The Lipid Droplet Associated Protein Perilipin 3 facilitates hepatitis C Virus-Driven Hepatic Steatosis

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Abbreviations: ADRP, adipocyte differentiation-related protein; ASO, antisense oligonucleotide; ATGL, adipose triglyceride lipase; CGI-58, comparative gene identification 58; DNV, dengue virus; ER, endoplasmic reticulum; HCV, hepatitis C virus; LD, lipid droplet; LXR, liver X
Abstract

Hepatitis C Virus (HCV) is an enveloped RNA virus responsible for 170 million cases of viral hepatitis worldwide. Over 50% of chronically infected HCV patients develop hepatic steatosis, and steatosis can be induced by expression of HCV core protein (core) alone. Additionally, core must associate with cytoplasmic lipid droplets (LD) for steatosis development and viral particle assembly. Given the LD is an important component of hepatic lipid storage, and serves as a platform for HCV particle assembly; this dynamic subcellular organelle is a gatekeeper in the pathogenesis of viral hepatitis. Here we hypothesized that core requires the host LD scaffold protein Perilipin 3 (PLIN3) to induce hepatic steatosis. To test our hypothesis in vivo, we have studied core-induced hepatic steatosis in the absence or presence of antisense oligonucleotide (ASO)-mediated knockdown of PLIN3. PLIN3 knockdown blunted HCV core-induced steatosis in transgenic mice fed either a chow or moderate fat diet. Collectively, our studies demonstrate that the LD scaffold protein PLIN3 is essential for HCV core-induced hepatic steatosis, and provide new insights into the pathogenesis of HCV.

Keywords: Hepatitis, Steatosis, Triglyceride, Lipid Droplet, Virus,
Introduction

The hepatitis C virus (HCV) affects 3% of the world’s population and is a leading cause of end stage liver failure, presenting a considerable global healthcare burden (1). Liver tissue is the primary target for HCV infections, where the virus elegantly co-opts the hepatic lipid metabolic processes to promote viral assembly (2, 3). Steatosis is a hallmark of HCV infection, occurring in 50-70% of chronically infected individuals (4). Importantly, there is an inverse correlation between steatosis and response to antiviral treatment and hepatic steatosis increases the risk for steatohepatitis, liver cirrhosis, and hepatocellular carcinoma (4). Hepatic steatosis is characterized by a pathological accumulation of liver lipids resulting in increases in the size and number of lipid droplets. Although it is well appreciated that HCV infection promotes hepatic steatosis, the exact mechanism is incompletely understood. The HCV core protein (core) is a viral structural protein that serves to form a capsid around the viral RNA genome and has been shown to induce steatosis in various models (5, 6). Core is the first viral protein translated and undergoes two proteolytic processing events at the endoplasmic reticulum (ER) membrane (7). Once core undergoes the second cleavage by signal peptide peptidase, it is able to associate with host cytoplasmic lipid droplets (LD) via a C-terminal binding domain composed of two amphipathic helices separated by a hydrophobic loop (8, 9). The processing events, allowing core to associate with LDs, have been shown to be essential for steatosis formation (10). Once core is associated with the LD, newly synthesized viral RNA is trafficked, by HCV non-structural protein NS5A, to core where core forms a protein capsule (nucleocapsid) around the HCV genome (11). Importantly, nucleocapsid formation by core occurs at the LD, which serves as the primary site of HCV viral particle assembly. The importance of the core-LD interaction is highlighted by independent studies demonstrating that
LDs are required for HCV virus production, and that disruption of core from the LD inhibits virus production (12, 13). There is now unequivocal evidence that the ability of core to interact with host LDs is critical for both viral particle assembly as well as associated hepatic steatosis (10, 12, 13).

Host liver LDs, which are essential for HCV particle production, are dynamic organelles storing neutral lipids within a hydrophobic core. The neutral lipid core, composed mainly of triglycerides and cholesterol esters, is surrounded by a phospholipid monolayer that is decorated by a unique proteome (14). LDs expand and contract based on cellular metabolic demand. When fatty acids are in excess, they can be incorporated into triglycerides (TG) by enzymes such as diacylglycerol acyl transferase (DGAT), which catalyze the terminal step in TG synthesis (15). When the cellular energy state is diminished, lipolytic enzymes can hydrolyze TGs from within the LD to release free fatty acids for beta-oxidation. One of the primary lipolytic enzymes for TG hydrolysis is adipose triglyceride lipase (ATGL), along with its cofactor comparative gene identification 58 (CGI-58), which cooperate to release fatty acids from the hydrophobic TG-rich LD core (16-18). Importantly, enzymes such as the ATGL-CGI-58 complex must gain access to the neutral lipid core of the LD in order to release fatty acids. Notably, certain LD proteins, specifically the PAT family proteins, can act as scaffolding proteins and help regulate LD size by serving as gatekeepers that regulate access to the neutral lipid core and allow for the assembly of lipogenic/lipolytic enzymes (19).

In liver, the two main lipid droplet associated scaffolding proteins are Perilipin 3 (PLIN3), also known as tail-interacting protein at 47kDa or TIP47, and Perilipin 2 (PLIN2), also know as adipocyte differentiation related protein or ADRP (19, 20). PLIN3 is a highly exchangeable protein involved in multiple processes including lipid storage, lipid mobilization, and LD
biogenesis (21, 22). PLIN2 is a non-exchangeable protein that is degraded if displaced from the LD (23). Expression levels of PLIN2 and/or PLIN3 on the LD surface can vary in response to changing cellular metabolic needs to alter TG storage levels in the cell (24-27) by regulating accessibility of enzymes involved in lipid synthesis or lipolysis. Interestingly, previous evidence has shown that expression of core alters the normal composition of LD scaffolding proteins. Core expression in hepatoma cells resulted in increased PLIN3 expression and decreased PLIN2 expression (28). Furthermore, core expression has been shown to cause a redistribution of LDs from the cell periphery to the perinuclear region, which is associated with the displacement of PLIN2 from redistributed LDs (29). The observation that core expression alters important LD scaffolding protein expression and changes the LD proteome may highlight a mechanism involved in core-induced steatosis, which is the focus of the current study.

