SHORT COMMUNICATION

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Small Hairpin RNAs Efficiently Inhibit Hepatitis C IRES–Mediated Gene Expression in Human Tissue Culture Cells and a Mouse Model

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Treatment and prevention of hepatitis C virus (HCV) infections remain a major challenge for controlling this worldwide health problem; existing therapies are only partially effective and no vaccine is currently available. RNA interference offers the potential of a novel therapeutic approach for treating HCV infections. Toward this end, we evaluated small hairpin interfering RNAs (shRNAs) targeting the conserved internal ribosome entry site (IRES) element of the HCV genome for their ability to control gene expression in human cells and animals. We used a reporter gene plasmid in which firefly luciferase (fLuc) expression is dependent on the HCV IRES. Direct delivery of HCV IRES shRNAs efficiently blocked HCV IRES-mediated fLuc expression in transfected human 293FT cells as well as in a mouse model in which nucleic acids were delivered to liver cells by hydrodynamic transfection via the tail vein. These results indicate that shRNAs, delivered as RNA or expressed from viral or nonviral vectors, may be effective agents for the control of HCV and related viruses.

Key Words: molecular imaging, luciferase, viral therapy, biophotonics

Hepatitis C virus (HCV) infects more than 170 million people worldwide and is the leading indication for liver transplantation. Existing treatments, including ribavirin and PEGylated interferon-α, are effective only in 50–60% of cases and have substantial side effects. The development of more effective HCV treatments is hampered by the lack of a good small animal model, the inability to culture the virus stably in tissue culture cells, and the high viral mutation rate [1,2]. However, the availability of an HCV replicon system has allowed the study of HCV replication and host–cell interactions and evaluation of antiviral agents, and more recently, a transgenic chimeric humanized mouse liver model that allows full HCV infection was developed [3,4]. Moreover, the use of *in vivo* imaging of HCV internal ribosome entry site (IRES)-dependent reporter systems has facilitated the evaluation of delivery and inhibition by anti-HCV agents in mouse liver over multiple time points using the same animals [5]. These tools have advanced our ability to develop and test novel therapeutic strategies such as the use of RNA interference.

RNA interference uses an evolutionarily conserved pathway that leads to down-regulation of gene expression. The discovery that synthetic short interfering RNAs (siRNAs) of ~19–21 bp can effectively inhibit gene expression in mammalian cells and animals without activating an immune response has led to a flurry of activity to develop these inhibitors as therapeutics. Chemical stabilization of siRNAs results in increased serum half-life [6], suggesting that intravenous administration may achieve positive therapeutic outcomes if delivery issues can be overcome. Furthermore, small hairpin RNAs (shRNA), as surrogates of siRNA, have also shown robust inhibition of target genes in mammalian cells and can be easily expressed from bacteriophage (e.g., T7, T3, or SP6) or mammalian Pol III promoters (e.g., U6 or H1), making them excellent candidates for viral delivery [7]. A substantial effort has been made to find effective nucleic acid-based inhibitors of HCV (reviewed in [3]). These efforts include "traditional" antisense oligonucleotides, antisense phosphorodiamidate morpholino oligonucleotides, ribozymes, and, more recently, siRNAs. A number of research groups have shown that

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siRNAs can effectively target HCV in human tissue culture cells [8–12] and in animal systems [13]. However, there have not been reports of the effects of direct delivery of shRNAs on target gene inhibition in animals to date.

We designed shRNAs (Fig. 1A) and synthesized them *in vitro* using DNA templates to target a conserved region of the HCV IRES (containing the AUG translation start site) and tested their ability to inhibit HCV IRES-mediated reporter expression in human tissue culture cells and a mouse model. The shRNAs contain a mir-23 micro-RNA loop structure that was previously suggested to facilitate cytoplasmic localization [14] and a 25-bp RNA stem with an additional 2 nucleotides at both the 5′ (2 guanosines) and 3′ (2 uridines) ends that may also interact though non-Watson–Crick G•U base pairings.

