Molecules and Cells

Nonstructural NS5A Protein Regulates LIM and SH3 Domain Protein 1 to Promote Hepatitis C Virus Propagation

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Hepatitis C virus (HCV) propagation is highly dependent on cellular proteins. To identify the host factors involved in HCV propagation, we previously performed protein microarray assays and identified the LIM and SH3 domain protein 1 (LASP-1) as an HCV NS5A-interacting partner. LASP-1 plays an important role in the regulation of cell proliferation, migration, and protein-protein interactions. Alteration of LASP-1 expression has been implicated in hepatocellular carcinoma. However, the functional involvement of LASP-1 in HCV propagation and HCV-induced pathogenesis has not been elucidated. Here, we first verified the protein interaction of NS5A and LASP-1 by both *in vitro* pulldown and coimmunoprecipitation assays. We further showed that NS5A and LASP-1 were colocalized in the cytoplasm of HCV infected cells. NS5A interacted with LASP-1 through the proline motif in domain I of NS5A and the tryptophan residue in the SH3 domain of LASP-1. Knockdown of LASP1 increased HCV replication in both HCV-infected cells and HCV subgenomic replicon cells. LASP-1 negatively regulated viral propagation and thereby overexpression of LASP-1 decreased HCV replication. Moreover, HCV propagation was decreased by wild-type LASP-1 but not by an NS5A binding-defective mutant of LASP-1. We further demonstrated that LASP-1 was involved in the replication stage of the HCV life cycle. Importantly, LASP-1 expression levels were increased in persistently infected cells with HCV. These data suggest that HCV modulates LASP-1 via NS5A in order to regulate virion levels and maintain a persistent infection.

Keywords: hepatitis C virus, LASP-1, NS5A, protein microarray, viral replication

INTRODUCTION

Hepatitis C virus (HCV) infection causes both acute and chronic hepatitis, which eventually leads to liver cirrhosis and hepatocellular carcinoma (HCC). HCV is an enveloped virus with a positive sense, single-stranded RNA that belongs to the genus *Hepacivirus* within the *Flaviviridae* family (Giannini and Brechot, 2003). The 9.6 kb genome encodes a single polyprotein that is precursor of 3,010 amino acids in length

which is sequentially processed by viral and host cellular proteases into 10 mature proteins. Core, E1, and E2 are structural proteins, p7 is an ion channel protein, and NS2-NS5B are nonstructural proteins involved in the replication of the viral genome (Bartenschlager et al., 2013; Lindenbach and Rice, 2005). Among these, nonstructural 5A (NS5A) is a multifunctional phosphoprotein consisting of 447 amino acid residues. We have previously reported that NS5A interacts with numerous host cellular proteins, including PI4KIIIα, DR6, pin1, pim1, RAD51AP1, and UBE2S to modulate viral propagation and host cellular signaling pathways (Lim and Hwang, 2011; Lim et al., 2011; Luong et al., 2017; Nguyen et al., 2018; Park et al., 2015; Pham et al., 2019). Since NS5A not only plays an important role in HCV replication but also contributes to HCV-mediated liver pathogenesis, this protein has begun to draw significant attention as a target for the development of antiviral drugs.

The LIM and SH3 domain protein 1 (LASP-1) gene was initially identified from a cDNA library of breast cancer metastases tissue, and the gene was mapped to human chromosome 17q21 (Tomasetto et al., 1995b). The Human LASP-1 gene encodes a membrane-bound protein that is 261 amino acids long and contains one N-terminal LIM domain followed by two actin-binding sites and a C-terminal src homology SH3 domain (Grunewald and Butt, 2008; Tomasetto et al., 1995a). The SH3 domain of LASP-1 serves as a binding motif to interact with zyxin. LASP-1 is involved in the regulation of cytoskeletal architecture and mainly localized within multiple sites of actin assembly including focal adhesions (Chew et al., 2002). LASP-1 regulates gene expressions of various molecules to stimulate cancer growth and the migration of various cancer cells (Zhao et al., 2010). LASP-1 expression is increased in many malignant tumors such as breast cancer, bladder cancer, and HCC (Ardelt et al., 2013; Grunewald et al., 2007; Wang et al., 2013). It has been previously reported that LASP-1 is upregulated in hepatocytes that overexpress HBV X protein through HBX-mediated c-Jun phosphorylation (Tang et al., 2012; You et al., 2018).

To identify cellular proteins involved in HCV propagation, protein microarray screening was employed using NS5A as a probe (Park et al., 2015). Among 90 cellular proteins interacting with NS5A, LASP-1 was selected for further study. Here we show that NS5A physically interacts with LASP-1 through the proline motif in domain I of NS5A and the tryptophan residue in the SH3 domain of LASP-1. Knockdown of LASP-1 increased both RNA and protein levels of HCV, whereas overexpression of LASP-1 decreased HCV replication. Interestingly, LASP-1 expression levels increased in cells persistently infected with HCV. We speculated that HCV may modulate LASP-1 to maintain chronic infection, and thus LASP-1 may contribute to HCV-mediated pathogenesis.

MATERIALS AND METHODS

Cell culture
All cell lines including HEK293T, Huh6, Huh7, and Huh7.5 were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum and 100 units/ml penicillin/streptomycin in 5% CO_2 at 37°C.

Huh7 cells harboring a subgenomic replicon derived from genotype 1b or Huh6 cells harboring a subgenomic replicon derived from genotype 2a were grown as reported previously (Lim et al., 2011).

Plasmid constructions
Myc-tagged wild-type and mutants of NS5A expression plasmids were generated by polymerase chain reaction (PCR) using the genotype 1b of HCV as a template and subcloned into the pEF6A vector. HCV NS5A mutants were constructed using full-length NS5A as a template. Total RNAs were isolated from Huh7.5 cells and full-length LASP1 was amplified from cDNA synthesized using a cDNA synthesis kit (Toyobo, Japan) according to the manufacturer's instructions. PCR products were inserted into the BamHI and EcoRI sites of the plasmid p3XFlag-CMV10 (Sigma-Aldrich, USA). Both LASP-1 and NS5A mutants were generated by site-directed mutagenesis (Enzynomics, Korea) according to the manufacturer's instructions.

