

# Repression of MicroRNA-30e by Hepatitis C Virus Enhances Fatty Acid Synthesis

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Chronic hepatitis C virus (HCV) infection often leads to end-stage liver disease, including hepatocellular carcinoma (HCC). We have previously observed reduced expression of microRNA-30e (miR-30e) in the liver tissues and sera of patients with HCV-associated HCC, although biological functions remain unknown. In this study, we demonstrated that HCV infection of hepatocytes transcriptionally reduces miR-30e expression by modulating CCAAT/enhancer binding protein  $\beta$ . *In silico* prediction suggests that autophagy-related gene 5 (ATG5) is a direct target of miR-30e. ATG5 is involved in autophagy biogenesis, and HCV infection in hepatocytes induces autophagy. We showed the presence of ATG5 in the miR-30e–Argonaute 2 RNA-induced silencing complex. Overexpression of miR-30e in HCV-infected hepatocytes inhibits autophagy activation. Subsequent studies suggested that ATG5 knockdown in Huh7.5 cells results in the remarkable inhibition of sterol regulatory element binding protein (SREBP)-1c and fatty acid synthase (FASN) level. We also showed that overexpression of miR-30e decreased lipid synthesis-related protein SREBP-1c and FASN in hepatocytes. **Conclusion:** We show new mechanistic insights into the interactions between autophagy and lipid synthesis through inhibition of miR-30e in HCV-infected hepatocytes. (*Hepatology Communications* 2019;3:943-953).

Chronic hepatitis C virus (HCV) infection is one of the etiologic causes of developing cirrhosis and eventually hepatocellular carcinoma (HCC).<sup>(1)</sup> Approximately 55% of patients infected with HCV develop steatosis because HCV uses host lipid metabolism for its life cycle.<sup>(2)</sup> Abnormal lipid metabolism influences metabolic disorders, cirrhosis, and liver carcinogenesis.<sup>(3-5)</sup> Histologically, HCV infection induces lipid droplet (LD) formation in hepatocytes.<sup>(6)</sup> We and others have shown that HCV infection induces autophagy<sup>(7,8)</sup> and modulates signaling pathways involved in lipogenesis, resulting in the accumulation of LDs,<sup>(9,10)</sup> although the precise mechanism is not fully understood.

MicroRNAs (miRNAs) are small noncoding RNAs that modulate the translation and stability of messenger RNAs (mRNAs) through a mechanism involving specific binding to mRNA based on base pair complementarity, controlling protein production. Most human miRNAs are processed into mature 20–26 nucleotide duplexes through endonucleolytic cleavage by ribonuclease (RNase) III enzymes and Argonaute (Ago) proteins. Dicer and Ago cooperate to form the RNA-induced silencing complex (RISC), consisting of Ago-bound single-strand miRNAs, which target host RNAs and induce a combination of translational repression.<sup>(11)</sup> miRNAs have a widespread impact on the etiologies of disease in viral infections<sup>(12)</sup> either

*Abbreviations:* Ago, Argonaute; ATG5, autophagy related gene 5; C/EBP- $\beta$ , CCAAT/enhancer binding protein  $\beta$ ; DAPI, 4',6-diamidino-2-phenylindole; FASN, fatty acid synthase; GFP, green fluorescent protein; HCC, hepatocellular carcinoma; HCV, hepatitis C virus; Ig, immunoglobulin; LC3B, microtubule-associated protein 1 light chain 3 beta; LD, lipid droplet; miR, microRNA; mRNA, messenger RNA; mTOR, mammalian target of rapamycin; p-mTOR, phosphorylated-mammalian target of rapamycin; RT-qPCR, reverse-transcription real-time polymerase chain reaction; siRNA, small interfering RNA; SREBP-1c, sterol regulatory element binding protein 1c; UPR, unfolded protein response; UTR, untranslated region.

Received January 11, 2019; accepted April 9, 2019.

Supported by the National Institutes of Health, National Institute of Diabetes and Digestive and Kidney Diseases (R01 DK081817) and Saint Louis University Liver Center Seed Grants.

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directly or indirectly through regulation of virus-associated host pathways.<sup>(11)</sup> Moreover, several miRNAs interact directly with host lipid metabolism.<sup>(13,14)</sup>

In the present study, we observed that miR-30e expression is inhibited at the transcriptional level following HCV infection by down-regulating the transcription factor CCAAT/enhancer binding protein  $\beta$  (C/EBP- $\beta$ ). miR-30e targets autophagy-related gene 5 (ATG5), a key molecule of autophagy signaling, and inhibits *de novo* lipogenesis-related protein, implicating its role in HCV-mediated pathogenesis.

## Materials and Methods

### CELL CULTURE, HCV INFECTION, AND TRANSFECTION

Huh7.5 cells and Huh7.5 cells harboring the HCV genotype 2a genome-length replicon (Rep2a cells; kindly provided by Hengli Tang, Florida State University, FL, USA) were used. Cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10 % fetal bovine serum and penicillin-streptomycin at 37°C in a 5% CO<sub>2</sub> atmosphere.

HCV genotype 2a (clone JFH1) was grown in Huh7.5 cells as described.<sup>(15)</sup> Virus released into cell culture supernatant was filtered through a 0.45- $\mu$ m pore cellulose acetate membrane and quantitated in standard IU/mL. For infection, cells were incubated with HCV genotype 2a (clone JFH1) (multiplicity of infection, 0.1) in a minimum volume of medium as described.<sup>(16)</sup> The cellular RNA was extracted 3 days postinfection.

