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Generation of hepatitis C virus–resistant liver cells by genome editing–mediated stable expression of RNA aptamer

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Hepatitis C virus (HCV) infections frequently recur after liver transplantation in patients with HCV-related liver diseases. Approximately 30% of these patients progress to cirrhosis within 5 years after surgery. In this study, we proposed an effective therapeutic strategy to overcome the recurrence of HCV. CRISPR-Cas9 was used to insert an expression cassette encoding an RNA aptamer targeting HCV NS5B replicase as an anti-HCV agent into adeno-associated virus integration site 1 (AAVS1), known as a "safe harbor," in a hepatocellular carcinoma cell line to confer resistance to HCV. The RNA aptamer expression system based on a dihydrofolate reductase minigene was precisely knocked in into AAVS1, leading to the stable expression of aptamer RNA in the developed cell line. HCV replication was effectively inhibited at both the RNA and protein levels in cells transfected with HCV RNA or infected with HCV. RNA immunoprecipitation and competition experiments strongly suggested that this HCV inhibition was due to the RNA aptamer–mediated sequestration of HCV NS5B. No off-target insertion of the RNA aptamer expression construct was observed. The findings suggest that HCV-resistant liver cells produced by genome editing technology could be used as a new alternative in the development of a treatment for HCV-induced liver diseases.

INTRODUCTION

Hepatitis C virus (HCV) is a small, enveloped virus of the family *Flaviviridae*. It has positive-sense single-stranded RNA as a genome. HCV can cause hepatitis C, liver fibrosis, cirrhosis, and liver cancer.[1] Thus, HCV infection is a major global public health concern. Newly developed treatments using pan-genotypic direct-acting antivirals have short duration (12–24 weeks), few side effects, and a 90% cure rate regardless of HCV genotype.[2] However, these treatments render patients susceptible to new resistant viruses. Approximately 70% of patients infected with HCV will progress to chronic hepatitis, about 50% of which will develop liver fibrosis and liver cirrhosis within 20–50 years, and this will ultimately lead to liver cancer.[3] Therefore, liver transplantation (LT) is performed to treat patients in the last stage of liver disease caused by HCV. However, HCV recurrence is frequently encountered after LT. Approximately 30% of patients will progress to cirrhosis within 5 years after surgery.[4,5] The current treatments to prevent these phenomena are insufficient.

Genome editing technology involves the use of CRISPR-CRISPR-associated (Cas) proteins, zinc-finger nucleases, and transcription activator–like effector nucleases to precisely manipulate specific genomic sequences.[6] This technology allows various manipulations, such as deleting a specific DNA or RNA sequence of the genomic locus or adding a specific sequence to a DNA locus.[7] The CRISPR-Cas system derived from *Streptococcus pyogenes* is the most commonly used system for gene editing.[8,9] This system can induce RNA-guided DNA cleavage using Cas9 endonuclease and single-guide RNA (sgRNA). sgRNA can target anywhere on the genome by Watson-Crick base pairing with a target sequence, which can induce a Cas9-mediated DNA double-strand break (DSB).[10,11] Cleaved DNA is repaired through nonhomologous end joining or homology-directed repair (HDR). The CRISPR-Cas9 system is easy to use because it can be applied to replace only the gRNA without having to create a new DNA binding domain each time.[6,7] Accordingly, the simplicity and flexibility of this technique offer many advantages for targeting various diseases.[12]

Aptamers are small single-stranded oligonucleotides that can form a three-dimensional shape and specifically bind to target molecules.[13,14] Aptamers are developed by the systematic evolution of ligands by exponential enrichment. Aptamers have many advantages clinically, including high affinity and specificity through the formation of their three-dimensional structure,[15,16] ease of synthesis by chemical methods, pharmaceutical flexibility, and poor immunogenicity.[17,18] Previously, we developed RNA aptamers with 2′-hydroxyl nucleotides specific to the HCV NS5B replicase of both genotypes 1b and 2a.[19] These RNA aptamers effectively inhibited HCV replication in cell culture without exhibiting cellular toxicity. They also suppressed

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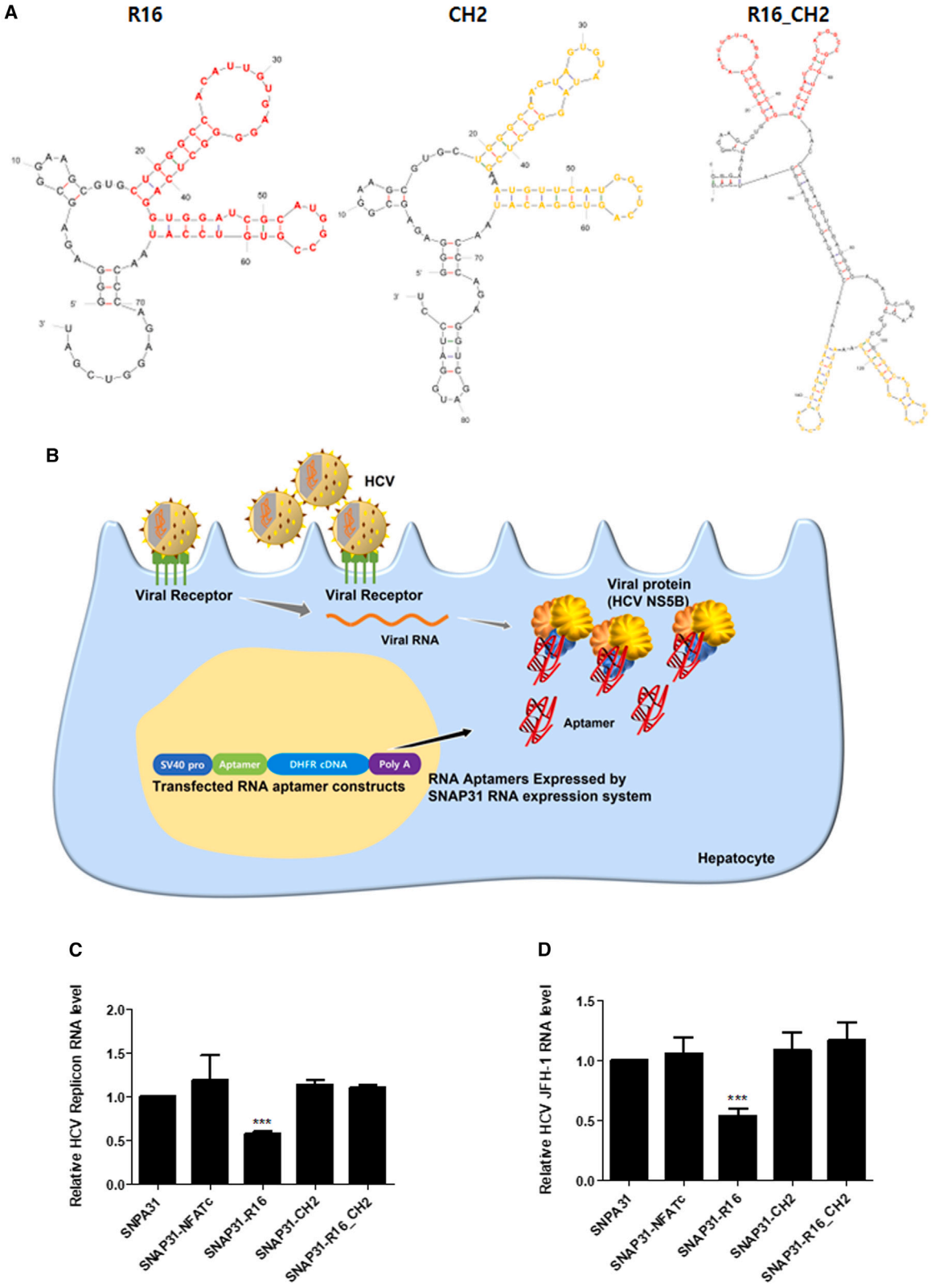

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HCV infectious virus particle formation without generating escape mutants of the viruses.

In this study, we proposed an effective therapeutic strategy to prevent the recurrence of HCV after LT. To this end, we established HCV-resistant hepatocellular carcinoma (HCC) cell lines by inserting an expression construct encoding an RNA aptamer against HCV NS5B replicase into adeno-associated virus integration site 1 (AAVS1) known as a "safe harbor" using a CRISPR-Cas9 system.[20,21] We used a dihydrofolate reductase (DHFR) minigene expression system as a cellular RNA expression system called SNAP31.[22] This system is based on RNA polymerase II (RNA Pol II). When the DHFR minigene is transduced into a cell, its short-size spliced transcripts are overexpressed in the cytoplasm. The fidelity and presence of off-targets of the insertion site and expression of the RNA aptamer were analyzed through whole-genome sequencing (WGS) and RT-PCR analysis, respectively, in the established cells. The anti-HCV efficacy was assessed using qRT-PCR, western blotting, and resistant HCV colony formation analysis after transfecting cells with HCV genomic RNA or infecting cells with HCV particles. RNA immunoprecipitation (RIP) and competition assay to analyze competitive aptamer binding to NS5B were performed to determine whether HCV inhibition was due to a decoy effect of the RNA aptamer.

