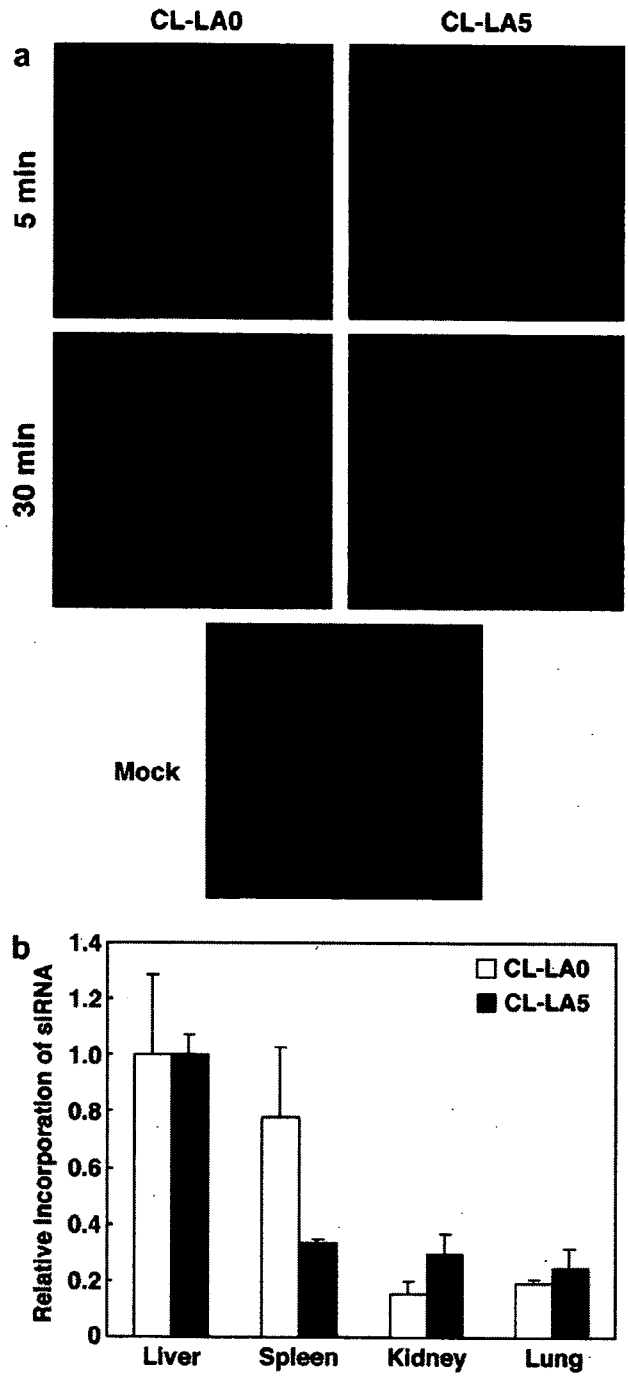

Fig. 3. siRNA delivery in mice. (a) Distribution characteristics of siRNA in liver. Alexa-546 labeled siE/CL-LA0 or siE/CL-LA5 (4.3 mg/kg) was injected intravenously into the orbital veins of BALB/c mice. The liver was observed by fluorescence microscopy at 5 or 30 min after injection. The nuclei were stained with DAPI. Mock; 10% (w/v) maltose solution. (b) Tissue distribution of Alexa-568 labeled siRNA delivered by CL-LA. The liver, spleen, kidney, and lung were examined at 30 min after injection, and the intensity of fluorescence of labeled-siE/CL-LA0 or siE/CL-LA5 was then calculated at 3 locations in each tissue specimen. The relative ratio for incorporation of siE was obtained by setting the liver intensity as control. Data represent means ± SD.

To examine whether a type I IFN response was caused by siRNA/CL-LA5, we measured IFN-β mRNA levels in the liver of CN2-29 mice. Poly(I):poly(C)/CL-LA5

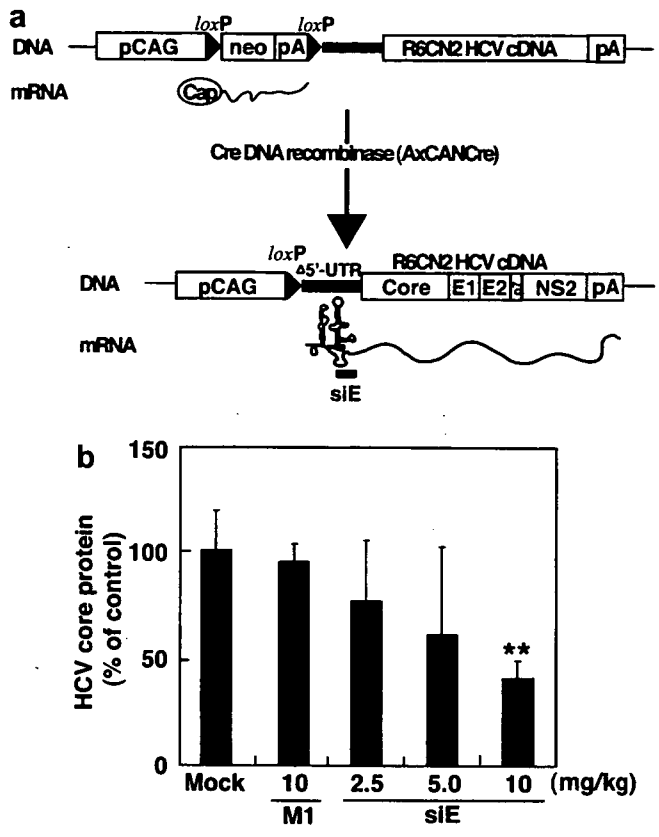

Fig. 4. Effect of siRNA on intrahepatic HCV expression in mice. (a) The CN2-29 transgenic mice contain part of the HCV gene (part of the 5′-UTR to NS2 protein). HCV protein expression can be controlled by infection of adenovirus encoding Cre DNA recombinase (AxCANCre). (b) siRNA/CL-LA5 was intravenously injected into CN2-29 mice at 1 h after AxCANCre infection. HCV core protein expressed in the liver was detected by ELISA after 48 h ($n = 3$). The average HCV core proteins were analyzed by Dennett's test. $^{**}P < 0.01$ vs. Mock control livers. Mock; 10% (w/v) maltose solution, M1; siGL3-M1.

drastically increased the IFN-β mRNA level to 10^6–10^7 copies per 1 μg total RNA, whereas siRNA/CL-LA5 induced only 10^1–10^2 copies per 1 μg total RNA

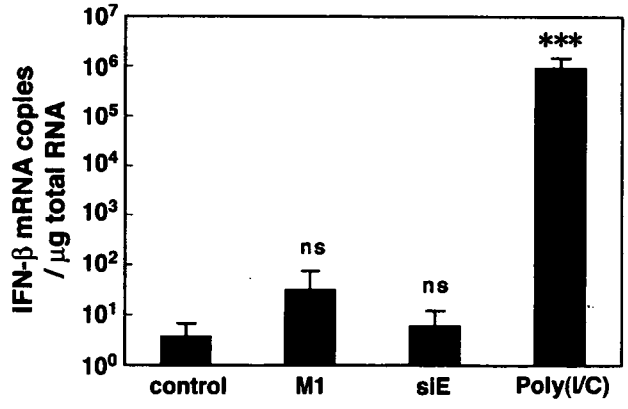

Fig. 5. IFN response *in vivo*. siRNA complexed with CL-LA5 was intravenously injected into CN2-29 mice ($n = 3$). At 6 h after injection, the IFN-β mRNA copy number was measured using real-time detection PCR. The average IFN-β mRNA copies were analyzed by Dennett's test. $^{***}P < 0.001$ and ns vs. non-treated control mice livers. ns, not significant; M1, siGL3-M1; Poly(I/C), poly(I):poly(C).

(Fig. 5). Moreover, siE/CL-LA5 induced only below 10^1 copies per 1 μg total RNA, equal to non-treated mouse liver. These results indicated that CL-LA5 was able to lead siRNA and poly(I):poly(C) to the liver by systemic intravenous injection. Subsequent administration of siE/CL-LA5 was then unable to activate the IFN response in mouse liver.

4. Discussion

Many studies of delivery systems for siRNA based on cationic liposomes have already been reported [24,34–40]. In those studies, the major problem of liposome as an siRNA carrier appears to have been a limitation to specific cell types, which resulted in unwanted tissue distribution *in vivo*. To address this problem, ligand or receptor mediated siRNA delivery systems were developed and these were able to increase uptake into the target cells [41–43]. In this study, to achieve liver specific delivery of siRNA, we designed a lactosylated cationic liposome as a carrier of siRNA and evaluated its delivery ability. The galactose terminus of lactose is a ligand of the asialoglycoprotein receptor, which is specifically expressed on the surface of hepatocytes. Thus, we expected liver specific delivery of siRNA would be enabled *via* this receptor-mediated endocytotic pathway [44]. As expected, CL-LA5, composed of cationic lipid and lactosylated phosphatidylethanolamine, effectively delivered the siE, which then inhibited HCV gene expression *in vitro* and *in vivo*.

siRNA is able to activate the cellular interferon pathway, especially when delivered with cationic liposome transfection reagents [32,33]. In addition, recent reports have revealed that siRNAs containing the 5′-UGUGU-3′ sequence are able to induce a toll-like receptor-mediated IFN response only when they are delivered *in vivo* with cationic lipid through intravenous administration [45,46]. These issues have raised concerns about the future of siRNA therapeutics. In fact, we found that the siE/CL-LA5 barely activated the type I IFN response, but that siGL3-M1/CL-LA5 weakly induced this response in mouse liver, although neither agent contained the 5′-UGUGU-3′sequence. Although the reasons for these phenomena are unclear, siE/CL-LA5 is likely to be tolerated by innate *in vivo* immunity and to have therapeutic potential for HCV.

