these studies whether the increase in miR-33 is dependent on increased transcription of the Srebp-2 gene or whether FXR can independently activate the expression of miR-33 and Srebp-2.

**Activation of Hepatic FXR Is Sufficient to Repress miR-33 Targets**

Previous studies have shown that hepatic miR-33 levels change 1.5- to 2.5-fold under physiological conditions that alter Srebp-2 expression. In contrast, overexpression of miR-33, either from adenovirus-mediated overexpression or from mimetics, can result in supraphysiologic changes of hepatic miR-33 that affect many targets. Consequently we were interested in determining whether the 1.6-fold increase in hepatic miR-33 levels that occurred after treatment of mice with GSK2324 (Figure 1) affected the mRNA and protein levels of known miR-33 targets.

To test this hypothesis, we treated wild-type and Fxr−/− mice with GSK2324 for 3 days before determining changes in specific hepatic mRNA and protein levels. The data of Figure 2A demonstrate that GSK2324 treatment of wild-type, but not of Fxr−/− mice, caused modest but significant decreases in Abca1, Cpt1a, and Hadhb mRNAs, 3 known miR-33 targets. However, mRNA levels of Nsf, a newly identified miR-33 target, were unchanged after GSK2324 treatment of either genotype (Figure 2A). Importantly, microRNAs often cause more robust decreases in protein levels than in the mRNA levels. Figure 2B and 2C shows that hepatic protein levels of ABCA1, carnitine palmitoyltransferase 1 (CPT1), carnitine O-octanoyltransferase 1 (CROT), HADHB, and N-ethylmaleimide-sensitive factor (NSF) were all decreased after treatment of wild-type mice with GSK2324. No repression of the 5 proteins was observed after treatment of Fxr−/− mice with the FXR agonist consistent with the absolute requirement for FXR in mediating the effects of GSK2324 (Figure 2B and 2C).

We have previously shown that FXR agonists also increase the hepatic expression of miR-144 and that miR-144 targets ABCA1 independent of miR-33. Therefore, we conclude that activation of hepatic FXR in vivo induces 2 microRNAs (miR-33 and miR-144) that independently target ABCA1 leading to decreased ABCA1 protein and function. Previous cell-based studies demonstrated that both miR-33 and miR-144 could act synergistically to target ABCA1.

**FXR Activation Causes Accumulation of pSREBP-2, but No Change in nSREBP-2**

SREBP-2 regulates gene expression by binding to sterol response elements in target genes. Among the most well-characterized SREBP-2 target genes are enzymes of the sterol biosynthesis pathway. We first wanted to determine whether SREBP-2 target genes were increased after FXR activation, because we demonstrated that Srebp-2 expression is increased under the same conditions. Despite the increase in Srebp-2 mRNA, the expression of SREBP-2 target genes, that included cytochrome P450 51 (Cyp51), farnesyl dipiphosphate synthase (Fdp), HMG-CoA reductase (Hmgcr), and squalene synthase (Sqle), was unchanged (Figure 3A). Western blots confirmed that GSK2324 treatment did not result in changes in farnesyl dipiphosphate synthase or HMG-CoA reductase protein (Figure 3B and 3C).

SREBP-2 protein is synthesized as an ER-membrane bound precursor that undergoes sterol-dependent proteolytic cleavage to generate nSREBP-2 to activate target genes. Having failed to observe a change in SREBP-2 activity, despite changes in Srebp-2 mRNA, we determined whether SREBP-2 protein was altered after GSK2324 treatment. We observed a 3-fold increase in pSREBP-2, but no change in nSREBP-2 (Figure 3B and 3C). Hepatic pSREBP-2 protein levels were unchanged after treatment of Fxr−/− mice with GSK2324, consistent with the absence of changes in Srebp-2 mRNA (Figures 1B and 3B and 3C). Taken together, these results demonstrate that FXR activation increases SREBP-2 expression and precursor protein, in the absence of proteolytic processing of SREBP-2 to the nucleus and subsequent activation of SREBP-2 target genes.

Two proteins, SCAP and INSIG, have been identified that regulate nSREBP-2 levels by controlling SREBP-2 retention in the ER. In the absence of SCAP, the translocation of pSREBP-2 to the Golgi for processing before nuclear localization is greatly increased. We demonstrate that GSK2324 treatment did not alter the abundance of SCAP or INSIG protein (Figure 3B and 3C). Taken together, these results demonstrate that FXR activation increases SREBP-2 expression and precursor protein, in the absence of proteolytic processing of SREBP-2 to the nucleus and subsequent activation of SREBP-2 target genes.

