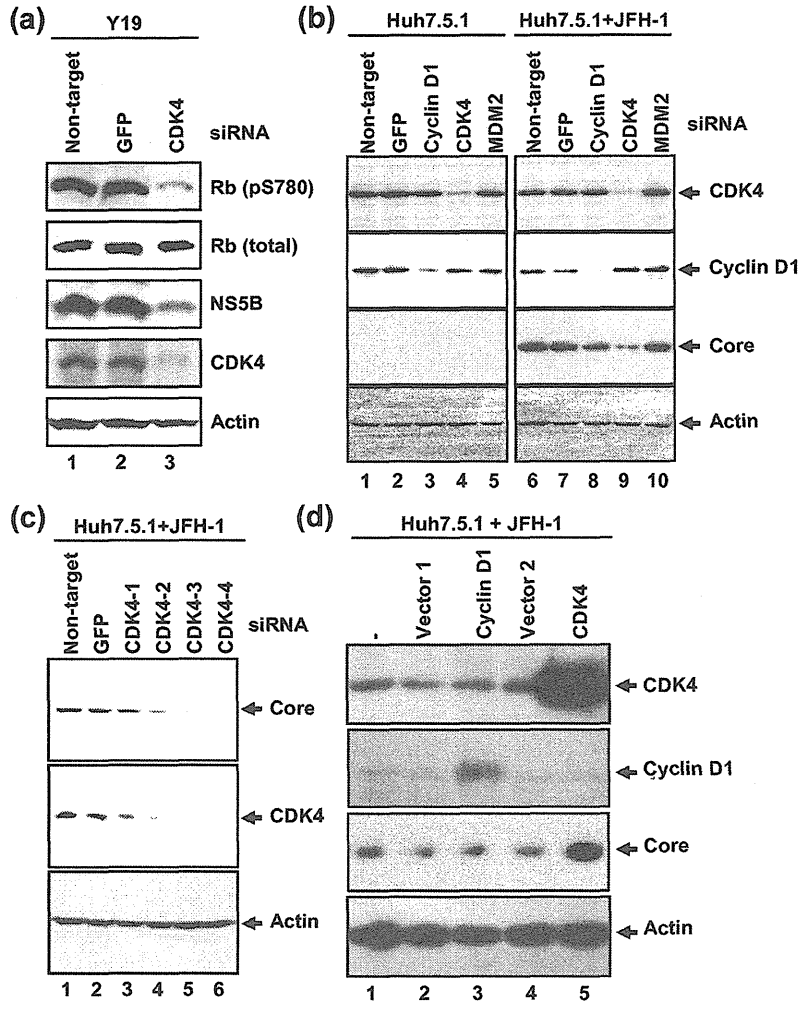

Fig. 4. CDK4 is required for efficient HCV replication. (a) Inhibition of HCV NS5B production by knockdown of CDK4 in HCV replicon cells. In Y19 cells, siRNA-mediated knockdown analysis was performed, and reduction of amounts of CDK4, phosphorylated Rb and HCV NS5B is shown by immunoblot. (b) Inhibition of production of HCV core by knockdown of CDK4, but not by knockdown of cyclin D1. To confirm the validity of inhibitor experiments, siRNA-mediated knockdown analysis was performed in both Huh7.5.1 and Huh7.5.1 + JFH-1 cells. Note that HCV core was not expressed in Huh7.5.1 cells. (c) Inhibition of the production of HCV core by knockdown of CDK4 using four separate siRNA molecules. Among the four, three siRNAs exhibited CDK4 knockdown and down-regulation of HCV core protein abundance. (d) Enhancement of the production of HCV core by over-production of CDK4, but not that of cyclin D1. For all 3 panels, total protein was isolated following siRNA exposure or transfection and subjected to immunoblot analysis for CDK4, cyclin D1, core protein, and actin (loading control).

Huh7.5.1 + JFH-1 cells separately with four distinct siRNAs. We again observed reductions in CDK4 and HCV core abundance in cells transfected with three of these four CDK4-specific siRNAs (CDK4-2, CDK4-3, and CDK4-4) (Fig. 4c). Notably, the siRNA (CDK4-1) that failed to impair HCV core abundance also failed to reduce CDK4 levels, indicating the specific requirement of CDK4 for HCV propagation.

In order to further clarify the role of CDK4 in the HCV life cycle, we overexpressed *CDK4* in Huh7.5.1 + JFH-1 cells, and observed increased levels of both CDK4 and HCV core protein (Fig. 4d). In contrast, overexpression of *cyclin D1* cDNA increased the levels of cyclin D1 protein, but not those of HCV core protein (Fig. 4d). These findings were consistent with the inhibitor assays, and suggested that CDK4 is a host factor required for HCV replication.

CDK4, along with CDK2 and CDK6, is classified as an interphase CDK (Malumbres and Barbacid, 2009). This fact, combined with the results of our CDK inhibitor analysis, suggested that CDK2 and CDK6 might also function as regulators of HCV. Therefore, we repeated our knockdown analysis with siRNAs against the transcripts encoding CDK2 and CDK6. As seen with CDK4, we found that cells with impaired production of CDK2 or CDK6 also exhibited reduced levels of HCV core protein (Fig. 5a and b). Interestingly, knockdown of the interphase CDKs induced over-production of the mitotic checkpoint protein BubR1 (Fig. 5b), suggesting that normal mitotic progression is required for HCV replication (Elowe, 2011).

3.4. CDK inhibition reduces serum HCV titer in mouse model of HCV infection

All of the above experiments were performed *in vitro*; we next addressed the potential *in vivo* activity of a CDK inhibitor in an animal model of HCV infection. Specifically, we tested the anti-HCV effect of inhibitor A (roscovitine; seliciclib;(McClue and Stuart, 2008; Nutley et al., 2005)) in HCV (genotype 1b)-infected chimeric mice with humanized livers (Nakagawa et al., 2007; Tateno et al., 2004). Although the data presented to date indicate that inhibitor B (CDK4 inhibitor) is the most effective in suppressing HCV

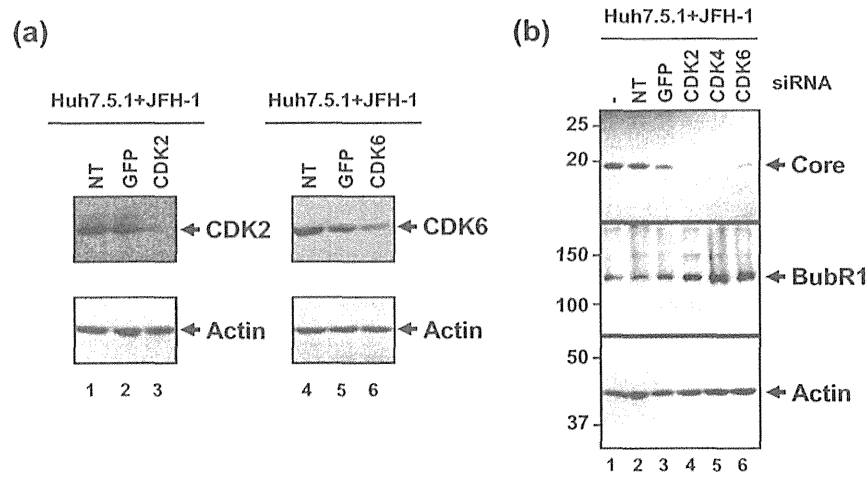

Fig. 5. Interphase CDKs are required for HCV replication. (a) Knockdown of CDK2 or CDK6 in Huh7.5.1 + JFH-1 cells was shown by immunoblot analysis. (b) Expression of HCV core protein was inhibited by knockdown of CDK2, CDK4, and CDK6 in Huh7.5.1 + JFH-1 cells. Immunoblot of BubR1 demonstrated that this cell-cycle checkpoint protein was induced by the reduced levels of each CDK.

replication, it has no valid data on *in vivo* use, which made it difficult to determine the dose for chimeric mice. As shown in Fig. 5, CDK2 was also found to be required for HCV replication, and roscovitine targets CDK2 (Table 1) and has lower EC50 for suppression of HCV replication than inhibitor B in R6FLR-N cells (Supplementary Fig. 1). In addition, this drug is undergoing a phase IIb clinical trial as monotherapy for non-small cell lung carcinoma, and is therefore well characterized, which facilitated our experiment in mice. We administered inhibitor A intravenously over a period of 14 days, both as monotherapy and in combination with subcutaneously-injected polyethylene glycol-conjugated interferon alpha (PEG-IFN). Inhibitor A significantly ($P < 0.05$) reduced serum HCV RNA levels when administered as a 50-mg/kg monotherapy (Fig. 6b), but did not demonstrate efficacy as a 25-mg/kg monotherapy (Fig. 6a). In addition, while treatment with PEG-IFN alone reduced HCV titer by more than one log at day 14, combined administration of PEG-IFN and inhibitor A (at either dose level) provided further reductions in serum HCV RNA levels (Fig. 6a and b). Detailed HCV titers in the serum of each chimeric mouse are shown in Supplementary Fig. 2. Judging from body weight, human albumin levels and ALT levels, our experiments did not significantly affect the status of chimeric mice (Supplementary Fig. 3). We also analyzed the liver tissues from a set of experiments on high dose of CDKI at day 14. We examined the HCV viral titers, CDK mRNA levels, and CDK protein levels in the liver tissues of three experimental groups (Supplementary Fig. 4). These results indicate that HCV titers in the liver of chimeric mice are clearly correlated with those in the serum at day 14, and that the mRNAs and proteins of CDK2, CDK4, and CDK6 are expressed in the end-point liver. These findings demonstrate that CDK inhibition suppresses HCV replication *in vivo*, suggesting that compounds of this class are candidate therapies for the treatment of HCV-infected individuals.

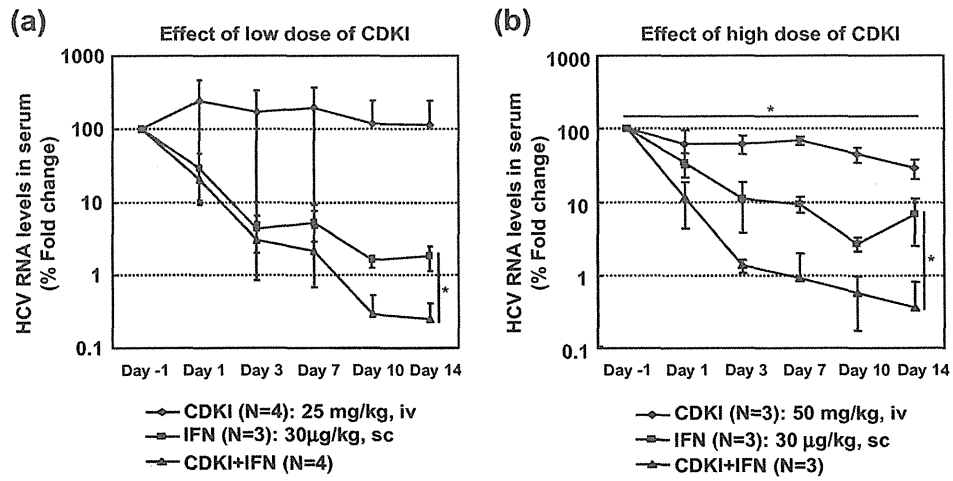

Fig. 6. *In vivo* anti-HCV activity of a CDK inhibitor. CDK inhibitor A (CDKI) and PEG-interferon α (IFN) were administered as single and combined therapies to HCV-infected chimeric mice with humanized livers. The results for low doses of CDKI are shown in (a), and those for high doses of CDKI are shown in (b). At the indicated time points, blood was sampled from each mouse. RNAs were prepared from the resulting sera and HCV RNA titers were measured by real-time RT-PCR. Each data point represents the mean ± SD from at least three mice. *$P < 0.05$.

4. Discussion

4.1. Molecular mechanisms for suppression of HCV replication by CDK inhibitors

CDKs play important roles in the regulation of the cell cycle, a process that is perturbed in many tumor cells. Inhibition of CDK activity has therefore been proposed as a strategy for the control of cancer development and the curing of cells with abnormal growth properties (Krystof and Uldrijan, 2010). In this paper, we report that small-molecule CDK inhibitors suppress HCV replication *in vitro* and *in vivo*. We also demonstrate that interphase CDKs, including CDK4, are required for the efficient replication of HCV. Previous reports have shown that HCV genome replication is strongly dependent on cellular proliferation, and that the activity of the HCV internal ribosomal entry site (IRES) is greatest in actively growing cells (Honda et al., 2000; Scholle et al., 2004). Thus, it is possible that regulation of cell cycle in HCV-infected cells by CDK inhibitors is responsible for the suppression of HCV replication. Alternatively, CDKs may regulate HCV replication via the Rb protein. Rb, which is a direct target of CDKs, is known to bind to and inhibit HCV NS5B polymerase (Munakata et al., 2005); CDK inhibitors might activate Rb by enhancing and maintaining the phosphorylated state of Rb. Indeed, hyper-phosphorylation of Rb is reported to inhibit the LxCxE-binding activity of Rb (Knudsen and Wang, 1996), which also mediates the interaction between Rb and NS5B.