RESULTS

Validation of RNA aptamer efficacy in HCV stable cells

Regarding anti-HCV reagents, we selected and validated the antiviral efficacy of the previously developed RNA aptamers CH2 and R16, which could specifically bind to HCV NS3 helicase and NS5B replicase, respectively.[19,23] These aptamers were observed to effectively inhibit replication of the viral genome. In particular, R16 effectively inhibited the replication of both HCV genotypes 1b and 2a in cells.[19] We also conjugated HCV NS3 and NS5B aptamers in a chimeric molecule (R16_CH2) to test whether the antiviral effect could be enhanced further in the cells. The secondary structures of each aptamer were checked with the Mulfold program (Figure 1A).[24] The SNAP31 system,[22] an RNA Pol II–based expression system, was used as an aptamer expression system to target HCV replication in the cytoplasm (Figure 1B). The antiviral efficacy of each aptamer was measured by transiently transfecting each construct encoding aptamers into Huh-7.5 cells supporting the stable replication of the HCV 1b subgenome or JFH-1 genome (Figures 1C and 1D). In the cell experiment, SNAP31-R16 showed 50% inhibition of HCV replication, whereas other aptamers had minimal effects. These results indicated that additional modification was required for the aptamer to effectively inhibit viral replication in HCV stable cells.

Modification of HCV NS5B-specific RNA aptamer and development of RNA aptamer stable cell lines

The expression level of the NS5B-specific aptamer was increased by cloning and expressing more than one copy of the aptamer sequence into one expression vector to increase anti-HCV efficiency of the aptamer in the cells. However, the NS3-specific RNA aptamer was excluded due to ineffectiveness and concerns about escape mutants.[25,26] The NS5B-specific R16 aptamer was minimized to a length of 70 bp. One copy (R70) or three or five consecutive copies (R70 ×3 or R70 ×5) of the minimized sequence were then cloned into a single expression cassette of the SNAP31 RNA expression vector. Each aptamer was expected to maintain its secondary structure based on the Mulfold program (Figure 2A).[24] Stably aptamer-expressing cell lines were developed by transfecting each expression construct into Huh-7.5, an HCC cell line capable of being infected with HCV, to examine the anti-HCV effect of each construct. As control, SNAP31 stably expressing the nuclear factor of activated T cells (NFATc) aptamer[27] was developed. A pool for each cell line was obtained, and aptamer expression was confirmed through RT-PCR analysis (Figure 2B). Resistance to HCV was verified in the stable cell line established for each aptamer construct.

Anti-HCV effect of HCV-specific RNA aptamer stable cell lines

HCV genotype 1b subgenomic replicon RNA and genotype 2a JFH-1 RNA were transfected into each cell line. HCV replication was effectively inhibited in cells stably expressing HCV NS5B-specific RNA aptamers R16, R70, R70 ×3, or R70 ×5 (Figures 3A and 3B). In contrast, no HCV inhibitory effect was observed in cells stably expressing NFATc or cells stably expressing R16_CH2. In addition, each established cell was infected with HCV genotype 2a JFH-1 virus. The cells showed effective resistance to HCV replication when they were infected with the JFH-1 virus at 0.1 or 0.5 MOI (Figures 3C and 3D). In contrast, cells stably expressing NFATc or R16_CH2 did not show resistance to HCV replication, similar to the results after transfection with HCV 1b subgenomic RNA or 2a JFH-1 RNA. These results indicated that stably NS5B aptamer–expressing cells acquired resistance to HCV.

A colony-forming assay was performed to determine whether the developed cell lines could induce drug-resistant escape viruses. The HCV 1b subgenomic replicon harbored a neomycin resistance gene,[28] and SNAP31 constructs contained a puromycin resistance gene. Cells stably expressing aptamers were transfected with HCV 1b subgenomic replicon RNA and selected using both G418 and puromycin. Of note, G418 and puromycin double selection almost completely inhibited colony formation in cells expressing HCV NS5B-specific RNA aptamers R70, R70 ×3, or R70 ×5 (Figure 3E).

Figure 1. Characterization of HCV-specific RNA aptamers and anti-HCV efficiency of RNA aptamer in HCV stable cells

(A) Secondary structure of aptamer R16, CH2, and R16_CH2. R16 is an HCV NS5B replicase-specific RNA aptamer. CH2 is an HCV NS3 helicase-specific RNA aptamer. R16_CH2 is a conjugated chimeric aptamer of R16 and CH2. The red color represents the predicted binding region of R16. The yellow color represents the predicted binding region of CH2. (B) Schematic diagram of the SNAP31 RNA expression system with HCV-specific RNA aptamers in a cellular model. (C and D) HCV genotype 1b subgenomic replicon cells (C) or HCV genotype 2a JFH-1 stable cells (D) were transfected with each SNAP31-aptamer construct. HCV RNA was assessed by qRT-PCR and expressed relative to the level in cells transfected with the SNAP31 vector. The results are presented as means ± SDs of triplicate measurements. ***$p < 0.0005$.

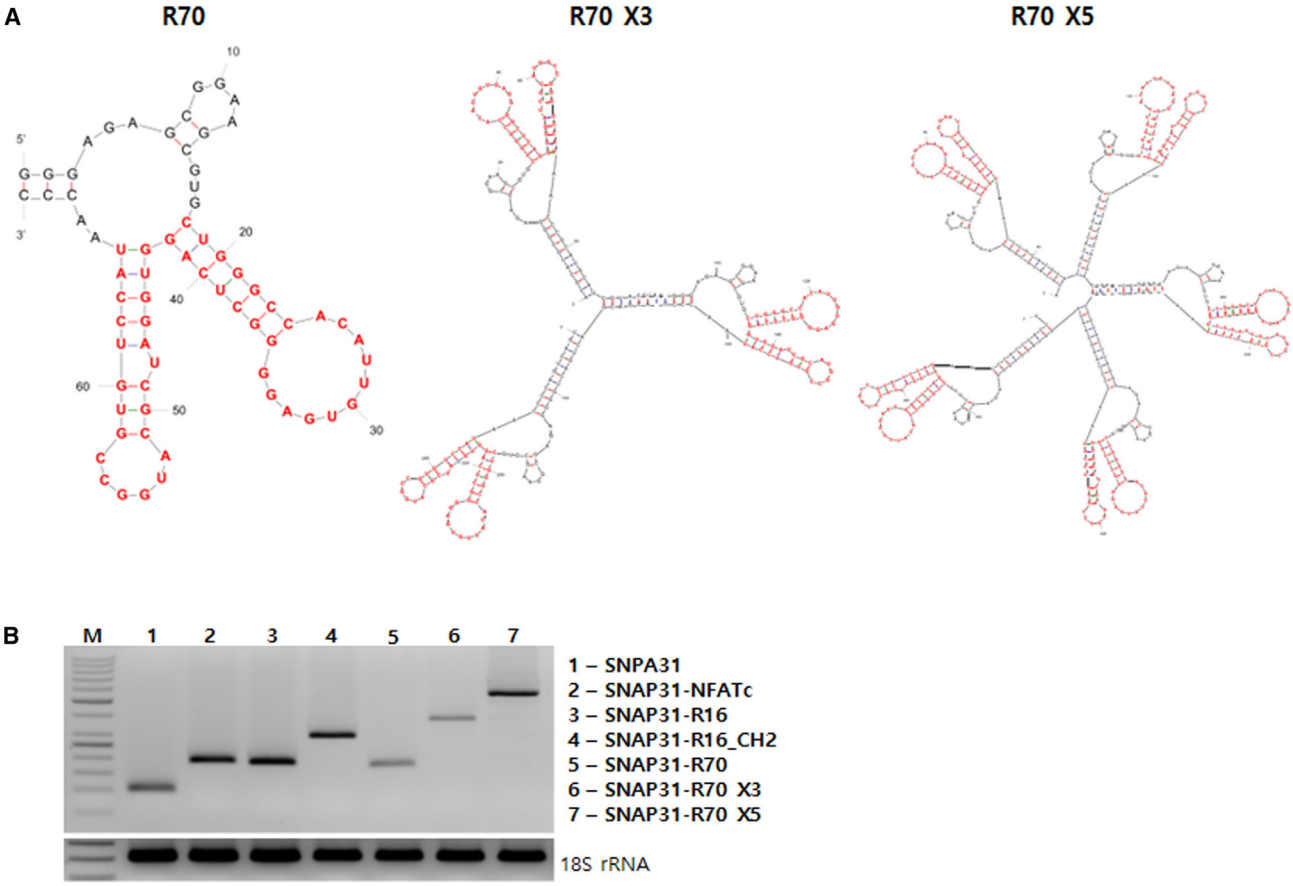

Figure 2. Characterization of HCV NS5B-specific RNA aptamers and development of aptamer stable cells

(A) Secondary structures of R70, R70 ×3, and R70 ×5. R70 is an HCV NS5B-specific RNA aptamer, which was a minimized form of R16. R70 ×3 is a conjugated form of three copies of R70. R70 ×5 is a conjugated form of five copies of R70. The sequences with red color represent the predicted binding region of R70. (B) The expression of RNA aptamer in each developed RNA aptamer stable cell was assessed by RT-PCR and 2% agarose gel electrophoresis. M indicates the DNA size marker. Human 18S rRNA was amplified as the internal control.

