Molecules and Cells

Hepatitis C Virus Nonstructural 5A Protein Interacts with Telomere Length Regulation Protein: Implications for Telomere Shortening in Patients Infected with HCV

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Hepatitis C virus (HCV) is a major cause of chronic liver disease and is highly dependent on cellular proteins for viral propagation. Using protein microarray analysis, we identified 90 cellular proteins as HCV nonstructural 5A (NS5A) interacting partners, and selected telomere length regulation protein (TEN1) for further study. TEN1 forms a heterotrimeric complex with CTC and STN1, which is essential for telomere protection and maintenance. Telomere length decreases in patients with active HCV, chronic liver disease, and hepatocellular carcinoma. However, the molecular mechanism of telomere length shortening in HCV-associated disease is largely unknown. In the present study, protein interactions between NS5A and TEN1 were confirmed by immunoprecipitation assays. Silencing of TEN1 reduced both viral RNA and protein expression levels of HCV, while ectopic expression of the siRNA-resistant TEN1 recovered the viral protein level, suggesting that TEN1 was specifically required for HCV propagation. Importantly, we found that TEN1 is re-localized from the nucleus to the cytoplasm in HCV-infected cells. These data suggest that HCV exploits TEN1 to promote viral propagation and that telomere protection is compromised in HCV-infected cells. Overall, our findings provide mechanistic insight into the telomere shortening in HCV-infected cells.

Keywords: hepatitis C virus, NS5A, protein microarray, telomere shortening, TEN1

INTRODUCTION

Hepatitis C virus (HCV) belongs to the *Hepacivirus* genus within the *Flaviviridae* family (Simmonds, 2004). HCV is an enveloped virus with a positive-sense, single-stranded RNA genome consisting of 9,600 nucleotides and its genome encodes 3,010 amino acids from a single open reading frame (Giannini and Brechot, 2003; Lindenbach and Rice, 2005). HCV causes both acute and persistent infections and increases the risk of developing severe liver diseases such as hepatic decompensation and hepatocellular carcinoma (McGivern

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and Lemon, 2011, Saito et al., 1990). Interestingly, studies have shown that patients with chronic HCV or chronic liver disease have shorter telomeres than healthy controls (Kitada et al., 1995; Wiemann et al., 2002). However, the mechanism of telomere length shortening in these patients is still largely unknown.

HCV nonstructural 5A (NS5A) protein is a multifunctional phosphoprotein consisting of 447 amino acids residues and is a pleiotropic protein involved in viral RNA replication and modulating cellular physiology in HCV-infected cells. NS5A is localized in the cytoplasm and forms part of the HCV RNA replication complex. It also interacts with both viral proteins and other cellular proteins to regulate host cellular signaling pathways and promote viral propagation (Choi et al., 2020; Lim and Hwang, 2011; Park et al., 2015; Tran et al., 2016).

Telomere length regulation protein (TEN1) is a component of the mammalian CST complex, which also contains CTC1 and STN1 proteins. The CST complex is a major regulator of telomere DNA synthesis and plays an important role in telomere protection, DNA metabolism, and telomerase activity (Chen et al., 2012). CST-telomeric DNA binding increases during the late S/G2 phase on telomerase action, coinciding with telomerase shut-off (Casteel et al., 2009; Miyake et al., 2009; Surovtseva et al., 2009). It has been previously reported that CST limits telomerase action at individual telomeres to approximately one binding and extension event per cell cycle (Chen et al., 2012). DNA polymerase α-primase (pol α) needs STN1 and TEN1 as cofactors to promote C-strand synthesis at the telomeres (Casteel et al., 2009; Huang et al., 2012; Nakaoka et al., 2012). CST malfunction in higher eukaryotes leads to accumulation of excessive G-strand telomere DNA and the formation of extrachromosomal telomeric circles (Gu et al., 2012; Miyake et al., 2009; Song et al., 2008). Moreover, a deficiency of CST continuously decreases C-strand length because of a lack of C-strand fill-in synthesis, leading to the gradual shortening of the telomeres.

We previously performed protein microarray analysis to identify cellular proteins interacting with HCV NS5A and selected TEN1 for further characterization in the present study. Protein binding between NS5A and TEN1 was verified by a coimmunoprecipitation assay. Silencing TEN1 expression resulted in a decrease in both HCV RNA and protein levels, suggesting that TEN1 was specifically required for HCV propagation. Importantly, we also noted that TEN1 was translocated from the nucleus to the cytoplasm, leading to telomere shortening in HCV-infected cells, and suggesting that HCV exploits TEN1 to promote viral propagation.

MATERIALS AND METHODS

Plasmid constructions

Total cellular RNAs were extracted from Huh7.5 cells by using RiboEx (GeneAll Biotechnology, Korea), and cDNA was synthesized by using a cDNA synthesis kit (Toyobo, Japan) according to the manufacturer's instructions. Full-length TEN1 was amplified by a primer set (Table 1). Polymerase chain reaction (PCR) products were inserted into the *Hin*dIII and *Eco*RI sites of the p3XFLAG-CMV10 vector (Invitrogen, USA). siRNA-resistant TEN1 mutant was constructed by introducing two silent mutations in the siRNA-binding site. cDNA corresponding to the NS5A coding sequence of HCV was amplified by PCR using the Korean isolate of HCV (genotype 1b) and subcloned into the pEF6 vector (Invitrogen).

Cell culture

All cell lines were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum and 100 units/ml penicillin/streptomycin and maintained in 5% CO_2 at 37°C. Huh7 cells harboring HCV subgenomic replicon derived from genotype 1b and interferon (IFN)-cured cells were grown as reported previously (Choi et al., 2020; Lim et al., 2011).