4.2. Roles of CDK4 in HCV replication

Among the CDK inhibitors tested in cell culture, a CDK4 inhibitor was the most effective in suppressing HCV replication, and RNAi analysis clearly showed that knockdown of CDK4 inhibited HCV replication. These data suggest that CDK4 is a novel host factor that regulates the HCV life cycle. CDK4 was originally identified as a catalytic partner of D-type cyclins (Matsushime et al., 1992). However, in contrast to our results for CDK4, we found that changes in cyclin D1 levels did not alter HCV replication. Knockdown and over-production of cyclin D1 appeared not to affect the regulation of HCV replication. We hypothesize that any cyclin D-specific regulation of HCV depends on some cyclin D activity distinct from protein level, or that the CDK4 dependence of HCV is mediated through a distinct binding partner in HCV-infected hepatocytes.

Knockout analysis in mice revealed that CDK4 is not essential for G1-phase progression, although CDK4-deficient mice exhibit a decreased growth rate and infertility (Rane et al., 1999). CDK4 is also suggested to play important roles in thymocyte maturation and pancreatic β-cell development (Chow et al., 2010). Thus, the CDK4-dependent aspects of HCV replication may reflect the functions of CDK4 other than cell-cycle regulation (Fig. 3b), consistent with the redundancy of CDK4 and CDK6 as interphase CDKs (Malumbres and Barbacid, 2009). At the same time, our data indicate that CDK2, CDK4, and CDK6 are each required for HCV replication in cultured cells, thus suggesting the significance of complete interphase progression during HCV replication. Normal hepatocytes are non-growing quiescent G0 cells, and enter the cell cycle after viral infection or during liver regeneration. The detailed molecular mechanisms underlying regulation of the interphase following HCV infection clearly deserves further investigation.

Interestingly, CDK4 has been demonstrated as a host factor required for HCV entry, using functional siRNA kinase screening (Lupberger et al., 2011). As our results clearly indicate that CDK inhibitors suppress HCV replication in the subgenomic replicon systems, the role of CDKs in our hypothesis is independent of HCV entry. Therefore, CDKs must have at least two distinct functions for supporting HCV replication and infection.

4.3. Relationship between endogenous CDK inhibitors and HCV replication

The presence of endogenous CDK inhibitors, such as p16, p21 and p27, helps normal hepatocytes to regulate the cell cycle (Kato et al., 1994; Toyoshima and Hunter, 1994). Production of these endogenous inhibitors is tightly regulated at every step from transcription to protein stability. Typically, p21 and p27 form a complex with CDK2 or CDK4 in quiescent hepatocytes, resulting in inhibition of kinase activity (Kwon et al., 2002). In place of cellular inhibitors, we used synthetic small-molecule inhibitors of CDKs to impair CDK function and suppress HCV replication. Given that CDK4 is required for HCV replication, up-regulation of p21 and/or p27 may be able to substitute the addition of exogenous CDK inhibitors. Indeed, HCV core is reported to stabilize p27 to arrest cell-cycle progression, and enhanced production of p21 is observed in hepatocytes of mice transgenic for HCV core (Chang et al., 2009; Yao et al., 2003). The potential role of p21 and p27 in HCV replication will require further analysis.

4.4. Conclusion

In conclusion, a novel host factor, CDK4, was identified as a potential target of HCV therapy. Small-molecule inhibitors of CDK, currently under development as anti-cancer therapies, may also find application in the treatment of individuals infected with HCV, particularly for viral genotypes 1 and 2. The combined administration of IFN and a CDK inhibitor was more effective than either alone, suggesting a possible future regimen for HCV treatment.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.antiviral.2014.05.011.

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Resistance to cyclosporin A derives from mutations in hepatitis C virus nonstructural proteins

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Debio-025

ABSTRACT

Cyclosporine A (CsA) is an immunosuppressive drug that targets cyclophilins, cellular cofactors that regulate the immune system. Replication of hepatitis C virus (HCV) is suppressed by CsA, but the molecular basis of this suppression is still not fully understood. To investigate this suppression, we cultured HCV replicon cells (Con1, HCV genotype 1b, FLR-N cell) in the presence of CsA and obtained nine CsA-resistant FLR-N cell lines. We determined full-length HCV sequences for all nine clones, and chose two (clones #6 and #7) of the nine clones that have high replication activity in the presence of CsA for further analysis. Both clones showed two consensus mutations, one in NS3 (T1280V) and the other in NS5A (D2292E). Characterization of various mutants indicated that the D2292E mutation conferred resistance to high concentrations of CsA (up to 2 μM). In addition, the missense mutation T1280V contributed to the recovery of colony formation activity. The effects of these mutations are also evident in two established HCV replicon cell lines—HCV-RMT ([1], genotype 1a) and JFH1 (genotype 2a). Moreover, three other missense mutations in NS5A—D2303H, S2362G, and E2414K—enhanced the resistance to CsA conferred by D2292E; these double or all quadruple mutants could resist approximately 8- to 25-fold higher concentrations of CsA than could wild-type Con1. These four mutations, either as single or combinations, also made Con1 strain resistant to two other cyclophilin inhibitors, N-methyl-4-isoleucine-cyclosporin (NIM811) or Debio-025. Interestingly, the changes in IC_{50} values that resulted from each of these mutations were the lowest in the Debio-025-treated cells, indicating its highest resistant activity against the adaptive mutation.

1. Introduction

The genome of the hepatitis C virus (HCV) is a single-stranded RNA with positive polarity and is classified in the *Flaviviridae* family. HCV frequently establishes chronic infections that lead to liver cirrhosis and hepatocellular carcinoma (HCC) [2]. An estimated 130–200 million people worldwide are now infected with HCV [3]. HCVs have been classified into six major genotypic groups (genotypes 1–6); genotype 1 is the most prevalent over most of the world. Treatments with alpha interferon (IFNα), together with the nucleoside analog ribavirin (RBV), greatly increased the percentage of HCV chronically infected patients able to reach a sustained anti-viral response (SVR). Covalent attachment of polyethylene glycol (PEGylated) IFN-α-plus-RBV therapy has a success rate of ~80% in patients with genotype 2 or 3 infections, but only ~50% in patients with genotype 1 infections [4,5]. The recently approved protease inhibitors boceprevir and telaprevir each improved the efficacy of IFN-α-plus-RBV therapy [6]. These direct-acting agents (boceprevir, simeprevir, sofosbuvir, faldaprevir and telaprevir, etc.) each have the advantage of being highly specific, but each may select for specific resistant mutations, limiting their long-time efficacy. Therefore, antiviral inhibitors targeting host factors crucial for viral replication should be developed to overcome these problems.

Abbreviations: HCV, hepatitis C virus; CsA, cyclosporine A; HCC, hepatocellular carcinoma; IFNα, alpha interferon; Cyp, cyclophilins; SVR, sustained anti-viral response.

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Reportedly, several HCV proteins interact with cyclophilins (Cyp) and modulate HCV replication [7–9]. To date, three Cyp inhibitors—Debio-025, NIM811, and SCY-635—have been deemed safe and effective for patients with HCV in phase I and II studies [10–12]. Development of Debio-025 has advanced the farthest through phase II studies, and Debio-025 has approved and showed a great deal of promise for decreasing HCV viremia in infected patients. However, emergence of drug-resistant HCV mutants could limit the therapeutic potential of CsA and Cyp inhibitors.

The HCV genome is a positive-sense, single-stranded RNA (about 9.6 kb) that encodes at least 10 viral proteins; these are categorized as structural core proteins (E1, E2) or nonstructural (p7, NS2, NS3, NS4A, NS4B, NS5A, and NS5B) [13,14]. The nonstructural proteins are involved in HCV RNA replication [14]. NS5A protein comprises three domains linked by two low-complexity sequences (LCS) that are either serine or proline rich; domain I is a highly structured zinc binding domain whose three-dimensional structure shows two dimeric conformations [15,16]. Domains II and III have been shown to be unstructured in their native states, but nuclear magnetic resonance and circular dichroism have shown that elements of secondary structure run throughout each of these domains [17–19]. NS5A is anchored to membranes by an N-terminal amphipathic helix and is an essential component of the viral genome replication complex; it also interacts with other non-structural proteins [20] or cellular factors. NS5A domain II is a substrate for the peptidyl-prolyl cis/trans isomerase activity of Cys A and B [21], and NS5A domain III is reportedly a substrate of CypA [22].

In this study, we used CsA to select for and isolate drug-resistant HCV mutants; we then performed virus genome sequencing to investigate the molecular mechanisms of this drug resistance.

2. Materials and methods

2.1. Cells, electroporation and ethics statement

HuH-7 cells were cultured in DMEM-GlutaMax-I (Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum, penicillin, and streptomycin (Invitrogen). Replicon cells were maintained in the same medium supplemented with 300 μg/ml G418 (Invitrogen). Cells were passaged three times a week, and at each passage, each culture was split into four subcultures. Electroporation of replicon RNA and G418 selection were performed as previously described [23]. All experimental protocol was approved by the regional research institute.

2.2. Establishment of cyclosporin A resistant replicon clones

FLR3-1 cells derived from Con1 (AJ238799)-based, luciferase-harboring HCV sub-genomic replicon cell were treated with both 2 μM of cyclosporin A and 0.5 mg/ml of G418 for 24 days. Surviving cells were further treated with 3 μM CsA for 2 days, 4 μM for 4 another days, and finally 6 μM for the last 10 days. Using limiting dilution cloning, we established nine clonal cell lines. Using real-time RT-PCR (ABI 7700 system, Applied Biosystems, Foster City, CA, USA) as described previously [24], we systematically measured HCV RNA copy number in each of these nine clonal lines.

2.3. Determination of consensus sequence of resistant clones

LongRange Reverse transcriptase (QIAGEN, Valencia, CA, USA) and an oligonucleotide primer (antisense sequence 9549–9569 of HCV-Con1) were used to reverse transcribe purified RNA (1 μg). The resulting cDNA, Phusion DNA polymerase (Finnzymes, Vantaa, Finland), and primers recognizing each non-coding region were used for PCR amplification of the entire non structural protein coding region of the sub-genomic replicon. The TA cloning kit (Invitrogen) was used to introduce each fragment into a separate plasmid; we picked up eight clones from each resistant cell line and their nucleotide sequences were determined.

2.4. Construction and RNA transcription

The pFK I389neo/NS3-3′/5.1 and pFK I389luc/NS3-3′/5.1 plasmids (ReBlikon, Baden-Württemberg, Germany) were used to generate HCV constructs with regions of the sub-genomic replicon with mutations (Fig. 2A). The QuikChangeII kit (Stratagene, La Jolla, CA, USA) was used to introduce specific mutations into the HCV sequences. To generate RNA, plasmids were digested with *XbaI* and used as a template for RNA transcription; RiboMax (Promega, Madison, WI, USA) was used for each transcription reaction.

2.5. Drug treatment

For the drug resistance assays, established CsA-resistant replicon clones were seeded onto 24-well tissue culture plates (10,000 cells/well) and cultivated overnight. Then cells were treated with various concentrations of CsA (0–8 μM) for 4 days. Surviving cells were stained with crystal violet.

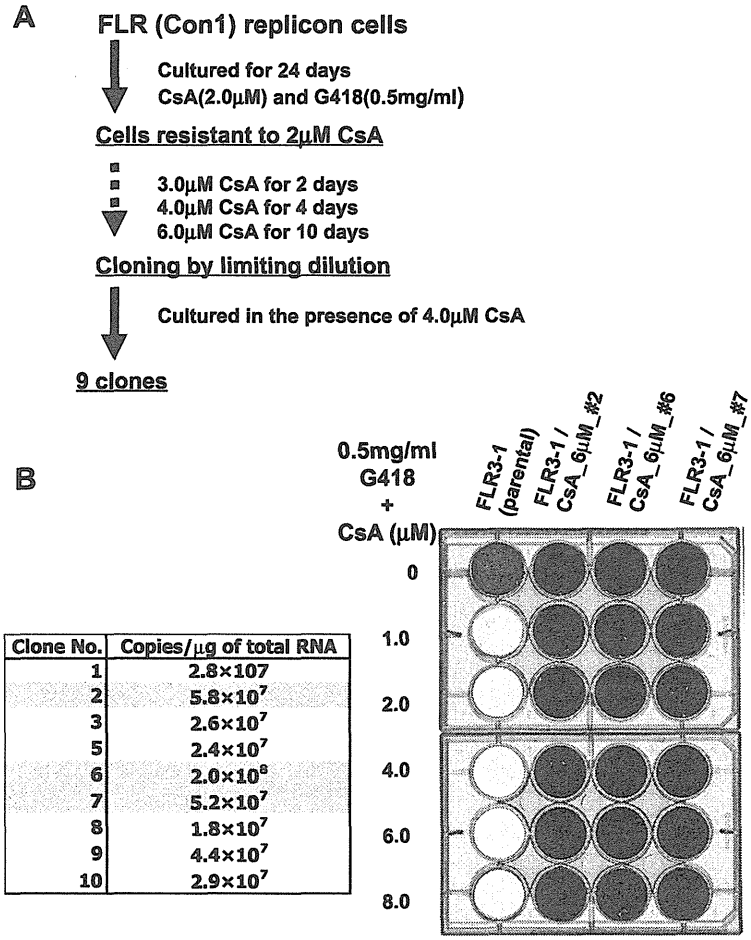
For HCV replication inhibition assays, replicon cells were seeded in 96-well tissue culture plates (5000 cells/well) and cultivated overnight. Serial dilutions of CsA (Fluka Chemie, Buchs, Switzerland) or NIM811 (Novartis) and Debio025 (Debiopharma) were then added to sets of wells. After incubation for 72 h, ABI prizm 6100 (Applied Biosystems) was used to extract total RNA from cells, and HCV-RNA was measured as described above. Each assay was carried out in triplicate.