On the other hand, we used transgenic mice expressing HCV RNA (encoding the IRES to NS2 protein region) to measure the knockdown efficiency of siE/CL-LA5. The target RNA is not replicable. During the course of an HCV infection, the virus exists as quasi-species composed of multiple variants [47]. Due to this physiological condition, mutants resistant to the siRNA may arise rapidly [48,49]. Although we believe that siE/CL-LA5 has the potential to silence natural HCV RNA, further investigations with an actual HCV infection system [50] are required.

Furthermore, siRNA/CL-LA5, a systemic method of delivery of siRNAs to liver tissue, would provide a means to introduce siRNAs into hepatocytes to achieve maximal therapeutic benefit, decrease the amount of drug required, and avoid nonspecific silencing and IFN response. Although further optimization of siRNA stability and safety profile characterizations are required for its practical application in humans, our delivery system of siRNA with CL-LA5 is a promising and feasible therapeutic strategy for liver disease associated with pathogenic gene products such as HCV.

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Therapeutic application of RNA interference for hepatitis C virus ☆

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Abstract

RNA interference (RNAi) is a sequence-specific post-transcriptional gene silencing by double-stranded RNA. Because the phenomenon is conserved and ubiquitous in mammalian cells, RNAi has considerable therapeutic potential for human pathogenic gene products. Recent studies have demonstrated the clinical potential of logically designed small interfering RNA (siRNA). However, there are still obstacles in using RNAi as an antiviral therapy, particularly for hepatitis C virus (HCV) that displays a high rate of mutation. Furthermore, delivery is also an important obstacle for siRNA based gene therapy. This paper presents the potential applications and the hurdles facing anti-HCV siRNA drugs. The present review provides insight into the feasible therapeutic strategies of siRNA technology, and its potential for silencing genes associated with HCV disease.

Keywords: RNAi; HCV; siRNA; Therapeutics; Escape; Delivery

Contents

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1. Introduction

An estimated 170 million people worldwide are persistently infected with hepatitis C virus (HCV) [1]. Although the initial infection is frequently asymptomatic, there are several subsequent clinical manifestations, including fibrosis of the liver, cirrhosis, and hepatocellular carcinoma. Presently combination therapy with pegylated interferon (IFN)-α and ribavirin has markedly improved the clinical outcome, but less than half of the patients with chronic hepatitis C can be expected to respond favorably to currently available agents [2]. Therefore, developing a new therapy for chronic HCV is a major public health objective.

HCV is a member of the Flaviviridae family, and has an approximately 9.6-kb single-stranded RNA genome with positive polarity [3,4]. The genome contains a single open reading frame which encodes a larger precursor protein of approximately 3000 amino acids. This precursor protein is post-translationally cleaved into three structural proteins (core, E1 and E2/p7) and six non-structural proteins (NS2, NS3, NS4A, NS4B, NS5A and NS5B) [5]. The open reading frame is flanked by 5′ and 3′ untranslated regions (UTR). The 5′ UTR is 324–341 nucleotides (nt) in length, is highly conserved in all viruses, is required for replication and functions as an internal ribosome entry site (IRES) to drive expression of the polyprotein [6]. The 3′ UTR is composed of three domains: a short variable region just downstream of the end of the open reading frame, a poly-U/UC tract of variable length, and a highly conserved 98 nt RNA element designated the X-tail [7]. HCV displays a high rate of mutation and is classified into distinct genotypes (1 to 6) and subtypes (> 100) whose distribution varies both geographically and between risk groups [8]. Furthermore, several distinct but closely related HCV sequences coexist within each infected individual. These are referred to as quasi-species and reflect the high viral replication rate and the lack of proofreading activity by the RNA-dependent RNA polymerase [9,10].

Since gene silencing by RNA interference (RNAi) became possible by using short interfering RNA (siRNA) of less than 30 base pair (bp) in mammalian cells, siRNA has become the tool for gene function studies. Because of the high knockdown efficiency and specificity of siRNAs, it is hoped they will become a useful therapeutic agent preventing pathogenic gene products associated with diseases, including cancer, viral infections and autoimmune disorders [11–16]. A number of groups have evaluated the antiviral potency of RNAi by using HCV-specific siRNA, exposing the possibilities and problems of siRNA-mediated antiviral agents.

In this review, we summarize the current status of anti-HCV therapy by RNAi technology. We also discuss the issues against using RNAi for HCV therapy, including escape mutants and specific transfer of siRNA to the liver, and propose some new ideas for methodological advances and directions in the field.

2. General background of RNAi

2.1. Discovery of RNAi

RNAi was originally discovered in *Caenorhabditis elegans* as a biological response to exogenous double-stranded RNA molecules (dsRNA) which induce sequence-specific gene silencing [17]. Subsequently, it was reported that RNAi could take place in a wide variety of organisms, such as fungi, plants, invertebrates and vertebrates [18]. In mammalian cells, it had been known that introduction of dsRNA activated the IFN pathway and RNA-binding protein kinase and induced non-specific degradation of RNA, translation inhibition and cell death [19–21]. However, Elbashir et al. succeeded in inducing RNAi machinery in mammalian cells by using 21 nt of small dsRNA (called small interfering RNA; siRNA) without a non-specific response against the dsRNA [22]. This finding was a major breakthrough for the application of RNAi. Thereafter, siRNA became a widespread tool for specific gene silencing.

2.2. Mechanism of action

It is generally accepted that the RNAi cascade is initiated when the host cell encounters a long dsRNA from a virus or endogenous source [14]. These long dsRNAs are cleaved into shorter dsRNA segments (siRNA) by the Dicer protein, ribonuclease III type protein [23]. The RNA segments are 19–22 nt containing 5′ phosphate and 3′ overhangs with 3′ hydroxyl termini in both the strands. These siRNA fragments are then incorporated into a large protein assembly, called the RNA-induced silencing complex, (RISC) [24], wherein the sense strand of siRNA is removed by a helicase associated with the RISC. The RISC with the antisense strand specifically cleaves mRNA which has a complementary sequence to the antisense strand [18,22]. The cleaved mRNA is then degraded immediately in the processing body [25] (Fig. 1).

In mammalian cells, dsRNA molecules, including siRNA and short hairpin RNA (shRNA) transcribed from a polymerase III promoter of a DNA fragment can efficiently induce RNAi activity. shRNA exported to the cytoplasm from the nucleus via Exportin-5 and the GTP-bound form of its cofactor Ran, [26,27] utilizes the RNAi cascade in the same fashion as siRNA.

2.3. Effective siRNA design

In mammalian cells, the knockdown efficacy of each siRNA depends on its sequence. Optimization of target sequence will increase siRNA potency and decrease estimated non-specific side effects because non-specific effects are dependent on siRNA concentration. Therefore, the prediction of a highly effective

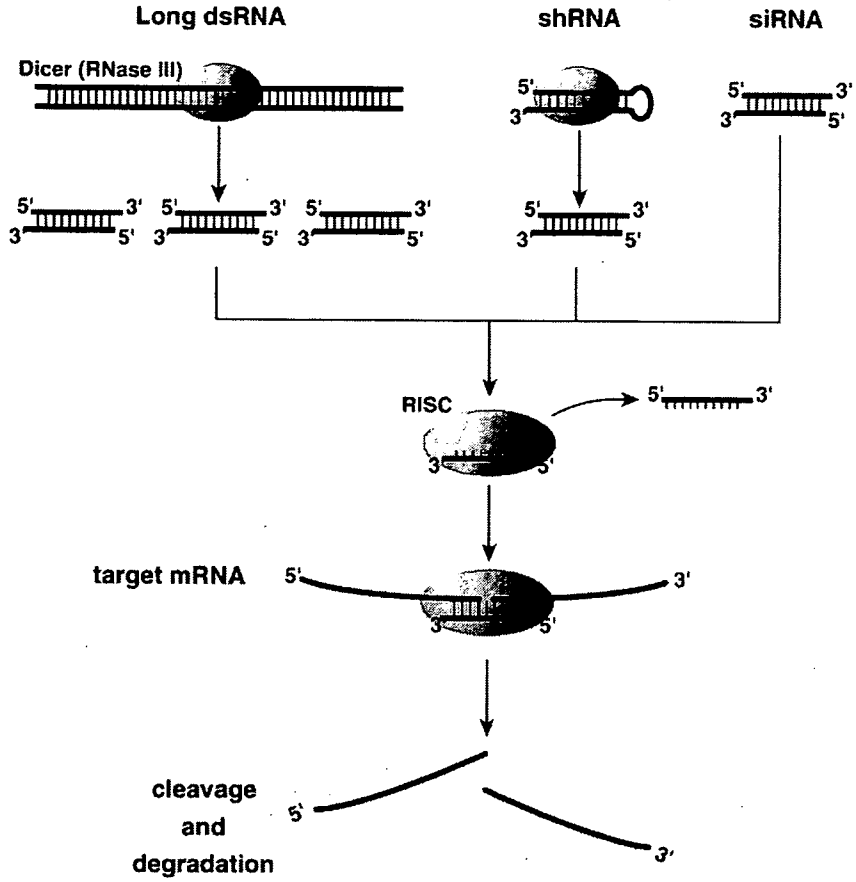

Fig. 1. RNA interference mechanism. Double-stranded RNA (dsRNA) is introduced into the cytoplasm of the cell via a DNA expression vector, long dsRNA, or small interfering RNA (siRNA). The long dsRNA is processed into ~23 nucleotide siRNAs by the enzyme Dicer and, in association with auxiliary protein(s), is transferred to the RNA-induced silencing complex (RISC). siRNAs of 19–23 nucleotides in length directly enter the RISC complex. Once bound by RISC, the siRNAs are unwound and one strand of the siRNA is discarded. The other (guide) strand anneals to the target sequence within the mRNA. This mRNA is cleaved by the "Silencer" component of RISC.

siRNA target site is needed for specificity of gene silencing and therapeutics.

