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Forkhead Box Transcription Factor Regulation and Lipid Accumulation by Hepatitis C Virus

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ABSTRACT

We have previously shown that hepatitis C virus (HCV) infection modulates the expression of forkhead box transcription factors, including FoxO1 and FoxA2, which play key roles in gluconeogenesis and β-oxidation of fatty acid, respectively. The aim of the present study was to determine the role of forkhead box transcription factors in modulating lipid metabolism. HCV infection or core protein expression alone in transfected Huh7.5 cells increased expression of sterol regulatory element binding protein 1c (SREBP-1c) and its downstream target, fatty acid synthase (FASN), which are key proteins involved in lipid synthesis. Knockdown of FoxO1 by small interfering RNA in HCV-infected cells significantly decreased SREBP-1c and FASN expression. Further, HCV infection or core protein expression in Huh7.5 cells significantly decreased the expression of medium-chain acyl coenzyme A dehydrogenase (MCAD) and short-chain acyl coenzyme A dehydrogenase (SCAD), involved in the regulation of β-oxidation of fatty acids. Ectopic expression of FoxA2 in HCV-infected cells rescued the expression of MCAD and SCAD. Oil red O and neutral lipid staining indicated that HCV infection significantly increases lipid accumulation compared to that in the mock-infected control. This was further verified by the increased expression of perilipin-2 and decreased activity of hormone-sensitive lipase (HSL) in HCV-infected hepatocytes, implying increased accumulation of neutral lipids. Knockdown of FoxO1 and ectopic expression of FoxA2 significantly decreased HCV replication. Taken together, these results suggest that HCV modulates forkhead box transcription factors which together increase lipid accumulation and promote viral replication.

IMPORTANCE

Hepatic steatosis is a frequent complication associated with chronic HCV infection. Its presence is a key prognostic indicator associated with the progression to hepatic fibrosis and hepatocellular carcinoma. Several mechanisms have been proposed to account for the development of steatosis and fatty liver during HCV infection. We observed that HCV infection increases expression of both SREBP-1c and FASN. Further investigation suggested that the expression of SREBP-1c and FASN is controlled by the transcription factor FoxO1 during HCV infection. In addition, HCV infection significantly decreased both MCAD and SCAD expression, which is controlled by FoxA2. HCV infection also increased lipid droplet accumulation, increased perilipin-2 expression, and decreased HSL activity. Thus, knockdown of FoxO1 (decreased lipogenesis) and overexpression of FoxA2 (increased β-oxidation) resulted in a significant disruption of the platform and, hence, a decrease in HCV genome replication. Thus, targeting of FoxO1 and FoxA2 might be useful in developing a therapeutic approach against HCV infection.

Hepatitis C virus (HCV) is an important cause of morbidity and mortality worldwide, causing a spectrum of disease ranging from an asymptomatic carrier state to end-stage liver disease (1–4). The most important feature of HCV infection is the development of chronic hepatitis in a significant number of infected individuals and the potential for disease progression to metabolic disorders, fibrosis/cirrhosis, and hepatocellular carcinoma (1–3, 5). We have previously shown that HCV modulates signaling pathways in inducing insulin resistance (6, 7).

Insulin regulates the expression of key enzymes involved in glucose and lipid metabolism by modulating the activity of specific forkhead box transcription factors (FoxO1 and FoxA2) in the liver. FoxO1 mediates the expression of genes involved in both glucose and lipid metabolism in the liver (8, 9). Insulin suppresses the expression of key gluconeogenic enzymes, including glucose 6-phosphatase (G6P) and phosphoenolpyruvate carboxykinase (PCK2), by stimulating the translocation of FoxO1 outside the nucleus (10). Increased glucose production can activate genes involved in lipid metabolism, including sterol regulatory element binding protein 1c (SREBP-1c) and fatty acid synthase (FASN).

On the other hand, FoxA2 controls hepatic lipid metabolism in type 2 diabetes, improving insulin resistance (11, 12). Thus, an interruption in insulin signaling may affect metabolic regulation. During chronic HCV infection, insulin resistance may elicit a vicious cycle for manipulating the functions of FoxA2, preventing optimal stimulation of normal metabolic functions of the liver.

Lipid homeostasis requires balancing metabolic vectors, including lipogenesis, export, and degradation (β-oxidation), a significant component of which is orchestrated by a family of membrane-bound master regulator transcription factors designated sterol regulatory element binding proteins (SREBPs) that regulate the expression of more than 30 genes involved in the synthesis and uptake of cholesterol, fatty acids, triglycerides, and phospholipids

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(13). Progression of chronic HCV infection involves development of fatty liver. Thus, it is very likely that HCV modulates signaling pathways involved in lipogenesis and lipid oxidation, resulting in excessive lipid deposition, which is manifested by the accumulation of lipid droplets (LDs).

Vertebrate LDs contain one or more of five related structural proteins: perilipin, adipophilin, TIP47, S3-12, and OXPAT/MLDP (14). The composition of LD coat proteins changes as the lipid droplet enlarges and matures, and work suggests that changes in protein composition may be a primary driver of hepatic steatosis (15).

In this study, we focused on determining the mechanisms by which HCV disrupts normal lipid metabolism. We have shown that HCV infection modulates both lipogenesis and β-oxidation, primarily by regulating forkhead box transcription factors. HCV increases the expression of SREBP-1c and FASN, which are involved in lipogenesis, and decreases the expression of medium-chain acyl coenzyme A dehydrogenase (MCAD) and short-chain acyl coenzyme A dehydrogenase (SCAD), key enzymes involved in β-oxidation. This is manifested by an increased expression of lipid droplets and perilipin-2, promoting lipid accumulation. Thus, targeting forkhead box transcription factors may provide new avenues for development of therapeutic modalities against HCV-mediated fatty liver generation.

