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Interferon-alpha-Induced Hepatitis C Virus Clearance Restores p53 Tumor Suppressor More Than Direct-Acting Antivirals

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The mechanism why hepatitis C virus (HCV) clearance by direct-acting antivirals (DAAs) does not eliminate the risk of hepatocellular carcinoma (HCC) among patients with advanced cirrhosis is unclear. Many viral and bacterial infections degrade p53 in favor of cell survival to adapt an endoplasmic reticulum (ER)-stress response. In this study, we examined whether HCV clearance by interferon-alpha or DAAs normalizes the ER stress and restores the expression of p53 tumor suppressor in cell culture. We found that HCV infection induces chronic ER stress and unfolded protein response in untransformed primary human hepatocytes. The unfolded protein response induces chaperone-mediated autophagy (CMA) in infected primary human hepatocytes and Huh-7.5 cells that results in degradation of p53 and induced expression of mouse double minute 2 (Mdm2). Inhibition of p53/Mdm2 interactions by small molecule (nutlin-3) or silencing Mdm2 did not rescue the p53 degradation, indicating that HCV infection induces degradation of p53 independent of the Mdm2 pathway. Interestingly, we found that HCV infection degrades p53 in a lysosome-dependent mechanism because lysosome-associated membrane protein 2A silencing restored p53 degradation. Our results show that HCV clearance induced by interferon-alpha-based antiviral therapies normalizes the ER-stress response and restores p53, whereas HCV clearance by DAAs does neither. We show that decreased expression of p53 in HCV-infected cirrhotic liver is associated with expression of chaperones associated with ER stress and the CMA response. *Conclusion:* HCV-induced ER stress and CMA promote p53 degradation in advanced liver cirrhosis. HCV clearance by DAAs does not restore p53, which provides a potential explanation for why a viral cure by DAAs does not eliminate the HCC risk among patients with advanced liver disease. We propose that resolving the ER-stress response is an alternative approach to reducing HCC risk among patients with cirrhosis after viral cure. (*Hepatology Communications* 2017;1:256-269)

Introduction

Chronic hepatitis C virus (HCV) infection is the major risk factor for hepatocellular carcinoma (HCC) in the United States. The risk is increased several fold among patients with advanced liver cirrhosis. The recent development of potent direct-acting antivirals (DAAs) is changing the therapeutic options for curing chronic HCV infection, and additional versions of highly effective DAA combination therapies are expected to be available in the future. This provides hope that HCV infection can be globally

Abbreviations: ANOVA, analysis of variance; ATF6, activating transcription factor 6; ATG5, autophagy related protein 5; BIP, binding immunoglobulin protein; CMA, chaperone-mediated autophagy; DAAs, direct-acting antivirals; eIF2, eukaryotic initiation factor 2; ER, endoplasmic reticulum; ERAD, ER-associated degradation; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; GFP, green fluorescent protein; HCC, hepatocellular carcinoma; HCQ, hydroxychloroquine; HCV, hepatitis C virus; HSC70, heat shock cognate protein complex 70; IFN, interferon; IRE1, inositol-requiring enzyme 1; LAMP-2A, lysosome-associated membrane protein 2A; LC3B, light chain 3B; Mdm2, mouse double minute 2; MOI, multiplicity of infection; mRNA, messenger RNA; PCR, polymerase chain reaction; peIF2, phospho-eukaryotic initiation factor 2; PERK, protein kinase-like endoplasmic reticulum kinase; PHHs, primary human hepatocytes; RBV, ribavirin; RT-PCR, reverse transcription polymerase chain reaction; SD, standard deviation; siRNA, small interfering RNA; TG, thapsigargin; TUDCA, tauroursodeoxycholic acid; UPR, unfolded protein response; XBP-1, x-box binding protein 1.

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eliminated and will require that all infected patients receive early diagnosis and access to antiviral treatment. However, individuals who are chronically infected but remain untreated have the highest risk of developing liver cirrhosis and HCC. Recent clinical studies show that an HCV cure using DAA-based antiviral therapy among patients with advanced liver cirrhosis does not eliminate HCC risk.[1-3] The incidence of HCC after a viral cure with a DAA-based therapy was found to be much higher than that of the earlier findings with an interferon (IFN)-based antiviral therapy.[4] The mechanism by which HCV cleared by an IFN-based antiviral therapy provides benefits in HCC reduction is unknown, but understanding the basic mechanism of how HCV actually causes HCC could answer this question and therefore remains an important area of future research.

HCV is a positive-strand, enveloped, RNA virus that replicates exclusively in the cytoplasm without integration into the host cell genome. Sustained RNA translation and replication in the hepatocytes results in an accumulation of large amounts of viral proteins in the endoplasmic reticulum (ER), which generates a substantial amount of stress response called the unfolded protein response (UPR). The UPR is orchestrated by three different cellular transcription factors: protein kinase-like endoplasmic reticulum kinase (PERK), activation of transcription factor 6 (ATF6), and inositol-requiring enzyme 1 (IRE1). These transcription factors maintain ER homeostasis. In general, infected hepatocytes cope with ER stress in four steps.[5] First, infected cells induce transcription of UPR to reduce the protein load in the ER. Second, the UPR improves ER function through inducing chaperone gene expression. Third, proliferation of ER compartments accommodates the viral protein load through initiating ER-associated degradation (ERAD) of unfolded proteins. The UPR uses two types of ERAD mechanisms: ubiquitin-proteasome-dependent degradation (type I) and autophagy-dependent lysosomal degradation (type II). Fourth, if the ERAD response fails to resolve the ER stress associated with the virus infection, then the UPR switches from cellular prosurvival signaling to apoptosis signaling. Infected cells also use different cellular surveillance mechanisms to block virus replication and spread.[6] One such mechanism involves the production of IFN, which directly inhibits virus replication.[7] The other mechanism involves blocking the spread of infection by inducing p53-mediated cellular apoptosis.[8]

Over the past several years, many investigators, including our own, showed that HCV-associated ER stress induced an autophagy response, which results in impaired host innate immunity through blocking endogenous IFN production[7,9] and also escapes from exogenously added IFN-α and ribavirin (RBV)-based antiviral therapy through degradation of interferon-alpha receptor 1 and RBV transporter.[10,11] In this report, we found that HCV infection induces chaperone-mediated autophagy (CMA) as a cell survival mechanism to avoid the ER-stress response. Our results show that HCV degrades both mutant p53 in a proliferative Huh-7.5 culture and wild-type p53 in a nonproliferative primary human hepatocytes (PHHs)

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culture using a mechanism that is independent of mouse double minute 2 (Mdm2). As the loss of p53 function is associated with HCC, our findings provide an explanation for why HCV clearance by DAAs does not eliminate the HCC risk among patients with advanced cirrhosis due to unresolved ER stress. In this study, we compared the restoration of the p53 tumor expression and stress response after viral eradication by interferon versus DAA therapies to determine whether unresolved ER stress could be one of the causes of HCC risk among patients with cirrhosis.

Materials and Methods

PRIMARY HUMAN HEPATOCYTES

PHHs were obtained from XenoTech, LLC (Kansas City, MO) and cultured with hepatocyte culture media supplemented with 10% human serum (Invitrogen, Brown Deer, WI). After 24 hours, they were infected with cell culture-grown HCV (JFH-ΔV3-Rluc virus, HCV genotype 2a) with a multiplicity of infection (MOI) of 0.1, using a standard protocol of our laboratory. After 18 hours of infection, hepatocytes were replaced with fresh hepatocyte culture media (XenoTech) supplemented with 10% human serum (Invitrogen). Uninfected or infected PHHs were harvested every 3 days, and cell pellets were used for RNA isolation and western blot analysis. The success of HCV replication in the infected PHHs was assessed by the detection of positive-strand HCV RNA levels by reverse transcription (RT) real-time quantitative polymerase chain reaction (PCR) and western blot analysis of HCV NS3 protein.

