Human Insulin Growth Factor 2 mRNA Binding Protein 2 Increases MicroRNA 33a/b Inhibition of Liver *ABCA1* Expression and Alters Low-Density Apolipoprotein Levels in Mice

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ABSTRACT Genome-wide association studies (GWAS) have linked *IGF2BP2* single-nucleotide polymorphisms (SNPs) with type 2 diabetes (T2D). Mice overexpressing *mIGF2BP2* have elevated cholesterol levels when fed a diet that induces hepatic steatosis. These and other studies suggest an important role for insulin growth factor 2 mRNA binding protein 2 (IGF2BP2) in the initiation and progression of several metabolic disorders. The ATPase binding cassette protein ABCA1 initiates nascent high-density apolipoprotein (HDL) biogenesis by transferring phospholipid and cholesterol to delipidated apolipoprotein AI (ApoAI). Individuals with mutational ablation of *ABCA1* have Tangier disease, which is characterized by a complete loss of HDL. MicroRNA 33a and 33b (miR-33a/b) bind to the 3' untranslated region (UTR) of *ABCA1* and repress its posttranscriptional gene expression. Here, we show that IGF2BP2 works together with miR-33a/b in repressing *ABCA1* expression. Our data suggest that IGF2BP2 is an accessory protein of the argonaute (AGO2)–miR-33a/b–RISC complex, as it directly binds to miR-33a/b, AGO2, and the 3' UTR of *ABCA1*. Finally, we show that mice overexpressing human *IGF2BP2* have decreased *ABCA1* expression, increased low-density lipoprotein-cholesterol (LDL-C) and cholesterol blood levels, and elevated SREBP-dependent signaling. Our data support the hypothesis that IGF2BP2 has an important role in maintaining lipid homeostasis through its modulation of *ABCA1* expression, as its overexpression or loss leads to dyslipidemia.

KEYWORDS lipid, fatty acids, ABCA1, IGF2BP2, LDL-C, SREBP, cholesterol, triglyceride, high-density lipoprotein, HDL, very low-density lipoprotein, VLDL, DICER, LDL, lipid, microRNA, reverse cholesterol transport

Reverse cholesterol transport is a complex process whereby peripheral cholesterol is transported back to the liver for metabolism (1). High-density lipoprotein (HDL) is required for reverse cholesterol transport, as it acts as a lipid carrier (1). Several intermediate forms of HDL that differ in size exist. HDL maturation requires the functions of the ATP binding cassette (ABC) family of proteins and apolipoprotein AI (ApoAI) (2). ABCA1 initiates HDL biogenesis by transferring phospholipid and cholesterol to delipidated ApoAI, resulting in the formation of nascent HDL particles. ABCG1 then transfers additional cholesterol, which nucleates the formation of mature HDL apolipoproteins. Individuals ablated for *ABCA1* have Tangier disease (familial alpha-lipoprotein deficiency) and produce little to no HDL (<5 mg/dl), are hypocholester-

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olemic, mildly hypertriglyceridemic, accumulate cholesterol ester in many tissues, and have an increase in the production of foam cells (3).

ABCA1 expression is regulated at the transcriptional, posttranscriptional, and post-translational levels (4–6). Specific microRNAs (miRs) modulate *ABCA1* mRNA translation and stability (2). miRs are short single-stranded noncoding RNA nucleotide sequences (~22 nucleotides [nt]) that are critical for mRNA silencing (7). miR-33a and -33b (miR-33a/b) bind to miR response elements (MREs) within the 3' untranslated regions (UTR) of *ABCA1* and suppress gene expression (7). miR-33b knock-in mice have reduced *ABCA1* expression and a 35% reduction in HDL levels (8). Mice treated with anti-miR-33a show a 50% increase in HDL (9–11), while miR-33a knockout mice have increased *ABCA1* expression and a concomitant 30 to 50% increase in HDL (12).

Studies have shown that the insulin growth factor 2 mRNA binding protein (IGF2BP) family of RNA binding proteins regulates RNA localization, stability, and translation (13–15). IGF2BPs function to stabilize ribonucleoprotein complexes (15) and are highly expressed in early development in the brain, limb buds, muscle, and epithelia of many organs (16). IGF2BP1/3 expressions decline over time, while IGF2BP2 expression is seen in most adult organs (13, 16). Emerging evidence suggests a link between IGF2BP2 function and metabolic diseases. Mice overexpressing *IGF2BP2* have elevated cholesterol levels when fed a methionine-choline-deficient diet that induces hepatic steatosis (17). Mice lacking *IGF2BP2* are resistant to diet-induced obesity and fatty liver disease and are more glucose tolerant and insulin sensitive (18). Moreover, several genome-wide association studies (GWAS) have linked *IGF2BP2* single-nucleotide polymorphisms (SNPs) to type 2 diabetes (T2D) (19).

Here, we show a role for human IGF2BP2 (*hIGF2BP2*) in regulating *ABCA1* mRNA expression through its being a member of an AGO2-miR-33a/b-RISC complex that binds directly to *ABCA1* mRNA and inhibits its expression.

RESULTS

IGF2BPs bind to miRs regulating lipid homeostasis. We used a proteomics approach to identify proteins binding to miRs regulating lipid homeostasis. miR-24, miR-33a/b, miR-122, and miR-185 were used as baits. A total of 48 proteins that bound all miRs were identified (see Table S1 in the supplemental material). STAU1 (staufen homolog 1), G3BP1/2 (RAS GTPase-activating protein-binding protein), IGF2BP1/2/3 (insulin growth factor 2 mRNA binding protein), HSP70/71 (heat shock protein), and heterogeneous nuclear ribonucleoproteins were among those identified. A number of the proteins identified were previously shown to bind a small activating RNA (saRNA) involved in AGO2 transcriptional activation (20).

