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Inhibition of hepatitis C virus infection by NS5A-specific aptamer

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ABSTRACT

To increase efficacy of hepatitis C treatment, future regiments will incorporate multiple direct-acting antiviral drugs. HCV NS5A protein was expressed and purified. Aptamers against NS5A were screened and obtained by the selective evolution of ligands by exponential enrichment approach and the antiviral actions of the aptamers were tested. The mechanisms through which the aptamers exert their antiviral activity were explored. The aptamers NS5A-4 and NS5A-5 inhibit HCV RNA replication and infectious virus production without causing cytotoxicity in human hepatocytes. The aptamers do not affect hepatitis B virus replication in HepG2.2.15 cells. Interferon beta (IFN-β) and interferon-stimulated genes (ISGs) are not induced by the aptamers in HCV-infected hepatocytes. Further study shows that domain I and domain III of NS5A protein are involved in the suppression of HCV RNA replication and infectious virus production by NS5A-4. Y2105H within NS5A is the major resistance mutation identified. NS5A aptamer disrupts the interaction of NS5A with core protein. The data suggest that the aptamers against NS5A protein may exert antiviral effects through inhibiting viral RNA replication, preventing the interaction of NS5A with core protein. Aptamers for NS5A may be used to understand the mechanisms of virus replication and assembly and served as potential therapeutic agents for hepatitis C.

1. Introduction

Hepatitis C virus (HCV) infects 170 million people worldwide and up to 80% of those infected become chronic infection. HCV infection can cause chronic hepatitis, liver cirrhosis and even hepatocellular carcinoma (Laucer and Walker, 2001). There is no vaccine available and peginterferon alpha (Peg-IFN-α)-based therapy is the current treatment for the patients (Hoofnagle and Seeff, 2006). Many patients do not response to the therapy. It is necessary to develop safe, well-tolerated and effective therapeutic agents against HCV infection (Sakamoto and Watanabe, 2009).

HCV is a single positive-strand RNA virus that has a genome about 9.6 kb in length. It encodes a single long polyprotein which is processed into core, E1, E2 structural proteins and non-structural proteins consisting of the p7 ion channel, NS2, NS3, NS4A, NS4B, NS5A and NS5B by cellular and viral enzymes. NS5A protein is a multifunctional protein and essential for HCV replication and virus production (Hughes et al., 2009; Jiang and Luo, 2012; Kim et al., 2011; Tellinghuisen et al., 2008). The essential role of NS5A in HCV lifecycle makes it an attractive target for the development of specific antiviral drugs (Gao et al., 2010; Rice, 2011). The recent development of infectious HCV clone JFH1 and genotype 1a (H77S) provides powerful tools for the study of virus lifecycle and discovery of inhibitors of viral infection (Lindenbach et al., 2005; Wakita et al., 2005; Yi et al., 2006; Zhong et al., 2005). Although NS5A is essential for HCV RNA replication and virion morphogenesis, its precious roles in the HCV lifecycle are unknown.

Aptamers are isolated from highly diverse starting library of synthetic oligonucleotides by selective evolution of ligands by exponential enrichment (SELEX) approach, an in vitro molecular evolution procedure (Ellington and Szostak, 1990; Tuerk and Gold, 1990). SELEX involves the repetitive reduction of the library complexity via rounds of selective binding to the target and amplification. Aptamers can specifically recognize the targets or regulate their functions. So aptamers can be used to explore the function of the targets. Aptamers possess many advantages over antibody as

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therapeutic reagents, including low cost, no immunogenicity, easy synthesis and modification (Keefe et al., 2010; Tan et al., 2011).

In the study, we obtained aptamers for HCV NS5A protein using in vitro SELEX. The data demonstrate that aptamers against NS5A protein inhibit HCV RNA replication, and infectious virus production through binding different domains of NS5A and blocking their functions. The data indicate that the aptamers may hold promise for understanding the mechanisms of viral replication and the development of novel approach for the treatment of chronic hepatitis C.

2. Materials and methods

2.1. Cells, plasmids and reagents

Huh7.5 cells and mouse monoclonal anti-NS2 antibody (6H6) were kindly provided by Charles Rice (Rockefeller University, New York, NY) (Blight et al., 2002). FCA1, a HCV genotype 1b replicon cell line, was a gift from Christoph Seeger (Fox Chase Cancer Center, Philadelphia, PA). pJFH1 and pJFH1/GND plasmids were generously provided by Takaji Wakita (National Institute of Infectious Diseases, Tokyo, Japan). The pH77-S and pH77-S/ΔE1P7 plasmids were obtained from Stanley M Lemon (University of North Carolina, Chapel Hill, NC) (Yi et al., 2006). Mouse monoclonal anti-NS5A antibody was a gift from Chen Liu (University of Florida, Gainesville, FL). The cells were grown in Dulbecco's modified Eagle's medium (Gibco) supplemented with 10% fetal bovine serum.

2.2. Expression and purification of HCV NS5A protein

The full-length of NS5A sequence was PCR amplified from the plasmid pJFH1, digested with NdeI and EcoRI, and inserted into pET-28b(+) (Novagen, Madison, MI) to produce pET28b-NS5A. NS5A protein was expressed in *Escherichia coli* BL21 (DE3) cells (Invitrogen, Carlsbad, CA). Cells were grown in LB medium and induced with 1 mM of IPTG. The NS5A protein was purified through His-tag. The protein was eluted with imidazole in phosphate-buffered saline (PBS) buffer and identified using anti-His antibody (Sigma, St. Louis, MO) or anti-NS5A antibody via western blot described below. The expression of HCV core protein was published previously (Shi et al., 2014).

2.3. In vitro selection of aptamers against HCV NS5A

The synthesized DNA library pool with an overall complexity of $\sim 10^{14}$ was purchased from Takara and used for in vitro selection. The sequence of the library is 5′-ACGCTCGGATGCCACTACA G(N40)CTCATGGACGTGCTGGTGAC-3′, where N40 represents 40 nucleotides with equal molar incorporation of A, G, C, and T at each position. The procedure was reported previously (Shi et al., 2014). Briefly, His-tagged NS5A or control protein was preincubated with nickel beads (Invitrogen, Carlsbad, CA), respectively. The DNA library was incubated with 20 μL of beads conjugated with control protein in 100 μL binding buffer (30 mM Tris–HCl, pH 7.5, 150 mM NaCl, 1.5 mM $MgCl_2$, 2 mM dithiothreitol, and 1% BSA) for 30 min at room temperature with shaking. The DNA–bead complexes were then precipitated and discarded to remove DNAs with nonspecific binding activity to agarose beads. The precleared supernatant was transferred to a new tube and further incubated with 2 μg of his-tagged NS5A protein for 30 min. NS5A–DNA complexes were precipitated with beads, and pellets were washed five times with binding buffer. DNAs were recovered, amplified with PCR, and used for next rounds of selection. Eight subsequent rounds of selection were carried out. After 8 rounds of selection, the amplified DNA was cloned and several clones were sequenced.