MATERIALS AND METHODS

Generation of cell culture-grown HCV. HCV genotypes 1a (clone H77) and 2a were grown in immortalized human hepatocytes (IHHs) or Huh7.5 cells, as previously described (16). Virus released in cell culture supernatant was filtered through a 0.45-μm-pore-size cellulose acetate membrane (Nalgene, Rochester, NY) to remove cell debris. The HCV RNA in the cell culture supernatant was quantified by real-time PCR (in an ABI Prism 7000 real-time thermocycler), using HCV analyte-specific reagents (ASRs; Abbott Molecular), at the Department of Pathology, Saint Louis University. The virus infectivity titer in the cell culture supernatant was measured using a fluorescent focus-forming assay. The average peak HCV titer ranged from $\sim 10^4$ to 10^5 focus-forming units/ml.

Reagents. Commercially available antibodies to FoxA2, SREBP-1, FASN, MCAD, and SCAD (Santa Cruz Biotechnology, Inc., Santa Cruz, CA), antibody to FoxO1 and a lipolysis activation antibody sampler kit (Cell Signaling Technology, Danvers, MA), horseradish peroxidase (HRP)-conjugated antibody to actin (Sigma-Aldrich, St. Louis, MO), and an antibody to perilipin-2 (Fitzgerald Industries International, Acton, MA) were procured. FoxO1 small interfering RNA (siRNA; Cell Signaling Technology, Danvers, MA) and FoxA2 plasmid (clone HsCD00330288; DNASU Plasmid Repository, Arizona State University, Tempe, AZ) were used in this study. Transfection experiments were performed using 1 μg siRNA against FoxO1 or 0.5 μg FoxA2 plasmid DNA per well of a 6-well plate.

Western blot analysis. Proteins from cell lysates in sample-reducing buffer were resolved by SDS-PAGE and transferred onto a nitrocellulose membrane, and the blot was blocked with 3% nonfat dry milk. The membrane was incubated with a primary antibody, followed by a secondary antibody coupled to horseradish peroxidase to detect protein bands by chemiluminescence (Amersham, Piscataway, NJ). Cellular actin was detected, using a specific antibody, for comparison of the protein load in each lane. Antibodies recognizing protein bands were stripped using Re-Blot Plus Strong solution (Millipore), and the blot was reprobed with other specific antibodies to determine their relative expression status in the same experiment.

Oil red O staining of lipid droplets. Control and HCV-infected hepatocytes were formalin fixed, washed with distilled water, and treated with 60% isopropanol for 5 min. After aspirating off the isopropanol, cells were treated with oil red O for 5 min and washed thoroughly before viewing under a phase-contrast microscope. The intensities of stained cells were quantified using ImageJ software. The diameters of the lipid droplets from at least 3 different fields were measured using accessory tools provided in a Leica Application Suite (Advanced Fluorescence Lite 2.6.3 build 8173; Leica Microsystems).

Immunofluorescence for neutral lipid and perilipin-2. HCV-infected cells were fixed with 2% formaldehyde in phosphate-buffered saline before staining with perilipin-2 as described previously (17). Fluorescence intensities were quantified using ImageJ software.

Real-time PCR and HCV replication. Huh7.5 cells were infected with HCV 2a followed by treatment with FoxO1 siRNA or overexpression of FoxA2 plasmid. Cellular RNA was isolated by use of the TRIzol reagent (Invitrogen, Carlsbad, CA), and cDNA was synthesized by using random hexamers and ThermoScript II RNase H reverse transcriptase (Invitrogen). The presence of HCV RNA was determined by real-time PCR (Applied Biosystems, Foster City, CA) using specific oligonucleotide primers (HCV primer targeted toward the 5′ untranslated region; assay identification number AI6Q1GI; Invitrogen), and the results were normalized to those for 18S rRNA. All reactions were performed in triplicate in an ABI Prism 7500 Fast analyzer.

Luciferase assay for determining HCV replication. Huh7.5 cells stably expressing the HCV genotype 2a full-length replicon tagged with a renilla luciferase gene (kindly provided by Hengli Tang, Florida State University, Tallahassee, FL) were used for determining HCV replication by the luciferase assay. Cells were either transfected with FoxO1-specific siRNA at different doses (0.2 μg, 1 μg, 5 μg) or transfected with FoxA2-overexpressing plasmid at different doses (8 ng, 40 ng, 200 ng) in a 24-well plate. Cells were lysed using reporter lysis buffer (Promega, Madison, WI) at 48 h posttransfection, and the clarified lysates were subjected to the luciferase reporter assay using a luminometer (Opticomp II; MGM Instruments, Hamden, CT).

Statistical analysis. Experiments were performed in at least triplicate sets. The significance of the results was determined by GraphPad Prism software using an unpaired two-tailed Student *t* test. A *P* value of <0.05 was considered significant.

RESULTS

HCV infection or core protein expression in hepatoma cells upregulates SREBP-1c and FASN via FoxO1. SREBP-1c and its downstream target, FASN, are the key enzymes involved in fatty acid synthesis. HCV is known to upregulate SREBP-1c and FASN at the transcriptional level (18, 19). We observed that HCV 2a (clone JFH1) infection of Huh7.5 cells or transfection with the core gene from HCV under the control of a cytomegalovirus promoter results in increased activated (cleaved-form) protein expression of SREBP-1c and FASN by Western blotting (Fig. 1A and C) compared to the level of expression for mock-infected control Huh7.5 cells. The Western blot was scanned densitometrically using ImageJ software (Fig. 1B and D). These results from three independent experiments suggested that HCV core protein plays a role in the modulation of key enzymes involved in lipogenesis.