For another HCV replication inhibition assay, mutant replicon RNA derived from pFK I389luc/NS3-3′/5.1 plasmid were introduced into HuH7 cells via electroporation, and the transformed/transfected cells were seeded to 96-well tissue culture plates. Drugs were added 24 h after electroporation. Luciferase activities were evaluated 4 h or 72 h after electroporation, which corresponded to 20 h before drug treatment or 48 h after drug treatment, respectively; the Blight-Glo kit (Invitrogen) and Envision (Perkin Elmer, Waltham, MA, USA) were used to take all measurements, and values at 72 h were normalized relative to the values from 4 h.

3. Results

3.1. Establishment of CsA-resistant clones

To establish CsA-resistant clones, we treated HCV FLR-N replicon cells with CsA (Fig. 1A) and obtained nine resistant clonal cell lines. We measured the amount of HCV RNA in each resistant clonal line and chose for further study the three lines that consistently had the largest amount of HCV RNA (Fig. 1B). We then determined the entire HCV sequence from 16 subclones; we isolated two groups of eight subclones (one group each from clones #6 and #7), because we could not establish clone #2; each subclone was isolated by treating a CsA-resistant clone (#6 or #7) with 6 μM CsA (Table 1). Although there were several mutations in the NS3–NS5B protein-coding regions, common mutations were isoleucine (I) to valine (V) at amino acid 1280 (T1280V) and aspartic acid (D) to glutamic acid (E) at amino acid 2292 (D2292E). At 1280, original Con1 has threonine (T) and was mutated into (I) in Con1 replicon cells.



Clone No.	Copies/μg of total RNA
1	2.8×10^{7}
2	5.8×10^{7}
3	2.6×10^{7}
5	2.4×10^{7}
6	2.0×10^{8}
7	5.2×10^{7}
8	1.8×10^{7}
9	4.4×10^{7}
10	2.9×10^{7}

Fig. 1. Basic characteristics of the nine cyclosporin A-resistant clones. (A) Flow chart outlining the selection of cyclosporin A-resistant HCV replicon clones. (B) Real-time PCR was used to determine the copy number of each Cys A-resistant clone. The three clones with the highest HCV genome copy number are highlighted in green (Left). Colony formation assay of mutant #2, 6 and 7 (Right). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

3.2. Identification of mutations responsible for CsA resistance

To define the mutations responsible for CsA resistance, we constructed various chimeric clones that each contained specific mutation that arose from CsA selection (Fig. 2A). We could thereby evaluate each mutation with regard to its effect on CsA resistance. We found that mutations in two proteins—NS5A and NS4A—significantly enhanced the resistance against CsA treatment (Fig. 2B). We also cultured replicon cells with these mutants in the presence of CsA (up to 2 μM); we found that cells with a D2292E mutation could survive, but cells with wild-type NS5A or T1280V mutation could not (Fig. 3A).

The effect of T1280V mutation on colony formation was further evaluated (Fig. 3B). Introduction of the T1280V mutation in *cis* to the D2292E mutation rescued the colony-formation defect of the D2292E mutant replicon cells; specifically, the T1280V–D2292E double-mutant replicon cells had the same colony-forming ability as the parental replicon cells.

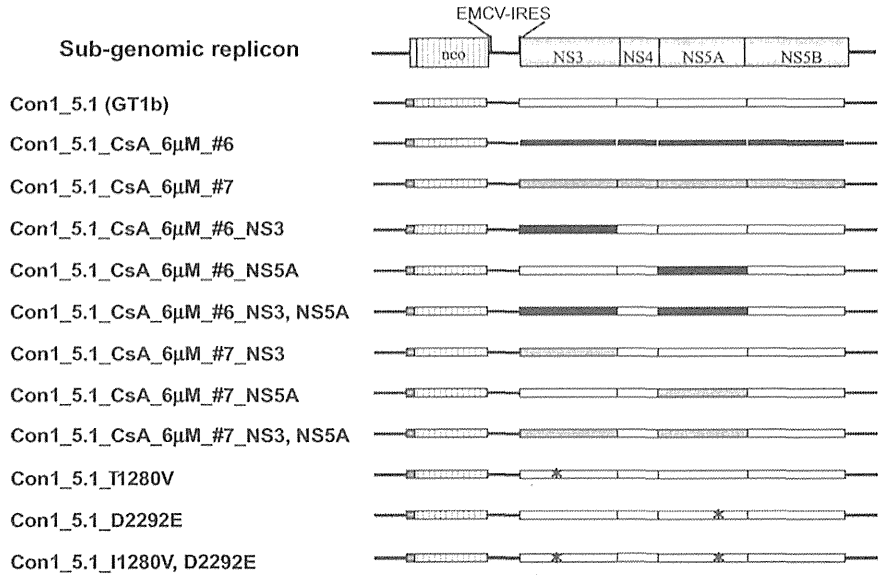
3.3. Evaluation of mutations for CsA resistance in other HCV genotypes

We evaluated whether the mutations that conferred CsA resistance to the HCV Con1 strain (genotype 1b) also conferred CsA resistance to the RMT (genotype 1a; AB520610) and JFH1 (genotype 2a; AB047639) strains (Fig. 4A, B and Table 2) [1]. D2292E conferred CsA resistance to the HCV strains RMT and JFH1, but T1280V did not (Table 2), as observed with HCV Con1 strain (Fig. 2E). The amino acid sequences surrounding mutations other than D2292E showed some differences among three genotypes (1a, 1b, and 2a) (Fig. 4B). D2292E mutants of these three genotypes showed resistance to CsA (Fig. 2E, Table 2) but the fold increase of resistance in genotype 1a and 2a was lower than that of genotype 1b (Tables 2 and 3). Therefore, there might be some residue(s) other than D2292E to influence the resistance to CsA.

3.4. Efficacy of mutations in NS5A for conferring CsA resistance

Although D2292E clearly conferred CsA resistance to HCV, other mutations in NS5A may also have had an effect because constructs with all four of the original NS5A mutations found in clone #6 mutations were more resistant to CsA than were constructs with only the D2292E mutation (Fig. 2B and E). We constructed HCV-luciferase replicons, each with one or more of four mutations (D2292E, D2303H, S2362G, and E2414K). HuH-7 cells were transiently transfected with RNA of each construct; we then treated the transfected cells with CsA (Table 3). Of the four single mutants, all but S2362G conferred some CsA resistance to HCV-luciferase replicons; notably, combinations of mutations had additive effects

A



B

Cyclosporin A	Replacement or Mutation			IC_{50} (μM)	Fold change
	NS3	NS4A	NS5A		
Con1_5.1 (GT1b)	-	-	-	0.18	1.0
	Cs6#6	-	-	0.21	1.2
	-	-	Cs6#6	2.10	11.7
	Cs6#6	-	Cs6#6	2.20	12.2
	Cs6#6	V1681A	Cs6#6	5.40	30.0

C

NIM811	replacement or mutations			IC_{50} (μM)	Fold change
	NS3	NS4A	NS5A		
Con1_5.1 (GT1b)	-	-	-	0.09	1.0
	Cs6#6	-	-	0.10	1.1
	-	-	Cs6#6	0.49	5.4
	Cs6#6	-	Cs6#6	0.50	5.6
	Cs6#6	V1681A	Cs6#6	1.80	20.0

D

Debio025	Replacement or mutation			IC_{50} (μM)	Fold change
	NS3	NS4A	NS5A		
Con1_5.1 (GT1b)	-	-	-	0.05	1.0
	Cs6#6	-	-	0.04	0.8
	-	-	Cs6#6	0.43	7.8
	Cs6#6	-	Cs6#6	0.40	8.0
	Cs6#6	V1681A	Cs6#6	0.75	13.7

E

Cyclosporin A	Replacement or mutation			IC_{50} (μM)	Fold change
	NS3	NS4B	NS5A		
Con1_5.1 (GT1b)	-	-	-	0.54	1.0
	Cs6#7	-	-	0.59	1.1
	-	-	Cs6#7	2.36	4.4
	Cs6#7	-	Cs6#7	2.51	4.6
	Cs6#7	I1797V	Cs6#7	2.44	4.5
	T1280V	-	-	0.57	1.1
	-	-	D2292E	1.71	3.1
	T1280V	-	D2292E	1.76	3.3

Fig. 2. (A) Schematic representations of 12 Con1 replicon-derived constructs. (B–D) Evaluation of Cs6#6 constructs with regard to resistance to CsA or to each of two CsA derivatives (NIM811 and Debio025). Real-time PCR was used to measure HCV sub-genome copy number in cells, and IC_{50}s were then determined from the copy number values. For each construct, the fold change represents the ratio of IC_{50} values from the construct and the parental Con1 replicon (IC50Construct:IC50Parental). (E) Resistance to CsA of three Cs6#7 derivative constructs that represent the T1280V and D2292E mutations as each single mutation or as a double mutation.

and conferred greater CsA resistance than any single mutation. The HCV replicon with all four mutations showed the strongest CsA resistance.

3.5. Evaluation of CsA-resistant mutants for resistance to cyclophilin inhibitors

We further evaluated each of the NS5A mutants for their ability to confer resistance to each of two other cyclophilin inhibitors, N-methyl-4-isoleucine-cyclosporin (NIM811, Table 4) and Debio-025 (Table 5). Of the four single mutants, D2292E conferred the highest resistance, and the combination of all four mutations conferred the overall highest resistance to NIM811 and to Debio-025. When we compared CsA, NIM811, and Debio-025, the mutation-mediated increases in IC_{50} values were lowest with the Debio-025 treatment (Tables 3–5).

4. Discussion

Here, we investigated two of nine HCV sub-genomic replicon cell clones (CsA-resistant HCV mutants) isolated following long-term dual treatment with CsA and G418. Comparing the HCV sequences of these two clones (#6 and #7), only two of many mutant sites were shared between the mutant HCV sequences. Specifically, both clones #6 and #7 had a D2292E missense mutation in NS5A and a T1280V missense mutation in NS3. D2292E is

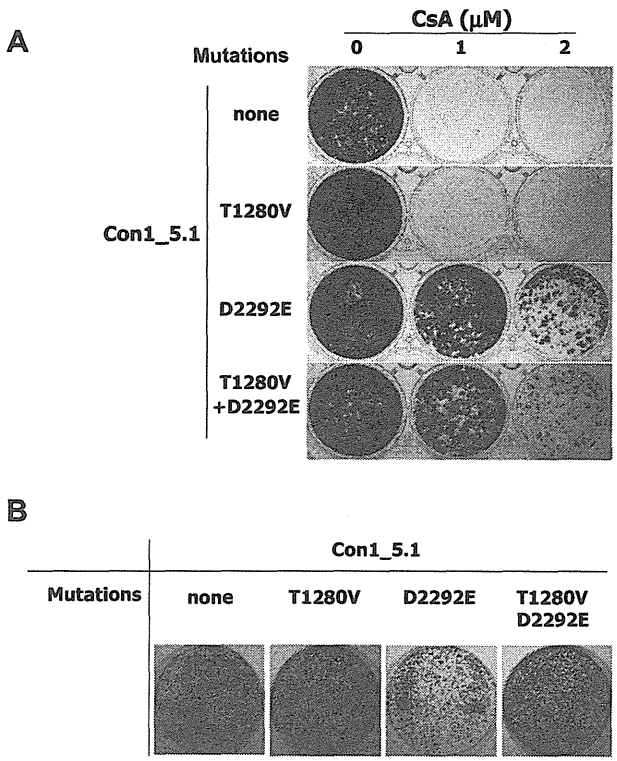


Fig. 3. (A) Resistance to CsA of T1280V and D2292E mutants. While under G418 selection, established replicon cells were treated with CsA at the indicated doses. (B) Standard methods described in Section 2 were used to determine the colony-forming abilities of T1280V and D2292E mutants.

known to confer CsA resistance to some HCV genotypes [25–28], and as a single mutation, it conferred CsA resistance to three separate HCV strains in our hands. In contrast, T1280V in NS3 was not previously identified as a CsA-resistance mutant, and in our hands, it had no impact on CsA resistance as a single mutation (Figs. 2E and 3A).

D2292E was the most significant resistance mutation in this study (Fig. 4C). This mutation is also significant in the regulation of HCV genome replication [29], and close to the CypA binding region [30] (Supplementary Fig. 1). With several genotypes (1a, 1b, 2a, 3, 4, and 6), D2292E is frequently observed after Debio-025 selection [28,31]. Other different mutations in NS5A and NS5B were identified in other studies of CsA resistance [7]; therefore, various mutations could influence HCV resistance to CsA.