Antibodies
Antibodies were purchased from the following sources: LASP-1, glyceraldehyde 3-phosphate dehydrogenase (GAPDH), Myc, and LAMIN A/C antibodies were from Santa Cruz Biotechnology (USA); Flag and actin antibodies were from Sigma-Aldrich; HCV core, NS3, and NS5A antibodies were described elsewhere (Lim et al., 2011).

Preparation of infectious virus
pFK-Jc1 construct was kindly provided from Dr. Ralf Bartenschlager (University of Heidelberg, Germany). Infectious cell culture grown virus was produced as described previously (Lim et al., 2011).

Generation of LASP-1 stable cell lines
To make the cell lines that stably express Flag-tagged LASP-1, Huh7 cells were transfected with the p3xFlag-CMV10-LASP-1 expression plasmid and cultured for eight weeks in the presence of 300 μg/ml of G418. Single positive clones were selected by immunoblot analysis using an anti-FLAG monoclonal antibody. Huh7 cell lines stably transfected with an empty vector (p3xFlag-CMV10 only) were selected as described above and used as a control.

Immunoprecipitation assay
HEK293T cells were cotransfected with 1 μg of Myc-tagged NS5A and 1 μg of Flag-tagged LASP-1. Total amounts of DNA were adjusted by adding an empty vector. Thirty-six hours after transfection, cells were harvested and an immunoprecipitation assay was performed as we reported previously (Lim et al., 2011).

In vitro pulldown assay
His-tagged NS5A protein purified from Escherichia coli was incubated with 30 μl of Ni-NTA agarose beads for 1 h at 4°C accompanied by gentle shaking. The beads were then washed three times in buffer (50 mM Na_2HPO_4 [pH 8.0], 100 mM NaCl, 1 mM PMSF, 1% protease cocktail inhibitor) and were incubated with cell lysate expressing Flag-tagged

LASP-1 for 2 h at 4°C. The sample was washed five times in lysis buffer (50 mM HEPES, pH7.6, 120 mM NaCl, 5 mM EDTA, 0.2% NP-40) and then bound protein was detected by immunoblotting analysis using an anti-Flag monoclonal antibody.

Immunoblot analysis
Equal amounts of the proteins were subjected to SDS-PAGE electrophoresis and electrotransferred to a nitrocellulose membrane. The membrane was blocked in TBST (20 mM Tris-HCl [pH 7.6], 500 mM NaCl, and 0.25% Tween 20) containing 5% nonfat dry milk for 1 h and was then incubated overnight at 4°C with the indicated antibodies in TBST, which contained 1% nonfat dry milk. Following three washes in TBST, the membrane was incubated for 1 h at room temperature with either horseradish peroxidase-conjugated goat anti-rabbit antibody or goat anti-mouse antibody (Jackson ImmunoResearch Laboratories, USA) in TBST. Proteins were detected using an ECL kit (Abfrontier, Korea).

Luciferase reporter assay
Huh7.5 cells were cotransfected with a pRL-HL plasmid containing the *Renilla* luciferase gene, which is under the control of the cytomegalovirus (CMV) promoter, the firefly luciferase gene, which is under the control of the HCV internal ribosome entry site (IRES), and the pCH110 reference plasmid. At 48 h after transfection, cells were harvested and then dual-luciferase assays were performed as described previously (Lim et al., 2011).

RNA interference
The siRNAs that target LASP-1 (sense 5′-GGA GUU UGA GAA GAA CAA G-3′; antisense 5′-CUU GUU CUU CUC AAA CUC C-3′) and the universal negative siRNA were purchased from Bioneer (Korea). The siRNA targeting the 5′ nontranslated region (NTR) of the Jc1 (5′-CCU CAA AGA AAA ACC AAA C-3′) was used as a positive control (Lim et al., 2011). The siRNA transfection was performed using a Lipofectamine RNAiMax reagent (Invitrogen, USA) according to the manufacturer's instructions.

Quantification of RNA
Total RNAs were isolated from either HCV-infected cells, cell culture media, or replicon cells using RiboEx LS reagent (Geneall Biotechnology), and cDNA was synthesized by using a cDNA synthesis kit (Toyobo) according to the manufacturer's instructions. Quantitative real-time PCR (qRT-PCR) experiments were performed using a CFX Connect real-time system (Bio-Rad Laboratories, Hercules, CA) as we reported previously (Pham et al., 2019).

MTT assay
Huh7 cells were seeded at 4×10^4 cells in a 24 well plate and transfected with the indicated siRNAs. At 4 days after transfection, cells were further incubated with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reagent (Sigma-Aldrich) for 2 h at 37°C. Cell viability was determined as we reported previously (Lim et al., 2011).

Immunofluorescence assay
Huh7.5 cells seeded on cover slides were fixed with 4% paraformaldehyde in phosphate-buffered saline (PBS) for 12 min at room temperature. After two washes in PBS, fixed cells were permeabilized with 0.1% Triton X-100 in PBS for 15 min and were then blocked with 0.5% bovine serum albumin (BSA) for 2 h. The cells were then incubated with the indicated antibodies overnight at 4°C. After three washes in PBS, cells were incubated with tetramethylrhodamine isothiocyanate (TRITC)-conjugated donkey anti-rabbit immunoglobulin G (IgG) for 2 h at room temperature. Cells were counterstained with 4′,6′-diamidino-2-phenylindole (DAPI) to label the nuclei. After three washes in PBS, cells were analyzed using the Zeiss LSM 700 laser confocal microscopy system (Carl Zeiss, USA).

Statistical analysis
Data are presented as mean ± SD. Student's *t*-tests were used for statistical analysis. The asterisks in the figures indicate significant differences ($*P < 0.05$, $**P < 0.01$, $***P < 0.001$). Experiments were carried out in triplicate. All statistical analysis in the study was carried out using Microsoft Excel 2016 (Microsoft, USA).