We have previously shown that insulin-induced FoxO1 translocation from the nucleus to the cytoplasm is impaired in HCV-infected hepatocytes (20). FoxO1 in the nucleus activates the expression of gluconeogenic enzymes like glucose 6-phosphatase (G6P) and phosphoenolpyruvate carboxykinase (PCK2). We have also observed an increased expression of G6P and PCK2 in HCV infection (7, 20). Increased glucose production can, in turn, stimulate expression of genes involved in lipid synthesis (21). This prompted us to examine the role of FoxO1 in regulating SREBP-1c and FASN expression, key enzymes involved in lipid synthesis. For this, the expression of FoxO1 in Huh7.5 cells was

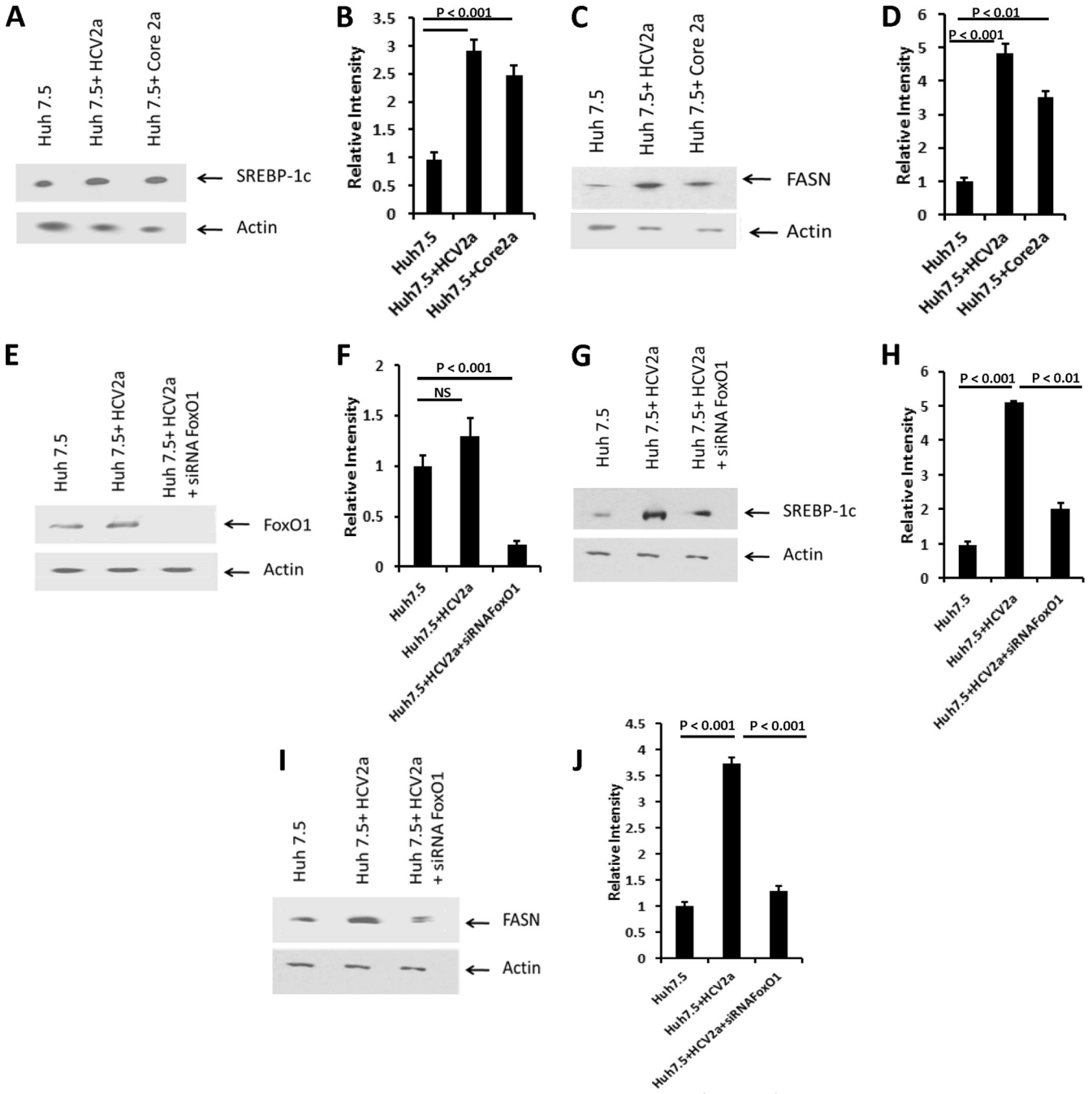

FIG 1 Hepatitis C virus infection or core protein expression increases SREBP-1c and FASN expression via FoxO1. (A and C) Huh7.5 cells were mock treated, infected with HCV genotype 2a, or transfected with core protein. The status of SREBP-1c and FASN was determined after 3 days by Western blotting. The level of actin expression in each lane was determined as a loading control for comparison of the results. (B and D) Western blots were scanned densitometrically using ImageJ software. (E and F) Expression of FoxO1 was greatly reduced following treatment of Huh7.5 cells with an siRNA specific to FoxO1. Mock-infected, HCV-infected, and HCV-infected and FoxO1 siRNA-treated Huh7.5 cells were analyzed by Western blotting and subjected to densitometric scanning to determine the expression status of FoxO1. (G and I) siRNA knockdown of FoxO1 greatly reduced the expression of SREBP-1c and FASN in a Western blot analysis. Mock-treated, HCV-infected, and HCV-infected and FoxO1 siRNA-treated Huh7.5 cells were analyzed by Western blotting to determine the expression of SREBP-1c. The level of actin expression in each lane was determined as a loading control for comparison of results. (H and J) Western blots were scanned densitometrically.

knocked down using specific siRNA (Fig. 1E and F) and infected with HCV 2a. Our results indicated that HCV infection increases expression of SREBP-1c and FASN, while knockdown of FoxO1 by siRNA attenuated the increased expression of SREBP-1c and FASN in virus-infected cells (Fig. 1G and I). The Western blot was scanned densitometrically using ImageJ software (Fig. 1H and J). These results suggest that the expression of SREBP-1c and FASN may be regulated by FoxO1 during HCV infection.