In addition to the D2292E mutation, the T1280V mutation in NS3 was present in both clones #6 and #7. Despite its presence in both clones, it did not confer CsA resistance as a single mutant, nor did it enhance the effects of the NS5A CsA-resistant mutants (Fig. 2E). Instead, it partially rescued the colony-forming defect caused by D2292E (Fig. 3B). We used three assays—colony formation assay without CsA treatment (Fig. 3B), cell survival assay of established replicon cells with CsA and G418 dual-treatment (Fig. 3A), and HCV replication inhibition assay without G418 treatment (Fig. 2E and Table 2)—to evaluate the HCV replication competence of each of these two mutations (D2292E, T1280V). It is difficult to fully explain all of the results, and comparison of the two CsA-resistant clones (clone #6 and #7) leaves some questions unanswered. These clones were similar to each other when considering survival during CsA and G418 dual-treatment (Fig. 1B), but they show differences in their resistance in HCV sub-genome replication assay (Fig. 2B and E). Apparently, each mutation in clone #7, except for D2292E, had no effect on the results of the HCV sub-genome replication inhibition assay with CsA. These findings might suggest that these mutations were important to G418 resistance, but not to the resistance of HCV to CsA treatment. In contrast, each of three other mutations in NS5A (D2303H, S2362G, and E2414K) that were found in clone #6 were required for the maximum level of drug resistance conferred by a mutant NS5A in this study. To our knowledge, D2303H is a novel CsA-resistant mutation, and as a single mutation, it conferred CsA resistance comparable to D2292E. D2303H, like D2292E, was located in carboxy-terminal of domain II of NS5A, which is reportedly a CypA binding site [9]. S2362G and E2414K were mutations in domain III of NS5A, and these mutations may have influenced the peptidyl-prolyl isomerase enzymatic catalytic activity of CypA [22]. The V1681A mutation in NS4A identified in clone #6 greatly enhanced the CsA resistance of a HCV construct that had NS3 and NS5A replaced with Cs6#6 sequences (Fig. 2B–D). Though

Table 1
The list of each mutated amino acid sequences in 16 clones throughout whole non-structural region.

		NS3						4A	4B	NS5A										5B
A.A. No.		1062	1275	1280	1560	1609	1612	1681	1797	2109	2179	2197	2231	2269	2292	2303	2320	2362	2387	2414	2992
p5.1		V	D	T	S	K	I	V	I	D	S	P	L	S	D	D	K	S	S	E	M
Cs6#6	1	V	D	V	G	K	T	A	I	D	S	P	L	S	D	D	K	G	S	K	M
	2	V	D	V	S	E	I	V	I	N	S	P	L	S	D	D	K	S	S	E	M
	3	V	D	V	G	K	T	A	I	D	S	P	L	S	E	H	K	G	S	K	M
	4	V	D	V	G	K	T	A	I	D	S	P	L	S	E	H	K	G	S	K	M
	5	V	D	V	G	K	T	A	I	D	S	P	L	S	E	H	K	G	S	K	M
	6	V	D	V	G	K	T	A	I	E	S	P	L	S	E	H	K	G	S	K	M
	7	V	D	V	G	K	T	A	I	E	S	P	L	S	E	H	K	G	S	K	M
	8	V	D	V	G	K	T	A	I	D	S	P	L	S	E	H	K	G	S	K	M
Cs6#7	1	I	G	V	S	E	I	V	I	N	S	P	P	P	E	D	K	S	P	G	T
	2	V	D	V	S	E	I	V	I	N	S	P	P	P	E	D	K	S	P	G	T
	3	I	G	V	S	K	I	V	V	N	P	L	L	S	E	D	T	S	S	E	M
	4	I	G	V	S	K	I	V	V	D	P	L	L	S	E	D	M	S	S	E	M
	5	I	G	V	S	K	I	V	V	D	P	L	L	S	E	D	T	S	S	E	M
	6	I	G	V	S	K	I	V	V	D	P	L	L	S	E	D	T	S	S	E	T
	7	I	G	V	S	K	I	V	V	D	P	L	L	P	E	D	K	S	P	G	T
	8	V	D	V	S	E	I	V	I	N	S	P	P	P	E	D	K	S	P	G	T

The two gray-highlighted lines were selected as the representative sequences of CsA_6μM_#6 and #7 and used to generate the derivative constructs.

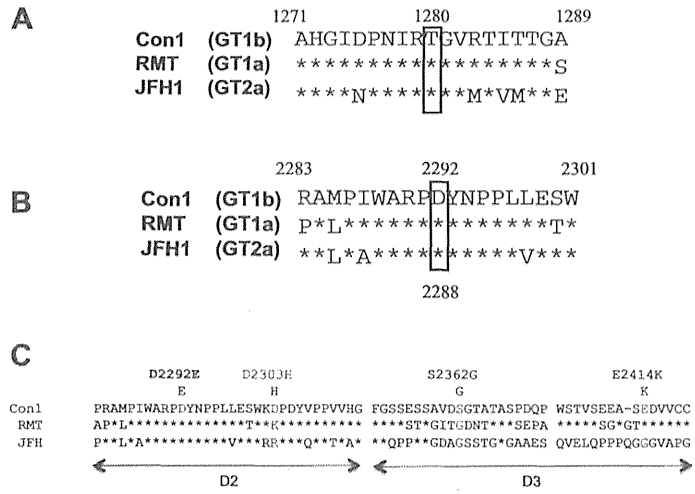


Fig. 4. Amino acid sequences of HCV-RMT-tri (GT1a) and HCV-JFH1 (GT2a) around (A) T1280V and (B) D2292E. (C) Location of the CsA resistant mutations in NS5A. Amino acid sequences around the positions of four CsA resistant mutations.

Table 2
Evaluation of resistance to CsA of mutants that have single mutations or combinations of multiple mutations.

	Mutations		IC_{50} (μm)	Fold change
	NS3	NS5A		
RMT-tri (RMT, GT1a)	–	–	0.79	1.0
	–	D2292E	2.1	2.7
	T1280I	–	0.96	1.2
	T1280I	D2292E	2.46	3.1
	T1280V	–	0.91	1.2
	T1280V	D2292E	2.54	3.2
JFH1 (JFH1, GT2a)	–	–	0.49	1.0
	–	D2292E	1.3	2.7
	T1280I	–	0.51	1.0
	T1280I	D2292E	1.38	2.8
	T1280V	–	0.69	1.4
	T1280V	D2292E	1.2	2.4

Threonine at site 1280 (RMT-tri or JFH1) were mutated to isoleucine (adaptive mutation of Con1 replicon) or valine (major mutation of CsA resistant clones). Aspartic acid at 2292 was mutated to glutamic acid.

Table 3
Evaluation of amino acid mutations in NS5A that conferred CysA resistance.

	Mutations in NS5A				IC_{50} (μm)	Fold change
	D2292E	D2303H	S2362G	E2414K		
Con1_5.1 (GT1b)					0.11	1.0
	○				0.88	7.9
		○			0.52	4.7
			○		0.12	1.0
				○	0.30	2.7
		○	○	○	1.0	9.4
	○	○			1.8	16.6
	○		○		0.95	8.5
	○			○	1.5	13.1
	○	○	○	○	2.80	25.7

we have not assessed V1681A as single mutant, analyzing its mechanism of CsA resistance and its cooperation with other mutations in NS3 and NS5A must be worthwhile because V1681A greatly enhanced the CsA resistance of some constructs.

In all, we evaluated three cyclophilin inhibitors—CsA, NIM811, and Debio-025. Among them, Debio-025 showed the strongest inhibition (IC_{50} values to any mutants) and was tolerated by CsA-resistant mutations (IC_{50} index change values, Fig. 2 and

Table 4
Evaluation of amino acid mutations in NS5A that conferred NIM811 resistance.

	Mutations in NS5A				IC_{50} (μm)	Fold change
	D2292E	D2303H	S2362G	E2414K		
Con1_5.1 (GT1b)					0.054	1.0
	○				0.324	6.0
		○			0.184	3.4
			○		0.056	1.0
				○	0.125	2.3
		○	○	○	0.455	8.4
	○	○			0.635	11.8
	○		○		0.403	7.5
	○			○	0.599	11.1
	○	○	○	○	0.923	17.1

Table 5
Evaluation of amino acid mutations in NS5A that conferred Debio-025 resistance.

	Mutations in NS5A				IC_{50} (μm)	Fold change
	D2292E	D2303H	S2362G	E2414K		
Con1_5.1 (GT1b)					0.024	1.0
	○				0.095	4.0
		○			0.074	3.1
			○		0.028	1.2
				○	0.024	1.8
		○	○	○	0.139	5.8
	○	○			0.198	8.3
	○		○		0.139	5.8
	○			○	0.185	7.8
	○	○	○	○	0.263	11.0

Table 3–5). It was interesting that the resistant mutants differed so greatly in their tolerance of these three inhibitors because all three inhibitors have the same mode of action. Garcia-Rivera et al. concluded that CsA resistance of HCV mutants were solely derived from dependence of the NS5A proteins on cyclophilins [28]. Our results might indicate that other factors are important to CsA resistance, in addition to residual cyclophilin activity.

Drugs that are intended to treat chronic HCV infection and that target important nonstructural HCV proteins—the serine protease NS3/4A, the large phosphoprotein NS5A, or the RNA-dependent RNA polymerase NS5B—have reached the clinical trial stage of drug development [32–34]. Two oral HCV protease inhibitors were approved by the FDA, and some of the drugs could achieve a sus-

tained virologic response (SVR) [35]. However, to develop treatments that eradicate individual chronic HCV infections, additional studies on the emergence of drug-resistant HCV mutants and on the molecular interactions at HCV replication complexes are necessary.

Our new findings provided insights into the way by which HCV acquires resistance to cyclophilin inhibitors, and these insights will facilitate the development of this type of anti-HCV drug for clinical use.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2014.04.053.

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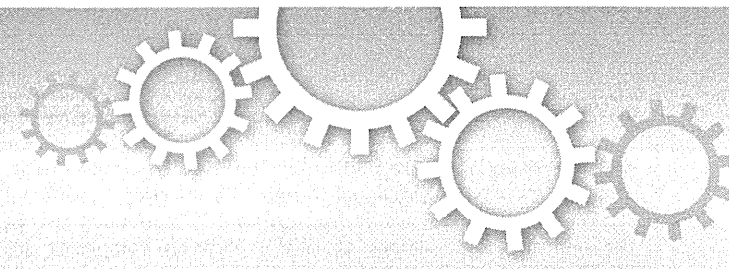

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In vivo therapeutic potential of Dicer-hunting siRNAs targeting infectious hepatitis C virus.

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The development of RNA interference (RNAi)-based therapy faces two major obstacles: selecting small interfering RNA (siRNA) sequences with strong activity, and identifying a carrier that allows efficient delivery to target organs. Additionally, conservative region at nucleotide level must be targeted for RNAi in applying to virus because hepatitis C virus (HCV) could escape from therapeutic pressure with genome mutations. *In vitro* preparation of Dicer-generated siRNAs targeting a conserved, highly ordered HCV 5′ untranslated region are capable of inducing strong RNAi activity. By dissecting the 5′-end of an RNAi-mediated cleavage site in the HCV genome, we identified potent siRNA sequences, which we designate as Dicer-hunting siRNAs (dh-siRNAs). Furthermore, formulation of the dh-siRNAs in an optimized multifunctional envelope-type nano device inhibited ongoing infectious HCV replication in human hepatocytes *in vivo*. Our efforts using both identification of optimal siRNA sequences and delivery to human hepatocytes suggest therapeutic potential of siRNA for a virus.

Hepatitis C virus (HCV) is a major etiological agent that causes chronic hepatitis, liver cirrhosis, and hepatocellular carcinoma. Despite clinical improvements provided by combination therapy with interferon-alpha and ribavirin, this therapeutic approach fails in about half of the patients[1]. Clinical proof-of-concept studies for new therapeutic agents have been reported, and several compounds have progressed into preclinical studies[2,3]. However, drug-resistant viruses appear to emerge readily in response to pharmacological selection by novel protease and RNA polymerase inhibitors. Therefore, the development of new therapies for refractory and diverse HCV genotypes represents a major public health objective.

RNA interference (RNAi) results from sequence-specific post-transcriptional gene silencing by double-stranded RNA[4,5]. The effectors of RNAi are short interfering RNA (siRNA) duplexes (~21–23 nt), which play a key role in the specific degradation of target mRNA. Recent studies have shown that a critical challenge in therapeutic application of RNAi is the identification of potent siRNAs; the functionality of these molecules is affected by the duplex nucleotide base preference and the accessibility of the target RNA[6,7,8,9,10]. Additionally, in the treatment of HCV, the emergence of "escaped/resistant" viruses that harbor point mutations in the target region is a major concern in the potential clinical application of RNAi[11,12,13]. Although current algorithms for the selection of anti-viral siRNAs consist of the guidelines previously derived for conserved sequences of viral genomes[14], there is compelling evidence that one siRNA-resistant human immunodeficiency virus (HIV)-1 had no mutations in the target site of the mRNA, but instead contained a point mutation 7 nt upstream of the target site[15]. This observation means that non-target mutations altering the local RNA secondary structure could ablate the RNAi activity without mutation in the siRNA target sequence. Additionally, there are reports that the secondary structure of target sites in mRNAs strongly reduce siRNA-mediated RNAi activity[16,17], hence the accessibility of certain local target structures on the mRNA is an important determinant in the gene silencing ability of siRNAs[18]. Therefore, the prediction of an effective siRNA targeting to virus genome cannot be identified

in the same fashion. Here, we attempt to predict active siRNA sequences using Dicer recognition in a conserved and highly ordered region of the HCV genome.