**Figure 2.** Farnesoid X receptor activation leads to reduction in miR-33 targets. A, Hepatic expression of miR-33 target genes Abca1, Cpt1a, Hadhb, and Nsf in wild-type or farnesoid X receptor knockout (KO) mice treated with either vehicle or GSK2324 (30 mpk/d) for 3 days (n=8 mice per group). B, Western blotting analysis of ABCA1, CPT1, CROT, HADHB, NSF, and β-actin in total liver lysates from wild-type or farnesoid X receptor KO mice treated with either vehicle or GSK2324 (30 mpk/d) for 3 days (n=5 mice per group). C, Densitometry analysis of Western blots (n=10 mice per group; combined analysis from C and Figure IIIA in the online-only Data Supplement). Gene expression was normalized to Tbp. Data are presented as mean±SEM. Significance was measured with 2-way ANOVA followed by Student t test. Bars with different letters (a, b, and c) are significantly different from one another at the level P<0.05. ABCA1 indicates ATP binding cassette transporter A1; CPT1, carnitine palmitoyltransferase 1; CROT, carnitine O-octanoyltransferase 1; FXR, farnesoid X receptor; KO, knockout; NSF, N-ethylmaleimide-sensitive factor; and WT, wild-type.
attenuated. In contrast, increased levels of INSIG in the ER impair translocation of pSREBP-2 to the Golgi. A previous report demonstrated that treatment of mice for 1 to 10 days with the FXR agonist GW4064 resulted in increased levels of Insig-2a mRNA, but no change in SREBP-2 target genes. Our data in Figure 3A through 3C demonstrate that treatment of wild-type mice with FXR-specific agonists induces both Srebp-2 mRNA and miR-33 levels. However, it remained unclear whether the FXRE within intron 10 of the Srebp-2 gene activated miR-33 directly or activated Srebp-2 transcription before splicing of the intronic miR-33.

In addition, under the experimental conditions of FXR activation described in Figures 1 through 3, the nucleus contains detectable nSREBP-2 protein, despite impairment of the maturation of pSREBP-2 (Figure 3B and 3C). Further, nSREBP-2 is known to bind to the promoter of the Srebp-2 gene and to act as a feed-forward activator of Srebp-2 transcription.1,2

Induction of miR-33 by FXR Agonists Requires nSREBP-2
Some microRNAs are generated as a result of splicing from introns of mRNAs after transcription of the host gene. One example is that miR-33 is localized within exon 16 of the Srebp-2 gene. Other microRNAs, such as miR-144, have their own promoter that controls the synthesis of the primary microRNA, independent of the expression of adjacent genes. The data presented in Figures 1 through 3 demonstrate that treatment of wild-type mice with FXR-specific agonists induces both Srebp-2 mRNA and miR-33 levels. However, it remained unclear whether the FXRE within intron 10 of the Srebp-2 gene activated miR-33 directly or activated Srebp-2 transcription before splicing of the intronic miR-33.

In addition, under the experimental conditions of FXR activation described in Figures 1 through 3, the nucleus contains detectable nSREBP-2 protein, despite impairment of the maturation of pSREBP-2 (Figure 3B and 3C). Further, nSREBP-2 is known to bind to the promoter of the Srebp-2 gene and to act as a feed-forward activator of Srebp-2 transcription.1,2