In contrast, cells with SNAP31 and cells stably expressing the NFATc aptamer or R16_CH2 generated numerous colonies that supported HCV replication. These results indicate that the stable expression of the NS5B aptamer could inhibit HCV replicon replication with the least generation of escape mutant clones.

Development of HCV-specific RNA aptamer stable cell lines using CRISPR-Cas9 system

An aptamer stable cell line was constructed by knocking in the SNAP31 RNA expression system into AAVS1 using the CRISPR-Cas9 system. AAVS1 is located on chromosome 19, where the protein phosphatase 1 regulation subunit 12c (PPP1R12C) gene is encoded. The site is known as a safe harbor. Thus, gene integration into this site is stable without side effects, even if a specific construct is knocked in.[20,21] Intron 1 region of the PPP1R12C gene was selected as a target, and three sgRNAs targeting this site were designed (Figures 4A and 4B).[29] The T7 endonuclease 1 (T7E1) assay was performed to analyze the genome editing efficiency of each sgRNA (Figure 4B), and T2 sgRNA with the AAVS1 T2 protospacer was observed to be the most efficient. Hence, it was used for knocking in the aptamer constructs.

We co-transfected each SNAP31 construct encoding the aptamer with sgRNA and Cas9 in plasmid form into Huh-7.5 to develop a cell line in which the aptamer was knocked in to AAVS1. An HDR donor vector for each aptamer construct was also cotransfected into Huh-7.5. Donor vectors have specific arms homologous to AAVS1. Therefore, HDR events following a CRISPR-Cas9-induced DSB will knock-in aptamer constructs at AAVS1. Because the SNAP31-aptamer constructs had a puromycin resistance gene, each cell clone was obtained through puromycin selection. RT-PCR analysis was performed to determine whether the aptamer was properly expressed in the obtained cell clone (Figure 4C). The RT-PCR results showed that the aptamers were well expressed in several cell clones. These cell clones expressing aptamers were subsequently subjected to genomic DNA-PCR to confirm AAVS1 knockin. If knockin occurred in AAVS1, a 4.3-kb band should appear. If not, a 2-kb band should appear. In addition, if the knockin was in the

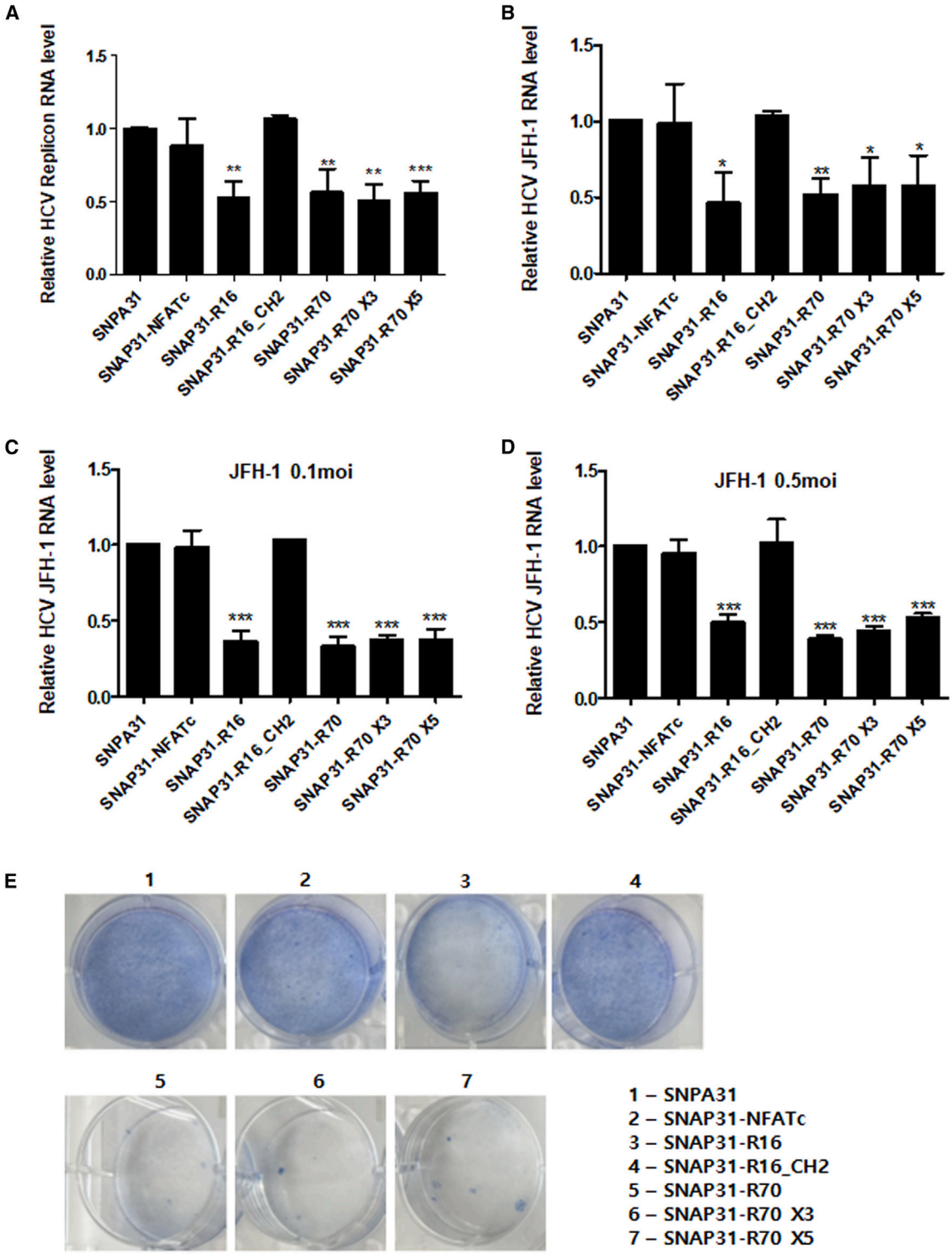

Figure 3. HCV inhibitory effect of the developed RNA aptamer stable cells

(A and B) HCV genotype 1b subgenomic replicon RNA (A) or HCV genotype 2A JFH-1 RNA (B) was transfected into each aptamer stable cell line. (C and D) HCV genotype 2A JFH-1 virus was used for infection at an MOI of 0.1 (C) or 0.5 (D) into each aptamer stable cell line. HCV genomic RNA levels were assessed by qRT-PCR and expressed relative to the level in SNAP31 stable cells. The results are presented as means ± SDs of triplicate measurements. *$p < 0.05$; **$p < 0.005$; ***$p < 0.0005$. (E) Each aptamer stable cell was transfected with HCV genotype 1b subgenomic replicon RNA and selected with puromycin and G418. Colony formation is shown.