Separately, delivering siRNAs to intracellular targets also remains a major obstacle. Over the last several years, significant efforts have been devoted to exploring novel delivery strategies; examples include cationic liposomes and polymer-based nanocarriers[19]. To overcome the problems associated with *in vivo* delivery of siRNAs, both biodistribution to the target organ and intracellular trafficking in target cells of nanocarriers need to be addressed. High siRNA-encapsulation efficacy and uniform particle size also are required. We describe here an improved delivery system consisting of a multifunctional envelope-type nano device (MEND)[20], in which siRNA is encapsulated by cationic charged lipid envelope. To avoid the undesired interaction of cationic MENDs with biological components and subsequent loss of activity, a pH-sensitive property was incorporated into the lipid envelope of MEND by using a novel pH-sensitive cationic lipid, YSK05[21]. For enhanced delivery of cargos into cells, pH-sensitive liposomes have been investigated since the mid-1980s[22,23]. Recently, significant progress has been made in *in vivo* systemic siRNA delivery with lipid nanoparticles (LNPs) composed of ionizable cationic lipids. These LNPs represent neutral surface at physiological pH, but convert to a cationic form under acidic conditions (as expected in the endosome); siRNAs delivered by this mechanism provide efficient reduction of target gene expression in liver[24]. In this study, we describe the development of liver-targeted MENDs containing YSK05 for delivery of the active siRNAs, a system with therapeutic potential for the treatment of HCV-infected liver.

Results

Dicer-hunting siRNA targeting the HCV IRES has powerful silencing efficacy. The most conserved sequences among different HCV genotypes are 5′ untranslated region (UTR). The HCV 5′ UTR forms an RNA folded structure which has functional for the internal ribosome entry site (IRES)[25], and then allows protein synthesis to proceed in a cap-independent manner, implying an important role in key step of the viral translation and replication. Therefore, siRNAs targeting the IRES are expected to reduce the chances of viral mutational escape because the conserved 5′ UTR are likely to contain both structurally and functionally constrained elements (**Fig. 1a**). As the HCV IRES has local higher order structures at the RNA level, random sequence of siRNA targeting to the region might not exclusively induce RNAi activity. To identify an effective siRNA targeting the IRES sequences, we previously tested the efficacy of several synthetic siRNAs using an *in vitro* HCV-replicon assay, revealing that the siE sequences had an IC50 of 167 pM in this assay[26]. In addition, we found that the Dicer-generated siRNAs (d-siRNAs) targeting in the IRES not only provided silencing for heterogeneous target mRNA, but also exhibited even stronger silencing for homogeneous target HCV RNA[26] (**supplementary Fig.1**). Thus, we suspected that d-siRNAs contain powerful siRNA sequences, and/or that d-siRNAs, comprised of a library of several siRNAs, are additive for silencing activity. Previous work has shown that dsRNAs that are longer than 21-mer siRNAs (e.g., 27-mer dsRNAs[27] or 29-mer shRNAs[28]) display enhanced potency in RNAi. We speculated that these longer dsRNAs serve as substrates for the Dicer endonuclease, directly linking the production of siRNA to incorporation into the RNA-induced silencing complex (RISC), for example via the RISC-loading complex[29]. Therefore, we sought to identify the active siRNA sequences in a library of d-siRNAs. Specifically, we screened an siRNA cleavage site of the target HCV genome by using two distinct 5′-rapid amplification of cDNA ends (RACE) methods. The first RACE method employed RNA oligo ligation, such that the 5′-end of a HCV RNA (following cleavage by RISC) was ligated to an RNA oligomer (44 bases); the resulting molecule then was subjected to cDNA synthesis, nested-PCR amplification, and sequencing (**Fig. 1b**). The second RACE method employed C-tailing, such that a series of C nucleotides were attached at the 3′-end of the synthetic cDNA; the resulting molecule was then annealed with an abridged anchor primer and subjected to nested-PCR amplification and sequencing (**Fig. 1c**). We first validated the ability of these RACE methods to detect siRNA-mediated cleavage. Synthetic siE was transfected into REF cells[30] (which harbor the divided-full genome replicon), and total RNA was purified from the transfected cells. Using the two independent 5′-RACE methods, we demonstrated that a unique site, at the 5′-end of the HCV genome, was cleaved following treatment with the siE (**Fig. 1d**). The cleavage site on the target HCV RNA genome was located 10 nt downstream of the 5′-end of the guide siE in 41 of 48 clones. Previous reports show that RISC cleaves the mRNA at a site precisely 10 or 11 nt downstream of the 5′ end of the siRNA guide strand[31]. Thus, our results confirm the utility of RACE for identifying the 5′-end of the cleaved target mRNA during silencing. Next, we determined the 5′-end of HCV genome cleaved by the d-siRNAs, which consist of several kinds of siRNA products generated *in vitro* by activity of the Dicer endonuclease. Treatment with the d-siD5-50 yielded six separate cleavage sites across the 50-nt-long target region of the HCV RNA genome (**Fig. 1e**). Treatment with the d-siD5-197 yielded five separate cleavage sites across the 197-nt-long target region of the HCV RNA genome (**Fig. 1f**). Notably, the si197-1 site, which was observed among the majority of d-siD5-197 clones, was identical to the si50-15 site. Additionally, note that we could not detect the HCV-specific siE site in cloning of d-siD5-50 and d-siD5-197 cleavage sites, although the siE site had exhibited superior silencing efficacy in previous studies[26].

siRNAs, which consist of duplexes of 21-nt RNAs that are base-paired with 2-nt 3′ overhangs, were designed based on the cleavage site defined by d-siRNAs; these siRNAs therefore were designated as Dicer-hunting siRNAs (dh-siRNA). We synthesized a series of 10 individual dh-siRNAs and transfected each into R6FLR-N replicon cells having with the reporter gene (**Fig. 1g** and **1h**). Compared to the efficacy of the siE, several of these individual dh-siRNAs showed strong silencing activity against HCV replication. The efficacy of the si197-1 was three times higher than that of siE; the other two dh-siRNAs silenced HCV replication with efficacy two times higher than that of siE.

Currently, software for efficient siRNA design of antiviral RNAi are available (siVirus[14], siDirect[32], Block-iT (Invitrogen), and the web-based antiviral siRNA design software). These programs make prediction based on highly conserved regions of divergent viral sequences, and claim to minimize off-target effects that might result from effects on the molecular mechanism of RISC assembly or from the sequence preferences of the RISC endonuclease. In the HCV RNA genome sequences, we predicted an effective siRNA targeting the IRES (nt 199-395) using the three commercial software programs. We compared a software-proposed siRNA as previously reported with dh-siRNAs we report here. The values of IC50 for the predicted siRNAs in HCV-replicon assays are presented in **Fig. 1i**. In the IRES region, the siBlock-iT software proposed four kinds of siRNAs; siDirect proposed seven kinds of siRNAs; and siVirus proposed no siRNA. Five kinds of dh-siRNA were proposed by either siBlock-iT or siDirect software, and the dh-si50-11 was proposed by both siBlock-iT and siDirect software. However, the first- and second-most effective siRNAs, dh-si197-1 and dh-si197-6, were proposed by neither siBlock-iT nor siDirect software. These corresponded to molecules with IC50s of 61 and 66 picomolar (respectively) in our replicon assays (**Fig. 1i**). These results indicated that dh-siRNA prepared based on the cleavage site of the target mRNA treated with Dicer-generated siRNAs were powerful siRNA target sites; commercial design software programs were not of service for siRNA prediction in the HCV IRES region. In the case of siRNAs targeting endogenous mRNA, precise processing by Dicer is not

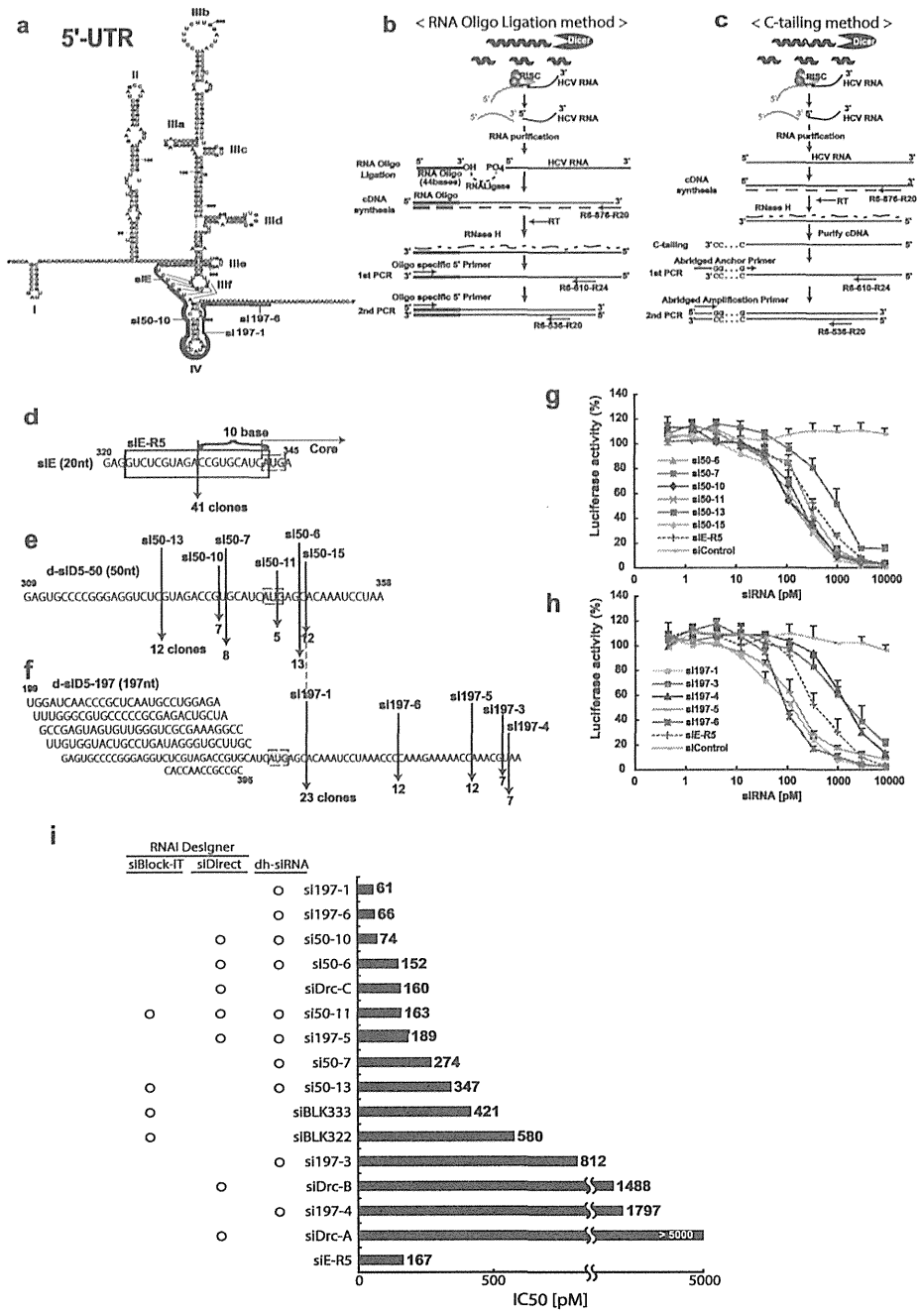

Figure 1 | Generation and efficacy of dicer-hunting siRNAs (dh-siRNAs). (a) Complicated secondary structure of the 5′-UTR in the HCV genome[50] and representation of the potent siRNA sequences we identified (si50-10, si197-1, and si197-6). (b, c) 5′-RACE strategy for identifying the RNAi cleavage sites of the target HCV genome using the RNA oligo ligation method (b) and the C-tailing at the 3′-end of RNA method (c). (d–f) Representation of the cleavage sites by siRNA and the number of clones in the sequences of HCV RNA genome that were used as templates. Cleavage site of the HCV RNA genome by siE (d), d-siD5-50 (e), and d-siD5-197 (f) was identified using the two RACE methods (2b and 2C). (g, h) Evaluation of silencing efficacy of dh-siRNAs. The HCV-replicon cells with reporter genes were transfected with the dh-si50 series (si50-6, 7, 10, 11, 13, and 15) (g) or the dh-si197 series (si197-1, 3, 4, 5, and 6) derived from d-si197 (h). Luciferase activity was measured after 48 hr. Data are presented as mean ± s.d. (n = 5) of values normalized to those obtained with mock-transfected cells. (i) Comparison of IC50 for inhibition of HCV replication by siRNAs that were derived from dh-siRNA or predicted by siRNA web design tools. Based on the HCR6 (genotype 1b) sequence, commercial software (siBlock-iT, siDirect) predicted several siRNA sequences (Supplementary table 1). IC50 values represent the mean for independent determinations (n = 5) using HCV replicon cells harboring subgenomic HCR6 sequences.