To test the ability of the HCV shRNAs to inhibit HCV IRES-mediated gene expression, we cotransfected human 293FT cells with a pHCV Dual Luc expression plasmid, secreted alkaline phosphatase expression plasmid (pSEAP2; to control for efficiency of transfection and nonspecific effects), and *in vitro* synthesized shRNAs. The pHCV Dual Luc expression plasmid contains the hepatitis C IRES target sequence; firefly luciferase (fLuc) activity is dependent on internal translational initiation from this IRES. The HCV shRNA (HCVwt) targeting the region of the IRES immediately downstream of the AUG translation start site strongly inhibits HCV IRES-mediated fLuc expression in human 293FT cells (Fig. 1B). This inhibition was observed regardless of whether the shRNA was delivered directly as RNA (Fig. 1B) or expressed from a transfected Pol III expression plasmid (data not shown). Dose–response experiments revealed the high potency of HCVwt shRNA inhibition of HCV IRES–dependent gene expression, yielding an IC_{50} of ~200 pM (Fig. 1B, diamonds). We observed little or no inhibition with HCVmut1/2 shRNA below the 9 nM level nor any effect on SEAP levels (Fig. 1B, squares, and data not shown). We also observed no inhibition with an unrelated 229 (TNF) shRNA (data not shown). The 229 shRNA is highly effective at inhibiting TNF expression in 293FT and other cell lines ([15] and data not shown), suggesting that this shRNA is utilized effectively by the RNAi apparatus. We observed no inhibition when the HCVwt shRNA was codelivered with a similar luciferase construct (pEMCV Dual Luc) in which the HCV IRES element was replaced by the EMCV IRES (Fig. 1C). To confirm that the shRNAs were acting by degrading target mRNA, we performed a Northern blot analysis (Fig. 1D). We separated equal amounts of total RNA isolated from cells transfected with no inhibitor or HCVwt, HCTmut1/2, or 229 shRNAs by gel electrophoresis. We transferred the separated RNA to a membrane and hybridized it to radiolabeled cDNA probes specific for fLuc, SEAP, and translation elongation factor 1A (EF1A). HCVwt shRNA (Fig. 1D, lane 3) specifically inhibited fLuc mRNA accumulation (63% inhibition compared to 229 shRNA (lane 2) when corrected for SEAP and EF1A mRNA levels); we observed no inhibition for HCVmut1/2 (lane 4). We detected no fLuc mRNA in untransfected cells (data not shown). We observed a similar robust inhibition of HCV–dependent gene expression by HCV shRNA in human hepatocyte Huh7 cells; HCVwt shRNA inhibited HCV–dependent reporter expression with an IC_{50} of less than 30 pM, and full inhibition was observed at ~300 pM (unpublished data). Single base-pair changes in the hairpin stem region, at either the upstream or the downstream position (see Fig. 1A for location), had partial inhibitory effects (unpublished data), suggesting that at least two base-pair changes are necessary to relieve the majority of inhibition.

We extended the ability of the HCVwt shRNA to inhibit target gene expression to a mouse model system using hydrodynamic delivery of the nucleic acids to mouse liver [13,16]. Fig. 2 shows the results of injecting a large volume of PBS (1.8 ml) containing pHCV Dual Luc, pSEAP2, and shRNA (10-fold excess shRNA/plasmid on a mass basis) into the tail veins of mice. Subsequently, at the time points shown, we injected luciferin intraperitoneally and imaged the mice with a high-sensitivity, cooled CCD camera. Fig. 2A shows representative mice chosen from each set (four or five mice per set) at the 84-h time point. At all time points tested, HCVwt shRNA robustly blocked fLuc expression, ranging from 94 to 98% inhibition compared to mice injected with pUC18 in place of shRNA inhibitor (Fig. 2B). Mutant (HCVmut1/2) or irrelevant (229) shRNAs had little or no effect. It should be noted that luciferase activity decreases with time, likely due to loss of DNA or to promoter silencing [5], and that the data are normalized to the pUC18 sample for each time point (see legend to Fig. 2).