Figure 3. Farnesoid X receptor activation results in the increased expression of Insig-2a and accumulation of precursor sterol response element binding protein 2 (SREBP-2) in the absence of changes in nuclear SREBP-2 protein. A, Hepatic expression of SREBP-2 target genes Cyp51, Fpds, Hmgcr, and Sqle in wild-type or farnesoid X receptor knockout (KO) mice treated with either vehicle or GSK2324 (30 mpk/d) for 3 days (n=8 mice per group). B, Western blotting analysis of HMG-CoA reductase, FDPS, and precursor SREBP-2 and β-Actin in total liver lysate, or nuclear SREBP-2 in nuclear extracts (n=5 mice per group). C, Densitometry analysis of Western blots displayed in (B). D, Insig-2a mRNA levels in livers of wild-type mice treated with either vehicle, or a single dose of GSK2324 (30 mpk) for 2 or 4 hours (n=6–7 mice per group). Hepatic expression of Insig-2a in wild-type or farnesoid X receptor knockout mice treated for 3 days with either vehicle or GW4064 (30 mpk/d; E; n=8 mice per group), or (F) GW4064 (60 mpk/d) (n=7–9 mice per group). G, Diagram summarizing SREBP-2 processing pathway regulated by farnesoid X receptor. Gene expression was normalized to Tbp. Data are presented as mean±SEM. Significance was measured with 2-way ANOVA followed by Student t test. Bars with different letters (a, b, and c) are significantly different from one another at the level P<0.05. FDPS indicates farnesyl diphosphate synthase; FXR, farnesoid X receptor; GSK, GlaxoSmithKline; HMGCR, HMG-CoA reductase; KO, knockout; SERBP-2, sterol response element binding protein 2; and WT, wild-type.
Therefore, whether the induction of miR-33 in response to FXR activation was dependent or independent of Srebp-2 transcription and nSREBP-2 protein remained unresolved. To distinguish between these possibilities, we generated and then used mice that lack hepatic SCAP (Scap<sup>L-KO</sup>).

Previous studies have shown that in the absence of SCAP, processing of pSREBP-2 in the Golgi is greatly attenuated and results in extremely low nuclear levels of SREBP-2. Our initial analysis showed that mRNA and protein levels of farnesyl diphosphate synthase (Fpds) and Srebp-2, both known targets of nSREBP-2, were reduced >90% in untreated Scap<sup>L-KO</sup> when compared with that in Scap<sup>fl/fl</sup> mice (Figure 4A and 4B). We next treated Scap<sup>fl/fl</sup> and Scap<sup>L-KO</sup> mice for 3 days with GSK2324 or vehicle. GSK2324 treatment of both Scap<sup>fl/fl</sup> and Scap<sup>L-KO</sup> mice led to a robust induction of Shp, Insig-2a, and miR-144 (Figure 4C and 4D). Therefore, we conclude that loss of SCAP does not interfere with the normal induction of hepatic FXR target genes by FXR agonists. In contrast, treatment of Scap<sup>L-KO</sup> mice with GSK2324 failed to induce either Srebp-2 (Figure 4E) or miR-33 (Figure 4F). Together, these data support the hypothesis that the FXR-dependent increase in hepatic miR-33 levels requires increased transcription of the Srebp-2 gene. The data also suggest that the FXRE within the Srebp-2 locus does not directly activate miR-33 expression but rather functions to increase transcription of the Srebp-2 gene with the subsequent excision and processing of miR-33 from intron 16.

**Reduction in Plasma Lipids After FXR Activation Involves Multiple Mechanisms**

Previous reports have shown that activation of hepatic FXR leads to decreased plasma HDL levels and that this was likely a result of increased hepatic expression of both the HDL receptor (Scarb1), that clears HDL from the plasma, and miR-144, that reduces hepatic ABCA1 protein thus limiting HDL generation. However, this study demonstrates that FXR agonists also induce hepatic expression of miR-33 that subsequently targets ABCA1 and lowers plasma HDL levels.

In an attempt to determine the relative importance of miR-33 and miR-144 in regulating hepatic ABCA1 and plasma HDL after FXR activation, we performed the experiments described in Figure 5. Both plasma total and HDL cholesterol levels are reduced in untreated Scap<sup>L-KO</sup> when compared with that in Scap<sup>fl/fl</sup> mice (Figure 5A). GSK2324 treatment of both Scap<sup>fl/fl</sup> and Scap<sup>L-KO</sup> mice resulted in further decreases in both total plasma cholesterol and HDL cholesterol (Figure 5A). However, the decrease in plasma HDL was greatest in the GSK2324-treated Scap<sup>fl/fl</sup> mice (Figure 5A). These latter mice responded to GSK2324 by the combined induction of miR-33, miR-144 (Figure 4D and 4F), and Scarb1 (Figure 5B). In contrast, GSK2324 treatment of Scap<sup>L-KO</sup> mice induces miR-144 (Figure 4D) and Scarb1 (Figure 5B), but not miR-33 (Figure 4F). Consistent with this lack of induction, miR-33 target genes CPT1, CROT1, HADHB, and NSF were not reduced in Scap<sup>L-KO</sup> mice treated with GSK2324 (Figure 5D and 5E). The changes in plasma HDL observed in Scap<sup>L-KO</sup> mice (Figure 5A) were paralleled by similar changes in hepatic Abca1 mRNA and protein levels.