Antibodies

Antibodies were purchased from the following sources: anti-glyceraldehyde 3-phosphate dehydrogenase (GAPDH), and Myc antibodies were from Santa Cruz Biotechnology (USA); anti-Flag and actin antibodies were from Sigma-Aldrich; goat anti-TEN1 antibody was from Santa Cruz Biotechnology. HCV core, NS3, and NS5A antibodies have been described elsewhere (Lim et al., 2011). Rabbit anti-TEN1 antibody was kindly provided by Professor Fuyuki Ishikawa (Kyoto University, Japan).

Table 1. List of primers used in this study

Primer	Primer sequence	Enzyme site	Purpose
			Cloning of HCV NS5A 1b into pEF6A
TEN1-F	GCAAGCTTATGCTGCCCAAACCT GGG	*Hin*dIII	Cloning of human TEN1 into p3XFLAG-CMV-10
TEN1-R	GCGAATTCCTACTGGCTGCCGCCCCG	*Eco*RI	
TEN1 resist-F	TGATGGGAGCACGCTGAAAATATTTGGCAGGTTGTGC	*Hin*dIII	Generation of siRNA-resistant mutant of TEN1
TEN1 resist-R	GGCACAACCTGCCAAATATTTTCAGCGTGCTCCCATCA	*Eco*RI	
STN1-F	GCGGTACCATGCAGCCTGGATCC	*Kpn*I	Cloning of human STN1 into pV5-EF6B
STN1-R	GCTCTAGATCAGAACGCTGTGTA	*Xba*I	
5′NTR-F	TGAGTGTCGTACAGCCTCCA		Quantitative real-time PCR
5′NTR-R	ACGCTACTCGGCTAGCAGTC		
qActin-F	TGACAGCAGTCGGTTGGAGCG		Quantitative real-time PCR
qActin-R	GACTTCCTGTAACAACGCATCTCATA		
qTEN1-F	GCTCCCTGTACATCGTCCTC		Quantitative real-time PCR
q-TEN1-R	CAACAAGGGCAGGTTCAT		

Protein array screening

HCV NS5A protein expressed from *Escherichia coli* was purified using Invitrogen Ni-nitrilotriacetic acid (Ni-NTA) agarose beads according to the manufacturer's instructions. Firstly, ProtoArray® Human Protein Microarray v5.0 (Invitrogen) was incubated with blocking buffer (50 mM HEPES [pH 7.5], 25% glycerol, 0.08% Triton X-100, 200 mM NaCl, 20 mM reduced glutathione, and 0.1 mM dithiothreitol [DTT]) for 1 h at 4°C. Next, purified NS5A protein diluted in probing buffer (phosphate-buffered saline [PBS] containing 0.1% Tween 20) was added to the protein microarray. Following incubation at 4°C for 1.5 h, the array was washed five times in ice-cold buffer and treated with Anti-V5-Alexa Fluor 647 antibody (Invitrogen) for 1.5 h at 4°C. The images were scanned using a PerkinElmer ScanArray Ex-press HT system and analyzed by the Invitrogen Prospector software (ver. 5.2).

Immunoprecipitation

HEK293T cells were cotransfected with 2 μg of Myc-tagged NS5A and 2 μg of Flag-tagged TEN1 plasmids. The total DNA amounts were adjusted by adding an empty vector. At 48 h after transfection, cells were harvested, and an immunoprecipitation assay was performed as reported previously (Lim et al., 2011).

Immunoblot analysis

Equal amounts of proteins were subjected to SDS-PAGE and electro-transferred to a nitrocellulose membrane. The membrane was blocked in blocking buffer (20 mM Tris-HCl [pH 7.6], 150 mM NaCl, and 0.25% Tween 20) containing 5% nonfat dry milk for 1 h and then incubated overnight at 4°C, with the indicated antibodies, in TBS-Tween 20 containing 1% non-fat dry milk. After three washes with TBS-Tween 20, the membrane was incubated with either horseradish peroxidase-conjugated goat anti-rabbit antibody or goat anti-mouse antibody (Jackson ImmunoResearch Laboratories, USA) in TBS-Tween 20 for 1 h at room temperature. Proteins were detected using an ECL kit (Amersham Biosciences, UK).

RNA interference

siRNAs targeting four different regions of TEN1 were purchased from Dharmacon (USA) and the universal negative control was purchased from Bioneer (Korea). An siRNA targeting the 5′NTR of the Jc1 virus (5′-CCU CAA AGA AAA ACC AAA CUU-3′) was used as a positive control siRNA (Lim et al., 2011). siRNA transfection was performed using Lipo-

