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HCV Core Protein affects Lipid Metabolism in a Genotype-Dependent Manner

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HCV Core Protein affects Lipid Metabolism in a Genotype-Dependent Manner

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HCV Core Protein affects Lipid Metabolism in a Genotype-Dependent Manner

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Chronic HCV infection is the leading cause of steatosis (fatty liver disease) and hepatocellular carcinoma (HCC). The virus establishes a chronic infection in 70% of patients and infects approximately 71 million people worldwide. Genotypes (gt) 1 and 3 are the most prevalent, with gt3 HCV being associated with more severe disease. It is known that core protein plays a role in the development of steatosis, but the precise mechanism is not yet understood. In this study, we investigate the role of genotypes in core-mediated differential regulation of lipid metabolism. Prior studies described the lipid induction by HCV infection or ectopic expression of core derived from gt1a and 3a HCV qualitatively. The goal of this thesis is to define the gt1a and 3a HCV core-mediated lipid regulation in a quantitative manner. Using FACS analysis, and immunofluorescence analysis by using confocal microscope, we found that gt3a core protein induces larger lipid droplet formation, although the quantity of lipids remains similar to that induced by gt1a core protein. We then attempted to determine the difference in fatty liver-associated gene expression levels induced by gt1a and gt3a core proteins. To do this, we utilized microarray to analyze the gene expression in cells transfected with different HCV core proteins. The analysis showed that both gt1a and gt3a core upregulated SOCS3; this upregulation has

been shown to influence the liver response to previously used antiviral therapies, as well as the induction of insulin resistance. Additionally, genes that were downregulated by gt1a and gt3a core are largely associated with insulin resistance, which plays a role in the development of diabetes. Interestingly, more genes appear to be downregulated by gt3a than gt1a core protein, which could suggest that gt3a core protein induces insulin resistance and steatosis in a different manner, or to a more severe degree. Overall, these results present a potential mechanism for the relationship between insulin resistance and lipid metabolism deregulation induced by gt1a and gt3a core proteins.

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LIST OF ABBREVIATIONS

HCV	Hepatitis C Virus
CHC	Chronic Hepatitis C
HCC	Hepatocellular carcinoma
GT	Genotype
LD	Lipid Droplet
LVP	Lipoviroparticle
VLDL	Very Low-Density Lipoprotein
LDL	Low-Density Lipoprotein
APOB	Apolipoprotein B
APOE	Apolipoprotein E
MTP	Microsomal Triglyceride Transferase Protein
NAFLD	Non-alcoholic Fatty Liver Disease
NASH	Non-alcoholic steatohepatitis
FACS	Fluorescence-Activated Cell Sorting
SOCS3	Suppressor of cytokine signaling 3
E1/E2	Envelope protein 1/2
FAS	Fatty acid synthase
FOXO1	Forkhead box O1
PCK2	Phosphoenolpyruvate Carboxykinase 2
CEBPB	CCAAT Enhancer Binding Protein Beta
SERPINE1	Serpin Family E Member 1

PI3KR1	Phosphoinositide-3-Kinase Regulatory Subunit 1
IGFBP1	Insulin Like Growth Factor Binding Protein 1
HSPG	Heparan suflate proteoglycan

INTRODUCTION

Chapter 1

HEPATITIS C VIRUS AND STEATOSIS

Hepatitis C virus (HCV) is a widespread virus that infects an estimated 71 million people worldwide [1], causing chronic disease in 70-85% of patients [1, 2]. This chronic disease causes steatosis (fatty liver disease), cirrhosis, and hepatocellular carcinoma [3]. Although there is an effective treatment available, many barriers still prevent its widespread application.

Steatosis occurs at an average of 55% in patients infected with chronic HCV (CHC) [4]. HCV genotypes have also been shown to play a role in the severity of steatosis [5]. Genotype 3 (gt3) HCV is associated with steatosis at a higher frequency than other genotypes, in about 73% vs. 50% of infected patients, respectively [4, 6]. Steatosis occurs when there is an accumulation of lipids (specifically triglycerides) within the cell. This state can be found in a number of liver diseases, including non-alcoholic steatohepatitis (NASH), non-alcoholic fatty liver disease (NAFLD), and HCV infection [7]. Metabolic steatosis has been associated with gt1 HCV and is often accompanied by other comorbidities such as obesity, hyperlipidemia, or diabetes mellitus [7]. However, gt3 HCV has been associated with “viral fat” [4], or viral steatosis, which is alleviated by treatment for HCV.

The lipids induced in the disease state steatosis are used in infectious HCV particle assembly [8]. HCV proteins, such as the structural protein core, have been shown to surround the lipid droplets (LD) and sequester them for their role in virus particle maturation. The LDs are an essential part of the HCV life cycle and are a component of the mature virus particle. HCV uses LDs in their infectious forms, lipoviroparticles (LVPs). These LVPs are a hybrid between virus particles and lipoproteins. Lipoproteins are proteins that bind to lipids and play roles in lipid metabolism within and outside the cell. They are categorized into 5 major categories: chylomicrons, very-low-density lipoproteins (VLDL), intermediate-density lipoproteins (IDL), low-density lipoproteins (LDL), and high-density lipoproteins (HDL). The liver is a major source of VLDL assembly and secretion into the circulation, where they are later catabolized into IDLs by lipoprotein lipases, which hydrolyzes the triglycerides in the core [9]. Triglycerides are packaged in droplets surrounded by apolipoproteins to form VLDL to move lipids from the liver to other organs. HCV LVPs have been shown to contain apolipoproteins like apoE and apoB [10, 11]. Surprisingly, not only the liver-specific isoform of apoB (apoB-100) is found in subsets of LVPs—in fact, intestinally-derived isoforms of apoB (apoB-48) have been found on the surface of LVPs, suggesting that lipoproteins on the virions could be exchanged [12]. Lipoproteins have been shown to play a role in HCV attachment to cell surfaces, as well as in the evasion of the immune system.

HCV LIFE CYCLE AND THE VLDL PATHWAY

HCV is a hepatotropic virus that belongs to the *Flaviviridae* family. It is an enveloped, positive-sense single-stranded RNA virus with a 9.6kb genome consisting of 5' and 3' non-coding regions surrounding a single open reading frame [10] that encodes for a single polyprotein [13, 14]. HCV utilizes an internal ribosome entry site (IRES) for translation of the viral polyprotein, which is processed by the host and viral proteases into 10 proteins—3 structural (core, E1, and E2) and 7 nonstructural (p7, NS2, NS3, NS4A, NS4B, NS5A, NS5B) [15].

The HCV life cycle includes attachment, entry, uncoating, assembly, and release. HCV enters cells by utilizing multiple entry factors, including claudin-1, occludin, SR-B1, and CD81 on the hepatocyte surface [13]. The viral RNA is then released into the cytoplasm [13], where it is translated into a polyprotein. This polyprotein is then processed at the ER [13], and the nonstructural proteins NS3, NS4A, NS4B, NS5A and NS5B form the replication complex [10]. The E1 and E2 HCV glycoproteins are transported into the ER, while core protein remains in the cytoplasm [16]. HCV core protein coats the lipid droplet surface via its domain 2 [17], and, through an unknown mechanism, is recruited to the virus assembly site in the ER detergent resistant membrane (DRM). The subsequent HCV assembly and virion maturation steps are unique in that the HCV assembly step includes the fusion of HCV immature virions with lipoproteins to form a lipoviroparticle (LVP) [2]. Molina *et al.* reported that a lower density LVP is more infectious than that of higher density indicating that increased lipidation of virus particles enhances viral infectivity [18].