Reynolds et al. defined characteristics of highly active siRNAs based on their analysis of 180 siRNAs targeting the firefly luciferase and human cyclophilin B genes. According to their report, highly effective siRNA includes 30–52% G/C content, at least 3 A/U bases at positions 15–19 in the sense strand, absence of inverted repeats, and sense strand base preference (an A at position 3, a U at position 10, not a G at position 13 and an A not G/C at position 19) [28]. Another group has also proposed a highly effective siRNA sequence reflecting the molecular mechanism of RISC assembly with an A/U at the 5′-end of the antisense strand, a G/C at the 5′-end of the sense strand, an AU-richness in the 5′ terminal third of the antisense strand, and the absence of any GC stretch over 9 bp in length [29]. The relationship between siRNA sequence and RNAi effect was analyzed in experiments using 62 targets of four exogenous (firefly luciferase, EGFP, ECFP and DsRed) and two endogenous genes (vimentin and Oct 4) [29]. Moreover, several algorithms to predict effective siRNA have been developed in recent years [30–36]. These reports show that optimal design of siRNA reflects the molecular mechanism of RISC assembly or the sequence preferences of the RISC endonuclease. In addition, it has been argued that local higher order structures in target mRNAs might restrict the accessibility of RISC, and attenuate or abolish RNAi activity [37–43].

There are reports that the secondary structure of target sites in local mRNA strongly reduce RNAi activity due to siRNA [38,44]. Moreover, there is compelling evidence that one siRNA-resistant Human immunodeficiency virus (HIV)-1 had no mutations in the target site of the mRNA, however, contained a point mutation 7 nt upstream of the target site, proving conformational change to stabilize a secondary structure sequestering the viral mRNA target [39]. Therefore, the methodology for siRNA design should be based on a combination of RNA target accessibility prediction, siRNA duplex thermodynamic properties and empirical design rules. The design of effective siRNA against HCV in particular will need these methodologies and/or a more restricted sequence manner because of the highly ordered structure of the genome and potential for quasi-species.

On a different note, Kim and colleagues tested the enhanced potency and efficacy of longer duplexes of 27-mer dsRNA [45]. The enhanced potency of longer duplexes is attributed to the fact that they are substrates of the Dicer endonuclease, directly linking the production of siRNA to incorporation in the RISC. Another study found synthetic shRNAs with a 29 base-pair stem to be more potent inducers of RNAi than siRNAs [46].

3. HCV targeting by RNAi

3.1. Advantage of applying RNAi to HCV

Currently, drugs against HCV, such as IFN, are influenced by HCV genotypes, which means that IFN-α with or without ribavirin therapy is more effective in genotype 2 than genotype 1 patients [47]. Therefore, genotype independent reagents, as few as possible dependent on quasi-species, are needed for HCV therapy. Usually, much time and effort is spent in the discovery of new types of therapeutic drugs. However once a target is identified and validated, siRNA itself can be directly exploited as a therapeutic for human disease, bypassing the costly, complex and long process of drug discovery. Therefore, the use of RNAi technology could be applied to inhibit viral disease, especially those viruses for which therapy at present is not sufficient, such as HCV.

In general, HCV RNA should be highly susceptible to RNAi because HCV replication occurs in the cytoplasm of liver cells without integration into the host genome, and the HCV genome is a single-stranded RNA, which looks like mRNA. Destruction of HCV RNA could induce failure of HCV replication and propagation. Therefore, utilization of RNAi technology is appropriate as a novel therapy against HCV.

3.2. In vitro model of HCV replication

Most studies of *in vitro* HCV replication have been conducted using the sub-genomic or full-genomic replicon system [48,49] because HCV cannot be propagated in conventional cell cultures. This system supports robust HCV replication, but not the production of infectious virions. Recent studies have reported that a full-length genome isolated from a Japanese patient with fulminant hepatitis (JFH-1; genotype 2a) could replicate in a sub-cell line of Huh-7 and produce virions with up to 1×10^5 infectious units per ml [50–52]. This cell culture-generated HCV system provides a powerful tool for studying the viral cycle and developing antiviral strategies, especially for HCV-specific RNAi.

3.3. RNAi for HCV

Several reports using siRNA target sequences have shown potent RNAi activity against HCV in the replicon model system. siRNAs directed against NS4B and core regions specifically decreased HCV RNA in a dose-dependent and specific manner [53]. Silencing HCV RNA was exponential with an 80-fold decrease in HCV RNA after 4 days [53]. siRNAs targeting the NS3 and NS5B regions can specifically inhibit replication of the HCV replicon and protein expression without an IFN response [54]. Another study has shown that two siRNAs targeting the NS5B region dramatically reduced protein expression from the replicon and the replication rate of HCV replicon RNA was reduced by more than 90% [55]. These same siRNAs protected naïve Huh-7 cells from challenge with HCV replicon RNA [55]. Yokota and coworkers demonstrated efficient inhibition of HCV replication and protein synthesis by siRNAs targeted at the 5′ UTR of the HCV genome, showing that siRNA at concentrations as low as 2.5 nM can suppress HCV replicon replication by approximately 80% [56]. siRNA targeting either the 5′ UTR or the luciferase reporter gene of HCV sub-genomic replicons can also reduce the level of luciferase activity in a dose-responsive manner by up to 85 to 90% [57]. There have also been reports using siRNA expression vectors to regulate HCV replication. Both strands of siRNA transcribed from two separate H1 [55] or U6 [56] promoters, and shRNA transcribed by a U6 promoter [56] inhibited the HCV replicon by RNAi activity. Recombinant lentiviruses expressing shRNA that targets the conserved region of NS3 among clinical isolates of HCV genotype 1b specifically suppressed HCV replication [58]. A study describing the transfection of a transcription plasmid DNA encoding the full-length HCV 1a genome, driven by the T7 promoter into Huh-7 cells together with adenoviruses carrying the gene for the T7 RNA polymerase, demonstrated that three siRNAs targeting the E2, NS3 and NS5B regions effectively inhibited core protein expression [59]. Using another cell line, other workers have shown that siRNA targeted against the NS5A region inhibited NS5A and core protein expression in human hepatoma (HepG2) cells transfected with the HCV full-length cDNA [60]. In another approach using cell culture-grown HCV genotype 1a and 2a, Kanda et al. demonstrated that the shRNA targeted to the 5′ UTR inhibited virus replication and infectivity titers [61]. While the relative efficiencies of individual target sequences of siRNA vary, probably due to transfection or expression efficiencies, it appears that all regions of HCV RNA are accessible to the RNAi machinery.

3.4. Resistant/escaped viruses against siRNA

By comparison of the consensus sequence isolated from an individual HCV patient over a time interval of 8 to 13 years, the overall rate of fixation of mutations throughout the HCV genome has been estimated at approximately 1.44 to 1.92×10^{-3} base substitutions per genome site per year [62,63]. This rate is very similar to that of other RNA viruses causing persistent infection, such as HIV and foot-and-mouth disease virus [64].

It is possible therefore, that a given siRNA becomes ineffective after a long time of treatment with the same siRNA. Indeed, the silencing activity of the siRNA targeting HIV [65,66] or poliovirus [67] was abolished due to the emergence of viral quasi-species harboring a point mutation in the target region. In the case of HCV, treatment with siRNA targeted against the NS5A region results in the development and presence of mutations in the target sequences of siRNA after the first two weeks of treatment [68]. There is experimental evidence that sequential treatment of one siRNA to an HCV replicon, results in accumulation of multiple point mutations within the target sequence [69]. These findings indicate that another developmental approach for evading the presence of an escape mutation is needed.

4. Prevention of escape mutation

4.1. Combination treatment with several siRNAs

Endoribonuclease-prepared siRNAs, digested from long dsRNA with recombinant human Dicer *in vitro* in order to mimic the RNAi pathway that is initiated when Dicer processes

dsRNA into siRNA, have been used to simultaneously target multiple sites of the viral genome [70]. Because endoribonuclease-prepared siRNAs can simultaneously target multiple sites of targeted sequences for degradation, these siRNAs can be used to inhibit the replication of a heterogeneous population of related viruses. In particular, we have shown that Dicer-generated siRNAs targeting the 5′ UTR region 197 nt in length, derived from HCV genotype 1b, were actually able to efficiently inhibit HCV genotype 2a replication (the sequence homology between genotype 1b and 2a was 92% within the 197-bp region) [71]. Using this idea, other workers have demonstrated that initiating the RNAi response using long dsRNA (in cells that lack an IFN response) or using a pool of siRNAs, produced by *in vitro* bacterial RNase III digestion of a 1000 bp dsRNA fragment, delayed viral escape of poliovirus [72]. These approaches generate a mixture of different siRNAs and the results suggest that the use of at least two defined siRNAs will be able to delay the escape of a target virus [69]. The combination of siRNA targeting not only the HCV 5′ UTR but also the coding sequences of the HCV genome leads to enhanced reduction of HCV RNA levels in replicon systems [73]. Indeed, BLT-HCV (Benitec Ltd, Australia), the first candidate to treat clinical HCV infection through RNAi, uses three components targeting different HCV sequences, underlining the importance of a multi-targeted approach to prevent the development of resistance [74].