This is the first demonstration of RNAi-mediated gene inhibition in an animal model following direct delivery of an RNA hairpin (not expressed *in vivo* from a plasmid or viral vector). The effectiveness of shRNA delivered directly to mouse liver following hydrodynamic injection was surprising in view of the high levels of nucleases found in blood. The observation that these shRNAs effectively knocked down gene expression in liver indicates that they (1) are very potent and not needed at high levels in mouse liver to cause gene inhibition, (2) are delivered very rapidly to the liver before they can be cleaved by nucleases, or (3) are inherently much more stable to nuclease degradation than linear RNA (or a combination of these characteristics). It should be pointed out that others have also observed that functional RNA can survive under similar hydrodynamic delivery conditions [16,17], including messenger RNAs [18]. In contrast to the experiments presented here, however, most of

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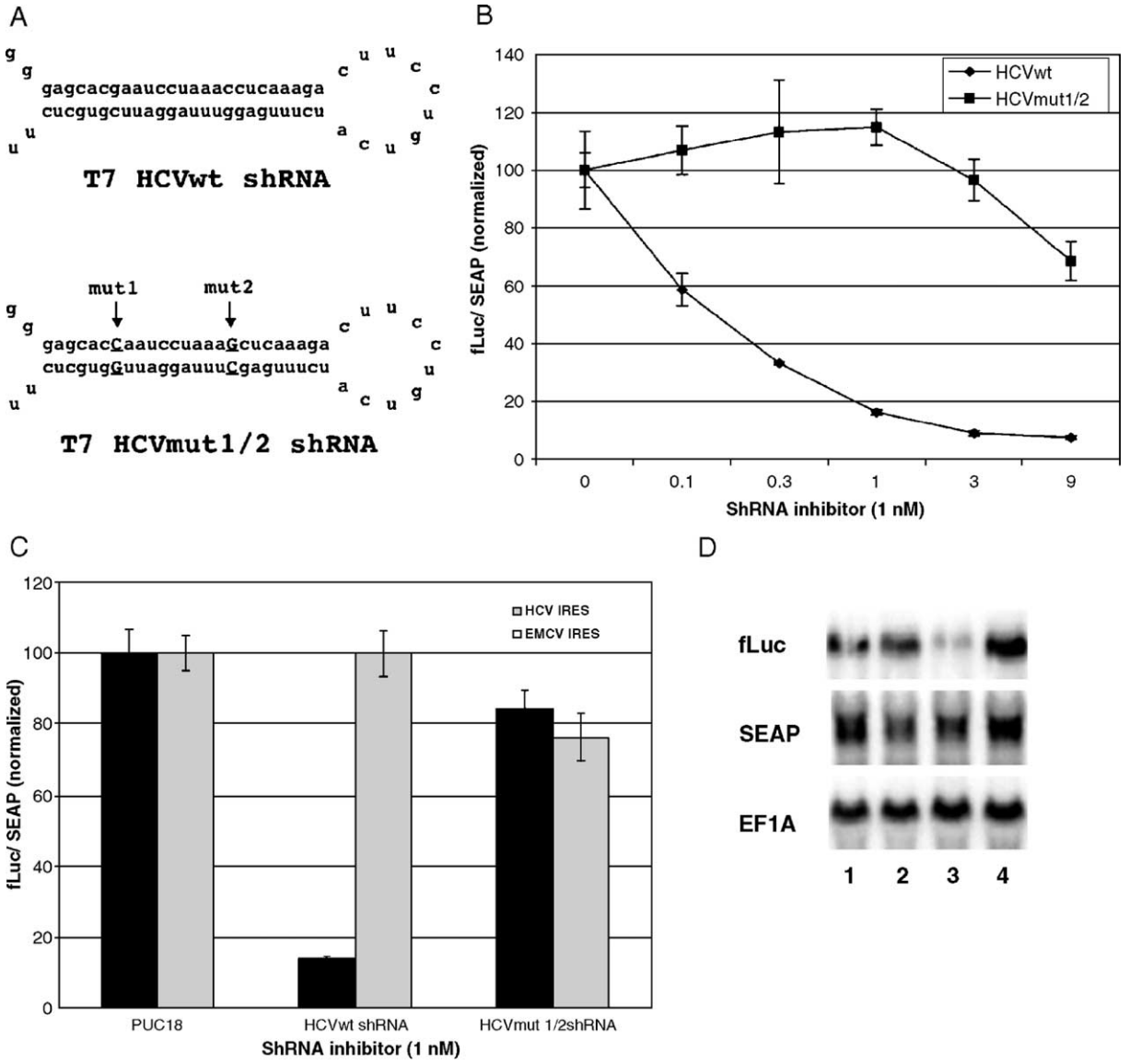