As mentioned above, the LVP is a very low-density particle that contains a virus component and a lipoprotein component. The lipoprotein component contains apolipoproteins (apo) E and B [19]. ApoE is a key lipoprotein that aids in HCV entry by interacting with SR-B1 and low-density lipoprotein receptor (LDL-R) [20]. ApoE and apoB are major components of the very low-density lipoprotein (VLDL), which suggests that HCV hijacks the VLDL secretion pathway and utilizes nascent VLDLs [2, 21]. HCV has been shown to interact with the VLDL pathway through the modulation of genes such as microsomal triglyceride transferase protein (MTP) [22]. HCV core protein has been shown to affect the synthesis of MTP and subsequently impact VLDL maturation [23]. The importance of lipid synthesis pathways in the formation and export of HCV LVPs has been shown throughout the literature, and many qualities of LVPs reflect those of mature VLDLs.

ApoB, which is essential for VLDL secretion, has been found on mature LVPs, which supports the hypothesis that the VLDL pathway is hijacked by HCV. ApoE, found on the LVP surface, was shown to be incorporated by HCV particles during maturation, and plays a role in HCV entry into hepatocytes [24, 25] by binding to heparan sulfate proteoglycans (HSPGs) on the cell membranes [25]. In the absence of ApoE, the literature shows that HCV particles are unable to bind to the cell surface and subsequently infect hepatocytes. Surprisingly, the presence of ApoB is not necessary for entry, although it is present on the LVP surface. An argument can be made that although ApoB is not necessary for LVP entry, it is required for LVP formation.

The secretion of ApoB is regulated by MTP in the context of HCV assembly [26]. Specifically, HCV core protein is responsible for the decrease of MTP functionality during

HCV infection [27]. However, Gastaminza *et al*. showed that HCV assembly and secretion are reliant on MTP function [11]. These conflicting reports suggest that core protein may inhibit MTP until a certain point to prevent VLDL secretion, to redirect this pathway later toward HCV assembly and secretion. Regardless, this core-mediated MTP inhibition likely impacts the VLDL pathway leading to reduced VLDL secretion, which may contribute to steatosis [11]. However, different HCV genotypes may have differential effects on the VLDL pathway, since while HCV gt3 infection was associated with hypobetalipoproteinemia, which suggests a decrease in the secretion of VLDLs [28, 29], HCV gt1b infection led to increased ApoE levels in the serum [20].

HCV CORE PROTEIN AND LIPID METABOLISM

By using the liver samples, Campana *et al.* demonstrated that gt1a and gt3a HCV infection did not alter the frequency of lipid droplet-containing cells [30]. However, compared to gt1a HCV infection, gt3a HCV infection was associated with higher histological steatosis grades and larger LDs within hepatocytes, consistent with previous reports [30, 31]. Importantly, ectopically expressed gt3a core was shown to induce larger LDs compared to those induced by gt1b core [32]. The similar induction of *in vivo* HCV infection-mediated lipid phenotype by the core expression in cell culture indicates that core is mainly responsible for disrupting host lipid metabolism [33] and likely plays a major role in the development of steatosis in the liver. To support this notion, Moriya *et al.* demonstrated that transgenic mice expressing HCV core induced hepatic steatosis [34]. The cause of more severe steatosis induction by gt3a HCV remains unclear. One potential mechanism could be the gt3a core-mediated preferential enrichment of cholesterol esters

in LDs compared to that from other genotypes [35]. Another mechanism suggested was the higher up-regulation of fatty acid synthase (FAS) promoter by gt3a core than gt1b core [36]. FAS plays a role in the synthesis of triglycerides in hepatocytes and modulating the FAS expression has led to the development of steatosis [37].

CLINICAL IMPORTANCE

As described above, the HCV genotype is a contributing factor to the severity of steatosis. Adinolfi *et al.* showed that patients with significant steatosis had higher fibrosis scores than non-steatotic patients [38]. Additionally, patients infected with gt3a had higher steatosis scores than those infected with non-gt3 HCV [38]. Gt1 HCV has been associated with metabolic steatosis, correlated with other preexisting factors such as obesity, diabetes, and alcohol usage. Gt3 HCV has been associated with viral steatosis, in which the steatosis correlates to the levels of viremia [6, 38]. Therefore, antiviral treatment for gt3 HCV could be sufficient to alleviate steatosis, while lifestyle changes in addition to antiviral treatment might be necessary to treat the steatosis in non-gt3 HCV infected patients [4].

Bugianesi *et al.* showed that HCV and non-alcoholic fatty liver disease (NAFLD) pathology is similar in regard to steatosis [5], but the liver fibrosis scores from chronically gt3 HCV infected patients are significantly higher than those from NAFLD patients [5]. They also found that while metabolic factors are typically associated with NAFLD, none of those metabolic factors affected steatosis in chronic gt3 HCV livers. The hypobetalipoproteinemia in HCV infected patients was associated with a significant increase of steatosis compared to those without [29]. Additionally, hypobetalipoproteinemia was associated preferentially with gt3a HCV infection compared

to other groups, and gt3a viral clearance partially corrected the steatosis and hypobetalipoproteinemia [29]. These results support the hypothesis of viral steatosis caused by gt3 HCV infection as opposed to metabolic steatosis.

GOAL AND IMPORTANCE

The goal of this thesis is to analyze the effect of gt1a and gt3a HCV core proteins on steatosis induction, specifically paying attention to gene regulation and the implications of said gene regulation. Additionally, in this thesis, we discuss the potential mechanisms leading to differential LD size, focusing on large LDs induced by gt3a core protein.

HCV core protein has been shown to interact with proteins involved in the VLDL secretion pathway for the formation of HCV-LVPs [10]. However, it is unknown how HCV core plays a role in this process, or whether the VLDL secretion pathway is differentially affected by different genotypes of the core. The literature review suggests that there are potentially many mechanisms by which core protein deregulates lipid metabolism for the benefit of the virus. For instance, in one particular study, MTP mRNA was decreased as a result of high concentrations of HCV proteins within the cell [39]. This in turn facilitates the accumulation of lipids within the cell, contributing to steatosis. Beside MTP, we anticipate that HCV core protein regulates the function of other genes associated with lipid metabolism to enhance HCV assembly and egress capabilities. *We hypothesize that HCV core protein affects hepatic steatosis development through alteration of lipid metabolism via differential gene regulation in a genotype-dependent manner.* It is important to determine whether there are differential mechanisms of steatosis induced by different genotypes of core, as this would further our knowledge of core protein's effect on lipid

metabolism. It is important to note that knowledge of these mechanisms would potentially contribute to identifying novel targets to treat the HCV-induced metabolic diseases, including steatosis, insulin resistance, etc.