Transfection of ATG5 small interfering RNA (siRNA) into Huh7.5 cells was performed using lipofectamine RNAiMAX (Invitrogen, Carlsbad, CA). Briefly, Huh7.5 cells were plated at a density of approximately  $1 \times 10^5$  cells/well in a 12-well plate and transfected with 50 nM ATG5 siRNA (sc-41445; Santa Cruz, Dallas, TX) or control siRNA and lysed for western blot analysis at 48 hours after transfection. Transfection of an miR-30e-5p mimic (002223; ThermoFisher Scientific, Waltham, MA) into Huh7.5 cells was performed using lipofectamine (Invitrogen). Briefly, Huh7.5 cells were plated at a density of approximately  $1 \times 10^5$  cells/well in a 12-well plate and transfected with 20 nM of miR-30e-5p mimic or control miR. Cells were lysed for western blot analysis 48 hours after transfection. Total RNA was prepared in a separate transfection.

### RNA QUANTITATION AND REVERSE-TRANSCRIPTION REAL-TIME POLYMERASE CHAIN REACTION

Total RNA was isolated by using a TRIzol reagent (Invitrogen). RNA was quantified by using a NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific). Complementary DNA was synthesized using miR-30e- or a U6-specific primer (Thermo Fisher Scientific) with a TaqMan miRNA reverse transcription (RT) kit or random hexamers and a Superscript III RT kit (Invitrogen). Real-time polymerase chain reaction (qPCR) was performed with a 7500 real-time PCR system

DOI 10.1002/hep4.1362

Potential conflict of interest: Nothing to report.

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(Applied Biosystems, Foster City, CA). TaqMan universal PCR master mix and a 6-carboxyfluorescein (FAM)-minor groove binder (MGB) probe for ATG5 (Hs00169468\_m1; Thermo Fisher Scientific) and 18S ribosomal RNA (rRNA; Hs03928985\_g1; Thermo Fisher Scientific) were used. Relative expression level was calculated by normalizing with U6 or 18S rRNA, using the  $2^{-\Delta\Delta CT}$  formula ( $\Delta\Delta CT = \Delta CT$  of the sample -  $\Delta CT$  of the untreated control).

## WESTERN BLOT ANALYSIS

Cells were lysed by using a sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) sample loading buffer. The lysates were subjected to PAGE and transferred onto a nitrocellulose membrane. The membranes were blocked with 5% nonfat dried milk and probed with the following specific primary antibodies: C/EBP- $\beta$ , sterol regulatory element binding protein (SREBP)-1 (2A4), and fatty acid synthase (FASN) (A-5; Santa Cruz); ATG5 (Proteintech, IL); and mammalian target of rapamycin (mTOR; 7C10) and phosphorylated-mTOR (p-mTOR; D9C2) (Cell Signaling Technology, Danvers, MA). After washing, the blots were incubated with secondary antibody for 1 hour. Proteins were detected by using an enhanced chemiluminescence western blot substrate (Pierce; ThermoFisher Scientific). Membranes were reprobed with antibody to actin (Santa Cruz) as an internal control for normalization of the protein load. ImageJ software (National Institutes of Health [NIH]) was used for densitometric scanning of western blot images.

## LUCIFERASE REPORTER ASSAYS

The miR-30e promoter (nucleotides -1813 to +1; P0) or promoter fragment of the deletion mutant of miR-30e (P1) were PCR amplified from Huh7.5 genomic DNA by using specific primers (Table 1). The fragments were digested with MluI and HindIII and cloned into the pGL3-Basic luciferase vector (Promega, Madison, WI). Huh7.5 cells and Rep2a cells were transfected with a luciferase reporter plasmid containing the miR-30e promoter (1  $\mu$ g/mL) by using Lipofectamine 3000 (Invitrogen). Luciferase activity was determined as described.<sup>(17)</sup>

Huh7.5 cells were plated at a density of  $0.8 \times 10^5$  cells/well in a 24-well plate and cotransfected with 25 nM C/EBP- $\beta$  siRNA (Santa Cruz) or control

**TABLE 1. PRIMER SEQUENCES USED IN THIS STUDY**

Primers	Sequence
miR-30e-full promoter_For	5'-ATTAGCCTCTGACGCGTGTGTTCTCTTAC-3'
miR-30e-F1 promoter_For	5'-ATTCAATACGCGTGAACCAAAGG-3'
miR-30e promoter_Rev	5'-TGACAGAAGCTTGCTAATCC-3'

siRNA and the promoter construct of miR-30e using Endofectin (GeneCopoeia, Rockville, MD). A luciferase reporter assay was performed 48 hours after transfection.

## Ago2-RNA CO-IMMUNOPRECIPITATION

The hepatocyte RNA-miR-30e complex was precipitated as described.<sup>(18)</sup> Briefly, miR-30e transfected Huh7.5 cells were lysed with lysis buffer (150 mM KCl, 25 mM Tris-HCl [pH 7.4], 5 mM ethylene diamine tetraacetic acid, 1% Triton X-100, 5 mM dithiothreitol, protease inhibitor mixture, and 100 U/mL RNase-OUT [Invitrogen]). Lysates were clarified, incubated with an anti-Ago2 monoclonal antibody (11A9; Sigma-Aldrich, St. Louis, MO) or isotype control immunoglobulin (Ig)G2a at 4°C for 7 hours, and mixed with protein G-Sepharose (GE Healthcare, Piscataway, NJ) overnight. The beads were washed 4 times, and RNA was isolated by using TRIzol reagent (Invitrogen). Target RNA was quantitated by RT-qPCR using specific primers.

## IMMUNOFLUORESCENCE

Huh7.5 cells were transfected with miR-30e mimic and infected with green fluorescent protein (GFP)-tagged HCV. Cells were fixed for 30 minutes with 4% paraformaldehyde after 72 hours of infection and then treated with 0.2% Triton X-100 for 5 minutes. After blocking with 3% bovine serum albumin, cells were incubated with primary antibody rabbit polyclonal microtubule-associated protein light chain 3B (LC3B; Sigma-Aldrich) and then conjugated to Alexa Fluor 594 (Molecular Probes, Eugene, OR) with anti-rabbit Ig. 4',6-Diamidino-2-phenylindole (DAPI) was incubated for nuclear staining. Three-channel optical images (green for HCV, red for LC3, and blue for nucleus) were collected using the sequential scanning mode of the Olympus FV1000 confocal system

(Olympus, Center Valley, PA). Images were superimposed digitally for fine comparisons.