RESULTS

HCV NS5A interacts with LASP-1 through the proline motif in domain 1 of NS5A and the tryptophan residue in the SH3 domain of LASP-1
To verify the protein microarray data, we performed an *in vitro* binding assay with His-tagged NS5A protein purified from *E. coli* and cell lysates expressing Flag-tagged LASP-1. As shown in Fig. 1A, NS5A interacted with LASP-1. To further verify the protein microarray data, HEK293T cells were cotransfected with Myc-tagged NS5A and Flag-tagged LASP-1 expression plasmids. At 3 days after transfection, total cell lysates were immunoprecipitated with an anti-Flag monoclonal antibody, and the bound protein was analyzed by immunoblotting with an anti-Myc antibody. Figure 1B shows that NS5A selectively interacted with LASP-1. To further demonstrate the protein interplay between NS5A and LASP-1 in HCV replicating cells, Huh7.5 cells were electroporated with Jc1 RNA. At 4 days after RNA electroporation, total cell lysates were immunoprecipitated with either control IgG or an anti-NS5A antibody, and the bound protein was immunoblotted with an anti-LASP1 antibody. The results shown in Fig. 1C demonstrate that NS5A interacted with endogenous LASP-1. These data suggest that NS5A may colocalize with LASP-1 in HCV-infected cells. To investigate this possibility, Huh7 cells were transfected with GFP-tagged LASP-1 plasmid and then infected with Jc1. At 2 days postinfection, cells were analyzed by an immunofluorescence assay. As demonstrated in Fig. 1D, LASP-1 protein was widely expressed in the cytoplasm of the HCV-infected cells, and both HCV NS5A and LASP-1 were colocalized in the cytoplasm as indicated by the yellow fluorescence in the merged image. These data indicate that NS5A specifically interacts with LASP-1 in HCV replicating cells.

To determine which region of NS5A was responsible for

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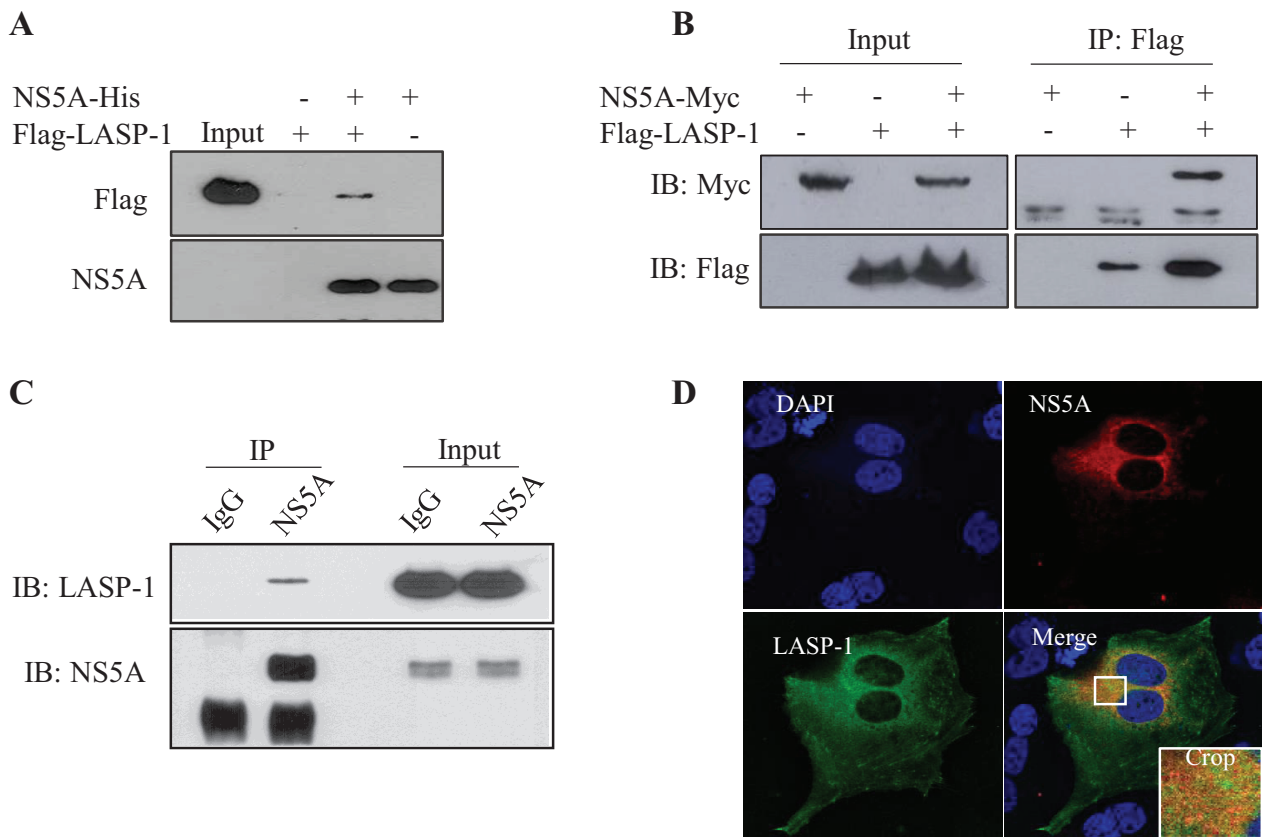
Fig. 1. HCV NS5A protein interacts with LASP-1. (A) HEK293T cells were transiently transfected with either vector (-) or Flag-tagged LASP-1 expression plasmid. Total cell lysates harvested at 36 h after transfection were incubated with His-tagged NS5A protein purified from *E. coli*. Bound protein was precipitated by Ni-NTA beads and detected by immunoblotting with an anti-Flag monoclonal antibody. NS5A protein was verified with an anti-NS5A antibody. (B) HEK293T cells were cotransfected with Myc-tagged NS5A and Flag-tagged LASP-1 expression plasmids. At 48 h after transfection, cell lysates were immunoprecipitated with an anti-Flag monoclonal antibody, and bound protein was detected by using an anti-Myc monoclonal antibody. Protein expressions of Myc-tagged NS5A and Flag-tagged LASP-1 were verified by immunoblotting with an anti-Myc monoclonal antibody and an anti-Flag monoclonal antibody, respectively. (C) Huh7.5 cells were electroporated with 10 μg of Jc1 RNA. Cell lysates harvested at four days after electroporation were immunoprecipitated with either control IgG or an anti-NS5A antibody. Bound protein was immunoblotted with an anti-LASP-1 antibody. Immunoprecipitation efficiency was verified by immunoblotting with an anti-NS5A antibody. IB, immunoblot; IP, immunoprecipitation. (D) Huh7 cells were transfected with GFP-tagged LASP-1 plasmid and then infected with Jc1 for 4 h. At 48 h postinfection, cells were fixed in 4% paraformaldehyde, and immunofluorescence staining was performed by using rabbit anti-NS5A antibody and TRITC-conjugated donkey anti-rabbit IgG to detect NS5A protein (red). Dual staining showed colocalization of NS5A and LASP-1 as yellow fluorescence in the merged image. Cells were counterstained with DAPI to label nuclei. The slides were examined under confocal fluorescence microscopy at 400× magnification.