PATIENTS AND LIVER SAMPLES

A total of 16 explant livers (eight HCV infected with liver cirrhosis, eight HCV-negative HBV-negative explant livers with cirrhosis) were collected from the Ochsner Medical Center (New Orleans, LA) for this study. The liver tissues derived from HCV-negative and HBV-negative patients with a history of alcohol intake; cryptogenic cirrhosis as well as nonalcoholic steatohepatitis were used as control. Explant liver tissues were collected with institutional review board approval from Tulane University Health Sciences Center and Ochsner Medical Center. Informed written consent was obtained from each patient.

STATISTICAL ANALYSIS

The immunostaining and immunofluorescence images were quantified using a computer image analysis software package (ImageJ; National Institutes of Health, Bethesda, MD).[12,13] All measurements were made at least in triplicate (n = 3). To compare means within groups, we performed one-factor analysis of variance (ANOVA) using the GraphPad Prism software. We assumed that all measurements have normal probability distributions, which is expected for these types of data. When the overall P value for the ANOVA analysis was significant ($P < 0.05$), we applied Dunnet's post hoc test to compare control samples with experimental samples. When performing comparisons between multiple groups, each analyzed with ANOVA, we used the Bonferroni correction to determine a revised cutoff for statistical significance that gives a combined 5% type I error probability ($^{*}P < 0.05$, $^{**}P < 0.005$, $^{***}P < 0.0005$).

Results

PERSISTENT HCV REPLICATION INDUCED CHRONIC ER STRESS IN HEPATOCYTES

To study the effect of HCV replication on hepatocyte growth and survival, we developed a replication model in nonproliferative PHHs. PHHs seeded in a six-well tissue culture plate without collagen were infected with JFH-ΔV3-Rluc virus (MOI 0.1) overnight. Replication of HCV in PHHs was confirmed by the measurement of viral NS3 protein by western blot analysis (Fig. 1A) and immunostaining for HCV core and NS3 (Fig. 1B). Huh-7.5 cells were also infected with JFH-ΔV3-Rluc virus (MOI 0.1), and HCV replication was studied over a month by the measurement of Renilla luciferase activity. We found an increased expression of Renilla luciferase in HCV-infected Huh-7.5 cells (Fig. 1C). These results were supported by immunostaining for viral core protein (Fig. 1D) and western blot for NS3 protein (Fig. 1E). HCV RNA translation, replication, and virus assembly occurs at the ER and the ER-derived membranes. Accumulation of large amounts of viral proteins during persistent replication induces a stress response in infected PHHs. Prior studies have shown that the UPR was either partially activated or suppressed to attenuate ER-stress-mediated hepatocellular apoptosis using transformed hepatoma cells (Huh-7.5) soon after virus infection.[9,10] It is

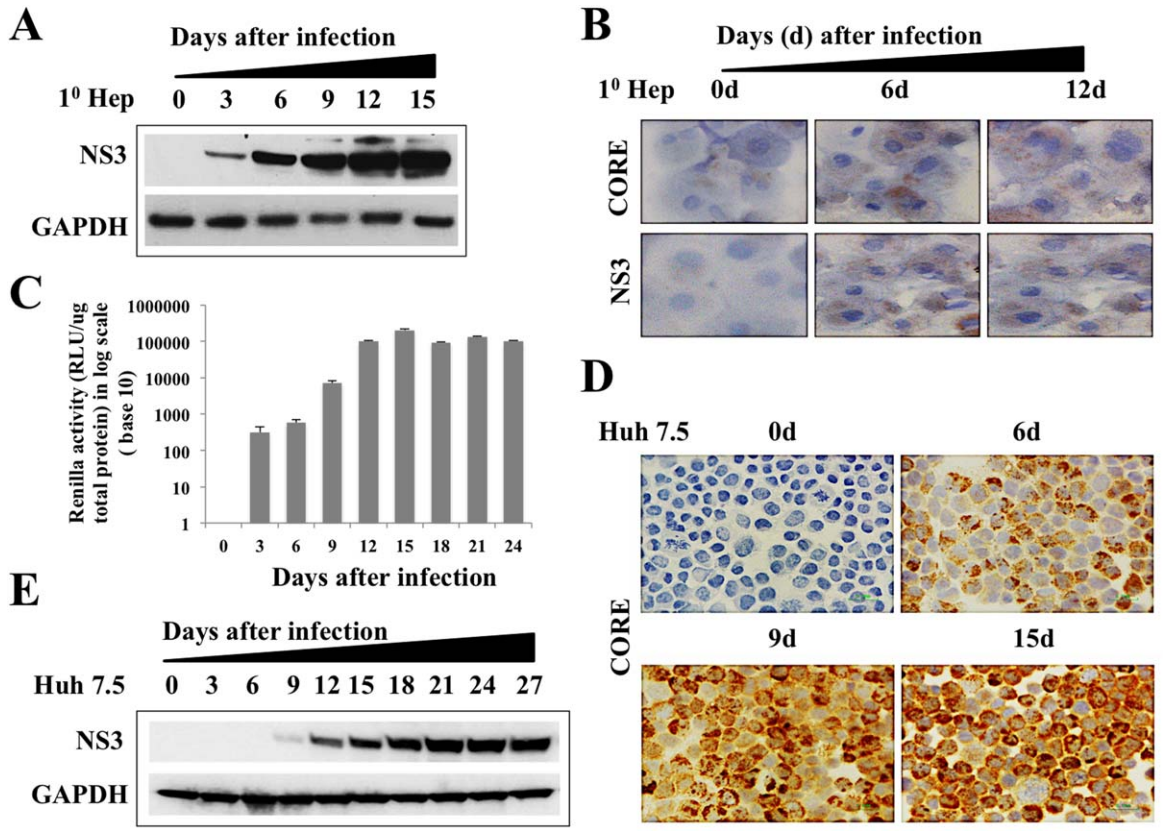

FIG. 1. Persistent HCV replication in nonproliferative PHHs and proliferative Huh-7.5 cell cultures. PHHs were infected with an HCV pJFH-ΔV3-Rluc clone at an MOI of 0.1 by overnight incubation. The next day cells were incubated with fresh media with 10% human serum. (A) HCV replication was confirmed by the measurement of NS3 protein levels by western blot. (B) Immunostaining of infected PHHs using a monoclonal antibody to the HCV core and rabbit polyclonal antibody to the NS3 protein. (C) Renilla luciferase activity of infected Huh-7.5 cells over 24 days. (D) Expression of HCV core protein by immunostaining. (E) Expression of HCV NS3 protein in infected Huh-7.5 cells over 27 days by western blot analysis. Range bars in the graph are SD. Images were taken at magnification ×40. Abbreviation: GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

unclear whether persistent HCV replication induced chronic or adaptive ER stress in untransformed hepatocytes. Using an HCV-infected PHH model, we found that the expression of all three branches of the UPR were induced and autophagy markers were also induced by western blot analysis for 15 days (Fig. 2A). We noticed that the expression of UPR markers remained high in the infected culture over 15 days compared to uninfected PHHs, indicating that HCV replication induces chronic ER stress. The UPR results in the production of chaperone to increase the folding capacity of the ER and also enhances the ERAD pathway and autophagy. We found that the autophagy response is induced in PHHs secondary to ER stress, which was confirmed by the measurement of autophagy-related 5 and light chain 3B-II levels by western blot analysis (Fig. 2A). We did not see any significant activation of ER-stress markers in uninfected PHHs when cultured for similar time points without HCV infection (Fig. 2B). The status of the ER-stress/UPR response was examined by comparing the expression of binding immunoglobulin protein (BIP), ATF6, and IRE1α in Huh-7.5 cells with or without HCV infection for similar time periods (Fig. 2C,D). These results are consistent with PHHs, which indicate that HCV infection induces chronic ER stress and the UPR response.