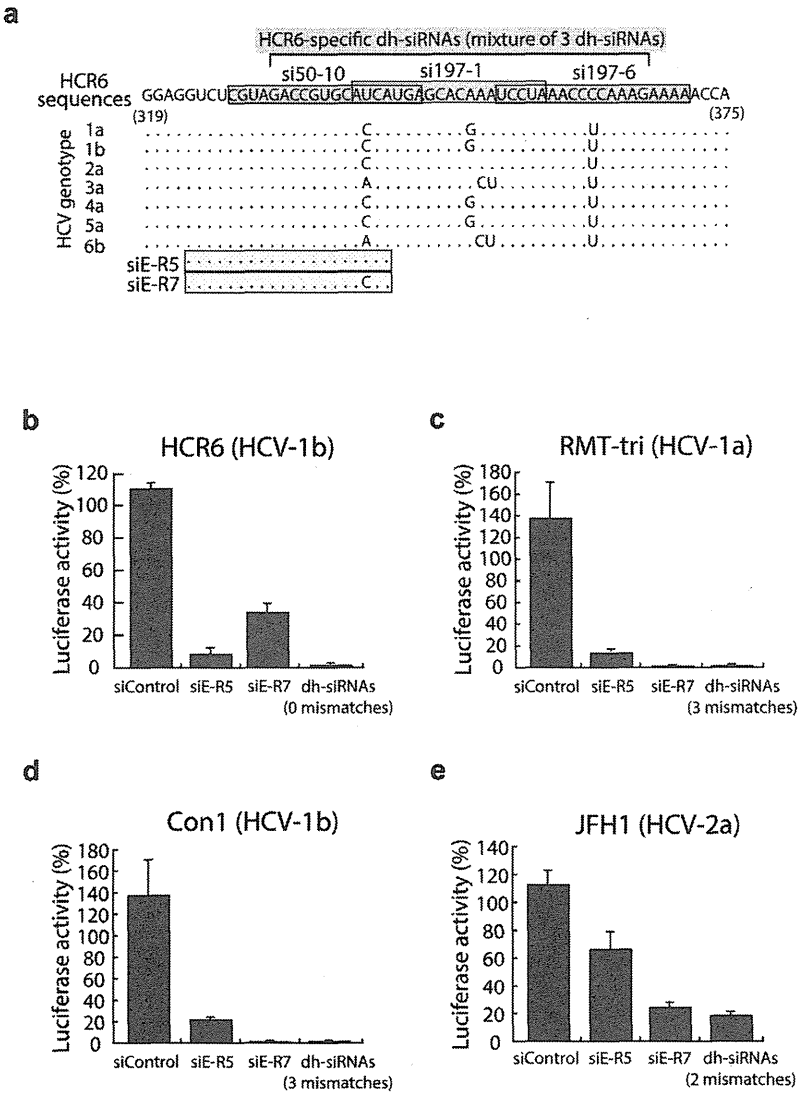

Figure 2 | Efficacy against heterogeneous viruses by mixtures of dh-siRNAs. (a) Alignment of HCV genotype sequences in the region targeted by the dh-siRNAs and siRNA sequences. Aligned sequences for siRNAs are indicated by shaded boxes; dots indicate matches to the consensus (HCR6); individual bases indicate genotype-specific differences. (b–e) Inhibition efficacy by mixture of three HCR6-based dh-siRNAs against homogeneous replicon cells (b), heterogeneous replicon cells with RMT-tri sequences (genotype-1b; 3 mismatches) (c), heterogeneous replicon cells with Con1 sequences (genotype-1b; 3 mismatches) (d), and heterogeneous replicon cells with JFH1 sequences (genotype-2a; 2 mismatches) (e). All samples were assessed at 72 hr after transfection. Data are presented as mean ± s.d. (n = 5) of values normalized to those obtained with mock-transfected cells.

critical because any cleavage frame would result in functional siRNAs; however the correlation of siRNA sequence with structurally/functionally constrained elements of HCV RNA is expected be critical for RNAi function.

Combination of the Dicer-hunting siRNA leads to enhanced reduction for heterogeneous HCV RNA. The notoriously error-prone replication of RNA viruses is a severe challenge for the development of siRNA-based anti-viral therapies. Indeed, HCV displays a high rate of mutation and is classified into distinct genotypes (1 to 6) (**Fig. 2a**). Therefore, to examine whether a mixture of dh-siRNAs can silence the replication of heterogeneous HCV RNA, we transfected HCR6 sequence-specific dh-siRNAs into heterogeneous replicon cells, specifically cells that harbor RMT-tri (genotype 1a), Con1 (genotype 1b), or JFH1 (genotype 2a) replicons (**Fig. 2b–e**). The combination treatment included si197-1, si197-6, and si50-10. All three of these sequences are HCR6-specific (genotype 1b), and the combination silenced the homogeneous HCV replication more effectively than the HCR6-specific siE-R5 alone (**Fig. 2b**). We next transfected a mixture of the dh-siRNAs into RMT-tri replicon cells, which harbor heterogeneous sequences compared to the HCR6 sequences. Treatment with the combination of three dh-siRNAs targeting the HCR6 sequences yielded a single mutation within the RMT-tri (genotype 1a) target sequences (**Fig. 2a**). The combination treatment (dh-si197-1, dh-si197-6, and dh-si50-10) represented a total of 3 mismatches versus the heterogeneous RMT-tri replicon but still provided silencing of heterogeneous RMT-tri replication. For comparison, siE-R5, which represents a single mismatch versus the RMT-tri target sequence, exhibited reduced silencing against the heterogeneous RMT-tri genome (**Fig. 2c**). Moreover, the replication of Con1 (another virus of genotype 1b) and JFH1 (a virus of genotype 2a) were silenced by the dh-siRNA combination treatment, with efficacy equal to or exceeding that of replicon-specific siE-R7 (**Fig. 2d** and **2e**). These results indicate that

a

	Initial MEND	Optimized MEND
Diameter (nm)	105±19	81±3
Polydispersity index	0.12±0.07	0.13±0.04
Zeta-potential (mV)	0.7±4.3	6.1±1.8
Encapsulation efficiency (%)	97±1	98±1
Apparent pKa	6.6	6.4

Data points are expressed as mean±s.d. (n=3).

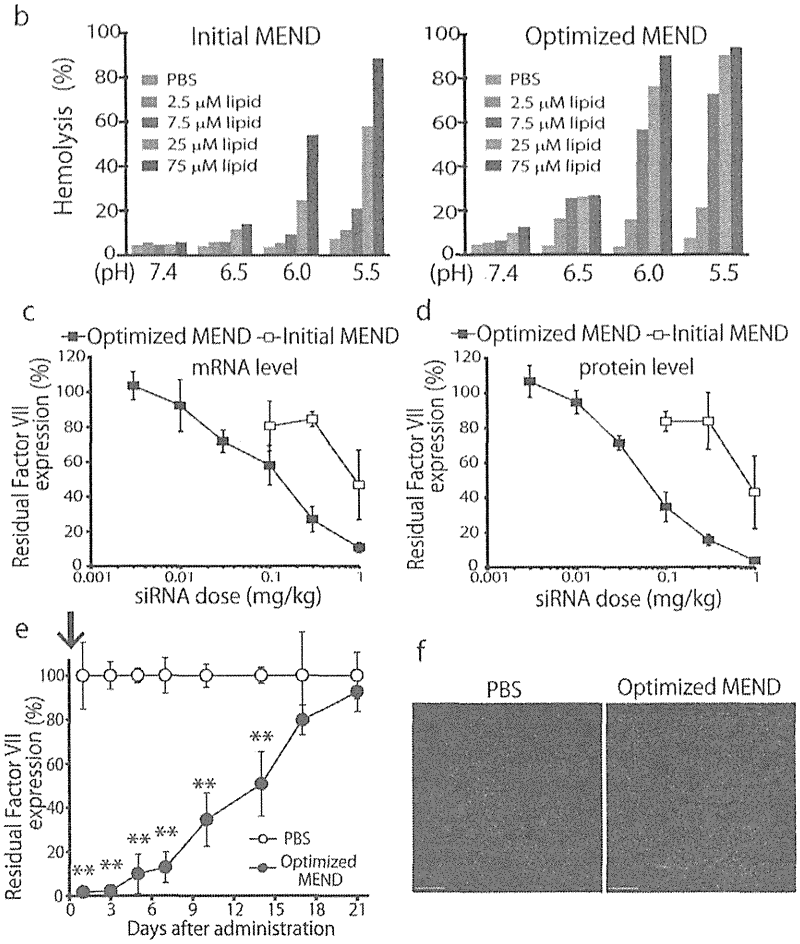

Figure 3 | *In vitro* and *in vivo* characterization of the optimized MEND. (a) Physical properties of multifunctional envelope-type nano devices (MENDs). (b) The membrane fusion activity of initial and optimized MENDs was assessed by an *in vitro* hemolysis assay. Values (normalized to hemolysis with known lysing agent (Triton)) are presented as the mean (n=3). N.C.; negative control (treated with PBS). (c, d) *In vivo* hepatic *Factor VII* mRNA (c) and serum Factor VII protein (d) levels at 48 hr after the administration of the initial and optimized MENDs. Data points are presented as the mean ± s.d. (n=3) of values normalized to those obtained with uninjected mice. (e) *In vivo* persistence of knockdown was investigated by monitoring serum Factor VII levels at the indicated number of days after administration of optimized MENDs at a dose of 1.0 mg siRNA per kg. **P<0.01. Data points are presented as the mean ± s.d. (n=3) of values normalized to those obtained with uninjected mice. (f) Liver tissues were collected after single injection of PBS or the optimized MENDs encapsulating Cy5-siRNA (red) and stained with FITC-isolectin B4 (green) and Hoechst33342 (blue) to detect blood vessels and nuclei (respectively). Bars represent 100 μm.

combination treatment with dh-siRNAs targeting the IRES sequence can silence and reduce HCV replication, even when applied to heterogeneous HCV genomes.

Characterization and optimization of pH-sensitive MENDs containing YSK05 for efficient siRNA delivery to hepatocytes. One of the challenges in developing RNAi therapeutics is the efficient delivery of siRNA into the cell affected by a given disease, for instance into the cytoplasm of HCV-infected hepatocytes. As a possible solution, we attempted dh-siRNAs delivery to hepatocytes using a pH-sensitive MEND containing YSK05, a pH-sensitive cationic lipid. The physical properties of siRNAs formulated in MENDs are shown in **Fig. 3a**. To examine the utility of MENDs containing YSK05 for the *in vivo* systemic delivery of siRNA to liver, mice were treated with siRNAs formulated in MENDs. We first screened the lipid composition to achieve efficient knockdown (**supplementary Fig. 2**). We found that MEND composed of YSK05, cholesterol, and PEG-DMG (at ratios of 70 : 30 : 3) provided the most efficient knockdown of target *srbI* gene in liver (**supplementary Fig. 2b**). Next, the membrane fusion activity of the optimized MEND was assessed by hemolysis assay[21] at the indicated pH to evaluate the potency for escape from endosomes via membrane fusion

(Fig. 3b). Membrane fusion activity for optimized MEND was increased compared to initial MEND at acidic pH. On the other hand, the apparent pKa of optimized MEND was comparable to that of the initial MEND (Fig. 3a). Factor VII is a secreted protein that can be readily measured in plasma, providing an index for the level of target gene knockdown in liver[24,33]. Therefore, we evaluated Factor VII knockdown using MENDs. As shown in Fig. 3c and 3d, initial and optimized MENDs decreased both liver mRNA and plasma level of Factor VII protein in a dose-dependent manner, with ED50s of approximately 0.8 mg/kg and 0.06 mg/kg (initial and optimized MENDs, respectively) for plasma levels of Factor VII. The results suggested that optimized MEND successfully induced the efficient knockdown in liver after systemic administration in mice. Next, the durability of knockdown of plasma Factor VII was determined. Single injection of optimized MEND was capable of mediating knockdown for at least 14 days at 50% inhibition (Fig. 3e). Finally, the distribution of siRNA was observed in the livers of animals treated with optimized MEND encapsulating Cy5-labeled siRNA. As shown in Fig 3f, MEND uniformly delivered siRNA to parenchymal cells in liver. These results suggested that optimized MENDs loaded with HCV-targeting dh-siRNAs can induce silencing against cytosolic replicating HCV. Therefore, further investigation was performed using the optimized MEND.

Silencing infectious HCV *in vivo*. We recently demonstrated the use of a novel mouse model for *in vivo* infection with hepatitis viruses[34] (Fig. 4a). Specifically, the model consisted of severe combined immunodeficient (SCID) mice, transgenic for urokinase-type plasminogen activator (uPA), which were transplanted with human hepatocytes. (In the following text, this model will be referred to simply as "chimeric mice".) We also have reported that these chimeric mice are a robust animal model to evaluate the efficacy of interferon and other anti-HCV agents[35].

First, optimized MENDs encapsulating DY547-labeled siRNAs were administered intravenous (IV) to chimeric mice (Fig. 4b). After 30 minutes, accumulation of siRNA was observed in the livers of the chimeric mice. We noted that the intracellular distribution of siRNA was not patchy, but was instead diffusely organized in the cytoplasm, presumably reflecting endosomal escape following endocytosis[21].