IGF2BPs can bind multiple miRs. To further validate our mass spectrometry (MS) results, we again determined if IGF2BPs could bind these same miRs. All three IGF2BPs bound to all miRs tested, with various affinities (Fig. 1A). None of the IGF2BPs bound to a scrambled miR (Fig. 1A, scrambled).

The loss of IGF2BP expression increases *ABCA1* mRNA expression. miR-33a/b regulate *ABCA1* expression. We determined if the loss of each IGF2BP had any effect on *ABCA1* silencing. *ABCA1* expression was determined in HepG2 cells expressing miR-33a/b that were transfected with small interfering RNAs (siRNAs) for each IGF2BP. Each *IGF2BP* siRNA reduced its cognate IGF2BP protein level without affecting the expression of others (Fig. 1B). Cells treated with miR-33a/b alone had reduced *ABCA1* expression levels compared to control cells transfected with a scrambled siRNA (Fig. 1C, control [Con] versus miR-33a/b). Loss of each IGF2BP resulted in an increase in *ABCA1* mRNA expression compared to control or miR-33a/b-treated cells (Fig. 1C, Con versus IGF2BP1/2/3si). Increased mRNA expression resulted in an increase in *ABCA1* protein level (Fig. 1D). Thus, all three IGF2BPs bind to miR-33a/b and regulate the silencing of *ABCA1* expression.

We decided to focus our studies solely on how IGF2BP2 regulates *ABCA1* expression, based on GWAS data linking *IGF2BP2* SNPs to T2D, as well as the fact that expression

FIG 1 IGF2BP1/2/3 bind miRs regulating lipid homeostasis. (A) Biotinylated miRs were immunoprecipitated using streptavidin beads, and levels of bound IGF2BP proteins were determined. scrambled, nonspecific miR. (B) IGF2BP levels were determined in cells with siRNA knocked down for the indicated IGF2BP protein (indicated by "si" suffix). scrambled, nonspecific miR. (C) qRT-PCR analysis was used to determine the relative mRNA expression levels of *ABCA1* mRNA in miR-33a/b-expressing cells knocked down for the indicated IGF2BP protein. *ABCA1* mRNA expression values are compared to *GAPDH* mRNA expression, which was set at 1. (D) *ABCA1* protein level was determined in miR-33a/b-expressing cells treated with siRNA against the indicated IGF2BP protein. The total cell lysate actin level was used as a loading control. The data represent the averages from five independent experiments. *, $P < 0.01$; **, $P < 0.001$; ***, $P < 0.0001$. Values are the means $\pm$ SEM.

of IGF2BP1/3 in adult organs in low and thus their physiological relevance in regulating *ABCA1* expression in adult individuals is lacking.

IGF2BP2 binds to the mRNA 3' UTR of *ABCA1*. miR-33a/b bind to two tandem MREs within the 3'-UTR region of human *ABCA1* mRNA (Fig. 2A and B). miR-33a/b binding causes decreased *ABCA1* 3'-UTR expression (1). To construct a similar system, we used the pSwitchLight system, which expresses luciferase activity as a readout for 3'-UTR-driven *ABCA1* mRNA expression.

HepG2 cells were first transfected with a wild-type (WT) (Fig. 2A) or mutant (MUT) pSwitchLight *ABCA1* 3'-UTR luciferase reporter (Fig. 2C, bold condensed letters), and luciferase activities were assayed in the presence of a control miR (Con-miR) or miR-33a/b. Cells cotransfected with the control miR and control luciferase plasmid had luciferase activity (Fig. 3A, bar A versus B). The expression of miR-33a/b alone did not affect control activity (Fig. 3A, bar B versus C) but caused an ~4-fold decrease in WT

FIG 2 *ABCA1* 3'-UTR sequences containing miR-33a/b MRE sites. (A) Wild-type *ABCA1* 3'-UTR sequence; (B) wild-type miR-33a/b MRE sequence; (C) mutant miR-33a/b sequences within the *ABCA1* 3'-UTR sequence. Italics indicate miR-33a/b binding sites within the *ABCA1* 3'-UTR sequence; boldface indicates miR-33a/b binding sites; condensed boldface indicates mutations within miR-33a/b binding sites in the *ABCA1* 3' UTR.

FIG 3 IGF2BP2 is required for miR-33a/b-dependent silencing of ABCA1 mRNA expression. (A) Luciferase activity was quantitated in HepG2 cells transfected with a control luciferase reporter (Con-Luc), ABCA1 3'-UTR-containing reporter (3' UTR), ABCA1 3'-UTR mutant reporter (MUT-3' UTR), pre-miR-33a/b (miR-33a/b), or control pre-miR (Con-miR). Luciferase activity was measured as described in Materials and Methods. (B) Luciferase reporter activity was measured in cells treated with IGF2BP2 siRNA (IGF2BP2si) or transfected with pCMV-IGF2BP2 (IGF2BP2 OE). (C) qRT-PCR analysis was used to determine relative endogenous ABCA1 mRNA expression in miR-33a/b-expressing cells treated with IGF2BP2 siRNA or transfected with pCMV-IGF2BP2. ABCA1 mRNA expression values are compared to GAPDH mRNA expression, which was set at 1. (D) ABCA1 protein level was determined in miR-33a/b-expressing cells treated with IGF2BP2 siRNA or transfected with pCMV-IGF2BP2. Actin was used as a loading control. (E) qRT-PCR analysis was used to determine relative endogenous ABCA1 mRNA expression in miR-33a/b-expressing cells transfected with a miR-33a/b anti-miR, treated with IGF2BP2 siRNA, or transfected with pCMV-IGF2BP2. ABCA1 mRNA expression values were compared to GAPDH mRNA expression, which was set at 1. (F) ABCA1 protein level in miR-33a/b-expressing cells were transfected with a miR-33a/b anti-miR, treated with IGF2BP2 siRNA, or transfected with pCMV-IGF2BP2. Actin was used as a loading control. (G) Relative miR-33a/b mRNA abundance. (H) Relative ABCA1 mRNA abundance. **, $P < 0.001$; ***, $P < 0.0001$. The qRT-PCR data are the averages from five independent experiments. Values are the means $\pm$ SEM. The Western blot panels represent the average results of five independent experiments.