2.4. Enzyme-linked oligonucleotide assay (ELONA)

Streptavidin-precoated microtiter plates were coated with biotin-labeled aptamer in PBS and incubated at room temperature for 2 h. The plates were washed thrice with PBS containing 1 mM $MgCl_2$, 0.1% BSA, 0.05% Tween-20. His-tagged NS5A protein or the lysate from HCV-infected cells was added into the plates and incubated at 37 °C for half an hour. After washing to remove unbound target protein, mouse monoclonal antibody against His or NS5A was added into the plates. After incubation at 37 °C for 1 h, HRP-conjugated goat anti-mouse secondary antibody was added and incubated at 37 °C for 30 min. Color development was performed by addition of freshly prepared substrate solution for 10 min at room temperature. The plates were read with Envision plate reader and the absorbance of each sample was measured at 450 nm.

2.5. Construction of plasmids expressing different domains of NS5A

Domains I, I-II, I-III, II and III of NS5A were amplified from pJFH1 and cloned into the expression vector pEF6/V5-His-TOPO using the primers. NS5A (F1) 5′-CGCGCGGTACCATGTCCGGATCCTGGCT-3′ and NS5A (639R) 5′-CTGCAGTCTAGACTAGTCTCCGCCGTGAT-3′ are for domain I of NS5A. NS5A (F1) 5′-CGCGCGGTACCATGTCCGGA TCCTGGCT-3′ and NS5A (1068R) 5′-CTGCAGTCTAGACTTGTCCGGC GTCTCCTT-3′ are for the domain I-II of NS5A. NS5A (F1) 5′-CGCGCGGTACCATGTCCGGATCCTGGCT-3′ and NS5A (1398R) 5′-CTGCAGTATAGACTGCAGCACACGGTGGTAT-3′ are for the domain I-III of NS5A. NS5A (F751) 5′-CGCGCGGTACCATGGACGTG GACATGGT-3′ and NS5A (1068R) 5′-CTGCAGTCTAGACTTGTCCG GCGTCTCCTT-3′ are for domain II of NS5A. NS5A (F1069) 5′-CGCGCGGTACCATGGTGGGTCTGAGCGAGA-3′ and NS5A (1398R) 5′-CTGCAGTATAGACTGCAGCACACGGTGGTAT-3′ are for domain III of NS5A.

2.6. Real-time PCR

Aptamer or library was delivered into the viral-infected cells by transfection with lipofectamine 2000 for different time periods. The protocol was published previously (Yang et al., 2011). QPCR primers for HBV DNA are 5′-GGCTTTCGGAAAATTCCTA-3′ and 5′-AGCCCTACGAACCACTG-3′.

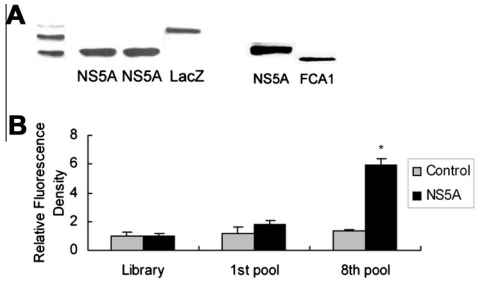

Fig. 1. Purification of HCV NS5A protein and binding affinity of DNA pools from round 1 and round 8 to NS5A protein. (A) Western blot analysis of purified NS5A protein. His-tagged NS5A was expressed by IPTG induction in *E. coli* BL21 (DE3) and then confirmed using mouse anti-His monoclonal antibody (left) or anti-NS5A monoclonal antibody (right) via western blot. (B) Comparison of the fluorescence of different pools of FITC-labeled DNA binding to NS5A protein or control protein LacZ. FITC-labeled DNA pools from Library, round 1 and round 8 were incubated with nickel beads conjugated with NS5A or LacZ protein in binding buffer in the presence of excess yeast tRNA. The density of the fluorescence was measured and normalized to the library. Results are the average of three independent experiments performed in triplicate. $^*P < 0.05$ verse library.

Table 1
Sequences of NS5A aptamers.

Name	Sequence
NS5A-1	ACGTACACTAGTGGTCCGAGCGAGGCTCATTGTCC
NS5A-2	GCGGATTCTCCATTGAACCTTCGTCAACTATGCGG
NS5A-3	CACGCCAGACCAGCCGTCCTCTCTTTATCCGAGCCTTCATCCGAG
NS5A-4	GCTATCTTATGGAAATTTCGTGTAGGGTTTGGTGTGGCGGGGCTA
NS5A-5	CTGGATCGAAAAGTATCCACGTTTGTCTGAAAATAGTGGC

2.7. MTS assay

The procedure was described previously (Yang et al., 2013). Briefly, one day before treatment, 1.0×10^3 cells were seeded in triplicate in a 96-well plate. The cells were cultured with or without aptamer for indicated time periods at 37 °C. Twenty microliter of the CellTiter AQ Solution containing MTS and an electron coupling reagent (Promega) was added to each well. After 2 h incubation at 37 °C, the absorbance at 490 nm was measured. Cell viability was calculated with respect to the control samples. Three independent experiments were performed.

2.8. Western blot analysis

The protocol was reported previously (Shi et al., 2014). Briefly, cells were washed with PBS and lysed in RIPA buffer (150 mM sodium chloride, 1% Nonider P-40, 0.5% sodium deoxycholate, 0.1% SDS and 50 mM Tris–HCl [pH 8.0] supplemented with 2 μg/mL of aprotinin, 2 μg/mL of leupeptin, 40 μg/mL of phenylmethysulfonyl fluoride, and 2 mM DTT). Twenty micrograms of protein were resolved by SDS/PAGE, transferred to a PVDF membrane and probed with appropriate primary and secondary antibodies. The bound antibodies were detected by ECL reagent (Pierce, Rockford, IL) according to the manufacturer's instruction.

