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Therapeutic short hairpin RNA expression in the liver: viral targets and vectors

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Over 500 million people worldwide are infected with one or more different and unrelated types of human hepatitis virus. Such individuals are at a high risk of developing acute or chronic hepatic disease, and ultimately dying from sequelae. Although a vaccine is available for hepatitis A and B virus, treatment options for chronically infected patients are limited, and particularly ineffective in case of hepatitis C virus (HCV) infection. A promising new avenue currently being explored is to harness the power of RNA interference for development of an antiviral therapy. The timing to pursue this particular approach is excellent, with the first in vivo *animal models for HCV infection becoming available, and the technology for liver-specific expression of short hairpin RNAs advancing at a rapid pace. Here, we critically review these important current developments, and discuss the next steps to bring this novel approach into the clinics.*
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Introduction

The leading cause for acute and chronic human liver disease is infection with one of the two major types of hepatitis virus – hepatitis B virus (HBV) or hepatitis C virus (HCV). An estimated 350 million people worldwide are carriers of HBV, which makes it one of the most prevalent chronic viral infections in humans.[1,2] Over one million people die annually from HBV-associated liver failure, end-stage cirrhosis or hepatocellular carcinoma (HCC), with up to 1% of all deaths occurring in the US and Europe.[3–5] The risk of eventually succumbing to HBV-related diseases is between 15 and 25% among the chronic carriers.[6] HBV is in fact the major causative agent of HCC, and as such represents the third leading cause of overall cancer death.[7] In hyperendemic areas such as China, the numbers are even more dramatic, with a 60% prevalence of total HBV infection, and an estimated 10% of the chinese population being chronically infected.[8]

Like HBV, HCV is one of the main causes of liver-associated morbidity and mortality, and is the leading indication for liver transplantation in the western world.[9] It is estimated that HCV infects more than 170 million people worldwide, including 2–4% of Americans.[10] Typically, the virus is transmitted percutaneously and thus persists as a particular problem among drug users. In about 70% of all (usually asymptomatic) acute infections, HCV establishes persistency, which is frequently (40–60%) characterized by chronic liver inflammation and fibrogenesis, and ultimately progresses to cirrhosis, end-stage liver failure and HCC.[11] Notably, the virus exists in at least six unique genotypes differing from each other by 31–34%, as well as in subtypes with further sequence diversity. The major genotypes in Western Europe and the US are HCV 1a and b, followed by 2 and 3, whereas genotypes 3–6 are rare and endemic to unique regions of the world.[12]

Despite their largely similar clinical sequelae, the two viruses differ dramatically in their prevention or treatment options, which directly relates to their differences in genetic structures and viral life cycles (see below). Most notably is that for HBV, but not HCV, a preventive recombinant vaccine (HBV surface antigen) has been available since 1981.[2] Nonetheless, HBV infection remains a challenging problem for human societies, largely because therapeutic intervention options for chronically infected HBV carriers are rare and usually of limited success. These options include treatment with immune modulators such as recombinant interferons α or γ, or nucleoside or nucleotide analogs such as lamivudine or adefovir, respectively, which inhibit the viral reverse transcriptase and thus impede HBV replication.[13] However, even with different drug combinations there is limited effectiveness not suited to achieve complete cure from the virus. This is because they do not promote HBV eradication from the infected host, thus resulting in relapse and recurrence of viremia after cessation of treatment.[4] An additional complication arises from the virus' ability to form escape mutants with prolonged treatment which are resistant to existing drugs,[14] a phenomenon that is an even greater problem with HCV infection.

In fact, the success rates of treatment of chronic HCV infection are at best 50–60%, using the most effective currently available regimen in the form of pegylated interferon α, alone or in combination with ribavirin.

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Nearly half of the patients do not respond to this treatment, and even in those who do, therapies are frequently prematurely discontinued due to severe adverse side effects.[15] Significantly hampering the development of urgently needed more effective regimens is the lack of tissue culture or small animal models to study replicating virus. A second inherent problem is the extreme mutation rate of the HCV genome, resulting from the high error rate of the viral RNA-dependent RNA polymerase in the range of 10^{-4}, and promoting the formation of viral 'quasi-species' swarms in infected individuals that can become resistant to current treatment options.[16]

The implementation of alternative safe, effective and specific therapeutics for chronic viral hepatitis is obviously a high-priority goal, along with the establishment of new *in vivo* models for HBV and HCV infection. Fortunately, there is now considerable hope that both these goals can soon be met. This hope is fueled by a plethora of papers from the past 3 years, consistently providing evidence that multiple steps in the HBV/HCV life cycles can be targeted by RNAi, using synthetic siRNAs (small interfering RNAs) or expressed shRNAs (short hairpin RNAs). Importantly, with proofs-of-concept initially obtained in tissue culture, first reports now also show RNAi efficacy in mice transgenic with the HBV genome, mimicking a chronic infection in humans.

In this article, we provide an overview over the current state-of-the-art technology in the field of anti-hepatitis virus RNAi. We characterize the two main targets (HBV and HCV) in more detail, with particular focus on aspects relevant to therapy, and briefly present available *in vitro* and *in vivo* test systems. We then summarize selected recent studies providing essential advances in the field, but restrict this part to vector-encoded shRNAs for reasons explained below. Finally, we review two potent viral vector systems for liver-directed shRNA transfer, AAV and adenovirus, and conclude with ideas for further methodological advances and directions for the field.