Host cell factors involved in infection are not prone to mutation and so can also be used as an effective therapeutic target for RNAi therapy. An adenovirus vector encoding siRNAs against La, polypyrimidine tract binding protein, the gamma subunit of human eukaryotic initiation factor 2B (eIF2Bγ) and human vesicle-associated membrane protein-associated protein of 33 kDa (hVAP-33) have all been tested [75]. These cofactors involved in HCV replication were suppressed by specific siRNA, and the inhibition resulted in less HCV replication in infected Huh-7 cells. The study demonstrated the feasibility of targeting cellular cofactors in HCV infection. Other studies have reported that the cellular genes, proteasome alpha-subunit 7 and Hu antigen R were knocked down using siRNA causing a 50% reduction in replicon RNA levels [76]. Combinations of siRNAs directed against cellular HCV cofactors and HCV itself have revealed additive HCV RNA inhibition effects [76]. One experiment used a lentiviral vector containing multiple cassettes expressing shRNAs to simultaneously target the IRES, NS5B, and the host cell receptor CD81 [77]. All were independently effective in reducing HCV replication, CD81 expression, and E2 binding; moreover additive effects were observed [77]. A significant number of studies have also focused on the cellular proteins that directly or indirectly interact with the UTRs of the viral genome [78–94]. Therefore, all of the proteins that affect the viral life cycle are candidates for siRNA targeting molecules.

Table 1
Reported candidates to prevent siRNA escape mutations

Target region	siRNA type	Provision	Ref
NS5B	Combination of siRNAs	Verification of mutations in siRNA target region Combination of two siRNAs together	Wilson [69]
Core	A synthetic siRNA	Verification of mutations in siRNA target sequence	Konishi [68]
NS4B to NS5B	Endoribonuclease-prepared siRNAs	Simultaneously target multiple sites	Kronke [70]
5′ untranslated region (UTR) domain III and IV	Dicer-generated siRNAs	Targeting the highly conserved region Simultaneously target multiple sites	Watanabe [71]
5′ UTR domain III and IV	U6-driven long hairpin RNA with mutations in the sense strand	Simultaneously target multiple sites Inducing intracellular Dicer substrate	Watanabe [71]
HCV various important regions (5′ UTR, NS3, NS4, NS5B, 3′ UTR)	Convergent opposing H1 and U6-driven siRNA	Combination of two siRNAs together	Krof [73]
5′ UTR domain III and host factors	U6-driven shRNA	Targeting against HCV and host factors (proteasome alpha-subunit 7 and/or Hu antigen R)	Krof [76]
Host factors	U6-driven shRNA	Targeting against host factors (La, PTB, eIF2Bγ and hVAP-33)	Zhang [75]
5′ UTR domain IV, NS5B and host factors	Lentiviral expressed multiple shRNA	Targeting against HCV and host factors (CD81) Simultaneously target triple sites	Henry [77]
5′ UTR domain III and 3′ UTR	Plasmid expressed hairpin ribozymes and U6-driven shRNA	Combination of ribozyme and siRNA technology	Jarczak [95]
5′ UTR domain IV	Retroviral expressed shRNA	Targeting the highly conserved sequence	Kronke [70]
5′ UTR domain IV	A synthetic siRNA	Targeting the highly conserved sequence	Watanabe [71]
5′ UTR domain III	H1.3-driven shRNA	Targeting the highly conserved sequence	Kanda [61]
5′ UTR domain II	Plasmid expressed shRNA	Targeting the highly conserved sequence	Prabhu [97]
5′ UTR domain IV	A T7-transcribed shRNA	Targeting the conserved region	Hamazaki [146]

Abbreviations: eIF2Bγ — gamma subunit of human eukaryotic initiation factor 2B.
HCV — hepatitis C virus.
hVAP-33 — human vesicle-associated membrane protein-associated protein of 33 kDa.
PTB — polypyrimidine tract binding protein.
RNAi — RNA interference.
shRNA — short hairpin RNA.
siRNA — small interfering RNA.
UTR — untranslated region.

Furthermore, in an attempt to enhance antiviral activity, Jarczak and colleagues investigated the simultaneous transfection of hairpin ribozymes targeting the 3′ UTR of the HCV genome and siRNAs targeting the 5′ UTR, reporting an efficient inhibition of replication of the HCV replicon [95].

These approaches targeting multiple sites of the HCV genome and/or host factors involved in HCV replication are, at present, a realistic and valid approach aimed at preventing the virus from developing resistance (see Table 1).

4.2. Targeting highly conserved viral sequences

An alternative approach to delay viral escape might be based on the choice of the target site. Certain regions of the viral genome are constrained by sequence conservation because they are essential for viral replication. According to the level of sequence conservation among different genotypes (Fig. 2a), target sequences located within the 5′ UTR have been studied in detail by many groups. The IRES region in the HCV 5′ UTR is highly conserved. In particular, the region around the AUG translation initiation codon is highly conserved, being invariant at position +8 to −65 (with the exception of a single nucleotide variation at position −2) in over 81 isolates from various geographical locations [96]. Targeting these regions by RNAi might prevent viral escape because point mutations in these structures might lead to loss of function.

To date, several reports have shown potent RNAi activity against HCV by using conserved target regions in the 5′ UTR (Table 1). Kronke et al. reported that retrovirus encoding shRNA targeting sequences in domain IV of the IRES, which is conserved among six genotypes, blocked viral replication for 8 days [70] while Watanabe and colleagues demonstrated that continuous transfection with siRNA targeting sequences in domain IV of the IRES also caused a gradual decline in the HCV replicon for up to 23 days [71]. In a report detailing six different HCV genotypes, siRNA targeted to domain II of the IRES effectively mediated degradation of HCV RNA and inhibited reporter protein expression [97]. Furthermore, the siRNA efficiently degraded HCV genomic RNA and inhibited core protein expression from infectious full-length HCV 1a and 1b clones [97]. Studies using shRNA targeted to the portion of domain IIId to IIIe in the IRES that is conserved perfectly among HCV genotypes 1a, 1b and 2a, demonstrated inhibition of virus replication and infectivity titers [61]. In these experiments, HCV genotypes 1a and 2a were examined using cell culture-grown HCV, while an inhibition of genome replication was observed with HCV genotype 1b [61]. Using two subsequent serial electroporations with the shRNA vectors to recover resistant colonies, a reduction in colony number was observed but using another shRNA targeted against domain II in the IRES, an increase in cell colony number was observed in 6 weeks.

It is well known that the 3′ UTR is also conserved among different HCV genotypes. If the 3′ UTRs of the HCV genome are used as target sites for RNAi, it is important to determine the susceptibility of the viral UTRs because interaction of the UTR with proteins or RNA might shield them from RISC-mediated recognition and cleavage. Harris et al. identified more than 70 cellular proteins that interact with the 3′ UTR of HCV using an original RNA affinity capture system used in conjunction with LC/MS/MS [94]. Indeed, it has been reported that the effects of siRNA and Dicer-generated siRNAs against the 3′ UTR are lower than those of the 5′ UTR (IRES) [71,76,95].

a

Regions	HCV Genotypes					
	1a/1b	1a/2a	1a/2b	1b/2a	1b/2b	2a/2b
5'-UTR	98	94	94	93	92	97
C	91	81	81	82	81	87
E1	75	62	56	59	55	69
E2	72	67	67	69	65	72
NS2	71	57	57	59	57	70
NS3	80	70	70	70	70	78
NS4	79	67	65	67	67	78
NS5	79	66	66	66	66	77
3'X	100	100	98	98	98	100
TOTAL	79	68	67	68	67	77