2.9. Co-immunoprecipitation (IP) and immunoblotting

HCV-infected Huh7.5 cells in 60 mm culture dish were washed thrice with 1 ml of ice-cold PBS. The cells were lysed in 100 μL of lysis buffer (25 mM Tris–HCl [pH 7.4] containing 150 mM NaCl, 1% NP-40, 1 mM EDTA and 5% glycerol) supplemented with protease inhibitors cocktail at 4 °C. Cell lysates were incubated at 4 °C for 30 min, and centrifuged at 12,000*g* for 15 min. The lysates were diluted to 2 μg/μl total cellular protein with PBS before beginning IP. Two hundred microgram of lysates was immunoprecipitated with 15 μL of anti-NS5A antibody by gently rocking the reaction mixture at 4 °C overnight. Capture the immunocomplex by adding 30 μL of washed Protein G Agarose bead slurry and gently rock the reaction mixture at 4 °C for 4 h. Wash the beads 3 times with PBS (10 s in the microcentrifuge at 14,000*g*). The protein binding to the beads was boiled in 60 μL of 2× Laemmli sample buffer (Bio-Rad, Hercules, CA) and then subjected to SDS-12% PAGE. The protocol of immunoblotting was described above.

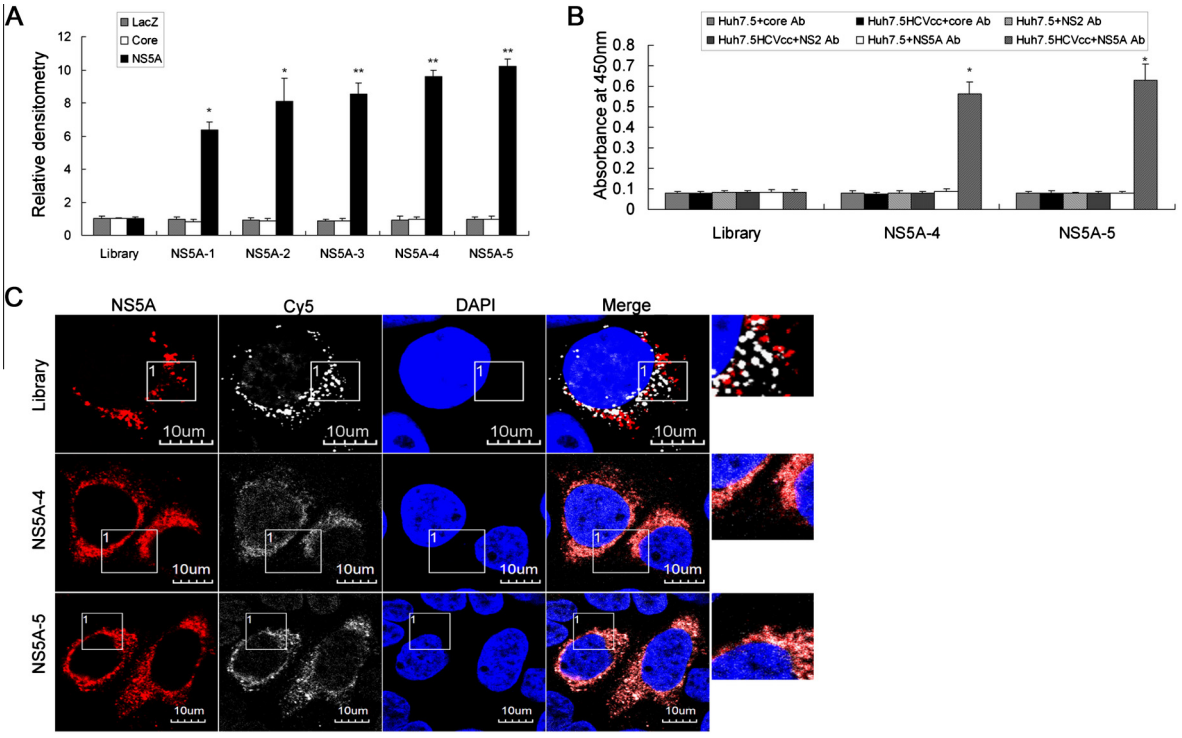

Fig. 2. Binding affinity of the selected aptamers for NS5A. (A) The binding affinity of aptamers NS5A-1, NS5A-2, NS5A-3, NS5A-4, and NS5A-5 to recombinant NS5A protein. Biotin-labeled aptamer or library was coated on the microtiter plate pre-coated with streptavidin. LacZ, core or NS5A protein was added to the plates and ELONA assay was performed. The densitometry was normalized to library. The data represented the means of three different experiments performed in triplicate. (B) The binding affinity of aptamer NS5A-4, NS5A-5 for NS5A protein in lysates of HCV-infected hepatocytes. Biotin-labeled NS5A-4 or NS5A-5 was coated on the microtiter plate pre-coated with streptavidin. Lysates of HCV-infected Huh7.5 or naive Huh7.5 cells were added to the plates. After washing, mouse anti-HCV core, NS2 or anti-NS5A monoclonal antibody was added and incubated at 37 °C for 1 h. HRP-conjugated goat anti-mouse IgG was added to the plates. The data were obtained as described in part A and represented the means of 3 independent experiments performed in triplicate. (C) Co-localization of NS5A-specific aptamers with NS5A protein in the HCV-infected hepatocytes. Cy5-labeled NS5A-4 or NS5A-5 was transfected into HCV-infected Huh7.5 cells for 24 h. The cells were fixed with ice-cold acetone for 10 min at −20 °C. The cells were washed with PBS, blocked with 1:50 goat serum for 30 min at room temperature and then incubated with mouse monoclonal anti-NS5A antibody for 1 h. The cells were stained with Texas Red-labeled goat anti-mouse for 45 min respectively at room temperature. The nuclei were counterstained with DAPI. Fluorescent images were obtained under fluorescent microscope. Identical setting was maintained for images capture. Representative images are shown. $^{*}P < 0.05$, $^{**}P < 0.01$ verse library.