Structure, life cycle and models of HBV and HCV

In the next two sections, we briefly summarize and compare the essential genetic structure of HBV and HCV, and describe how the particular virus features and life cycles are reflected in current experimental models of either virus. This part is not comprehensive, and the reader is referred to further review articles for breadth.[1,3,4,17]

Hepatitis B virus

HBV is a noncytopathic member and also the prototype of the family Hepadnaviridae, small (virion diameter of 42 nm) enveloped mammalian and avian viruses. The HBV genome is a single 3.2 kb, partially double-stranded (ds) DNA molecule with an extremely condensed organisation (Figure 1a). In fact, every single nucleotide is encoding, and the four different open reading frames (ORFs) overlap to an extent that at least half of the genome is simultaneously part of two of them. The ORFs are labeled C, P, S and X and transcribed into four capped and polyadenylated mRNAs, encoding the viral pre-core/core (capsid, C) and envelope (S) structural proteins, as well as the viral polymerase (P) and X protein, whose function is only partially understood. As will be described in detail below, all four viral transcripts represent accessible targets for RNAi. Perhaps most interesting is the longest HBV RNA, a 3.5 kb (+)RNA which is not only translated into the core and polymerase proteins, but also serves as a pre-genomic RNA for viral replication. As such, it becomes encapsidated into viral particles together with the viral polymerase, which subsequently mediates reverse transcription of the pre-genomic RNA into a single-stranded DNA. This in turn serves as a template for second-strand DNA synthesis to yield a covalently closed circular (ccc) molecule. Once these steps are completed in the cytoplasm of the infected hepatocyte, the particles traffic one of two possible routes: either to the nucleus to amplify the cccDNA genome, or to the endoplasmatic reticulum (ER), to engage the viral envelope proteins and exit the cell.

Important in view of HBV as a target for RNAi-based therapies is its ability to infect up to 100% of the hepatocytes in the liver. HBV spreads relatively slowly following the initial infection, and viral DNA expands logarithmically only after a prolonged lag phase, to reach high peak levels of up to 1×10^{13} virus genomes per ml. In most individuals, the virus is thus rapidly cleared due to its recognition by the adaptive immune system, which becomes activated by high-level HBV antigen expression. Still, the virus can manage to establish persistency in many cases, due to a combination of lack of induction of the innate immune response, and active evasion and inhibition of the adaptive branch of the immune system.[17]

HBV is very amenable to study as a target for antiviral RNAi (Figure 1a). This is because the full-length HBV genome has been cloned into plasmids, resulting in infectious molecular clones that can be readily introduced into cells, together with anti-HBV shRNA expression vectors. This strategy was exploited extensively in the past, either by transfecting hepatoma cells in culture, or via direct plasmid delivery into intact livers in adult mice, using the technique of high-pressure, hydrodynamic tail vein injection which typically results in transfection of up to 50% of hepatocytes.[18] In addition to such transient approaches, the HBV genome was also stably introduced into cultured cells, and several labs generated HBV-transgenic mice based on different strains.[19,20] High levels or replicative viral DNA in liver and robust serum titers of infectious particles are detected in these mice, but the virions cannot re-infect mouse liver due to lack of a receptor. Moreover, not all viral transcripts are detected and cccDNA is not produced. Available assays for the analyses of anti-HBV shRNA efficacy are straight-forward and efficient, and include detection of viral envelope (surface antigen, sAg; core antigen, cAg) proteins using specific antibodies, or quantification of replicated HBV DNA, viral transcripts or serum viral DNA titers.[18]

Hepatitis C virus

HCV is a noncytopathic member of the family Flaviviridae, enveloped viruses of about 50 nm in diameter which infect humans and chimpanzees. The HCV genome is strikingly different from that of HBV in many

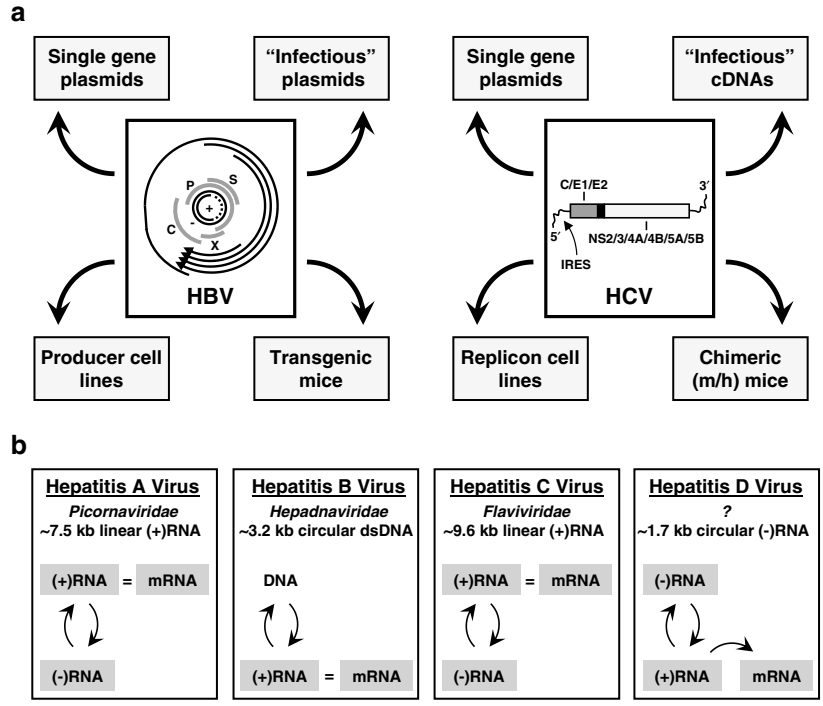

Figure 1 Structure and life cycle of hepatitis viruses. (**a**) Schemes of the HBV and HCV genomes and models for their *in vitro* or *in vivo* studies. Details of the two viral genomes are described in the text; briefly, HBV carries a partially double-stranded DNA genome endocing four major ORFs (C, P, S and X), resulting in four major transcripts (arrows) terminating at a common polyA site. In contrast, HCV carries a linear single-stranded RNA genome encoding 10 different proteins, structural (C, E1, E2) or non-structural (all NS proteins). The black box represents the p7 protein which has not yet been clearly assigned to the structural or non-structural group. The ends of the HCV genome are 5′ or 3′ untranslated regions, with the 5′UTR comprising the IRES. For both viruses, expression plasmids for individual genes or the full-length genomes are available, but fully infectious systems were only reported for HBV thus far. To date, the only *in vivo* system to study HCV replication are chimeric mice which harbor human hepatocytes in their livers.[25] (**b**) Comparison of all four major human hepatitis viruses, including HAV and HDV which are not discussed here. The arrows indicate replication cycles, while gray boxes highlight potential RNA targets for RNAi.