Both the efficacy and tolerability of these dh-siRNA-loaded optimized MENDs are critical issues for potential human clinical usage. Therefore, we examined the toxicology (in the "humanized" livers of chimeric mice) of optimized MEND-siRNAs during repeat-dose administration. Specifically, dh-siRNAs were formulated in optimized MENDs and administered to chimeric mice for a total of four IV infusions (on days 0, 2, 4, and 6) at siRNA doses of 1 mg/kg (Fig. 4a). This treatment appeared to be well tolerated; human hepatocytes in the chimeric mice remained viable, as demonstrated by tracking of serum human albumin levels and serum alanine aminotransferase (ALT) levels (an indicator of liver toxicity) (Fig. 4c and 4d). Although a slight increase in ALT values was observed, the change was not additive over the course of successive infusions. (Fig. 4d). Consistent with the results, a mixture of the dh-si197-1, dh-si197-6, and dh-si50-10 (siHCVs) provided silencing of HCV replication (Fig. 4e). In contrast, a non-targeting control siRNA (siControl), also formulated in optimized MENDs, did not induce silencing of HCV replication (Fig. 4e). To evaluate the long-term efficacy of dh-siRNA-mediated silencing of HCV replication, we dosed chimeric mice with combined (three siRNAs; administered as IV infusions on days 0 and 3) and followed the animals for 2 weeks (Fig. 5a). As seen above for knockdown duration by systemic optimized MEND treatment (Fig. 3c), the combined siHCVs (dh-si197-1, dh-si197-6, and dh-si50-10, also formulated in optimized MENDs) suppressed HCV replication for 2 weeks (Fig. 5b); no adverse effects were seen in human serum albumin levels or body weights (Fig. 5c). At day 14, livers were also harvested and screened by immunohistochemistry (IHC) and for quantitation (via real-time RT-PCR) of hepatic HCV RNA. As shown in Figure 5d, intrahepatic HCV core protein (detected by IHC) was dramatically reduced by the siHCV treatment (PXB283-27 mouse) compared with the siControl treated mouse, in which HCV core protein was detected within human hepatocytes. RT-PCR[36] demonstrated that the siHCV treatment provided an approximately 25-fold reduction in hepatic HCV RNA levels (treatment vs. siControl; 1.34×10^3 vs. 3.36×10^4 copies/μg total RNA, respectively).

Separate experiments, using another HCV pathogenesis mouse model[37], demonstrated that siHCV (dh-si197-1) treatment improved HCV-induced liver inflammatory responses in conditional HCV transgenic mice (Fig. 5e).

Discussion

The distinct effector phase of the RNAi pathway has been the focus of considerable research. This step depends on cleavage of the target mRNA by the RISC following base-pairing with the antisense strand of siRNA[38]. Therefore, the prediction of an effective siRNA had been evaluated based on the molecular mechanism of RISC assembly or the sequence preferences of the RISC endonuclease. Numerous strategies have been published to select siRNA sequences for targeting endogenous mRNA, and the resulting sequences have proved effective in some applications. However, the concept may not be appropriate to select target genes from other organisms, such as viruses.

Viruses typically exist as populations harboring multiple sequence variants, making these organisms notorious for the ability to develop resistance and escape control. Thus the prediction of effective antiviral siRNAs requires additional factors such as conservation of target sequence preferences. Based the level of sequence conservation among different HCV genotypes, the IRES of the 5′ UTR has been proposed as an RNAi target site. As highly conserved sequences are likely to contain structurally or functionally constrained elements, it has been argued that local higher order structures in target mRNAs might restrict accessibility to RISC, and attenuate or abolish RNAi activity. There are reports that the secondary structure of target sites in mRNAs strongly reduce siRNA-mediated RNAi activity[16,17], hence the accessibility of certain local target structures on the mRNA is an important determinant in the gene silencing ability of siRNAs[18]. However, in general, RNA folding program such as mfold or sfold can predict more than one secondary structures for the same mRNA; it is difficult to know which of the proposed structures represents the real or the most frequent fold employed in the cell. Therefore, target mRNA structure is a criterion that cannot be easily defined nor confidently scored solely on the bases of *in silico* calculations.

To facilitate the prediction of highly active siRNA molecules in the HCV structurally elements, we used human Dicer endoribonuclease activity for preparation of certain siRNAs. Because RISC loading takes place in the context of the RISC-loading complex, which consists of an Argonaute protein, Dicer, and the dsRBD-containing protein TRPB in human cells[39], Dicer favorable siRNA preference might be influence on loading to the complex. With mapping the corresponding cleavage site by the endoribonuclease-prepared siRNAs with the long dsRNA of IRES region, potent siRNA candidates in the structurally element have been identified by 5′ RACE methods. Thereby, we speculate that the accessibility of Dicer to target mRNA also may be a critical factor. Indeed, siRNA sequences derived by our hunting-by-Dicer method may be more potent inducers of RNAi than the siRNAs predicted by commercial design software.

The optimized MEND was accumulated in liver to around 90% of the injected dose within 30 min (data not shown). Recently, it was reported that neutral liposomal systems acquire ApoE in circulation, which enhances uptake of the liposomes into hepatocytes by low-density lipoprotein (LDL) receptor (LDLR) expressed on the surface

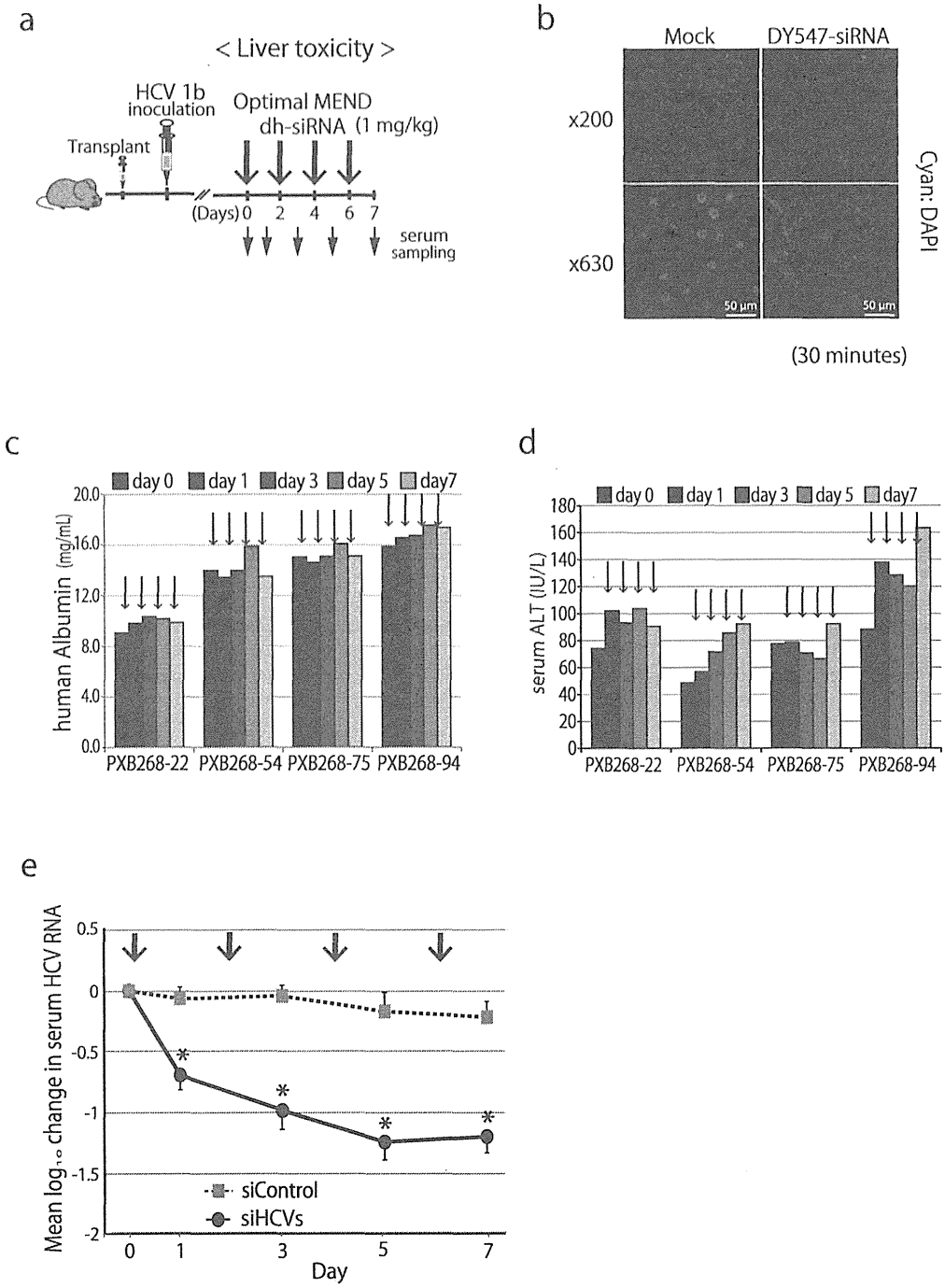

Figure 4 | Liver toxicity of siRNA formulated in optimized MEND in humanized chimeric mouse. (a) Schedule of treatment by dh-siRNA formulated in optimized MENDs in chimeric mice carrying human hepatocytes (PXB mice) infected with HCV genotype 1b (HCR6). The mice were administered intravenously with siRNA-loaded optimized MENDs by 4 repeat doses. All optimized MENDs were loaded with siRNA and administered to provide doses of 1 mg/kg. (b) Distribution characteristics of siRNA in chimeric mice carrying human hepatocytes. DY-547-labeled siRNA formulated in optimized MENDs was injected intravenously into the orbital veins of chimeric mice. The liver was observed by fluorescence microscopy at 30 min after injection. The nuclei were stained with DAPI. Mock: unlabeled siRNA formulated in optimized MEND. (c, d) Liver toxicity by administration of siHCVs (the HCV-specific dh-siRNAs formulated in optimized MEND). Four-time injection of siRNA formulated in optimized MEND into HCV-infected chimeric mice (individual animals: PXB268-22, PXB268-54, PXB268-75, and PXB268-94) was performed on days 0, 2, 4, and 6 (indicated by vertical arrows). Serum human albumin levels (c) and alanine aminotransferase (ALT) levels (d) were monitored for 1 week. (e) Four-time injection of siHCVs and siControl into HCV-infected chimeric mice was performed on days 0, 2, 4, and 6 (indicated by vertical arrows). The HCV genomic RNA change from baseline following treatment with siHCVs (n=4) or with siControl (non-targeting control siRNA formulated in optimized MEND, n=3) were shown; data points are presented as the mean ± s.d.