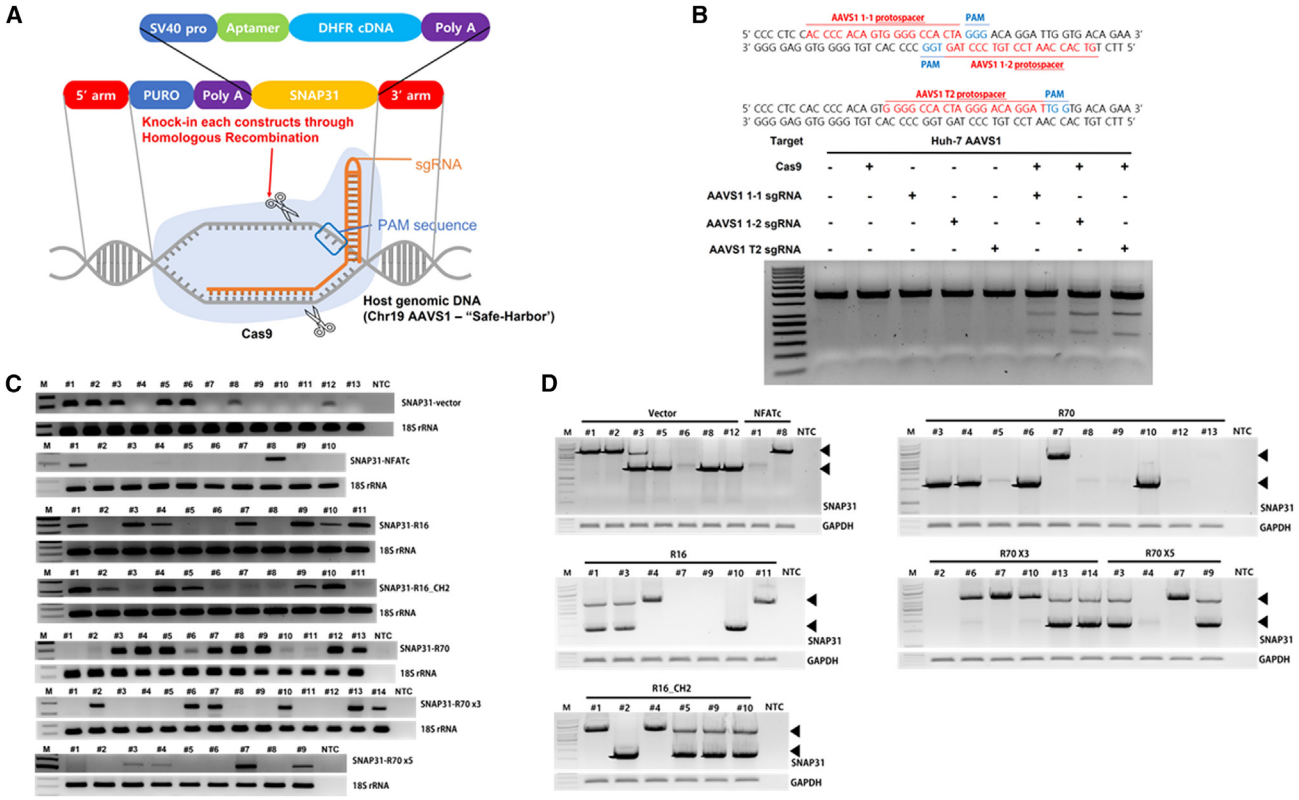

Figure 4. Development of AAVS1 HCV-specific RNA aptamer stable cells using the CRISPR-Cas9 system

(A) Schematic diagram of the SNAP31 construct encoding an HCV-specific RNA aptamer with a DHFR minigene and knockin of the construct into AAVS1 through HDR. (B) The sequence of AAVS1 sites targeted by sgRNAs (top) and the T7 endonuclease 1 (T7E1) assay with each sgRNA (bottom). (C) The expression of RNA aptamer was assessed by RT-PCR in each AAVS1 HCV-specific RNA aptamer knockin cell clone and 2% agarose gel electrophoresis. The numbers represent clone numbers for each aptamer stable cell. M indicates the DNA size marker. NTC denotes the PCR controls. Human 18S rRNA was amplified as the internal control. (D) Genomic DNA was assessed in each AAVS1 knockin cell clone expressing HCV-specific RNA aptamers by PCR and 1% agarose gel electrophoresis. The numbers represent clone numbers for each aptamer stable cell. The expected band size is indicated by an arrow. M indicates the DNA size marker. NTC denotes PCR controls. Human glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was amplified as the internal control.

homozygote form (i.e., knockin of two copies of a construct), only one PCR band with a 4.3-kb length would be observed. In contrast, if it was in the heterozygote form (i.e., knockin of one copy of a construct), two PCR bands with lengths of 2 and 4.3 kb would be observed. The result showed that the aptamer construct was knocked in in either a heterozygote or a homozygote form in several cell clones (Figure 4D). Among the developed cell clones, cell clones with homozygote aptamer knockins showed higher expression levels of the aptamer. A cell proliferation assay showed no difference in these cell clones compared to naive cells (Figure S1). Established cell clones with homozygote aptamer construct knockins into AAVS1 were then tested to verify resistance to HCV.

HCV resistance of AAVS1 HCV-specific RNA aptamer knockin cell clones

We transfected HCV genotype 1b subgenomic replicon RNA or genotype 2a JFH-1 RNA into each RNA aptamer cell clone knocked in into AAVS1 to test its resistance to HCV. Each cell clone stably expressing HCV NS5B-specific RNA aptamers R16, R70, R70 ×3, or R70 ×5 effectively inhibited HCV replication at the RNA level (Figures 5A and 5B). In contrast, no HCV inhibitory effect was observed in cell clones expressing NFATc or R16_CH2 aptamers. In addition, each cell clone was infected with increasing doses of HCV genotype 2a JFH-1 virus. Cell clones expressing HCV NS5B-specific RNA aptamers showed effective resistance to HCV replication at the RNA level except for cell clones expressing NFATc or R16_CH2 aptamers (Figures 5C and 5D). Moreover, the cell clone stably expressing HCV NS5B-specific RNA aptamers R70, R70 ×3, or R70 ×5 effectively inhibited HCV replication at the protein level when HCV genotype 1b subgenomic RNA and genotype 2a JFH-1 RNA were transfected (Figures 5E and 5F). These results indicate that HCV NS5B-specific RNA aptamers stably expressed in AAVS1 of each cell clone could confer cells with HCV replication resistance.

An RNA immunoprecipitation (RIP) analysis was performed to determine whether HCV resistance in the developed cell clones resulted from an interaction between the aptamer and HCV NS5B. Aptamers were immunoprecipitated with HCV NS5B in cell clones stably expressing HCV NS5B-specific RNA aptamers R16, R70, R70 ×3, or R70 ×5 after cells were transfected with HCV genotype 1b

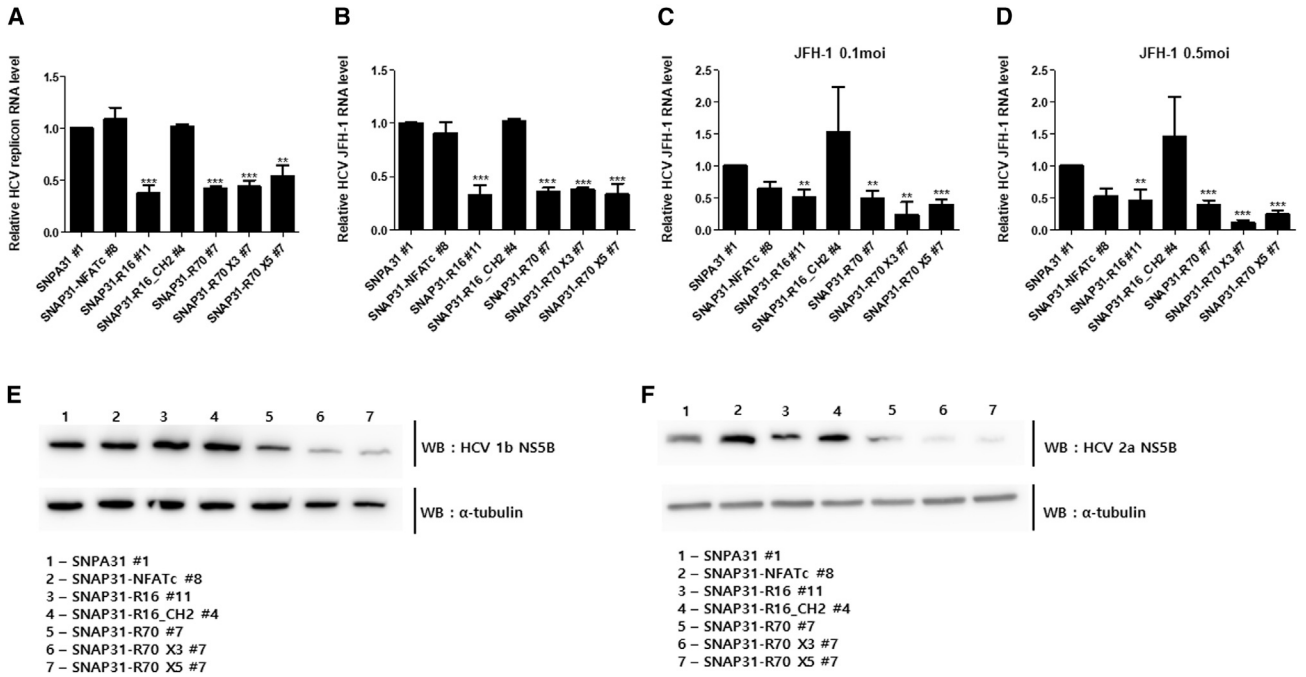

Figure 5. HCV inhibitory effect of developed AAVS1 RNA aptamer knockin cell clones