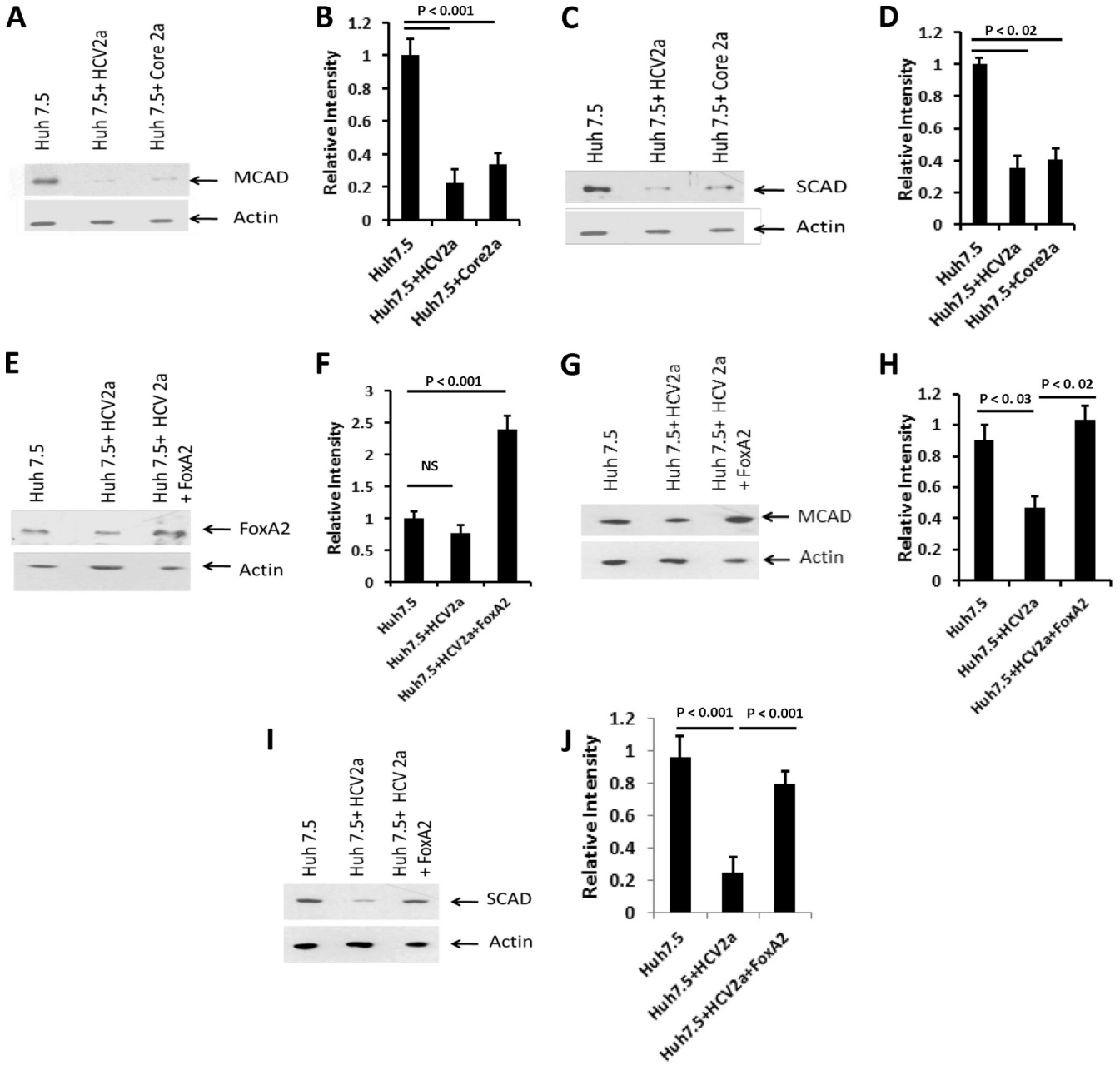

FIG 2 Hepatitis C virus infection or core protein expression decreases the expression of MCAD and SCAD via FoxA2. (A and C) Huh7.5 cells were mock treated, HCV infected, or transfected with the core protein gene. MCAD and SCAD expression status was determined after 3 days by Western blotting. The level of actin expression in each lane was determined as a loading control for comparison. (B and D) Western blots were scanned densitometrically. (E and F) Ectopic expression of FoxA2 was determined following transfection of plasmid DNA in Huh7.5 cells. Mock-treated, HCV-infected, and HCV-infected and FoxA2 plasmid-transfected Huh7.5 cells were separately analyzed by Western blotting and subjected to densitometric scanning to determine FoxA2 expression status. NS, not significant. (G and I) Overexpression of FoxA2 rescued MCAD and SCAD expression. Mock-treated, HCV-infected, and HCV-infected and FoxA2 plasmid-transfected Huh7.5 cells were analyzed by Western blotting for the status of MCAD and SCAD expression. The level of actin expression in each lane was determined as a loading control for comparison of results. (H and J) Western blots were scanned densitometrically.

HCV infection or core protein expression in hepatoma cells decreases MCAD and SCAD expression via FoxA2. HCV is reported to downregulate the transcription of MCAD and SCAD (22). We analyzed MCAD and SCAD protein expression in virus-infected cells. Our results suggested that HCV infection or core protein expression decreases MCAD and SCAD expression in Huh7.5 cells compared to that in mock-infected control Huh7.5 cells (Fig. 2A and C). The Western blot was scanned densitometrically using ImageJ software (Fig. 1B and D). Forkhead transcription factor FoxA2 activates genes involved in hepatic lipid metabolism and is regulated by insulin. Expression of constitutively active FoxA2-T156A in diabetic mice is known to elevate hepatic lipid metabolism by activating the expression of genes encoding enzymes involved in mitochondrial β-oxidation and ketogenesis,