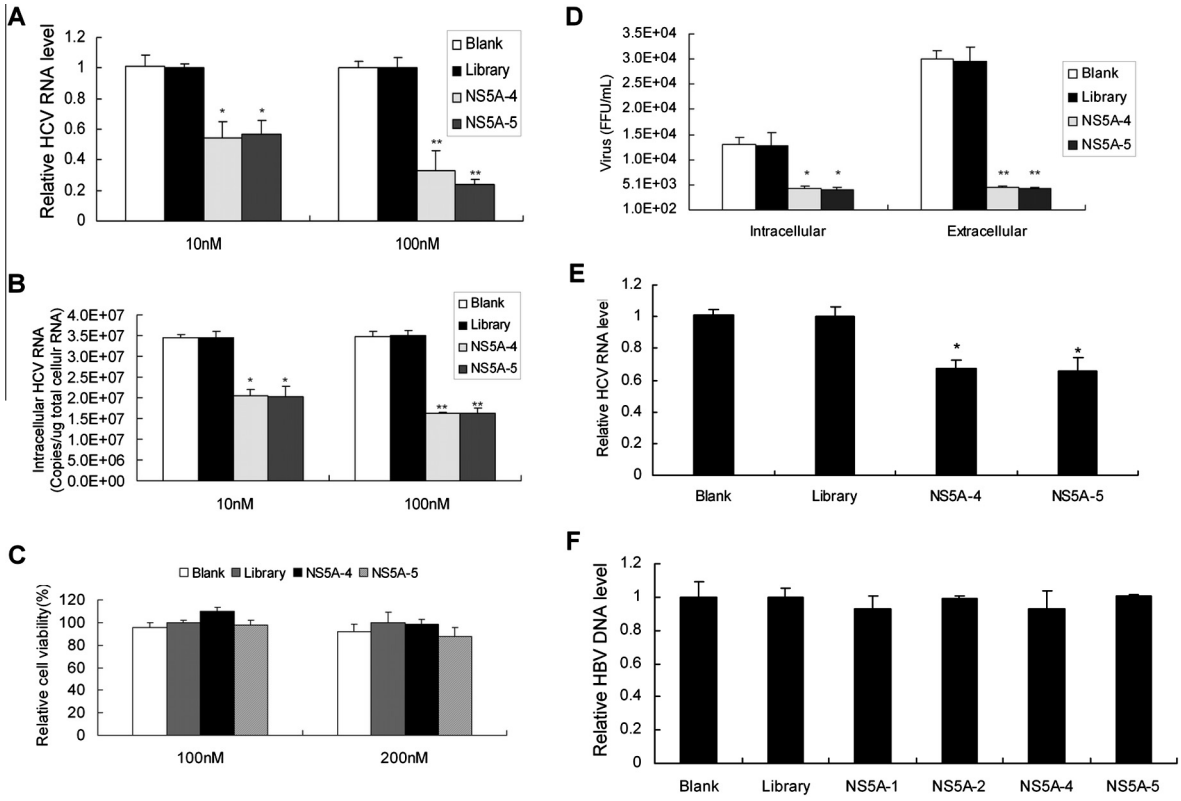

Fig. 3. Inhibition of HCV infection by the aptamers against NS5A. (A) Effect of NS5A aptamers on viral RNA replication in HCV replicon cell. FCA1 cells were treated with NS5A-4 or NS5A-5 for 72 h. Intracellular viral RNA was detected by real-time PCR and normalized with GAPDH. (B) Effect of NS5A aptamers on HCV RNA replication in infectious cell culture system. HCV-infected Huh7.5 cells were treated with NS5A-4 or NS5A-5 for 72 h. Intracellular viral RNA was detected by real-time PCR. (C) The effect of NS5A aptamer on viability of HCV-infected Huh7.5 cells. HCV-infected Huh7.5 cells were treated by 100 nM and 200 nM aptamers for 72 h. The effect of aptamer on cell viability was measured by MTS assay. The data were normalized with the library. (D) Effect of NS5A aptamers on the production of secreted and intracellular virus in HCV-infected hepatocytes. Huh7.5 cells were infected by JFH1 at MOI of 0.1 for 72 h. The cells were treated by 100 nM NS5A-4, NS5A-5 or control library for 72 h. The supernatants and virus particles inside the viral-infected cells were harvested and titered by FFU assay on naïve Huh7.5 cells. The infectivity titers in the supernatant or inside the cells are presented as the means and standard errors of three independent infections. (E) Effect of NS5A aptamers on H77-S virus replication in infectious cell culture system. H77-S virus suspension was used to infect Huh7.5 cells for 3 days. The cells were treated by 100 nM aptamer or control library treatment for 72 h. Intracellular viral RNA was detected by real-time PCR and normalized with GAPDH. (F) Effect of NS5A aptamers on hepatitis B viral DNA replication. HepG2.2.15 cells were treated with 100 nM of each aptamer for 72 h. Intracellular HBV DNA was detected by real-time PCR and normalized with GAPDH. If not stated otherwise bar graphs represent means of three independent experiments. $^{*}P < 0.05$, $^{**}P < 0.01$ verse library.

2.10. Intracellular virus preparation

The procedure was described previously (Shi et al., 2014).

2.11. Focus-forming units assay

The protocol was published previously (Shi et al., 2014).

2.12. Selection of resistance-conferring mutations

HCV-infected Huh7.5 cells were treated with 100 nM NS5A-4 or library for 3 weeks. NS5A cDNA was recovered from cells by RT-PCR. NS5A amplicons were used to generate cDNA clones with a TOPO-TA cloning kit (Invitrogen). The substitution Y2105H was introduced into pJFH1 plasmid by using QuickChange site-mutagenesis kit (Stratagene). In vitro transcripts of wild type JFH1 and selected mutated Y2105H JFH1 were generated and transfected into Huh7.5 cells, respectively.

2.13. Statistical analysis

Differences between means of reading were compared using Student *t*-test. Error bars represent S.D.

3. Results

3.1. Purification of HCV NS5A and selection of NS5A aptamers

To generate aptamers against HCV NS5A, cDNA fragment encoding the entire NS5A gene of JFH1 was amplified by PCR and cloned into the expression vector. NS5A protein was expressed and purified by its N-terminal His-tag. The purified NS5A protein was confirmed by immunoblot analysis (Fig. 1A).

A nucleotide library was obtained from a pool of $\sim 10^{14}$ single-stranded DNA molecules containing a random segment of 40 nucleotides flanked by 5′ and 3′ common primers as conserved linkers to amplify the selection process. After 8 rounds of selection, there was a significant increase in the binding of round 8 DNA pools to NS5A protein compared to that of the round 1 pools in the presence of excess yeast tRNA in the binding buffer (Fig. 1B). The selected aptamers were cloned and a total of 55 clones from the DNA pools of round 8 were sequenced. The topmost sequences were used as representative members for the study. The aptamers were named NS5A-1, NS5A-2, NS5A-3, NS5A-4 and NS5A-5 (Table 1).