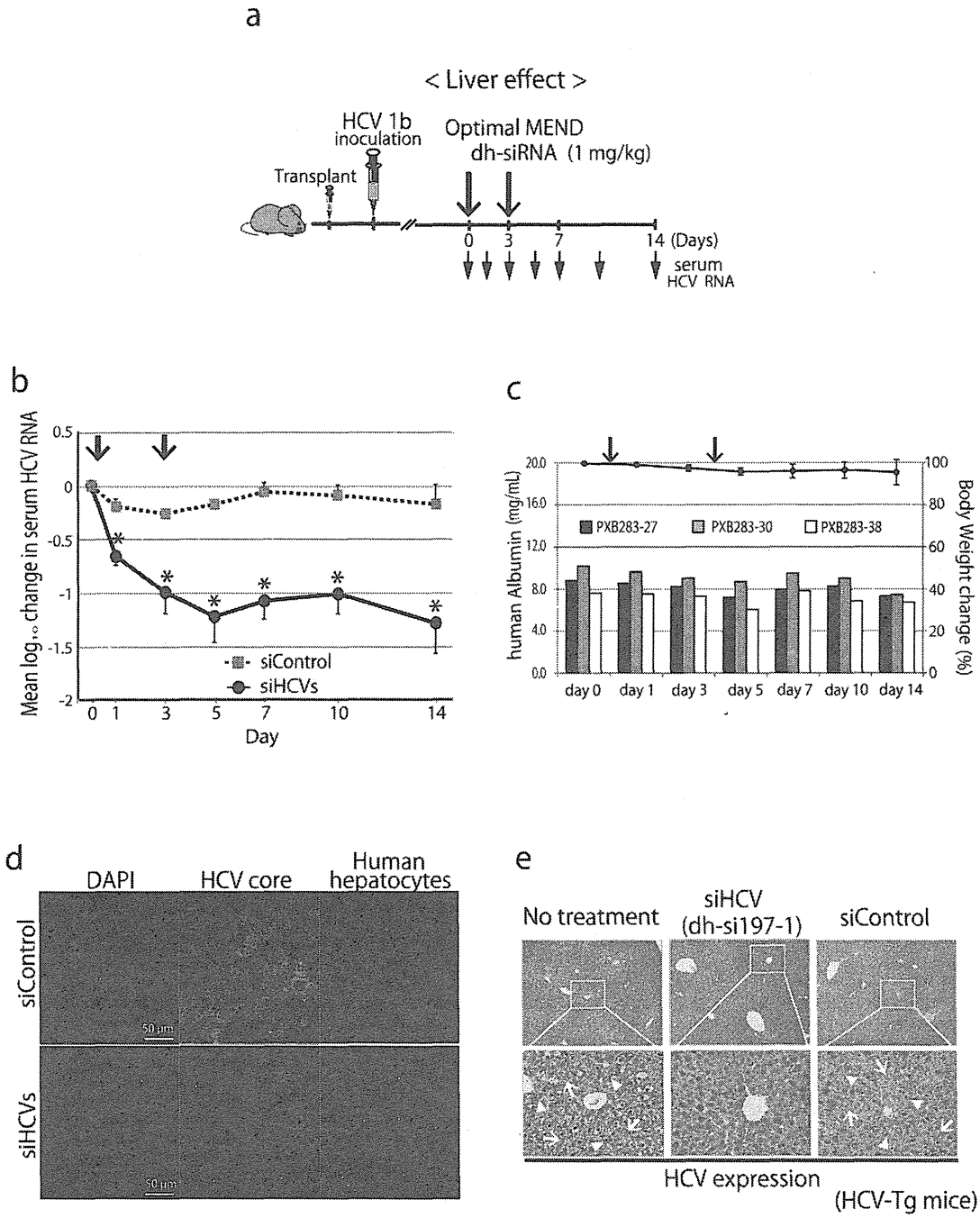

Figure 5 | Silencing efficacy of siRNA formulated in optimized MEND against ongoing infectious HCV RNA. (a) Schedule of treatment by dh-siRNA formulated in optimized MENDs in chimeric mice carrying human hepatocytes (PXB mice) infected with HCV genotype 1b (HCR6). The mice were administered intravenously with siRNA (1 mg/kg)-loaded optimized MENDs by 2 repeat doses. (b) Long-term silencing efficacy of siHCVs (the HCV-specific dh-siRNAs formulated in optimized MEND) against ongoing infectious HCV RNA. The HCV genomic RNA change from baseline in individual mice following treatment with siHCVs (n=3) or with siControl (n=3) were monitored for 2 weeks. (c) The serum human albumin levels (indicated as bar in left y-axis) in individual animals, as well as the change of body weight (indicated as line in right y-axis; plotted as mean + s.d. (across 3 animals)) over 2 weeks. (d) Intrahepatic analysis of chimeric mice infected with HCV. Two weeks after administration of siHCVs or siControl (injected on day 0 and day 3), chimeric mouse liver was harvested. The presence of human hepatocytes and HCV core protein were evaluated by immunohistochemistry. (e) dh-siRNA-mediated amelioration of HCV-induced liver damage in a murine model of inducible HCV. The inducible-HCV transgenic mouse model (HCV-Tg mice; see Materials and Methods) provides conditional expression of HCV core, E1, E2, and NS2 proteins. Six months after HCV induction, mice were treated by injection (on days 0 and 2) with optimized MENDs loaded with a single species of dh-siRNA (si197-1). On day 4, livers were harvested and assessed histologically (hematoxylin and eosin staining) for HCV-induced liver inflammatory responses. Degenerated liver tissue with diffuse inflammation and spotty necrosis was observed in the livers of the "no treatment" and siControl mice; treatment with si197-1-loaded optimized MENDs reduced HCV-induced liver damage. Arrows indicate necrosis; arrowheads indicate inflammation.

of hepatocytes[40,41]. It also was reported that the average diameters of sinusoidal fenestrae in C57CL/B mice and healthy human are 141 nm and 107 nm, respectively[42]. The average diameter of optimized MENDs was around 80 nm (Fig. 3a), which would permit optimized MENDs to pass through fenestrae and access hepatocytes. Therefore, we assume that MENDs containing YSK05 are taken up by hepatocytes by a process mediated by ApoE-LDL receptor association. This association presumably follows extravasation of MENDs from sinusoidal lumen to Disse through fenestrae, which results in widespread delivery of siRNAs to hepatocytes in healthy and transgenic mice, as well as to human hepatocytes in chimeric mice. The lipid composition for the initial MEND *in vivo* was chosen at YSK05/DSPC/cholesterol/PEG-lipid=50 : 10 : 40 : 3 mol%, as described previously[24,43]. Since DSPC, a helper lipid, was not essential to exert silencing in liver, DSPC was eliminated from lipid envelope (**supplementary** Fig. 2a). The pH-sensitive YSK05 lipid is presumed to be responsible for the endosomal release of the MEND cargo. Hence we tested the silencing activity of siRNA-loaded MENDs with increasing ratios of YSK05; activity was maximized at approximately 70 mol% YSK05. Based on our testing, a MEND composed of YSK05, cholesterol, and PEG-lipid at 70 : 30 : 3 was regarded as the optimized version. For systemic siRNA delivery using ionizable LNPs, lipid pKa value was identified as an important parameter, with optimum pKa in the range of 6.2–6.5[44]. In the present study, the pKa values of initial and optimized MENDs were determined as 6.6 and 6.4 respectively, which fell within this optimal pKa range. The endosomal escape of MEND is presumably mediated by membrane fusion. According to hemolysis activity, optimization of MEND improved fusion ability at acidic pH, but did not affect fusion at neutral pH. This optimization contributed to a 10-fold increase in silencing activity, yielding an ED50 of ~0.06 mg/kg in liver. Our results suggested that efficient siRNA delivery depends on the use of lipid-based nano-carriers that provide both optimized pKa and highly fusogenic characteristics. We also successfully delivered anti-miRNA oligonucleotide against miRNA-122 (miR-122) into hepatocytes using optimized YSK05-MEND, which resulted in efficient miR-122 knockdown and reduced plasma cholesterol level[45,46]. Our results permitted us to attempt delivery of dh-siRNAs to HCV-infected liver using the optimized MENDs. Our steady progress with a liver-targeting delivery system should facilitate the development of a safe and effective strategy for targeting HCV in hepatocytes in the near future.

Although our results were conducted in mouse models for HCV pathogenesis, results from these technologies are expected to provide therapeutic potential against infectious HCV *in vivo*, while also providing a new siRNA design tool for targeting viral seqeunces. Despite other obstacles (e.g., off-target effects), RNAi using these technologies provides a new potential therapeutic application that may effectively treat HCV infection.

Methods

Ethics statements. All *in vivo* experiments were approved by the Institutional Animal Care and Use Committee, and protocols for animal experiments were approved by the local ethics committee. The animals received humane care according to guidelines of the National Institutes of Health. Human patients provided informed written consent before sampling (collection of HCV-containing blood samples).

siRNAs. The design of HCV-directed siRNAs has been described previously[26]. Briefly, we designed nine siRNAs that target the 5′-UTR and 3′-UTR of the HCV genome and examined their efficacy in the *in vitro* inhibition of HCV replication. Of these nine siRNAs, the most effective siE was one directed toward nucleotides 323 to 342 of the HCV genome. The sequences for the sense and antisense strands of the siRNA are as follows: siE sense: 5′-GUC UCG UAG ACC GUG CAU CAU U-3′; antisense: 5′-UGA UGC ACG GUC UAC GAG ACU U-3′. siRNAs were generated by annealing equimolar amounts of complementary sense and antisense strands.

Anti-luciferase siRNA (siLuc) (sense: 5′-CCG UCG UAU UCG UGA GCA ATT-3′; antisense: 5′-UUG CUC ACG AAU ACG ACG GTT-3′) was purchased from Sigma (Ishikari, Japan). Anti-FVII siRNA (siFVII) (sense: 5′-GGAucAucucAAGucuuAcT*T-3′; antisense: 5′-GuAAGAcuuGAGAuGAuccT*T-3′; lower case letters indicate 2′-fluoro-modified nucleotides, asterisks indicate phosphorothioate linkages) were purchased from Hokkaido System Science Co., Ltd. (Sapporo, Japan).

Dicer-generated siRNAs. We generated the HCV-specific long dsRNA template for *in vitro* transcription by PCR-amplified DNA templates and synthesized Dicer-generated siRNAs (d-siRNAs) by cleavage with recombinant human Dicer (rhDicer; Gene Therapy Systems, San Diego, CA)[26]. By comparison with the silencing efficiency of d-siRNAs, d-siD5-50 and d-siD5-197 silenced the HCV replication more efficiently than synthetic siE (Supplementary information Fig. 1). The template dsRNAs of d-siD5-50 and d-siD5-197 were located at nucleotides 309–358 and 199-395 of HCR6 sequence (GenBank accession number AY045702).

siRNAs predicted by the commercial software. The siRNA design algorithms for antiviral RNAi, siVirus, siDirect and Block-iT (Invitrogen), were used for the selection of the target sequence for siRNA within the specified target HCV genome (nucleotides 199–395).

Cell culture and HCV-replicon assay. We used four HCV subgenomic replicon cell lines, FLR3-1 (genotype 1b, Con-1)[47], R6FLR-N (genotype 1b, strain N)[26], JFH-1/FLR/K4 (genotype 2a)[48], and RMT-tri (genotype 1a)[49], which have the firefly luciferase gene for the sensitive and precise quantification of the HCV replication levels using a luciferase assay. We also used REF cells[30] which harbor the divided-full genome replicon for analysis of the HCV 5′ UTR sequences. Each cell line was seeded at a density of 5×10^3 per well in 96-well tissue culture plates, and grown (at 37°C and 5% CO_2) in complete Dulbecco's modified Eagle's medium supplemented with Glutamax I (Invitrogen, Carlsbad, CA) and containing 5% fetal calf serum (Invitrogen). Cells were transfected with 30 nM siRNA using RNAiMax (Invitrogen, Carlsbad, CA). After 72 hours, luciferase activity was determined in triplicate using the Steady-Glo or Bright-Glo luciferase assay kit (Promega Madison, WI). The luciferase signal was measured using an LB940 luminometer (Berthold, Freiburg, Germany) and the results were expressed as the mean percentage of control. IC_{50} values of siRNA were calculated by nonlinear curve-fitting using the equation: $Y=100-(Y_{Bottom} \times X/(IC_{50} + X))$, where Y represents percent inhibition and X represents the concentration of siRNAs.

5′-rapid amplification of cDNA ends (RACE) analysis. Replicon cells were transfected with synthetic HCV-specific siRNA (siE) and Dicer-generated siRNAs using Lipofectamine RNAiMAX or Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol. At 6 h post-transfection, total RNA from replicon cells was extracted using the acid guanidinium-phenol-chloroform method[26].

For the RNA oligo ligation method, 5 μg of total RNA was ligated to the GeneRacer RNA adapter (Invitrogen, 5′-CGA CUG GAG CAC GAG GAC ACU GAC AUG GAC UGA AGG AGU AGA AA-3′) without any prior processing. Ligated RNA was reverse transcribed into cDNA using the HCV-specific reverse primer (R6 876-R20 reverse primer: 5′- AGA GGA AGA TAG AGA AAG AG-3′). To detect cleavage product, semi-nested-PCR was performed as follows: first-run PCR used a primer complementary to the RNA adapter (GeneRacer 5′ Nested Primer: 5′-GGA CAC TGA CAT GGA CTG AAG GAG TA-3′) in combination with an HCV-specific primer (R6 610-R24 reverse primer: 5′-CCC TCG TTG CCA TAG AGG GGC CAA-3′); second-run PCR used the same oligo specific primer (GeneRacer 5′ Nested Primer) in combination with a second HCV-specific primer (R6 536-R20 reverse primer: 5′-GAT AGG TTG TCG CCT TCC AC-3′).

For the C-tailing at the 3′-end of RNA method, first-strand cDNA synthesis was performed by using SuperScript II reverse transcriptase (Promega Corporation, Madison, WI) to transcribe total RNA (5 μg) with the HCV-specific reverse primer (R6 876-R20 reverse primer) according to the manufacturer's protocol. The resulting first-strand cDNA was subjected to another round of 5′-RACE using a distinct RACE kit (Cat. 18374-058, Invitrogen, Carlsbad, CA). The first-strand cDNA was tailed at the 3′-end by terminal transferase TdT and dCTP. The primer set consisted of an HCV-specific reverse primer (R6 610-R24) and the Abridged Anchor Primer for the first-run PCR, and an HCV-specific reverse primer (R6 536-R20) and the Abridged Universal Amplification Primer for the second-run PCR.

Amplification fragments obtained by the two independent 5′-RACE methods were resolved on 3.0% agarose and sized using a 1-kb Plus DNA Ladder (Invitrogen). Specific cleavage sites were further confirmed by DNA sequencing.

Dicer-hunting siRNA sequences design. Based on the cleavage site defined by d-siRNAs, reverse genetic approach was applied to the design of Dicer favourable siRNA sequence. A dh-siRNA consists of duplexes of 21-nt RNAs that are base-paired with 2-nt 3′ overhangs.

Preparation of MENDs. Cholesterol, 1,2-distearoyl-sn-3-phosphatidylcholine (DSPC), 1,2-dimyristoyl-sn-glycerol, and methoxyethyleneglycol 2000 ether (PEG-DMG) were purchased from Avanti Polar Lipid (Albaster, AL). The synthesis of YSK05 was performed as previously described[21]. MENDs encapsulating siRNAs were prepared by a *t*-BuOH dilution procedure. Lipid in 90% (v/v) *t*-BuOH was mixed with siRNA in 20 mM citrate buffer (pH 4.0) at siRNA/lipid ratio of 0.1 (wt/wt) under strong agitation to yield a final *t*-BuOH concentration of 60% (v/v). Then, the lipid/siRNA mixture was added into 20 mM citrate buffer (pH 4.0) under strong agitation to yield a final *t*-BuOH concentration of <12% (v/v). Ultrafiltration was performed to remove *t*-BuOH, replacing external buffer with phosphate-buffered saline (PBS, pH7.4) and concentrating the MENDs. A lipid envelope of initial MEND was