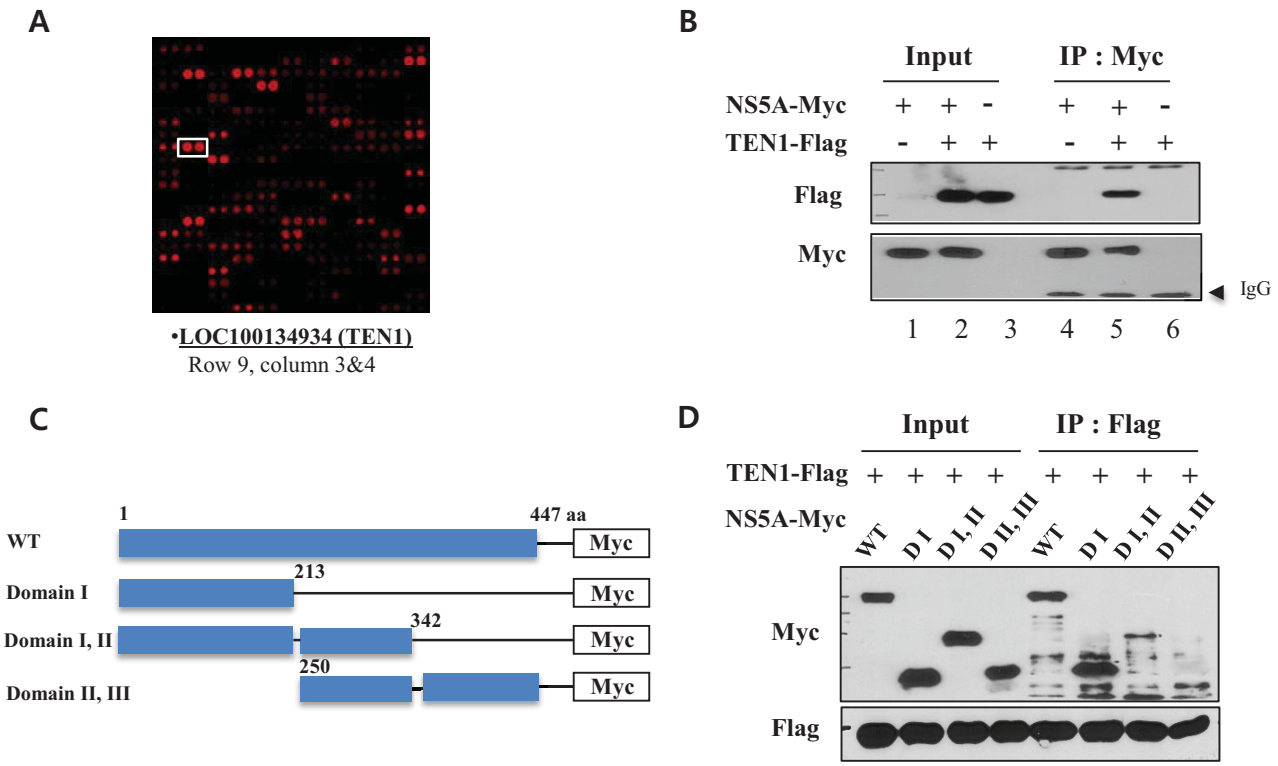

Fig. 1. HCV NS5A interacts with TEN1 through the domain I of NS5A. (A) Identification of TEN1 as a binding partner for NS5A in protein microarray. (B) HEK293T cells were cotransfected with Myc-tagged NS5A and Flag-tagged TEN1 expression plasmid. At 48 h after transfection, total cell lysates were immunoprecipitated (IP) with an anti-Myc monoclonal antibody, and then bound protein was detected by immunoblot analysis with an anti-Flag monoclonal antibody. Arrowhead indicates IgG heavy chain. (C) Schematic illustration of both wild-type and mutants of HCV NS5A expression plasmid. WT, wild type; aa, amino acids. (D) HEK293T cells were cotransfected with Flag-tagged TEN1 and Myc-tagged NS5A expression plasmids. At 48 h after transfection, cell lysates were immunoprecipitated with an anti-Flag polyclonal antibody, and bound proteins were immunoblotted with an anti-Myc polyclonal antibody. Protein expressions of Myc-tagged NS5A and Flag-tagged TEN1 were verified by immunoblotting with an anti-Myc or anti-Flag monoclonal antibody using the same cell lysates.

fectamine RNAiMax Reagent (Invitrogen) according to the manufacturer's instructions.

Quantification of TEN1 and HCV RNAs

Total RNAs were isolated from cell culture-grown HCV (HCVcc)-infected cells using RiboEx LS Reagent (GeneAll Biotechnology) and cDNAs were synthesized by using a cDNA synthesis kit (Toyobo) according to the manufacturer's instructions. Quantitative real-time PCR (qRT-PCR) experiments were performed as reported previously (Lim et al., 2011).

Immunofluorescence assay

Huh7.5 cells grown on cover slides were infected with HCVcc and were then fixed with 4% paraformaldehyde in PBS at room temperature for 12 min. After two washes with PBS, the fixed cells were permeabilized with 0.1% Triton X-100 in PBS for 15 min and blocked with 0.5% bovine serum albumin for 2 h at room temperature. The cells were incubated overnight with a goat anti-TEN1 antibody and a rabbit anti-NS5A antibody, respectively. After three washes with PBS, the cells were incubated with either fluorescein isothiocyanate (FITC)-conjugated anti-goat IgG or tetramethylrhodamine isothiocyanate (TRITC)-conjugated donkey anti-rabbit IgG for 2 h at room temperature. The cells were then counterstained with 4′,6-diamidino-2-phenylindole (DAPI) to label the nuclei. After three washes with PBS, the cells were analyzed using the Zeiss LSM 700 laser confocal microscopy system (Carl Zeiss, USA).

Telomere length assay

The quantitative PCR procedure to measure telomere length was based on Cawthon's technique, which used primers that hybridize the telomeric hexamer repeats. The number of binding sites for the primers increases as the average telomere length increases. For each DNA sample, the factor by which the sample differed from a reference DNA sample is its ratio of telomere repeat copy number to single gene copy number. This ratio should be proportional to the average telomere length. We designed a pair of telomere primers and a single-copy albumin primer as reported previously (Cawthon, 2009). Primers designed for single-copy gene albumin amplicons would melt at a much higher temperature than telomere amplicons.

Statistical analysis

Data are presented as mean ± SD. Student's *t*-test was used for statistical analysis. The asterisks on the figures indicate significant differences, as noted in the figure legends. All statistical analysis in the study was performed using Microsoft Excel 2016 (Microsoft, USA).