FIG. 1. Design of HCVwt shRNA inhibitors and efficacy in human tissue culture cells. (A) HCV shRNA sequences. HCVwt shRNA was transcribed from a DNA template comprising hybridized DNA oligonucleotides (IDT, Coralville, IA, USA) T7-HCVwt-For, TAATACGACTCACTATAGGGAGCAC**G**AATCCTAAA**C**CTCAAAGACTTCCTGTCATCTTTGAG**G**TTTAGGATT**C**GTGCTCTT, and T7-HCVwt-Rev, AAGAGCAC**G**AATCCTAAA**C**CTCAAAGATGACAGGAAGTCTTTGAG**G**TTTAGGATT**C**GTGCTCCCTATAGTGAGTCGTATTA. The template contains T7 promoter sequences (underlined), as previously described for the TNF-α 229 shRNA irrelevant control [15]. T7 transcripts for HCVmut1/2 (double mutation) shRNA were identical, with the exception that nucleotide changes (G → C and C → G) were incorporated into the synthesized oligonucleotides at the capitalized residues. (B and C) Inhibition of HCV IRES-mediated gene expression by HCV shRNAs in human tissue culture cells. 293FT cells (1.7×10^5 cells/well) were cotransfected (Lipofectamine 2000; Invitrogen) with 40 ng pHCV Dual Luc (B; firefly expression dependent on HCV IRES [5,13]) or a matched control pEMCV Dual Luc reporter construct (C; HCV IRES replaced by EMCV IRES, which does not contain the HCVwt shRNA target site; both kindly provided by Peter Sarnow, Stanford University), 50 ng pSEAP2-control plasmid (BD Biosciences Clontech), and the indicated amount of HCVwt, HCVmut1/2, or 229 T7-generated shRNAs. PUC18 plasmid was added to the transfection mix to give a final total nucleic acid concentration of 800 ng per transfection per well (24-well tissue culture plates). 48 h later, supernatant was removed for SEAP analysis (pNPP system; Sigma) and cells were lysed and fLuc activity was measured (Dual-Luciferase Reporter assay system; Promega). In B and C, fLuc values were divided by SEAP values and normalized to the no-inhibitor control, pUC18. (D) Northern blot analysis of cotransfected 293FT cells. 10 μg of total RNA, isolated from cells transfected with pHCV Dual Luc, pSEAP, and no inhibitor (lane 1), 229 (lane 2), HCVwt (lane 3), or HCVmut1/2 (lane 4) shRNAs, was separated by denaturing gel electrophoresis, transferred to membrane, and hybridized sequentially to ^{32}P-labeled fLuc, SEAP, or elongation factor 1A (EF1A) cDNA probes. The RNA blot was exposed to an imager screen for visualization and quantitation (Bio-Rad FX Molecular Imager).