Region-dependent sequence homology of four representative HCV genotypes (%)

b

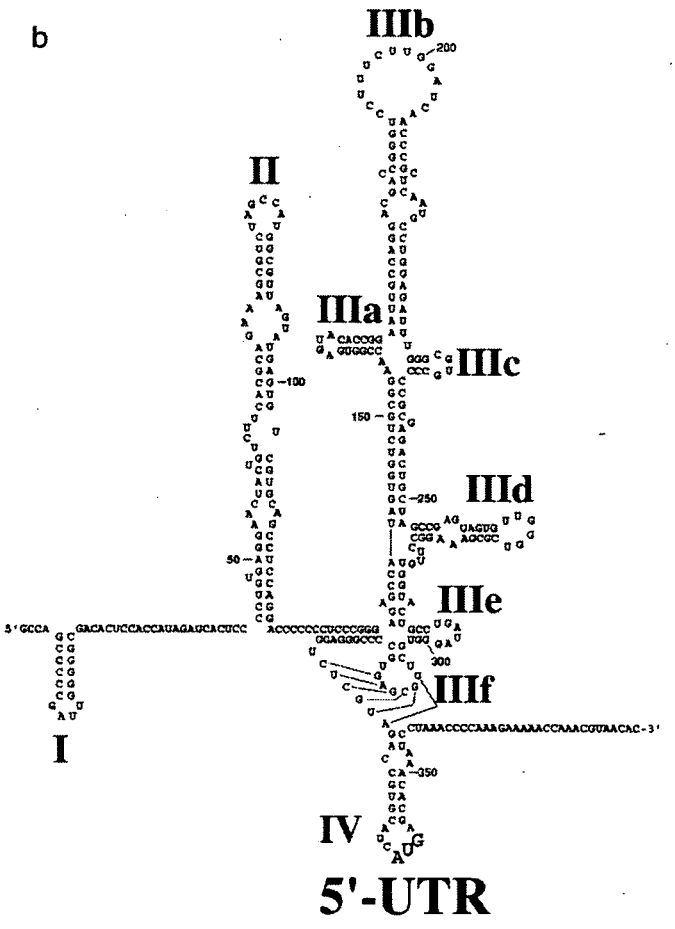

Fig. 2. (a) Region-dependent sequence homology of four representative hepatitis C virus (HCV) genotypes (%) [63]. (b) Structure of the 5′ untranslated region (UTR) domain of HCV. Schematic representation of the secondary structure of the HCV internal ribosome entry site (IRES) [104]. The translation start codon is shown in bold.

4.3. Long modified hRNA

Possible strategies to reduce the development of viral escape could include the simultaneous use of multiple siRNAs or the use of long-hairpin RNA (lhRNA), which would be recognized by Dicer, resulting in multiple active siRNAs in the cytoplasm. Watanabe and colleagues have proposed a novel approach using lhRNAs with multiple designed-point mutations within the sense strand (Fig. 3), which are transcribed from a U6 promoter and cause potent and specific silencing of HCV replicon RNAs [71]. In mammalian cells, exposure of cells to a 50 bp *in vitro* synthesized dsRNA induces the production of class I IFNs, but not when such molecules are expressed in the cell from a DNA construct with the U6 promoter. Modification of lhRNAs, expressed from a polymerase III promoter by inclusion of multiple G:U wobbles only in the sense strand of the stem sequence, induced RNAi without any non-specific effects [71,98]. Because the IFN response is attenuated in Huh-7 cells [71,99], HepG2 cells were used to test the IFN response, and this sequence-specific silencing effect was still apparent without any non-specific effects related to an IFN response [71]. This strategy for evading the IFN response has been confirmed using Hela S3 cells, HEK293 cells and non-transformed mouse embryonic fibroblasts [98]. This finding may have a major impact on the further development of RNAi-based antiviral strategies and has already been applied to other error-prone viruses. DNA constructs encoding virus-specific lhRNAs are capable of inhibiting HIV-1 production in a sequence-specific manner without an IFN response [100]. In addition, Nishitsuji et al. have shown that lhRNA, containing multiple mutations spanning 50 nt in the HIV-1 target region, effectively suppresses wild-type and shRNA-resistant HIV-1 [101]. Using an *in vivo* murine hydrodynamic injection model, Weinberg and coworkers have reported that the U6 vector encoding anti-hepatitis B virus (HBV) lhRNA, with a 62 bp stem sequence containing multiple mutations, diminishes markers of HBV replication by 70–90% without an IFN response [102]. Thus the use of lhRNA could become a future method for siRNA escape-virus therapeutics due to its efficacy and ability to overcome genotype variation in HCV and other viruses.

5. Highly effective target sites for anti-HCV RNAi

Software for efficient siRNA design for antiviral RNAi is currently available (siVirus: web-based antiviral siRNA design software) [103]. The novel algorithm is based on highly conserved regions of divergent viral sequences and has minimum off-target effects. As mentioned above, there have been many studies where the highly conserved region, the 5′ UTR of HCV, was targeted for RNAi (Fig. 2a). A highly conserved region (especially the 5′ UTR region) is among one of the most suitable target sites, but any portion in the region is not susceptible to RNAi activity [56,70,71]. Because the 5′ UTR contains an IRES element it has a highly ordered structure (Fig. 2b). The proposed secondary structure of the HCV IRES is thought to contain four major domains (I to IV) [6,104,105]. The IRES sequence may be conserved among HCV and related to flaviviruses and pestiviruses and folds into a highly ordered complex structure with multiple stem-loops for structure-function relationships. The ternary interaction of the IRES, the 40S ribosomal subunit, and eukaryotic initiation factor 3 are essential for translation initiation of the HCV genome RNA [106–109]. Furthermore, several reports suggest that many cellular proteins bind to the IRES of HCV for translation initiation (reviewed in [5]). Watanabe et al. examined siRNA-mediated silencing in various positions of the 5′ and 3′ UTR [71]. Targeting the siE region in domain IV of the IRES produced the most effective inhibition of replication of the HCV replicon. However shifting the siRNAs one or more nucleotides towards either the 5′ or 3′ end of the siE target position, reduced the efficacy of RNAi activity (Fig. 4). Therefore, not all portions of the IRES are susceptible to RNAi activity probably due to the structure and function interacting with the host factor, and so predicting the most effective siRNA target sequence is very difficult. Taking viral function and experimental data into account, designing functional siRNAs targeting the IRES of HCV is not the same for endogenous and exogenous universal genes. Ultimately at present, only experimental testing can determine the most effective siRNA for the HCV genome. Other regions, such as the 3′-UTR and the NS3 and NS5B protein coding regions, are controversial siRNA target sites due to the degree of conservation and the accessibility of the RISC.

6. Targeting HCV by siRNA *in vivo*

6.1. Delivery of siRNA

Delivery remains a major obstacle for RNAi-based therapy, because siRNAs do not cross the mammalian cell membrane

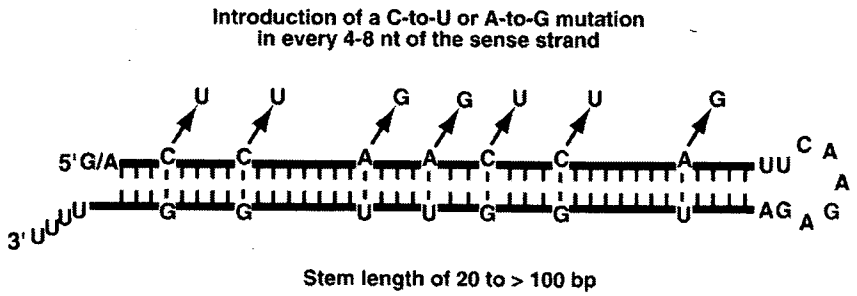

Fig. 3. Schematic representation of the long-hairpin RNA (lhRNA). U6 promoter-driven expression of lhRNA with multiple point mutations in the sense strand can cause RNA interference without triggering an interferon response or cell death (for details, see text and references [71,98]). Modified from [98].

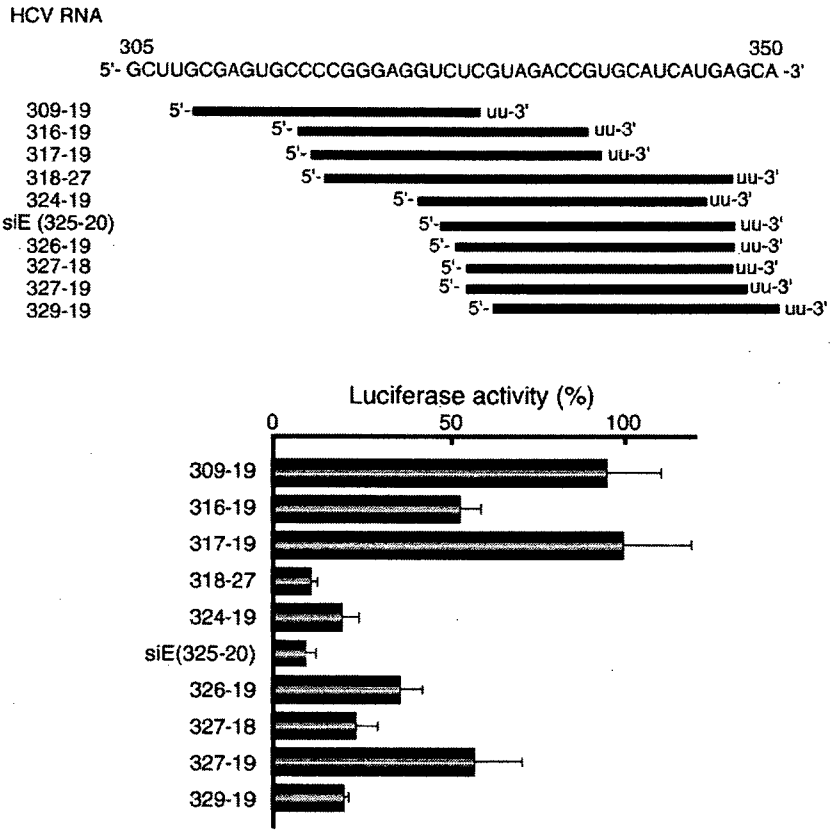

Fig. 4. Effect of positional variations in the active siE region. Hepatitis C virus (HCV) replicon cells (R6FLR-N replicon containing luciferase reporter gene) were transfected using Lipofectamine 2000 with small interfering RNAs (siRNAs) in which the target position was shifted towards either the 5′- or 3′-end of the siE region located in sub-domain IV of the 5′ untranslated region. Luciferase activity assay measured 48 h after transfection with 1 nM siRNAs. Figure adapted from [71].

unaided and most of the transfection methods used for *in vitro* studies cannot be used *in vivo*. One solution is to express siRNA precursors in viral vectors and the other is to deliver synthetic siRNAs by complexing or linking them to delivery lipids. For viral vector based siRNA gene delivery, there are potential virus-associated immunogenicity problems. Although retrovirus mediated RNAi for liver delivery has been reported, the safety of such a vector in a long-term treatment program is always a concern due to the virus being integrated into the host cell chromosome [110,111]. Therefore viral vector based siRNA delivery still needs further investigation.