LASP-1 binding, the interaction between LASP-1 and various deletion mutants of NS5A (Fig. 2A) was investigated with a transfection-based coimmunoprecipitation assay. As shown in Fig. 2B, LASP-1 interacted with domain I and with domains I and II but not with domains II and III. These results indicate that domain I of NS5A was responsible for binding with LASP-1. Next, we determined the region of LASP-1 responsible for NS5A binding using truncated mutants of LASP-1 (Fig. 2C). LASP-1 has a highly conserved tryptophan residue in the SH3 domain and this tryptophan residue mediates interaction with proline-containing peptides (Larson and Davidson, 2000; Winters and Pryciak, 2005). We, therefore examined whether the tryptophan residue of LASP-1 was involved in

binding with NS5A. Figure 2D shows that NS5A interacted with wild-type LASP-1 but not with SH3-deleted mutants (ΔSH3). Of note, NS5A was unable to bind the tryptophan mutant (W239A) of LASP-1, indicating that tryptophan residue in SH3 domain of LASP-1 plays a crucial role in NS5A binding. Since NS5A contains a PXXP motif in domain I (Tan et al., 1999), we further investigated whether proline-rich sequences in NS5A were responsible for binding with LASP-1. For this purpose, two proline residues in the PXXP motif were substituted with alanine (P/A), and then HEK293T cells were cotransfected with either LASP-1 and wild-type NS5A or LASP-1 and proline motif mutant (P/A) of the NS5A expression plasmids. Figure 2E shows that LASP1 interacted
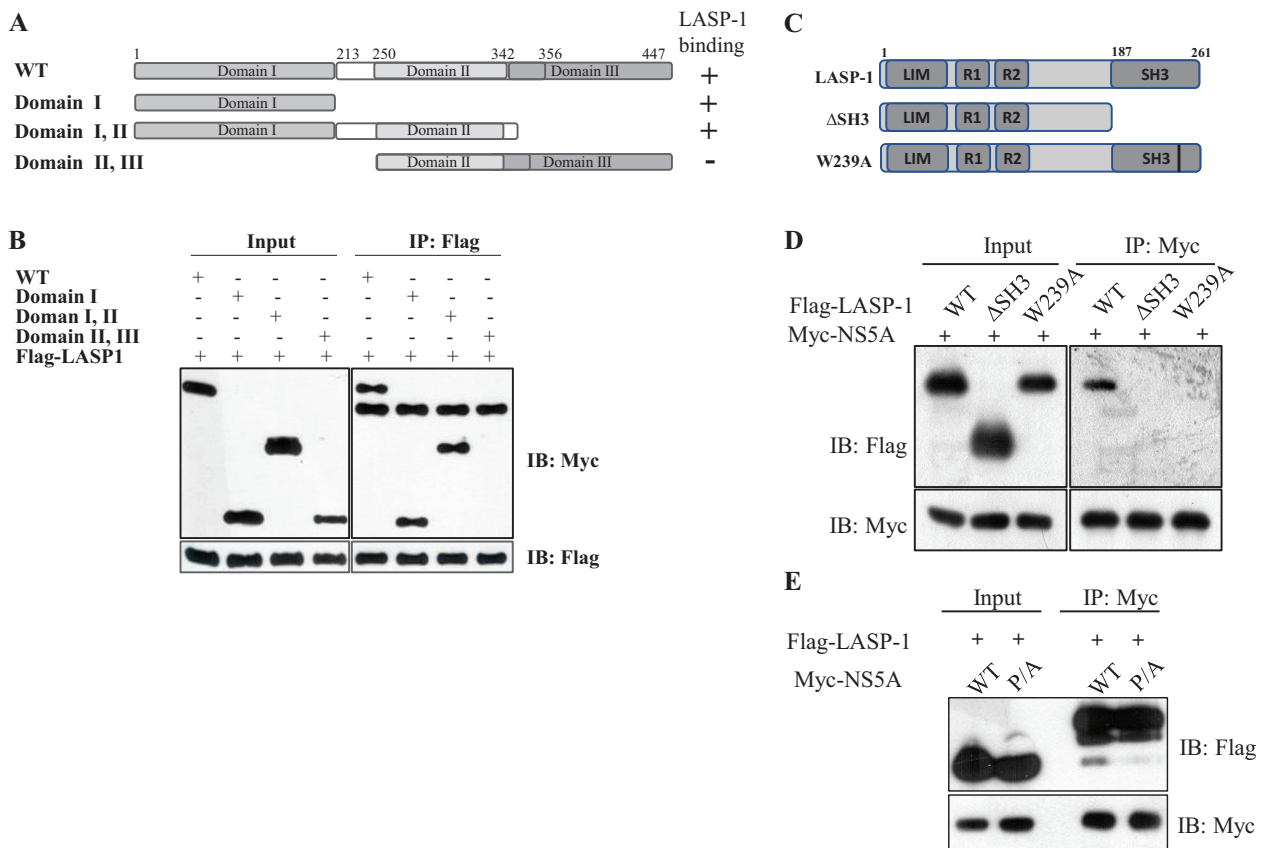
Fig. 2. NS5A interacts with LASP-1 through the proline motif in domain 1 of NS5A and the tryptophan residue in the SH3 domain of LASP-1. (A) Schematic representation of NS5A mutants. (B) HEK293T cells were cotransfected with Flag-tagged LASP-1 and Myc-tagged wild-type (WT) or with mutants of NS5A expression plasmids. At 36 h after transfection, total cell lysates were immunoprecipitated with an anti-Flag monoclonal antibody and bound proteins were immunoblotted with an anti-Myc antibody. Protein expressions of Flag-tagged LASP-1 and Myc-tagged wild-type or mutants of NS5A were verified by immunoblotting with an anti-Flag antibody and an anti-Myc antibody, respectively. (C) Schematic diagram of LASP-1 mutants. (D) HEK293T cells were cotransfected with Myc-tagged NS5A and Flag-tagged mutants of LASP-1. At 36 h after transfection, total cell lysates were immunoprecipitated with an anti-Myc monoclonal antibody and bound proteins were immunoblotted with an anti-Flag antibody. Protein expression of input plasmids was verified by immunoblot analysis with the indicated antibodies. (E) HEK293T cells were cotransfected with Flag-tagged LASP-1 and Myc-tagged wild type plasmids or class I, proline-motif mutants (P/A) of NS5A expression plasmids. At 36 h after transfection, total cell lysates were immunoprecipitated with an anti-Myc antibody and bound protein was immunoblotted with an anti-Flag antibody. IB, immunoblot; IP, immunoprecipitation.

with wild-type NS5A. However, mutation in the PXXP motif of NS5A impaired LASP-1 binding, indicating that the proline-rich sequence in domain I of NS5A was responsible for binding with LASP-1. Collectively, HCV NS5A interacts with LASP-1 via domain 1 of NS5A and the SH3 domain of LASP-1.