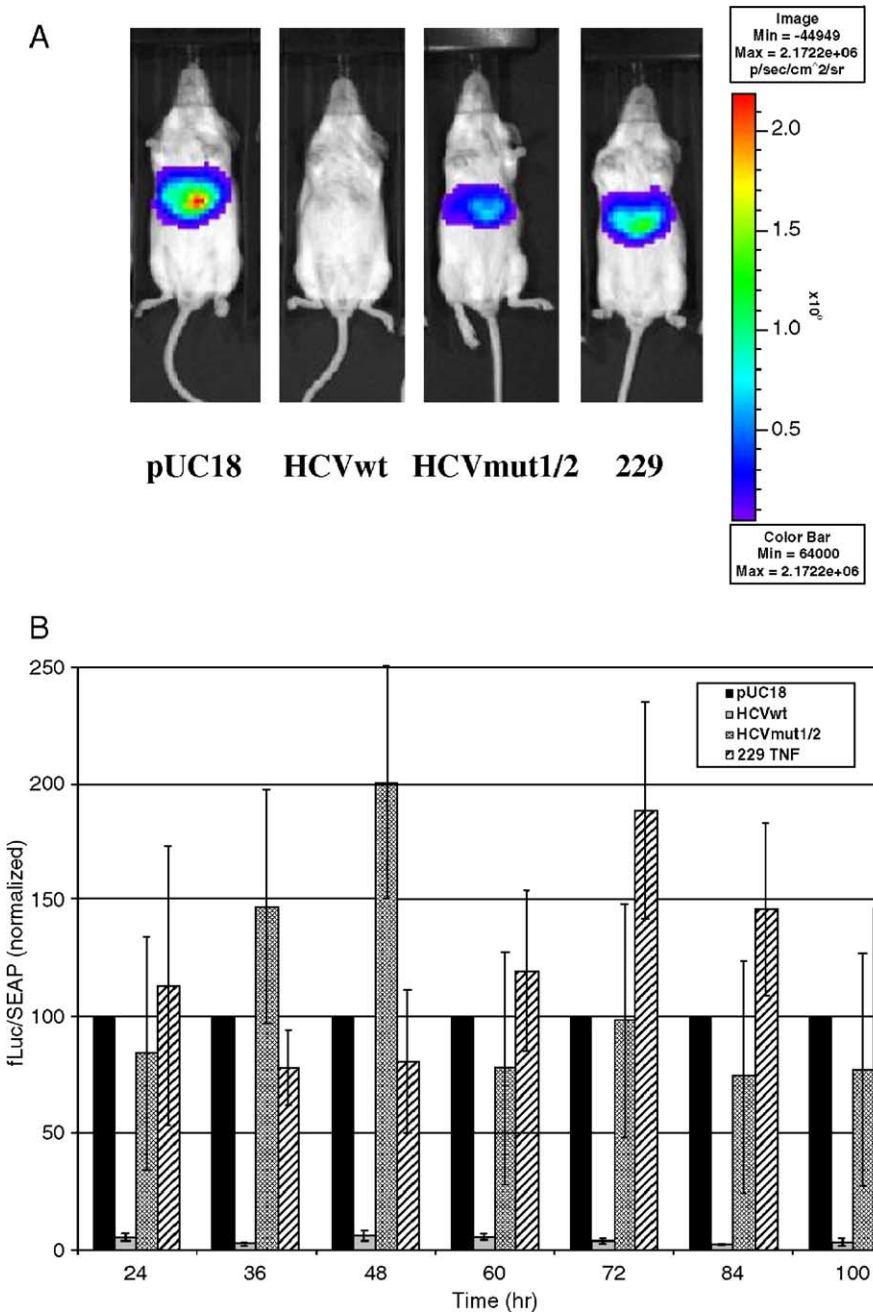

FIG. 2. Inhibition of HCV IRES-mediated reporter gene expression in mice. pHCV Dual Luc reporter (10 μg) and 2 μg pSEAP2 (SEAP plasmid was added to control for injection efficiency and nonspecific inhibition) plasmids were co-injected at constant high pressure (1.8 ml over 4–5 s) into the tail veins of 6-week-old Balb/c mice (animals were treated according to NIH guidelines for animal care and the guidelines of Stanford University) with 98 μg of the indicated HCV shRNA inhibitors (or irrelevant 229 shRNA inhibitor). Sufficient pUC18 plasmid was added to give equivalent (110 μg) nucleic acid concentrations for each mouse. At the indicated postinjection time points, luciferin was administered intraperitoneally, and the mice were imaged using the IVIS *in vivo* imaging system ([30]; representative mice from the 84-h time point are shown in (A), red color indicates highest luciferase expression) and quantitated (B) using LivingImage software (Xenogen) as an overlay on Igor image analysis software (WaveMetrics, Inc., Lake Oswego, OR, USA). Raw values were collected as photons/second. 96 h following injection, the mice were bled and the amount of SEAP activity was determined by pNPP assay (Sigma). The quantitated data are presented as fLuc divided by SEAP activity, normalized to pUC18 control mice (100%), and standard errors of the mean for each group (n = 4–5 animals) are shown.