The other option for delivery of siRNA is to use a non-viral vector, such as cationic liposomes. These vehicles consist of positively charged lipid bilayers which can form complexes with negatively charged siRNA molecules (Fig. 5a). The physical properties of cationic liposomes are well understood and cationic liposomes do not elicit an immune response, which is a great advantage for drug targeting as multiple doses of siRNA are possible, which is crucial for an siRNA therapeutic effect. Moreover, because cationic liposomes are easily modified and improved, the use of these liposomes holds the best promise for clinical applications [112]. Zimmermann et al. described a successful intravenous injection of lipid-encapsulated siRNA targeting apolipoprotein B (apoB) into cynomolgus monkeys, which caused decreased serum cholesterol levels with a single injection [113]. The siRNA-treated animals were evaluated for pharmacokinetics, efficacy and safety of this compound. The siRNA specifically reduced liver apoB mRNA by >90% by 48 h after treatment which was maintained for 11 days after a single dose of 2.5 mg/kg. Moreover, this silencing effect occurred as a result of *APOB* mRNA cleavage at precisely the site predicted for the RNAi mechanism. This was the first report demonstrating that single-dose systemic delivery of siRNA to non-human primates could achieve a silencing effect, which provided important insight into the systemic delivery of clinical RNAi drugs.

6.2. Effects of RNAi in liver

The majority of *in vivo* experiments analyzing the effects of siRNA in animal models have been performed using hydrodynamic transfection procedures where high volumes of siRNA are rapidly injected by high pressure into the tail vein [114]. This method leads to siRNA uptake by highly vascularized tissues including the liver, kidneys and lung. Up to 40% of liver hepatocytes can be transfected with the bulk of the DNA taken up by hepatocyte nuclei [115,116]. When multiple nucleic acid molecules are co-administered by a hydrodynamic procedure, co-localization of these molecules within the same subset of hepatocytes occurs with high frequency, making co-transfection with multiple nucleic acid molecules possible. Effects of RNAi in the liver have been examined by hydrodynamic transfection methods. McCaffrey and colleagues fused the NS5B gene with a luciferase reporter gene and introduced them as a plasmid into mouse livers by hydrodynamic injection [114]. By bioluminescence assays, the

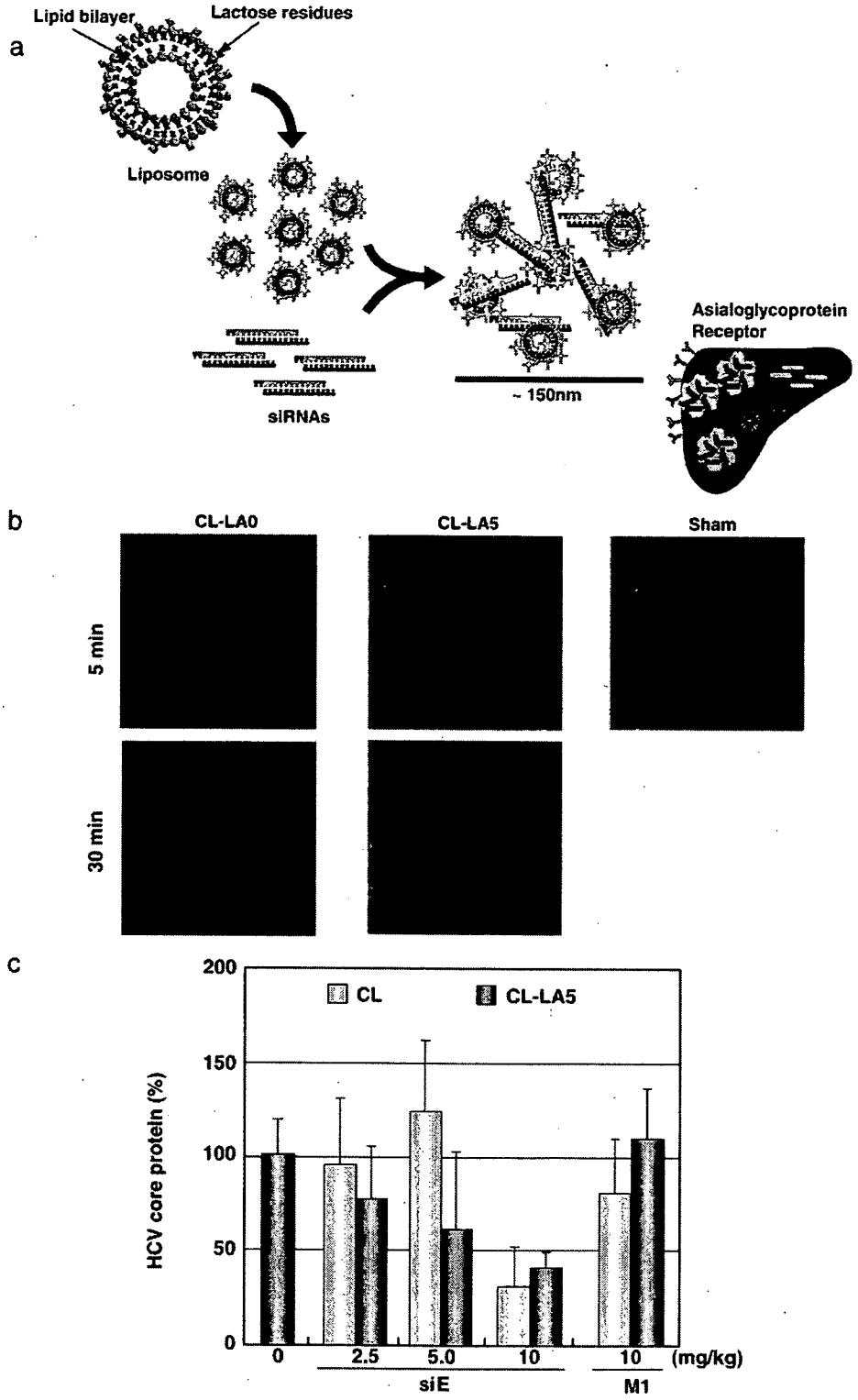

Fig. 5. Effect of small interfering RNA (siRNA) on intrahepatic hepatitis C virus (HCV) expression in mice after intravenous injection with lactosylated cationic liposomes. (a) Schematic representation of liposomes conjugated with lactose residues as a carrier of siRNA into hepatocytes possessing asialoglycoprotein receptors, which recognize compounds bearing terminal galactose moieties [120]. (b) Distribution characteristics of siRNA in liver. BALB/c mice were injected intravenously into the orbital vein with 4.3 mg/kg Alexa-546 labeled siE/lactosylated cationic liposome. The liver at 5 min or 30 min after injection was observed by fluorescence microscopy. CL-LA0, non-lactosylated cationic liposome; CL-LA5, lactosylated cationic liposome; Sham, 10% (w/v) maltose solution. The nuclei are stained with DAPI. (c) siRNA/CL-LA5 was intravenously injected into CN2-29 HCV-transgenic mice containing part of the HCV gene (part of the 5′ untranslated region to NS2 protein) [147], 1 h after induction of HCV gene expression. HCV core protein expressed in the liver was detected by ELISA after 48 h. HCV-specific siE/CL-LA5 decreased the amount of core protein in a dose-dependent manner, whereas non-lactosylated CL-LA0 combined with siE and unrelated M1/CL-LA5 did not inhibit the expression of HCV core protein except using a high dose of siRNA/CL-LA0. Sham, 10% (w/v) maltose solution; M1, negative control.

authors found that a co-injected anti-NS5B shRNA expression plasmid reduced luciferase levels by almost two orders of magnitude, indicating that the HCV fusion gene was efficiently targeted and degraded in the liver. Another study demonstrated that shRNA targeted against the IRES region, which contains the AUG translation start site, blocked IRES-mediated gene expression in liver cells after delivery by hydrodynamic transfection via the mouse tail vein [117]. Synthetic siRNA targeted against the core region can also inhibit gene expression in the liver after hydrodynamic co-transfection in a transient mouse model. In mouse livers the 27-mer dsRNA enhanced the gene silencing effect which was reduced by weak G–U mismatch base pairing [118]. These studies demonstrate the *in vivo* effectiveness and selectiveness of RNAi against HCV sequences when hydrodynamic injection is employed to deliver the effecter RNA together with DNA to hepatocytes. However, hydrodynamic delivery is not suitable for human therapy at present [119].