**Figure 4.** The farnesoid X receptor–dependent regulation of Srebp-2 and miR-33 is abolished in the absence of sterol response element binding protein cleavage activated protein (SCAP). A, Western blotting analysis of HMG-CoA reductase, FPDS, precursor sterol response element binding protein 2 and β-actin in total liver lysate from Scap<sup>fl/fl</sup> or liver-specific Scap knockout (Scap<sup>L-KO</sup>) mice (n=5 mice per group). B, Densitometry analysis of Western blots displayed in A. C, Hepatic expression of Shp and Insig-2a. D, miR-144, E, Srebp-2, F, miR-33 in wild-type Scap<sup>fl/fl</sup> or liver-specific Scap knockout (Scap<sup>L-KO</sup>) mice treated with either vehicle or GSK2324 (30 mpk/d) for 3 days (n=8 mice per group). Data are presented as mean±SEM. Significance was measured with 2-way ANOVA followed by Student t test. Bars with different letters (a, b, and c) are significantly different from one another at the level P<0.05. FPDS indicates farnesyl diphosphate synthase; GSK, GlaxoSmithKline; HMGCR, HMG-CoA reductase; KO, knockout; SCAP, SERBP cleavage activated protein; and SREBP-2, sterol response element binding protein 2.
ABCA1 protein (Figure 5C through 5E). These results demonstrate cross talk and coregulation of FXR and SREBP-2 for the specific regulation of the SREBP-2 locus, but not at other genes.

Together, our previous studies and this study demonstrate that the reduction in ABCA1 protein after FXR activation in C57BL/6 mice involves both miR-144 and miR-33. Indeed, GSK2324 treatment of mice pretreated with an antisense oligonucleotide to miR-144 resulted in a small but significant decrease in ABCA1 protein and plasma total and HDL cholesterol levels (Figure IV in the online-only Data Supplement). Therefore, suppression of the FXR-dependent induction of miR-144 or miR-33 alone is not sufficient to abolish the regulation of ABCA1 by FXR.

**Discussion**

Here, we show that the Srebp-2 gene is a direct FXR target gene that contains a functional FXRE within intron 10. Importantly, we demonstrate that FXR agonists lead to a rapid and sustained induction of both Srebp-2 and miR-33 and that the changes in miR-33 are sufficient to repress known targets that include ABCA1, CPT1a, CROT1, HADHB, and NSF.

Whether other miR-33 targets are also regulated by FXR remains to be established. However, among the many newly identified miR-33 targets is Srebp1, which is known to be repressed by FXR. Therefore, our results suggest that the repression of Srebp1 by FXR may also involve miR-33. To understand the mechanism of induction of miR-33 by FXR agonists, we used mice that lack hepatic expression of SCAP. As a result, nSREBP-2 levels and the levels of most SREBP-2 target genes are significantly repressed when compared with wild-type mice. Treatment of Scap KO mice with FXR agonists failed to induce Srebp-2 mRNA or miR-33, although known FXR target genes that include Shp, Insig-2a, and miR-144 were induced normally. These data are consistent with a model in which agonist activation of FXR bound to the FXRE within the Srebp-2 gene, leads to increased transcription of the Srebp-2 locus and the subsequent increase in miR-33 is a result of splicing the RNA from intron 16 (Figure 5F). Further, these data do not support a model in which the FXRE functions independently to directly induce miR-33.

This report confirms and extends an earlier study in which Hubbert et al showed that activation of FXR induced Insig-2a mRNA. They proposed that the increased levels of INSIG-2a