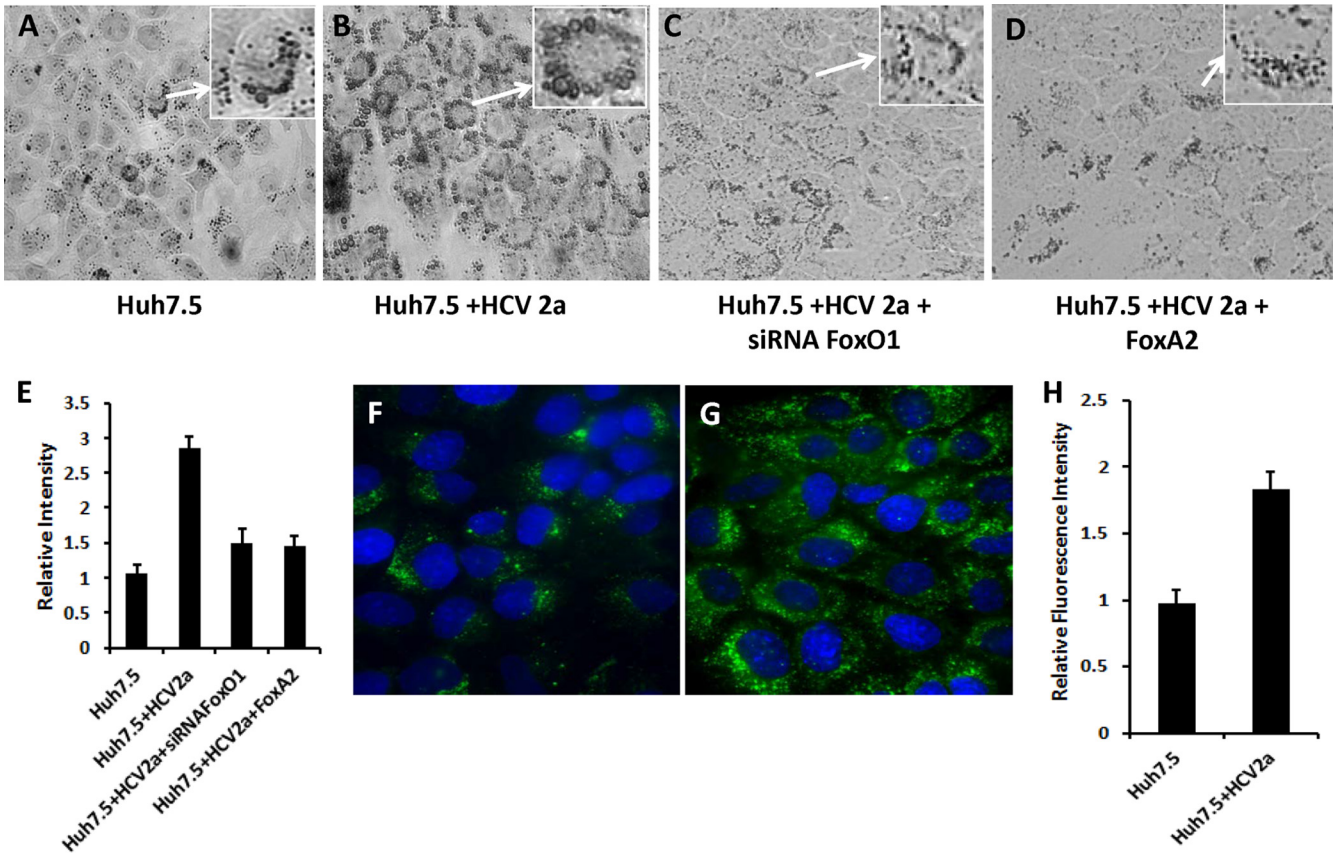

FIG 3 HCV increases lipid accumulation in Huh7.5 cells. (A and B) Mock-treated control and HCV 2a-infected Huh7.5 cells were stained with oil red O after 3 days to determine lipid droplet accumulation. (Insets) Magnified images of randomly selected cells. (C and D) Huh7.5 cells were infected with HCV 2a, followed by transfection with FoxO1 siRNA (1 μg) or FoxA2 plasmid DNA (0.5 μg) after 24 h. Cells were stained with oil red O after 3 days to determine lipid droplet accumulation. (Insets) Magnified images of randomly selected cells. (E) The relative intensities of lipid droplets in at least 4 different randomly chosen fields were determined by ImageJ software. (F and G) Mock-treated or HCV-infected Huh7.5 cells were stained for neutral lipid by immunofluorescence. Green stain, neutral lipid; blue color, 4′,6-diamidino-2-phenylindole-stained cell nucleus. (H) The relative fluorescence intensity of neutral lipid stain in at least 4 different randomly chosen fields of control and HCV 2a-infected cells was determined by ImageJ software.

including CPT1a, MCAD, and very-long-chain acyl coenzyme A dehydrogenase (12). In addition, we have previously shown that HCV-infected hepatocytes reduce nuclear accumulation of FoxA2 (20), which would imply decreased β-oxidation. Based on the decreased expression of MCAD and SCAD following HCV infection of Huh7.5 cells, we examined whether forced overexpression of FoxA2 would rescue the expression of MCAD and SCAD. Overexpression of FoxA2 (Fig. 2E and F) increased MCAD and SCAD expression (Fig. 2G to J), suggesting a role for HCV in modulation of FoxA2 expression leading to a decrease in fatty acid degradation.

HCV infection increases lipid droplet formation. Since HCV infection modulates forkhead box transcription factors to increase lipogenesis and decreases lipid degradation, we examined the accumulation of lipid droplets by oil red O staining in two different cell lines (IHHs and Huh7.5 cells). HCV infection significantly increased lipid droplet formation in both cell types compared to that in mock-infected control cells. The results are illustrated with Huh7.5 cells (Fig. 3A and B). Since knockdown of FoxO1 decreased expression of lipogenic genes in HCV-infected cells and overexpression of FoxA2 increased expression of enzymes involved in β-oxidation of fatty acids, we next determined if the forkhead box transcription factors play a role in modulating expression of lipid droplets. For this, Huh7.5 cells were infected with HCV 2a and transfected after 1 day with siRNA for specific inhibition of FoxO1 or transfected with plasmid DNA for ectopic expression of FoxA2. Cells were stained after 3 days with oil red O to determine lipid accumulation. Knockdown of FoxO1 or overexpression of FoxA2 significantly decreased lipid droplet formation in HCV-infected cells (Fig. 3C and D). The results were quantified using ImageJ software and are also shown (Fig. 3E). The average diameter of the majority of the lipid droplets was 1.4 ± 0.08 μm for HCV 2a-infected cells and was reduced to 0.6 ± 0.05 μm for siRNA against FoxO1-treated cells, 0.68 ± 0.04 μm for cells ectopically expressing FoxA2, and 0.5 ± 0.04 μm for untreated control cells. To further verify lipid accumulation, immunofluorescence staining of neutral lipids was performed using mock-treated control and HCV-infected hepatocytes. HCV-infected cells displayed a significant increase in expression of neutral lipids (Fig. 3F to H).