ABCA1 3'-UTR luciferase expression (Fig. 3A, bar B versus E). miR-33a/b-dependent repression was lost when the seed sequences for miR-33a/b binding were mutated (Fig. 3A, bar F versus G). Expression of either the ABCA1 3' UTR or the ABCA1 MUT 3' UTR had no effect on Con-miR luciferase activity (Fig. 3A, bar D versus F).

Next, HepG2 cells expressing miR-33a/b were transfected with a pSwitchLight ABCA1 3'-UTR luciferase reporter and IGF2BP2 expression was either knocked down with siRNA (IGF2BP2si) or overexpressed. Once again, miR-33a/b expression did not alter the level of luciferase activity of the control luciferase reporter (Fig. 3B, bar A), while its overexpression reduced WT ABCA1 3'-UTR luciferase activity (Fig. 3B, bar B). The knockdown of IGF2BP2 abolished miR-33a/b-dependent inhibition of luciferase expression (Fig. 3B, bar C), whereas overexpressing IGF2BP2 further decreased WT ABCA1 3'-UTR luciferase activity to a level that was lower than what was seen in the presence of miR-33a/b alone (Fig. 3B, bar B versus D).

We next looked at the effects of altering IGF2BP2 expression levels on endogenous ABCA1 mRNA expression. We found that miR-33a/b expression repressed the mRNA

expression of endogenous *ABCA1* (Fig. 3C, Con versus miR-33a/b). In contrast, knocking down *IGF2BP2* expression resulted in an induction in *ABCA1* mRNA expression (Fig. 3C, Con versus IGF2BP2si), while its overexpression resulted in a dramatic reduction (Fig. 3C, Con versus IGF2BP2 OE). *ABCA1* protein level was reduced in miR-33a/b-expressing cells (Fig. 3D, Con versus miR-33a/b), while we observed an ~4-fold increase in *IGF2BP2* knockdown cells (Fig. 3D, Con versus IGF2BP2si). In contrast, overexpressing *IGF2BP2* caused an ~5-fold decrease in protein (Fig. 3D, Con versus IGF2BP2 OE).

Inhibition of *ABCA1* expression by miR-33a/b requires IGF2BP2. Our data suggested that miR-33a/b works with IGF2BP2 to inhibit *ABCA1* expression. Thus, we overexpressed *IGF2BP2* in the absence or presence of an anti-miR-33a/b and determined endogenous *ABCA1* mRNA expression. Overexpressing *IGF2BP2* reduced the *ABCA1* expression level ~3-fold (Fig. 3E, Con versus IGF2BP2 OE). Anti-miR-33a/b expression alone blocked miR-33a/b-dependent inhibition (Fig. 3E, Con versus anti-miR). In addition, *IGF2BP2* overexpression in the presence of the anti-miR-33a/b was unable to reduce *ABCA1* mRNA expression (Fig. 3E, anti-miR versus anti-miR-33a/b IGF2BP2 OE), whereas knocking down *IGF2BP2* mRNA expression in the presence of endogenous miR-33a/b caused an increase in *ABCA1* expression (Fig. 3E, IGF2BP2si).

ABCA1 protein directly correlated with the level of *ABCA1* mRNA expression (Fig. 3F). *IGF2BP2* overexpression resulted in a 3-fold decrease in *ABCA1* protein (Fig. 3F, Con versus IGF2BP2 OE), while a 2-fold increase was observed in anti-miR cells (Fig. 3F, Con versus anti-miR-33a/b). *ABCA1* protein levels were similar between cells overexpressing *IGF2BP2* and anti-miR and cells expressing anti-miR alone (Fig. 2F, anti-miR-33a/b versus anti miR-33a/b IGF2BP2 OE). Finally, knocking down *IGF2BP2* caused an ~5-fold increase in *ABCA1* protein (Fig. 3F, IGF2BP2si).

To show that IGF2BP2 physically interacted with miR-33a/b to inhibit *ABCA1* mRNA expression, we immunoprecipitated IGF2BP2 and determined the level of miR-33a/b or *ABCA1* mRNA bound. IgG control did not pull down miR-33a/b (Fig. 3G) or *ABCA1* mRNA (Fig. 3H), while both coimmunoprecipitated with IGF2BP2 (Fig. 3G and H). These results strongly suggest that IGF2BP2 directly associates with miR-33a/b and *ABCA1* mRNA to negatively regulate *ABCA1* mRNA level and *ABCA1* protein expression.

IGF2BP2 interacts with the endonuclease AGO2. Several factors regulating mRNA processing were enriched in our proteomic assay. These included the G3BP1/2 RAS GTPase binding proteins and the staufen homolog 1, STAU1. Thus, we immunoprecipitated IGF2BP2 and determined if G3BP1/2 and/or STAU1 directly bound and whether any associations observed were required for miR-33a/b silencing of *ABCA1* expression. While we did not find AGO2 in our proteomic assay, we still tested whether it physically bound IGF2BP2 under our conditions, as it is a core protein within the RISC complex.

Ten percent of the total lysate used in our immunoprecipitation experiments is shown in Fig. 4A. IGF2BP2, G3BP1/2, and STAU1 physically bound to miR-33a/b but did not bind to a scrambled miR sequence (Fig. 4B, scrambled versus miR-33a/b). Interestingly, AGO2 also physically interacted with miR-33a/b (Fig. 4B, scrambled versus miR-33a/b).