aspects; most notably, HCV is an RNA virus with a single-stranded, linear, positive-sense (+)RNA genome of about 9.6 kb in length (Figure 1a). This RNA molecule lacks a 5′ cap, but instead carries an internal ribosome entry site (IRES) within the 5′ untranslated region (UTR), which binds eukaryotic ribosomal subunits and initiation factors to ultimately assemble the translationally active 80S complex. Translation of the entire genome then results in a large polyprotein precursor which becomes proteolytically cleaved into distinct processing intermediates, and finally into 10 individual viral proteins. These proteins fall into two categories, structural (C, E1, E2, p7) and non-structural (NS2, NS3, NS4A, NS4B, NS5A and NS5B), and specific functions were ascribed to most of them. Briefly, E1 and E2 are glycoproteins embedded into the lipid membrane surrounding the viral nucleocapsid, which itself is formed by Core, an RNA-binding protein. E1 and E2 might be involved in binding to extracellular parts of potential HCV receptors and thus mediate HCV entry into hepatocytes.[21] The various multifunctional NS proteins are mostly required for coordinated amplification of the viral RNA and as such perform complex actions in the infected cell, often requiring formation of heterodimers or interactions with cellular factors. Most remarkable NS genes/proteins, and very promising targets for RNAi-based therapies, are (1) NS3 with its dual N-terminal serine protease and C-terminal RNA helicase/NTPase activities, (2) NS5A, which exists in different phosphoforms whose functional relevance remains unknown, but could involve inactivation of the innate immune response, as well as (3) NS5B, which is an RNA-dependent RNA polymerase (RdRP) and as such the key player in virus replication.

HCV replication occurs in the cytoplasm of infected cells, where viral proteins and RNA together with cellular factors form the so-called 'membranous web', which serves as a scaffold for the genome replication complex. HCV replication is most likely semiconservative and asymmetric, that is, the single-stranded positive RNA serves as a template for synthesis of a negative progeny strand, forming a transient ds RNA intermediate together with the (+)RNA. The negative strand is next reverse transcribed into multiple (+)RNA genomes by the viral RdRP enzyme, and the nascent RNA molecules then serve as templates for either a new replication cycle, or for translation, or packaging (Figure 1b). Assembled virions finally bud into the ER and exit the cell through the secretory pathway.

In contrast to HBV, HCV spreads rapidly in the infected host, and the viral RNA expands logarithmically already within the first 2 weeks, although the viral loads in chronically infected patients are much lower and only range from 1×10^3 to 1×10^7 viral genomes per ml.[17] Nonetheless, it was estimated that early after inoculation, swarms of up to 1×10^{12} particles are produced per day, which exceeds the rate reported for HIV by two orders of

magnitude.[21] It is still unknown what percentage of hepatocytes becomes infected with the virus, and it remains controversial whether it replicates at high levels in a few hepatocytes, or at low levels throughout the entire liver. Likewise, it is unclear why the immunological responsiveness to HCV is largely variable between individuals, but it generally appears that the virus has developed evasion strategies to escape both the innate and adaptive immune reponse.[17] An important factor in defeating the host immune system is the previously mentioned extreme mutation rate of HCV (10^{-3} per nucleotide per year, i.e., 100 times higher than for HBV), due to the lack of proof-reading activity of the viral RdRP enzyme, and resulting in evolution of quasi-species in infected patients.[17]

In addition to rapid mutation, another fundamental hurdle to the study of HCV as an RNAi target is posed by the fact that since its cloning 16 years ago,[22] the virus has remained notoriously hard to propagate *in vitro* (Figure 1a). A surrogate strategy was developed by several investigators, the first about 6 years ago when Bartenschlager's and Rice's groups designed the HCV subgenomic replicons. These are molecules derived from a cloned HCV 1b genome in which the structural genes were replaced with a selectable marker (neomycin phosphotransferase), upstream of a second heterologous viral IRES to direct expression of the non-structural proteins.[23,24] Following transfection of replicon-derived RNA into human hepatoma cells and selection with G418, cell lines grew out that contained self-replicating HCV RNAs. Interestingly, replicon RNAs frequently harbor the so-called adaptive mutations in the NS3, NS4B and NS5A/B genes, which increase RNA replication by up to 10 000-fold to levels sufficient to confer G418 resistance to the cell line.[24] It is believed that these mutations shift the balance between viral RNA replication, translation and packaging, and might thus explain some of the difficulties in growing the virus *in vitro*. Notably, replicons are now also available for genotypes 1a and 2a, and some subgenomic constructs were used to study HCV RNA replication in non-hepatoma cell lines.[21] Thus, replicon systems provide an excellent and widely used means to dissect viral replication and protein functions, and in particular to develop and test antiviral RNAi.

Importantly, very recent work now implies that the replication of authentic virus in cell culture, and even the *in vivo* study of infectious HCV in small-animal models, might no longer remain elusive. Firstly, mice were reported which contain chimeric mouse/human livers and which support HCV infection and replication within the human hepatocytes, thus representing a fully infectious *in vivo* system.[25] Secondly, a series of three papers showed that a full-length genome from an HCV 2a isolate (JFH-1, from a Japanese patient with fulminant hepatitis) replicates in cell culture, and depending on the Huh-7 subline used, produced robust titers of up to 1×10^5 infectious units per ml.[26–28]

Towards antiviral RNAi therapeutics

Over the past 3 years, a plethora of reports demonstrated the feasibility to target HBV and HCV *in vitro* and *in vivo*, initially using synthetic siRNAs, or more recently, vector-expressed shRNAs. In the next three sections, we describe the various possibilities to target the two viruses, directly or indirectly (through host cell factors), and then summarize a series of relevant proof-of-principle studies.

We focus exclusively on shRNA approaches in this review for two reasons: firstly, attempts for siRNA-mediated inhibition of hepatitis viruses have already been reviewed extensively in the recent past.[17,29–38] Secondly, at this time, we believe that vectored shRNA delivery represents a more promising means of efficient and sustained viral gene silencing in the liver, as compared to chemically synthesized siRNAs, which are limited by their shorter half-life and inherent problems of higher costs and less effective *in vivo* delivery.