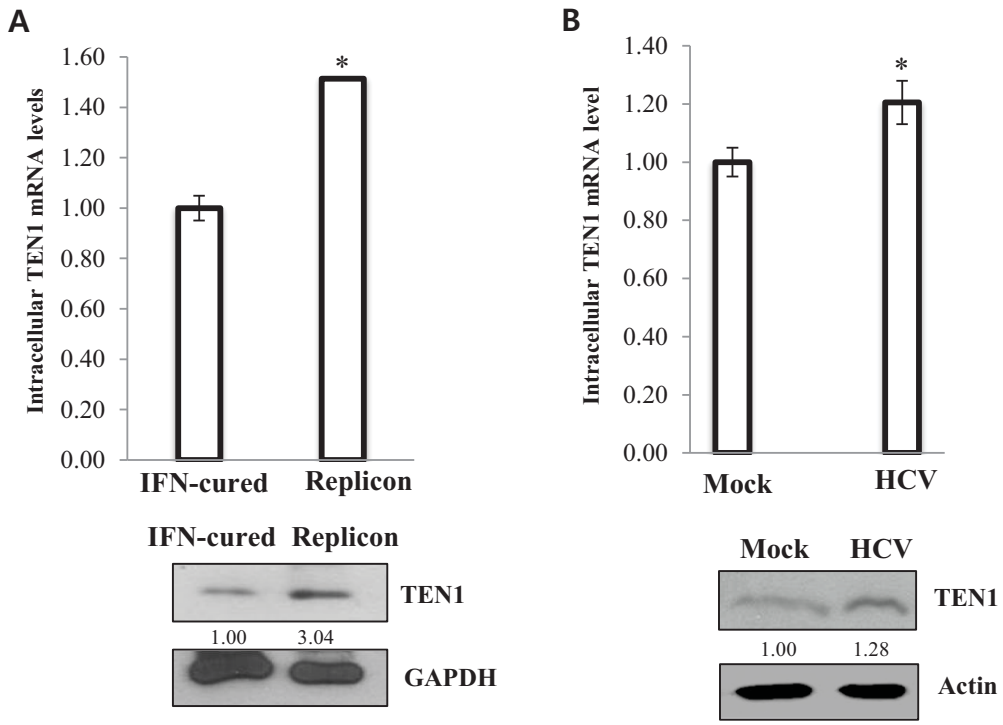

Fig. 2. HCV increases TEN1 expression level. (A) (Top) TEN1 mRNAs isolated from either IFN-cured Huh7 cells or Huh7 cells harboring replicon derived from HCV genotype 1b were quantified by qRT-PCR. The asterisk indicates a significant difference ($*P < 0.05$) from the value for the control. Error bars indicate SD. (Bottom) Total cell lysates harvested from either IFN-cured or subgenomic replicon derived from HCV genotype 1b were immunoblotted with the indicated antibodies. (B) (Top) Huh7.5 cells were either mock infected or infected with HCV Jc1 for 4 h. At 96 h postinfection, TEN1 mRNA levels were determined by qRT-PCR. Data from two independent experiments were quantified. (Bottom) Total cell lysates harvested at 96 h postinfection were immunoblotted with the indicated antibodies.

RESULTS

HCV NS5A interacts with TEN1

We previously identified the cellular proteins interacting with HCV NS5A protein (Park et al., 2015). Among these, we selected and characterized the TEN1 (Fig. 1A). We first confirmed the protein interaction between NS5A and TEN1 by performing a coimmunoprecipitation assay using a transient expression system. HEK293T cells were cotransfected with a plasmid expressing HCV NS5A protein derived from genotype 1b and a plasmid expressing TEN1. As shown in Fig. 1B, NS5A selectively interacted with TEN1 (lane 5). To determine the region in NS5A responsible for TEN1 binding, the interactions between TEN1 and various deletion mutants of NS5A (Fig. 1C) were determined by a transfection-based coimmunoprecipitation assay. The results shown in Fig. 1D demonstrate that TEN1 interacted with domain I and domain I, II but not with domain II, III, indicating that NS5A interacts with TEN1 through domain I, encompassing amino acid residues 1-213 of NS5A.

HCV upregulates TEN1 expression level

To investigate whether the TEN1 expression level was modulated by HCV, we determined the mRNA and protein levels of TEN1 in HCV replicating cells. Figure 2A shows that the intracellular mRNA level of TEN1 increased significantly in Huh7 cells harboring HCV replicon derived from genotype 1b compared with IFN-cured Huh7 cells. Additionally, the protein level of TEN1 increased markedly and consistently in HCV replicon cells (Fig. 2A, lower). We then investigated whether the TEN1 expression level was altered in HCV-infected cells. Similar to HCV replicon cells, the intracellular mRNA level of TEN1 increased significantly in HCV-infected cells compared with the mock-infected cells (Fig. 2B, upper). We further