LASP-1 expression level is increased in HCV-infected cells
To investigate whether HCV modulated LASP-1 expression level, Huh7.5 cells were either mock-infected or infected with Jc1, and then mRNA levels of LASP-1 were determined by quantitative PCR (qPCR). As shown in Fig. 3A, the mRNA level of LASP-1 at 10 days postinfection was significantly higher in Jc1-infected cells as compared with mock-infected cells. Consistent with these results, protein levels of LASP-1 were

also markedly increased in Jc1-infected cells as compared with mock-infected cells (Fig. 3B). Of note, both mRNA and protein levels of LASP-1 were not altered during the early stage of HCV infection (data not shown). To further confirm these results, Huh7.5 were either mock-infected or infected with Jc1, and then protein expression levels of LASP-1 were analyzed at various time points after HCV infection. Figure 3C shows that protein level of LASP-1 did not change in the first four days postinfection. Importantly, protein levels of LASP-1 gradually increased from day 8 to day 12 postinfection. However, the NS5A protein level gradually decreased during this same period of HCV infection. Since LASP-1 protein levels increased in cells persistently infected with HCV, these data suggest that LASP-1 may be involved in chronic infection, thereby affecting liver pathogenesis of HCV.

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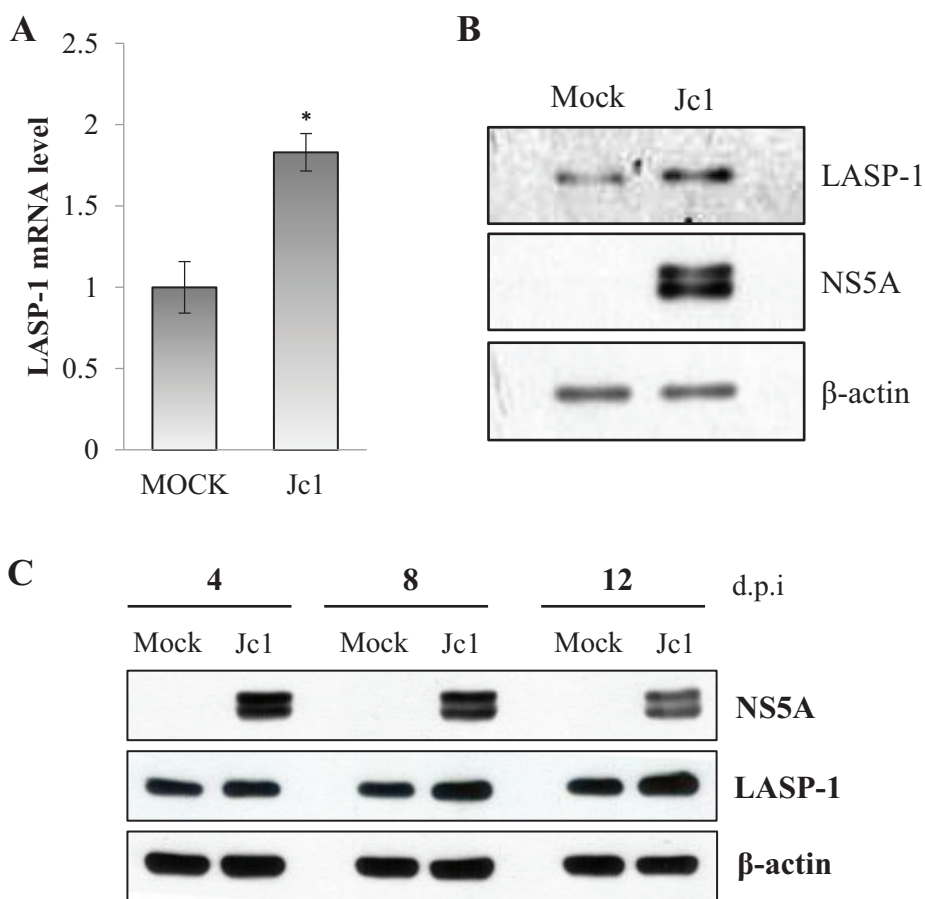
Fig. 3. LASP-1 expression level is increased in cells persistently infected with HCV. (A) Huh7.5 cells were either mock-infected or infected with Jc1 (multiplicity of infection [MOI] = 0.5) for 4 h. At 10 days postinfection (d.p.i), mRNAs levels of LASP-1 were analyzed by qPCR. The asterisk in the figure indicates significant difference ($*P < 0.05$). Experiments were carried out in triplicate. Error bars indicate SD. (B) Total cell lysates harvested at 10 days postinfection were immunoblotted with the indicated antibodies. (C) Huh7.5 cells were either mock-infected or infected with Jc1 for 4 h. Total cell lysates harvested at various time points were immunoblotted with the indicated antibodies.
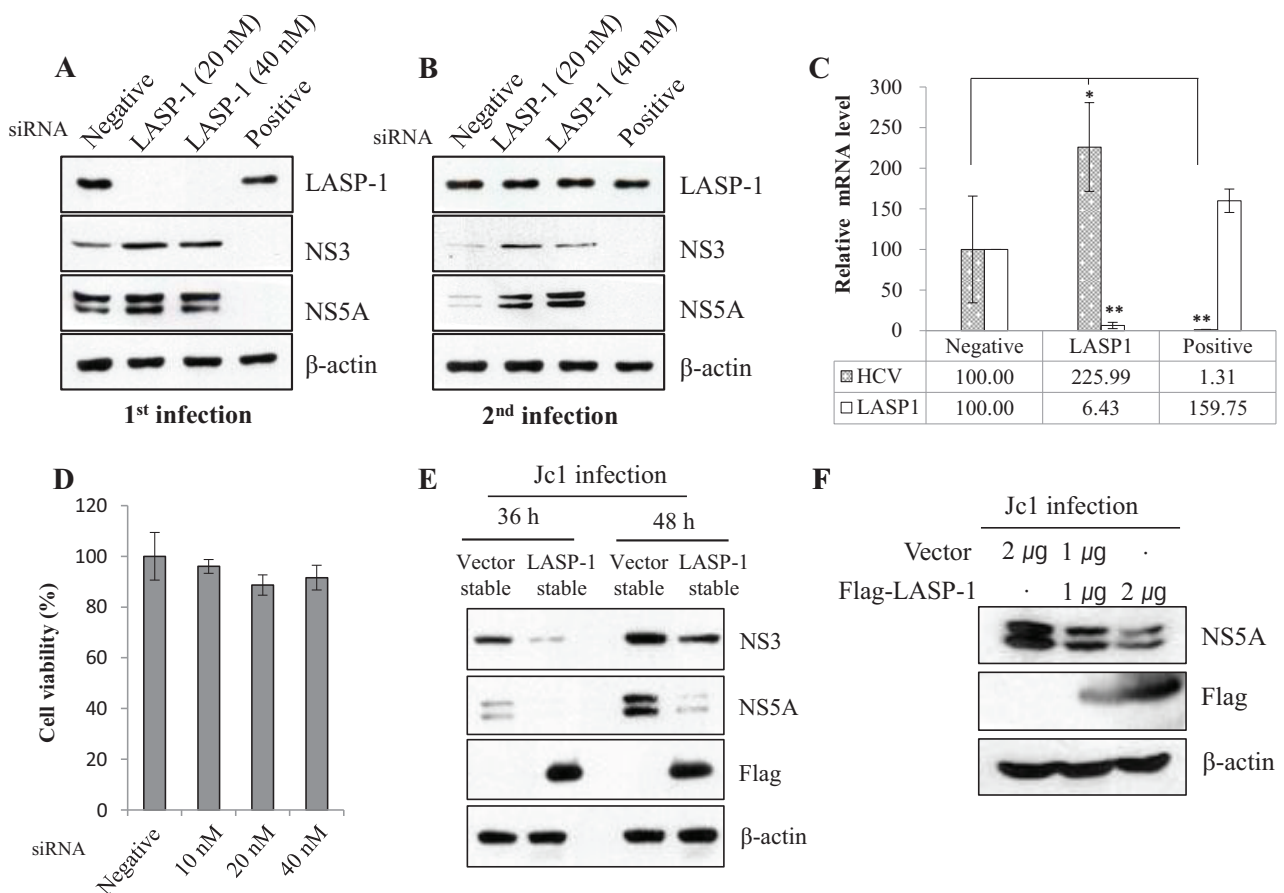
LASP-1 negatively regulates HCV propagation