(A–D) HCV genotype 1b subgenomic replicon RNA (A) or HCV genotype 2A JFH-1 RNA (B) was transfected into each AAVS1 aptamer stable cell clone. HCV genotype 2A JFH-1 virus was used for infection at an MOI of 0.1 (C) and 0.5 (D) into each AAVS1 aptamer stable cell clone. HCV genomic RNA level was assessed by qRT-PCR and expressed relative to the level in the AAVS1 SNAP31 knockin cell clone. The results are presented as means ± SDs of triplicate measurements. $^{**}p < 0.005$; $^{***}p < 0.0005$. (E and F) HCV genotype 1b subgenomic replicon RNA (E) or HCV genotype 2A JFH-1 RNA (F) was transfected into each AAVS1 aptamer stable cell clone. The NS5B protein level of each cell clone was measured by immunoblotting. α-Tubulin was immunoblotted as the internal control. WB, western blot.

subgenomic replicon RNA or genotype 2a JFH-1 RNA (Figures 6A and 6B). In qRT-PCR analysis, R70 ×3 exhibited a more pronounced inhibitory effect on HCV 2a RNA than either R70 or R70 ×5 (Figure 5). In contrast, R70 ×3 showed an inhibitory effect on HCV 1b RNA that was similar to that of both R70 and R70 ×5. Accordingly, the RIP analysis results indicated that R70 ×3 showed more binding to HCV 2a NS5B than 1b NS5B. Of note, no RNA IP with HCV NS5B was observed in cell clones expressing NFATc or R16_CH2 aptamers. The results indicated that the developed cell clones became resistant to HCV replication through the specific RNA aptamer-mediated sequestration of NS5B protein. We performed competition assays for the aptamer by cotransfecting HCV RNA with plasmids expressing HCV NS3 or HCV NS5B into each of the aptamer stable cells to confirm further that the HCV inhibition effect of the aptamer stable cells was caused by the aptamer targeting of HCV NS5B. When HCV NS5B was overexpressed, HCV resistance of the cell clones stably expressing HCV NS5B-specific RNA aptamers R70 ×3 and R70 ×5 was competitively and efficiently reduced (Figures 6C and 6D). In contrast, the reduction of aptameric activity was not observed upon the overexpression of NS3. The results indicated that the aptamers inhibited HCV replication by specifically binding to HCV NS5B in cells.

A colony-forming assay was performed to determine whether the developed cell clones could induce drug-resistant escape viruses (Figure 6E). Each cell clone was transfected with HCV genotype 1b subgenomic replicon RNA and selected using G418 and puromycin. G418 and puromycin double selection efficiently inhibited colony formation in cell clones expressing HCV NS5B-specific RNA aptamers. In contrast, cell clones with SNAP31 or cell clones stably expressing the control NFATc aptamer or R16_CH2 generated more colony formation, indicating efficient HCV replication. Cell clones expressing R70 ×3 or R70 ×5 showed almost complete inhibition of colony formation, which was more efficient than the established pools of cells shown in Figure 3E. These results indicate that the stable expression of NS5B RNA aptamer in AAVS1 could inhibit HCV replicon replication and generate the fewest escape mutant clones.

Off-target analysis of AAVS1 HCV-specific RNA aptamer knockin cell clones

We performed WGS to determine whether each developed cell clone was affected by a potential off-target effect of the CRISPR-Cas9 system. WGS was conducted on AAVS1 knockin cell clones stably expressing HCV NS5B-specific RNA aptamers R70 ×3 and R70 ×5, which showed the highest inhibitory effect on HCV replication. An off-target search was performed through Cas-OFFinder.[30] Targeted sites that differed from the target sequence of sgRNA by one to three sequences were analyzed. No change was found in a total of 21 potential off-target sites analyzed in the developed cell clones, compared to naive cells (Table 1; and Figure S2). Therefore, the CRIPSR-Cas9 system did not induce any off-target effects in developing cell clones

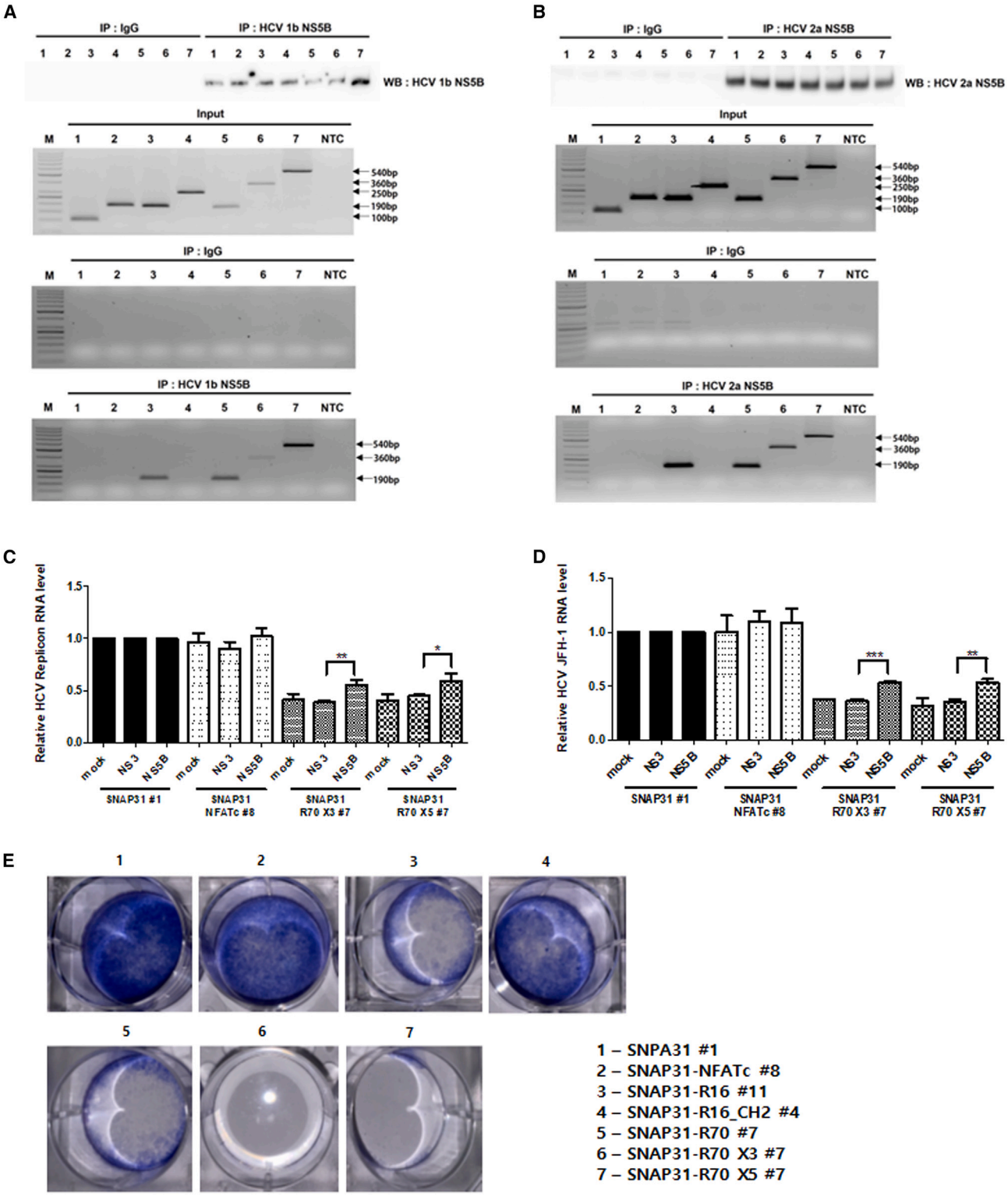

Figure 6. Inhibitory mechanism of RNA aptamer of each AAVS1 knockin cell clone

(A and B) HCV genotype 1b subgenomic replicon RNA (A) or HCV genotype 2A JFH-1 RNA (B) was transfected into each AAVS1 aptamer stable cell clone, and RNA IP was performed for HCV RNA-treated cells. NS5B proteins in cell extracts after IP with IgG or NS5B antibody were assessed by immunoblotting (top). RNA was amplified by RT-

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stably expressing NS5B RNA aptamers in AAVS1. The results indicate that these developed cell clones could accurately and safely express HCV NS5B-specific RNA aptamers in AAVS1 and that the expressed aptamers could effectively induce resistance to HCV.