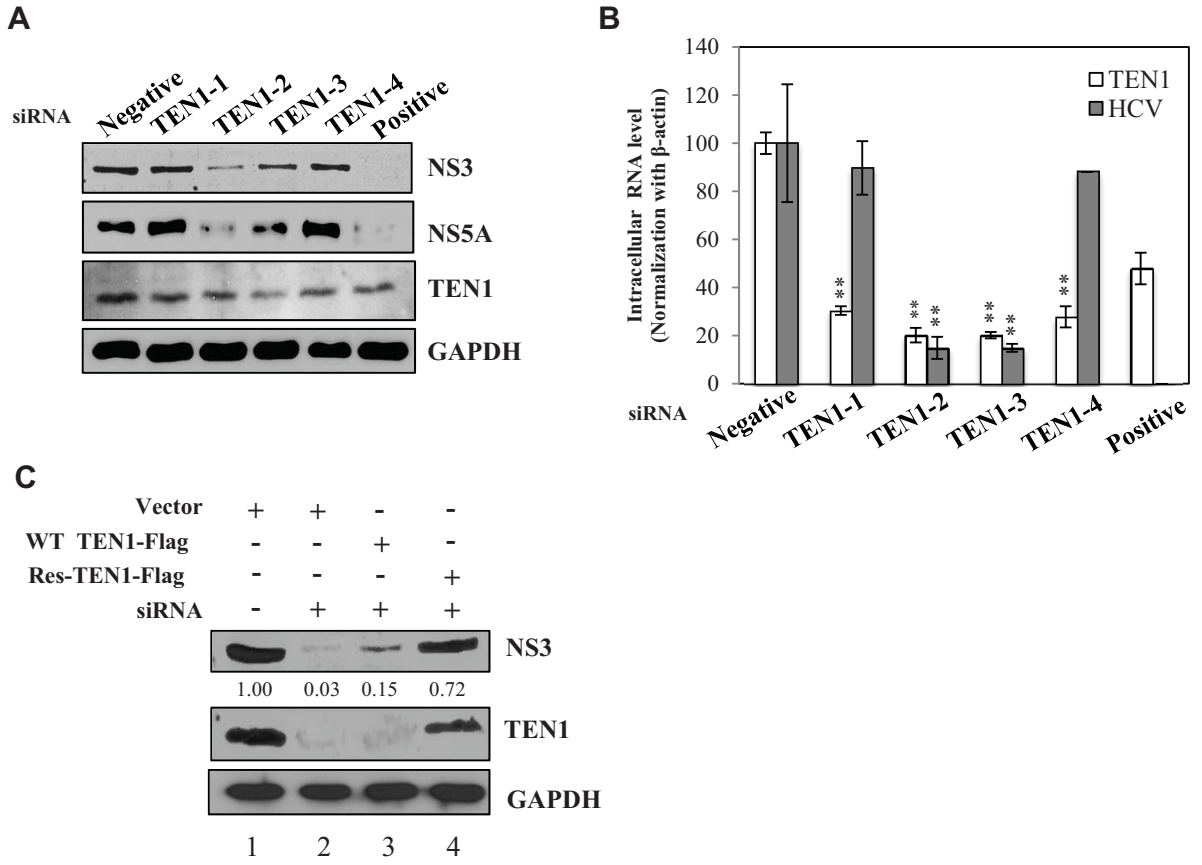

Fig. 3. Knockdown of TEN1 impairs both RNA and protein expression levels of HCV. (A) Huh7.5 cells were transfected with a 20 nM concentration of the indicated siRNA constructs. Total cell lysates harvested at 72 h after transfection were immunoblotted with the indicated antibodies. (B) Total RNAs were extracted from cells harvested at 72 h after siRNA transfection and then both TEN1 mRNAs and intracellular HCV RNAs were quantified by qPCR. Negative, universal negative-control siRNA. Positive, HCV-specific siRNA. TEN1-1, TEN1-2, TEN1-3, and TEN1-4 are the siRNAs targeting differerent regions of TEN1. The asterisks indicate significant differences (**$P < 0.01$) from the value for the negative control. Experiments were performed in duplicates. Error bars indicate SD. (C) Huh7 cells were transfected with siRNAs targeting TEN1. At 24 h after transfection, cells were further transfected with either Flag-tagged wild-type TEN1 or Flag-tagged siRNA-resistant mutant TEN1 plasmid for 24 h and then infected with Jc1. At 48 h postinfection, HCV protein levels were determined by immunoblot analysis. siRNA-resistant mutant TEN1 contains two silent mutations in the siRNA binding sites. Res, siRNA-resistant TEN1 mutant. WT, wild type.