Cationic liposomes are a good candidate for liver specific delivery because the physicochemical properties that determine the tissue disposition of the gene carrier, such as the particle size and specific ligand, can be easily controlled. Thus, to specifically and effectively transfer siRNA into hepatocytes, we designed lactosylated cationic liposomes because the galactose terminus of lactose is a ligand of the asialoglycoprotein receptor, which is specifically expressed on the surface of hepatocytes (Fig. 5a). Liver specific delivery of siRNA would be enabled via this receptor-mediated endocytotic pathway [120,121]. These lactosylated cationic liposomes display excellent delivery into mouse liver hepatocytes (Fig. 5b). Furthermore, anti-HCV siRNA delivered by complexes with these lactosylated cationic liposomes can decrease HCV protein expression in the HCV-transgenic mouse (Fig. 5c). This indicates that this delivery system is able to interrupt ongoing intrahepatic HCV expression *in vivo*, not by co-transfection with siRNA and target templates (unpublished observations).

Several reports have shown expression of HCV-specific siRNA molecules using retroviral vectors [58,70,77]. Expression of the shRNA, resulting in a siRNA, is usually performed by the RNA polymerase III promoters U6 or H1. In the case of the HBV replication model system [122], studies have reported that shRNA can be delivered to the livers of mice by using an adenovirus delivery system [123]. Using these vectors a significant suppression of HBV replication was seen for at least 26 days. These experiments have yet to be performed using HCV itself.

6.3. Stability of siRNA

Improving serum stability is a key in the development of a systemic delivery system for siRNA, because rapid degradation of unmodified RNA [124,125] and to a lesser extent DNA [126] in serum by serum nuclease occurs. Not only are protective vehicles, such as liposomes, being designed to escort and target intact siRNA effectors for cellular uptake, but chemical modification of siRNAs is also being investigated as a means of directly stabilizing the molecules. Importantly, several groups have shown that inhibition of gene expression by siRNAs is compatible with a broad spectrum of chemical modifications [125,127–131]. Some groups have also used chemical modification of siRNAs to reduce unintended regulation, so called off-target effects [132–134]. Indeed, Soutschek et al. demonstrated that systemic administration of modified siRNAs can silence an endogenous gene [124]. The siRNA was a cholesterol conjugated, double-stranded oligonucleotide that was chemically stabilized through a partial phosphorothioate backbone and 2′-*O*-methyl sugar modification. The siRNA targeting apoB mRNA was administered to mice by systemic injection using a small volume and normal pressure and an assay was performed after 24 h. Cleavage of apoB mRNA occurred specifically at the predicted site [124].

6.4. IFN response due to siRNA

Initially, it was reported that a siRNA less than 30 bp in length was expected to evade the IFN response. However, during development of effective *in vivo* siRNA delivery techniques, it was shown that siRNAs could activate the immune system and induce the production of cytokines both *in vivo* and *in vitro* [135,136]. This mechanism appears to work through Toll like receptor 7 and 8 which recognize siRNA in a sequence-dependent manner [137,138]. Moreover, it has been shown that Toll like receptor 7 is essential for induction of cytokines due to siRNA [110,139,140]. The IFN response can be avoided by carefully tailoring the design and selection of siRNA [28], by incorporating two 3′ adenosines to prevent base pairing with the initiating guanines [138], and by using the lowest effective dose of the siRNA vector. Alternatively, immunostimulatory side effects can be masked using chemically-modified nucleotides [139,141,142]. In HCV therapy however, an IFN response, if any, would be desirable and may enhance therapeutic effects, because current treatment of HCV is based on IFN-α therapy [143]. Therefore, it is important that all events induced by siRNA, including silencing and unwanted effects, occur only at the selected location.

6.5. Exportin-5 depletion

Since siRNA-mediated RNAi shares components of endogenous micro RNA (miRNA)-induced gene-inactivation [144], there is a possibility that a large amount of exogenous siRNA may interfere with the miRNA pathway. The long-term effects of sustained high-level shRNA expression have been tested in the livers of adult mice [145]. Forty-nine kinds of shRNA targeting 6 different genes were evaluated using an adeno-associated virus vector. Dose-dependent liver injury was found in 36 mice and 23 mice died. Interestingly, the morbidity was associated with the down-regulation of miRNAs in the liver, suggesting that large amounts of exogenous shRNA expression compete with the endogenous miRNA for binding to exportin-5. Optimizing the shRNA dose and design sequence should help minimize the risk of over saturating the endogenous miRNA pathways. Thus cautious analysis will be required before RNAi can be applied to HCV therapeutics, especially in terms of off-target effects.

7. Conclusions and perspectives

Currently logically designed siRNA methodology is a potent and useful tool for endogenous genes. However logical design

is more difficult in the case of HCV therapeutics as the virus has original sequence-function or structure-function relationships. HCV is also notorious for its resistance and escape ability. Therefore, the application of RNAi-mediated therapeutics for HCV is restricted compared to those for endogenous genes. However, the evidence available to date clearly suggests that the technology can overcome these problems. Recent advances in the optimization of siRNA stability and safety profile characterization are promising. Although the form of delivery is another problem for siRNA based therapy, steady progress with a liver targeting delivery system will enable a safe and effective strategy for targeting HCV in hepatocytes in the near future. Despite these and other obstacles, RNAi provides a new potential therapeutic application that may effectively treat HCV infection.

Acknowledgements

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Survival of liver failure pigs by transplantation of reversibly immortalized human hepatocytes with Tamoxifen-mediated self-recombination

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***Background/Aims*: Hepatocyte transplantation and bioartificial liver treatment are attractive alternatives to liver transplantation. The availability of well-characterized human hepatocyte lines facilitates such cell therapies.**

***Methods*: Human hepatocytes were immortalized with a retroviral vector SSR#197 expressing catalytic subunit of human telomerase reverse transcriptase (hTERT) and enhanced green fluorescent protein (EGFP) cDNAs flanked by a pair of loxP recombination targets. Then, Tamoxifen-dependent Cre recombinase was expressed in SSR#197-immortalized hepatocytes. Cre/LoxP recombination was performed in the established cells by simple exposure to 500 nM Tamoxifen for a week. Then, the reverted population of the cells was recovered by EGFP-negative cell sorting and characterized in vitro and in vivo using a pig model of acute liver failure (ALF) induced by D-galactosamine (0.5 g/kg) injection.**

***Results*: A human hepatocyte cell line 16T-3 was established. Reverted 16-T3 cells showed the increased expression of hepatic markers in association with enhanced levels of transcriptional factors. Compared to normal human hepatocytes, albumin production and lidocaine-metabolizing activities of reverted 16-T3 cells were 0.32 and 0.50-fold, respectively. Transplantation of reverted 16T-3 cells significantly prolonged the survival of ALF pigs.**

***Conclusions*: Here we demonstrate the usefulness of Cre/LoxP -mediated reversible immortalization of human hepatocytes with Tamoxifen-mediated self-recombination.**

***Keywords*: Hepatocytes; Hepatocyte transplantation; Acute liver failure; Reversible immortalization**

1. Introduction

Acute liver failure (ALF), which affects approximately 2000 persons per year in the United States, is characterized by severe and sudden liver cell dysfunction that results in coagulopathy and hepatic encephalopathy in previously healthy individuals [1]. This catastrophic illness can rapidly progress to coma and death from

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cerebral edema and multiorgan dysfunction. Although orthotopic liver transplantation (OLT) remains the only curative therapy for patients with acute or terminal liver failure, this procedure is highly costly, complex, and limited by the scarcity of donor livers [2]. Since Berry and Friend established a method to isolate hepatocytes from whole livers in 1969 [3], laboratory animal studies and limited clinical trials have demonstrated that hepatocyte transplantation (HT) can potentially be used for the treatment of liver failure [4–6] and inborn errors of liver metabolism [7,8]. HT presents distinct advantages over OLT for organ replacement therapy: (i) HT is technically simpler than OLT, requiring only intraportal injection of a cell suspension, (ii) hepatocytes can be cryopreserved for future use and (iii) hepatocytes obtained from one donor can be used for multiple patients [4,9]. Yet, major obstacles to the broad clinical use of HT include the competition with OLT for the few suitable donor livers and the fact that primary hepatocytes cannot be readily expanded *in vitro* [4,9].

To address these issues, we have previously shown that primary human hepatocytes can be expanded *in vitro* through a procedure of "reversible immortalization" by retrovirus-mediated transfer of an immortalizing oncogene (simian virus 40 large T antigen (SV40 T)) that can be subsequently excised by Cre/Lox-mediated site-specific recombination [10,11]. Reverted hepatocytes were capable of protecting partially hepatectomized rats from acute liver failure after intrasplenic transplantation [11], and this procedure was then extended by us and others to various cell types [12–15]. However, the requirement for expression of the Cre recombinase at the completion of the expansion phase in order to trigger irreversible excision of the integrated oncogene necessitated secondary virus-mediated gene transfer in a large number of cells. This technical requirement has presented a formidable hurdle to make this approach practical for human patients, and proof-of-principle in a large animal was lacking.

Here, we have extensively modified this approach by utilizing human telomerase reverse transcriptase (hTERT) as the immortalizing cDNA and making it self-excising in the presence of Tamoxifen without the need for secondary gene transfer. Unlimited supplies of reverted human hepatocytes were obtained, thus allowing large-scale production and the first demonstration of efficacy in a large animal model of ALF.