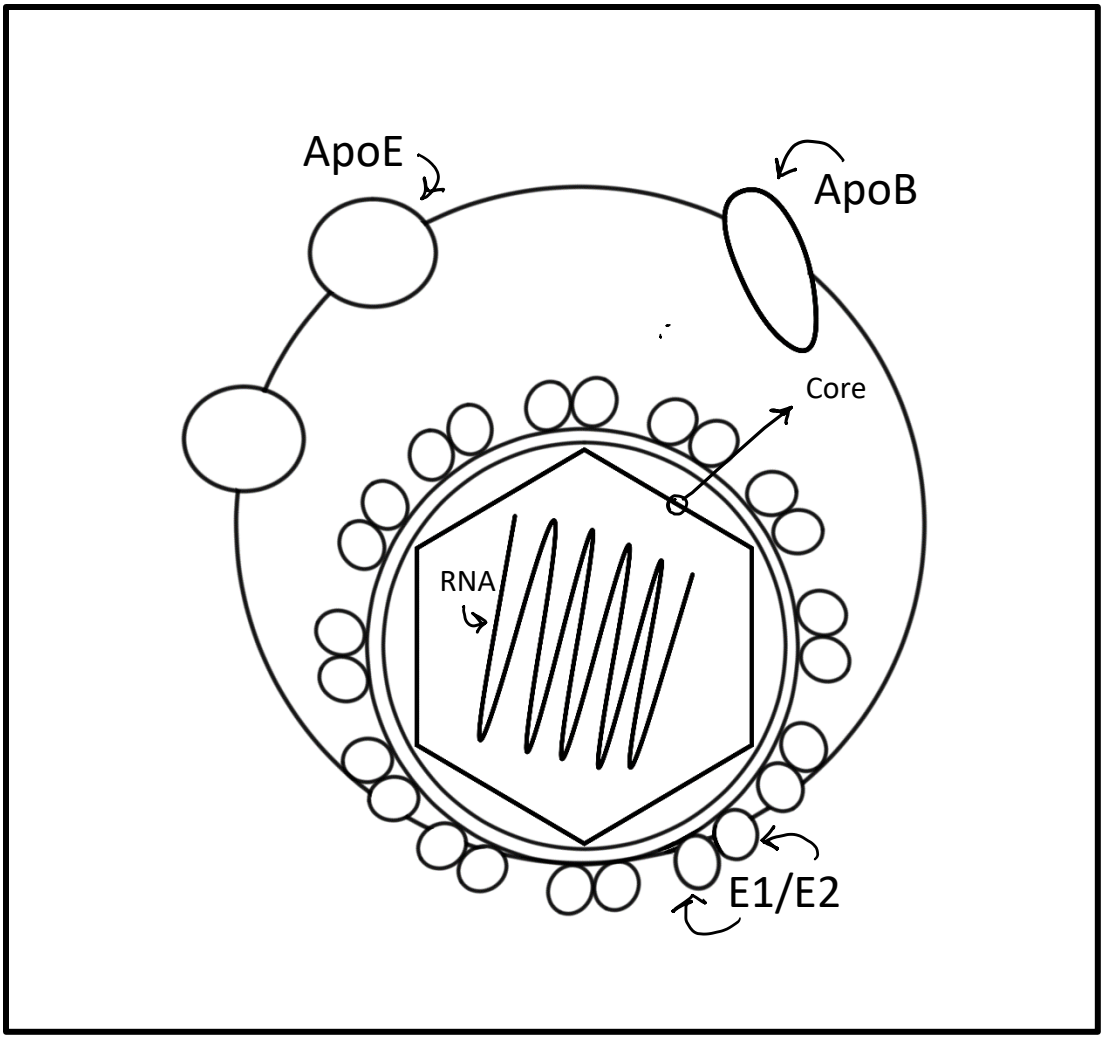

Figure 1. Mature lipoviroparticle (LVP).

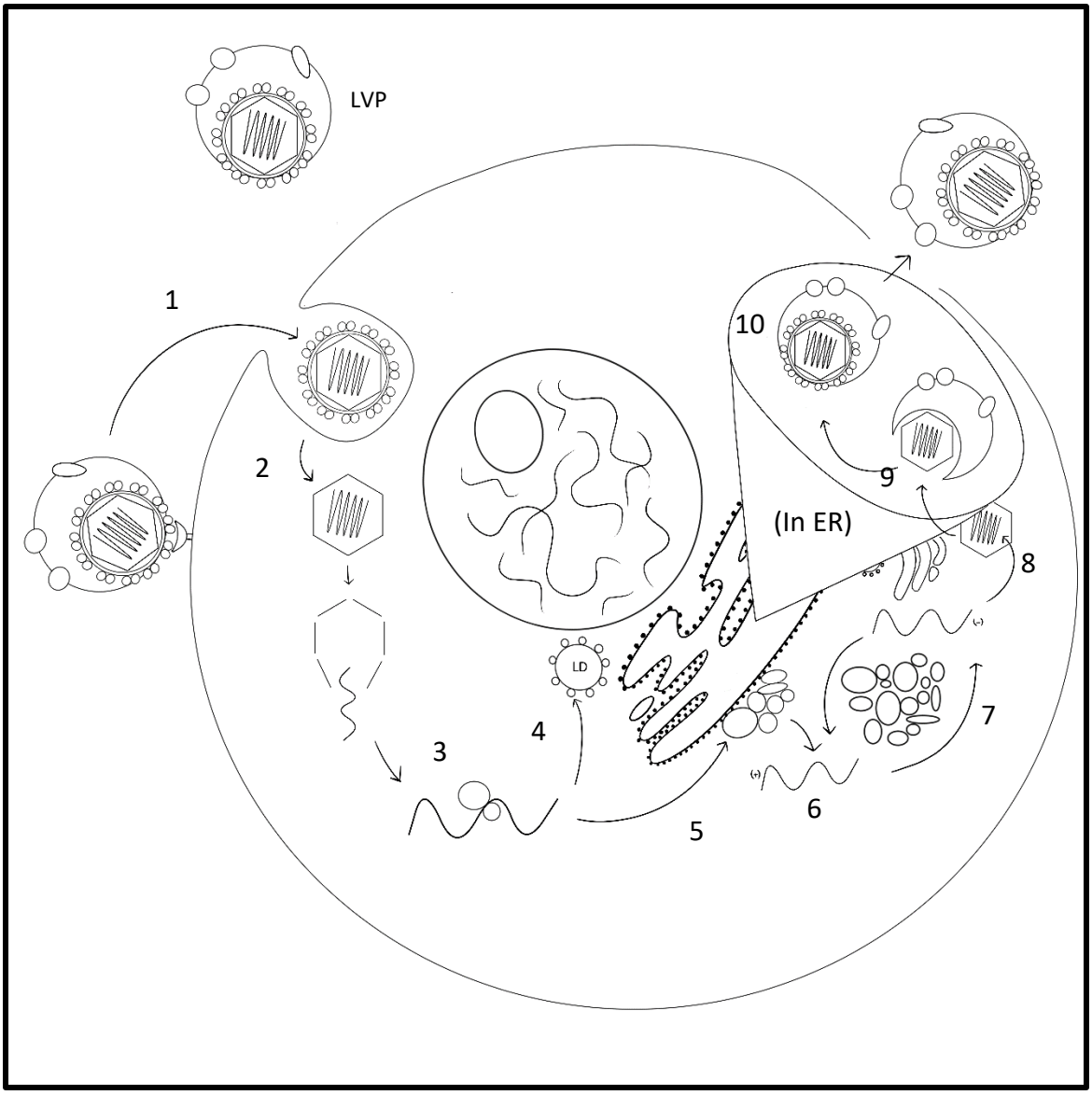

Figure 2. HCV Life Cycle. 1) Virus entry via cell receptors and endocytosis. 2) Uncoating. 3) Translation. 4) The core protein surrounds lipid droplet. 5) NS proteins form replication complexes on the ER membrane. 6) (+) and (-) sense RNA synthesized. 7) on the membranous web. 8) Nucleocapsid assembly. 9) Envelopment and budding into the ER. 10) Release from ER followed by maturation before secretion.

METHODS

Chapter 2

TRANSFECTION

8-well chamber slide or 12- plates were seeded with a hepatocellular carcinoma cell line (FT3-7) in Dulbecco's modified Eagle's minimal essential medium (DMEM, Gibco™) containing 10% fetal bovine serum (FBS, Gibco™) and transfected (once cells reached 90-100% confluency) with HCV gt1a core, HCV gt3a core, or BIND vector expressing plasmid, according to Mirus TransIT®-IL1 protocol (Mirus). A mixture of plasmid DNA, Mirus TransIT®-IL1 reagent, and Opti-MEM reduced serum medium (Gibco™) was incubated according to protocol with the following modifications: transfection solution was incubated for 30 min at room temperature, added to cells and incubated for an additional 4 minutes before covering in fresh DMEM+10% FBS. For 8-well chamber slide, following 24 hours post-transfection, the medium was replaced with 10% FBS-DMEM containing streptavidin/penicillin.