HCV infection increases perilipin-2 expression and decreases HSL activity. Since we observed increased accumulation of lipid droplets and decreased expression of enzymes involved in β-oxidation, we next determined the status of perilipin in HCV-

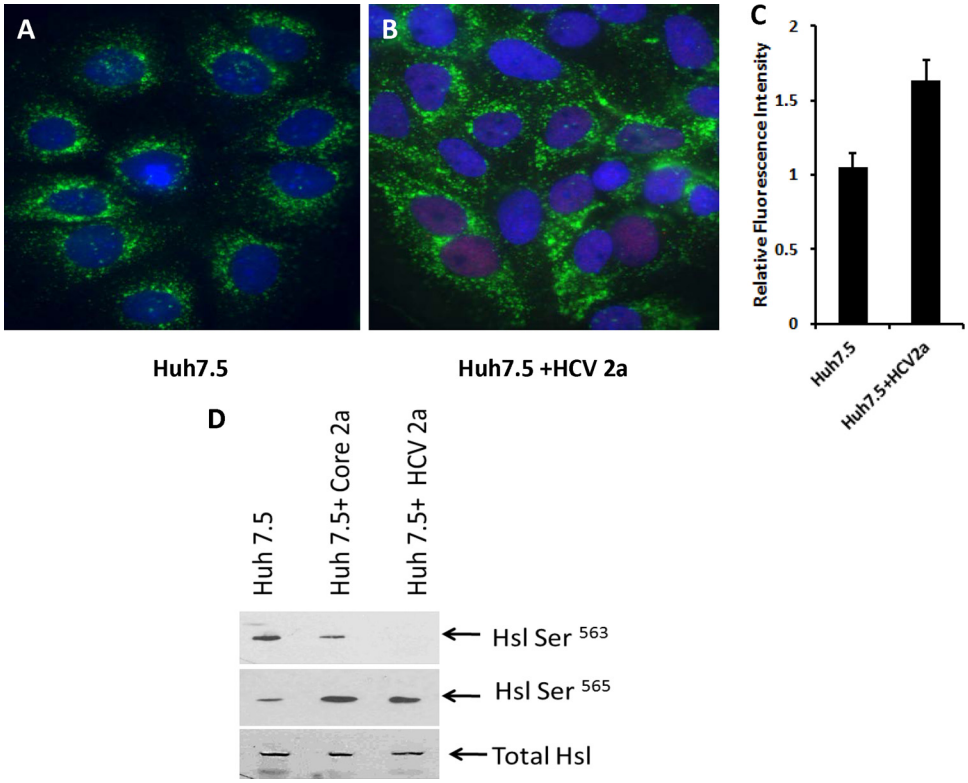

FIG 4 HCV infection increases perilipin-2 expression and decreases HSL activity. (A and B) Mock-treated or HCV 2a-infected Huh7.5 cells were stained for perilipin-2 by immunofluorescence (green) and for nuclear staining by 4′,6-diamidino-2-phenylindole (blue). (C) The relative fluorescence intensity of perilipin-2 stain in at least 4 different randomly chosen fields of control and HCV 2a-infected cells was determined by ImageJ software. (D) Mock-treated, HCV-infected, or core-transfected Huh7.5 cells were examined after 3 days for expression of phospho-HSL (serine 563 and serine 565) and total HSL by Western blotting. The amount of total HSL was similar in control and experimental cells and acted as a loading control.

infected hepatocytes. The perilipins are a family of proteins that associate with the surface of lipid droplets and protect degradation of lipids. Phosphorylation of perilipin is essential for the mobilization of fats. HCV infection significantly increased perilipin-2 expression compared to that in mock-treated control Huh7.5 cells (Fig. 4A to C), which further supports the observation of increased lipid accumulation in HCV-infected hepatocytes.

Perilipin-2 protects lipid droplets from the action of hormone-sensitive lipase (HSL) (23, 24). Protein kinase A (PKA) increases the hydrolytic activity of HSL by phosphorylation of a single site identified as Ser^{563}. Phosphorylation at Ser^{565} impairs the phosphorylation of Ser^{563} by PKA and negatively regulates the activity of HSL. We found that HCV infection decreased the phosphorylation of Ser ^{563} HSL, implying less activation of HSL (Fig. 4D). HCV infection also increased Ser^{565} HSL phosphorylation, implying inactivation of HSL activity, while total HSL abundance was unchanged. These results suggest that HCV infection increases perilipin-2 expression and decreases HSL activity through pathways that include decreased lipolysis and increased lipid accumulation.

FoxO1 and FoxA2 transcription factors contribute to HCV replication. Transcription factors FoxO1 and FoxA2 play important roles in the regulation of lipogenesis and β-oxidation of lipids during HCV infection. Here, we determined whether FoxO1 and FoxA2 also modulate HCV replication. Knockdown of FoxO1 by specific siRNA resulted in a significant decrease of HCV RNA by real-time PCR analysis (Fig. 5A). On the other hand, overexpression of FoxA2 resulted in a significant decrease of HCV RNA (Fig. 5B), suggesting its involvement in HCV genome replication. To further verify the role of these transcription factors in HCV genome replication, we used a full-length replicon system tagged with luciferase. FoxO1 knockdown or FoxA2 overexpression resulted in a significant decrease in luciferase activity in a dose-dependent manner (Fig. 5C and D), thereby confirming the suggestions that FoxO1 and FoxA2 may each play a critical role in HCV replication.