General strategies

In most HBV/HCV silencing studies published to date, the RNAi machinery was directly targeted towards the viral RNA, in attempts to degrade these molecules which can serve as viral genome (HCV), or replication intermediates and transcripts (both HBV and HCV). Moreover, in the primarily suggested therapeutic scenario, antiviral shRNAs would be delivered to patients who are chronically infected with a hepatitis virus, with the aim to permanently silence viral gene expression, or ideally, to clear the pathogen from the individual. Nonetheless, there are multiple other treatment avenues that appear worth pursuing, and which we describe in the following and summarize in Figure 2.

Firstly, several groups suggested that in addition to targeting an acute or chronic viral infection, RNAi may be particularly useful as a prophylactic treatment. Here, the specific idea is to pre-treat cells or tissues with an shRNA expression vector *ex vivo*, prior to re-introduction into the patient.[39] It is possible to imagine that such a pre-treatment would render the cells/tissues immune to infection, thus one potential application would be to protect a donor liver prior to transplantation into an HCV-infected recipient. While this idea is certainly promising, it remains to be shown whether the current or future generations of viral or non-viral shRNA delivery vectors are capable of sufficiently transducing entire livers *ex vivo*, and whether a 'vaccination' strategy based on stably expressed shRNAs in newly transplanted livers is really effective.

A second controversy regards the percentage of hepatocytes that needs to be transduced with the shRNA vector in order to obtain a therapeutically relevant

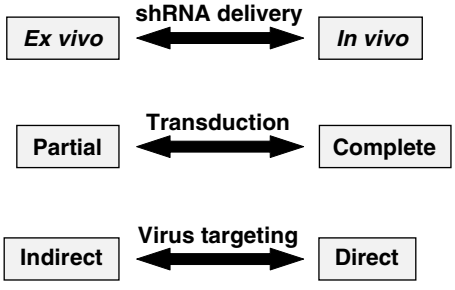

Figure 2 General strategies for anti-HBV/HCV RNAi therapies. Shown are three levels at which current or potential future antiviral RNAi strategies differ fundamentally. See text for details.

antiviral effect. Ideally, one would aim for complete liver transduction to ensure maximum silencing of viral gene expression and suppression of replication in all cells. However, findings from chronically infected HBV patients suggest that transduction of a limited number of cells might already induce viral clearance.[40] This is because those individuals maintain an HBV-specific CTL response throughout the infection which continually destroys HBV antigen-expressing cells. This could theoretically result in liver repopulation with cells in which HBV was successfully silenced, and which accordingly have a survival advantage. Likewise, Uprichard *et al*.[40] suggested scenarios in which efficient RNAi in a limited cell number would lead to an initial reduction in viral antigen levels, which are otherwise thought to blunt the T-cell response in chronically infected patients. A transient drop of overwhelming HBV antigenemia could in theory allow for recovery of the T-cell response, and/or allow the patient to mount a more vigorous immune response to a therapeutic vaccine, likely to eventually clear the virus. These ideas could for instance be tested by partially silencing HBV gene expression in HBV-transgenic mice, followed by adoptive transfer of virus-specific CTLs or by therapeutic immunization. If successful, the feasibility to induce pathogen clearance by transducing a limited hepatocyte population is highly desirable for clinical purposes, as it would allow the use of lower doses of the viral shRNA delivery vehicles, and thus reduce the risks of vector-associated side effects.

Thirdly, while direct targeting of virus-associated RNAs appears as the obvious strategy to combat infection, several groups suggested, or already provided proof-of-principle, that an alternative efficient strategy would be to silence host cell factors required for viral infection/replication, or causing subsequent subacute liver failure. The value of this approach was for instance demonstrated by Song *et al*.,[41] who studied the outcome of high-pressure tail vein injection of siRNAs targeting Fas RNA in mice. Fas is a cell-death receptor and a key mediator of T-cell-mediated apoptosis in liver cells during viral hepatitis. The authors found that anti-Fas RNAi not only protected mouse hepatocytes from apoptosis, but also saved the majority ($>80\%$) of animals from death by fulminant hepatitis induced by injection of anti-Fas antibody. Similar findings were obtained by Zender *et al*.[42] who silenced Caspase 8-encoding mRNA. Together, these studies imply that inhibition of hepatocellular necrosis during acute liver inflammation periods will have additive or synergistic therapeutic effects, when combined with targeting of viral RNAs. Further approaches in a similar direction would be to target the viral receptors, once identified, or other cellular cofactors for the viral life cycle, as for instance shown by Zhang *et al*.[43] They repressed a series of endogenous genes thought to be required for HCV propagation, and indeed observed inhibition of virus replication. An interesting target for HCV RNAi might also be the cellular microRNA miR-122, which was recently shown to be essential for HCV replication in liver cell lines.[44] Generally, the advantage of all these approaches is that they are independent of the viral genome and can thus overcome the virus' ability to escape from RNAi by mutation, which is a particular problem with HCV. On the other hand, it remains to be investigated whether depletion of endogenous genes will have pleiotropic detrimental effects.

HBV as an shRNA target

HBV makes extensive use of overlapping reading frames within its DNA genome, suggesting that while the viral DNA itself cannot be targeted, the multiple HBV RNAs will make the virus highly susceptible for RNAi. HBV is in fact an excellent candidate for therapeutic RNAi, as its unusually compact genome with lack of redundancy results in very limited sequence plasticity and prevents the virus from evading RNAi by mutation. Thus, ideally, a single shRNA can potentially target multiple viral transcripts simultaneously, and efficiently inhibit not only viral gene expression, but also DNA replication, because HBV amplifies through an RNA intermediate (Figure 1b).

In Table 1, we comprehensively summarize all recent studies that assessed the use of shRNAs to target the transcripts from all four major HBV ORFs, *in vitro* and in a few cases also *in vivo* in mouse models of HBV replication.[8,14,18,40,45–52] The *in vitro* studies comprise transiently shRNA-transfected cells, which were either co-transfected with plasmids encoding a full-length HBV genome, or already contained the viral DNA. The *in vivo* approaches were similar, in that the HBV DNA was either transiently introduced by hydrodynamic tail vein injection of plasmid DNA, or stably integrated into the genome of transgenic mice. The shRNA expression cassettes were introduced by injection, or delivered by a recombinant adenovirus.