CHOLESTEROL IFA

FT3-7 cells seeded in 8-well chamber slide or 6-well plates were transfected with HCV gt1a core, HCV gt3a core, or vector expressing plasmid. A mixture of plasmid DNA, Mirus TransIT®-IL1 reagent, and Opti-MEM reduced serum medium (Gibco™) was incubated according to protocol with the following modifications: reagent was incubated at 30 min benchtop and added to the cells following the addition of DMEM+10% FBS. After 24

hours, the medium was replaced with Serum-free DMEM (Gibco™) supplemented with our without TopFluor Cholesterol stain (Avanti Polar Lipids, Inc). The cells were washed once with PBS and harvested with Accutase after an additional 24 hours.

CELL LYSIS AND PROTEIN EXTRACTION

FT3-7 cells were transfected with HCV gt1a or gt3a core plasmid to overexpress HCV core protein. After 48 hours, cells were harvested and lysed (on ice) with a solution of 1% Triton-X-100, EDTA (pH 8.0), and 100X Protease inhibitor. The cells were incubated on ice for 10 minutes, then harvested and resuspended in Eppendorf 1.5mL tubes. The tubes were then centrifuged at 12,000rpm at 4℃ for 5 minutes, and the supernatant was collected and stored at -80C.

WESTERN BLOTTING

HCV gt1a and gt3a core protein containing cell lysates were thawed on ice and mixed 1:3 with 4X Loading Buffer. The samples were then heated at 100℃ for 10 minutes, centrifuged down and loaded into a 12% SDS-PAGE gel. The gel was run for 30 min at 50V, then run at 110V for 1 hr 20 minutes. The gel was then washed, and the proteins were transferred to a membrane at 100V for 1 hr in a cold room or an ice-cooled condition. The membrane was then removed and blocked with a 1:1 mixture of Blocking Buffer (LI-COR) and PBS for 1 hr with agitation. The membrane was then incubated with anti-HCV core antibody (Invitrogen) at 1:1000 dilution in PBS for 4 hours benchtop with agitation or overnight at 4℃. Following incubation with the primary antibody, the membrane was then washed in PBS-T three times for 10 minutes per wash. After washing step, the membrane was placed in anti-mouse 800 at 1:10000 dilution for 1 hour at room temperature in darkness and gentle rocking. Following incubation with the secondary antibody, the

membrane was washed as described above. Images were obtained with Odyssey imager and analyzed using Odyssey imaging software.

FACS ANALYSIS

At 48h post-transfection, FT3-7 cells were harvested using Accutase® cell detachment solution (Innovative Cell Technologies, Inc.). The cells were then centrifuged at RT for 5 min at 2500rpm. Following centrifugation, the Accutase was then aspirated from each tube. The cells were then washed with PBS by adding 200ul of PBS to resuspend the cells and centrifuged at RT for 5 min at 2500rpm. PBS was then aspirated and cells were permeabilized and fixed with 200ul of a 4% paraformaldehyde (PFA) and 0.01% Triton-X-100 solution at RT for 20 min. Cells were then centrifuged at 2500rpm for 5 min in swinging bucket centrifuge at 4℃. Cells were washed with 1ml PBS by aspirating the liquid and resuspending the cells in PBS, then centrifuging in swinging bucket centrifuge for 5 min at 2500rpm. This was done twice. Then, the cells were stained with 200ul of 1:2000 diluted anti-HCV Core antibody overnight at 4℃. Cells were washed twice using the above method, and then stained with 1:1000 diluted AlexaFluor anti-mouse 488 (Invitrogen) fluorescent stain for 1-2 h benchtop. After washing 2X with PBS the above method, cells were stained with 200ul of 1:10000 diluted LipidTOX neutral lipid stain. Cells were washed once and resuspended in PBS, kept in darkness until imaged using C6 Flow Cytometer.

IMMUNOFLUORESCENCE AND CONFOCAL MICROSCOPY

Cholesterol-stained cells: FT3-7 cells in an 8-well chamber plate were pre-stained with 1:1000 diluted TopFluor Cholesterol in Serum-Free DMEM overnight. After one wash with PBS, they were then fixed with 4% paraformaldehyde, permeabilized with 0.1%

Triton-X-100 for 10 min, washed twice and stained with 1:1000 diluted anti-HCV Core Ab (Invitrogen) overnight and 1:1000 diluted AlexaFluor 405 secondary antibody (Invitrogen) for two hours. Following 2X washes, cells were stained with 1:1000 LipidTOX Neutral Lipid stain (Invitrogen). Cells were washed, mounted using DAPI-Free Fluoromount-G mounting solution, and imaged with Confocal Microscopy.

LipidTOX-only stained cells: FT3-7 cells seeded in an 8-well chamber plate were transfected with gt1a core, gt3a core, and ApoE-GFP, with a BIND vector negative control. The cells were fixed after 48 hours with 4% paraformaldehyde and permeabilized with 0.1% Triton-X-10. After 2X washes, cells were covered with 1:1000 diluted anti-HCV Core Ab overnight. The following day the slide was stained with 1:1000 diluted AlexaFluor 405 secondary antibody for 1-2 hours benchtop. Following two washes with PBS, the cells were then stained with 1:1000 diluted LipidTOX neutral lipid stain. Cells were then washed with PBS and mounted with DAPI-Free Fluoromount-G mounting solution. After allowing time to solidify, cells were imaged with confocal microscopy. Antibodies were diluted with a solution of PBS and 3% BSA. LipidTOX was diluted in PBS.

RNA EXTRACTION

350ul of RW1 buffer was added to the RNeasy column (Qiagen) and centrifuged for 1 min at top speed (17k x g). Flow-through was discarded. 500ul of RPE was added to the column and incubated for 1 min. The column was then centrifuged at top speed for 1 min. Flow-through was discarded and the collection tube changed. 500ul of RPE was added to the column and then centrifuged at top speed for 1 min. Flow-through was discarded. The column was then centrifuged at top speed with an empty column for 2 minutes. Following centrifugation, 50ul of Nuclease-free water was added to the column and incubated for 4

minutes. Then the column was centrifuged at top speed for 2 minutes. The concentration was then measured, and the RNA integrity was analyzed by using an agarose gel.

IMAGEJ AND STATISTICAL ANALYSIS

All confocal images were imaged using ImageJ software, including calculating the size through the total area of each lipid droplet within each boundary. Cell counting was performed through the ImageJ software by calculating the number of individual droplets detected and data were in GraphPad Prism 8. Statistical analysis was performed using a one-way ANOVA.

RESULTS

Chapter 3

HCV CORE PROTEIN INCREASED THE LIPID LEVEL IN FT3-7 CELLS

The literature shows that core protein alone is capable of inducing steatosis, but the core-mediated lipid level increase per cell has not been quantified. Also, although gt3a HCV infection was associated with more severe hepatic steatosis than that caused by gt1a HCV infection, it remains unclear whether this is due to the different levels of lipid induction by different genotype-derived core or not. To address this gap in knowledge, we performed the experiment to quantify the gt1a and gt3a core expression-mediated lipid induction in FT3-7 hepatoma cells, by using the fluorescence-activated cell sorter (FACS) analysis. We first determined the level of core protein expression by performing the Western blot analysis following transfection with core-expressing plasmids and vector controls (Fig. 3). The anti-core antibody that we used strongly reacted with gt1a core but not with gt3a core after protein denaturation, potentially due to the amino acid sequence difference between these two proteins (Fig. 3A, upper panel). Therefore, we introduced the FLAG epitope tag at the N-terminal of core and performed the Western blot analysis by using an anti-FLAG antibody to detect gt1a and gt3a core proteins. As shown in Fig 3, bottom panel, we detected similar levels of gt1a and gt3a core protein by using an anti-FLAG antibody.