3.2. Binding affinity of the aptamers against HCV NS5A protein

To determine the binding affinity of individual aptamer to NS5A protein, ELONA assay was performed with individual aptamer and

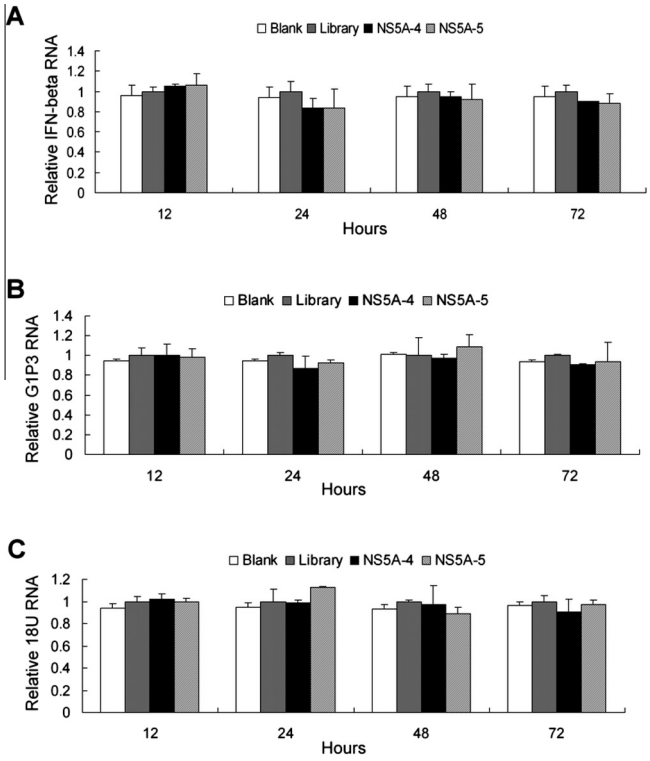

Fig. 4. NS5A-specific aptamers do not trigger innate immune response in HCV-infected hepatocytes. NS5A-specific aptamers do not induce IFN-β (A), G1P3 (B) and 18U (C) in HCV-infected hepatocytes. Virus suspension at MOI of 0.1 was used to infect Huh7.5 cells for 3 days. The cells were treated by 100 nM of each aptamer or library for 12, 24, 48, 72 h. IFN-β, G1P3 or 18U mRNA was detected by real-time PCR and normalized with GAPDH. Results are the average of three independent experiments.

NS5A protein. As shown in Fig. 2A, the aptamers NS5A-1, NS5A-2, NS5A-3, NS5A-4 and NS5A-5 showed high binding affinity for recombinant NS5A protein compared to control library while they displayed no apparent binding affinity for recombinant core or LacZ protein. Various aptamers bound to NS5A protein and this interaction was retained in the presence of excess yeast tRNA in binding buffer, indicating that their binding to NS5A was specific. We examined whether the aptamer NS5A-4 and NS5A-5 can bind NS5A protein in the lysates of HCV-infected hepatocytes. NS5A-4 and NS5A-5 showed specific high binding affinity to NS5A protein in the lysates of viral-infected cells in comparison with the library while they displayed no apparent binding affinity for core or NS2 protein in the lysates of viral-infected cells (Fig. 2B). Co-localization of NS5A-specific aptamers with NS5A protein in the HCV-infected hepatocytes was showed in Fig. 2C, supporting the interaction of NS5A-specific aptamers and NS5A protein in the viral-infected cells.

3.3. Inhibition of HCV infection by NS5A-specific aptamers

To examine whether the aptamers for NS5A inhibit HCV replication, we treated HCV replicon cell FCA1 with NS5A-4 or NS5A-5. HCV RNA level was lower in the cells with NS5A-4 or NS5A-5 treatment than in library-treated cells (Fig. 3A). Next, we decided to test whether the aptamers against NS5A inhibit HCV infection in the context of infectious cell culture system. JFH1 virus suspension at multiplicity of infection (MOI) of 0.1 was used to infect Huh7.5 cells for 72 h and the cells were treated by NS5A aptamers. Viral RNA in aptamer-treated cells was significantly decreased 1-fold in comparison with library-treated cells (Fig. 3B), although there was no difference of viral protein level (Fig. 6A). The concentration of the aptamers used in our experiments and even high concentration of aptamers showed no apparent toxic effect to the cells (Fig. 3C), suggesting that inhibition of viral replication is not due to the cytotoxic effect.

To test whether the aptamers against NS5A inhibit the production of viral particles, we harvested the supernatant of the HCV-infected hepatocytes with aptamers treatment. The viruses in the supernatant were titered by FFU assay on naïve Huh7.5 cells. The number of NS5A-positive foci (FFU) decreased 6-fold in Huh7.5 cells infected by secreted virus from HCV-infected hepatocytes with NS5A-4 or NS5A-5 treatment in comparison with library treatment (Fig. 3D). We want to understand whether the decrease in the extracellular infectious virions brought about by the aptamers is due to defective virus assembly. The level of intracellular infectious virions was reduced 3-fold in the cells with NS5A-4 or NS5A-5 treatment (Fig. 3D) although intracellular viral RNA decreased 1-fold in NS5A-4 or NS5A-5-treated cells (Fig. 3B). These data suggested that NS5A aptamers may cause reduction of virus assembly and release.

In addition, we examined the effect of NS5A aptamer on HCV genotype 1a infection in human hepatocytes. H77-S RNA was in vitro transcript from the plasmid pH77-S. H77-S RNA was transfected into Huh7.5 cells and the virus was collected according to reported previously (Yi et al., 2006). H77-S virus was used to infect Huh7.5 cells and the cells were treated by NS5A-4 or NS5A-5. As shown in Fig. 3E, viral RNA in the aptamer-treated cells decreased in comparison with cells with library treatment. Aptamers did not affect hepatitis B viral replication (Fig. 3F), implying that the aptamer against NS5A specifically inhibits HCV infection.

3.4. Aptamers against NS5A do not induce IFN-β and IFN-stimulated genes in HCV-infected hepatocytes

The presence of DNA molecules inside or outside the cells may cause a nonspecific induction of IFN, which may cause antiviral effect. To exclude the possibility that inhibition of HCV infection by NS5A-specific aptamers is due to the DNA-induced innate immune response, we examined the expression of IFN-β and ISGs in aptamer-treated cells. IFN-β was not induced in NS5A-4 or NS5A-5-treated cells (Fig. 4A). IFN antiviral activity is exerted by IFN-stimulated genes (ISGs). Our published results demonstrated that G1P3 and 1-8U play a role in the establishment of intracellular antiviral state (Zhu and Liu, 2003; Zhu et al., 2003). NS5A-4 or NS5A-5 did not induce G1P3 and 1-8U in viral-infected cells (Fig. 4B and C). The data clear show that aptamers against NS5A do not induce IFN-β and ISGs in viral-infected hepatocytes, indicating that inhibition of HCV infection by NS5A aptamers is not due to the innate immunity.