these experiments included codelivery of RNase inhibitors with the RNA.

To determine the relative effectiveness of the shRNA inhibitors compared to siRNAs, we made a direct comparison in both the tissue culture and animal systems (Fig. 3). SiRNAs containing 25- or 19-bp duplexes directed against the same target site in the HCV IRES had inhibitory activity comparable to that of shRNAs in 293FT (Fig. 3A) and Huh7 cells (data not shown). In the hydrodynamic mouse model system (Fig. 3B), both the 19-mer and the 25-mer HCV siRNAs showed inhibition at the early 6-h time point but not at later times. In contrast, HCVwt shRNA showed only partial inhibition at the 6-h time point, and full inhibition was not reached until 24–48 h. Beyond 24 h, the treatment with shRNA showed greater inhibition than with either of the siRNAs. The lack of sustained inhibition by the siRNAs tested may be explained by the limited stability of siRNAs in blood or cells [6]. The presence of the loop structure in the shRNAs may increase stability or possibly facilitate transport in

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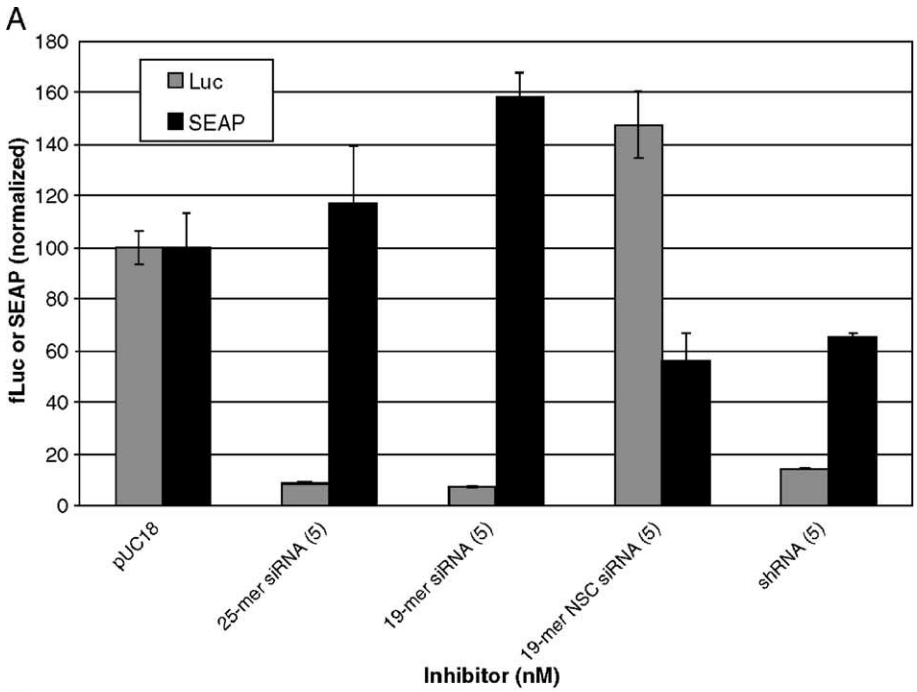