DISCUSSION

The current study is a direct continuation of our previously published work (20) with forkhead box transcription factors in which we evaluated their role in metabolic gene regulation during HCV infection. In this report, we have shown that HCV infection modulates both lipogenesis and β-oxidation processes primarily by regulating forkhead box transcription factors. HCV increases the expression of SREBP-1c and FASN, which are involved in lipogenesis, and decreases the expression of key enzymes, MCAD and SCAD, which are involved in β-oxidation (Fig. 6). These are manifested by an increased expression of lipid droplets (LDs) and perilipin-2, which promotes lipid accumulation. We have also observed that HCV infection decreases lipolysis by inactivating HSL. Overall, these results suggest that HCV infection promotes lipid accumulation and prevents lipid degradation, leading to increased lipid accumulation. Increased accumulation of LDs is often observed in chronically HCV-infected patients in the form of fatty

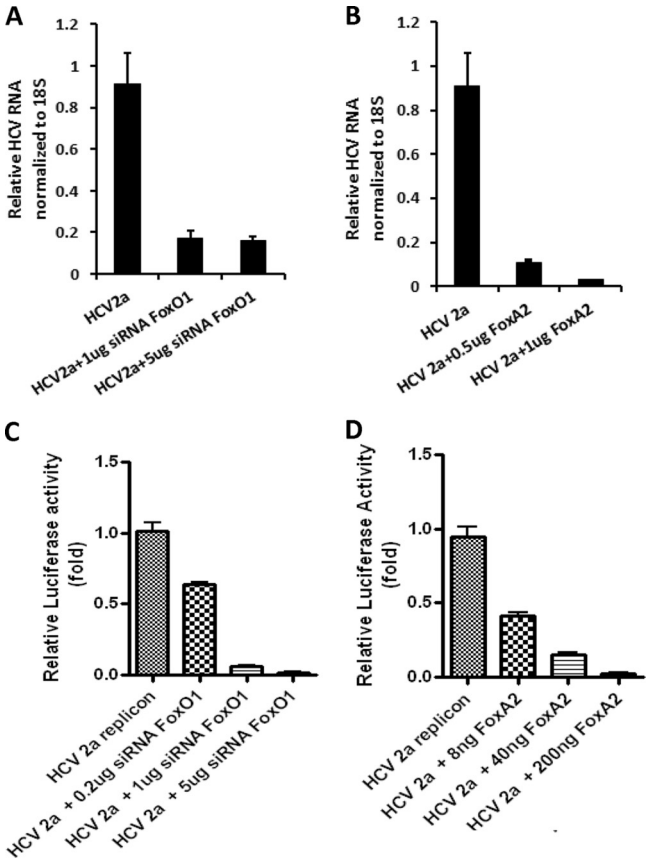

FIG 5 FoxO1 and FoxA2 transcription factors are necessary for HCV replication. (A) HCV replication in HCV 2a-infected Huh7.5 cells was determined by real-time PCR after treatment with siRNA against FoxO1 (1 μg and 5 μg) or after ectopic expression of FoxA2 plasmid DNA (0.5 μg and 1 μg). (B) HCV replication was determined by luciferase assay in an HCV 2a replicon-bearing cell line transfected with either siRNA against FoxO1 (0.2 μg, 1 μg, and 5 μg) or ectopically expressing FoxA2 (8 ng, 40 ng, and 200 ng).

liver. In addition, we have observed that HCV modulates forkhead box transcription factors to its advantage for supporting virus genome replication. We further show that knockdown of FoxO1 or overexpression of FoxA2 significantly decreases HCV replication. Thus, HCV increases lipid accumulation in hepatocytes by modulating both FoxO1 and FoxA2, which in turn favors virus genome replication.

HCV NS5A activates SREBP-1c, resulting in increased lipogenesis (19, 25). FASN is upregulated during HCV infection and regulates virus entry and production (26, 27). While this work was in progress, Sun et al. (28) reported the involvement of FoxO1 in modulating SREBP-1c, FASN, and multiple genes promoting HCV replication. In this study, we have focused on understanding the key mechanisms of lipid accumulation in HCV-infected cells for promotion of virus replication. Our observations extend those findings by demonstrating (i) upregulation of lipogenesis by HCV via FoxO1, (ii) downregulation of fatty acid β-oxidation via FoxA2, (iii) the effects of HCV infection on MCAD and SCAD expression involved in β-oxidation, (iv) modulation of forkhead box transcription factors for promotion of HCV replication, and (v) upregulation of perilipin-2 and inhibition of HSL activity. These observations provide further in-depth information on the mechanism by which modulation of lipids by HCV promotes its own genome replication.

LDs are cytosolic lipid storage organelles consisting of neutral lipids (triacylglycerides and sterol esters) surrounded by a phospholipid monolayer and a growing list of associated proteins. HCV utilizes LDs for production of infectious virus (29–31). LDs are thought to act as a platform for HCV replication and assembly. HCV genome replication, similar to that in some other positive-strand RNA viruses, occurs within a membranous web derived from intracellular vesicles (29). HCV particles were observed in close proximity to LDs, indicating that some steps of virus assembly take place around LDs. HCV core protein associates with lipid droplets (32, 33). Envelope glycoproteins E1 and E2 reside in the endoplasmic reticulum lumen (34), and the viral replicase localizes on endoplasmic reticulum-derived membranes. HCV core protein recruits HCV RNA, nonstructural proteins, and replication complexes to LD-associated membranes, and this recruitment is critical for infectious virus particle production (29). HCV NS4B is known to play a crucial role in virus replication at the site of virion formation, namely, the microenvironment associated with LDs (35). Mutations of HCV core and NS5A result in a failure to associate with LDs and impair the production of infectious virus (26), indicating the importance of these proteins in HCV replication and assembly. It has recently been shown that lipid droplet-binding protein TIP47 regulates HCV RNA replication through interaction with the HCV NS5A protein (36). TIP47 serves as a novel cofactor for HCV infection possibly by integrating LD membranes into the membranous web (36). On the other hand, NS5A also associates with Rab18 (37) and physically recruits sites of HCV replication to LDs. Thus, the HCV NS5A protein may play a role in interaction with LDs, possibly promoting a site for viral replication in infected cells.