3.5. Domains I and III of NS5A protein are involved in the antiviral effects of NS5A-specific aptamer NS5A-4

To identify the residues of NS5A involved in aptamer binding and inhibition of viral infection, we generated truncated versions of NS5A. The expression of different domains of NS5A protein could be confirmed using western blot (Fig. 5A). ELONA assay was used to determine which truncated version of NS5A binds to the aptamer. Deletion of domains II and III of NS5A did not affect the affinity for aptamer NS5A-4 and domain I of NS5A is essential for the binding of NS5A-4 to the protein (Fig. 5B). The data suggest that the binding region of aptamer NS5A-4 may localize inside domain I of NS5A protein. To further confirm that aptamer NS5A-4 binds to domain I of NS5A and inhibits viral infection, we conducted competition experiments. HCV-infected Huh7.5 cells were transfected with plasmids expressing different domains

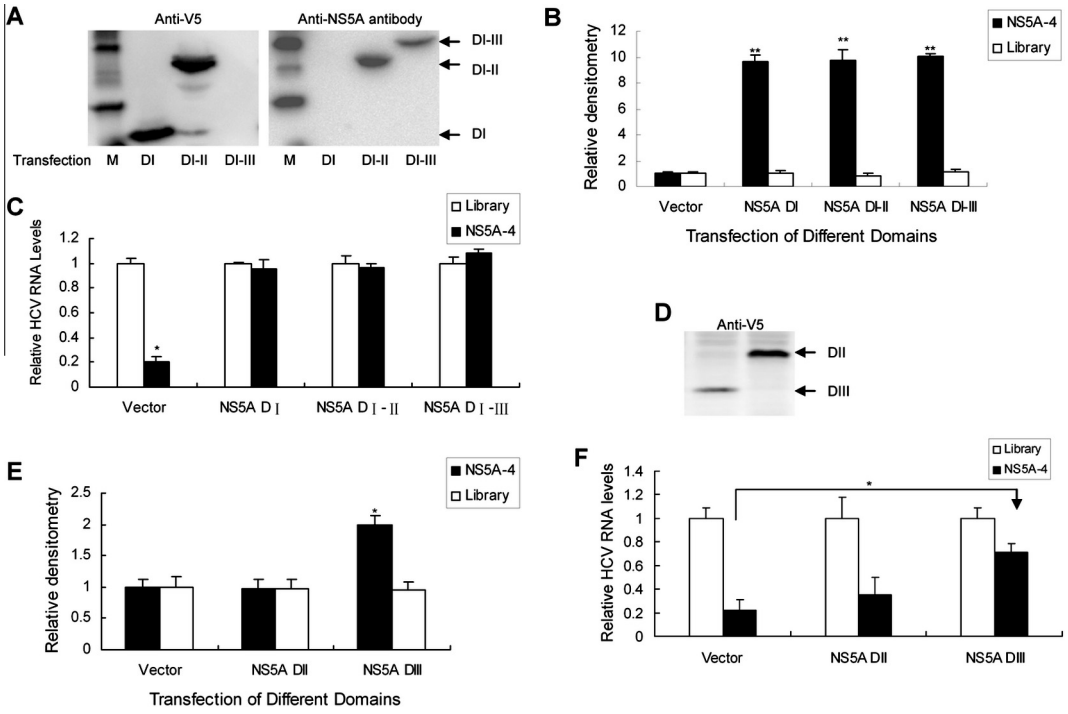

Fig. 5. Domains I and III of NS5A protein are involved in the antiviral effects of NS5A-specific aptamer NS5A-4. (A) Confirmation of the expression of different truncated versions of NS5A protein. Different truncated versions of NS5A were amplified and cloned into pEF6/V5-His-TOPO. The plasmids were delivered into Huh7.5 cells. Cellular protein was isolated and different truncated versions of NS5A were confirmed using anti-V5 or anti-NS5A antibody by western blot. (B) Binding affinity of different truncated versions of NS5A to the aptamer NS5A-4. The cells were treated as described in part A. The protein was isolated from the cells and binding affinity of the truncated versions of NS5A to aptamer NS5A-4 was determined by ELONA. The vector pEF6/V5-His-TOPO was used as a control. (C) Domain I of NS5A protein is involved in the inhibition of HCV infection by NS5A-4. JFH1 virus was used to infect the naïve Huh7.5 cells, followed by transfection with plasmid expressing different truncated versions of NS5A. The cells were treated by NS5A-4 or library for 72 h. Intracellular viral RNA was determined by real-time PCR and normalized with GAPDH. (D) Confirmation of the expression of domains II and III of NS5A protein. Domain II or domain III of NS5A was cloned into pEF6/V5-His-TOPO and the plasmids were delivered into Huh7.5 cells. The expression of domains II and III of NS5A was confirmed by western blot using anti-V5 antibody. (E) Binding affinity of domain II or domain III of NS5A to the aptamer NS5A-4. The cells were treated as described in part D. The protein was isolated from the cells and the binding affinity of different domains of NS5A to aptamer NS5A-4 was determined by ELONA. The vector was used as a control. (F) Domain III of NS5A protein is involved in the antiviral effect by NS5A-4. JFH1 virus was used to infect the naïve Huh7.5 cells, followed by transfection with plasmid containing domain II or III of NS5A. The cells were treated by NS5A-4 or library for 72 h. Intracellular viral RNA was determined by real-time PCR and normalized with GAPDH. If not stated otherwise bar graphs represent means of three independent experiments. $^{*}P < 0.05$, $^{**}P < 0.01$ verse vector-transfected cells.

of NS5A. Then the cells were treated by NS5A-4 aptamer or control library. As shown in Fig. 5C, HCV RNA level in the cells transfected with plasmid containing domain I of NS5A was markedly higher than in control cells. Taken together, the data suggest that domain I of NS5A protein may be involved in the antiviral effects of aptamer NS5A-4. To test whether domain II or III of NS5A is essential for the inhibition of HCV infection by NS5A-4, we performed similar experiments. The domain II or III of NS5A was cloned and the expression of domains II and III of NS5A protein could be confirmed using Western blot (Fig. 5D). NS5A-4 bound to the domain III of NS5A (Fig. 5E). The competition experiments showed that viral RNA level was higher in cells transfected with plasmid containing domain III of NS5A than in control cells (Fig. 5F). Taken together, the data suggest that domains I and III of NS5A protein may be involved in the antiviral effects of aptamer NS5A-4.