Previous studies have shown that core’s association with the LD is an indispensable part of HCV replication and persistence making it an attractive target for discovery of new targets to inhibit HCV infections (12, 13, 30). Although the core-LD association is a critical part of the HCV life cycle, it is still unclear as to how the viral protein interacts with the LD resulting in a pathological accumulation of lipids in the liver. In order to understand mechanisms involved in HCV-mediated pathogenesis we have focused our studies on core-induced hepatic steatosis in vivo, given that hepatoma cell models have dramatically altered lipid metabolic processes when compared to primary hepatocytes. Core serves as a critical component in the viral life cycle of HCV and by defining how core interacts with the LD we should be able to identify innovative targets to disrupt core’s association with the LD and impair HCV infections. Given core expression has been shown to increase expression of the LD scaffolding protein PLIN3 (28), we hypothesized that PLIN3 is an important component for HCV core protein to
induce steatosis. To test our hypothesis we have generated a hepatocyte-specific HCV core transgenic mouse model, and studied core-induced steatosis with normal or diminished levels of PLIN3.

Materials and Methods

Generation of HCV core transgenic mice

Although HCV genotype 3a is most closely related to hepatic steatosis risk in people, we chose to use genotype 1b to allow comparisons to the vast majority of cell biology work with HCV core. The full-length coding sequence of HCV core (genotype 1b) was subcloned from the pCAGC191 vector (31) into the MluI- and Clal-digested pLiv11 vector (32). The pLiv11 vector contains the human apolipoprotein E promoter along with a 3’-hepatic control region, which selectively drives transgene expression in hepatocytes (32, 33). The pLiv11-HCVcore transgenic cassette was separated from the vector backbone by digestion with restriction enzymes NotI (5’) and SpeI (3’). This linearized vector was then microinjected into fertilized embryos of B6D2 F1J mice. PCR was used to confirm presence of the HCV core gene, apoE promoter, and hepatic control region (data not shown) in founder mice. Genotyping was performed by PCR analysis on genomic DNA isolated from ear snips as previously described (34) using primers specific for the core construct as follows: primer 1, 5’-GAG CAC AAA TCC TAA ACC CCA AAG-3’, and primer 2, 5’-GAT GGT CAA ACA GGA CAG CAG AG-3’. From this effort, we established three independent founder strains with low (HCVcore$^{Tg29}$), medium (HCVcore$^{Tg15}$), or high (HCVcore$^{Tg3}$) core protein expression.
Animal studies

At the age of 6-8 weeks, subgroups of mice were fed *ad libitum* with either chow or a moderate fat diet (MFD) containing 20% of energy as lard and added cholesterol, 0.1% (wt/wt), for a total of 6 or 8 weeks. Mice treated with ASO, received 50 mg/kg of either PLIN3 ASO or control (non-targeting) ASO via intraperitoneal injection once a week (total dose of 50 mg/kg per week) for 6-8 weeks. Second-generation ASOs were synthesized by Ionis Pharmaceuticals (Carlsbad, CA) and formulated in PBS (35). The PLIN3 ASO, Ionis 409003 (5’-CACAGTGTTGTCTAGGGCCT-3’), is a second-generation oligonucleotide that incorporates several chemical modifications to improve potency, duration of action, and tolerability. All of the internucleotide phosphates are chemically modified with a phosphorothioate substitution, in which one of the nonbridging oxygen atoms is substituted with sulfur. Additionally the compound incorporates five 2’-O-(2-methoxyethyl) (2’-MOE) modified ribonucleosides at the 3’ and 5’ ends with ten 2’-O-deoxyribonucleosides in between to support RNaseH-1 mediated target mRNA degradation. These modifications improve the binding affinity for target mRNA as well as stability against exonuclease-mediated degradation. A control oligonucleotide, Ionis 141923 (5’-CCTTCCCTGAAGGTTCCTCC-3’), contains the same chemical modifications, with no complementarity to known genes, including lipid droplet proteins (26). For LXR agonist and fasting studies, 6-week-old C57BL/6 mice were fed *ad libitum* with chow for a period of 6 weeks while receiving treatment with either Control or PLIN3 ASO’s. For studies with LXR agonist, T0901317 was suspended in a vehicle containing 1.0% carboxymethylcellulose and 0.1% Tween 80. For a period of seven days mice were gavaged once daily with either vehicle or 25 mg/kg T0901317 as previously described (36, 37). For fasting studies, subgroups of mice were fasted for 18 hours prior to necropsy.
All mice used in the studies were housed in a pathogen-free barrier facility at Wake Forest University School of Medicine or the Cleveland Clinic with the approval of the American Association for Accreditation of Laboratory Animal Care. The Institutional Animal Care and Use Committee from Wake Forest University or the Cleveland Clinic approved all protocols before execution of the studies.

**Immunoblotting and real-time PCR**

Proteins were resolved by SDS-PAGE, transferred to PVDF membrane (Millipore), and were detected after incubation with indicated antibodies using LiCor Odyssey Infrared Imaging system. Antibodies used include: anti-protein disulfide isomerase (PDI) rabbit polyclonal (Cell Signaling #2446), anti-glyceraldehyde-3-phsophate dehydrogenase (GAPDH) rabbit monoclonal (Cell Signaling #5174), and anti-α tubulin mouse monoclonal (Cell Signaling #3873) and anti-perilipin 3 rabbit polyclonal (Proteintech #10694-1-AP). The monoclonal HCV core antigen antibody (C7-50) was obtained from Thermo Scientific. Densitometry was determined using Image Studio Version 4.0.21. Tissue RNA extraction and quantitative PCR was conducted as previously described (38, 39). Cyclophilin A was used for an invariant control and expression levels were calculated based on the $\Delta\Delta Ct$ method. qPCR was conducted using the Applied Biosystems 7500 Real-Time PCR System. All qPCR primers are available upon request.