DISCUSSION

Considering the rapid progression of HCV infection in LT patients and the lack of donated organs, it is necessary to develop effective treatments that can prevent liver disease caused by HCV recurrence after LT. In this study, we proposed an effective treatment for HCV recurrence after LT. To this end, we developed HCV-resistant liver cells by safely and stably integrating a construct encoding an HCV NS5B-specific RNA aptamer (Figure 7). The Huh-7.5 HCC cell line capable of being infected with HCV[31,32] was used as a model system. Drug-resistant HCV could emerge from a stable but heterogeneous pool of cells, each with different aptamer expression levels. Homogeneous and strong expression is especially important for antiviral applications because cells with weak expression of the aptamer can become places for viruses to hide and develop resistance. This study stably integrated the expression cassette of the aptamer into the safe harbor AAVS1 locus[20,21] using genome editing tools to achieve more homogeneous expression among knockin cells, unlike other gene delivery methods, including lentivirus, which could place the expression cassette in the wrong loci and possibly induce weak expression in the cells. The CRISPR-Cas9 system was used to knock-in the expression cassette for HCV NS5B replicase-specific RNA aptamers into the AAVS1 site of Huh-7.5 cells. A DHFR minigene-based SNAP31 RNA expression system[22] was used for high levels of RNA aptamer expression to target HCV that replicates in the cytoplasm. The HCV inhibitory effect was more efficient in AAVS1 knocked in cells than in randomly integrated cells.

Cell clones with homozygous knockins and the stable expression of HCV NS5B-specific RNA aptamers were selected and tested for their resistance to HCV replication by the HCV challenge assay. The developed cell clones showed efficient resistance to HCV at both the RNA and protein levels. RIP analysis and competition experiments indicated that HCV resistance in the cells was most probably due to NS5B replicase sequestration by HCV NS5B-specific RNA aptamers. More important, long-term colony-forming analysis revealed that these cell clones showed the least generation of drug-resistant escape mutant virus. No off-target effect was observed in AAVS1 aptamer knockin cell clones in terms of the integration site of the RNA aptamer construct through CRISPR-Cas9-mediated genome editing. Taken together, these results indicate that the AAVS1 knockin of an anti-HCV reagent could stably, effectively, and safely confer liver cells resistant to HCV replication.

In contrast to the NS5B-specific RNA aptamer, cell clones stably expressing HCV NS3 helicase-specific RNA aptamer did not show resistance to HCV. Moreover, many drug-resistant virus clones were generated in the colony-forming assay when the HCV NS3 helicase-specific RNA aptamer was used. This could have been due to the frequent detection of adaptive mutations in the NS3 region of the HCV RNA genome,[25,26] resulting in the stable expression of the NS3 aptamer, which may have induced drug-resistant virus clones. According to RIP analysis, the chimeric aptamer in which the NS5B aptamer was linked to an NS3 aptamer failed to sequester NS5B. This could have been the main cause of inefficient resistance to HCV replication in the developed cell lines stably expressing the chimeric aptamer. However, the reason why the chimeric aptamer failed to inhibit HCV replication remains unclear. The tertiary structure of the chimeric aptamer may have been disrupted, preventing NS5B from binding to the chimeric aptamer.

In summary, in this study, we obtained cell clones in which an aptamer was stably expressed by knocking in an HCV-specific RNA aptamer construct into AAVS1 and confirming that these cell clones were resistant to HCV. Except for studies that developed cells resistant to the human immunodeficiency virus (HIV) by modifying T cells using genome editing tools,[33,34] we believe that this was the first study to create a cell line with resistance to a specific virus by knocking in a specific antiviral reagent into a safe site using genome editing tools. It is noteworthy that none of the 21 expected off-target sites that could be identified in the cell clones showed off-target effects.

Several recent studies showed that mature hepatocytes could be differentiated and developed from stem cells, including induced pluripotent stem cells.[35,36] Although this study was conducted using an HCC cell line as a model, HCV-specific RNA aptamers could be safely knocked in to stem cells using a genome editing tool. Hepatocytes derived from stem cells could be produced and used as a source of autologous or allogenic donor liver cells that are resistant to HCV for transplantation. Cell lines that are resistant to other specific viruses could be created by this method for use in antiviral regimens.

However, there are several caveats to using genome editing tools, especially off-target effects, which may affect various stages of cell integrity.[37,38] The use of truncated and less active sgRNAs that are shortened at the 5′ end by two to three nucleotides can be used to decrease undesired mutagenesis at some off-target sites because this sgRNA structure has a higher sensitivity to mismatches than those of full length.[39] Moreover, DSB during genome editing can induce damage signals in cells.

PCR from input or immunoprecipitate using IgG or NS5B antibody in each cell extract. The expected band size is indicated by an arrow. M indicates the DNA size marker. NTC denotes PCR controls. Lane 1: SNAP31 #1 cell, lane 2: SNAP31-NFATc #8 cell, lane 3: SNAP31-R16 #11 cell, lane 4: SNAP31-R16_CH2 #4 cell, lane 5: SNAP31-R70 #7 cell, lane 6: SNAP31-R70 ×3 #7 cell, lane 7: SNAP31-R70 ×5 #7 cell. (C and D) HCV genotype 1b subgenomic replicon RNA (C) or HCV genotype 2a JFH-1 RNA (D) was cotransfected into each aptamer stable cell line with the HCV NS3 or NS5B expression plasmid. HCV genomic RNA level was assessed by qRT-PCR and expressed relative to the level in SNAP31 stable cells. The results are presented as means ± SDs of triplicate measurements. *$p < 0.05$; **$p < 0.005$; ***$p < 0.0005$. (E) Each AAVS1 aptamer stable cell clone was transfected with HCV genotype 1b subgenomic replicon RNA and selected with puromycin and G418. Colony formation is shown.

Table 1. Expected off-target site and off-target status

Chromosome	Position	Gene/region[a]	Sequence (PAM)[b]	Strand	Mismatches	InDel[c]
chr2	60,163,423	-/intergenic	5′-GGG*C*CCACTAGGG*T*CA*A*GAT (AGG)-3′	+	3	No
chr2	231,959,829	-/intergenic	5′-GGGGCCA*G*T*G*GGGACAGGA*A* (GGG)-3′	–	3	No
chr5	170,084,408	-/intergenic	5′-G*A*GGCCAC*C*AGGGACAGG*C*T (GGG)-3′	–	3	No
chr6	36,797,686	CPNE5/intron	5′-GGG*A*CCA*TC*AGGGACAGGAT (GGG)-3′	+	3	No
chr8	22,778,067	PEBP4/intron	5′-GGGGC*A*ACTAG*A*GACAGGA*A* (GGG)-3′	–	3	No
chr6	143,802,949	SCRIB/intron	5′-GG*T*GCCACTAGG*C*ACAGGA*G* (CGG)-3′	+	3	No
chr11	47,425,037	PSMC3/intron	5′-GGGGCCACTAGGGA*A*AG*AG*T (GGG)-3′	–	3	No
chr12	108,187,898	WSCD2/exon	5′-*T*GGGCCACTA*T*GGACAGGA*A* (TGG)-3′	–	3	No
chr13	36,622,469	-/intergenic	5′-GGGGCCACTAG*A*GA*AG*GGAT (GGG)-3′	+	3	No
chr12	105,960,562	-/intergenic	5′-GGGGCCA*A*T*TA*GGACAGGAT (GGG)-3′	+	3	No
chr15	25,222,747	SNHG14/intron	5′-GGG*A*CCACT*G*GG*C*ACAGGAT (CGG)-3′	–	3	No
chr15	45,535,694	HMGN2P46/intron	5′-GGGG*T*CACT*G*GGGACA*A*GAT (TGG)-3′	–	3	No
chr16	32,025,807	-/intergenic	5′-GGGGCCA*G*TAGGGA*G*AGGAT (AGG)-3′	–	2	No
chr16	32,969,368	-/intergenic	5′-GG*A*GCCA*G*TAGGGA*G*AGGAT (AGG)-3′	–	3	No
chr16	33,884,562	-/intergenic	5′-GG*A*GCCA*G*TAGGGA*G*AGGAT (AGG)-3′	+	3	No
chr19	16,064,176	-/intergenic	5′-GGGGC*TT*CTA*A*GGACAGGAT (GGG)-3′	–	3	No
chr19	34,665,010	SCGB1B2P/intron	5′-GGGGCCAC*C*AGG*T*ACA*T*GAT (GGG)-3′	–	3	No
chr20	32,447,026	NOL4L/exon	5′-GGGGCCA*G*TAGGG*G*CAGGA*C* (AGG)-3′	–	3	No
chr20	43,709,922	MYBL2/intron	5′-GGGG*GA*ACTAG*T*GACAGGAT (AGG)-3′	–	3	No
chr21	41,521,014	-/intergenic	5′-GGGGCC*C*CT*G*GGGACAG*A*AT (GGG)-3′	–	3	No
chr22	44,303,217	SHISAL1/intron	5′-GG*T*GCCAC*C*AGGGA*G*AGGAT (GGG)-3′	–	3	No

[a]Gene/region indicates the position of the expected off-target site on the chromosome by the name of each gene and region.
[b]The italic and underlined sequences indicate nucleotide mismatches with the sequence of the on-target site in each position.
[c]In the InDel section, "No" means that no change was found at each expected off-target site in AAVS1 knockin cell clones stably expressing HCV NS5B-specific RNA aptamers compared to naive cells.