Huh7.5 cells were transfected with ATG5 siRNA (sc-41445; Santa Cruz) or control siRNA for 48 hours. Cells were stained by boron-dipyrromethene (BODIPY) 493/503 (Invitrogen) for 1 hour, and DAPI was incubated for nuclear staining. Lipid accumulation was observed by using the Olympus FV1000 confocal system (Olympus) in cells with DAPI-stained nuclei. Images were superimposed digitally for fine comparisons. ImageJ software (NIH) was used for densitometric scanning of immunofluorescence images.

## STATISTICAL ANALYSIS

Results are presented as mean  $\pm$  SD. Data were analyzed by the Student *t* test with a two-tailed distribution. *P* < 0.05 was considered statistically significant.

## Results

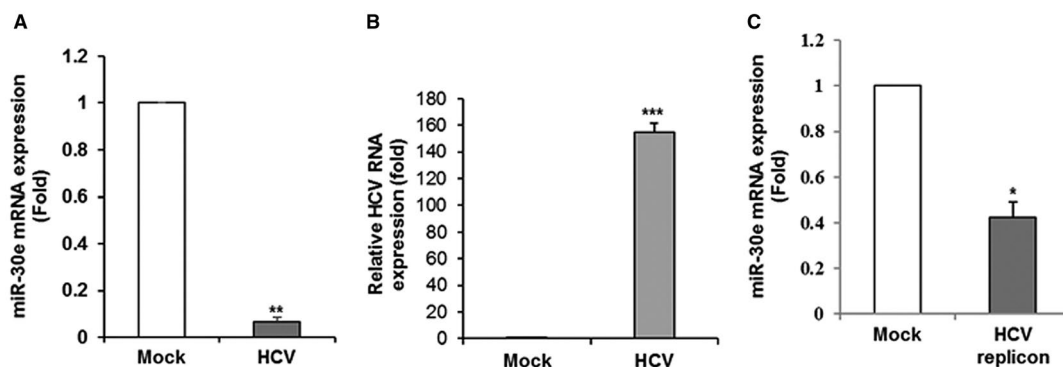
### HCV INFECTION DOWN-REGULATES miR-30e EXPRESSION

We have previously shown that miR-30e expression was significantly lower (*P* < 0.003) in the sera of patients with HCV-infected HCC compared with healthy volunteers.<sup>(19)</sup> We also observed

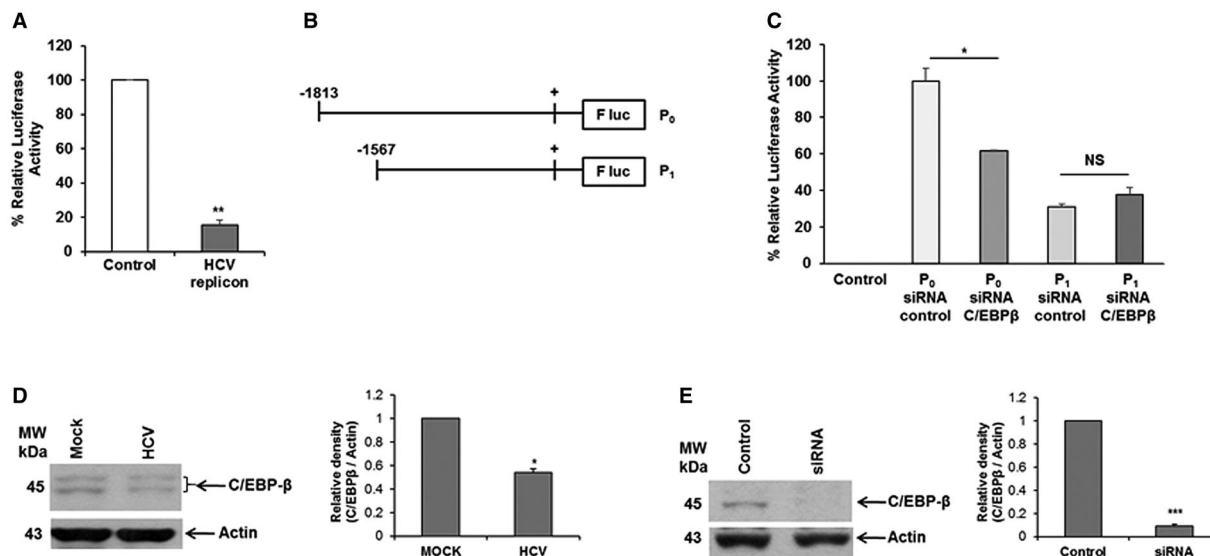
down-regulation of miR-30e in liver samples from patients with HCV-associated HCC, although the mechanism of miR-30e down-regulation remains unknown. We examined the expression level of miR-30e in HCV-infected human hepatocytes. The level of miR-30e was significantly reduced following HCV infection in Huh7.5 cells (Fig. 1, panel A). HCV RNA was measured from HCV-infected Huh7.5 cells by RT-qPCR (Fig. 1, panel B). A similar result was obtained when we examined the level of miR-30e in Huh7.5 cells harboring the genome-length HCV replicon (Rep2a cells) compared to parental Huh7.5 cells (Fig. 1, panel C). Together, our results indicate that HCV infection suppresses miR-30e expression in hepatocytes.