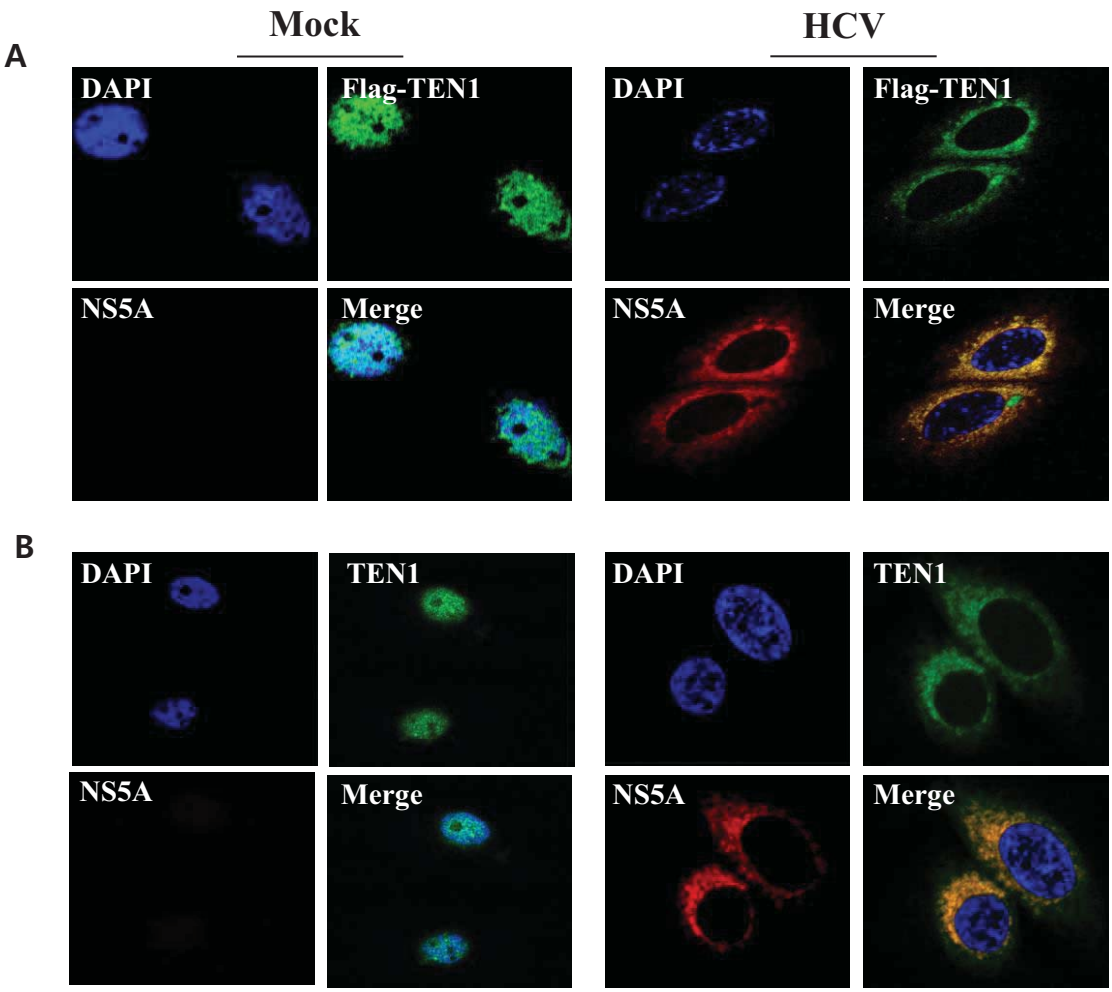

Fig. 4. TEN1 is translocated from the nucleus to the cytoplasm in HCV-infected cells. (A) Huh7.5 cells were transfected with Flag-tagged TEN1. At 24 h after transfection, cells were either mock-infected or infected with Jc1 virus for 4 h. At 24 h postinfection, cells were fixed in 4% paraformaldehyde, and immunofluorescence staining was performed by using an anti-Flag polyclonal antibody and fluorescein isothiocyanate-conjugated mouse anti-goat IgG to detect Flag-TEN1 (green) and a rabbit anti-NS5A antibody and TRITC-conjugated donkey anti-rabbit IgG to detect NS5A (red). Dual staining showed colocalization of TEN1 and NS5A as yellow fluorescence in the merged image. Cells were counterstained with 4',6-diamidino-2-phenylindole (DAPI) to label nuclei (blue). (B) Huh7.5 cells were either mock-infected or infected with Jc1 for 4 h. At 48 h postinfection, cells were fixed in 4% paraformaldehyde, and immunofluorescence staining was performed by using an anti-TEN1 polyclonal antibody and fluorescein isothiocyanate-conjugated goat anti-goat IgG to detect TEN1 (green) and a rabbit anti-NS5A antibody and TRITC-conjugated donkey anti-rabbit IgG to detect NS5A (red).

demonstrated that the protein level of TEN1 increased in HCV-infected cells compared with the mock-infected cells (Fig. 2B, lower). Notably, the protein level increase in HCV-infected cells was relatively lower than in replicon cells, which suggests that HCV upregulates the TEN1 expression level to promote HCV propagation.

TEN1 is required for HCV propagation

To investigate the functional implication of protein interplay between NS5A and TEN1, we examined whether TEN1 is required for HCV propagation. Therefore, Huh7.5 cells were transfected with either control siRNA or siRNA pools containing four siRNA constructs targeting different sites of TEN1. At 24 h after transfection, the cells were infected with Jc1 viruses. Figure 3A shows that knockdown of TEN1 by siRNA constructs #2 and #3 impaired HCV protein expression, whereas knockdown of TEN1 by siRNA constructs #1 and #4 had no effect. We then investigated the silencing effect of TEN1 on HCV RNA replication by qRT-PCR. As shown in Fig. 3B, silencing of TEN1 by siRNA constructs #2 and #3 significantly reduced HCV RNA levels in HCV-infected cells. It was also notable that HCV RNA levels were reduced by ~90% in cells transfected with TEN1-specific #2 and #3 siRNAs compared with cells transfected with the negative control siRNA. We confirmed that treatment with the same concentration of siRNAs displayed no cytotoxicity in Jc1-infected cells, indicating that silencing effect was specific to TEN1 (data not shown). To rule out the off-target effect of TEN1 siRNA #2,

we generated an siRNA-resistant TEN1 mutant. As shown in Fig. 3C, exogenous expression of the siRNA-resistant TEN1 mutant, but not the wild-type TEN1, restored the HCV protein expression level (lane 3 vs lane 4). Taken together, these data indicate that TEN1 is necessary for HCV propagation.

TEN1 is translocated from the nucleus to the cytoplasm in HCV-infected cells

Since NS5A interacted with TEN1, we further examined whether NS5A could colocalize with TEN1. Huh7.5 cells transfected with Flag-tagged TEN1 were either mock-infected or infected with the Jc1 virus and the TEN1 localization was examined by indirect immunofluorescence. As shown in Fig. 4A, the TEN1 protein was mainly localized to the nucleus in mock-infected cells (left panel). Surprisingly, the TEN1 translocated from the nucleus to the cytoplasm in Jc1-infected cells (Fig. 4A, right panel). Moreover, NS5A and TEN1 were colocalized in the cytoplasm, as indicated by the yellow fluorescence. To verify this result, the Huh7.5 cells were transfected with TEN1 expression plasmid and were either mock-infected or infected with Jc1. As shown in Fig. 4B, endogenous TEN1 translocated from the nucleus to the cytoplasm before colocalizing with NS5A in Jc1-infected cells. These data suggest that HCV exploits TEN1 to promote viral propagation and meaning that TEN1 functions as a proviral host factor.