**Liver histology**

Portions of livers were fixed in 10% buffered formalin and processed for hematoxylin and eosin (H&E) staining by the Clinical Pathology Lab in the Department of Pathology at
Hepatic lipid quantification

Liver lipid extracts were made and triglyceride, total cholesterol, free cholesterol, and cholesterol ester were measured using detergent solubilized enzymatic assays as previously described (34, 38, 40).

Plasma Lipid and Lipoprotein Analyses

Plasma triacylglycerol levels were quantified enzymatically (L-Type TG M, Wako Diagnostics, Richmond, VA, USA). Plasma lipoproteins were fractionated by size from 2.5µl of individual plasma samples using a Superose 6 PC 3.2/30 column (GE Healthcare Bio-Sciences AB, Uppsala, Sweden) followed by on-line determination of triglycerides and cholesterol as previously described (41). The lipid concentrations of the different lipoprotein fractions were calculated after integration of individual chromatograms.

Liver lipid droplet isolation

Hepatic lipid droplets were isolated by sucrose gradient centrifugation essentially as described in (42). Approximately 100mg of tissue was minced with a razor blade on a cold surface. Minced tissue was transferred to a Potter-Elvehjem homogenizer, and then 200µl of 60% sucrose was added to the tissue sample and incubated on ice for 10 min. Next, 800µl of lysis buffer was added and mixed then incubated on ice for 10 min. Samples were homogenized with five strokes of Teflon® pestle and transferred to a 2 ml centrifuge tube.
600uL of lysis buffer was carefully layered on top of homogenate and centrifuged for 2 h at 20,000 g at 4°C. The tube was then frozen at -80°C freezer then cut at the 1000uL mark. The bottom piece of the centrifuge tube contained the non-lipid droplet fraction, which was allowed to thaw before being transferred to a new tube. The lipid droplet fraction was collected by cutting an ~4-6mm piece from the top of the ice cylinder and placed in a new 2mL tube. To increased purity of lipid droplet fraction, this process was repeated once more. Briefly, 200uL of 60% sucrose was added to the lipid droplet fraction. Next, 800uL of lysis buffer was added and mixed followed by carefully layering with 600uL of lysis buffer then centrifuged for 2 h at 20,000 g at 4°C. After freezing at -80°C the tube was cut and the lipid droplet fraction was collected by cutting an ~4-6mm piece from the top of the ice cylinder and placed in a new tube. Protein analyses was performed using the modified Lowry assay as previously described (43). Lipids in the LD fraction were extracted according to the Folch method (44), then determined using detergent solubilized enzymatic assays as previously described (34, 38, 40).

Statistical analyses

All graphs were plotted by GraphPad Prism 6.0e (45). Unless indicated, data are expressed as the mean ± S.E.M., and were analyzed using either a one-way or two-way analysis of variance (ANOVA) followed by Tukey’s post hoc analysis using JMP version 10.0.2d1 software (SAS Institute, Cary, NC) as previously described (36, 46).

Results
Expression of HCV core protein in liver induces a dose-dependent increase in liver lipid accumulation

In order to study hepatic steatosis induced by HCV core protein (core) in vivo, we generated multiple lines of mice with hepatocyte-specific transgenic expression of core. To ensure hepatocyte specific expression of core, we inserted the full-length sequence, of genotype 1b, into the pLiv11 vector (21). We used the sequence of genotype 1b since it is the most common genotype in chronic HCV infections (47). After backcrossing founder mice to a C57BL/6 background we analyzed liver lysates for expression of core and identified three distinct HCV core transgenic lines with increased core expression (Fig. 1A-B): HCVcore\textsuperscript{Tg29} (low core expression); HCVcore\textsuperscript{Tg15} (moderate core expression); and HCVcore\textsuperscript{Tg3} (high core expression). To examine the effect of core expression on liver lipid accumulation we performed biochemical analysis of liver triglycerides (Fig. 1C) and found that HCVcore\textsuperscript{Tg15} and HCVcore\textsuperscript{Tg3} had significantly increased liver triglycerides relative to WT mice, with a 2.29-fold and 4.23-fold increase, respectively. While there was only a slight decrease in body weight (Fig. 1D), only reaching significance in HCVcore\textsuperscript{Tg3} mice relative to control, there is increased liver size in both HCVcore\textsuperscript{Tg15} and HCVcore\textsuperscript{Tg3} mice (Fig. 1E). H&E staining on fixed liver sections also showed a dose-dependent increase in liver lipid accumulation with increasing levels of HCV core protein (Fig. 1F).

HCVcore\textsuperscript{Tg15} mice exhibit hepatic steatosis, which is exacerbated after feeding a moderate fat diet.

Initial studies were performed to determine the optimal dietary background to study HCV core-induced hepatic steatosis. In order to differentiate between core-induced steatosis
and diet-induced steatosis we elected to feed mice a moderate fat diet (MFD) containing 20% kcal from lard and 0.1% added cholesterol (w/w) or maintained on a chow diet for six weeks (Fig. 2). There was no significant difference in body weight between wild-type littermates (WT) and HCVcoreTg15 on chow or MFD although both groups had 5-12% increased body weight after 6 weeks of MFD (Fig. 2A). Additionally, MFD feeding did not alter liver size in WT mice, measured by liver to body weight ratio, however HCVcoreTg15 mice consistently displayed a larger liver by 26% on chow and 74% on MFD (Fig. 2B). In addition, we found that HCVcoreTg15 mice had a significant increase in liver triglycerides after addition of MFD with a 4.64-fold increase relative to WT mice on MFD (Fig. 2C). Western blot analysis showed HCVcoreTg15 mice had 30% increased PLIN3 expression on chow diet compared to WT, however HCVcoreTg15 mice had a 2-fold increase in PLIN3 expression relative to WT mice on MFD (Fig. 2D-F). H&E staining of liver sections confirmed the increase in liver triglyceride storage in HCVcoreTg15 mice compared to WT, which was greatly exacerbated by MFD feeding as seen by an increase in LD size and number (Fig. 2G).