Figure 5. Farnesoid X receptor activation in Scap KO mice reduces hepatic ATP binding cassette transporter A1 protein and plasma high-density lipoprotein cholesterol levels but not other miR-33 target genes. A, Plasma lipid levels from wild-type (Scapflox/flox) or littermate liver-specific Scap knockout (ScapL-KO) mice treated with either vehicle or GSK2324 (30 m.p.k.d) for 3 days (n=8 mice per group). B, Hepatic expression of Scarb1 and Abca1 in wild-type (Scapflox/flox) or littermate liver-specific Scap knockout (ScapL-KO) mice treated with either vehicle or GSK2324 (30 m.p.k.d) for 3 days (n=8 mice per group). C, Western blotting analysis of ATP binding cassette transporter A1, CPT1, CROT, HADHB, NSF, and β-actin in total liver lysate from Scapflox/flox or littermate liver-specific Scap knockout (ScapL-KO) mice (n=5 mice per group). D, Densitometry analysis of Western blots (n=10 mice per group; combined analysis from D and Figure IIIB in the online-only Data Supplement) displayed in E. E, Graph showing the regulation of ABCA1, CPT1, CROT, HADHB, NSF, and β-actin (fold change). F, Diagram summarizing the regulation of ABCA1, CPT1, CROT, HADHB, NSF, and β-actin (fold change).
might be sufficient to attenuate the maturation of pSREBP-2 to nSREBP-2. Our new analysis of chromatin immunoprecipitation sequencing data now identifies multiple FXREs within the Insig-2α locus consistent with this gene being a direct target of FXR. Further, the demonstration that treatment of wild-type mice with FXR agonists results in significant increases in pSREBP-2 protein in the absence of changes in nSREBP-2 protein is entirely consistent with a model in which the increase in INSIG-2α protein prevents the translocation of the pSREBP-2:SCAP complex to the Golgi, thus preventing the normal maturation of pSREBP-2 to nSREBP-2. Therefore, FXR activation does not increase sterol synthesis, the major process regulated by nSREBP-2, as maturation of the accumulating pSREBP-2 is impaired (Figure 2). Increased sterol synthesis would seem undesirable in conditions, where FXR is activated. FXR is active when bile acids, the breakdown products of cholesterol, are elevated, and a further elevation in cholesterol under those conditions would seem physiologically counter productive.

So what would be the physiological reason for FXR agonists to induce Srebp-2 mRNA expression without increasing the functional level and activity of nuclear SREBP-2? We propose that the answer lies with the microRNA, miR-33, that is generated from intron 16 of Srebp-2. We show that expression and activity (ie, reduction of target genes) of miR-33 are both increased after FXR activation (Figure 3). The most responsive miR-33 target gene identified thus far is ABCA1, which contains ≥2 miR-33 binding sites.9–11 We have observed more subtle regulation of other miR-33 target genes, consistent with miR-33 fine-tuning the FXR response. In the case of the regulation of ABCA1, it seems that FXR uses 2 independent microRNAs to reduce ABCA1 levels. We previously showed that the reduction in ABCA1 is part of a larger FXR response to enhance cholesterol movement into the gall bladder.12 The relative contribution of miR-33 in the FXR-dependent regulation of ABCA1 remains to be established. The dual microRNA regulation of ABCA1 by miR-33 and miR-144 would likely ensure a more robust regulation of ABCA1, consistent with our observations.

The regulation of miR-33 by FXR is modest, ~50%. This is in line with the physiological response of expression Srebp-2, which changes no >2-fold under conditions of cellular sterol deprivation (high SREBP-2 activity) or sterol excess (low SREBP-2 activity).13 Therefore, the changes observed in miR-33 target genes after FXR activation are reflective of the physiological role of miR-33, which is different from the supraphysiological conditions that would be expected when miR-33 is overexpressed using mimetics or adenovirus particles.

Finally, we also demonstrate that the FXR-dependent coregulation of Srebp-2 mRNA and miR-33 requires SCAP. SCAP is required for transportation of pSREBP-2 from the endoplasmic reticulum to the Golgi where pSREBP-2 is processed to nSREBP-2 protein, before nuclear localization and subsequent activation of target genes that include Srebp-2 itself. The most facile interpretation is that the increase in miR-33 after FXR activation requires Srebp-2 transcription and that the latter process is dependent on nuclear levels of SREBP-2 protein. Whether FXR and SREBP-2 physically interact to regulate the SREBP-2 locus remains to be determined, but there is a corequirement for both, at least in this specific context. The end result of this intricate and complex regulation is that FXR uncouples the actions of SREBP-2 from those of miR-33. Although both are coregulated by FXR, it seems that the requirement for the actions of miR-33 have outweighed the actions of Srebp-2.

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Disclosures
Dr Vallim has patents and disclosures related to the use of anti-miR-144 as a therapeutic agent, which are owned by the University of California Los Angeles. The other authors report no conflicts.