NS5A forms a trimeric complex with STN1 and TEN1

TEN1 directly interacts with STN1 in the CST complex and functions in telomere length protection (Miyake et al., 2009). We conducted an immunoprecipitation assay to verify the protein interplay between STN1 and TEN1 (Fig. 5A, lane 6) and it was notable that that NS5A did not interact with STN1 (Fig. 5B). To investigate the mechanism underlying protein interaction between cytoplasmic NS5A and nuclear TEN1 in HCV-infected cells, we first investigated the effect of NS5A on TEN1 and STN1 interaction. HEK293T cells were cotransfected with Flag-tagged TEN1, V5-tagged STN1, and Myc-tagged NS5A. We demonstrated that TEN1 interacted with either NS5A (Fig. 5C, lane 4) or STN1 (Fig. 5C, lane 5). Surprisingly, TEN1 coprecipitated both NS5A and STN1 (Fig. 5C, lane 6) in immunoprecipitation assay. Since NS5A did not interact with STN1, this result indicated that NS5A formed the complex with STN1 via interaction with TEN1. Consequently, NS5A interacts with TEN1 as a trimeric complex in the cytoplasm of the HCV-infected cells. However, we were still unsure how the cytoplasmic NS5A was able to contact with nuclear TEN1 in the cytoplasm. Kelich et al. (2021) report that protein levels of CTC and STN1 in the CST complex are reduced in the nucleus during the G2 and M phases of the cell cycle. This may suggest that either newly synthesized TEN1 protein is retained in the cytoplasm or nuclear-localized TEN1 is re-localized to the cytoplasm as the cell cycle progresses in HCV-infected cells.

Telomere length is shortened in HCV-infected cells

Previous studies have reported that patients with chronic liver diseases have shorter telomere lengths compared with those in healthy controls (Kitada et al., 1995; Wiemann et al., 2002). To investigate whether telomere length was altered by HCV infection, we compared telomere lengths between mock-infected and HCV-infected cells by a qPCR assay using telomere-specific primers. As shown in Fig. 6A, the telomere lengths of HCV-infected cells were consistently shorter compared with the mock-infected cells. On day 18 postinfection, telomere length was significantly decreased in HCV-infected cells compared with mock-infected cells (Fig. 6B). These results suggest that telomere length is shortened in persistently infected cells with HCV meaning that telomere shortening may be indicative of chronic liver diseases.

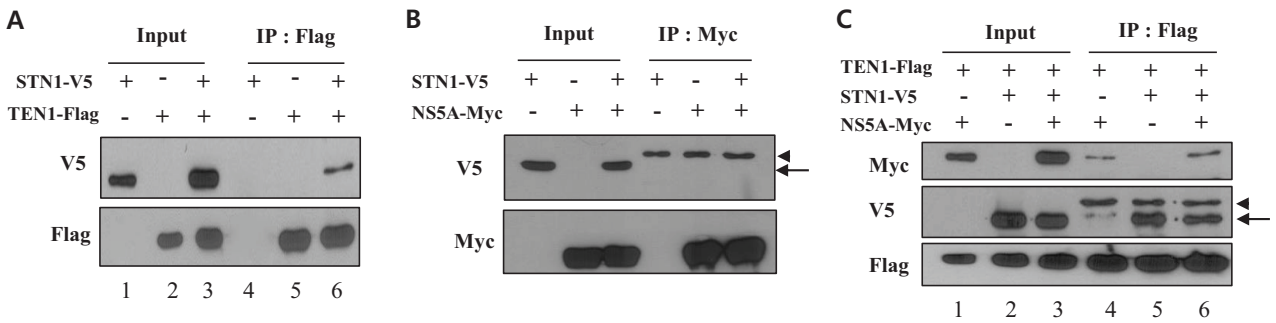

Fig. 5. NS5A interacts with TEN1/STN1 complex. (A) TEN1 directly interacts with STN1. Huh7.5 cells were cotransfected with Flag-tagged TEN1 and V5-tagged STN1 expression plasmids. At 48 h after transfection, cell lysates were immunoprecipitated (IP) with an anti-Flag monoclonal antibody, and bound proteins were detected by immunoblot analysis using an anti-V5 monoclonal antibody. (B) NS5A does not interact with STN1. Huh7.5 cells were cotransfected with V5-tagged STN1 and Myc-tagged NS5A expression plasmids. At 48 h after transfection, total cell lysates were immunoprecipitated with an anti-Myc monoclonal antibody, and bound proteins were then detected by immunoblot analysis using an anti-V5 monoclonal antibody. Protein expression of Myc-tagged NS5A was verified by immunoblot analysis using rabbit anti-NS5A polyclonal antibody. Arrowhead indicates IgG heavy chain and arrow denotes STN1-V5. (C) Huh7.5 cells were cotransfected with Flag-tagged TEN1, V5-tagged STN1, and Myc-tagged NS5A expression plasmids. At 48 h after transfection, total cell lysates were immunoprecipitated with an anti-Flag monoclonal antibody, and then bound proteins were detected by immunoblot analysis using either an anti-Myc monoclonal antibody or an anti-V5 monoclonal antibody. Immunoprecipitation efficiency was verified by immunoblot analysis using an anti-Flag antibody. Arrowhead indicates IgG heavy chain and arrow denotes STN1-V5.