Thus, cells surviving after genome editing may harbor tumorigenic potential.[40,41] Therefore, instead of the DSB approach, attempts have been made to induce more sophisticated corrections by using nickase.[42] The vector transfection and antibiotic selection methods used in this study for the selection of stable knockin cell clones are not appropriate for use in clinics. Therefore, an efficient delivery method for genome editing tools is desired.[12] Several groups have tried to deliver genome editing tools to *in vitro* or *in vivo* models using viral delivery systems.[43,44] In addition, nonviral delivery systems, such as those using polyethylene glycol (PEG)ylated liposomes, are also being developed.[45,46] Although there are challenges to overcome in using genome editing tools, the simplicity and flexibility of the technique reported here show great potential for advancement.

MATERIALS AND METHODS

Construction of RNA aptamer expression vectors

SNAP31 RNA expression vector-based constructs were generated using previously described methods.[22] This vector has an early SV40 promoter, a DHFR cDNA encoding region, and a poly(A) region for small RNA expression. We cloned RNA aptamer sequences NFATc, R16, R16_CH2, R70, R70 ×3, and R70 ×5 between the early SV40 promoter and the DHFR cDNA-encoding region of the SNAP31 vector using an In-Fusion Cloning kit (TaKaRa, Otsu, Japan) following the manufacturer's instructions. The aptamer sequences are listed in Table S1.

Construction of CRIPSR-Cas9 system expression vectors

Information on *Streptococcus pyogenes* Cas9 endonuclease was obtained from the National Center for Biotechnology Information. Cas9 DNA was synthesized by Mbiotech Co. (Seoul, South Korea). Cas9 DNA was then cloned into a mammalian expression vector pcDNA4/TO/Myc-His A (Thermo Fisher Scientific, Waltham, MA) for intracellular expression. sgRNA had two components: CRISPR RNA (crRNA, a 17- to 20-nt sequence complementary to the target AAVS1 site) and a tracrRNA that served as a binding scaffold for Cas nuclease. We designed sgRNA sequences (Table S1) with only 20-nt-long crRNA. DNA encoding sgRNAs was cloned into a pU6 +1 plasmid, an RNA Pol III promoter-derived RNA expression vector for the nuclear expression of sgRNA.[47] A donor vector was constructed using a pzDonor-AAVS1-puro vector (Sigma-Aldrich, St. Louis, MO). This vector has specific arms homologous to AAVS1 for HDR and a puromycin resistance gene for the clonal selection of stable cells. We cloned the region from the early SV40 promoter to poly(A) of each aptamer-containing SNAP31 vector between both homologous arms of the donor vector. All of the cloning procedures were carried out using

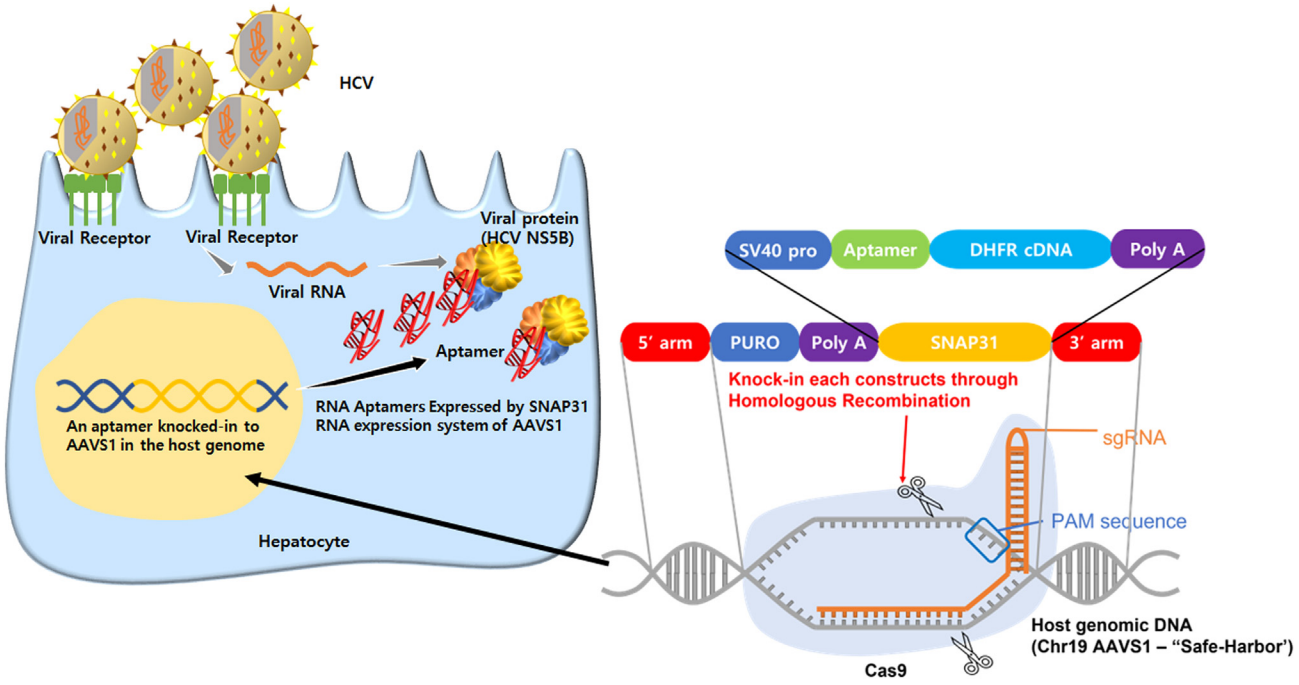

Figure 7. Development of AAVS1 knockin cells stably expressing HCV-specific aptamer

DSBs in the genome were induced with the CRISPR-Cas9 system targeting AAVS1. HDR occurs by homology of donor constructs to the target, and knockin of the donor constructs occurs. The RNA aptamer is stably expressed in the cytoplasm of the knockin cells. It inhibits HCV replication by targeting HCV viral proteins. PAM, protospacer adjacent motif.

an In-Fusion Cloning kit (TaKaRa) following the manufacturer's instructions.

Cells and HCV constructs

The human HCC cell line Huh-7.5 was maintained in DMEM with high glucose (HyClone, Thermo Fisher Scientific) supplemented with 10% fetal bovine serum. Huh-7.5 cells have mutational inactivation of the retinoic acid–inducible gene I. Therefore, they are highly permissive of HCV replication initiation.[32] The genotype 1b (con 1 strain) plasmid pFKI389*neo*/NS3–3′/5.1 containing two cell culture–adaptive mutations in NS3 and one in NS5A (provided by R. Bartenschlager) was restricted with *Ase*I and *Sca*I or genotype 2a plasmid pJFH-1 containing full-length JFH-1 cDNA downstream of the T7 RNA promoter construct (provided by T. Wakita) was digested with *Xba*I to synthesize HCV RNA. HCV RNA was synthesized in a test tube with digested plasmids using T7 RNA polymerase (TaKaRa). The HCV genotype 1b subgenomic replicon cell line, genotype 2a JFH-1 genomic replicon cell line, and infectious genotype 2a JFH-1 virus were generated using previously described methods.[28,48] HCV NS3 and NS5B DNA were synthesized by Mbiotech Co. NS3 and NS5B DNA were then cloned into the mammalian expression vector pcDNA4/TO/Myc-His A (Thermo Fisher Scientific) for intracellular expression.

Development of RNA aptamer stable cells

Huh-7.5 cells were seeded into 35-mm plates at a density of 2×10^5 cells/plate. The next day, 3 μg of each aptamer expression plasmid was transfected into the plated cells using Lipofectamine 3000 (Thermo Fisher Scientific) according to the manufacturer's instructions. Forty-eight hours after transfection, the plated cells were replated into 100-mm dishes. The medium was then replaced with fresh medium containing 1 μg/mL puromycin (Gibco, Thermo Fisher Scientific) every 3 days for 2 weeks until viable colonies were obtained. When a viable colony was created, a pool of colonies was obtained. Total cellular RNA was then purified from the obtained pools and used to verify the expression of the aptamers. Total RNA was extracted using Trizol (Invitrogen, Waltham, MA). cDNA was then synthesized by reverse transcription with a random hexamer. Two μL of the synthesized cDNA was used for PCR with primers (5′-CGATTCTAGCG CGTCCGC-3′ and 5′-GGTCTTGTACCCGTAGCCG-3′) at 95°C for 30 s, 58°C for 30 s, and 72°C for 30 s for 35 cycles with a PCR instrument (Applied Biosystems, Waltham, MA) and 2× PCR premix (Meridian Bioscience, Cincinnati, OH).