ASO-mediated knockdown of PLIN3 expression decreases steatosis in HCVcoreTg15 mice on a chow and moderate fat diet.

Next, to test the importance of PLIN3 expression in HCV core-induced hepatic steatosis, we used antisense oligonucleotides (ASO) to knockdown expression of PLIN3. We observed PLIN3 ASO reduced expression of liver PLIN3 protein to ~12-25% of the respective control ASO groups (Fig. 3A-B), however HCVcoreTg15 mice given PLIN3 ASO did have 2.4-fold increased expression relative to WT mice on PLIN3 ASO (Fig. 3B). On Control ASO, HCVcoreTg15 mice had 51% more liver triglycerides than WT mice, however knockdown of
PLIN3 reduced liver triglycerides to similar levels of WT mice given Control ASO (Fig. 3C). Additionally, PLIN3 ASO reduced liver triglycerides in WT mice by approximately 66% (Fig. 3C). Interestingly, despite the significant decreases in liver triglycerides in both WT and HCVcore<sup>Tg15</sup> mice, there was no decrease in liver size (Fig. 3D). In fact, HCVcore<sup>Tg15</sup> mice had a small but significant 10% increase in liver/body weight ratio when given PLIN3 ASO (Fig. 3D). Liver sections with H&E staining did show some decreased lipid storage, however sections were mostly unremarkable from mice on chow (Fig. 3E).

Our previous studies showed the MFD exacerbated the steatosis phenotype in HCVcore<sup>Tg15</sup> (Fig. 2) so we wanted to determine the effect of PLIN3 ASO on this exacerbated phenotype. Analysis of protein expression showed that HCVcore<sup>Tg15</sup> mice had a 2.19-fold increase in PLIN3 expression compared to WT mice, however both genotypes had expression levels reduced to 25-34% of that of WT mice when given PLIN3 ASO (Fig. 3F-G). On Control ASO, HCVcore<sup>Tg15</sup> mice had an approximately 3-fold increase in liver triglycerides compared to WT (Fig. 3H). Importantly, knockdown of PLIN3 significantly reduced liver triglycerides by almost 50% in HCVcore<sup>Tg15</sup> mice. WT mice receiving PLIN3 ASO also had reduced liver triglycerides however this did not reach significance. Interestingly, while both groups did have reduced liver triglycerides with PLIN3 ASO treatment, there was no change in liver size with transgenic mice maintaining an approximately 50% increase in liver/body weight ratio (Fig. 3I). Liver sections with H&E staining showed that HCVcore<sup>Tg15</sup> mice on Control ASO had much greater lipid accumulation compared to WT mice, as evidenced by increased lipid droplet size and number, however treatment with PLIN3 ASO completely blunted this effect (Fig. 3J), which was confirmed by a pathologist histological examination (data not shown).
Antisense Oligonucleotide-mediated knockdown of PLIN3 expression decreases steatosis in HCVcore\textsuperscript{Tg3} mice on chow diet.

In order to confirm our results we performed a similar study in a separate line of HCV core transgenic mice using HCVcore\textsuperscript{Tg3} (high core expression) fed chow. Female HCVcore\textsuperscript{Tg3} mice were maintained on a chow diet for eight weeks while receiving biweekly injections with either a non-targeting Control ASO or an ASO directed against PLIN3. As expected, HCVcore\textsuperscript{Tg3} mice had higher levels of PLIN3 mRNA in the liver (2.1 fold increased) compared to WT (Fig. 4A). However, both WT and HCVcore\textsuperscript{Tg3} receiving PLIN3 ASO had significantly reduced PLIN3 mRNA which was ~5-10% of that in respective Control ASO treatment groups (Fig. 4A). Western blotting confirmed knockdown of PLIN3 protein expression in both WT and HCVcore\textsuperscript{Tg3} mice receiving the PLIN3 ASO (Fig. 4B). Interestingly, HCVcore\textsuperscript{Tg3} mice with PLIN3 knockdown had a 38% reduction in liver triglycerides, and triglyceride mass was statistically similar to that of WT mice (Fig. 4C). There was no significant difference between body weight of WT and HCVcore\textsuperscript{Tg3} on either ASO treatment regimen (data not shown), however, HCVcore\textsuperscript{Tg3} mice exhibited an ~2-fold increase in liver/BW ratio compared to WT mice, which was not altered by PLIN3 knockdown (Fig. 4D). Next, to assess the effect of PLIN3 knockdown on liver morphology, we performed H&E staining on fixed liver sections and observed increased steatosis in HCVcore\textsuperscript{Tg3} mice compared to WT mice in the Control ASO group (Fig. 4F). Remarkably, HCVcore\textsuperscript{Tg3} mice receiving PLIN3 ASO had diminished steatosis and lipid accumulation that was comparable to WT mice (Fig. 4F). Collectively, knockdown of PLIN3 effectively blunted HCV core-induced hepatic steatosis in our transgenic mice that have the highest amount of core expression.
Knockdown of PLIN3 reduces hepatic steatosis due to LXR agonist but not steatosis due to fasting.