### HCV INFECTION DOWN-REGULATES C/EBP- $\beta$ -MEDIATED miR-30e PROMOTER ACTIVATION

We next examined how HCV modulates miR-30e expression. For this, an miR-30e promoter sequence (nucleotides -1,813 to +1; P<sub>0</sub>) was cloned into pGL3 luciferase reporter plasmid DNA, using specific primers (Table 1). Huh7.5 cells and Huh7.5 cells harboring the HCV genome-length replicon cells were transfected with the P<sub>0</sub> construct to examine promoter activity. miR-30e promoter activity was significantly down-regulated in HCV replicon-expressing cells compared to control parental cells (Fig. 2, panel A). To determine



**FIG. 1.** HCV inhibits miR-30e expression in hepatocyte and replicon (genotype 2a) cells. (A) Huh7.5 cells were left uninfected (mock) or infected with JFH1 for 48 hours. Expression of the miR-30e gene was examined by RT-qPCR. U6 RNA was used as an internal control. miR-30e was down-regulated in JFH1-infected Huh7.5 cells. (B) Expression of HCV RNA from virus-infected Huh7.5 cells was measured by RT-qPCR. (C) miR-30e was down-regulated in Huh7.5 cells harboring the HCV genome-length replicon cells. Values represent data from three independent experiments, mean  $\pm$  SD. Statistical significance was analyzed using the two-tailed Student *t* test; \**P* < 0.05, \*\**P* < 0.01, \*\*\**P* < 0.001.



**FIG. 2.** HCV transcriptionally suppresses miR-30e promoter activity. (A) Huh7.5 cells (control) and Huh7.5 cells harboring the HCV genome-length replicon (HCV replicon) were transfected with the miR-30e-luciferase reporter construct ( $P_0$ ), and promoter activity was measured by a relative luciferase assay after 48 hours of transfection. Data are presented as mean  $\pm$  SD from at least three independent experiments. (B) Schematic diagram of the miR-30e promoter region (nucleotides  $-1813$  to  $+1$ ;  $P_0$ ) cloned into the luciferase reporter plasmid ( $P_0$ ) and a deletion mutant of the miR-30e promoter construct (nucleotides  $-1567$  to  $+1$  [ $P_1$ ]). (C) Huh7.5 cells transfected with the miR-30e-luciferase reporter constructs ( $P_0$  or  $P_1$ ) knocked down C/EBP- $\beta$  using a specific siRNA, and promoter activity was measured by a relative luciferase assay after 48 hours of transfection. Data are presented as mean  $\pm$  SD from three independent experiments. (D) Huh7.5 cells were left uninfected (mock) or infected with HCV for 48 hours. Cell lysates were analyzed for C/EBP- $\beta$  expression by western blot using specific antibodies. The blot was reprobbed with antibody to actin for protein load. Densitometric scanning results are presented. (E) Huh7.5 cells transfected to control or siRNA to C/EBP- $\beta$ . Cell lysates were prepared 48 hours after transfection and analyzed for C/EBP- $\beta$  expression by western blot using specific antibodies. The blot was reprobbed with antibody to actin for protein load. Densitometric scanning results are presented. Statistical significance was analyzed using the two-tailed Student  $t$  test; \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$ . Abbreviations: F luc, firefly luciferase; NS, not significant.

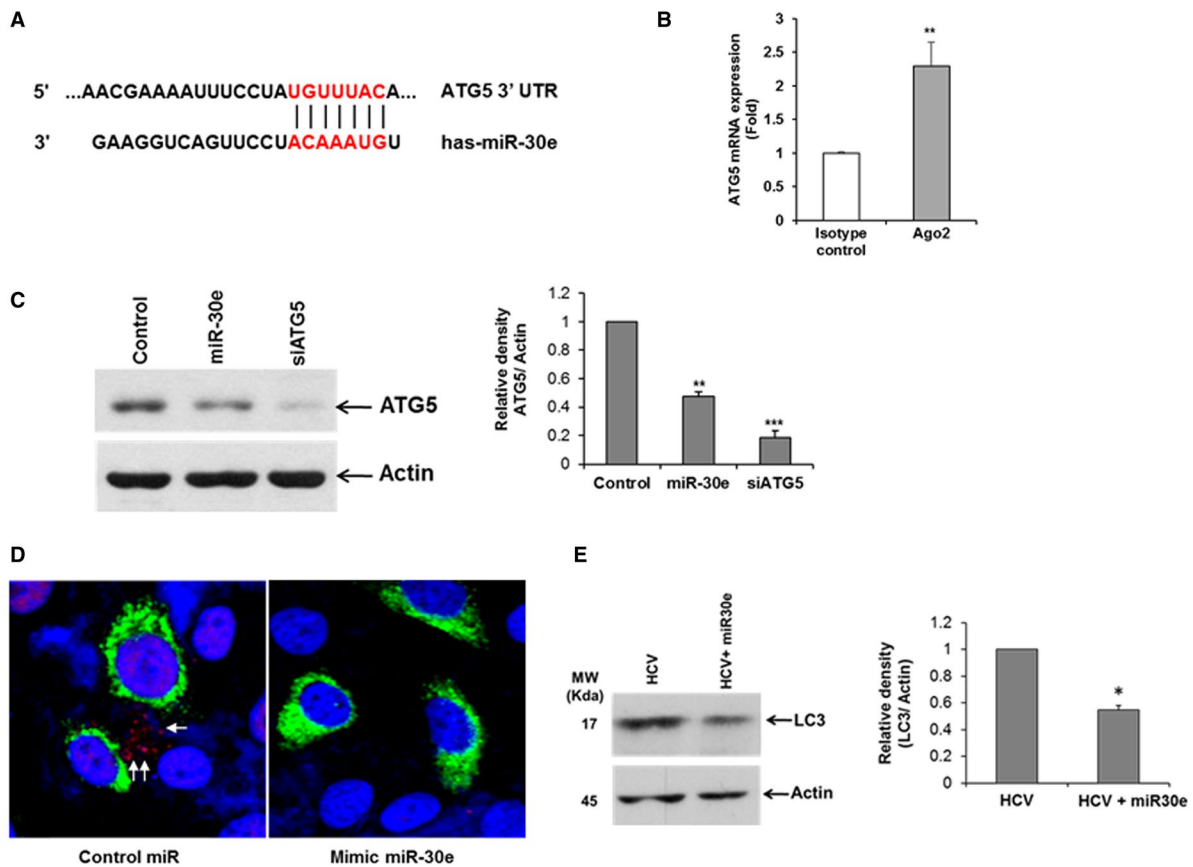
the *cis*-regulatory elements present in the miR-30e promoter region (nucleotides  $-1,813$  to  $+1$ ), we searched PROMO, using version 8.3 of TRANSFAC. Several regulatory elements were found in the designated consensus sequence of the miR-30e promoter, including binding sites for C/EBP- $\beta$ . C/EBP- $\beta$ , which belongs to the leucine zipper family, is an important transcriptional regulator.<sup>(20)</sup> Moreover, we have previously shown that HCV down-regulates promoter activity of complement C3 and miR-181c by C/EBP- $\beta$ .<sup>(21,22)</sup>