T7E1 assay

Huh-7.5 cells were seeded into 35-mm plates at a density of 2×10^5 cells/plate. The next day, 1 μg of sgRNA expression plasmid and 1 μg of Cas9 expression plasmid were cotransfected into plated cells using Lipofectamine 3000 (Thermo Fisher Scientific) according to the manufacturer's instructions. Forty-eight hours after transfection, the transfected cells were harvested, and genomic DNA was purified using the QuickExtract DNA extraction solution (QE09050, Lucigen, Hoddesdon, UK) according to the manufacturer's instructions. Two

microliters of the purified genomic DNA was used for PCR with primers (5′-CCGGGCCCCTATGTCCACTT-3′ and 5′-AATCTGCC TAACAGGAGGTG-3′) at 95°C for 30 s, 58°C for 30 s, and 72°C for 30 s for 35 cycles on a PCR instrument (Applied Biosystems) using 2× PCR premix (Meridian Bioscience). PCR products were eluted from the gel in 30 μL of water and a PCR clean-up kit (Thermo Fisher Scientific). The eluted PCR products were then denatured at 95°C for 5 min. After denaturation, annealing was performed by decreasing 2°C/s from 95°C to 85°C and 0.1°C/s from 85°C to 25°C. T7E1 (New England Biolabs, Ipswich, MA) digestion was then performed with the annealed 200 ng of DNA at 37°C for 1 h using a PCR instrument (Applied Biosystems) in a reaction volume of 20 μL. The digested mixture was analyzed by gel electrophoresis.

Development of AAVS1 HCV-specific RNA aptamer knockin cell clones

Huh-7.5 cells were seeded into 35-mm plates at a density of 2×10^5 cells/plate. The next day, 1 μg of each aptamer expression plasmid (donor plasmid), 1 μg of sgRNA expression plasmid, and 1 μg of Cas9 expression plasmid were cotransfected into plated cells using Lipofectamine 3000 (Thermo Fisher Scientific) according to the manufacturer's instructions. Forty-eight hours after transfection, the plated cells were replated into 100-mm dishes. The medium was then replaced with fresh medium containing 1 μg/mL puromycin (Gibco, Thermo Fisher Scientific) every 3 days for 2 weeks until viable colonies were obtained. When viable colonies were created, each colony was picked, replated onto a 96-well plate, and grown in 60-mm dishes. Total cellular RNA was obtained from the cell clones and used to confirm the expression of the aptamers. Total RNA was extracted using Trizol (Invitrogen). cDNA was then synthesized by reverse transcription with a random hexamer. Two microliters of the synthesized cDNA was used for PCR with the same primers and conditions as were used for the RNA aptamer stable cells. Genomic DNA was extracted from each clone and used for PCR to confirm knockin at AAVS1. Genomic DNA purification was performed using QuickExtract DNA extraction solution (QE09050, Hoddesdon) according to the manufacturer's instructions. Two μL of purified genomic DNA was used for PCR with primers (5′-CCC CTTACCTCTCTAGTCTGTGC-3′ and 5′-CTCAGGTTCTGGGAG AGGGTAG-3′) at 95°C for 30 s, 58°C for 30 s, and 72°C for 30 s for 35 cycles on a PCR instrument (Applied Biosystems) using 2× PCR premix (Meridian Bioscience).

Cell proliferation assay

Each AAVS1 knockin cell clone was seeded into 60-mm plates at a density of 1×10^5 cells/plate on seven different plates and harvested every day for 7 days. Cell numbers were counted each day using a hemocytometer, and differences in proliferation from naive Huh-7.5 cells were observed.

HCV challenge assay

RNA aptamer stable cells or AAVS1 RNA aptamer knockin cells were seeded into 35-mm plates at a density of 2×10^5 cells/plate. The next day, 3 μg of HCV genotype 1b subgenomic replicon RNA or genotype 2a JFH-1 RNA was transfected into the plated cells using Lipofectamine 3000 (Thermo Fisher Scientific), with or without HCV NS3 or NS5B expression plasmid according to the manufacturer's instructions. Alternatively, the next day, 0.1 or 0.5 MOI of HCV genotype 2a JFH-1 virus was used to infect the plated cells. Transfected or infected cells were harvested after 96 h using Trizol (Invitrogen) for total RNA extraction or RIPA buffer (50 mM Tris-HCl [pH 8.0], 150 mM NaCl, 0.2% sodium azide, 0.1% SDS, 0.1% NP-40, and 0.5% sodium deoxycholate) for total protein extraction.

cDNA was synthesized from total RNA by reverse transcription with primers specific to each HCV genotype (1b: 5′-CTGTTGTTGCCC AGTGATAGC-3′; 2a: 5′-AGCACCTTACCCAGGCCTAT-3′). Two microliters of the synthesized cDNA was used for qRT-PCR with primers (genotype 1b: 5′–ACCAAACGTAACACCACCGG-3′ and 5′-CTGTTGTTGCCCAGTGATAGC-3′; genotype 2a: 5′-CGACCAG TCCCACCATCCTT-3′ and 5′-AGCACCTTACCCAGGCCTAT-3′) at 95°C for 30 s, 58°C for 30 s, and 72°C for 30 s for 40 cycles using a StepOne Plus Real-Time PCR instrument (Applied Biosystems) and SYBR Green 2× real-time PCR premix (Meridian Bioscience). Threshold levels obtained from the HCV assay were adjusted to those of 18S rRNA PCR to correct for minor variations in cDNA loading.

Immunoblotting was performed with total protein as previously described[49] using the following primary antibodies: mouse-polyclonal anti-1b NS5B (ab100895, Abcam, Cambridge, UK), mouse-polyclonal anti-2a NS5B (D3KY46, Kerafast, Oxford, UK), and mouse-monoclonal anti-α-tubulin (DM1A, Abcam). Protein bands were visualized by an enhanced chemiluminescence protocol (Amersham Pharmacia, Amersham, UK).

Colony-forming assay

Each RNA aptamer stable cell line and AAVS1 RNA aptamer knockin cells (2×10^5) on 35-mm dishes were transfected with 2 μg of HCV genotype 1b subgenomic replicon RNA using Lipofectamine 3000 reagent (Thermo Fisher Scientific) according to the manufacturer's instructions to observe the long-term effects of stably expressed aptamers on HCV replication. The medium was replaced after transfection with fresh medium containing 1 μg/mL puromycin (Gibco, Thermo Fisher Scientific) every 3 days. After puromycin-resistant pools were obtained, the cells were treated with 500 μg/mL G418 (Gibco, Thermo Fisher Scientific) every 2 or 3 days for 1 month to select cells that could support HCV subgenomic RNA replication. Each cell line was grown in 35-mm dishes until viable colonies were obtained. Colony formation was confirmed by methylene blue staining.

RIP analysis

Each AAVS1 RNA aptamer knockin cell was seeded into 100-mm plates at a density of 2×10^6 cells/plate. The next day, 10 μg of HCV genotype 1b subgenomic replicon RNA of HCV genotype 2a JFH-1 RNA was transfected into the plated cells using Lipofectamine 3000 (Thermo Fisher Scientific) according to the manufacturer's instructions. Twenty-four hours after transfection, the cells were

washed with cold 1× PBS, incubated with 1% formaldehyde for 10 min, and treated with glycine (pH 7.0) at a final concentration of 0.25 M for 5 min. The cells were then lysed with RIPA buffer and sonicated. The sonicated lysates were immunoprecipitated with normal immunoglobulin G (IgG), mouse polyclonal anti-1b NS5B (ab100895, Abcam), or mouse polyclonal anti-2a NS5B (D3KY46, Kerafast). The pellets and supernatants were incubated at 70°C for 1 h to reverse cross-links. RNA was then purified, reverse-transcribed using a random hexamer, and subjected to PCR for aptamer detection with the same primers and conditions as were used for RNA aptamer stable cells.

WGS

Naive Huh-7.5 cells or AAVS1 knockin Huh-7.5 cell clones with R70 ×3 or R70 ×5 were seeded into 24-well plates at a density of 10^5 cells/plate. The next day, the cells were harvested using QuickExtract DNA extraction solution (QE09050, Lucigen). Genomic DNA was then extracted from the harvested cells following the manufacturer's instructions, sequenced, and analyzed by Macrogen (Seoul, South Korea).

Statistical analysis

The significance of the differences between the mean values within groups was tested using a paired one-tailed t test (Student's t test). All of the data are expressed as averages ± SDs. Differences were considered statistically significant at $p < 0.05$.

DATA AND CODE AVAILABILITY

The data supporting the findings of this study are available within the article and its supplementary materials.

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.omtm.2023.101151.

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AUTHOR CONTRIBUTIONS

S.-W.L. designed the experiments; T.H.K. conducted the experiments; and T.H.K. and S.-W.L. analyzed the data and wrote the manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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