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 patients1. Clinical proof-of-concept studies for new therapeutic agents have been reported, and several compounds have progressed into preclinical studies2,3. 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 RNA4. 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 RNA5,6,7,8,9,10. 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 RNAi11,12,13. Although current algorithms for the selection of anti-viral siRNAs consist of the guidelines previously derived for conserved sequences of viral genomes14, 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 site15. 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 activity16,17, hence the accessibility of certain local target structures on the mRNA is an important determinant in the gene silencing ability of siRNAs18. 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 nanocarriers20. 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)29, 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, YSK0521. For enhanced delivery of cargos into cells, pH-sensitive liposomes have been investigated since the mid-1980s22,23. 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 liver24. 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 assay26. 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 studies26-29.

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 (siVirus4, siDirect5, 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 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.
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 to 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 (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.

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.
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. 3). Next, the membrane fusion activity of the optimized MEND was assessed by hemolysis assay at the indicated pH to evaluate the potency for escape from endosomes via membrane fusion.

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

| Diameter (nm)       | Initial MEND | Optimized MEND |
|---------------------|--------------|----------------|
| 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 pH | 6.6 | 6.4 |

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**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.
silencing of HCV replication (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. 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. 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.

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 mouse. 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.

Both the efficacy and tolerability of these dh-siRNA-loaded optimized MENDs are critical issues for potential human clinical usage. Therefore, we examined the toxicity of 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 demonstrated that the siHCV treatment provided an approximately 25-fold reduction in hepatic HCV RNA levels (treatment vs. siControl; 1.34 X 10^4 vs. 3.36 X 10^6 copies/μg total RNA, respectively).

Separate experiments, using another HCV pathogenesis mouse model, 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. 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, hence the accessibility of certain local target structures on the mRNA is an important determinant in the gene silencing ability of siRNAs. 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, 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 hepatocytes20,21. It was also reported that the average diameters of sinusoidal fenestrae in C57Cl/B mice and healthy human are 141 nm and 107 nm, respectively22. 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 facilitates 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 previously24,25. Since DSPC, a helper lipid, was not essential for 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 6.2–6.524. 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 levels18,26. 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 sequences. 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 previously26. Briefly, we designed nine siRNAs that target the 5'-UTR and 3'-UTR of the HCV genome and examined the efficacy of HCV RNAi replication. Of these nine siRNAs, the most effective siRNA 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: sense: 5'-GUCC UCG UAG AAC GGU AAC AAU C AU-3'; antisense: 5'-UGA UGC ACG GUC GUC AGC ACU U-3'. siRNAs were generated by annealing equimolar amounts of complementary sense and antisense strands.

Anti-luciferase siRNA (siluc) (sense: 5'-CCG UGG UAU UGG GUA AGA ATT-3'; antisense: 5'-UGU CUG ACG AAU ACG GGT T-3') was purchased from Sigma (Ishikari, Japan). Anti-FVII siRNA (siFVII) (sense: 5'-GGAGAUCuGAA-GACuAuT*T-3'; antisense: 5'-GuAGAAGuCuGAGAuT*G-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). After transfection of cells with the siRNAs, dsRNA was cleaved by Dicer with the silencing efficiency of d-siRNAs, d-siD5-30 and d-siD5-197 silenced the HCV replication more efficiently than synthetic siRNA (Supplementary information Fig. 1). The template diRNAs of d-siD5-30 and d-siD5-197 were located at nucleotides 309–358 and 199–359 of HCR6 sequence (GenBank accession number AT1405702).

siRNAs predicted by the commercial software. The siRNA design algorithms for antiviral siRNA, 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)*, RF6LR-N (genotype 1b, strain N), JFH-1/FLR/K4 (genotype 2a)*, and MRT-1 (genotype 1a)*, 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 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 × 10^5 per well in 96-well tissue culture plates, and grown at 37°C and 5% CO2 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 RNAmax (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. IC50 values of siRNA were calculated by nonlinear curve-fitting using the equation: Y = 100-(Ymax × X/IC50 + 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 (siF) 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 method27.