Because NS5A interacted with LASP-1, we investigated the possible involvement of LASP-1 in HCV propagation. For this purpose, Huh7.5 cells were either mock-infected or infected with Jc1 and then further transfected with the indicated siRNAs. At 48 h after transfection, protein levels were analyzed by immunoblotting with the indicated antibodies. Figure 4A shows that HCV protein levels increased in LASP-1 knockdown cells. To confirm this result, naïve Huh7 cells were infected with Jc1 harvested from the culture supernatant of the first infection. As shown in Fig. 4B, HCV protein levels were markedly increased in cells infected with released virion found in the supernatant of LASP-1 knock-down cells. We further demonstrated that the intracellular HCV RNA levels were also significantly increased in LASP-1-silenced cells (Fig. 4C), but this change did not induce cellular toxicity (Fig. 4D). These data suggest that LASP-1 may be a negative regulator for HCV propagation. To further clarify the involvement of LASP-1 in HCV propagation, we established a cell line stably expressing either vector or LASP-1 protein. Both vector stable cells and LASP-1 stable cells were infected with Jc1. Total cell lysates harvested at two different time points were immunoblotted with the indicated antibodies. As shown in Fig. 4E, HCV protein levels decreased in LASP-1 stable cells as compared to the vector stable cells. To verify these results, Huh7.5 cells were transiently transfected with either empty vector or Flag–tagged LASP-1 expression plasmids and then further in-

fected with Jc1. Consistently, ectopic expression of LASP-1 in Jc1-infected cells led to a decrease in HCV protein level (Fig. 4F). Together, these data suggest that LASP-1 may function as an antiviral host factor against HCV infection.

LASP-1 is involved in the replication step of the HCV life cycle

To investigate which step of the HCV life cycle was negatively regulated by LASP-1, Huh 7 cells harboring HCV subgenomic replicon derived from genotype 1b were transfected with LASP-1-specific siRNA. As shown in Fig. 5A, knockdown of LASP-1 significantly increased the protein (upper panel) and RNA (lower panel) levels of the HCV replicon derived from genotype 1b. Consistently, siRNA-mediated knockdown of LASP-1 led to a significant increase in the protein (upper panel) and RNA (lower panel) levels of the HCV replicon derived from genotype 2a (Fig. 5B). Since knockdown of LASP-1 enhanced HCV replication in subgenomic replicon cells, we asked whether LASP-1 was also involved in HCV IRES-mediated translation. To address this question, Huh7.5 cells were transfected with either negative control siRNA or LASP-1-specific siRNA together with pRL-HL dual luciferase (Fig. 5C, upper panel) and pCH110 β-galactosidase plasmid (Lim et al., 2011). At 48 h after transfection, the relative luciferase activity was determined. We showed that LASP-1 was not involved in HCV IRES-dependent translation (Fig. 5C, lower panel). We further showed that overexpression of LASP-1 displayed no