References
MicroRNAs are known to play important roles as fine-tuning regulators of gene expression. Here, we show that the bile acid-regulated nuclear receptor farnesoid X receptor (FXR) controls the hepatic expression of both miR-33 and Srebp-2. We identify a functional FXR response element in intron 10 of the Srebp-2 gene allowing us to identify the molecular mechanism for the FXR-dependent regulation of Srebp-2/miR-33. Further we show that FXR activation uncouples the activities of the parent gene Srebp-2 from the microRNA (miR-33) via the induction of Insig-2a. Therefore, we have identified a physiological context where miR-33 and Srebp-2 are coregulated (i.e., after FXR activation) but the net effect of this regulation is a specific induction of miR-33.
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SUPPLEMENTAL MATERIAL

The Nuclear Receptor FXR Uncouples the Actions of miR-33 from SREBP-2

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ONLINE FIGURES

Online Figure I. Activation of FXR in mice regulates the expression of hepatic microRNAs. Wild-type C57BL/6 mice (6-9/group) were treated daily for 2 days with vehicle or GSK2324 (30 mpk/day) as previously described. Fold change indicates the change in miRNA expression after comparison of GSK2324 with vehicle treatment. Open circles (black) represent the significantly altered miRNAs with a fold change >25% (adjusted P<0.05) as previously identified. Open circles (red) represent additional significantly altered miRNAs using less stringent criteria (adjusted P<0.05).
Online Figure II. ChIP-Seq data identifies multiple FXR binding sites at the Insig-2a locus. ChIP-seq analysis of genome-wide FXR binding sites\textsuperscript{3,4} identifies multiple putative FXREs at the Insig-2a locus on Chromosome 1.
**Online Figure III. Western blot analysis of miR-33 targets.** Western blot analysis of miR-33 targets in total liver lysate from (A) wildtype (WT) and *Fxr*−/− (KO) mice or (B) *Scap*^Flox/Flox^ and littermate liver-specific *Scap* knockout (*Scap*^L-KO^) mice treated with either vehicle or GSK2324 (30 mpk/day) for 3 days (n=5 mice/group).
Online Figure IV. The FXR-dependent Regulation of ABCA1 Is not Abolished in the Absence of miR-144. (A) Western blotting analysis of ABCA1 and β-Actin in total liver lysate from wildtype mice treated with either control (Con) anti-miR or anti miR-144 ASO for 7 days on days 1 and 4, and then treated daily with either vehicle or GSK2324 (30 m,p,k/day) for 3 days on days 5-7 (n=7 mice/group). (B) Densitometry analysis of Western blots (n=6 mice/group) displayed in (A). (C) Plasma total and HDL cholesterol levels measured on day 7 in mice treated as described in (A). (D) Hepatic expression of Abca1, Insig-2a, Scarb1 and Shp in wild-type mice treated with control anti-miR or anti-miR-144 for 7 days, treated with either vehicle or GSK2324 (30m.p.k.d) for 3 days (n=6 mice/group). Data are presented as mean ± SEM. Significance was measured with two-way ANOVA followed by Student t test. Bars with different letters (a, b, c) are significantly different from one another at the level p < 0.05.

ONLINE MATERIALS AND METHODS

Mice: All animals were bred and housed in a pathogen-free animal facility and unless otherwise stated, were male and maintained on a C57BL/6 background (Jackson Laboratories, Bar Harbor ME). Floxed Scap$^{−/−}$ mice were purchased from The Jackson Laboratory (Strain 004162), and were crossed to Albumin-Cre mice (Jackson Laboratories). Liver-specific Scap$^{−/−}$ mice and their respective littermate wild-type (flox) controls were used for experiments. The generation of liver-specific Fxr$^{−/−}$ mice and their respective littermate wild-type (flox) controls, and whole body Fxr$^{−/−}$ mice was as previously described.1 Mice were fed a standard chow diet (NIH31 modified mouse/rat diet, catalog No. 7013, Harlan Teklad) ad libitum. For treatments with FXR agonists, GSK2324 was dissolved in water and administered once daily to mice via intraperitoneal injection (I.P.) at 30 mg/kg body weight (mpk) unless otherwise stated in the figure legend. In experiments where GW4064 and GSK2324 were compared, agonists were dissolved in water containing 0.5% Tween 80, and mice were treated once daily with either drug or vehicle alone at 60 mpk for 3 days via I.P. injection. For anti-miR treatments, mice were treated with control (Con) anti-miR or anti-miR-144 (Regulus Therapeutics) for 7 days on days 1, 4, and then treated daily with either vehicle or GSK2324 (30 mpk/day) for 3 days on days 5-7. Unless otherwise stated, mice were fasted for 4-6 hours after the last treatment with FXR agonists prior to removal of tissues. All experiments were carried out according to NIH guidelines and were approved by the UCLA Office of Animal Research Oversight (OARO).