Principally, in summary these studies consistently proved the feasibility to achieve great levels of suppression of viral gene expression, often in a range of 80 or 90% as compared to controls such as non-related shRNAs. There was however no clear pattern, which viral transcript might be most susceptible to RNAi and the results between studies varied, most probably attributable to fundamental differences in the experimental settings. Most noteworthy findings were obtained by McCaffrey *et al*.,[18] Shlomai and Shaul,[45] and Uprichard *et al*.,[40] who independently assessed anti-HBV shRNAs in different *in vivo* models and found high efficiencies of their constructs, albeit for only relatively short periods (up to 26 days[40]). An important consistent finding in many studies was that inhibition of viral gene expression did not require active viral replication, suggesting that RNAi strategies are excellent options as adjuvants to conventional anti-HBV therapies, for example with inhibitors of the viral reverse transcriptase.

HCV as an shRNA target

HCV is a particularly attractive target for RNAi therapies due to its genetic structure and life cycle: as a (+)RNA virus that replicates via a (−)RNA intermediate, successful shRNA treatment could theoretically eliminate the genomic template, as well as replicative forms together with mRNA transcripts, and thus completely resolve HCV infection. However, to date, this remains a hypothesis due to the historical lack of *in vitro* or *in vivo* systems fully supporting HCV replication, although with the recent isolation of a particular 2a genotype (see above), experimental proof may come soon.

A second general handicap with HCV as a target was also mentioned before, which is the extreme viral

Table 1 Studies using expressed shRNAs to target HBV

Gene	*shRNA*[a]	*Promoter*	*Vector*	*Study design and results*[b]	*Comments*	*Relevance*[c]	*Reference*
C, S, P, X	25	hU6	Plasmid	Plasmid (HBV/shRNA) co-injection in mice 77–92% reduction of HBV DNA and RNA 85–99% reduction of HBsAg and cAg	First report of *in vivo* RNAi against HBV Evidence for side effect from one shRNA Only transient effects studied (7 days)	+++	McCaffrey *et al.*[18]
C, X	19	hH1	Plasmid	Studies in cultured Huh7 or 2.2.15 cells 68–95% reduction of HBV DNA and RNA 63–89% reduction of HBcAg	One of first reports of *in vitro* anti-HBV RNAi Transient ($\leqslant$5 days) *in vitro* transfections Demonstrated shRNA specificity	+++	Shlomai and Shaul[45]
C, S, P, X also DR	21	hU6	Plasmid	Studies in stably transfected HepG2 cells 90–97% reduction of HBV DNA and RNA	Best effects with combined shRNA/lamivudine	++	Chen *et al.*[46]
C, S, P, X	19	mU6	Plasmid	Studies in Huh-7 cells $\sim$90% reduction of HBV RNAs 83–86% reduction of HBsAg and eAg	Identified a potent RNAi target region	+	Zhang *et al.*[43]
S	19	hU6	Plasmid	Studies in 2.2.15 cells 44% reduction of HBsAg	Identified a potent RNAi target region	+	Liu *et al.*[48]
hLa	21	hU6	Plasmid	Studies in HepG2 and 2.2.15 cells 8–66 $\times$ reduction of HBV RNAs 26–51% reduction of HBsAg and eAg	hLa protein involved in HBV metabolism? Might suggest indirect approach to target HBV	++	Ni *et al.*[49]
S	19	hH1	PFV, AAV	Studies in 293T.HBs or 2.2.15 cells 80–90% reduction of HBV/sAg mRNA 71–98% reduction of HbsAg	Created stable HBsAg cell line (293T) Stable (5 months) silencing in cell clones Proof-of-concept for viral anti-HBV RNAi	++	Moore *et al.*[50]
S, P, X	NR	mU6	1st g Ad	Use of adenovirus in HBV-transgenic mice $>$90% reduction of HBV DNA and RNA Up to 100 $\times$ reduction of HBsAg and cAg	Adenovirus-mediated *in vivo* anti-HBV RNAi Transient RNAi due to Ad clearance ($\sim$day 20) Evidence for RNAi-resistant pgRNA species	+++	Uprichard *et al.*[40]
S	21	hU6	Plasmid	Studies in HepG2 and 2.2.15 cells $\sim$90% reduction of sAg-gfp fusion gene 43–64% reduction of HBsAg and eAg	Inhibition for up to 9 days	+	Yang *et al.*[51]
C, S, P, X also DR	19–25	mU6	Plasmid	Studies in 2.2.15 cells 2 $\times$ reduction of HBV DNA 56–72% reduction of HbsAg and eAg	Identified a potent RNAi target region	+	Ren *et al.*[8]
S	19	hH1, hU6	Plasmid	Plasmid (HBV/shRNA) co-injection in mice Also tests in HBsAg-transgenic mice Reduction of HBV DNA, RNA, HBsAg, cAg	Focused on major S region Identified a potent RNAi target region	+	Cheng *et al.*[14]
P, S, X	19	hH1	Plasmid	Plasmid injection in HBV-transgenic mice 95–99% reduction of HBsAg and cAg Rare mutation allowed escape from shRNA	Found potent shRNAs for HBV genotypes A-G Isolated a resistant quasispecies from patient	+++	Wu *et al.*[52]

Abbreviations: C, core antigen; S, surface antigen; P, polymerase; X, X protein; DR, direct repeat; hLa, human La protein; NR, not reported; PFV, prototype foamy virus; AAV, adeno-associated virus; 1st g Ad, first-generation adenovirus; hU6, human U6; hH1, human H1; mU6, mouse U6.
[a]Listed are shRNA stem lengths.
[b]Listed are most important findings and maximum knockdown results only.
[c]Study relevance was subjectively classified as very important (+++), important (++), or rather confirmative of previous work (+).

heterogeneity and evolution of quasi-species resulting from the high error rate of the NS5B protein. In fact, Wilson and Richardson[39] recently provided experimental evidence that sequential treatment of an HCV replicon with one siRNA results in accumulation of multiple point mutations within the target sequence, allowing the replicon to escape RNAi activity. Their findings also suggest that a single base pair change in the target region is not sufficient to confer resistance, and importantly, they moreover show that the evolution of escape replicons (or HCV genomes) can be severely limited through the use of two or more siRNAs in combination.[39]