Fig. 4. LASP-1 is a negative regulator for HCV propagation. (A) Huh7 cells were infected with Jc1 for 4 h and then transfected with the indicated siRNAs. At 48 h after transfection, total cell lysates were immunoblotted with the indicated antibodies. Negative: universal negative-control siRNA; Positive: HCV-speci c siRNA targeting the 5' NTR of the Jc1 (5'-CCU CAA AGA AAA ACC AAA CUU-3'). (B) Naïve Huh7 cells were infected with Jc1 harvested from culture supernatant from the first infection described in panel A. At 48 h postinfection, total cell lysates were harvested and immunoblotted with the indicated antibodies. (C) Huh7 cells were infected with Jc1 for 4 h and then transfected with either the negative, 20 nM of LASP-1-specific siRNA, or the positive control siRNA. At 48 h after transfection, intracellular RNA levels were analyzed by qPCR. The asterisks in the figures indicate significant differences ($*P < 0.05$, $**P < 0.01$). Experiments were carried out in triplicate. Error bars indicate SD. (D) Huh7 cells were transfected with either negative siRNA or various concentrations of LASP-1-specific siRNAs, and then cell viability was determined by MTT assay. (E) Huh7 cells stably expressing either vector or LASP-1 protein were infected with Jc1 for 4 h. At two different time points, cell lysates were immunoblotted with the indicated antibodies. (F) Huh7 cells were infected with Jc1 for 4 h. At 24 h postinfection, cells were further transfected with either empty vector or two different concentrations of Flag–tagged LASP-1 expression plasmid. At 48 h after transfection, cell lysates were immunoblotted with the indicated antibodies.

effect on HCV IRES-dependent translation (Fig. 5D). All these data indicate that LASP-1 is specifically involved in the replication step of the HCV life cycle.

Protein interplay between NS5A and LASP-1 plays a crucial role in HCV propagation

To verify the functional significance of protein interplay between NS5A and LASP-1, we explored the binding effect of LASP-1 and NS5A on HCV replication. To answer this question, Huh7 cells infected with Jc1 were transfected with an empty vector, Flag-LASP-1, or Flag-W239A-LASP-1 expression plasmid. As shown in Fig. 6A, HCV protein levels were significantly decreased by wild-type LASP-1, but not NS5A

binding-defective LASP-1, in HCV infected cells. We further confirmed that HCV protein levels were significantly reduced by wild-type, but not by NS5A binding-defective LASP-1, in subgenomic replicon cells (Fig. 6B). Collectively, these data indicate that LASP-1 negatively regulates HCV replication via the protein interplay between NS5A and LASP-1.

DISCUSSION

NS5A is a multifunctional viral protein, which not only contributes to viral replication and assembly, but also interacts with multiple host proteins to modulate signal transduction pathways and host immune responses. Although NS5A has
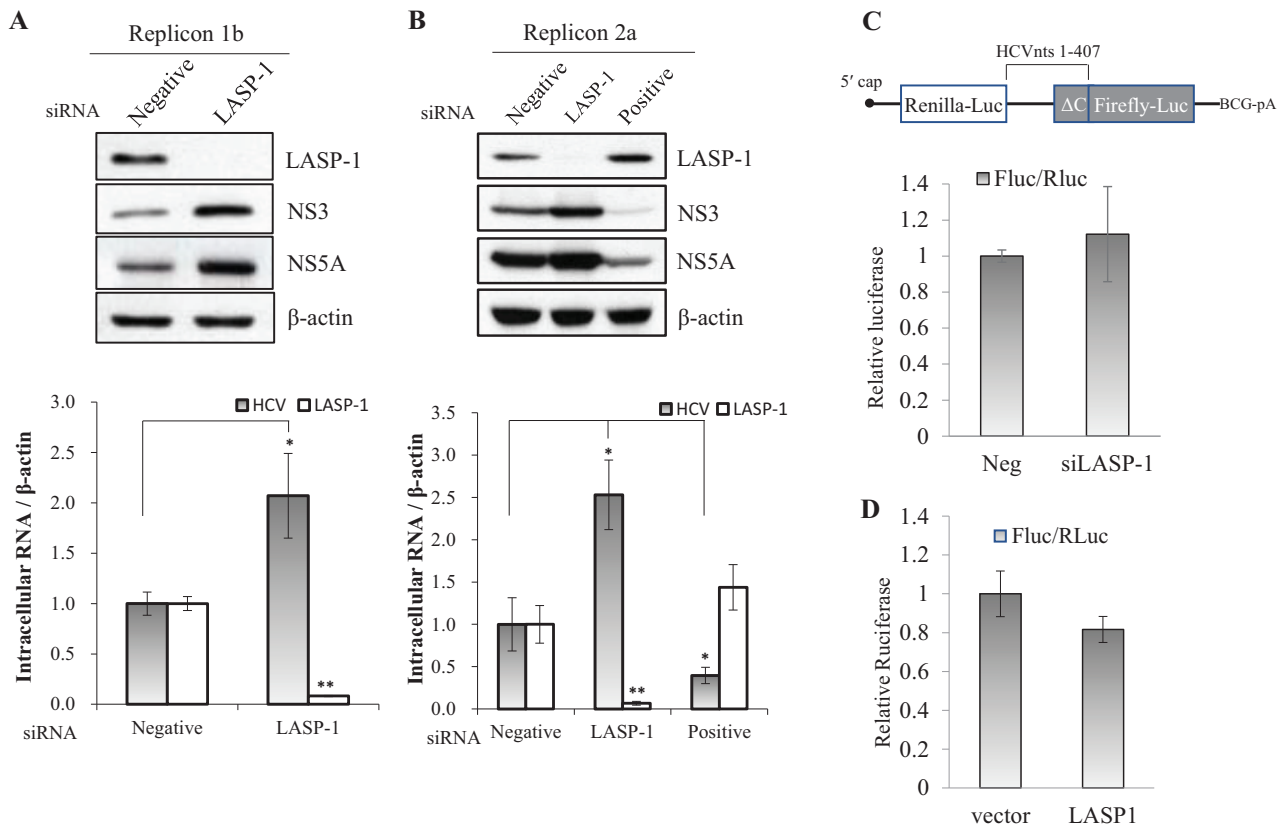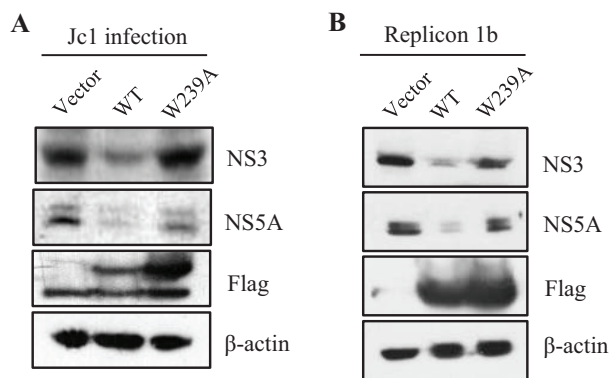
Fig. 5. LASP-1 is involved in the replication stage of the HCV life cycle. (A) (Upper panel) Huh 7 cells harboring the HCV subgenomic replicon derived from genotype 1b were transfected with either the negative or LASP-1-specific siRNA. At 72 h after transfection, total cell lysates were immunoblotted with the indicated antibodies. (Lower panel) Cells treated as described in the upper panel were harvested at 72 h after transfection, and intracellular RNA levels were analyzed by qPCR. (B) (Upper panel) Huh 6 cells harboring HCV subgenomic replicon derived from genotype 2a were transfected with the indicated siRNAs. At 72 h after transfection, total cell lysates were immunoblotted with the indicated antibodies. (Lower panel) Cells treated as described in the upper panel were harvested at 72 h after transfection, and intracellular RNA levels were analyzed by qPCR. Negative: universal negative-control siRNA; Positive: HCV-specific siRNA targeting the 5' NTR of the Jc1. (C) Huh7.5 cells were transfected with either the negative or LASP-1-specific siRNA. Cells were further cotransfected with the pRL-HL dual-reporter plasmid and the pCH110 β-galactosidase plasmid. At 48 h after transfection, luciferase activities were determined. (D) Huh7.5 cells were cotransfected with either vector or Flag-tagged LASP1 with pRL-HL and pCH110 plasmids. At 48 h after transfection, luciferase activities were determined and then normalized to β-galactosidase activities. HCV IRES activity was expressed as FLuc/RLuc ratio. The asterisks in the figures indicate significant differences (*$P < 0.05$, **$P < 0.01$). Experiments were carried out in triplicate. Error bars indicate SD.