For the RNA oligo ligation method, 5 µg of total RNA was ligated to the GeneRacer RNA adapter (Invitrogen, 5'-CGA CUG AGC GAC GAC ACU AGC AUG GCU UUA AUA-3') without any prior processing. Ligated RNA was reverse transcribed into cDNA using the HCV-specific reverse primer (R6 876-820 reverse primer: 5'-AGA GGA GAG TAG AGA AGG AG-3'). To detect cleavage product, semi-purified-PCR was performed as follows: first-run PCR used a primer complementary to the RNA adapter (GeneRacer 5' Nested Primer: 5'-GGC CAC CAA TAG TCT TTA GAG CAT A-3') in combination with an HCV-specific primer (R6 610-624 reverse primer: 5'-CTT ACC CGG ATG AGG GCA CAA-3'); second-run PCR used the same oligo specific primer (GeneRacer 5' Nested Primer) in combination with a second HCV-specific primer (R6 536-520 reverse primer: 5'-GAT TGG TCT GCC TCC AC-3'). In the C-tailing at the 5'-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 876-820 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 tailored at the 5'-end by terminal transferase TdT and cDTP. The primer set consisted of an HCV-specific reverse primer (R6 610-624) and the Abridged Anchor Primer for the first-run PCR, and an HCV-specific reverse primer (R6 536-520) 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 was a duplex of 21–23 nt that are base-paired with 2–3 3' overhangs.

Preparation of MENDs. Cholesterol, 1,2-distearyl-sn-3-phosphatidylcholine (DSPC), 1,2-dimyristoyl-sn-glycero-3-phosphocholine (DMPC), 1,2-distearoyl-sn-3-phosphatidylglycerol (DEG-DMG) were purchased from Avanti Polar Lipid (Alabaster, AL). The synthesis of YSK05 was performed as previously described28, 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 (w/w) under strong agitation to yield a final t-BuOH concentration of 60% (v/v). Then, the lipid/siRNA mixture was exposed to 20 mOsm NaCl solution and anti-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, pH 7.4) and concentrating the MENDs. A lipid envelope of internal MEND was
prepared using YSK05, DSPC, cholesterol, and PEG-DMA at a molar ratio of roughly 10:3:4:4 as described previously19,20, and optimized MEND was prepared using YSK05, cholesterol, and PEG-DMA at a molar ratio of 70:30:3.

Characterization of MEND. The average diameter and zeta-potential of MENDs were determined using a ZetaSizer Nano ZS ZEN5600 (MALVERN Instrument, Worcestershire, UK). siRNA encapsulation efficiency was determined using endogenous Ribogreen assay (Invitrogen Carlsbad, CA). MENDs were diluted in 10 mM HEPES buffer (pH 7.4) containing 20 µg/mL dextran sulfate and Ribogreen in the presence or absence of 0.1% (v/v) Triton X-100. Fluorescence was measured by Varispec Flash (Thermo scientific) with λex = 500 nm, λem = 525 nm. siRNA calculation was based on a siRNA standard curve. siRNA encapsulation efficiency was calculated by comparing siRNA concentration in the presence and absence of Triton X-100. The pKa of YSK05 in each MEND was determined using 6-(p-Toluidino)-2-naphthalenesulfonic acid (TNS). Thirty µM of MEND lipid and 6 µM of TNS were mixed in 200 µL of 20 mM citrate buffer, 20 mM sodium phosphate buffer, or 20 mM Tris-HCl buffer containing 130 mM NaCl at a pH ranging from 3.6 to 9.0. Fluorescence was measured using a Varispec Flash with λex = 321 nm, λem = 447 nm, at 37°C. The pKa values were measured as the pH giving rise to half-maximal fluorescent intensity.