We next determined whether IGF2BP2 physically interacted with AGO2, STAU1, and/or G3BP1/2. IGF2BP2 antibodies were able to coimmunoprecipitate with AGO2 but not STAU1 (Fig. 4C), while AGO2 antibodies did coimmunoprecipitate with IGF2BP2 and STAU1 (Fig. 4C). STAU1 antibodies were only able to coimmunoprecipitate with AGO2 (Fig. 4C). Both of the G3BP1/2 antibodies did coimmunoprecipitate with each other but did not pull down IGF2BP2, AGO2, or STAU1 (data not shown).

AGO2 and STAU1 are required for IGF2BP2-dependent inhibition of *ABCA1* expression. We next determined if AGO2, STAU1, or G3BP1/2 were required for IGF2BP2 inhibition of *ABCA1* mRNA expression. We assayed for *ABCA1* mRNA expression levels in cells transfected with miR-33a/b and either knocked down for or overexpressing *IGF2BP2*, *AGO2*, *STAU1*, or *G3BP1/2* (Fig. 5A and C). Knocking down *IGF2BP2*, *AGO2*, or *STAU1* in miR-33a/b-expressing cells resulted in an increase in *ABCA1* mRNA expression, while no changes were seen in *G3BP1/2* knockdown cells (Fig. 5A). *ABCA1* protein

FIG 4 IGF2BP2 binds the RISC endonuclease AGO2. miR-33a/b was biotinylated and incubated with cell lysates. Bound proteins were pulled down using streptavidin beads, resolved by SDS-PAGE, and analyzed by Western blotting. (A) Ten percent of the total protein level in the cell lysates is shown. (B) miR-33a/b was biotinylated and incubated with cell lysates. Bound proteins were pulled down using streptavidin beads, resolved by SDS-PAGE, and analyzed by Western blotting. Lysate, total cell lysate; scrambled, nonspecific miR control. (C) Cell lysates were incubated with polyclonal antibodies (IP; indicated at the top of the blots), and the levels of bound proteins were determined. The figure represents the average results of five independent experiments.

levels directly correlated with the levels of *ABCA1* mRNA expression, with the exception of G3BP2, whose knockdown did cause a reduction in *ABCA1* protein level (Fig. 5B, Con versus G3BP2si).

We further found that overexpressing *IGF2BP2*, *AGO2*, or *STAU1* reduced *ABCA1* mRNA expression to levels that were lower than that observed for miR-33a/b-expressing cells (Fig. 5C). Once again, the levels of *ABCA1* protein directly correlated with *ABCA1* mRNA expression levels. Finally, we found that overexpressing *G3BP1/2* partially reduced *ABCA1* mRNA expression compared to control cells (Fig. 5C, Con versus *G3BP1* OE and *G3BP2* OE). The reduction in *ABCA1* mRNA expression in this case reduced the *ABCA1* protein level (Fig. 5D).

We next determined how knocking down or overexpressing *AGO2*, *STAU1*, or *G3BP1/2* affected *IGF2BP2* repression of *ABCA1* mRNA expression in cells that were either knocked down for (Fig. 6A) or overexpressing (Fig. 6C) *IGF2BP2* and expressing

FIG 5 Both AGO2 and STAU1 are required for IGF2BP2-dependent miR-33a/b *ABCA1* silencing. (A) qRT-PCR analysis was used to determine relative endogenous *ABCA1* mRNA expression in miR-33a/b-expressing cells treated with siRNAs targeting the indicated genes (indicated by “si” suffix). *ABCA1* mRNA expression values were compared to *GAPDH* mRNA expression, which was set at 1. (B) *ABCA1* protein level was determined in miR-33a/b-expressing cells treated with the indicated siRNA. Actin was used as a loading control. (C) qRT-PCR analysis was used to determine the relative endogenous *ABCA1* mRNA expression in miR-33a/b-expressing cells transfected with pCMV plasmids expressing the indicated genes (OE). (D) *ABCA1* protein level was determined in miR-33a/b-expressing cells transfected with pCMV plasmids expressing the indicated genes (OE). Con, control. **, $P < 0.001$; ***, $P < 0.0001$. The qRT-PCR data are the averages from five independent experiments. Values are the means $\pm$ SEM. The Western blot panels represent the average results of five independent experiments.

miR-33a/b. miR-33a/b expression once again decreased *ABCA1* mRNA expression (Fig. 6A, Con versus miR-33a/b). Overexpressing *IGF2BP2* in miR-33a/b cells further reduced *ABCA1* mRNA expression compared to cells expressing only miR-33a/b (Fig. 6A, miR-33a/b versus *IGF2BP2* OE). Strikingly, repression of *ABCA1* mRNA expression in miR-33a/b cells overexpressing *IGF2BP2* required AGO2 and STAU1, as loss of either caused *ABCA1* mRNA expression to return to levels similar to that seen in control cells (Fig. 6A, *IGF2BP2* OE versus AGO2si or STAU1si). Knocking down *G3BP1/2* expression had no effect on *ABCA1* mRNA expression in miR-33a/b cells overexpressing *IGF2BP2* (Fig. 6A, *IGF2BP2* OE versus *G3BP1*si or *G3BP2*si). *ABCA1* protein levels directly correlated with *ABCA1* mRNA expression levels (Fig. 6B). Along the same lines, overexpressing AGO2, STAU1, or *G3BP1/2* had no effect on *ABCA1* mRNA expression when *IGF2BP2* was knocked down, even when expressing miR-33a/b (Fig. 6C). *ABCA1* protein levels correlated with *ABCA1* mRNA expression levels once again (Fig. 6D).

miR-33a/b overexpression in mice results in alterations in apolipoprotein levels. In order to decipher the physiological effects of overexpressing *IGF2BP2* on lipid metabolism, we first overexpressed miR-33a/b in mice and determined *ABCA1* expression and liver lipid composition. These results gave us a control background of what the effects are of miR-33a/b on various lipid species. miR-33a/b was overexpressed using an adenoviral overexpression system through tail vein injection ($n = 3$). Mice received a single injection at day 0 and were sacrificed 7 days postinjection.