Given that treatment with PLIN3 ASO effectively diminished hepatic steatosis due to core protein expression and moderate fat diet, we wished to address whether knockdown of PLIN3 could prevent hepatic steatosis that occurred by alternative mechanisms. To do this we first used the synthetic LXR agonist T0901317 to induce steatosis after treating male C57BL/6 mice with either Control or PLIN3 ASO for 6 weeks. Upon examination of liver triglycerides, Control ASO-treated mice receiving the LXR agonist had a greater than 6-fold increase in liver triglycerides (Fig. 5A). However, when mice given the LXR agonist were treated with PLIN3 ASO there was a significant reduction of liver triglycerides by 56%. Next, we tested whether PLIN3 knockdown would have an effect on hepatic steatosis induced fasting. After treating male C57BL/6 mice with either Control or PLIN3 ASO for 6 weeks we fasted subgroups of mice for 18 hours. As expected, in the control ASO-treated group, fasted mice had a 5-fold increase in liver triglycerides compared to fed mice (Fig. 5B). Amazingly, treatment with PLIN3 ASO had no effect on liver triglycerides in fasted mice with similar values to the control ASO group. Overall these results indicate that ASO-mediated knockdown of PLIN3 selectively reduces hepatic steatosis due to certain conditions.

Knockdown of PLIN3 alters plasma VLDL and HDL lipids.

Expression of core in the liver has previously been shown to impair the secretion of VLDL (48) therefore we wanted to examine whether knockdown of PLIN3 had an effect on plasma lipoprotein profiles in our mice. To do this we examined the mass of lipids in different fractions of lipoproteins isolated by HPLC in male WT and HCVcoreTg15 mice fed a moderate
fat diet (Fig. 6). Overall, both WT and HCVcore$^\text{Tg15}$ mice treated with PLIN3 ASO had significantly reduced plasma triglycerides, primarily due to a decrease in the VLDL fraction (Fig. 6A-C). Additionally, HCVcore$^\text{Tg15}$ mice did show a trend for slightly increased LDL triglycerides in the Control ASO group, however there were no significant changes in LDL or HDL triglycerides within groups (Fig. 6D-E). Interestingly, when looking at lipoprotein fractions of total cholesterol, HCVcore$^\text{Tg15}$ mice seemed to have increased amounts of intermediate particles, seen as small LDL/large HDL (Fig. 6F). While WT mice treated with PLIN3 ASO have an overall trend for decreased plasma total cholesterol, VLDL particles from these mice had increased total cholesterol while HDL cholesterol trended to decrease (Fig. 6E-J). Overall, these data indicate that knockdown of PLIN3 reduces overall plasma triglycerides while having an inverse effect on plasma total cholesterol between WT and HCVcore$^\text{Tg15}$ mice on a moderate fat diet.

**PLIN3 knockdown reduces core expression on the lipid droplet.**

Given that both PLIN3 and HCV core interface with LDs, we examined LD proteins in core transgenic mice with normal or reduced PLIN3 levels. To do this we performed LD isolation from livers of HCVcore$^\text{Tg15}$ mice fed a MFD. As expected, in the LD fraction we saw an enrichment of LD proteins, PLIN3 and core, compared to the non-LD fraction and whole liver (Fig. 7A). Furthermore, the LD fraction contained little to no expression of the endoplasmic reticulum protein PDI, or the cytosolic protein GAPDH. Additionally, administration of PLIN3 ASO resulted in substantial knockdown of PLIN3 in all three fractions. To examine the changes in LD proteins more closely, we performed western blot on the LD fraction of individual animals (Fig. 7B). Consistently, PLIN3 protein expression is dramatically
decreased in the LD of WT and HCVcore\textsuperscript{Tg15} animals with PLIN3 ASO treatment. Interestingly, HCVcore\textsuperscript{Tg15} mice have a significant reduction of 44% in expression of core on the LD when treated with PLIN3 ASO (Fig. 7C). Both WT and HCVcore\textsuperscript{Tg15} have approximately 85-91% reduced expression of PLIN3 on the LD (Fig. 7D). Intriguingly, HCVcore\textsuperscript{Tg15} mice have a 3.85-fold increase in CGI-58 expression compared to WT animals in the Control ASO group, however knockdown of PLIN3 reduces the expression of CGI-58 on the LD to similar levels between HCVcore\textsuperscript{Tg15} and WT animals (Fig. 7E). Analysis of lipids extracted from the liver LD fraction show HCVcore\textsuperscript{Tg15} mice given control ASO have an overall significant increase in triglycerides, phosphatidylcholine, total cholesterol, free cholesterol and esterified cholesterol (Fig. 7F-J).

**HCV core-induced hepatomegaly does not involve PLIN3**

Although PLIN3 knockdown protects against HCV core-induced hepatic steatosis (Fig. 3-4), hepatomegaly driven by HCV does not depend on PLIN3 (Fig. 8). HCVcore\textsuperscript{Tg15} mice exhibit significant increases in liver weight (Fig. 8A) even when normalized to body weight (Fig. 8B). However, PLIN3 knockdown does not alter HCV core-induced hepatomegaly (Fig. 8A-B). To examine PLIN3-independent mechanisms underlying HCV core-induced hepatomegaly we examined hepatic glycogen storage and activation of transcriptional programs that are known to regulate liver size (49, 50). HCVcore\textsuperscript{Tg15} mice have modestly increased levels of hepatic glycogen, which are significantly elevated with PLIN3 ASO treatment (Fig. 8C). Given the key role that the Hippo/Yap/Taz pathway plays in liver size (49), we examined the hepatic expression of key genes driven by this pathway. HCVcore\textsuperscript{Tg15} mice did not have significant increases in Hippo/Yap/Taz target genes (Cyr61, NF2, or Gata4), yet PLIN3 knockdown was
unexpectedly associated with decreased expression of Hippo/Yap/Taz target genes (Fig. 8D-8F). Another key transcriptional pathway known to regulate liver size is activation of the nuclear hormone receptor pregnane X receptor (PXR) (50) and HCVcoreTg15 mice exhibited marked increases in several PXR targets genes (Fig. 8G-8I). Collectively, these data suggest that HCV core-induced hepatomegaly is independent of PLIN3, and may involve increased glycogen storage and aberrant activation of PXR signaling.