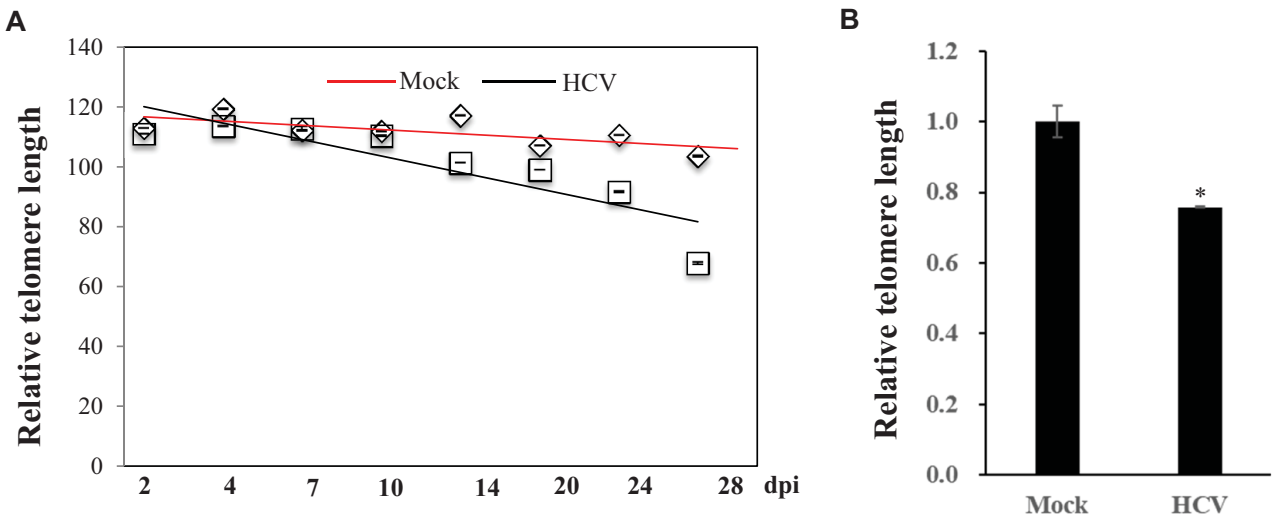

Fig. 6. Telomere length is shortened in HCV-infected cells. (A) Huh7.5 cells were either mock-infected or infected with HCV Jc1 and then harvested at the indicated time points to measue telomere length from the genomic DNA. The amount of input genomic DNA is quantified by qPCR product of a single copy gene (S). It is used to normalize the signal from the telomere (T) reaction. Relative T/S ratio is proportional to the average telomere length per genome. dpi, days postinfection. (B) Huh7.5 cells were either mock-infected or infected with Jc1. At 18 days postinfection, genomic DNAs were purified to measure the telomere length. The asterisk indicates a significant difference ($*P < 0.05$) from the value for the control. Error bars indicate SD.

DISCUSSION

HCV NS5A plays a critical role in many aspects of the viral life cycle. As a multifunctional phosphoprotein that interacts with various host cellular proteins, NS5A is an essential component of the HCV replicase and is required for HCV assembly (Appel et al., 2008; Kim et al., 2011; Macdonald and Harris, 2004; Tellinghuisen et al., 2008). We identified TEN1 as a binding partner of NS5A by conducting a protein microarray assay and confirmed protein interaction between NS5A and TEN1 by a coimmunoprecipitation assay. TEN1 is a nuclear-localized protein that is complexed with STN1 in normal cells. However, our study show that TEN1 is colocalized with NS5A in the cytoplasm of HCV-infected cells. We hypothesized that nuclear TEN1 could be translocated to the cytopasm as a complex with STN1. However, further studies are necessary to determine the molecular mechanism of the nuclear to cytoplasmic translocation of TEN1 in HCV-infected cells.

TEN1 is a 13-kDa protein that is ubiquitously expressed in human tissue, including the liver. TEN1 is a component of the mammalian CTC1-STN1-TEN1 complex that is involved in telomere length protection (Feng et al., 2017; Kasbek et al., 2013; Lim and Cech, 2021). Human TEN1 functions as a specialized replication factor for the C-strand fill-in reaction for maintaining telomere integrity. Reduced levels of TEN1 will therefore result in increased telomere loss. TEN1 has an additional role in the CST complex that helps in solving various challenges to the replication machinery. Our study demonstrated that siRNA-mediated knockdown of TEN1 impaired both RNA and protein expression of HCV. We also showed that HCV increased TEN1 expression level in both HCV subgenomic replicon cells and HCV-infected cells and that HCV upregulated TEN1 expression through NS5A protein. However, the detailed molecular mechanism underlying TEN1 upregulation in HCV-infected cells requires further investigation.

Telomeres are nucleoprotein structures located at the termini of eukaryotic chromosomes which protect the chromosome ends from DNA damage during DNA-replication. Telomeres consist of tandem repeats of the DNA sequence TTAGGG ranging from 10 to 15 kb in humans (Srinivas et al., 2020; Turner et al., 2019). Telomere shortening is part of the natural aging process but can be facilitated by chronic disease such as chronically active HCV, chronic liver disease, and liver cirrhosis (Biron-Shental et al., 2013; Kitada et al., 1995; Kitay-Cohen et al., 2008; Wiemann et al., 2002). However, the mechanism of telomere length shortening in HCV replicating cells has not been studied yet. TEN1 is localized to the nucleus in normal human cells and is essential for telomere maintenance and homeostasis. However, our study demonstrate that HCV infection induces TEN1 to translocate from the nucleus to the cytoplasm. Since nuclear TEN1 depletion impairs telomere maintenance and integrity, telomere length is reduced in HCV-infected cells. Collectively, our data provide unprecedented insights into the molecular mechanism of telomere shortening in patients with chronic HCV infection.

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AUTHOR CONTRIBUTIONS

All authors have given approval to the final version of the manuscript. Y.S.L. performed experiments, analyzed data, and wrote the manuscript. M.T.N.N., T.X.P., T.T.X.H., and E.M.P. performed experiments. D.H.C., S.M.K., and D.T. provided reagents and expertise. Y.S.L. and S.B.H. designed experiments and secured funding. S.B.H. supervised the study and wrote the manuscript.

CONFLICT OF INTEREST

The authors have no potential conflicts of interest to disclose.

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