FIG 6 Overexpression of *IGF2BP2* cannot restore miR-33a/b-dependent *ABCA1* silencing in cells lacking *AGO2* or *STAU1* expression. (A) qRT-PCR analysis was used to determine relative endogenous *ABCA1* mRNA expression in miR-33a/b-expressing cells transfected with pCMV-*IGF2BP2* (OE) and treated with siRNAs targeting the indicated genes ("si" suffix). *ABCA1* mRNA expression values were compared to *GAPDH* mRNA expression. (B) *ABCA1* protein level was determined in miR-33a/b-expressing cells transfected with pCMV-*IGF2BP2* (OE) and treated with the indicated siRNAs. Actin was used as a loading control. (C) qRT-PCR analysis was used to determine relative endogenous *ABCA1* mRNA expression in miR-33a/b-expressing cells transfected with pCMV plasmids expressing the indicated genes (OE) and treated with *IGF2BP2* siRNA. (D) *ABCA1* protein level was determined in miR-33a/b-expressing cells transfected with pCMV plasmids expressing the indicated genes (OE) and treated with *IGF2BP2* siRNA. Actin was used as a loading control. Con, control. **, $P < 0.001$; ***, $P < 0.0001$. The qRT-PCR data represent the average results of five independent experiments. Values are the means $\pm$ SEM. The Western blot panels represent the average results of five independent experiments.

We observed that overexpressing miR-33a/b in mice resulted in an ~65% reduction in liver *ABCA1* mRNA expression (Fig. 7A) and *ABCA1* protein level (Fig. 7B). Interestingly, reduced *ABCA1* mRNA expression had no effect on serum cholesterol or triglyceride levels (Fig. 7C) but caused significant increases in very low-density lipoprotein (VLDL) and low-density lipoprotein-cholesterol (LDL-C) levels, along with a reduction in the level of high-density lipoprotein (HDL) (Fig. 7D). We did not find any changes in fatty acid composition (Fig. 8A) or liver alkaline phosphatase (ALP), alanine transaminase (ALT), or aspartate aminotransferase (AST) levels (data not shown).

hGFP-IGF2BP2 overexpression in mice results in reduced levels of liver *ABCA1* mRNA expression and protein. We next overexpressed human *IGF2BP2* (hGFP-*IGF2BP2* [where GFP is green fluorescent protein]). Western analyses revealed that mice overexpressing hGFP-*IGF2BP2* produced a GFP-tagged protein of ~90 kDa, which is the approximate molecular weight of an hGFP-*IGF2BP2* chimera (Fig. 9A, adenovirus [Ad]-cytomegalovirus [CMV]-GFP-*IGF2BP2*). We did see an additional protein band in these mice at the approximate weight of green fluorescent protein (GFP) (Fig. 9A, GFP). This

FIG 7 miR-33a/b overexpression in mice results in loss of ABCA1 expression and defects in apolipoprotein homeostasis. (A) Liver RNA was isolated and used for determining ABCA1 mRNA expression by qRT-PCR analysis. Average values were from mice injected with control Ad-CMV or miR-33a/b (Ad-miR-33a/b). (B) ABCA1 protein levels were determined in animals injected with control Ad-CMV or miR-33a/b (Ad-miR-33a/b). (C) Serum cholesterol and triglyceride levels were determined using commercial kits. Filled circles, Ad-CMV; open circles, Ad-miR-33a/b. (D) Apolipoprotein levels were determined using the Lipoprint assay. Filled circles, Ad-CMV; open circles, Ad-miR-33a/b. **, $P < 0.001$; ***, $P < 0.0001$. Values are the means $\pm$ SEM ($n = 3$).

is most likely a degradative product of the *hGFP-IGF2BP2* protein. GFP-expressing vector control mice produced a protein of the approximate molecular weight of GFP (Fig. 9A, Ad-CMV-GFP). The endogenous expression levels of mouse IGF2BP2 were similar in both of these cohorts, indicating that any changes seen in lipid composition were due to overexpression of *hIGF2BP2* (Fig. 9A, IGF2BP2). Based on our Western blot results, we believe that mice overexpressing *hGFP-IGF2BP2* produce an *hGFP-IGF2BP2* chimeric protein.

We found that mice overexpressing *hGFP-IGF2BP2* had an $\sim 80\%$ reduction in ABCA1 mRNA expression in the liver (Fig. 9A), with a concomitant reduction in ABCA1 protein

FIG 8 Overexpression of *hIGF2BP2* in mice has a slight effect on fatty acid composition and $C_{16:0}/C_{18:0}$ ratio. (A) Total liver fatty acid compositions from the three cohorts were determined by analyzing fatty acid methyl esters by GC. Black bars, CMV-GFP vector control cohort; white bars, CMV-IGF2BP2 cohort; gray bars, CMV-miR-33a/b cohort. (B) The C_{16}/C_{18} ratio was determined by using the percentage of total $C_{16:0}$ and $C_{18:0}$ fatty acids.

FIG 9 Overexpressing *IGF2BP2* in mice results in loss of *ABCA1* expression, elevation in serum cholesterol, and defects in apolipoprotein homeostasis. (A) GFP-IGF2BP2 liver protein levels in mice injected with control CMV-GFP (Ad-CMV-GFP) or CMV-GFP-IGF2BP2 (Ad-CMV-GFP-IGF2BP2) were determined. Actin was used as a loading control. (B) qRT-PCR analysis was used to determine the relative endogenous *ABCA1* mRNA expression levels in mice injected with control CMV-GFP (Ad-CMV-GFP) or CMV-GFP-IGF2BP2 (Ad-CMV-GFP-IGF2BP2). (C) Serum cholesterol, triglyceride, and apolipoprotein levels were determined as described in the legend to Fig. 7. Filled circles, Ad-CMV-GFP; open circles, Ad-CMV-GFP-IGF2BP2. (D) Apolipoprotein levels were determined using the Lipoprint assay. Filled circles, Ad-CMV-GFP; open circles, Ad-CMV-GFP-IGF2BP2. **, $P < 0.001$; ***, $P < 0.0001$. (E) Liver enzymes activities were determined. ALP, alkaline phosphatase; AST, aspartate aminotransferase; ALT, alanine aminotransferase. Values are the means $\pm$ SEM ($n = 3$).