PERSISTENT HCV INFECTION ACTIVATES CMA

CMA is responsible for degradation of cytosolic proteins induced under ER-stress conditions through

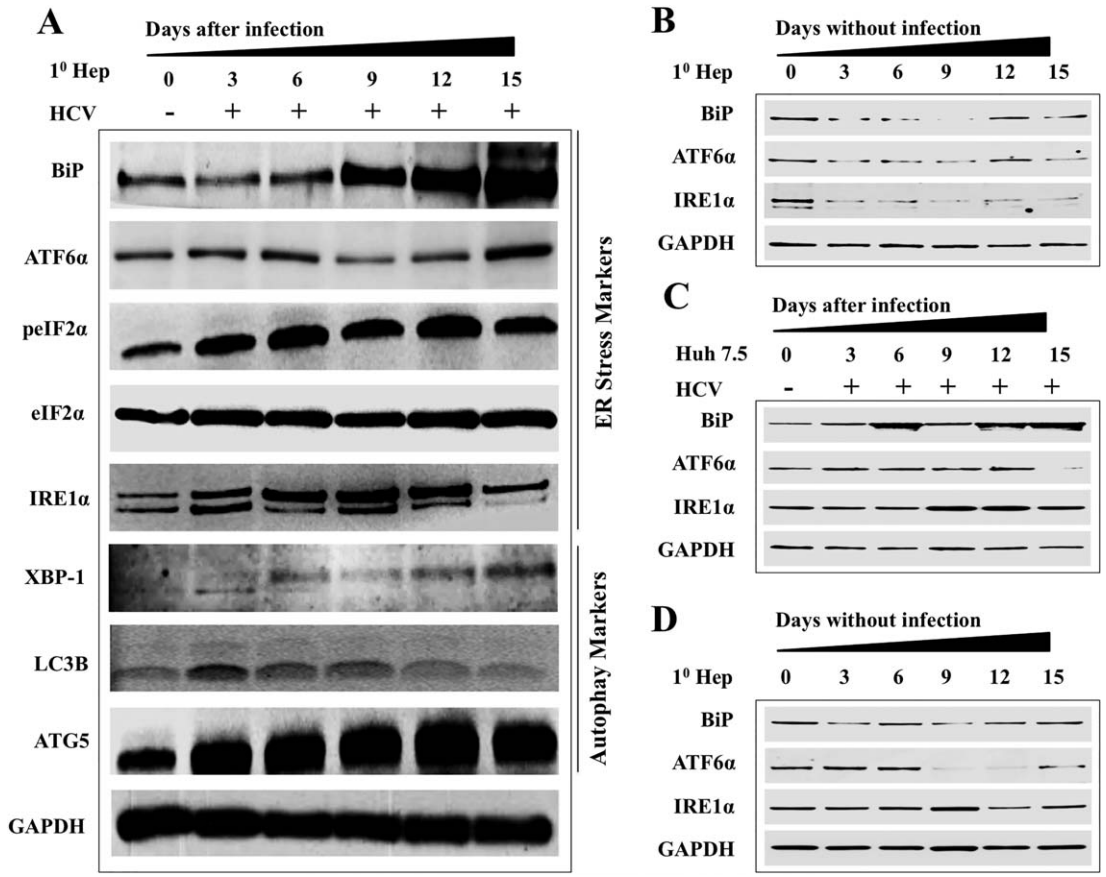

FIG. 2. Persistent HCV infection of PHHs and Huh-7.5 cells activates three branches of UPR gene expression and induces an autophagy response. (A) Cell lysates prepared from infected PHHs were examined for the expression of ER-stress markers and autophagy markers by western blot analysis. (B) UPR gene expression in uninfected PHHs examined at similar time points. (C) Expression of BIP, ATF6, and IRE1α in Huh-7.5 cells with HCV infection and (D) without infection. Abbreviation: GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

the PERK and IRE1 axes of the UPR.[14,15] All CMA substrates contain a consensus pentapeptide motif (KFERQ) that is recognized by a cytosolic chaperone; for example, heat shock cognate protein complex 70 (HSC70) binds to lysosome-associated membrane protein 2A (LAMP-2A), which results in the direct translocation of unfolded protein substrate across lysosomal membranes and subsequent degradation. The expression of HSC70 and LAMP-2A has been shown to be the rate-limiting step for the uptake of cellular substrates by lysosome-mediated protein degradation during the process of CMA. Western blot analysis shows that LAMP-2A and HSC70 expression in PHHs was induced nicely after HCV infection. The expression of LAMP-2A and HSC70 was not induced in PHHs cultured without HCV infection (Fig. 3A). Immunostaining verified the induced expression of LAMP-2A and HSC70 in infected PHHs at days 0, 3, and 15 (Fig. 3B). The immunostaining data were quantified using ImageJ software and were significantly induced after HCV infection (Fig. 3C). The impact of HCV replication on the expression of HSC70 and LAMP-2A was examined in infected Huh-7.5 cells (Fig. 3D). Western blot analysis showed that HSC70 and LAMP-2A levels were induced in persistently infected Huh 7.5 cell culture after day 9 and were maintained steadily throughout the infection. The expression of LAMP-2A and HSC70 in HCV-infected Huh-7.5 cells was confirmed by immunostaining (Fig. 3E). LAMP-2A and HSC70 expression was quantified using ImageJ software and was found to be induced after HCV infection (Fig. 3F). These results suggest that CMA is induced due to the accumulation of misfolded proteins in the ER during HCV replication.