Next, to determine the lipid level in core-expressing cells, we performed FACS analysis to detect the core expression and lipid levels at a single-cell level. To detect the core, we used the anti-core antibody, since this core antibody reacted strongly to both gt1a

and gt3a core protein following paraformaldehyde fixation, suggesting that this antibody may target conformational epitope in the core. In fact, based on 7 independent experiments, we determined that the mean fluorescence intensities for gt1a and gt3a core expression were similar (Fig. 4). We also determined that ~20% of cells on average expressed detectable levels of gt1s and gt3a core proteins following transfection (Fig. 5). These data suggest that gt1a and gt3a core protein transfection efficiency and core protein expression levels were similar in multiple experiments. To quantify the lipid, we stained the lipid with a LipidTOX deep red neutral lipid stain. In brief, following transfection with core-expressing plasmids and vector controls, the cells were fixed with PFA, permeabilized with Triton-X-100 in order to allow the anti-core antibody to enter the cells, and core was labeled using AlexaFluor anti-mouse 488-labeled secondary antibody. Following sufficient washing to remove all traces of serum, the cells were stained with LipidTOX deep red neutral lipid stain. Loss of lipid due to fixation was not a concern, as PFA causes minimal damage to cells. We also optimized cell damage at different incubation times for PFA and found that 20 minutes was sufficient to fix the cells and prevent cell damage.

As shown in Fig. 6, both gt1a and gt3a core protein significantly increase lipid levels in hepatocytes compared to control. Previous methods utilized Nile red staining and other staining methods of quantifying lipids, such as using PLIN2 [30], or using qualitative analysis, whereas in our experiments, we used a quantitative FACS analysis technique that enabled us to sort out the population of cells we are looking for and get a more accurate determination of lipid level without convolution. Our results were reproducible up to n=7, further supporting our hypothesis that core protein quantitatively and significantly induces lipid levels in hepatoma cells compared to the controls. We detected 60-70% increase in

lipid levels in gt1a and gt3a core expressing cells compared to control. However, there was no significant difference between the lipid levels induced by gt1a and gt3a core (Figure 6). With these results, we conclude that both gt1a and gt3a core proteins induced significant but similar levels of lipids in hepatoma cells.

GT1A AND GT3A HCV CORE PROTEIN INDUCES DIFFERENT SIZES OF LIPID DROPLETS

As discussed earlier, infection with gt3a HCV induced larger LDs than those induced by gt1a HCV in the liver [30], and *in vitro* study suggested that gt3a core alone may be responsible for inducing larger LDs in cells [32]. To verify this previous finding, we performed the confocal immunofluorescence microscopy by using the cells transfected with gt1a and gt3a core-expressing plasmids for 48hrs, which were then fixed and labeled with anti-core antibody and LipidTOX to detect core protein and LDs, respectively. As shown in Figure 7, we were able to confirm that gt3a core induced larger LDs than those induced by gt1a core, which is consistent with histopathological data and other *in vitro* studies. Next, we determined the number and size of LDs induced by gt1a and gt3 core proteins quantitatively by analyzing multiple confocal images of core positive cells using the ImageJ software to better understand the lipid induction phenotypes by these proteins. As shown in Figure 8A, the LDs found in gt3a core expressing cells were significantly larger than those in gt1a core-expressing or control cells. The gt1a core did not affect the average size of LDs compared to control (Fig. 8A). The numbers of LDs in cells were not significantly different, regardless of gt1a or gt3a core expression, compared to control. However, we detected the relatively higher number of LDs in gt1a core-expressing cells on average than control cells or those expressing gt3a core (Fig. 8B). This trend might

explain why the lipid induction levels in gt1a and gt3a core-expressing cells were similar, despite that gt3a core induced significantly larger LDs compared to gt1a core. In other words, our data suggest that gt1a core tends to induce a larger number of regular-sized LDs and gt3a core induces large LDs without significantly increasing the number of LDs per cells. Future studies will be needed to elucidate the gt3a core-specific mechanism to induce larger LDs and address how these larger LDs may contribute to a more severe form of hepatic steatosis.

HCV CORE PROTEIN REGULATES LIPID METABOLISM-RELATED GENES

In previous experiments, we found that core protein induces similar levels of lipids in hepatocytes regardless of genotypes, but there is a genotypic difference in phenotype regarding LD size. To determine whether the difference in lipid phenotypes induced by gt1a and gt3a core proteins were caused by gene regulation, we performed a Fatty Liver Disease microarray (RT^2 Profiler PCR Array, Qiagen). We chose this microarray to understand the basis of more severe steatosis linked to gt3 HCV infection compared to gt1a HCV by identifying the genes that are involved in fatty liver disease affected by core protein and determining their roles in hepatic lipid metabolism. To perform the microarray, we isolated total RNAs from cells transfected with gt1a and gt3a core-expressing plasmids or vector control at 48hr post-transfection.

Based on three independent experiments, we were able to generate a volcano plot of genes regulated because of core protein expression (Figure 9). Gt1a and gt3a both significantly upregulated SOCS3 (suppressor of cytokine signaling-3), which is responsible for regulating cytokines and subsequently inhibiting STAT3 activation [40]. Increased SOCS3 activation is associated with a lack of response to antiviral therapy, as

well as a reduction in insulin signaling [40]. This is consistent with the knowledge that HCV causes insulin resistance [41].

The most downregulated gene by gt1a core expression is IGFBP1, which is an important player in cell migration and metabolism. Interestingly, gt3a core downregulated many more genes, including IGFBP1, PCK2, CEBPB, SERPINE1, PIK3R1, and FOXO1. The functions of these genes are summarized in Table 1. Among these genes, FOXO1 is particularly interesting, despite the minimum level of downregulation, as it is a transcription factor that has a downstream effect on MTP involved in VLDL synthesis. Unlike SOCS3, which was significantly upregulated by gt1a and gt3a core expression, most of the genes downregulated by these two proteins were not significantly reduced compared to control due to variation in values in three different experiments, so these results represent a trend. Also, these results are likely an underestimation of the effect of the core on gene regulation, since only ~20% of cells were core positive following core plasmid transfection (Fig. 5). Despite these limitations, it is interesting to note that most of the genes that are mainly downregulated by gt3a core are involved in insulin resistance and inflammation in the liver. A future study will be needed to better understand how the HCV core genotype-dependent deregulation of these genes would lead to different HCV genotype-associated insulin resistance and steatosis phenotypes.