(Fig. 9B). Serum cholesterol levels, but not triglycerides, were increased in these mice (Fig. 9C, Ad-CMV-GFP versus Ad-CMV-GFP-IGF2BP2), and these mice had elevated and decreased levels of LDL-C and HDL, respectively (Fig. 9D and E, Ad-CMV-GFP versus Ad-CMV-GFP-IGF2BP2). VLDL levels were unchanged (Fig. 9D, Ad-CMV-GFP versus Ad-CMV-GFP-IGF2BP2). We found that in contrast to mice overexpressing miR-33a/b, those mice overexpressing *hGFP-IGF2BP2* had elevated alkaline phosphatase levels (Fig. 9E, Ad-CMV-GFP versus Ad-CMV-GFP-IGF2BP2).

Finally, we did not see any statistical differences in various fatty acid species among the three cohorts (Fig. 8A) but did find a trend toward a lower $C_{16}:C_{18}$ ratio ($P = 0.056$) between the CMV-GFP vector control cohort and either the CMV-GFP-IGF2BP2 and CMV-GFP-miR-33a/b cohort (Fig. 8B). This type of trend has been reported previously (21).

Mice overexpressing *hIGF2BP2* have increased SREBP signaling. Our data indicate that mice overexpressing *hGFP-IGF2BP2* have elevated serum cholesterol and LDL levels (Fig. 10), suggesting that SREBP signaling was activated. SREBP1 and SREBP2 transcriptional activities are required for fatty acid and *de novo* cholesterol gene expression, respectively (22).

FIG 10 Mice expressing *GFP-IGF2BP2* accumulate mature forms of SREBP1/2 and have elevated lipid protein expression. Protein levels were determined by Western analysis in mice injected with CMV-GFP (Ad-CMV-GFP) or CMV-GFP-IGF2BP2 (Ad-CMV-GFP-IGF2BP2). Actin was used as a loading control.

We found that the livers of *hGFP-IGF2BP2*-overexpressing mice had increased levels of mature SREBP1 (m-SREBP1) protein and an increase in fatty acid synthase protein (FASN) (Ad-CMV-GFP versus Ad-CMV-GFP-IGF2BP2) (Fig. 10). Mature SREBP2 (m-SREBP2) also accumulated, as did its transcriptional target, HMG-coenzyme A reductase (HMGCR) (Fig. 10, Ad-CMV-GFP versus Ad-CMV-GFP-IGF2BP2). Finally, we observed increases in ApoB levels in mice overexpressing *hIGF2BP2* compared to control mice (Ad-CMV-GFP versus Ad-CMV-GFP-IGF2BP2).

DISCUSSION

Using a biochemical proteomic approach, we identified miR-33a/b binding proteins that included IGF2BP2. Our studies showed that IGF2BP2 was required for miR-33a/b silencing of *ABCA1* mRNA expression in HepG2 cells, as knockdown of *IGF2BP2* resulted in the loss of miR-33a/b-dependent silencing, while its overexpression resulted in an increase. Coimmunoprecipitation experiments revealed that IGF2BP2 physically interacted with miR-33a/b and the RISC endonuclease AGO2, suggesting it is an integral part of an AGO2-miR-33a/b-IGF2BP2 complex that binds to the 3'UTR of *ABCA1*, resulting in reduced mRNA expression. Further validation of the existence of this complex arises from the fact that AGO2 was required for *IGF2BP2*- and miR-33a/b-dependent silencing of *ABCA1* mRNA expression and it directly interacted with this miR.

We found that STAU1 had no effect on silencing even though it physically interacted with AGO2. This may be due to the fact that it did not interact with IGF2BP2. STAU1 has double-stranded RNA binding activity and can associate with multiple miRs to regulate mRNA translation (23). The GTPase proteins G3BP1/2 also associated with miR-33a/b but did not physically interact with IGF2BP2. In contrast to STAU1, overexpressing or knocking down G3BP1/2 did have an effect on IGF2BP2-dependent silencing, which may suggest an AGO2-independent role for IGF2BP2 in repressing *ABCA1* mRNA expression. G3BP1 has been shown previously to have a role in the processing of a specific set of miRs that includes miR-1 in cardiomyocytes (24).

Mature forms of SREBP1 and SREBP2 accumulated in mice overexpressing *hIGF2BP2*,

and we observed increases in proteins involved in fatty acid (FASN) and *de novo* cholesterol (HMGCR) biosynthesis. There were concomitant elevations in cholesterol and in VLDL and LDL-C levels and a reduction in HDL levels. Increases in VLDL and LDL-C directly correlated with increased ApoB levels. ApoB binds multiple apolipoproteins (VLDL, LDL-C, chylomicrons), and elevated levels are associated with a higher risk of acquiring cardiovascular disease (25). Interestingly, antisense oligonucleotides targeting ApoB have been successfully used to treat individuals with familial hypercholesterolemia (26).

Mice overexpressing *IGF2BP2* in the liver showed a decrease in the ratio of C_{16}/C_{18} fatty acids (21). These same mice accumulated mature SREBP1, and there was an increase in the mRNA expression of the SREBP1 target gene, fatty acid elongase *ELOVL6*, which is required for C_{18} synthesis. Interestingly, these overexpressing mice had signs of hepatic steatosis. Although not significant, we also saw a downward trend in the C_{16}/C_{18} ratio in mice overexpressing *hIGF2BP2*. *ELOVL6* mRNA expression was not determined in our studies, so we cannot say whether short-term adenoviral overexpression of *hIGF2BP2* did or did not affect its expression. As stated above, *IGF2BP2*-overexpressing mice have steatosis of the liver. Whether short-term overexpression of *hIGF2BP2* also leads to steatosis or even fibrosis awaits liver histology analyses.