3.6. NS5A aptamer disrupts the interaction of NS5A with core protein

Interaction of NS5A with core protein is essential for infectious virus production (Masaki et al., 2008). Disruption the interaction of NS5A with core protein impairs infectious virus production (Jiang and Luo, 2012). To prove this, we analyzed the interaction of NS5A with core protein in intact cells by Co-IP experiments. The amounts of NS5A or core protein were comparable in aptamer-treated or library-treated cells (Fig. 6A). Core protein in immunoprecipitates with equal amount of NS5A protein in aptamer NS5A-4 or NS5A-5-treated HCV-infected Huh7.5 cells was lower than that in library-treated group (Fig. 6B). The results showed that NS5A-specific aptamer disrupted the interaction of NS5A with core protein. All the data indicate that NS5A-specific aptamers may inhibit HCV infection through disrupting the interaction of NS5A with core protein.

3.7. Isolation and characterization of JFH1-resistant variants

Viral target-based inhibitor allows for the selection of resistant viruses. To identify amino acid mutations conferring resistance to NS5A aptamer, JFH1-infected Huh7.5 cells were treated with 100 nM NS5A-4 or library for 3 weeks. Mutations within NS5A associated with reduced susceptibility to NS5A-4 were selected. Substitution at NS5A residue 2105 (Y2105H substitution) was identified. The aptamer NS5A-4 showed high binding affinity for wild type NS5A protein in the lysates of HCV-infected Huh7.5 cells while it displayed no apparent binding affinity for mutated Y2105H NS5A protein (Fig. 7A).

To evaluate the contribution of the selected specific amino acid substitution to resistance, the Y2105H substitution was introduction into JFH1. The sensitivity of the variant to NS5A-4 was assessed in the infectious cell culture system. Y2105H substitution resulted in decrease in NS5A-4 potency (Fig. 7B and C). There was no apparent difference of infectivity of Y2105H mutant compared to the wild type (Fig. 7B and C). The data suggested that selected Y2105H substitution within NS5A is the major resistance substitution identified.

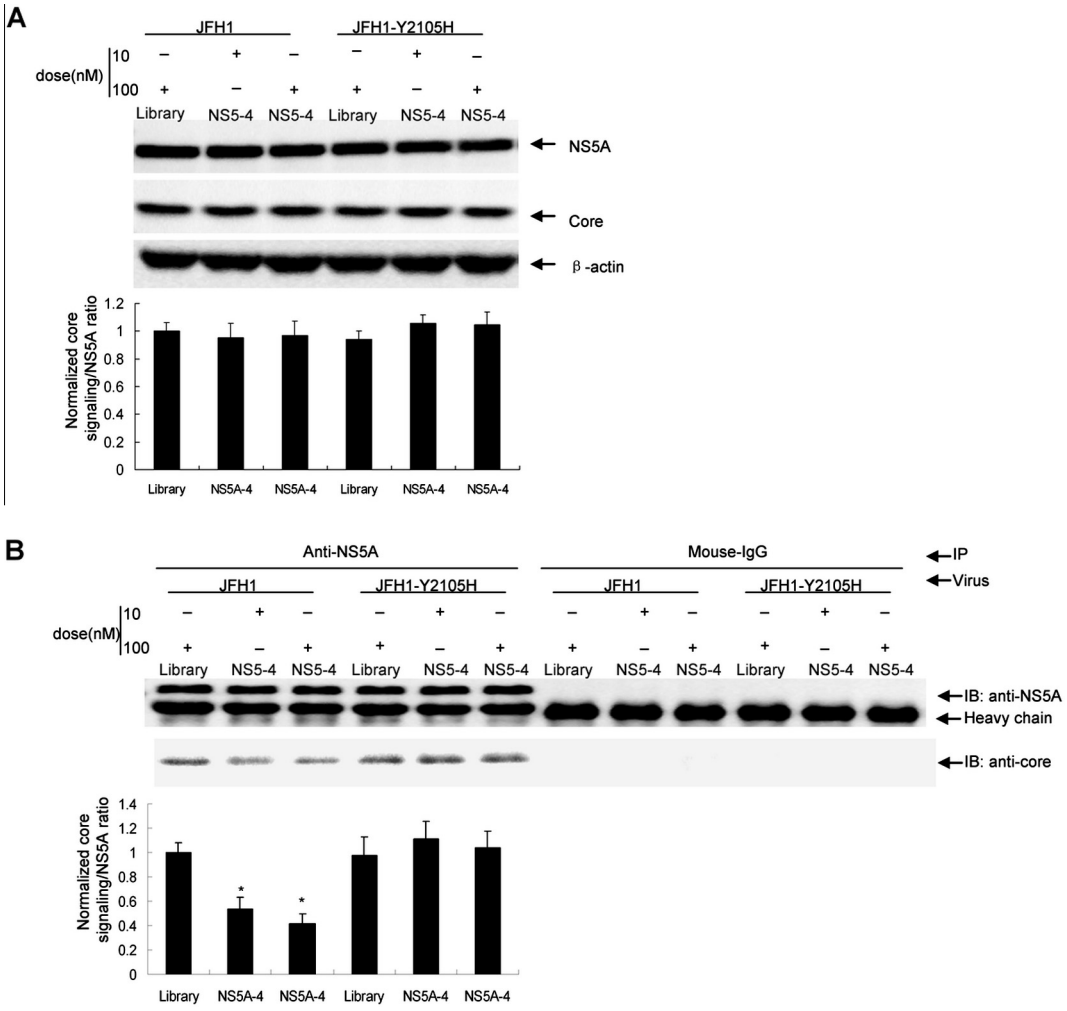

Fig. 6. NS5A aptamer disrupts the interaction of NS5A with core protein. (A) Protein was isolated from the HCV-infected Huh7.5 cells with aptamer or library treatment. The whole cell lysates were used as input control. The input NS5A or core protein was detected by Western blot using anti-NS5A or anti-core antibody, respectively. Core protein was quantified by densitometry in comparison with NS5A protein. (B) Effect of aptamer NS5A-4 on the interaction of NS5A with core protein. Protein was isolated from the HCV-infected Huh7.5 cells with aptamer or library treatment and immunoprecipitated with the antibody against NS5A conjugated with agarose beads, respectively. The protein binding to the beads was boiled and subjected to SDS–PAGE. The protein was transferred onto PVDF membrane and then reacted with primary and secondary antibodies. Core protein in the immunoprecipitates was detected with western blot and quantified by densitometry in comparison with NS5A protein in the immunoprecipitates. The data represented the means of 3 independent experiments. $^{*}P < 0.05$ verse library-treated cells.

4. Discussion

Many chronic hepatitis C patients do not response to current pegylated IFN-α-based therapy. To increase the efficacy of treatment, future regiments will incorporate multiple new agents directly targeting the virus. The protease inhibitors against HCV NS34A have been recently approved by FDA for patients with HCV genotype 1 infection (Pawlotsky, 2012). There are many patients resistant to the protease inhibitor (Pawlotsky, 2012; Halfon and Locarnini, 2011). It is desired to seek combination therapy for HCV infection, which targeted different steps of virus lifecycle.