A number of further lessons were learned from the series of anti-HCV RNAi studies summarized in Table 2.[43,53–61] Owing to the limitation posed by the lack of an infectious system, the primary target in these studies were HCV replicons, and accordingly, shRNAs were designed and tested against various sites in the genes encoding non-structural viral proteins, in particular NS3 and NS5B. These genes were frequently chosen because the encoded proteins are critical for viral replication and transcription, suggesting a limited sequence plasticity for these targets. Nonetheless, Wilson and Richardson[39] found that in particular the NS5B gene can tolerate mutations, partly due to the degeneracy of the genetic code, but also because the predicted amino-acid changes did not appear to affect polymerase function. Perhaps an even better target is represented by the HCV 5′UTR, which acts as an internal ribosomal entry site and whose activity seems to be determined by RNA structural characteristics. Consequently, this sequence is significantly less prone to mutation than any other part of the viral genome, exemplified by the fact that it is highly conserved among the six known HCV genotypes.[39,56] Thus, not surprisingly, several groups reported efficient inhibition of HCV replicons when targeting the 5′UTR with various shRNAs (Table 2).

In general, it remains controversial which viral RNA forms are susceptible to RNAi, and it has for instance been speculated that the genomic RNA may initially be protected from degradation due to encapsidation by core protein, but become accessible after uncoating.[39] From the limited experience with escape mutants forming under continuous RNAi pressure, there is evidence that the (−)RNA replicative intermediate is susceptible to RNAi, and clearly, efforts to inhibit replicon gene expression, or expression of reporters under the control of the HCV IRES, show that viral transcripts represent efficient RNAi targets. In summary, it thus appears that all three viral RNAs are appealing targets for developing RNAi-based therapies, but a conclusive answer will have to await the establishment of infectious *in vivo* test systems.

Until then, the most noteworthy *in vivo* analysis of anti-HCV RNAi came from McCaffrey *et al*.,[62] although they used a somewhat artificial assay. In their study, the NS5B gene was fused with a luciferase reporter gene and introduced as a plasmid into mouse livers by hydrodynamic injection. Using bioluminescence assays, the 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. It remains to be tested whether these *in vivo* findings will be reproducible in the context of an authentic HCV infection, where the other parts of the viral genome will be present and expressed as well, and viral replication will occur.

Liver-directed viral shRNA expression vectors

The two currently most promising viral vector systems for liver-directed shRNA expression are adenovirus and adeno-associated virus (AAV). The common property making these vectors so attractive is their ability to transduce hepatocytes extremely efficiently, and in particular in case of AAV, transduction is persistent and results in gene expression for the entire life span of the cell.[63] Moreover, adding to their appeal is the fact that both viruses and vectors derived thereof were studied extensively for several decades, resulting in ample data from *in vitro* and *in vivo* pre-clinical analyses (e.g., Ehrhardt *et al*.[64] and Grimm *et al*.[65]). These provide us with a very profound knowledge of the pros and cons of either vector system with regards to liver-directed gene transfer, which will help in the specific engineering of these tools as antihepatitis therapeutics.

Since both vectors were reviewed extensively in the past, the following section is not comprehensive and the reader should refer to the literature for further details.[63,66–69]

Adenovirus

Adenoviruses are non-enveloped viruses with a 100 nm capsid, carrying a ds linear DNA genome of approximately 36 kb. This genome encodes a set of 'immediate early' and 'early' genes at various locations on both strands of the DNA, together with a group of five 'late' mRNAs coding for the virion proteins. Replacement of the immediate early gene E1 with foreign DNA results in a first generation adenoviral vector (Figure 3), which were generated and studied extensively in the past, and found to result in highly efficient gene transfer to the liver in various animal models. However, a problem with these viruses is their tendency to show toxic effects *in vivo*, owing to the intra-cellular *de novo* production of immunogenic viral proteins, or to association of viral antigens with MHC I on the cell surface, resulting in CTL responses and ultimately in loss of transgene expression.[64,70–72]

In efforts to make the vector safer, highly attenuated 'gutted' viruses deleted for all adenoviral coding sequences were recently developed (Figure 3).[64,70,73,74] These particular vectors allow for transfer of up to 35 kb of foreign DNA and thus provide ample space for shRNA expression cassettes; in fact, they appear almost over-dimensioned and require long stuffer DNA to be incorporated along with the shRNA, to provide optimal vector genome encapsidation. In direct comparison to first-generation vectors, the gutted variants result in significantly reduced CTL activity, but the humoral immune response against the incoming capsid remains a major challenge. Nonetheless, gutted vectors were found to give stable transgene expression in various small and large animal models of liver-directed gene transfer for at least several months. However, transgene expression usually fell by >90% over a period of 1–2

Table 2 Studies using expressed shRNAs to target HCV (or HAV)