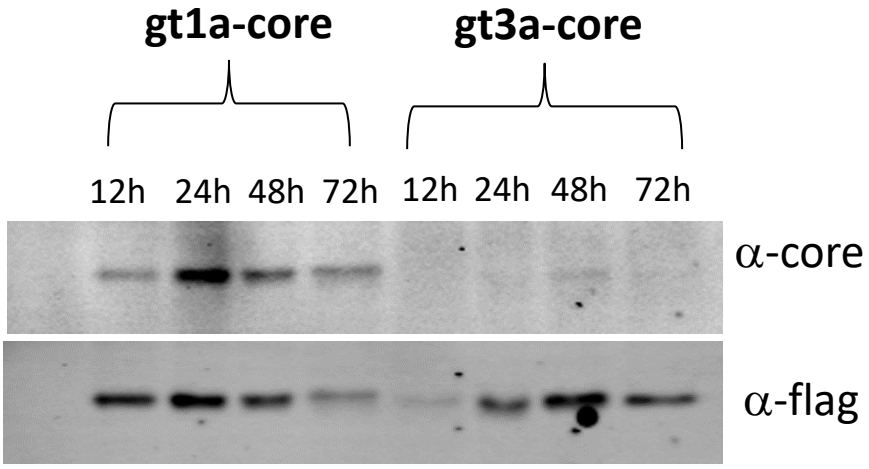

Figure 3. Western blotting time course. Transfection of FT3-7 cells after 12, 24, 48, and 72 hours. 48 hours shows the similar core protein expression level

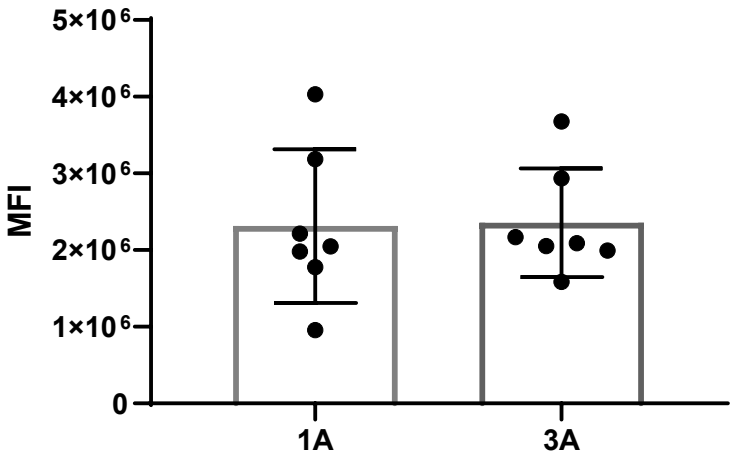

Figure 4. FACS analysis using an anti-core antibody. Core expression level measured by mean fluorescence intensity.

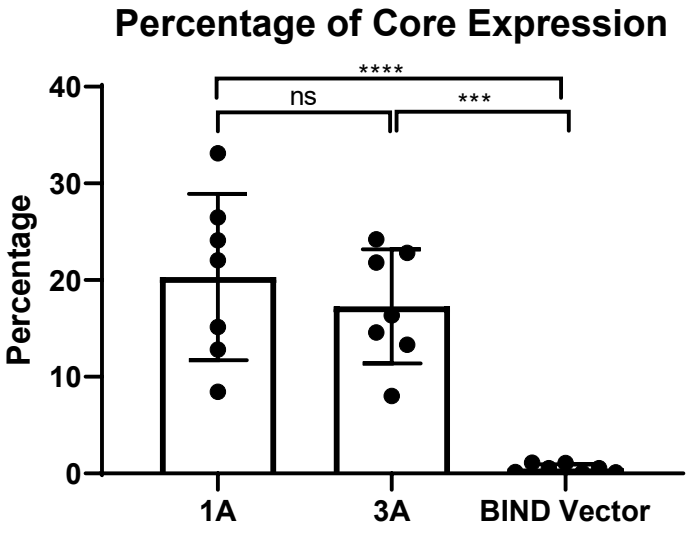

Figure 5. Percentage of core expression. FACS analysis. Statistical analysis using GraphPad Prism 8 and an Ordinary one-way ANOVA. (ns= no significance, p value=0.6244; ***= significant, p value=0.0002; ****= significant, p value <0.0001)

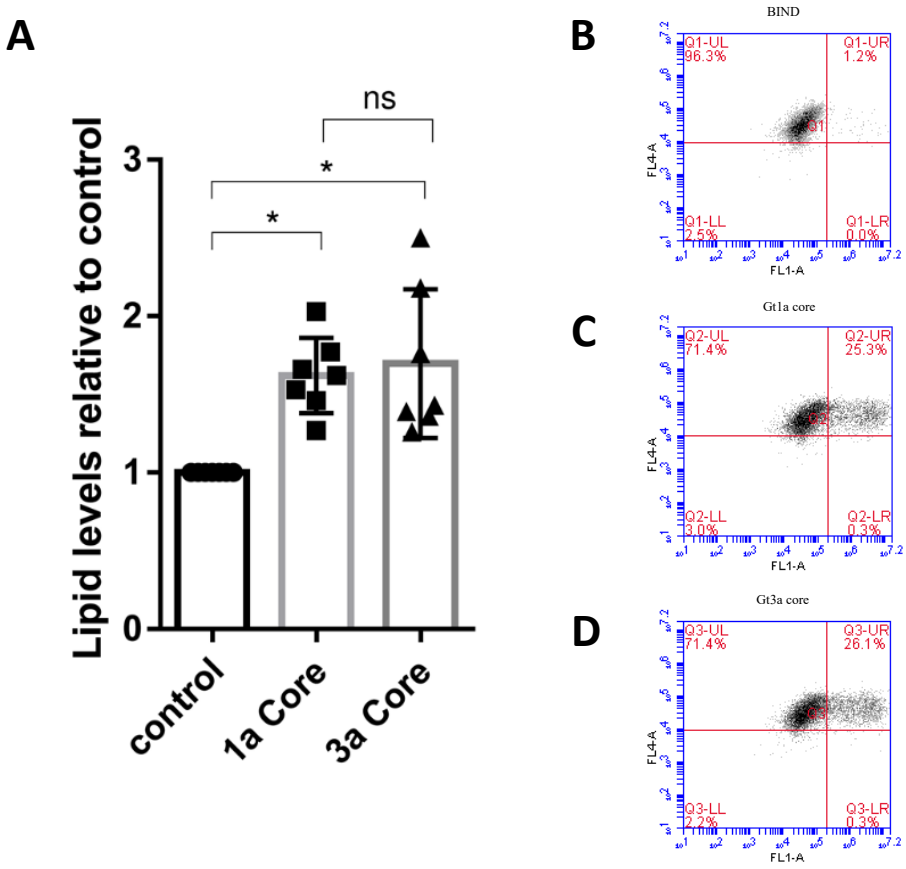

Figure 6. FACS analysis. Result of n=7 replicates. Cells transfected with core protein 1a and 3a had significantly more lipids compared to those transfected with BIND vector control. A) Non-significant difference between the genotypes. B-D) Flow cytometry data for BIND vector control, gt1a core and gt3a core. (x-axis: core staining, y-axis: LD staining.) Statistical analysis using Ordinary one-way ANOVA through GraphPad Prism 8.