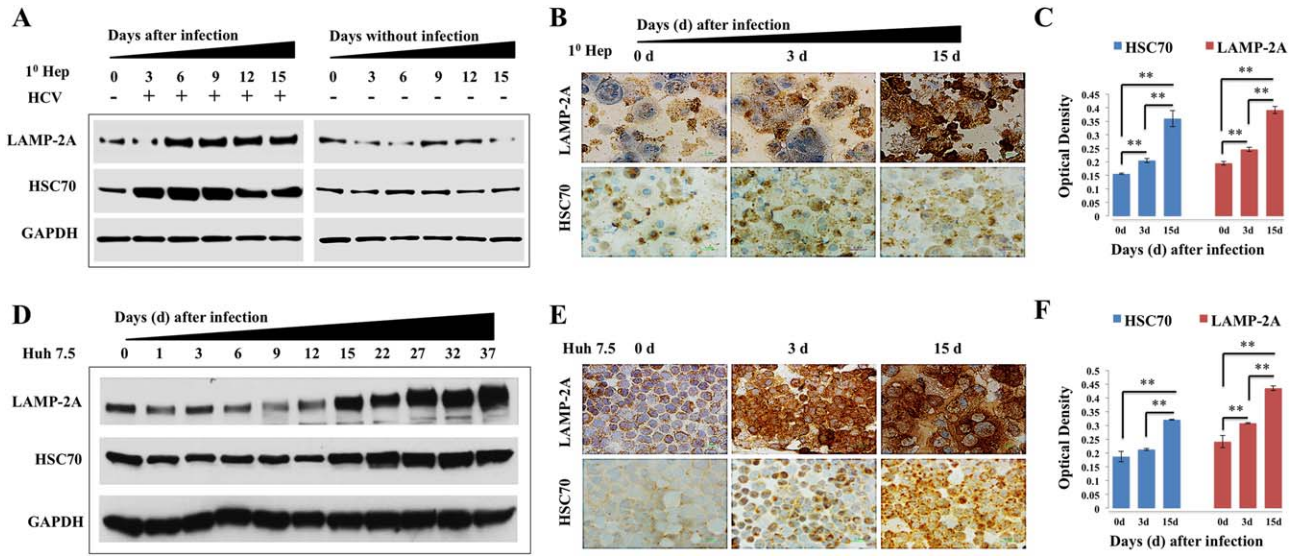

FIG. 3. Persistent HCV infection in PHHs and Huh-7.5 cells induces expression of LAMP-2A and HSC70. (A) Protein lysate of infected PHHs were made at the indicated time points, and the expression of LAMP-2A and HSC70 was analyzed by western blotting. (B) The expression of LAMP-2A and HSC70 in infected PHHs was examined by immunostaining of cytospin slides. (C) Quantitation of LAMP-2A and HSC70 staining by ImageJ software. ** $P < 0.005$. (D) Cell lysates were prepared from HCV-infected Huh-7.5 cells at different time points and expression of LAMP-2A, HSC70, and GAPDH levels was measured by western blotting. (E) Immunocytochemical staining of LAMP-2A and HSC70 of Huh-7.5 cells at day 0, 3, and 15 after HCV infection. (F) Quantification of LAMP-2A and HSC70 expression in Huh-7.5 cells after HCV infection using ImageJ software. Range bars in graphs are SD. ** $P < 0.005$. Images were taken at magnification X40. Abbreviation: GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

PERSISTENT HCV INFECTION DEGRADES WILD-TYPE p53 IN PHHs

The p53 protein is induced during cellular stress, leading to transcriptional up-regulation of genes that are involved in the regulation of apoptosis, proliferation, metabolism, and immune response. The expression level of p53 protein is regulated by Mdm2-mediated proteasome degradation.[16] A recent publication showed that p53 is a target of CMA because p53 harbors two pentapeptide motifs (200 NLRVE204 and 341FRELN345) that are similar to the HSC70 recognition sequence.[17] We examined whether HCV-induced CMA degrades wild-type p53 in infected PHHs. The expression of p53, Mdm2, and p21 levels was examined in HCV-infected PHHs by western blot analysis (Fig. 4A). A significant decrease in p53 expression was found to be associated with an increased level of Mdm2 and decreased expression of p21 in HCV-infected PHHs. PHHs cultured under similar conditions over 15 days showed stable expression of p53, suggesting that the degradation of wild-type p53 is not related to *in vitro* culture. The expression of p53 and p21 was induced and Mdm2 level decreased when PHHs were cultured at similar time points without HCV infection (Fig. 4B). We then examined the impact of infection on transcription of p53, p21, and Mdm2 levels by real-time RT-PCR. Results shown in Fig. 4C indicate that HCV infection induces transcription of p53, p21, and Mdm2 messenger RNA (mRNA) with time. There was an inverse relationship between protein and mRNA expression levels of p53 and p21 but a direct relationship with Mdm2. These results indicate that the decrease in the level of wild-type p53 is due to protein degradation.

To confirm that persistent HCV replication in Huh-7.5 cells also degrades mutant p53 protein by CMA, the expression of p53 was examined by western blot analysis. p53 levels were induced during early stages of infection (0-6 days), and the levels of p53 then significantly decreased after day 9 (Fig. 5A). We found that loss of p53 is associated with increased expression of Mdm2 and decreased expression of p21. The level of p14ARF expression was also decreased after HCV infection. The variation of p53 expression was not due to a lack of adequate amounts of protein in the lysate because levels of tubulin were comparable

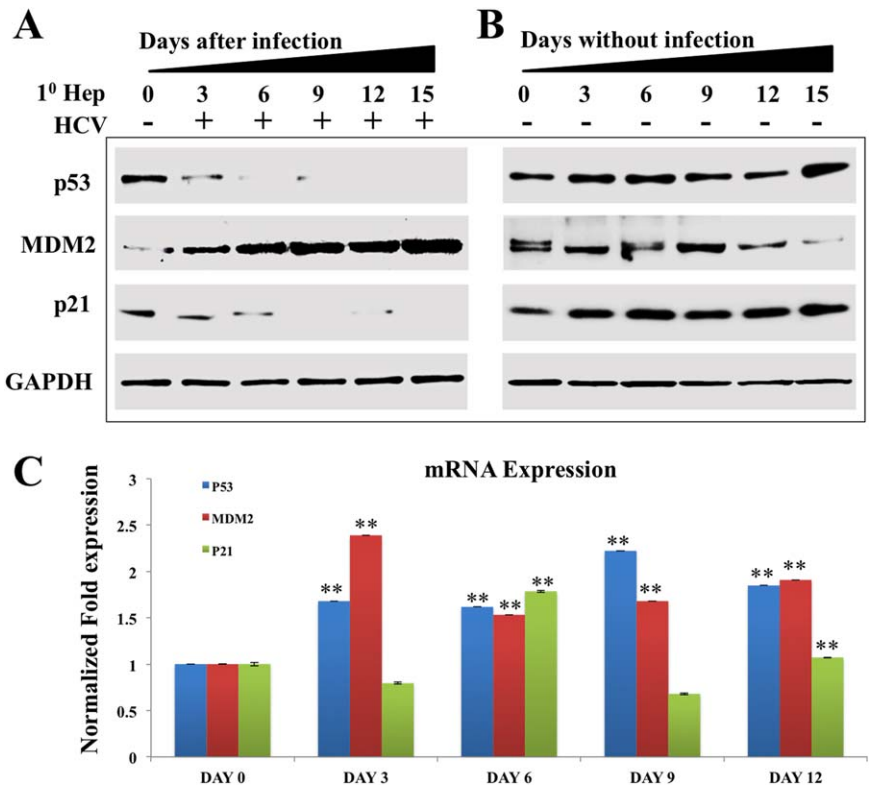

FIG. 4. Persistent HCV replication in primary human hepatocytes degrades p53 tumor suppressor and p53 target gene. (A) Cell lysates prepared from infected PHHs at indicated time points were examined for the expression of p53, Mdm2, and p21 by western blotting. GAPDH levels were examined for comparison. (B) Expression of p53, Mdm2, and p21 was measured in the uninfected PHHs at indicated time points by western blotting. (C) Real-time RT-PCR analysis of mRNA levels of p53, p21, and Mdm2 of uninfected and HCV-infected PHHs. ** $P <$ 0.005. Range bars in the graph are SD. Abbreviation: GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

among samples. Real-time RT-PCR results showed that mRNA levels of p53, Mdm2, and p21 increased in the infected culture, excluding the possibility that loss of p53 was not at the level of decreased mRNA transcription (Fig. 5B). The mRNA expression of p53 and p21 was inversely correlated with protein levels, whereas protein and mRNA levels of Mdm2 were induced after virus infection. The expression levels of p53, p21, and Mdm2 proteins in the infected Huh7.5 cells at day 15 were examined by immunocytochemical staining (Fig. 5C). Quantification of immunostaining data showed that the expression of p53 and p21 was decreased significantly with the HCV core expression, whereas the Mdm2 protein expression was induced (Fig. 5D).