We next identified the specific region on the miR-30e promoter responsible for HCV-mediated repression. We generated deletion mutants of the miR-30e promoter constructs (nucleotides  $-1567$  to  $+1$  [ $P_1$ ]) and cloned these into pGL3 luciferase reporter plasmid DNA (Fig. 2, panel B). We knocked down C/EBP- $\beta$  using a specific siRNA and examined the activity of the  $P_0$  or  $P_1$  construct of the miR-30e promoter in Huh7.5 cells to analyze the role of C/EBP- $\beta$

in the regulation of HCV-mediated miR-30e expression. Knockdown of C/EBP- $\beta$  inhibited the activity of the miR-30e  $P_0$  promoter, but the  $P_1$  fragment did not exert an effect (Fig. 2, panel C), suggesting that the trans-suppressor element may be located between regions  $-1,813$  to  $-1,565$ . We further confirmed that HCV infection in Huh7.5 cells reduces C/EBP- $\beta$  expression (Fig. 2, panel D), in agreement with previous reports.<sup>(21,22)</sup> Transfection of siRNA to C/EBP- $\beta$  in Huh7.5 cells reduced protein expression (Fig. 2, panel E). Together, these results suggest that HCV-mediated C/EBP- $\beta$  suppression is an important mechanism for miR-30e down-regulation.

## ATG5 IS A DIRECT TARGET OF miR-30e

*In silico* prediction suggests that ATG5 is a direct target of miR-30e (Fig. 3, panel A). A recent report



**FIG. 3.** miR-30e targets the ATG5 3' UTR. (A) Schematic representation of miR-30e-predicted binding sites in the 3' UTR of ATG5 mRNAs. (B) Huh7.5 cells transfected with miR-30e mimic cell lysates were immunoprecipitated with Ago2-specific monoclonal antibody or an unrelated IgG2a isotype control. RNA was isolated from immunoprecipitates, and ATG5 expression was analyzed by RT-qPCR. Statistical significance was analyzed using the two-tailed Student *t* test; \*\**P* < 0.01. (C) Huh7.5 cells were transfected to control, mimic miR-30e, or siRNA to ATG5. Cell lysates were prepared 48 hours after transfection and analyzed for ATG5 expression by western blot using specific antibodies. The blot was reprobbed with antibody to actin for protein load. Densitometric scanning results are presented. Statistical significance was analyzed using the two-tailed Student *t* test; \**P* < 0.05, \*\**P* < 0.01, \*\*\**P* < 0.001. (D) Hepatocytes were infected with GFP-HCV, and transfected miR-30e or control miR was used as the negative control. Cells were fixed and stained with LC3 antibody 72 hours after infection. Confocal microscopy examination for subcellular localization of LC3 visualized by LC3 (red), GFP-HCV (green), and nucleus (DAPI) in merged image panels. Representative image (60×) is shown, and punctate localization of LC3 on autophagic vacuoles (arrows) was clearly visible in HCV-infected hepatocytes. (E) HCV-infected Huh7.5 cells were treated with miR-30e, and cell lysates were subjected to western blot analysis using the LC3B-specific antibody. The blot was reprobbed with antibody to actin for normalization. Densitometric scanning results are presented. Statistical significance was analyzed using the two-tailed Student *t* test; \**P* < 0.05.

showed that miR-30e targets the 3'-untranslated region (UTR) of ATG5 and inhibits its translation in gastric cancer cells.<sup>(23)</sup> We examined the interaction between miR-30e and ATG5 in hepatocytes. For this, miR-30e-transfected Huh7.5 cell lysates were immunoprecipitated with an Ago2-specific monoclonal antibody or an unrelated isotype control. RNA was isolated from the immunoprecipitated lysates and

analyzed by RT-qPCR. Higher ATG5 level was noted in the miR-30e-Ago2 complex compared to immunoprecipitated control, suggesting its presence in their complex (Fig. 3, panel B). Overexpression of miR-30e inhibits ATG5 expression (Fig. 3, panel C). siRNA to ATG5 was used as a positive control. As reported,<sup>(23)</sup> we did not observe the inhibition of ATG5 mRNA by miR-30e in our experimental system. We and

others have shown that HCV induces autophagy.<sup>(17,24)</sup> ATG5 is an important molecule in the autophagy signaling pathway. We therefore predicted that overexpression of miR-30e in HCV-infected hepatocytes will reduce ATG5 expression and may impair the induction of autophagy. For this, we overexpressed miR-30e in GFP-tagged HCV-infected cells and stained these for LC3, a marker for autophagy. Our results demonstrated that overexpression of miR-30e inhibited HCV-induced LC3 lipidation compared to control miR-treated cells (Fig. 3, panel D). We also observed that overexpression of miR-30e inhibited HCV-induced LC3 lipidation by western blot analysis (Fig. 3, panel E).