GWAS data have established a genetic link between *IGF2BP2* and metabolic disorders, such as obesity, diabetes, and nonalcoholic fatty liver disease (NAFLD)/nonalcoholic steatohepatitis (NASH) (27–29). Multiple murine studies have validated the GWAS data. It has been shown that liver-specific overexpression of *IGF2BP2* causes hepatic steatosis in mice (17, 30, 31), an early pathology seen in NAFLD patients and a major risk factor for progression to a more severe form of fatty liver, NASH (32). Moreover, mice with a total ablation of *IGF2BP2* are (i) resistant to high-fat-diet-induced obesity, (ii) have increased glucose tolerance and insulin sensitivity, (iii) have lower serum cholesterol levels, and (iv) have a higher energy expenditure rate (18). In contrast, however, there is a recent report that shows that older mice with a liver-specific knockout of *IGF2BP2* have reduced fatty acid oxidation and modest elevations in serum glucose and triglycerides when fed a high-fat diet (≥ 6 months) (33). In addition, Greenwald et al. (34) very recently demonstrated that conditional knockout of *IGF2BP2* in pancreatic islet cells of mice fed a high-fat diet attenuated glucose-stimulated insulin secretion. The same authors used Hi-C assays to map chromatin structure and found that risk variants of *IGF2BP2* correlated with reduced chromatin accessibility and gene expression.

ABCA1 itself may have a role in suppressing hepatic steatosis under lipotoxic conditions. Overexpressing ApoA1 in mice fed a methionine-choline-deficient steatotic diet suppressed hepatic steatosis, but only when *ABCA1* was expressed (35). However, it has also been shown that mice lacking liver *ABCA1* were protected from acquiring hepatic steatosis, although these mice did have decreased hepatic insulin signaling (36). We must point out that a subset of our *in vivo* data conflicts with some data reported using *Abca1*^{−/−} mice. In one study, it was found that these mice had reduced serum cholesterol, LDL, and HDL levels but increased numbers of lipid-laden macrophages in their lungs (37). Moreover, it has also been shown that *ABCA1* liver-specific knockout mice have reduced LDL and HDL levels but increased VLDL levels (38). We also saw an elevation in VLDL level, increases in serum cholesterol and LDL levels, and a decreased HDL level when we overexpressed *hIGF2BP2* (Fig. 6). These conflicting data may stem from the fact that *IGF2BP2* regulates pathways other than *ABCA1* gene expression silencing. We also cannot rule out the possibility that *hIGF2BP2* is overexpressed in other tissues involved in lipid metabolism and that some residual *ABCA1* protein remains.

Overall, we have shown that *IGF2BP2* forms a complex with miR-33a/b and AGO2 that negatively regulates *ABCA1* mRNA expression. Reduction in *ABCA1* mRNA expression leads to a reduction in *ABCA1* protein and the lipotoxic accumulation of VLDL and LDL-C apolipoproteins, with a concomitant reduction in HDL. It now becomes important to further define the interaction between *IGF2BP2* and the RISC complex at a more

molecular level. Understanding the biochemistry regulating this complex will not only give rise to a more basic understanding of miR regulation but also give insight into possible new therapies for treating diseases associated with loss of *ABCA1* function.

Conclusion. Reverse cholesterol transport is a complex process whereby peripheral cholesterol is transported back to the liver for metabolism. The ATP binding cassette family protein *ABCA1* is an important factor involved in HDL biogenesis. It initiates HDL biogenesis by transferring phospholipid and cholesterol to delipidated ApoA1, resulting in the formation of nascent HDL particles. *ABCA1* expression is regulated at the posttranscriptional level by the microRNAs miR-33a and miR-33b, which repress *ABCA1* expression. The IGF2BP1/2/3 family of RNA binding proteins regulates RNA localization, stability, and translation. GWAS have linked *IGF2BP2* SNPs to type II diabetes. Using a proteomic approach, we identified IGF2BP2 as a protein that binds miR-33a/b. We have explored this interaction as to whether it has any influence on *ABCA1* expression and, if so, what are the *in vivo* consequences of altered IGF2BP2 expression. We have shown that a miR-33a/b–IGF2BP2–AGO2 complex negatively regulates *ABCA1* expression *in vitro*. We also found that overexpressing IGF2BP2 in mice results in (i) a decrease in *ABCA1* gene expression and protein levels, (ii) defects in apolipoprotein homeostasis and accumulation of VLDL and LDL-C, and (iii) activation of SREBP signaling in the liver. Overall, our data strongly suggest that IGF2BP2 plays an important role in miR-33a/b-dependent silencing of *ABCA1* expression.

MATERIALS AND METHODS

Cell culture studies. HepG2 cells were cultured in MEM medium (supplemented with 10% fetal bovine serum, 1% nonessential amino acids, 1% sodium pyruvate, 1% L-glutamine). Cells were incubated at 37°C with 5% CO₂.

Identification of proteins interacting with various miRs. A biotinylated labeled microRNA pull-down assay (LAMP) was used to identify proteins interacting with individual miRs. HepG2 cell lysates were incubated with individual biotinylated miRs. Protein-miR interacting proteins were UV cross-linked and immunoprecipitated using streptavidin beads. Protein bands were excised from SDS-PAGE gels, and proteins were identified by gas chromatography-mass spectrometry (GC/MS).

Protein determination. HepG2 cells were lysed in radioimmunoprecipitation assay (RIPA) buffer. Proteins concentrations were determined using the Bradford method and Ponceau S staining of nitrocellulose membranes.