ER STRESS AND AUTOPHAGY DEGRADES p53 INDEPENDENT OF Mdm2

We also noticed that treatment of ER-stress inducer, the luminal Ca^{2+} mobilizing agent thapsigargin (TG), promotes p53 degradation in HCV-infected culture in a concentration-dependent manner (Supporting Fig. S1A). We showed that autophagy induction in HCV culture by torin-1 treatment (a mammalian target of rapamycin inhibitor) increased degradation of p53 in a concentration-dependent manner (Supporting Fig. S1B). We tested whether the loss of p53 expression by HCV-induced ER stress/CMA could be restored by using an ER-stress inhibitor (tauroursodeoxycholic acid [TUDCA]) or a lysosome inhibitor (hydroxychloroquine [HCQ]). For this purpose, an HCV-infected culture at day 15 (p53 negative) was treated with an increasing concentration of TUDCA or HCQ, and the expression of p53 was examined by western blot analysis (Supporting Fig. S1C,D). The expression of HCV core protein levels in infected Huh-7.5 cells did not change significantly during the TG, torin-1, and TUDCA treatments. Mdm2 is a really interesting new gene finger domain-containing protein that is known to degrade p53 through the ubiquitin-proteasome pathway. Because Mdm2 levels are nicely induced in HCV-infected culture, we speculated that Mdm2 might be involved in the degradation of p53.

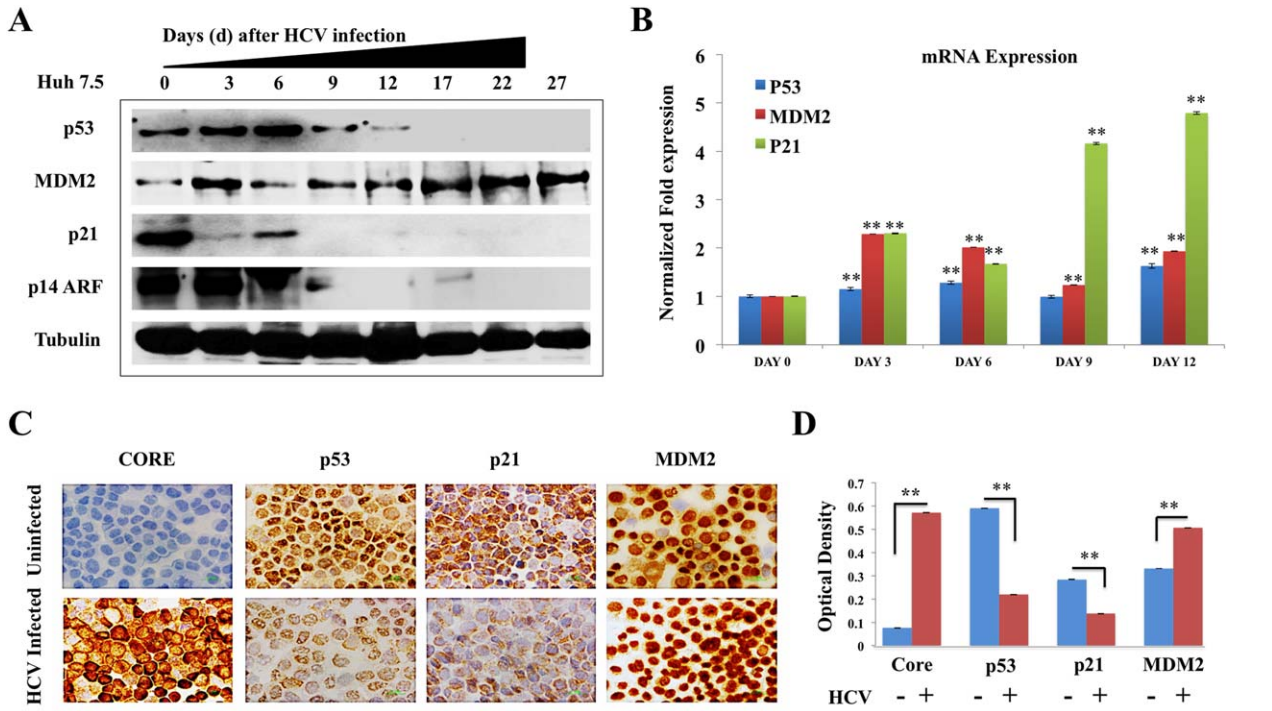

FIG. 5. Persistent HCV replication in Huh-7.5 cells degrades mutant p53, p21, and p14ARF protein independent of Mdm2. (A) Equal amounts of cell lysates prepared from HCV-infected Huh7.5 cells were used to measure the expression levels of p53, Mdm2, p21, p14ARF protein, and tubulin levels by western blot analysis. (B) Quantification of p53, p21, and Mdm2 mRNA levels by RT-PCR of infected PHHs. ** $P < 0.005$. (C) Immunostaining showing the expression of p53, p21, and Mdm2 in uninfected and HCV-infected culture at day 12. (D) Quantification of immunostaining of p53, p21, and Mdm2 by ImageJ software. ** $P < 0.005$. Images were taken at magnification X40. Range bars in graphs are SD.

It is known that Mdm2-mediated degradation of p53 is dependent on the direct interaction of these two proteins, so we examined whether disruption of the interaction between p53 and Mdm2 by using inhibitor nutlin-3 could result in p53 stabilization in HCV cell culture. It is also known that p53 is degraded through ubiquitin-proteasome pathways and that MG132 is an inhibitor of proteasome-mediated proteolysis that has been used extensively as an inhibitor for stabilization of p53. We found that treatment with either nutlin-3 or MG132 alone or in combination did not stabilize p53 expression levels in HCV-infected culture (Supporting Fig. S1E). These results were also confirmed by fluorescence microscopy after double staining. We found that the nuclear expression of p53 was minimal in HCV-infected Huh-7.5 cells and that the expression was not induced in nutlin-3-treated and MG132-treated culture but was induced in HCQ-treated culture, indicating that p53 degradation is independent of Mdm2-mediated degradation. Restoration of p53 by HCQ suggests that degradation of p53 is through the lysosomal pathway (Supporting Fig. S2). The expression of p53 was monitored with red fluorescence by using Alexa Fluor 647 goat anti-rabbit immunoglobulin G (Invitrogen), and cytoplasmic HCV expression was monitored by green fluorescence protein (GFP) expression using an HCV-GFP chimera virus.[18] The GFP and red fluorescence were quantified using ImageJ software. This analysis revealed that HCV replication decreased expression of p53 and nutlin3a and that MG132 treatment did not restore its expression but HCQ restored p53 expression significantly (Supporting Fig. S2B).