Gene	*shRNA*[a]	*Promoter*	*Vector*	*Study design and results*[b]	*Comments*	*Relevance*[c]	*Reference*
HAV-IRES	21	hH1	Plasmid	Transfection of Huh-7 cells 40–75% inhibition of IRES-luciferase ~60% inhibition of replicon	First report of anti-HAV shRNA expression Identified IRES as promising target region Domain IIIc might be good and universal target	+++	Kanda *et al*.[53]
5′U	19	hU6	Plasmid	Transfection of 293T or Huh-7 cells ~3× inhibition of fusion gene (luciferase) ~3× inhibition of replicon in Huh-7	First report of *in vitro* anti-HCV shRNA expression Identified very potent shRNA targeting loop IV Evidence that UTR secondary structure is crucial	+++	Yokota *et al*.[54]
5B	21	dual hH1	Plasmid	Transfection of Huh-7 cells Inhibition of NS3 expression from replicon Stable transfection inhibited HCV by 70%	Bi-cistronic vector for separate strand expression Technically not shRNA, but siRNA Identified NS5B as conserved and good target	+++	Wilson *et al*.[55]
5′U, C, 4B (1b)	19	hH1	Mo-MuLV	Retroviral shRNA delivery into Huh-7 cells 3 shRNAs anti-5′UTR-IV-blocked replicon Stable transfection yielded HCV resistance	Supports usefulness of retroviral vector for RNAi Found accessible region within 5′UTR (loop IV) Demonstrated feasiblity to confer HCV resistance	+++	Kronke *et al*.[56]
5′U, C, 3, 5B (1b)	19	hU6	Plasmid, lentivirus	Transfection/infection of Huh-7 cells Inhibition of NS3 expression with plasmid ~7× reduction of HCV RNA with lentivirus	Chose well-conserved target regions in 1b isolate shRNA against 5′UTR was surprisingly inefficient Suggested targeting NS3 for isolate 1b	++	Takigawa *et al*.[57]
La, PTB, eIF2Bγ, hVAP33	19	hU6	Plasmid, adenovirus	Transfection/infection of Huh-7 cells ~93% replicon inhibition with anti-La/PTB Anti-hVAP also inhibited translation	Strictly focused on cellular HCV cofactors Provides further evidence for role of the factors Additive/synergistic therapeutic effects possible	++	Zhang *et al*.[43]
IRES	19–25	T7	Naked shRNA	Transfection of *in vitro* transcribed shRNAs 63% inhibition of IRES-luciferase in 293FT 94–98% inhibition of luciferase in mice	*In vitro* rather than *in vivo* shRNA transcription Naked shRNAs were stable and effective in blood Differ in kinetics from siRNAs against same target	+++	Wang *et al*.[47]
CypA-C	NR	hU6	Plasmid, retrovirus	Transfection/infection of Huh-7 cells 30–60% inhibition of replicon expression ~10× inhibition in stable anti-Cyp cells	Indirect approach based on cyclosporin A Could explain cyclosporin A anti-HCV efficacy Has potential for novel anti-HCV therapy	++	Nakagawa *et al*.[59]
5′U, 3′U, PSMA7, HuR	19–21	hU6	Plasmid, retrovirus	Transfection/infection of Huh-7 cells 47–71% inhibition of replicon expression ~60% reduction of HCV RNA levels	Confirmed 5′UTR as very promising target Similar HCV inhibition when silencing cofactors Additive effects suggest a novel dual approach	++	Korf *et al*.[60]
E2, 3, 5B (1a)	19	hH1	Plasmid	Transfection/infection of Huh-7 cells Inhibition of core and NS5A expression ~80% reduction of HCV RNA levels	Expressed complete HCV 1a genome Expression required recombinant adenovirus All three HCV targets appeared equally efficient	+	Prabhu *et al*.[61]

Abbreviations: HAV-IRES, internal ribosomal entry site of hepatitis A virus; 5′U, 3′U, 5′UTR, 3′UTR (untranslated region); C, HCV core proten; E2, HCV structural gene; 3, 4B, 5B, HCV non-structural (NS) genes; IRES, HCV internal ribosomal entry site; PTB, polypyrimidine tract-binding protein; eIF2Bγ, subunit gamma of eukaryotic initiation factor 2B; human VAMP-associated protein of 33 kDa; CypA-C, cyclophilins A to C; PSMA7, proteasome α-subunit 7; HuR, Hu antigen R (all cellular non-HCV proteins). (1a, b) indicates that the sequences tested were specific for HCV genotype 1a or b; hU6, human U6; hH1, human H1; T7, T7 RNA polymerase; Mo-MuLV, Moloney murine leukemia virus; 'Naked shRNA', the shRNA was *in vitro* transcribed and purified.
[a]Listed are shRNA stem lengths; NR, not reported.
[b]Listed are most important findings and maximum knockdown results only.
[c]Study relevance was subjectively classified as very important (+++), important (++) or rather confirmative of previous work (+).

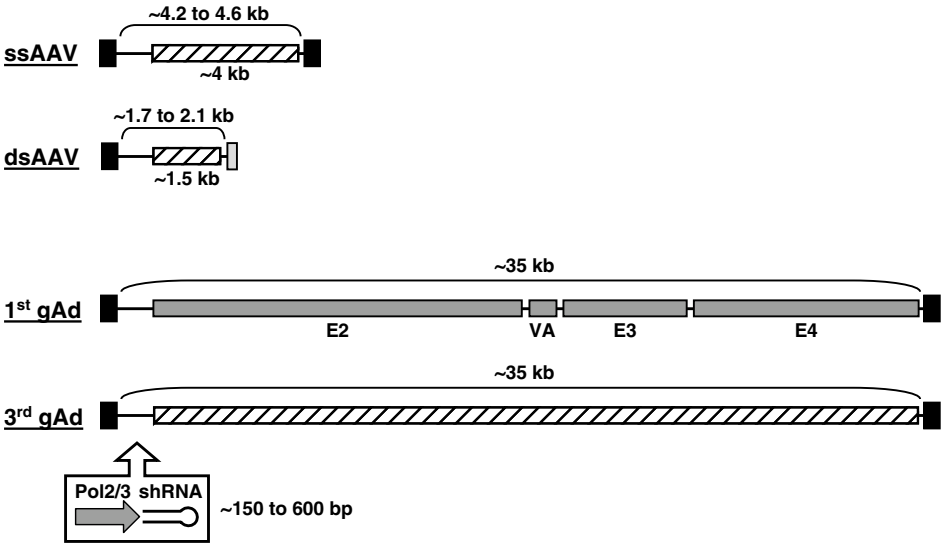

Figure 3 Viral gene transfer vectors for liver-directed shRNA delivery. Shown on top are a conventional single-stranded adeno-associated viral (AAV) vector, allowing insertion of up to 4.6 kb of foreign DNA (shRNA expression cassette plus stuffer DNA). Depicted below is a novel double-stranded variant, which packages two inverted copies of the insert, due to a mutation in one of the viral encapsidation elements (gray box, wild-type elements are shown in black). The bottom schemes show a conventional first-generation adenoviral vector, in which only the E1 gene is replaced with an shRNA cassette (plus stuffer), while the other viral genes are still expressed. In an optimized gutted (third generation) vector, the entire genome is deleted and replaced with a long stuffer sequence of about 35 kb of DNA. The inset shows a typical shRNA cassette, with the shRNA under the control of an RNA polymerase II or III promoter.