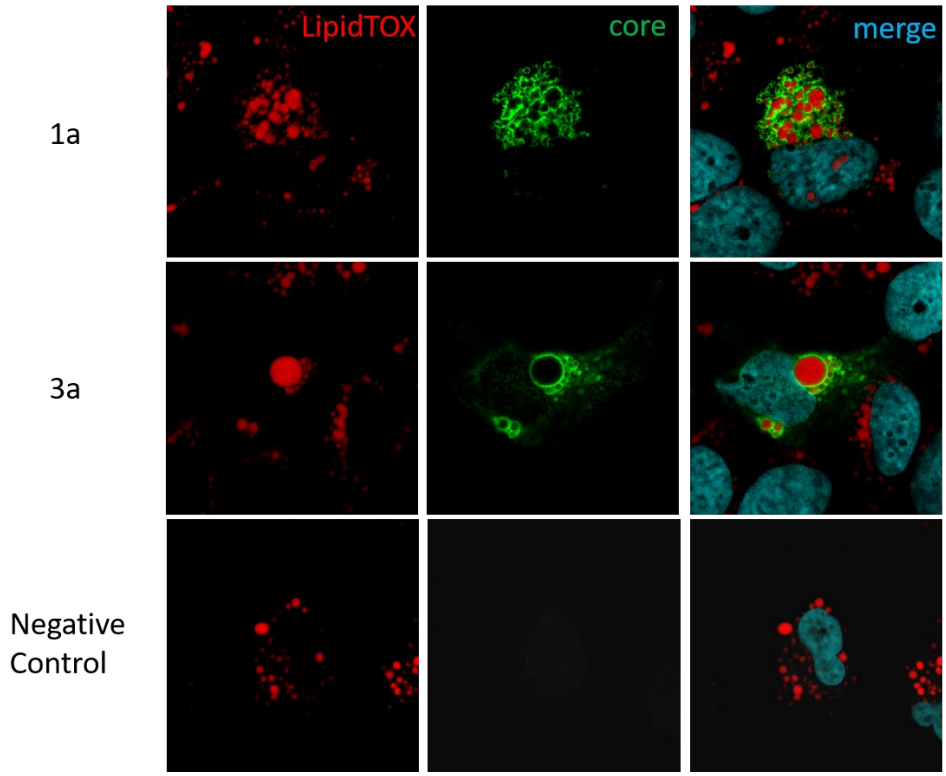

Figure 7. Confocal microscopy. The size and distribution of lipid droplets are shown in cells transfected with gt1a and gt3a core proteins and a BIND vector negative control. LipidTOX neutral lipid stain, HCV core protein, and merged images shown.

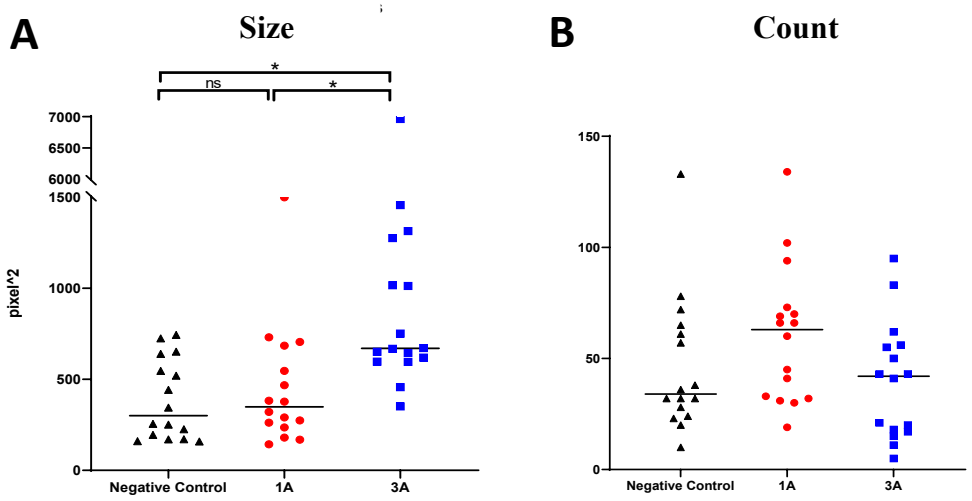

Figure 8. Size and count of LDs**.** A) Size of lipid droplets. Using ImageJ, the size of lipid droplets was analyzed from individual cells and quantified. Negative control used is BIND vector plasmid. * - significant, ns – not significant. B) Lipid droplet counts within the cells. Using ImageJ, the number of lipid droplets were analyzed from individual cells and quantified. Statistical analysis with One-way ANOVA in GraphPad Prism 8. Negative control used is BIND vector plasmid. * - significant, ns – not significant.

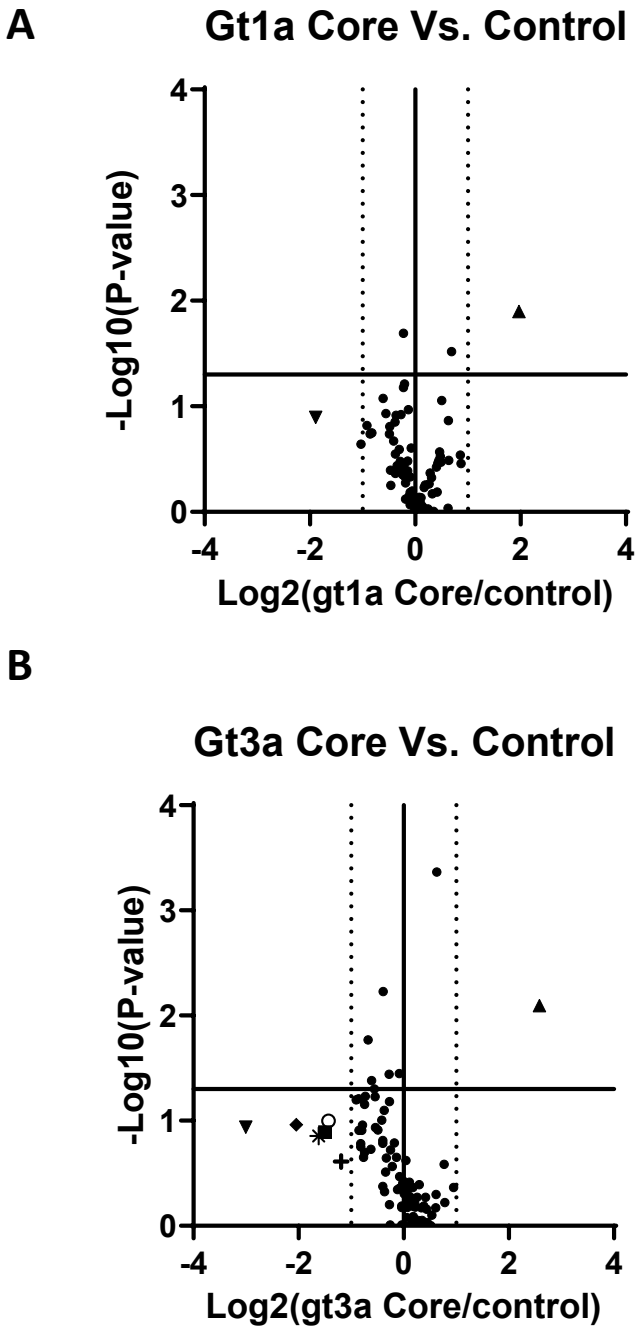

Figure 9. Fatty Liver Disease Microarray results. A) gt1a core protein transfected cells vs. BIND vector control transfected cells. B) gt3a core protein transfected cells vs. BIND vector control transfected cells. For more information on proteins which showed a fold change x > +/- 1.00, and represented with unique symbols, refer to Table 1. P-value < 0.05.

LEGEND	GENE	GT1a FOLD CHANGE	GT3a FOLD CHANGE	FUNCTION
▼	IGFBP1	-1.89	-3.00	Protein expressed is important in cell migration and metabolism; function is regulated by insulin
◆	PCK2	--	-2.04	Gluconeogenesis in the liver
*	CEBPB	--	-1.62	Activity of this protein is important in the regulation of genes involved in immune and inflammatory responses
■	SERPINE1	--	-1.50	Inhibitor of fibrinolysis
○	PIK3R1	--	-1.43	Subunit of PI3K enzyme. Functions in the regulation of several hormones, including insulin. PI3K signaling may also play a role in the maturation of fat cells
+	FOXO1	--	-1.19	Transcription factor that targets MTP
▲	SOCS3	1.97	2.58	Suppressor of cytokine signaling; upregulation induces insulin resistance

Table 1. Information regarding genes up- and down-regulated by gt1a and gt3a core protein expressing plasmid.