### DEPLETION OF ATG5 INHIBITS LIPID SYNTHESIS IN HEPATOCYTES

LDs have been implicated as an essential link for efficient autophagy in the yeast system,<sup>(25)</sup> and autophagy is inhibited following depletion of free fatty acids. In HCV infection, both autophagy and LDs are induced in hepatocytes. Because ATG5 is a direct target of miR-30e, we wanted to understand the interplay between ATG5 and lipid synthesis. ATG5 and ATG7 are essential molecules for the induction of autophagy; ATG7 conjugates ATG5 to ATG12 for autophagosome formation.<sup>(26)</sup> ATG7-deficient mice suppress LD formation,<sup>(27)</sup> and SREBP-1c forms LDs through *de novo* lipogenesis.<sup>(28)</sup> To investigate the relationship between ATG5 and SREBP-1c expression, we knocked down ATG5 using a specific siRNA and performed western blot analysis using specific antibodies. A significant down-regulation of SREBP-1c and FASN expression level was observed compared to that of the control cell after normalization with actin (Fig. 4, panel A). A similar result was obtained when we examined the expression of SREBP-1c and FASN in Huh7.5 cells harboring the genome-length HCV replicon (Rep2a cells) compared to parental cells (Fig. 4, panel B). We also observed a significant down-regulation of p-mTOR, an intermediate between ATG5 and SREBP-1c expression level, compared to that of the control cell (Fig. 4, panel C). We further showed an inhibition of LDs in ATG5-depleted cells (Fig. 4, panel D). Together, these data suggest that depletion of ATG5 inhibits SREBP-1c and FASN expression.

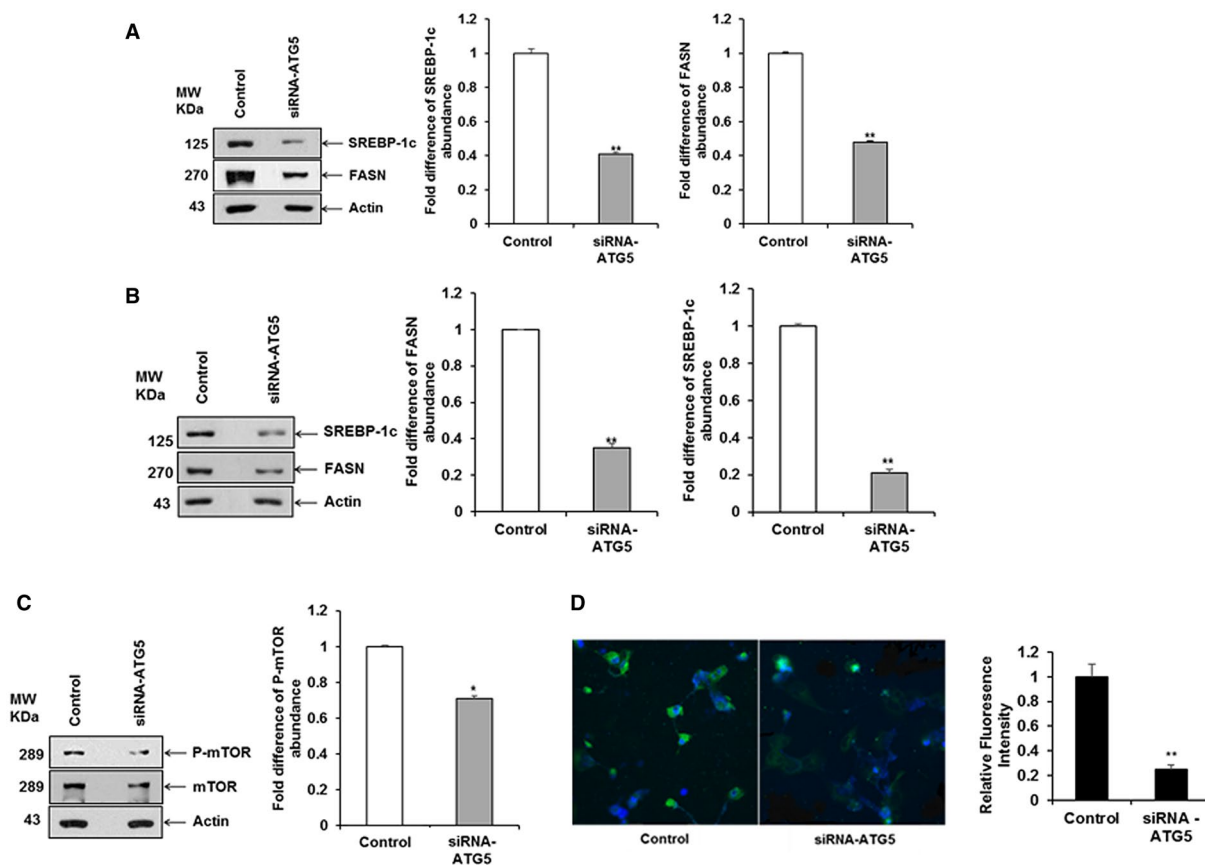
### OVEREXPRESSION OF miR-30e INHIBITS LIPID SYNTHESIS IN HEPATOCYTES

SREBP-1 and FASN are important players in lipid metabolism, and SREBPs are key transcriptional factors in lipogenesis and lipid uptake.<sup>(29,30)</sup> We observed that HCV infection in Huh7.5 cells increased expression of SREBP-1c and its downstream target FASN (Fig. 5, panel A). We also observed ATG5 depletion inhibits SREBP-1c and FASN expression. We therefore examined whether miR-30e overexpression modulates lipid synthesis in hepatocytes. For this, we performed western blot analysis in Huh7 cells transfected with an miR-30e mimic or control miR. A significant down-regulation of SREBP-1c and FASN expression level compared to that of the control parental cell was observed after normalization with the housekeeping protein actin (Fig. 5, panel B). A similar result was obtained when we examined the expression of SREBP-1c and FASN in Huh7.5 cells harboring the genome-length HCV replicon (Rep2a cells) compared to parental cells (Fig. 5, panel C). Together, our data suggest that miR-30e inhibits ATG5 and eventually SREBP-1c and FASN in hepatocytes.