Cell Culture and Luciferase Reporter Assay: Hep3B (ATCC) cells were cultured according to ATCC recommendations. For reporter assays, the mouse Srebp-2 promoter (2kb) was cloned upstream of the luciferase reporter gene in the pGL4.10 plasmid. The mouse FXRE from intron 10 of the Srebp-2 gene was cloned downstream of the luciferase reporter gene. The regulation of the Srebp-2 promoter by FXR was determined by co-transfecting a β-galactosidase expression plasmid and increasing amounts of pcDNA mouse FXRα2 expression plasmid into Hep3B cells in the presence or absence of GSK2324 (1 µmol/L). Promoter activity was normalized to β-galactosidase activity to correct for transfection efficiency.

Real Time PCR: Total RNA was isolated with the miRNAeasy kit (QIAGEN) according to the manufacturer’s instructions. Gene expression was determined from cDNA synthesized using High Capacity cDNA Reverse Transcription kit (Applied Biosystems) from 500ng of total RNA and using a Lightcycler480 Real-time qPCR machine and Lightcycler480 Mastermix (Roche). Relative gene expression was determined using an efficiency corrected method and efficiency was determined from a 3-log serial dilutions standard curve made from cDNA pooled from all samples. Primers were designed across exon-exon boundaries using Roche UPL guidelines. Primer sequences are available on request. Results were normalized to Tbp mRNA. Relative miRNA expression was determined by Taqman RT-PCR using pre-designed miR-144 (for miR-144-3p) or miR-33 probe sets (Applied Biosystems) from cDNA synthesized from 100ng total RNA (Applied Biosystems), and normalized to SnoRNA 202.

Western Blot Assay: Whole liver lysates were homogenized in RIPA buffer (1x PBS with 1% SDS, 5g/L sodium deoxycholate, 1% NP40) supplemented with protease inhibitor cocktail (Roche) fortified with additional PMSF, Leupeptin, Aprotinin and ALLN (Sigma) and quantified using the Bradford assay (BioRad). Nuclei were prepared as previously described.2 Equal amounts of protein were separated on 4-12% acrylamide gels (BioRad) and transferred to a PVDF membrane (Millipore). Membranes were blocked for 16 hours in 5% non-milk fat solution in Tris-buffered saline containing 0.1% Tween 20 and probed with antibodies to ABCA1 (Dr. John Parks), β-actin (Sigma), CPT1 (Genetex, GTX114337), CROT (Novus, NPBI-31441), HADHB
(Aviva Systems Bio, ARP48133), HMGCR and FPDS (Dr. Peter Edwards), NSF (BD BioSciences, 612272), and SREBP-2 (Dr. Tim Osborne) for 16 hours. HRP detection was carried out using ECL plus reagent (GE Healthcare) according to manufacturer’s instructions. HRP signal detection was determined electronically using GE Image Quant LAS 4000 system and parameters set strictly below the saturation point. Densitometric analysis was carried out using Quantity One software (BioRad). We determined the densitometry values for i) the miR-33 targets for each lane (representing the hepatic extract from individual mice) and ii) the actin control for the same lane, and then divided (normalized) the miR-33 target by the loading control. Relative protein levels were expressed as fold changes after normalization to β-actin protein levels, with vehicle treated wild-type mice set to 1.

**Plasma Lipid and Lipoprotein Analysis:** All plasma lipid samples were analyzed by the UCLA Atherosclerosis Research Unit Lipid Core Facility, which is certified by the Centers for Disease Control lipid standardization program (Laboratory identification number, LSP-251). Plasma total and HDL cholesterol were determined as previously described.1

**Statistical Analysis:** Statistical analysis was performed with Prism Graphpad software (version 5.0) or Microsoft Excel. Comparison between control and treatment group(s) was performed with either a Student t test or Two-way ANOVA followed by a Student t test or a Tukeys post-hoc test, and bars with different letters (a, b, c, d) are significantly different from one another at the level of p < 0.05.


METHODS

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