The Mdm2-independent degradation of p53 in HCV culture was also confirmed by two different approaches, inhibition of Mdm2 by small molecules and silencing by small interfering RNA (siRNA). Because the cyclin-dependent kinase inhibitor roscovitine has been shown to suppress the expression of Mdm2, we examined the effect of Mdm2 suppression on the stability of p53 in uninfected and HCV-infected culture. Inhibition of Mdm2 by roscovitine

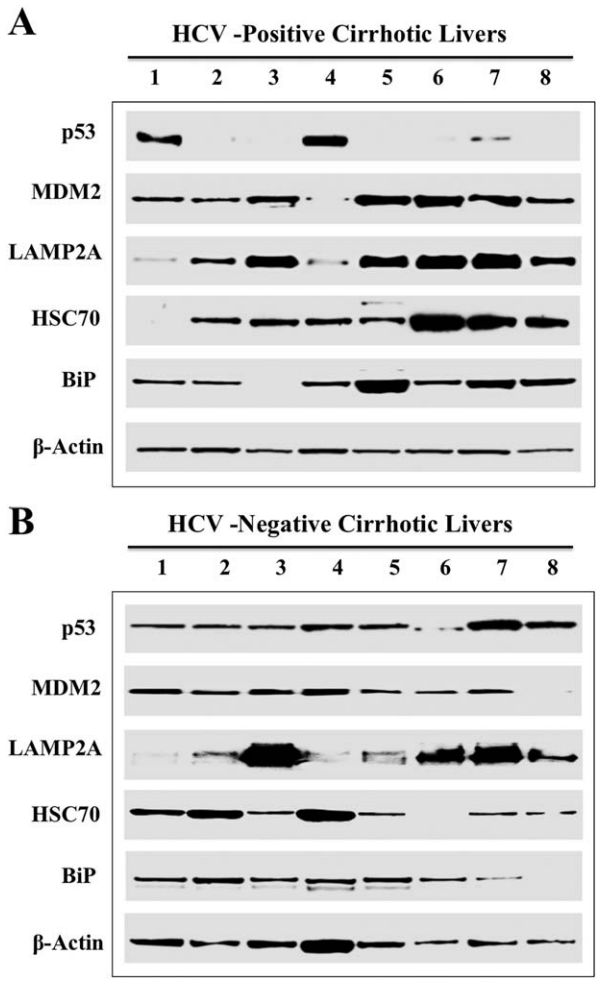

FIG. 6. Western blot analysis shows the expression levels of p53, ER-stress marker (BIP), and CMA markers (LAMP-2A and HSC70) in eight HCV-positive and eight HCV-negative explant cirrhotic livers. (A) HCV-positive explant cirrhotic livers. (B) HCV-negative explant cirrhotic livers.

stabilizes p53 in uninfected Huh-7.5 cells (Supporting Fig. S3A), but inhibition of Mdm2 levels in HCV culture under similar treatment did not stabilize p53 levels (Supporting Fig. S3B). In addition, transfection of Mdm2 siRNA in HCV-infected culture decreased Mdm2 expression but did not stabilize p53 expression in HCV-infected culture, suggesting that degradation of p53 in HCV culture occurs in an Mdm2-independent manner (Supporting Fig. S3C). Interestingly, silencing of LAMP-2A by siRNA transfection stabilizes p53 expression, suggesting that the loss of p53 in HCV culture occurs through lysosome-mediated degradation (Supporting Fig. S3D).

SERUM STARVATION DEGRADES p53 AND SILENCING LAMP-2A PREVENTS p53 DEGRADATION IN Huh-7.5 CELLS

Serum starvation is one of the established experimental models that have been used by many investigators to activate CMA. Uninfected Huh-7.5 cells were cultured in serum-free media for 0, 16, 24, and 48 hours, and the expression of mutant p53 was examined by western blot analysis (Supporting Fig. S4A). We found that p53 expression was significantly diminished after 16 hours of serum starvation and that this had no effect on the expression of Mdm2 levels. Serum starvation also induced the expression of LAMP-2A and HSC70 in Huh7.5 cells. Silencing LAMP-2A stabilized p53 expression under the serum starvation condition (Supporting Fig. S4B). We then examined whether p53 protein interacted with the CMA effector proteins HSC70 and LAMP-2A, which translocate to the lysosome for their degradation. Because LAMP-2A serves as a receptor for the selective uptake and degradation of the p53/HSC70 complex, we verified this interaction by immunoprecipitation using antibodies against p53 or LAMP-2A. A co-immunoprecipitation experiment verified an interaction between p53 with LAMP-2A or HSC70 during serum starvation (Supporting Fig. S4C). The expression of p53, Mdm2, and p21 levels under serum starvation conditions was examined by immunostaining of cultured Huh-7.5 cells (Supporting Fig. S4D). These results showed that both cytoplasmic and nuclear p53 staining decreased upon serum starvation. The expression of Mdm2 was induced in the nucleus under serum starvation. These results suggest that persistent HCV infection degrades p53 through CMA by inducing expression of cellular chaperones. The ER stress and CMA-mediated p53 degradation mechanisms shown in the infected cell culture were examined by western blot analysis using tissue extracts from cirrhotic explant livers with or without HCV infection. We found that the expression of p53 tumor suppressor was decreased among five out of eight HCV-infected explant livers (Fig. 6A). The expression of the ER stress chaperone (BIP), CMA-related proteins (HSC70, LAMP-2A), and Mdm2 was highly elevated in HCV-positive liver cirrhosis compared with HCV-negative cirrhotic samples (Fig. 6A,B). These results are consistent with our previous report showing the ER-stress response is induced in HCV-infected chronic liver diseases and liver cirrhosis.[19] In summary, we were able to verify the significance of our experimental findings using HCV-infected PHHs and persistently infected Huh-7.5 cells