DISCUSSION

Chapter 4

This thesis provides evidence of a phenotypic and quantitative difference in lipid level induced by core protein derived from gt1a and gt3a HCV. Many previous reports demonstrated that gt3a HCV induces larger size LDs than other HCV genotypes. However, analysis of this phenotype in a quantitative manner on an individual cell base and at the gene regulation levels have not been performed, as far as we know. Based on the current study, this thesis reports that a) this difference occurs when hepatocytes are exposed to core protein alone, verifying the previous report, b) there is a quantitative difference in the size and count of lipid droplets per cell, c) different genotypes of core induced similar levels of total lipids in cells, and d) gt3a core downregulated many more genes involved in insulin resistance compared to gt1a core. Our results are significant since the information we obtained provides insight that more severe hepatic steatosis induction by gt3 HCV, compared to other HCV genotypes, could be due to the quality of lipid induction associated with larger LD formation and/or deregulated insulin resistance, and not caused by a higher level of lipid induction.

INSULIN RESISTANCE, ROS PRODUCTION, AND OTHER MECHANISMS MAY CONTRIBUTE TO LARGE LIPID DROPLET FORMATION BY GT3A CORE PROTEIN

We determined that gt3a core protein is sufficient to induce large LD production in FT3-7 hepatoma cells. Not only is this shown phenotypically, but in this thesis, we have demonstrated that there are quantifiably larger lipid droplets present in gt3a core protein-expressing cells. Consistent with the literature [42], both gt1a and gt3a core efficiently localized to the LDs. However, gt1a core protein induced higher numbers of regular-sized

LDs clustered in the perinuclear space, whereas gt3a core protein induced larger LDs, also, localized in the perinuclear space, but without increasing their number significantly. The differential LD sizes and distribution patterns caused gt1a and gt3a core may explain our additional data showing that the level of lipids induced by core proteins were similar regardless of their genotypes.

The large size of LDs in gt3a core protein expressing cells could be a result of lipid droplet fusion. However, previous studies offered alternative mechanisms. Clément *et al.* showed that IRS1 downregulation by gt3a core contributed to the enlargement of LDs through PTEN depletion. In their data, they show that core protein alone is sufficient to affect mRNA translation of PTEN. They also showed that synergistic effect of downregulation of both PTEN and IRS1 resulted in increased LD size [43]. Of note, downregulated PTEN has been implicated in the promotion of steatosis.

On the other hand, it is also possible that the ROS production caused by HCV core protein [44] may increase the size of LDs as well. Previous studies have shown that increased oxidative stress has an effect on lipid metabolism, leading to an increase in hepatic lipids [45].

It is likely that the induction of large LDs by gt3a core protein is a result of a variety of factors. More data is needed to determine whether these factors are interconnected or are separate from one another. It is possible that the regulation of certain genes, lipid fusion, insulin resistance, and the presence of ROSs contribute to the production of large lipid droplets in gt3a core expressing cells.

MTP REGULATION IN HCV-INFECTED CELLS MAY OCCUR IN A GENOTYPE-DEPENDENT MANNER

VLDL formation requires MTP activity, which transfers lipids onto nascent ApoB, and thereby promotes the maturation of VLDLs [22]. To determine whether HCV infection results in the regulation of MTP gene expression, Banerjee *et al.* utilized RT-PCR and found that gt1a HCV infected hepatocytes did not change the expression of MTP [46]. This conflicts with the data from the Yamaguchi group [47], where they found that MTP is regulated by core protein following 48h post-transfection of core expressing plasmid. However, under the conditions of chronic HCV, MTP expression is downregulated [39]. The above studies were performed by using gt1a and gt2a HCV and there is little to no data demonstrating the effect of gt3 HCV on MTP level. Our data shows that there is some regulation of FOXO1 gene by gt3a core protein expression. FOXO1 is a transcription factor that regulates liver gluconeogenesis [48], plays a role in insulin signaling [49], and regulates MTP [49]. Therefore, gt3a core also potentially regulates MTP expression.

CORE INDUCED GENE REGULATION MAY AFFECT INSULIN RESISTANCE IN A GENOTYPE-DEPENDENT MANNER

The results show that there are a variety of genes that are regulated by HCV core proteins. In our data, as well as in other pieces of literature, gt1a [50] and gt3a [51] HCV core proteins both upregulate SOCS3 gene, which encodes for the SOCS3 protein. The upregulation of this protein promotes insulin resistance in hepatocytes, which is consistent with data indicating that both gt1a and gt3a HCV play a role in insulin resistance [5]. We found that core protein alone is enough to upregulate SOCS3, consistent with the data reported by Lerat *et al.*, who used HCV core to determine whether SOCS3 was upregulated to decrease expression of IRS-2 [52], thereby promoting insulin resistance.

IGFBP1 function is inhibited by the presence of insulin [53]. Insulin directly impacts the IGFBP1 promoter via FOXO1 [54] through its suppression. Alberstein *et al.*

showed that core induced insulin resistance by eliminating insulin's effect on IGFBP1. Interestingly, our data shows that IGFBP1 gene is downregulated not only by gt1a but also gt3a core, suggesting that core protein has a direct effect on IGFBP1 regulation, in addition to above indirect mechanism. Interference with the PI3K/Akt pathway can lead to insulin resistance, and HCV core protein has been shown to inhibit AKT [55]. Our data shows that gt3a core protein downregulates other insulin resistance-related genes in this pathway, such as PI3KR1, which encodes for the PI3K subunit. These data indicate that HCV core protein may directly downregulate PI3K as well. Aside from our data, core protein has not been shown to directly interact with PI3K or the PI3KR1 gene, although Liu *et al.* suggests that NS5A may bind to PI3K. Since core and NS5A have been shown to interact within the cell [56], it is possible that these two proteins together play a role in the inhibition of PI3K function.

According to our results, gt3a core protein alone was sufficient to downregulate CEBPB, or C/EBPβ gene. This data conflicts with Nishitsuji *et al.*'s findings, which showed that HCV upregulates C/EBPβ function and promotes the production of inflammatory cytokines [57]. However, the aforementioned study only used HCV-1b/JFH1(gt2a) chimera and JFH1 HCV; therefore, one could infer that downregulation of this gene is unique to gt3a virus.

SUMMARY AND FUTURE DIRECTIONS

In summary, HCV core protein induces different size of LDs in hepatocytes in a genotype-dependent manner, despite there being no difference in lipid level induction as determined by FACS analysis. Here we have shown that the gt3a-induced lipid phenotype is distinct and characterized by large LDs that are localized in the perinuclear space. Although gt1a core protein induces smaller LDs, these LDs also remain in the perinuclear space, in contrast to LDs present in control cells, which are localized throughout the cytoplasm. To our knowledge, there have been no studies that have investigated the effect

of core protein on lipid levels quantitatively at a single cell level such as the one in this report. FACS analysis was used and the number of LDs were analyzed with ImageJ and immunofluorescence. By using these methods, we determined that while there is no difference in the quantity of lipid induced by the gt1a and gt3a core, gt3a core protein induced significantly larger lipid droplets in FT3-7 cells.

The mechanism of the production of these large LDs is currently unknown, but I speculate that VLDL pathway may be involved in this. Based on the evidence provided by the microarray data, the downregulation of FOXO1 by gt3a core suggests that downstream proteins, such as MTP, which plays an integral part in the VLDL pathway, may have an impact on LD size.

To our knowledge, the effects of core protein alone on the gene expressions related to fatty liver disease have not been analyzed. In future experiments, virus-containing different genotypes of core can be used in both FACS analysis and microarray. By using these viruses, we can determine the effects of different genotype of core in the context of virus. I believe that the use of these viruses in FACS analysis, immunofluorescence, and microarray would elucidate the effect of different genotype of core in a way that core alone cannot provide. We do not know the effect of other proteins on the functionality of core protein and the effect these other proteins have on lipid metabolism. However, based on the literature and our results, I believe that the presence of other HCV proteins could result in significantly larger lipid droplets formation, and/or larger extent of gene regulation related to the VLDL pathway and insulin resistance.

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