2. Materials and methods

2.1. Hepatocyte immortalization and culture

A polycistronic retroviral vector, SSR#197, was constructed as described previously [15]. Human hepatocytes were purchased from Cell systems Co. (Seattle, WA) and cultured with chemically defined serum-free CS-C medium (Cell systems Co.). Hepatocytes (1×10^6) were transduced with 2 mL of ψCrip SSR#197-producing cell supernatant in the presence of 12 mg/ml polybrene (Sigma) at 37 °C for 4 h two times a day for 3 days. Two days after the final transduction, cells were subjected to flow cytometric cell sorting for recovering GFP-positive populations, as previously described [15]. These GFP-expressing cells were cloned in 96 well plates using a limiting dilution method. Based on the gene expression profile assessed by RT-PCR, as described below, and lack of tumorigenicity (Table 1), one of 24 clones, clone no.16, was selected for subsequent transfection.

2.2. Integration of the Tamoxifen-dependent Cre recombinase-expression cassette (MerCreMer)

Clone no. 16 was transfected by means of Lipofectamine 2000 (Invitrogen, Carlsbad, CA) with the plasmid pCAGMerCreMer/Puro that expresses a Cre recombinase protein fused to two mutant estrogen-receptor ligand-binding domains (MerCreMer) under the control of the CAG promoter (kindly provided by Michael Reth, MPI fur Immunobiologie, Freiburg, Germany), which is a composite of the Cytomegalovirus IE enhancer and the chicken β-actin promoter [16]. Seventy-two hours after transfection, selection with 5 μg/ml puromycin was initiated. Within 4 weeks, puromycin-resistant colonies emerged and a total of 40 clones were isolated using cloning rings. On the basis of Cre/LoxP recombination efficiency, cell line 16-T3 was selected for further study and was maintained in serum-free ISE-RPMI [17] medium containing 3 μg/ml puromycin.

2.3. Tamoxifen-mediated Cre/LoxP recombination

16-T3 cells were inoculated in T175-flasks, and 500 nM 4-hydroxytamoxifen (4-HT) (Sigma, Saint Louis, MI) was added 24 h later to the ISE-RPMI culture medium. The cells were maintained in the presence of 4-HT for a week and then subjected to flow cytometric analysis with a Moflo sorter (DakoCytomation, Tokyo, Japan). Then, the reverted population of 16-T3 cells was recovered by EGFP-negative cell sorting and further characterized (Fig. 1).

2.4. Telomerase activity

Telomerase activity in 16-T3 cells was assayed by the PCR-based telomeric repeat amplification protocol (TRAP) using a TRAPEZE telomerase detection kit (Serologicals, Norcross, GA) according to the manufacturer's protocol. Cellular extracts from 16-T3 cells were diluted to 100 ng/ml. Two microliters of each cellular extract was subjected to the TRAP assay before being loaded on a 10% polyacrylamide gel, and products were visualized by SYBR Green I staining (Molecular Probes, Eugene, OR) as described previously [14,15].

2.5. Assay for oncogenicity

To evaluate the potential tumorigenicity of immortalized or reverted 16-T3 cells, 1×10^7 cells were transplanted into the right shoulder or right thigh of SCID mice, respectively (KLEA Japan, Tokyo, Japan). As a positive control, 1×10^7 PLC/PRF/5 cells derived from a human hepatoma cell line [18] were transplanted into the left shoulder of the mice. Mice were observed for at least 6 months.

2.6. Gene expression of hepatocyte markers

Total RNA was isolated from 16-T3 or its reverted form and subjected to reverse transcription and polymerase chain reaction (RT-PCR) and Northern blot analyses with specific primers and probes, as previously described [11]. Primers used were as follows: albumin (576 bp), AAACCTCTTGTGGAAGAGCC (5′-primer) and CAAA GCAGGTCTCCTTATCG (3′-primer); asialoglycoprotein receptor (495 bp), TAGGAGCCAAGCTGGAGAAA (5′-primer) and ACCT GCAGGCAGAAGTCATC (3′-primer); bilirubin uridine diphosphate glucuronosyl transferase (495 bp), ATGACCCGTGCCTTTATCAC

Table 1
Characterization of SSR#197-transduced human hepatocyte clones

Clone No.	ALB	ASGPR	BUGT	CYP	GS	GST	HBCFX	Tumorigenicity[a]
1	–	–	+	–	+	+	–	–
2	+	–	+	+	+	+	–	–
3	+	–	+	–	+	+	–	–
4	+	–	+	+	+	+	–	+
5	+	–	+	+	+	+	–	+
6	–	–	+	+	+	+	–	+
7	+	–	+	+	+	+	–	+
8	–	–	+	–	+	+	+	+
9	–	–	+	+	+	+	+	+
10	–	–	+	+	+	+	–	+
11	–	–	+	+	+	+	–	–
12	+	–	+	–	+	+	+	–
13	+	–	+	–	–	+	–	–
14	+	–	+	–	+	+	–	–
15	+	–	+	+	+	+	–	–
16	+	+	+	+	+	+	+	–
17	+	–	+	+	+	+	+	–
18	+	–	+	+	+	+	+	–
19	–	–	+	+	+	+	–	+
20	–	–	+	+	+	+	–	–
21	+	–	+	+	+	+	–	+
22	+	–	+	+	+	+	–	+
23	+	–	+	+	+	+	+	+
24	+	–	+	+	+	+	–	+

ALB, albumin; AGPR, asialoglycoprotein receptor; BUGT, bilirubin-UDP-glucuronosyltransferase; CYP, cytochrome P450 3A4; GS, glutamine synthetase; GST, glutathione-S-transferase-π; HBCFX, human blood coagulation factor *X*. The expression of these hepatic markers was assessed by RT-PCR.

[a] Tumorigenicity was assayed using SCID mice.

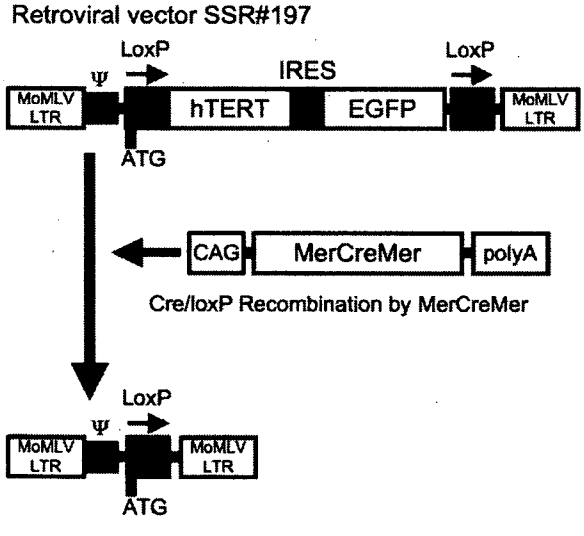

Fig. 1. Reversible immortalization of human hepatocytes with Tamoxifen-mediated self-excision of hTERT. Schematic drawings of the immortalizing retroviral vector SSR#197, before and after Cre/Lox recombination, and the plasmid pCAGMerCre/Puro^R encoding the MerCreMer Tamoxifen-dependent Cre recombinase fusion protein. MoMLV LTR, Moloney Murine leukemia Virus long terminal repeat; IRES, internal ribosomal entry site.

(5′-primer), TCTTGGATTTGTGGGCTTTC (3′-primer); glutamine synthetase (535 bp), ATGCTGGAGTCAAGATTGCG (5′-primer), TCATTGAGAAGACACGTGCG (3′ primer); glutathione-S-transferase π (496 bp), GCCCTACACCGTGGTCTATT (5′-primer), GG CTAGGACCTCATGGATCA (3′-primer); human blood coagulation factor X (493 bp), GTGCATGGAAGAGACCTGCT (5′ primer), GA AGTCAAGCAGGTCGAAGG (3′ primer); human β-actin (610 bp), TGACGGGGTCACCCACACTGTGCCCATCTA (5′-primer), CTA GAAGCATTTGCGGTGGACGATGGAGGG (3′-primer). The amplification reaction involved denaturation at 95 °C for 30 s, annealing at 58 °C for 30 min, and 72 °C for 30 min using a thermal cycler (Perkin-Elmer, Foster City, CA) at 30–35 cycles for all markers. The PCR products were resolved on 1% agarose gels and visualized by ethidium bromide staining. The intensity of the bands of liver markers to human β-actin, used as an internal control, in both immortalized and reverted cells, was comparatively presented using NIH Image.

2.7. Evaluation of hepatocyte-specific functions

Immunofluorescent staining for albumin was conducted with immortalized and reverted 16-T3 cells with a labeled anti-albumin antibody, as previously reported [19]. Staining with Oregon green phalloidin (Molecular Probes, Eugene, OR) and Hoechst dye (Sigma, Saint Louis, MI) was simultaneously performed for specific staining of actin filaments and nuclei, respectively. Ten microliters of culture medium from 1×10^6 immortalized and reverted 16-T3 cells cultured with ISE-RPMI medium for 24 h was subjected to albumin production analysis using the Human Albuwell II kit (Exocell Inc., Philadelphia, PA) according to the manufacturer's protocol. Immortalized, reverted, and normal human hepatocytes (1×10^6 cells) were inoculated in each well of a 6-well plate per 24 h per mg of cellular protein to evaluate metabolic activity of lidocaine (1 mg/mL) (Fujisawa Pharmaceutical Co., Osaka, Japan). Concentration of lidocaine was measured by SRL Co. (Okayama, Japan). Primary isolated human hepatocytes (kindly gifted by Dr. S. Suzuki at the Huamn Animal Bridge (HAB) Institute (Ichikawa, Japan) were used as a positive control.