Discussion

Here we demonstrate that transgenic expression of HCV core results in increased expression of the lipid droplet-associated protein PLIN3, in vivo. Furthermore, HCV core-induced hepatic steatosis is diminished by ASO-mediated knockdown of PLIN3. This result was confirmed in two distinct HCV core transgenic lines and under different dietary conditions. Additionally, we provide evidence that core expression is associated with increased expression of PLIN3 on the LD and that knockdown of PLIN3 expression results in a significant decrease in core expression on the LD. Lastly, knockdown of PLIN3 expression selectively reduces steatosis due to core expression and LXR agonist treatment, but has no effect on fasting-induced fatty liver. Overall, these studies reinforce the significant role the LD plays in HCV-mediated pathogenesis, and identifies the host LD protein PLIN3 as a factor driving HCV core-induced pathology.

It is well known that core expression alone is sufficient to induce hepatic steatosis (5), a common feature of chronically infected HCV patients. Uncovering the exact mechanism of core-induced steatosis has broad clinical implications, but is still not completely understood. Multiple mechanisms by which core induces steatosis have been proposed such as: increased
lipid synthesis (51); decreased lipolysis (10); decreased fatty acid oxidation (52); and
decreased secretion of lipids via very low density lipoproteins (53). Although core expression
can alter many lipid metabolic pathways one important aspect to consider is that core must
associate with LDs in order to stimulate LD accumulation and steatosis (9, 10). Previous work
by the Ott group showed core's association with cytosolic LDs blocked the turnover of TG
resulting in steatosis (10). In a later study examining lipid storage and release from LDs in
murine liver and cell culture, the group conclusively showed that core expression primarily
resulted in decreased TG hydrolysis and this required the lipolytic enzyme ATGL (54).
Paradoxically, in vitro studies revealed that although core inhibits lipolysis at LDs, core
expression was associated with increased abundance of the TG hydrolase complex ATGL-
CGI-58 on the LD (34). Based on this evidence, the authors concluded that core altered
properties of the LD, resulting in enhanced and prolonged interaction between ATGL and CGI-
58, thereby preventing access of the complex to TG within the LD (54). Similar to these cell-
based findings, we have found that transgenic expression of HCV core in vivo results in
increased LD-associated CGI-58 (Fig. 7E). Furthermore, HCVcore^{Tg15} mice treated with PLIN3
ASO had reduced CGI-58 expression on the LD, however this may be part of an overall trend
for decreases in LD protein expression on isolated liver LD with PLIN3 knockdown.

Although CGI-58 expression is not increased on the LD of HCVcore^{Tg15} mice treated
with PLIN3 ASO, it is possible that core expression causes an imbalance in localization of
scaffolding proteins on the LD, which prevents access of lipolytic enzymes to TG within the LD.
Previous evidence in cell models show that core expression alters the expression of LD
scaffolding proteins (28, 29) and our results confirm, in vivo, that liver PLIN3 expression is
increased in addition to increased PLIN3 on the LD (Fig. 7). Knocking down expression of
PLIN3 in HCVcore\textsuperscript{Tg} mice possibly restores the balance of LD scaffolding proteins, allowing for lipolytic enzymes to access the neutral lipid core. The Farese and Walther group have recently shown the importance of protein crowding on determining LD protein composition. Their seminal study showed that LD proteins compete for limited binding sites on the LD surface, and during lipolysis, the LD binding affinity plays a large role in determining localization (55). Additionally, this work suggests there is a competition between LD proteins, when the LD surface is at a steady state, where increasing levels of proteins with high affinities can change the composition of LD proteins and PAT proteins could possibly serve a regulatory function related to crowding (55). Accordingly, it seems reasonable to postulate that core outcompetes other LD proteins for the limited space on the LD due to the high affinity of core for the LD. As core expression on the LD increases it causes a change in the LD protein composition, which we observed as expression of the PAT family member PLIN3 increased. These changes likely alter the normal LD protein composition and prevent lipolytic enzymes from accessing lipids within the LD, resulting in lipid accumulation and steatosis formation.

Importantly, our studies show that only certain types of hepatic steatosis are resolved by PLIN3 knockdown. In addition to HCV core-induced hepatic steatosis, we have show that PLIN3 ASO effectively blunts LXR agonist stimulated fatty liver (Fig. 5A). Additionally, previous work has shown that PLIN3 ASO effectively reduces diet-induced fatty liver in addition to improving insulin sensitivity and glucose tolerance (26). Interestingly, knockdown of PLIN3 expression had no significant effect on hepatic steatosis due to fasting (Fig. 5B). It is likely that the source of lipids that accumulate in the liver play a major role in the type of LD scaffolding proteins that accumulate on the LD. Activation of LXR in the liver leads to an increase in genes involved in lipid synthesis such as SREBP-1, FAS and ACC (56).
Additionally, HCV core expression has also been shown to increase lipogenesis to some degree (51, 57). Previous studies using PLIN3 ASO have demonstrated a decrease in liver Gpat and Dgat2 expression (26), which are two major enzymes required for the incorporation of de novo synthesized fatty acids into monoacylglycerol and triacylglycerol, respectively (58, 59). Interestingly, PLIN3 ASO treatment was only shown to have a significant impact on Dgat2. This is unique because while both DGAT1 and DGAT2 are able to synthesize TG, only DGAT2 can be found localized to the surface of LD (15) and DGAT2 primarily synthesizes TG from endogenous fatty acids while DGAT1 plays a greater role in incorporating exogenous fatty acids into TG (58). Additionally, previous evidence shows that lipogenic enzymes can relocalize to the LD in order to facilitate LD growth (60). It is possible that PLIN3 is an important factor for localization of lipid synthetic enzymes on the LD, and knockdown via PLIN3 ASO reduces the incorporation of lipids into the LD and blunts steatosis.