### Discussion

In the present study, we demonstrated that (i) HCV infection transcriptionally down-regulates miR-30e, (ii) down-regulation of miR-30e targets ATG5 and modulates induction of autophagy, and (iii) overexpression of miR-30e or ATG5 knockdown inhibits *de novo* lipogenesis.

We previously showed that HCV infection increased lipid accumulation through metabolic gene regulation.<sup>(9)</sup> Increased accumulation of LDs frequently causes liver steatosis in patients chronically infected with HCV.<sup>(2,31)</sup> In this report, we observed that HCV infection modulates lipogenesis processes primarily by regulating miR-30e transcription. Overexpression of miR-30e inhibits the expression of SREBP-1c and FASN, which are known to be involved in *de novo* lipogenesis and an increased expression of LDs, promoting lipid accumulation. Together, these results suggest that HCV increases *de novo* lipogenesis in hepatocytes by inhibiting miR-30e expression.



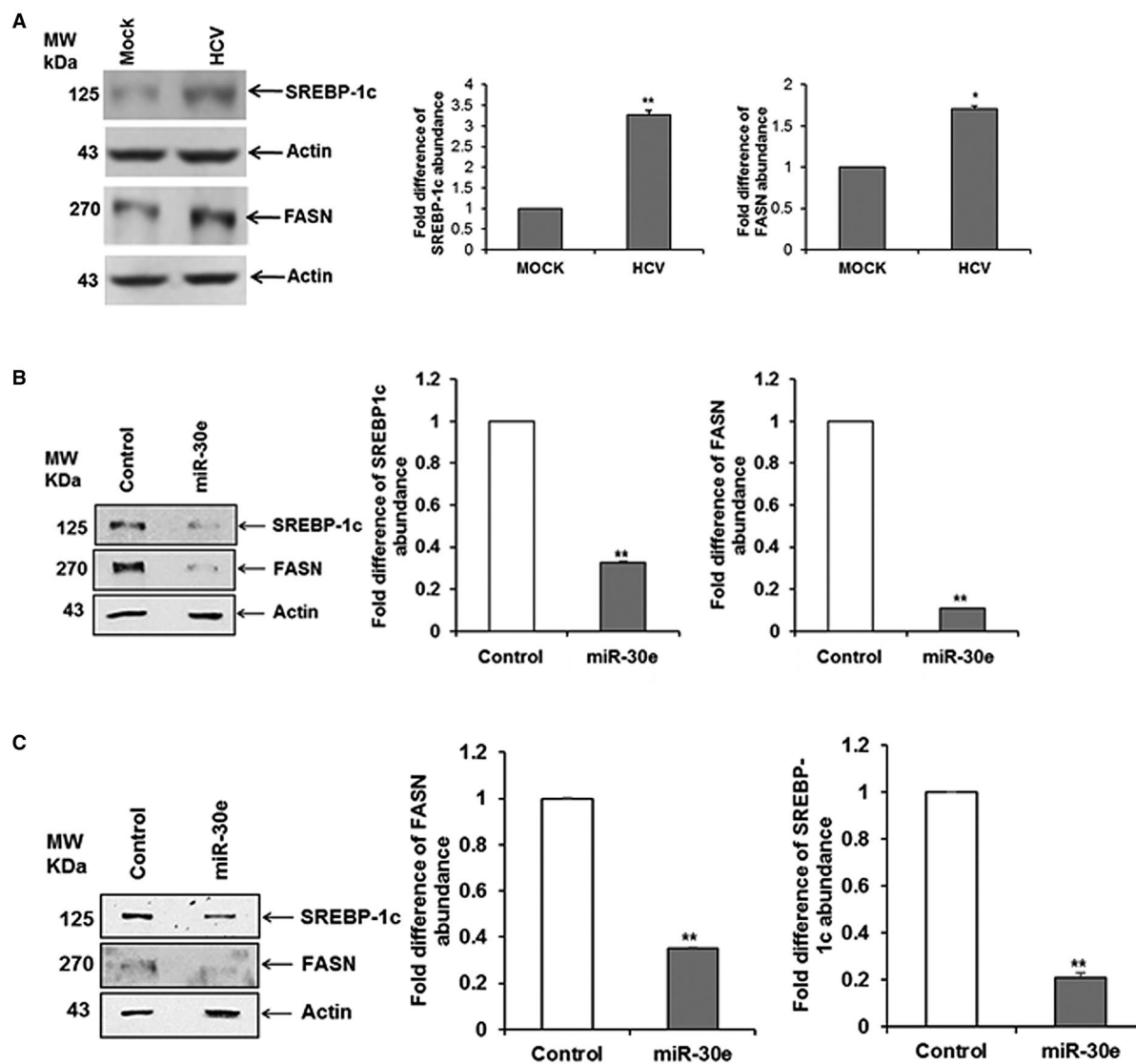
**FIG. 4.** Knockdown of ATG5 inhibits SREBP-1c and FASN in hepatocytes. (A) Huh7.5 cells were transfected with siRNA to ATG5 (siRNA-ATG5) or siRNA negative control, and cell lysates were prepared after 48 hour post-transfection. SREBP-1c and FASN were detected by western blot analysis using specific antibodies. The blot was re-probed with antibody to actin for loading control. (B) Huh7.5 cells harboring the HCV genome-length replicon cells were transfected for 48 hours with siRNA-ATG5 or siRNA negative control, and cell lysates were prepared. SREBP-1c and FASN were down-regulated in siRNA-ATG5-transfected hepatocytes. (C) A significant down-regulation of p-mTOR expression level in Huh7.5 cells transfected with siRNA-ATG5 compared to that of the control cells was observed after normalization with actin. A densitometry scan is shown on the right in all panels. (D) Hepatocytes were transfected for 48 hours with siRNA-ATG5 or siRNA negative control, and cells were stained 48 hours after transfection. Confocal microscopy examination for LDs visualized by LDs (BODIPY, green) and nucleus (DAPI, blue) in merged image panels (20 $\times$ ). A representative image is shown. Quantitation of the BODIPY staining is shown in the bar graph on the right. Statistical significance was analyzed using the two-tailed Student *t* test; \**P* < 0.05, \*\**P* < 0.01. Abbreviation: BODIPY, boron-dipyrromethene.