Western analysis. Protein samples were subjected to 10% SDS-PAGE and transferred onto nitrocellulose membranes. Immunoblot membranes were blocked in 10% milk and washed once with Tris-buffered saline plus Tween 20 (10 mM Tris [pH 7.4], 0.01% Tween 20). Membranes were incubated with primary antibody overnight. After five washes with Tris-buffered saline plus Tween 20 for 10 min each, membranes were incubated with secondary antibody for 1 h. After five washes with Tris-buffered saline plus Tween 20, membranes were immersed in chemiluminescence agent and exposed for 2 to 5 min; β -actin was used as a loading control. All antibodies were tested for specificity using cells treated with an siRNA specific for each target protein.

Antiactin polyclonal antibodies were from Abcam (ab8227) and used at a 1:1,000 dilution. Anti-*ABCA1* polyclonal antibodies were from Abcam (ab1880) and used at a 1:500 dilution; anti-IGF2BP1 monoclonal antibodies were from Santa Cruz (IGF2BP1 [sc-390149], IGF2BP2 [sc-377014], and IGF2BP3 [sc-365640]), and all were used at a 1:2,000 dilution; anti-AGO2 polyclonal antibodies were from Abcam (ab32381) and used at a 1:750 dilution; anti-STAU1 polyclonal antibodies were from Sigma-Aldrich (SAB1406490) and used at a 1:1,000 dilution; anti-G3BP polyclonal antibodies were from Thermo Fisher (G3BP1 [PA5-29455]; G3BP2 [PA5-53797]) and were used at a 1:500 dilution.

RNA isolation and qRT-PCR. Total RNA was extracted with TRIzol reagent (Invitrogen). cDNA was synthesized from total RNA using an RT² Easy first-strand kit (Qiagen). Quantitative real-time PCR (qRT-PCR) was carried out using a Stratagene MX3005P (Stratagene). Relative *ABCA1* expression levels were normalized to the *GAPDH* level.

Transfection and luciferase reporter assay. The LightSwitch wild-type *ABCA1* 3'-UTR luciferase reporter plasmid was obtained from Switchgear Genomics (Fig. 2A). The plasmid contains two miR-33a/b seed sites (Fig. 2B). The LightSwitch *ABCA1* 3'-UTR mutant plasmid (Fig. 2C) was constructed by mutating 2 nucleotides within the miR-33a/b seed sequences (Fig. 2C, condensed bold letters) using the QuikChange Lightning mutagenesis kit (Stratagene). siRNAs and control siRNA were obtained from Thermo Scientific. pCDNA3.1 was used to overexpress *IGF2BP2*. Cells were incubated in serum-free medium overnight before being transfected with pre-miR-33a/b, control pre-miR, LightSwitch *ABCA1* 3'-UTR reporter plasmid, LightSwitch *ABCA1* 3'-UTR mutant plasmid, *IGF2BP2* siRNA, control siRNA, and pCDNA3.1-*IGF2BP2* using Lipofectamine 2000 (Invitrogen). Luciferase activity was detected 24 h after transfection using the LightSwitch reporter assay system (Switchgear Genomics) as described in the manufacturer's protocol. Pre-miR-33a/b were transfected into cells at a concentration of 25 nM.

Coimmunoprecipitation assays. The ProFound mammalian coimmunoprecipitation kit (Pierce) was used per the manufacturer's instructions. Briefly, antibodies were treated with AminoLink plus coupling

gel slurry containing the beads overnight at 4°C by end-over-end mixing. Antibodies immobilized on beads were incubated with 300 µg of cell lysate at room temperature for 90 min. Beads were washed to remove all unbound proteins. Bound proteins were eluted using an elution buffer supplied with the commercial kit. Eluted proteins were resolved by SDS-PAGE and detected by Western blotting. Beads that were not immobilized with target antibodies served as the control for nonspecific interactions.

Mouse adenoviral studies. C57BL/6N (BL/6) mice were purchased from Jackson Laboratories. The Invivotek Institutional Animal Care and Use Committee (IACUC) approved all experiments. Eight-week-old male mice were injected via the tail vein with 1×10^9 PFU human GFP-IGF2BP2 (*hGFP-IGF2BP2*) or miR-33a/b-expressing adenovirus in phosphate-buffered saline (PBS) (Vector Biolabs, Malvern, PA). At 7 days postinjection, the mice were sacrificed and liver tissue and serum were collected for analysis.

Determination of cholesterol and triglyceride levels. Serum cholesterol levels were measured in BL/6 mice. Blood was collected from the retro-orbital sinus, and cholesterol concentrations were determined enzymatically from serum (Thermo DMA, Arlington, TX). Triglyceride levels were determined using a hand-held meter (CardioChek, PTS Inc., Indianapolis, IN) with test strips specific for triglyceride measurements. An unpaired *t* test was used to analyze differences between different cohorts (*n* = 3).

Determination of apolipoprotein levels. Apolipoprotein levels were measured using the LipoPrint LDL system (LipoPrint LDL subfraction kit, catalog no. 48-7002; Quantimetrix, Redondo Beach, CA) according to the manufacturer's instructions.

Liver fatty acid analysis. Fatty acids were extracted from frozen, homogenized tissue, converted to methyl esters, and analyzed by GC. The retention times of authentic standards (Nu-Chek Prep) were used, or fatty acid peaks were identified by GC/MS analysis.

Determination of liver enzyme activities. Serum was collected by a cardiac bleed. Liver enzymes were determined on an ACE Alera analyzer (Alfa Wasserman, Inc.) according to the manufacturer's instructions.

Statistical analysis. The data shown are the averages from at least five independent experiments. The data are the means $\pm$ standard errors of the mean (SEM). Statistical analysis was performed using Student's *t* test.

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

SUPPLEMENTAL FILE 1, XLSX file, 0.03 MB.

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J.T.N. and M.Y. cowrote the manuscript. J.T.N., M.Y., V.M., M.H., and C.G.-E. designed the experiments and interpreted the data. M.Y., W.L., V.M., and C.G.-E. performed the experiments.

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