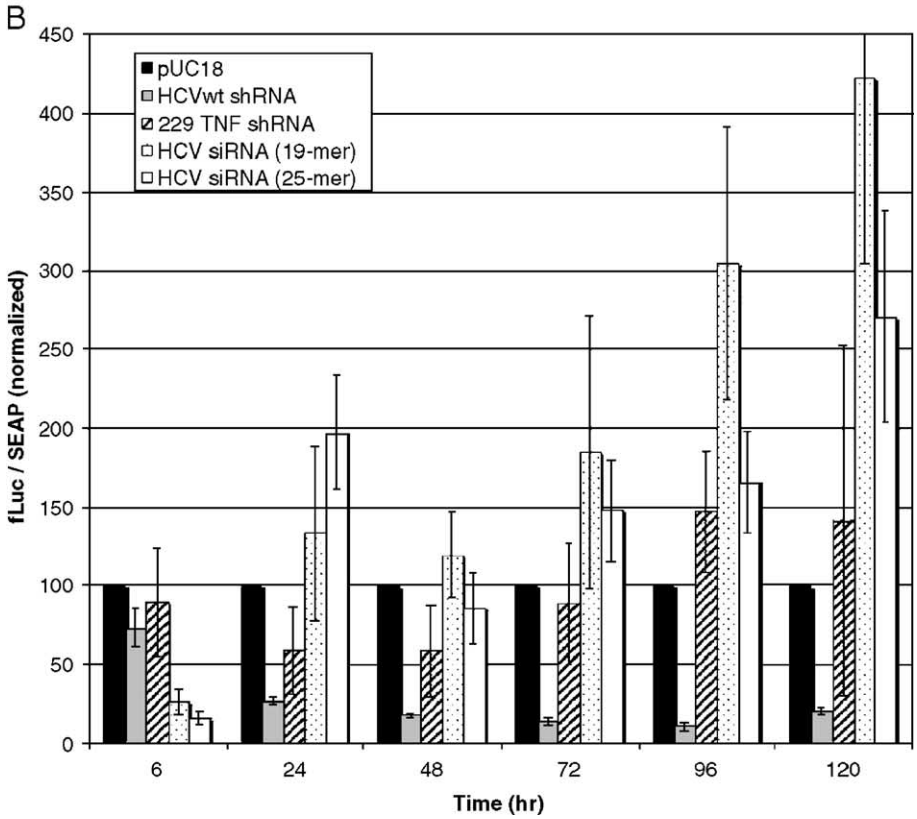

FIG. 3. Comparison of HCV IRES shRNA vs siRNA inhibitory activities. (A) The pHCV Dual Luc reporter and pSEAP2 expression plasmids were cotransfected into 293FT cells with 5 nM HCV shRNA or siRNAs (containing either 19 or 25 bp) and analyzed as described for Fig. 1. Chemically synthesized siRNAs were prepared using 2′-O-ACE-RNA phosphoramidites by Dharmacon (Lafayette, CO, USA). The sequences for the sense and antisense strands were 19-mer, sense, 5′-NNGAAUCCUAAACCUCAAAGAUU, and antisense, 5′-UCUUUGAGGUUUAGGAUUCUU; 25-mer, sense, 5′-GAGCACGAAUCCUAAACCUCAAAGAUU, and antisense, 5′-UCUUUGAGGUUUAGGAUUCGUGCUCUU. (B) For the animal experiment, equal amounts (5 nmol) of inhibitor (shRNA or siRNA) were co-injected with pHCV Dual Luc, pSEAP2, and pUC18 plasmids as described for Fig. 2B. Each bar represents the average of 3–5 mice.