miRNAs impact the regulation of many cellular processes, and viruses modulate the host miRNA expression either directly or indirectly to accelerate pathogenesis.<sup>(12,32)</sup> In reaction to viral infection, signaling pathways in hepatocytes direct a variety of intracellular events that generate *de novo* lipogenesis in the infected cells.<sup>(13,14)</sup> In earlier reports, miR-30e was implicated as a subtype-specific prognostic marker in breast cancer and ovarian cancer<sup>(33,34)</sup> and is inhibited in chronic myeloid leukemia, lung cancer, and patients with HCC.<sup>(19,35,36)</sup> miR-30 has been implicated as a tumor suppressor and inhibits

epithelial-to-mesenchymal transition in HCC through metastasis-associated protein 1 modulation.<sup>(37,38)</sup> In cancer development, fatty acids are understood to be the most relevant because lipid metabolism participates in the regulation of many cellular processes, such as cell growth, proliferation, differentiation, and survival.<sup>(39)</sup> Moreover, miR-30e has been implicated in the regulation of fatty acid metabolism in goat mammary epithelial cells.<sup>(40)</sup>

We observed inhibition of miR-30e expression by HCV infection or HCV replicon in human hepatocytes, suggesting direct evidence for an association





**FIG. 5.** miR-30e inhibits SREBP-1c and FASN in hepatocyte and replicon (genotype 2a) cells. (A) Huh7.5 cells were left uninfected (mock) or infected with HCV, and cell lysates were analyzed for SREBP-1c and FASN expression by western blot using specific antibodies. The blots were reprobated with antibody to actin for protein load. (B) Huh7.5 cells were transfected with miR-30e mimic or miRNA mimic negative control, and cell lysates were prepared after 48 hour post-transfection. SREBP-1c and FASN were detected by western blot analysis using specific antibodies. The blot was reprobated with antibody to actin for loading control. (C) SREBP-1c and FASN were down-regulated in Huh7.5 cells harboring the HCV genome-length replicon cells. A densitometry scan is shown on the right in all panels. Statistical significance was analyzed using the two-tailed Student *t* test; \**P* < 0.05, \*\**P* < 0.01.

with HCV infection. C/EBP- $\beta$  is an important transcriptional factor belonging to the leucine zipper family and is involved in several cellular functions, such as cell proliferation and differentiation.<sup>(20)</sup> We previously showed that C/EBP- $\beta$  is down-regulated following HCV infection.<sup>(21)</sup> In our experiments, we demonstrated that HCV inhibits miR-30e promoter activity by down-regulating C/EBP- $\beta$  expression.

Deletion of the C/EBP- $\beta$  binding site on an miR-30e promoter ( $P_1$  fragment) or knockdown of C/EBP- $\beta$  showed suppression of miR-30e promoter activity.

We demonstrated an interaction between miR-30e and ATG5 in our complex by RISC assay; this concurs with a report that ATG5 is a direct target of miR-30e.<sup>(23)</sup> ATG5 and ATG7 are autophagy-specific

genes involved in the initiation of autophagosome formation and completion through a ubiquitin-like conjugation system.<sup>(41)</sup> Interestingly, ATG7-deficient mice suppressed LD formation,<sup>(27)</sup> and liver-specific ATG5-knockout mice displayed impaired LD biogenesis.<sup>(42)</sup> HCV infection induces autophagy. We postulate that HCV-mediated inhibition of miR-30e facilitates ATG5 for autophagy induction following HCV infection. We showed previously that autophagy acts as an upstream positive regulator of mTOR complex 1 (mTORC1),<sup>(17)</sup> which regulates SREBP-1c at the transcriptional level<sup>(43)</sup>; however, further study is needed to dissect this mechanism. SREBP-1c forms LDs through *de novo* lipogenesis.<sup>(28)</sup> Furthermore, unfolded protein response (UPR) activates autophagy in HCV-infected hepatocytes,<sup>(44)</sup> and UPR activates SREBP maturation in cultured cells.<sup>(45,46)</sup> Moreover, the activation of multiple UPR responses and the SREBP pathway significantly overlapped.<sup>(47)</sup> Processing of SREBP-1c has been reported to depend on mTORC1.<sup>(48)</sup> In our study, SREBP-1c and FASN were inhibited by knockdown of ATG5 in hepatocytes. These results demonstrated that *de novo* lipogenesis is regulated through autophagy in hepatocytes following HCV infection. HCV causes steatosis, with the reported mean prevalence of HCV-related NAFLD being 55% and nonalcoholic steatohepatitis being 4%-10% of cases.<sup>(49)</sup> We previously showed that HCV-infected hepatocytes increased expression of SREBP-1c and its downstream target FASN, both of which are involved in lipogenesis.<sup>(9)</sup> We showed here HCV-mediated down-regulation of miR-30e is responsible, in part, for up-regulating SREBP-1c and FASN expression.

We observed that expression levels of *de novo* lipogenesis-related protein SREBP-1c and FASN are significantly inhibited in miR-30e-overexpressed hepatocytes. miR-30e also directly targets the 3' UTR of ATG5. Moreover, knockdown of ATG5 expression negatively regulates SREBP-1c and FASN protein expression level. In summary, we have demonstrated that HCV infection transcriptionally down-regulates miR-30e expression by targeting C/EBP- $\beta$ , which in turn enhances the expression of *de novo* lipogenesis-related protein SREBP-1c and FASN through autophagy. Our observations contribute to the understanding of the mechanistic role of miR-30e in HCV-host interactions toward pathogenesis.

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