Steatosis and abnormal lipid metabolism caused by chronic HCV infection may be linked to enhanced LD formation (38). Overproduction of LDs is induced by HCV core protein itself, and excessive core-dependent formation of LDs is suggested to produce the necessary microenvironment for virus production (29).

The requirement of lipids for viral replication is not unique for HCV. Rotaviruses associate with cellular lipid droplets for replication, and compounds disrupting lipid droplets inhibit rotavirus replication (39). Replication of another flavivirus, West Nile virus (WNV), is associated with intracellular membrane rearrangements and requires fatty acid synthesis (40). Positive-strand RNA viruses have evolved mechanisms to reprogram the host cells for their propagation by exploiting and hijacking host proteins, membranes, lipids, and even microRNAs during infection (41). Since positive-strand RNA viruses depend on intracellular membranes for their replication, perturbations in membrane lipid composition and/or protein lipidation are likely to impact viral replication. In addition, phospholipids have also been shown to influence alphavirus replicase protein activity (42, 43). Here, we have observed that HCV infection increases lipid droplet formation, which was significantly reduced upon knockdown of FoxO1 using specific siRNA or overexpression of FoxA2. These data correlated with a decrease in HCV replication, as determined by real-time PCR or a replicon-based luciferase reporter assay, upon FoxO1 siRNA treatment or FoxA2 ectopic expression. Thus, our results suggest that HCV utilizes lipids as a platform for viral genome replication.

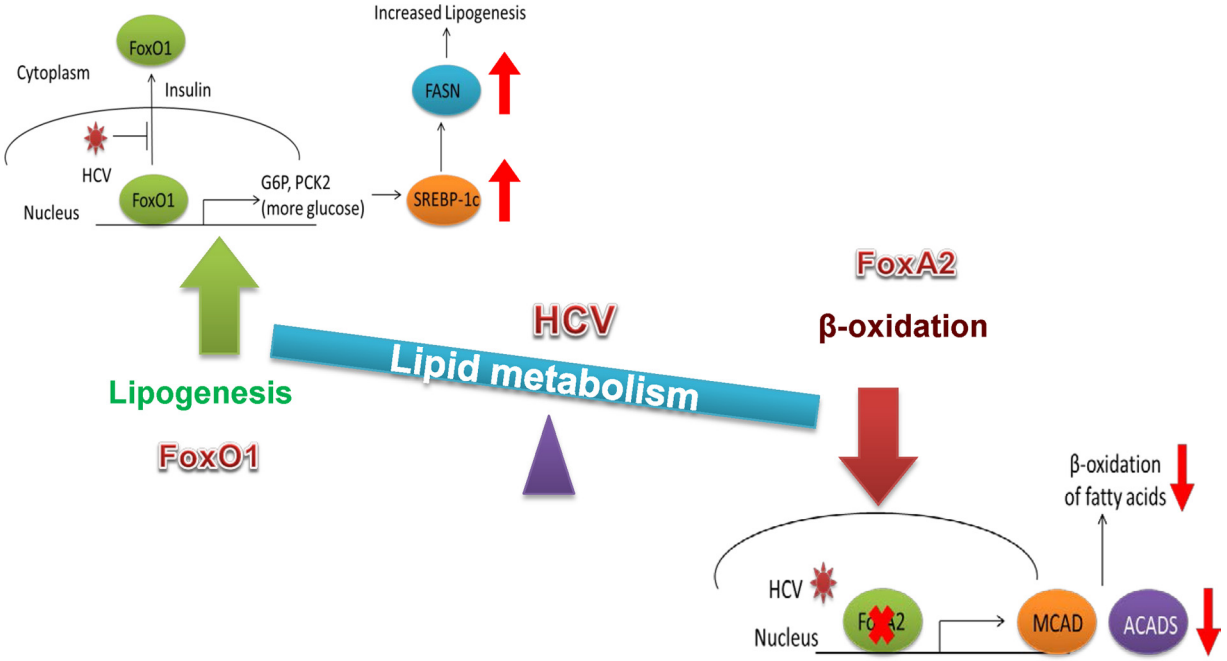

FIG 6 Schematic showing the potential of FoxO1 and FoxA2 in regulating lipogenesis and β-oxidation during HCV infection. HCV infection prevents insulin-stimulated translocation of FoxO1 from the nucleus to the cytoplasm, leading to increased gluconeogenesis (7, 17). Knockdown of FoxO1 by HCV significantly reduces SREBP-1c and FASN expression for increased lipogenesis. On the other hand, HCV infection reduces nuclear accumulation of FoxA2 (17). Overexpression of FoxA2 rescues the expression of MCAD and SCAD, indicating that HCV-mediated FoxA2 regulation may decrease β-oxidation.

Hepatic steatosis is a frequent complication associated with chronic HCV infection. Its presence is a key prognostic indicator associated with the progression to hepatic fibrosis and hepatocellular carcinoma (44). Several mechanisms have been proposed to account for the development of steatosis and fatty liver observed during HCV infection (45, 46). HCV infection enhances lipogenesis, reduces secretion of very-low-density lipoprotein, attenuates β-oxidation of lipid, and increases virus growth and replication through complex pathways that intersect via modulating host cell lipid metabolism (47, 48). We observed that HCV infection increases expression of both SREBP-1c and FASN. Further investigation suggested that the expression of SREBP-1c and FASN is controlled by the transcription factor FoxO1 during HCV infection. In addition, HCV infection significantly decreased both MCAD and SCAD expression. We further determined that the expression of MCAD and SCAD is controlled by FoxA2 during HCV infection (Fig. 6). HCV infection also increased LD accumulation, increased perilipin-2 expression, and decreased HSL activity. Thus, knockdown of FoxO1 (decreased lipogenesis) and overexpression of FoxA2 (increased β-oxidation) resulted in a significant disruption of the platform and, hence, a decrease in HCV genome replication. Thus, targeting of FoxO1 and FoxA2 might be useful in developing a therapeutic approach against HCV infection.

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