While knockdown of PLIN3 does significantly reduce HCV core- and LXR agonist-induced hepatic steatosis, there seems to be no impact on fatty liver that results from prolonged fasting. Previous work has shown that fasting seems to have little impact on PLIN3 expression in the liver, whereas PLIN2 is substantially upregulated in mice fasted for 24 hours (61). This possibly indicates that PLIN3 is primarily essential for the incorporation of de novo synthesized lipids whereas PLIN2 may be important in the storage of lipids taken up as a result of adipose lipolysis. Regardless of the mechanism, it is important to recognize that PLIN3 ASO effectively reduces many types of hepatic steatosis, in addition to improving insulin sensitivity, and may provide an effective means in treating patients affected by non-alcoholic fatty liver disease (NAFLD). HCV patients who develop steatosis respond poorly to treatment (4), and reduction of steatosis may be able to augment certain treatment regimens.
Furthermore, due to the evidence that hepatic steatosis may be considered a prerequisite for enhanced liver disease (62), PLIN3 ASO therapy might provide treatment against cirrhosis and hepatocellular carcinoma (HCC) although further studies are required.

While therapy with PLIN3 ASO may provide an effective means against many types of fatty liver disease, it is also critical to consider this as an alternative treatment for HCV-infected patients. Importantly, previous studies demonstrate that the association of core on the LD is essential for the assembly of infectious HCV particles (12, 13), and our data show that knockdown of PLIN3 expression significantly reduces core expression on the LD (Fig. 7B-C). Furthermore, studies using HCV infectious cell culture systems show PLIN3 is associated with released HCV particles (63), and silencing PLIN3 effectively abrogates the assembly and release of infectious viral particles (64, 65). Although recently developed direct acting antiviral treatments are very effective, Scheel and Rice point out that even if current therapies were to achieve a success rate of 95% in all infected patients, there would still be more than 10 million individuals requiring treatment (66). The statement points to the need for innovative cures incorporated to include HCV infections of all genotypes, in various disease states, and circumvent the appearance of drug-resistant strains. Furthermore, even though new direct acting antiviral treatments are successful, there is evidence that treatment with direct acting antivirals may enhance the prevalence of HCC (67), although studies are conflicting (68, 69).

Overall, by taking advantage of ASO technology, we demonstrate the importance of the LD scaffolding protein PLIN3 in a mouse model of HCV core-induced hepatic steatosis. Our studies further clarify mechanisms underlying the association of core with host LDs, and show core induces a change in LD proteins, in vivo. The change in LD proteins may be a major factor in the importance of the core-LD association for HCV particle assembly by forming a
stable assembly platform. Due to the importance of LD binding affinity for determination of protein localization on LDs, it seems reasonable why the core sequence is so highly conserved across multiple HCV genotypes (70, 71). Additionally, while core has not been shown to directly interact with PLIN3, previous in vitro studies have shown that PLIN3 is critical for trafficking of NS5A to LDs (65). Furthermore, it has been determined that the functionally similar capsid of dengue virus binds to LDs via PLIN3 (72). Since PLIN3 knockdown diminished HCV core-driven hepatic steatosis, coupled with the importance of the lipid droplet for assembly of HCV viral particles, it is tempting to speculate that knocking down PLIN3 may hold therapeutic benefit for other viruses that co-opt similar LD-associated viral particle assembly. Collectively, this works demonstrates that targeting host LD-associated proteins may be an effective means to prevent HCV-induced pathology. Although effective anti-HCV therapies are currently available (66), this work provides additional broader implications for other pathogens that use the LD for enhanced persistence (73).
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Figure Legends and Figures

Figure 1. Expression of HCV core protein in liver induces a dose-dependent increase in liver lipid accumulation. Male mice from each line maintained on a chow diet until 6-8 weeks of age. A: Western blot analysis of liver homogenate of each transgenic line from B: Quantitative analysis of liver immunoblot. C: Enzymatic determination of triglycerides from liver lipid extract (n=3-7 per group). All hepatic lipid values were normalized to tissue weight. D, E: Total body weight and liver size (expressed as a ratio to body weight) at necropsy. F: Liver sections of each line were stained with H&E for morphological analysis (20X Magnification). Data shown represent mean ± SEM. Levels not connected by same letter are significantly different.

Figure 2. HCVcore\textsuperscript{Tg15} mice have considerable increase in liver triglycerides after feeding with moderate fat diet. At 6 weeks of age, male WT or HCVcore\textsuperscript{Tg15} mice were placed on chow or moderate fat diet (20% kcal lard, 0.1% cholesterol) for a period of 6 weeks. A, B: Total body weight and liver size (expressed as a ratio to body weight) at necropsy. C: Enzymatic determination of triglycerides from liver lipid extract (n=4-5 per group). D: Western blot analysis of liver homogenates from mice fed chow or moderate fat diet. E, F: Quantitative analysis of liver immunoblot for core (E) and PLIN3 (F). G: Liver sections of chow and diet fed mice were stained with H&E for morphological analysis (20X Magnification). Data shown represent mean ± SEM. Levels not connected by same letter are significantly different.

Figure 3. ASO-mediated knockdown of PLIN3 expression decreases steatosis in HCVcore\textsuperscript{Tg15} mice on chow and moderate fat diet. At 6 weeks of age, male WT and
HCVcoreTg15 mice were treated with either control or PLIN3 ASO for 6 weeks while maintained on either a chow or moderate fat diet. A-B: Western blot (A) and quantitative analysis (B) of PLIN3 in liver homogenates from mice fed chow. C-D: Enzymatic determination of triglycerides from liver lipid extract (C) and liver size (expressed as a ratio to body weight) at necropsy from mice fed chow (D). E: Representative pictures of H&E staining performed on fixed liver sections (20X Magnification) from mice fed chow. F-G: Western blot (F) and quantitative analysis (G) of PLIN3 in liver homogenates from mice fed moderate fat diet. H-I: Enzymatic determination of triglycerides from liver lipid extract (H) and liver size (expressed as a ratio to body weight) at necropsy from mice fed moderate fat diet (I). J: Representative pictures of H&E staining performed on fixed liver sections (20X Magnification) from mice fed moderate fat diet. All hepatic lipid values were normalized to tissue weight. Data shown represent mean ± SEM. Levels not connected by same letter are significantly different.