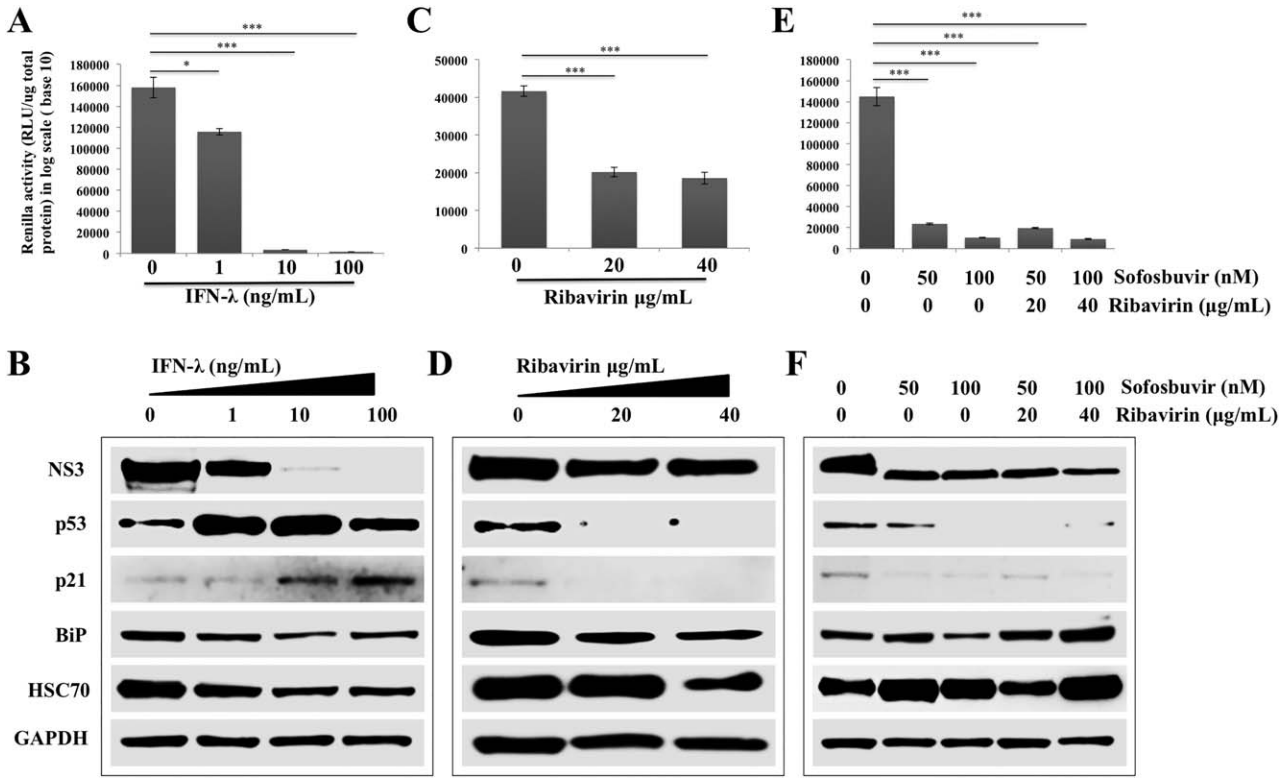

FIG. 7. ER-stress and p53 expression in HCV-infected Huh-7.5 culture after treatment with IFN-λ1, RBV, and sofosbuvir for 72 hours. (A) Dose-dependent antiviral effect of IFN-λ1 measured by Renilla luciferase activity (normalized to microgram protein). (B) Expression level of HCV NS3, p53, p21, ER-stress (BIP, HSC70), and GAPDH by western blotting. (C) Dose-dependent antiviral effect of RBV (20 μg/mL, 40 μg/mL) in HCV-infected Huh-7.5 cell culture measured by Renilla luciferase activity. (D) Equal amounts of protein lysates prepared from the treated and untreated culture were measured for the level of HCV NS3, p53, p21, ER-stress (BIP, HSC70), and GAPDH by western blotting. (E) HCV-infected Huh-7.5 cells were treated with sofosbuvir alone or in combination with RBV for 72 hours. Antiviral effect was measured by Renilla luciferase activity. (F) Expression level of HCV NS3, p53, p21, ER-stress (BIP, HSC70), and GAPDH measured by western blotting. * $P < 0.05$, *** $P < 0.0005$. Range bars in graphs are SD. Abbreviation: GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

with liver tissues derived from HCV-infected advanced cirrhosis.

IFN-INDUCED HCV CLEARANCE NORMALIZES ER-STRESS RESPONSE AND RESTORES p53 MORE THAN DAAs

Because the HCV-induced stress response degrades p53 by CMA mechanisms, we performed a comparative investigation to determine whether there is any difference in the p53 expression when HCV is cleared by either IFN or DAAs. First, persistently HCV-infected Huh-7.5 cells grown in six-well plates were given two rounds of IFN-λ1 treatment at 3-day intervals. Then, HCV clearance from the infected culture was measured by Renilla luciferase activity. We observed that IFN-λ1 significantly decreased HCV replication in the cell culture model (Fig. 7A). HCV clearance by IFN-λ1 significantly decreased HCV NS3 expression and restored the expression of p53 and p21 by reducing the expression of ER-stress chaperones (BIP and HSC70) (Fig. 7B). Levels of glyceraldehyde 3-phosphate dehydrogenase remained the same in all the lanes. Second, we examined whether HCV clearance by RBV treatment could restore p53 expression. Persistently infected HCV culture was treated with two different concentrations of RBV, and the effect of viral clearance on p53 response was measured. We found that RBV inhibited HCV replication, which was determined by

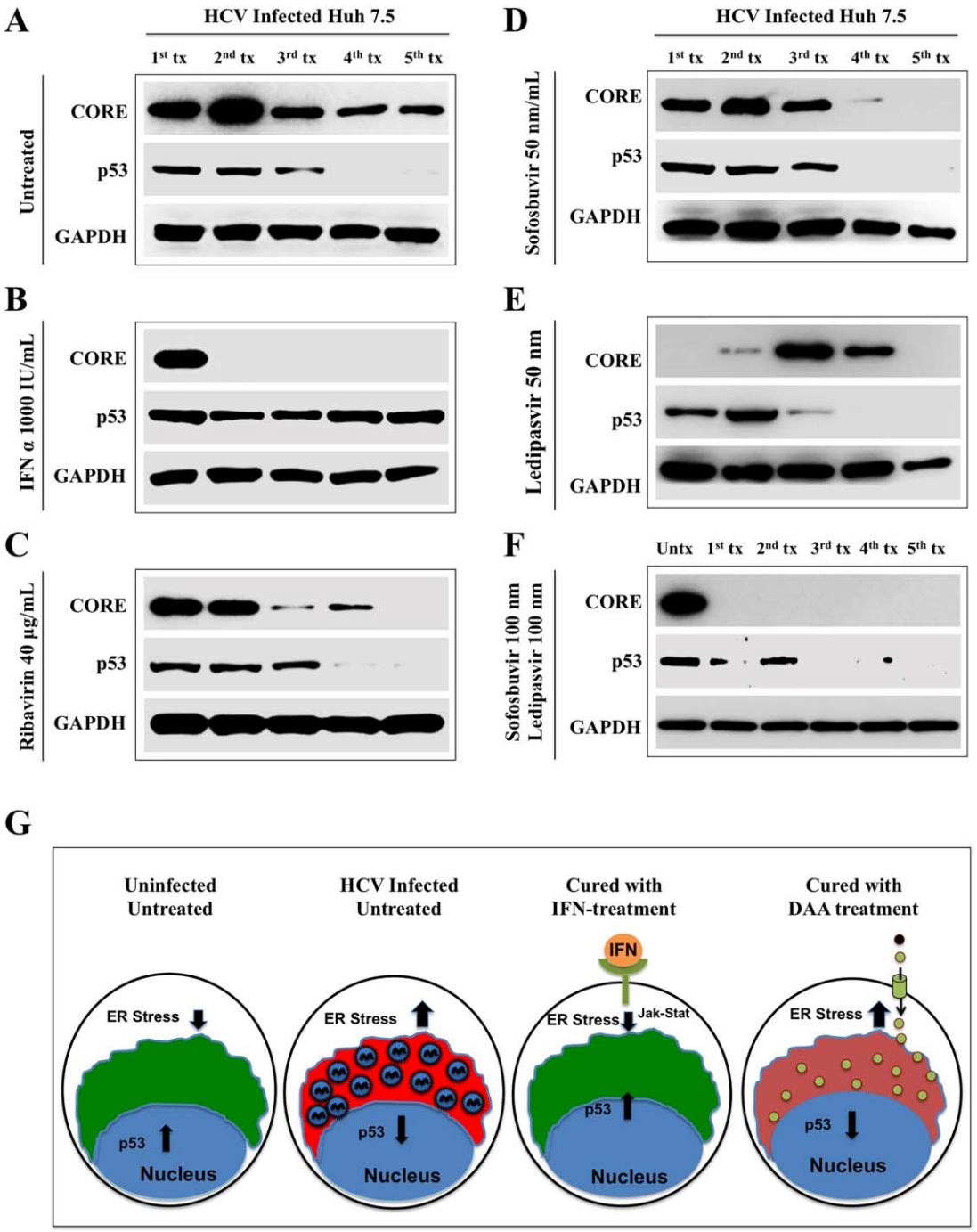

FIG. 8. Expression of p53 in infected Huh-7.5 cells after repeated antiviral treatment. HCV-infected Huh-7.5 cells at day 9 were treated with either IFN-α, RBV, sofosbuvir, ledipasvir, or combined sofosbuvir plus ledipasvir. After 72 hours, cells were split and then treated again with the same antiviral agent. After five rounds of antiviral treatment, the expression of HCV, p53, and GAPDH levels was measured by western blot analysis. (A) Untreated HCV-infected culture. (B) IFN-α treated culture. (C) Ribavirin-treated culture. (D) Sofosbuvir-treated culture. (E) Ledipasvir-treated culture. (F) Combination treatment with sofosbuvir plus ledipasvir. (G) Hypothetical model showing that HCV clearance by DAA does not restore p53 in hepatocytes because DAAs themselves induce ER-stress. Abbreviation: GAPDH, glyceraldehyde 3-phosphate dehydrogenase; tx, treatment.