NS5A protein is a master regulator of HCV replication and assembly (Tellinghuisen et al., 2005). NS5A was initially an unpopular target because it has no enzymatic activity despite its important roles in virus lifecycle. A compound targeting NS5A has recently been reported as one of the most potent and broadly active inhibitors of viral replication observed to date (Gao et al., 2010). NS5A has become a common target for the development of anti-HCV drugs.

Here we reported the selection of aptamers for HCV NS5A protein and inhibition of HCV infection by these aptamers. This is the first report of the NS5A aptamers mediating a significant inhibition of HCV infection. The study showed that the aptamers targeting NS5A not only bided HCV NS5A protein and inhibited the infection of genotype 2, but also inhibited genotype 1 infection. The data indicate that NS5A from these two genotypes of HCV may share aptamer binding sites.

There are relatively few data on how HCV assemble and release. It has been reported that NS5A is required for both viral RNA replication and production of infectious virus, although its precise roles in HCV lifecycle are unknown. This hints at coordination between the machinery involved in making new copies of the genome RNA and the machinery packaging it into virus particles. NS5A protein is an attractive candidate for these functions. Although the aptamer NS5A-4 is found to bind NS5A protein, the detail residues involved in the interaction between NS5A-4 aptamer and its target remain to be explored. Identification of these residues may give us a clue about the essential functional regions of NS5A. Aptamers against NS5A can be used to understand the molecular mechanisms of HCV replication and assembly as well as the interaction between NS5A and host factors. The aptamer targeting HIV Gag protein is used to examine the interaction of Gag protein with

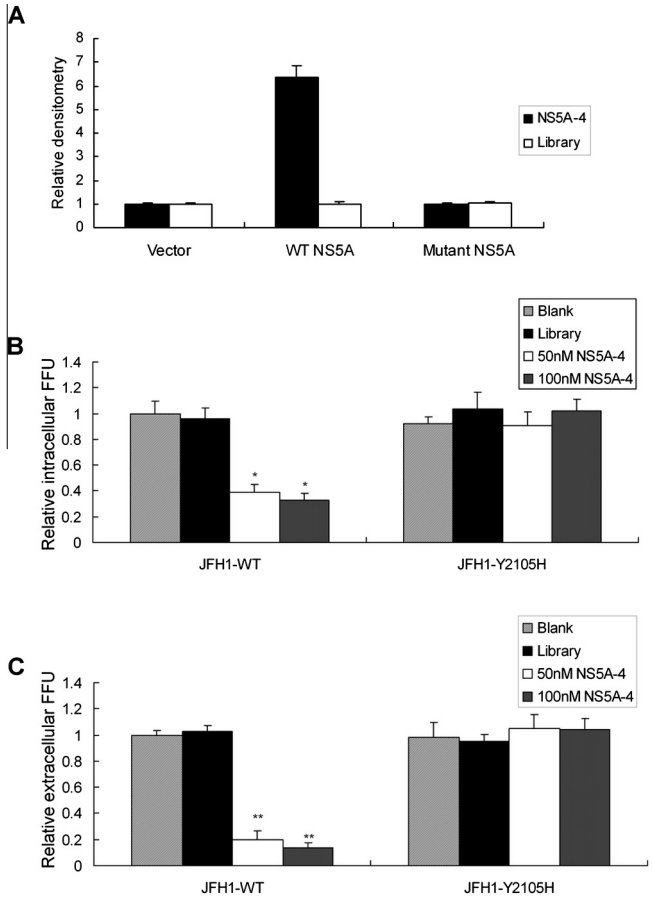

Fig. 7. Y2105H substitution in NS5A is the major selective resistance mutation identified. Selection of resistance-conferring mutations was performed. (A) Huh7.5 cells were infected with media from Huh7.5 cells transfected with in vitro transcripts RNA from wild type (WT) or selected Y2105H viral clone. The protein was isolated from the cells and the binding affinity of WT or mutant NS5A protein in the lystates to aptamer NS5A-4 was determined by ELONA. The densitometry was normalized to library. (B and C) Huh7.5 cells were infected with media from Huh7.5 cells transfected with in vitro transcripts RNA from WT or selected Y2105H viral clone, followed with NS5A-specific aptamer treatment for 72 h. The effect of aptamer NS5A-4 on the production of infectious WT or selected Y2105H mutated virus particles was tested as described in Fig. 3D. The infectivity titers inside the cells (B) and the supernatant (C) were measured by FFU assay. The data were normalized to WT virus-infected cells and represented three independent experiments. $^{*}P < 0.05$, $^{**}P < 0.01$ verse control cells.

HIV RNA (Ramalingam et al., 2011). The aptamers for HIV reverse transcriptase provide a useful tool for the study of the mechanisms of how these aptamers act as broad-spectrum inhibitors of reverse transcriptase (Ditzler et al., 2011). All the examples illustrate the potential use of aptamers in the studies of virus lifecycle and exploring the interaction between virus and host factors. Continue research will aim to elucidate the modes of action of NS5A aptamers to further our understanding the functions of NS5A and to develop novel NS5A inhibitors with a higher genetic barrier to resistance.

In the present study, we demonstrated that the aptamers against NS5A protein disrupt the interaction of NS5A with core protein. Further study will be required to dissect the complex interplay between viral RNA replication and virion assembly and to disclose the multiple roles of NS5A using the aptamers against this protein.

The antiviral activities of aptamer might be due to the aptamer-induced innate immune response (Hwang et al., 2012). It is one exceptional example because RIG-I aptamer was designed to have specific motifs to bind and activate RIG-I in 5′-triphosphate-independent manner. Several studies suggest that inhibition of virus infection by aptamers targeting viral proteins is due to suppression of the activities of viral proteins by the aptamers (Bentham et al., 2012; Moore et al., 2011; Wheeler et al., 2011). In consistent with these studies, our study showed that the aptamers for NS5A do not induce IFN-β and ISGs and supported that inhibition of HCV infection by aptamers for NS5A is not due to the innate immunity.

In summary, our study provides the first evidence of direct antiviral activity of aptamer against NS5A in infectious cell culture system. The data demonstrate that the aptamers against NS5A protein exert antiviral effects through inhibiting viral RNA replication and disrupting the interaction of NS5A with core protein. Aptamers for NS5A may be used to understand the mechanisms of viral replication and assembly and served as potential therapeutic agents for HCV infection.

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