the blood and/or cellular uptake. The increased potency of siRNAs in the hydrodynamic mouse system at the early 6-h time point opens the intriguing possibility that a combination of siRNAs and shRNAs may be beneficial by combining the faster action of the siRNAs with the longer lasting activity of the shRNAs. Using stabilized siRNAs

may overcome the limitations seen in these experiments [6]. Our preliminary experiments suggest that chemically stabilized siRNAs have inhibitory activities very similar to those of unmodified siRNAs in both tissue culture cells and mice (data not shown).

A recent report demonstrates that *in vitro*-synthesized transcripts from bacteriophage promoters can potently induce interferon-α and -β due to the presence of an "unnatural" 5′ triphosphate [19]. Furthermore, shRNAs expressed from Pol III expression vectors may also induce interferon (IFN) [20]. How this IFN induction would affect the use of shRNAs in a clinical setting for HCV infection is unclear. Current HCV therapy includes treatment with interferon-α, suggesting that if interferon is induced by shRNA, it may have a positive effect [21]. To date, no interferon-related side effects have been reported in animals following administration of RNAi, although this may simply reflect an absence of proper testing [2]. Clearly, further studies are needed to determine the extent that IFN-α and -β is induced by shRNAs in animals. In preliminary tissue culture studies, no interferon-β was induced in the 293FT cells at 24, 48, or 96 h following transfection with HCVwt shRNA (1, 3, or 9 nM; data not shown) as determined using a human IFN-β ELISA (R&D Systems). Furthermore, we were unable to observe induced oligoadenylate synthetase (OAS) levels in 293FT cells 48 h following shRNA administration (data not shown) using a PCR-based protocol and published OAS primer sequences [22].

Additional concerns have been raised regarding off-target effects of siRNA [23–25] as well as potential cytotoxic effects when RNAi is delivered by lentiviral vectors [22]. As with other pharmaceuticals, proper testing of several potential siRNA drugs will be required to identify those having the highest activities and reject those that have unacceptable off-target effects.

The IRES region in the HCV 5′-UTR is highly conserved and has several segments that appear to be invariant, making the IRES a prime target for nucleic acid-based inhibitors. The region around the AUG translation initiation codon is particularly highly conserved, being invariant at positions +8 to −65 (with the exception of a single nucleotide variation at position −2) in over 81 isolates from various geographical locations [26]. Despite the conservation of sequence in the IRES motif, it is uncertain whether targeting a single sequence, even if highly conserved, will be sufficient to prevent escape mutants. RNA viruses, including HCV, are notorious for their high mutation rates due to the high error rate of the RNA polymerase and the lack of proofreading activity. On average, each time HCV RNA is replicated, one error is incorporated into the new strand. This error rate is compounded by the prodigious production of viral particles in an active infection (up to a trillion per day in patients with active infections) [27]. Therefore, it is likely that several conserved sites will need to be targeted or, alternatively, single shRNAs could be used as a component of a combination treatment, such as with ribavirin and PEGylated interferon. It should be noted that a single mismatch of the HCVwt shRNA (at either the mut1 or the mut2 position, see Fig. 1A) does not completely block shRNA activity (unpublished data); thus each shRNA may have some activity against a limited number of mutations.

The key remaining issues for development of effective RNAi therapeutics are "delivery, delivery, delivery." Although the liver appears to be a prime candidate due to its ability to take up nucleic acids readily, systemic delivery through hydrodynamic injection does not appear to be feasible in humans; limited success has been achieved, however, in hydrodynamic delivery to primate muscle [28,29]. The high potency of "naked" shRNAs to block liver gene expression, as well as the ability of shRNAs to be readily expressed from Pol III promoters, makes shRNAs attractive candidates for viral treatment in a clinical setting.

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