no enzyme activity, it has become an attractive target for the development of DAAs. The currently available NS5A inhibitors are ombitasvir, ledipasvir, elvasvir, velpatasvir, daclatasvir, and pibrentasvir (Gitto et al., 2017; Gottwein et al., 2018). Since monotherapy of DAAs generates drug-resistant mutant of HCV, combination therapies of NS5A-targeting DAAs and NS3/4A- or NS5B-targeting DAAs have shown over 90% sustained virological response (SVR). In addition, NS5A-interacting host proteins, such as cyclophillin A, have attracted much attention as host targeted antivirals (HTAs) (Pawlotsky et al., 2015).

We have previously identified LASP-1 as one of NS5A interacting cellular partners from protein microarray screening (Park et al., 2015). NS5A interacted with LASP-1 through domain 1 of NS5A and the tryptophan residue in the SH3 do-

main of LASP-1. In fact, the C-terminal SH3 domain of LASP-1 is involved in protein-protein interactions at focal adhesions (Li et al., 2004). SH3 domains predominantly bind proline-rich sequences containing a short and conserved PXXP motif (Saksela et al., 1995). Since the SH3 domain-deleted mutant of LASP-1 lost its ability to bind to NS5A, we further investigated whether PXXP sites in NS5A were responsible for interactions with LASP-1. NS5A has two highly conserved PXXP SH3-binding sites, class I (amino acid 26-32) and class II (amino acid 350-356) motifs, among HCV genotypes (Tan et al., 1999). Our data showed that the class I, proline-rich motif in domain I of NS5A displayed an important role in binding with LASP-1. We further demonstrated that NS5A interacted with LASP-1 through a highly conserved tryptophan residue in the SH3 domain. Human LASP-1 is phosphorylated at

A Jc1 infection
Vector WT W239A

NS3
NS5A
Flag
β-actin

B Replicon 1b
Vector WT W239A

NS3
NS5A
Flag
β-actin

Fig. 6. Protein interplay between LASP-1 and NS5A plays a crucial role in HCV propagation. (A) Huh7 cells were infected with Jc1 for 4 h. At 24 h postinfection, cells were transfected with vector, Flag-LASP-1, or Flag-LASP-1-W239A, respectively. At 48 h after transfection, cell lysates were immunoblotted with the indicated antibodies. (B) Huh 7 cells harboring HCV subgenomic replicon derived from genotype 1b were transfected with vector, Flag-LASP-1, or Flag-LASP-1-W293A, respectively. At 48 h after transfection, total cell lysates were immunoblotted with the indicated antibodies.

serine 146 by cAMP- and cGMP-dependent protein kinases (Keicher et al., 2004; Mihlan et al., 2013). It has been previously reported that nuclear localization and accumulation of LASP-1 are related with poor long-term survival in breast cancer (Ardelt et al., 2013). However, we noticed that both phosphorylation status and cytoplasmic localization of LASP-1 were not altered in HCV infected cells (data not shown).

Viruses rely heavily on host cell machinery to propagate, whereas the host cell's defense against viral propagation is the activation of the cellular immune system. In the present study, we showed that LASP-1 suppressed HCV propagation and functioned as an antiviral host factor against HCV. Therefore, silencing of LASP-1 increased HCV replication, whereas overexpression of LASP-1 restrained HCV propagation. Importantly, this negative effect of LASP-1 on HCV propagation was mediated through the interaction with NS5A. Indeed, ectopic expression of NS5A binding-defective LASP1 (W239A) failed to suppress HCV propagation. All of these data suggest that HCV may self-regulate virion level through NS5A and LASP-1 in order to maintain a persistent viral infection.

LASP-1 plays an important role in cell migration, invasion, proliferation, migration, and protein-protein interaction. Alterations of LASP-1 expression have been implicated in HCC (Tang et al., 2012; Wang et al., 2013). In HBV, HBX protein promotes LASP-1 expression through activation of c-Jun in hepatoma cells (Tang et al., 2012; You et al., 2018). In the present study, we showed that both RNA and protein levels of LASP-1 were increased in cells that were persistently infected with HCV, suggesting that LASP-1 may be implicated in HCV-induced liver pathogenesis. Collectively, our data suggest that HCV may self-regulate virion level via NS5A and LASP-1 in order to maintain a persistent infection. However, further research is required to elucidate the detailed mechanism of NS5A and LASP-1 interaction in HCV-replicating cells.

AUTHOR CONTRIBUTIONS

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