Figure 4. ASO-mediated knockdown of PLIN3 expression decreases steatosis in HCVcoreTg3 mice on chow diet. At 8 weeks of age, female WT and HCVcoreTg3 mice were treated with either control or PLIN3 ASO for 8 weeks while maintained on a chow diet. A: Relative levels of liver PLIN3 mRNA was quantified by real-time PCR, normalized to levels of Cyclophilin A, and expressed relative to levels in WT mice given control ASO (n=4 per group). B: PLIN3 protein expression determined by western blot analysis of liver homogenates. C: Enzymatic determination of triglycerides from liver lipid extract (n=4-6 per group). D: Liver size (expressed as a ratio to body weight) of mice at necropsy. E: Measurement of plasma triglycerides determined enzymatically. F: Representative pictures of H&E staining performed on fixed liver sections (20X Magnification). All hepatic lipid values were normalized to tissue...
weight. Data shown represent mean ± SEM from four to six mice per group. Levels not connected by same letter are significantly different.

Figure 5. Knockdown of PLIN3 reduces LXR agonist-induced hepatic steatosis but not steatosis due to fasting. A: Liver triglycerides were measured enzymatically from extracts made from male C57BL/6 mice fed a chow diet and treated with either a control or PLIN3 ASO for 6 weeks. During the last week of treatment, mice were also gavaged orally with either a vehicle or exogenous LXR agonist (T0901317) (n=5 per group). B: Enzymatic determination of liver triglycerides from lipid extracts made from male C57BL/6 mice fed a chow diet and treated with either a control or PLIN3 ASO for 6 weeks and necropsied following an 18 hour fast (n=5 per group).

Figure 6. Knockdown of PLIN3 alters plasma VLDL and HDL levels. Plasma from male WT and HCVcore Tg15 mice, 6 weeks of age, treated with either control or PLIN3 ASO for 6 weeks while maintained on a moderate fat diet (n=4-6 per group). A: Plasma lipoprotein profile of triglycerides. B: Total plasma triglycerides. C: Total plasma VLDL triglycerides. D: Total plasma LDL triglycerides. E: Total plasma HDL triglycerides F: Lipoprotein profile of plasma total cholesterol. G: Total plasma cholesterol. H: Total plasma VLDL cholesterol. I: Total plasma LDL cholesterol. J: Total plasma HDL cholesterol

Figure 7. Decreased core accumulation on hepatic lipid droplets of HCVcore Tg15 mice with ASO-mediated knockdown of PLIN3. Lipid droplet isolation from liver of male WT and HCVcore Tg15 mice, 6 weeks of age, treated with either control or PLIN3 ASO for 6 weeks while
maintained on a moderate fat diet (n=3 per group). A: Western blot analysis was performed on fractions. B-E: Western blot analysis was performed on liver lipid droplet fraction of individual animals and quantitative analysis was performed, relative to the WT control group, on expression of core (C), PLIN3 (D), and CGI-58 (E). F-J: Lipids were extracted from lipid droplet fractions and lipid content was determined enzymatically for triglycerides (F), phosphatidylcholine (G), total cholesterol (H), free cholesterol (I), and esterified cholesterol (J). Data shown represent mean ± SEM. Levels not connected by same letter are significantly different.

Figure 8. HCV core-induced hepatomegaly does not involve PLIN3. At 8 weeks of age, male WT and HCVcoreTg15 mice were treated with either control or PLIN3 ASO for 8 weeks while maintained on a moderate fat diet. A-B: Total liver weight (A) and liver size (expressed as a ratio to body weight) of mice at necropsy (B). C: Enzymatic determination of glycogen from liver extracts and normalized to tissue weight (n=5 per group). D-F: Relative levels of liver mRNA was quantified by real-time PCR, normalized to levels of Cyclophilin A, and expressed relative to levels in WT mice given control ASO (n=5 per group). G-I: At 6 weeks of age, male WT or HCVcoreTg15 mice were placed on chow or moderate fat diet for a period of 6 weeks and relative levels of liver mRNA was quantified by real-time PCR, normalized to levels of Cyclophilin A, and expressed relative to levels in WT mice given control ASO (n=4-5 per group). Data shown represent mean ± SEM. Student’s t-test analysis was performed. *Significantly different from WT within each group (p<0.05). †Significantly different from WT in Control ASO group (p<0.05).
Figure 1
Figure 2

A

Body Weight (g)

Chow Diet

WT Tg15

B

Liver/BW Ratio

Chow Diet

C

Liver TG (mg/g)

Chow Diet

WT Tg15

D

HCVcore PLIN3 α-Tubulin

Chow Diet WT Tg15 WT Tg15

E

HCVcore/α-Tubulin

Chow Diet

F

PLIN3/α-Tubulin

Chow Diet

G

WT HCVcore Tg15

Chow Diet

Diet
Figure 3
Figure 4

A. PLIN3 mRNA levels in WT and Tg3 mice.

B. Western blot analysis of HCVcore, PLIN3, and α-Tubulin in Control ASO and PLIN3 ASO groups.

C. Liver TG levels (mg/g) in WT and Tg3 mice.

D. Liver/BW ratio in WT and Tg3 mice.

E. Plasma TG levels (mg/dL) in WT and Tg3 mice.

F. Histological images of liver tissue in WT and HCVcore^Tg3^ mice treated with Con ASO and PLIN3 ASO.
Figure 5
Figure 8

A. Liver Weight (g)  
- Con, WT vs. PLIN3, Tg

B. Liver Body Weight
- Con, WT vs. PLIN3, Tg

C. Liver Glycogen (µg/mg)
- Con, WT vs. PLIN3, Tg

D. Cyp3a11 mRNA
- Con, WT vs. PLIN3, Tg

E. Gata4 mRNA
- Con, WT vs. PLIN3, Tg

F. Mdr1a mRNA
- Con, WT vs. PLIN3, Tg