Hemolysis assay. Fresh red blood cells (RBCs) were collected from ICR mice and suspended in PBS. The RBC suspension was mixed with indicated amount of MEND, incubated at 37°C for 30 min, and then centrifuged (4°C, 400 g, 5 min). The absorbance of the supernatant was measured at 545 nm. Positive and negative control samples were prepared by incubation of RBCs with 0.5% (v/v) Triton X-100 or PBS, respectively. The %hemolysis was expressed as the % of the absorbance of the positive control.

In vivo mouse Factor VII knockdown experiments. Male ICR mice (5–6 weeks old) were purchased from Japan SLC (Shizuoka, Japan). MENDs encapsulating siFVII were diluted to the appropriate concentrations in PBS (pH 7.4) and administered intravenously (IV); via the tail vein at a dose volume of 10 to 15 µL. At the indicated time points, blood and liver were collected. The blood was processed to plasma, and plasma levels of Factor VII protein were determined using a colorimetric Biophen VII assay kit (Aniaera) according to the manufacturer’s protocol. The standard curve for Factor VII plasma levels was generated using plasma collected from PBS-treated mice. Total RNA in liver was isolated using TRIzol (Invitrogen) according to the manufacturer’s protocol. The resulting RNA was reverse transcribed using a High Capacity RNA-to-cDNA kit (ABI) according to the manufacturer’s protocol. For each specimen, quantitative PCR analysis was performed on 2 ng of cDNA using Fast SYBR Green Master Mix (ABI) and a Lightcycler480 system II (Roche). Results were performed in a volume of 15 µL. The primers for mouse FVII were (forward) 5’-AGC GAT CCA TCA CCT TGA CAG AAC GGT GTG -3’ and (reverse) 5’-CCG ACC TTC AAA GTC TAG GAG GCA-3’.

In vivo microscopic observation. Optimized MENDs encapsulating Cy5-labeled siRNA were administered into male ICR mice (5–6 weeks old). Five mice were projected sacrifice, FITC-conjugated isocyanate B4 (40 µg/mouse) was intravenously injected via the tail vein to stain blood vessels. At 30 min after intravenously injection of optimized MEND, each animal was perfused with PBS to remove blood from the liver, and then with 4% paraformaldehyde for 5 min. Liver tissues were collected, fixed in 4% paraformaldehyde for 12 h at 4°C, dehydrated in ethanol, and embedded in paraffin. Sections were cut in 3 µm-thick sections (LEICA CM3000, Leica Microsystems, Wetzlar, Germany). The sections were stained with Hoescht33342 to detect nuclear DNA, and observed at an excitation wavelength of 633 nm using a laser-equipped Nikon A1 (Nikon Co., Ltd., Tokyo, Japan) with a 60x objective lens.

In vivo HCV infection experiments. We purchased chimeric mice from PhenixBio (Hiroshima, Japan). The chimeric mice were generated by transplanting human primary hepatocytes and a combination of CD45-negative, CD34-negative, CD14-negative cells using the week 6 mouse passagem activator transgene controlled by an albumin promoter (uPA/SCID). Six weeks after hepatocyte transplantation, each mouse was injected IV with patient serum containing 106 copies of HCV genotype 1b (HCR6; accession SCIENTIFIC). Each mouse was injected IV with patient serum containing 106 copies of HCV genotype 1b (HCR6; accession SCIENTIFIC). Six weeks after hepatocyte transplantation, each mouse was injected IV with patient serum containing 106 copies of HCV genotype 1b (HCR6; accession SCIENTIFIC).

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Author contributions
M.K. conceived the study. T.W., H.H., C.M. and Y.S. conducted the study equally. T.W. and H.H. coordinated the analysis and manuscript preparation. M.S. and H.H. had input into the study design and A.T., Y.H. and T.O. accomplished mouse management. M.A. and K.I. revised the manuscript for intellectual content. T.W., H.H., C.M. and Y.S. contributed equally.

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