Renilla luciferase activity (Fig. 7C). Western blot analysis of the lysate prepared from the RBV-treated and untreated cultures showed that expression of p53 and p21 was not induced and levels of the ER stress markers BIP and HSC70 were not inhibited (Fig. 7D). Third, we examined the expression of p53, p21, and the stress response in an HCV-infected cell culture after sofosbuvir treatment. Infected culture was treated with sofosbuvir alone or sofosbuvir along with two different concentrations of RBV. Antiviral activity was measured by Renilla luciferase after two rounds of treatment (Fig. 7E). Cell lysates prepared from the treated and mock-treated cultures were examined for expression levels of p53, p21, BIP, and HSC70 by using western blot analysis. HCV clearance by either sofosbuvir alone or in combination with RBV did not restore p53 or p21 expression and did not reduce the expression of ER-stress chaperones (BIP and HSC70) (Fig. 7F). We examined whether HCV clearance by long-term repeated treatment with IFN-α could also decrease stress response compared with a DAA combination (Harvoni) currently approved for HCV treatment. In this experiment, HCV-infected cultures were given five rounds of antiviral treatment to see whether or not p53 expression could be restored after long-term HCV clearance. HCV replication degraded p53 over time (Fig. 8A), and IFN-α-induced HCV clearance induced p53 expression at all time points (Fig. 8B). The expression of p53 was not restored in RBV-treated culture (Fig. 8C), sofosbuvir-treated culture (Fig. 8D), ledipasvir-treated culture (Fig. 8E), or the combined treatment of sofosbuvir plus ledipasvir (Fig. 8F). The expression of HCV core was inhibited completely during the DAA combination treatment compared to the untreated culture, but the p53 expression was not restored (Fig. 8F). The lack of restoration of p53 expression after HCV cleared by DAA tempted us to examine the impact of DAA treatment on the ER-stress response in uninfected Huh-7.5 cells. We found that the expression of ER-stress chaperones was induced in uninfected Huh-7.5 cells after treatment with sofosbuvir and ledipasvir (Supporting Fig. S5). Based on these data, we hypothesized that HCV clearance induced by DAAs is unable to restore p53 expression because these agents themselves induce ER stress in hepatocytes (Fig. 8G). In summary, our results indicated that there was a significant difference in the restoration of p53 tumor suppressor expression and resolution of ER stress when HCV clearance was induced by IFN compared to DAAs.

Discussion

We performed this study to gain an insight into how the tumor suppressor p53 expression is restored in infected hepatocytes when HCV is cleared by IFN-based antivirals compared to DAAs. During the first phase of our investigation, we studied the basic mechanism for how the HCV-induced ER-stress response alters the expression of p53 tumor suppressors. We used HCV-infected nonproliferative PHHs and persistently infected Huh-7.5 cells as a model system to determine whether HCV replication produces an adaptive or chronic ER-stress response. We found that all three branches of UPR were induced and the expression of the UPR gene did not decrease to a baseline level, suggesting that HCV replication in PHHs creates a chronic-type ER-stress response. These results are consistent with previous publications that show a high expression of UPR genes in chronic liver disease and liver cirrhosis is related to HCV infection.[19,20] We examined how HCV-infected hepatocytes manage ER stress without inducing cell death. Our results showed that persistent HCV replication improved cell survival pathways by inducing an autophagy response. It is not well established that HCV uses autophagy (macroautophagy) to promote viral RNA replication, translation, and virus production.

In this report, we found that persistent HCV replication induces CMA, as evidenced by the steady expression of HSC70 and LAMP-2A in an HCV culture after day 12. CMA induced by HCV degrades both wild-type p53 in PHHs and mutant p53 in Huh-7.5 cells. These results were verified using chronic HCV-infected liver tissue from patients with liver cirrhosis. Our findings are in agreement with a report showing that CMA degrades mutant p53 in a lysosome-dependent manner.[17] We showed that inhibition of p53/Mdm2 interaction using nutlin-3 or silencing Mdm2 did not prevent degradation of p53 in an HCV-infected culture. LAMP-2A silencing restored p53 degradation in an HCV culture, suggesting that p53 degradation in an HCV-infected culture is mediated by lysosomal degradation. Our results provide a potential novel mechanism for how HCV regulates p53 in favor of its continued survival.

Chronic HCV infection is one of the risk factors for developing liver cirrhosis and HCC. The risk of HCC is very high among patients with advanced liver fibrosis/cirrhosis. Recent reviews summarize the importance of p53 in HCV-induced HCC mechanisms and how

HCV infection alters p53 functions by many different pathways.[21,22] Rusyn and Lemon[22] claimed that the majority of publications show that HCV replication represses p53 functions. Tomohiro et al.[23] showed that 24-dehydrocholesterol reductase is elevated in response to HCV infection and inhibits p53 by stimulating the accumulation of the Mdm2-p53 complex in the cytoplasm and by inhibiting the acetylation of p53 in the nucleus. A study by Duong et al.[24] reported that p53 can be inactivated through induced expression of protein phosphatase 2A. They showed HCV transgenic mice that overexpress protein phosphatase 2A develop larger tumors after diethylnitrosamine treatment. We now provide new evidence suggesting that HCV-induced ER-stress response promotes rapid degradation of p53 to improve cellular surveillance. The degradation of p53 was also seen when nonviral agents induced ER stress and autophagy. As the loss of p53 function is associated with the majority of human cancers, our findings may contribute to understanding the HCV-induced HCC mechanism.

The availability of new IFN-free DAAs dramatically increased sustained virological response among patients with hepatitis C virus infection. It is anticipated that an increased number of patients will be able to clear infection with this new medication. At present, only a few studies have investigated the risk factor for HCC after eradication of the virus infection by DAA-based therapy among patients with advanced liver cirrhosis. These clinical studies show that the risk of HCC is greater after HCV cure by DAAs compared to IFN-based antiviral therapy. In this study, we compared the fate of the ER-stress response and restoration of the p53 tumor suppressor in infected culture after HCV clearance. We found that HCV clearance by IFN-α or IFN-λ is able to induce expression of p53 and normalize the ER-stress response. However, DAA treatment inhibited HCV replication but did not inhibit the ER-stress response or restore p53 expression. We noted that IFN-induced HCV clearance restores the p53 level significantly better than ribavirin, sofosbuvir, and ledipasvir. Our results suggest that unresolved ER stress after HCV clearance by DAAs continues to suppress p53 expression, which could increase the risk for HCC development.

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Supporting Information

Additional Supporting Information may be found at onlinelibrary.wiley.com/doi/10.1002/hep4.1025/suppinfo.