years, associated with a substantial loss of vector DNA in the liver, for reasons unclear.[64,70,75,76]

In a proof-of-principle study for the use of first-generation adenoviral vectors to treat HBV infection, Uprichard *et al*.[40] expressed two of their most efficient anti-HBV shRNAs from such a vector in HBV-transgenic mice. Therefore, these mice were injected with 2×10^9 adenoviral particles encoding either the anti-HBV shRNAs, or a scrambled control, and serum HBsAg was measured as a downstream indicator of HBV gene expression. Impressively, already at day 4 post-infection, the anti-HBV vectors led to a $\sim$5-fold reduction in sAg, and by day 20 (length of experiment), the drop was about 100-fold. HBV inhibition was confirmed on the RNA level via Northern blot analyses, showing a five- to 50-fold reduction of pre-genomic or envelope transcripts. To investigate the vector effect on HBV DNA replication, the authors then repeated the injections in HBV-transgenic mice that were deficient for the expression of IFNγ and the IFNα/β receptor, because in wild-type mice, adenovirus infection induces interferons which clear HBV DNA from the liver. Similar to the initial experiment, the anti-HBV shRNA vectors led to significant reductions of HBV proteins (sAg and cAg) and RNA, with the latter nearly eliminated from the liver at day 26. In addition, Southern blot analyses showed that HBV replicative intermediates were virtually undetectable in livers of mice treated with one adenoviral construct, proving that clearance of viral transcripts was sufficient to abolish HBV DNA replication.

A strength of this study was that it was the first to demonstrate the feasibility to suppress ongoing HBV gene expression and replication *in vivo*, in a model which mimicks the situation in chronically infected humans, where every hepatocyte may contain an HBV genome. However, it is largely questionable whether the particular adenoviral approach will be useful in humans. This is exemplified by the fact that the adenoviral DNA was almost completely (90%) cleared from infected mice at day 20, which likely resulted from the above-mentioned toxicity from a first-generation vector, and which also explains why the reported study was ended at this timepoint.

Moreover, the vector also causes an interferon response, which might in fact be beneficial as it aids in HBV suppression. On the other hand, uncontrolled IFN (and CTL) activation must be regarded as an adverse effect that would prevent application of the vector in humans. In fact, the risks associated with the use of first-generation adenoviral vectors became dramatically clear when a few years ago, a 19-year-old patient treated with a high dose of an E1/E4-deleted variant died from acute liver failure.[77] Hopefully, these risks will be diminished with newer generations of these vectors, in particular the gutted variants. Once the technology to produce these vectors has been improved, and optimal stuffer DNA sequences have been identified, gutted adenoviruses might indeed become an important tool for liver-directed shRNA transfer.[78]

Adeno-associated virus

AAVs are small (virion diameter of about 20 nm), non-enveloped members of the family Parvoviridae with a single-stranded DNA genome of approximately 4700 nucleotides. The genome is extremely simple and comprises only two genes, encoding seven non-structural or structural proteins, flanked by short packaging sequence elements (inverted terminal repeats, ITRs) (Figure 3). To make a recombinant AAV, plasmids are used which contain a full-length viral genome with convenient restriction sites located next to the ITRs, allowing replacement of the viral genes with a transgene

expression cassette of up to 4.6 kb.[79] The resulting construct is then encapsidated into shells from the portfolio of over 100 known AAV serotypes (a process referred to as pseudotyping), with AAV-2 representing the prototype and best-studied family member.[68]

A variety of properties make AAV vectors very interesting tools for liver-directed shRNA expression, including the lack of pathogenicity of the wild-type virus, and the ease and efficiency of vector production due to highly advanced technology. Perhaps most important with respect to their use as anti-HBV/HCV RNAi vectors is AAV's ability to persist in the transduced hepatocyte, typically as an episomal, transcriptionally active DNA molecule.[80] This property could essentially be exploited to achieve life-long antiviral shRNA expression in patients, thus providing a persistent cure from HBV/HCV and/or protection from (re-)infection in a 'vaccination' strategy (see above).

As proof-of-concept for this idea, Moore *et al*.[50] recently engineered an AAV-2-based vector to express their most effective shRNA-directed against the HBsAg gene, and then evaluated RNAi persistency in tissue culture. Therefore, they also generated a 293T-based cell line stably expressing the target gene, or used hepatoma cells containing a full-length-integrated HBV genome. At 7 days after transduction of both cell lines with their AAV/shRNA vector, the authors detected a 89–98% reduction of sAg protein in the cell supernatant, as compared to an irrelevant shRNA control. An 80% reduction in pre-genomic HBV RNA levels in transduced hepatoma cells further corroborated AAV vector efficiency. Notably, when single cells were cloned from the transduced pool, ~83% inhibition of sAg expression were still found 5 months post-AAV treatment, demonstrating the stability of the antiviral RNAi.

While this work highlights the potential of AAV vectors to mediate long-term anti-HBV RNAi, several improvements are needed to translate the approach into humans. One problem is that the vector used in this study was based on AAV-2, which is highly prevalent in the human population and thus frequently neutralized by antibodies.[63] Moreover, AAV-2 is not very efficient in the liver, where it results in transgene expression in not more than 5–10% of all hepatocytes, at least in mice.[81] It will therefore be important to evaluate different AAV serotypes for liver-directed shRNA expression, such as the recently described AAV-8 which at high doses transduces 100% of hepatocytes in murine livers.[82] As mentioned before, complete and stable liver transduction will most likely be beneficial for anti-HBV/HCV approaches.

A second hurdle is the need for extreme vector doses of greater than 5×10^{12} even with the efficient AAV-8 pseudotype,[82] which in humans might translate into 10^{14} particles and more. Perhaps this problem can be alleviated by the use of 'ds' AAV genomes (Figure 3), which transduce liver 10–100× more efficiently than conventional single-stranded AAVs, and thus allow the use of significantly lower doses.[83–86] Thus far, dsAAVs were limited by their reduced packaging capacity of only ~2.4 kb foreign DNA, but regarding the usually small size of shRNA expression cassettes of only a few hundred basepairs, these vectors might in fact be ideally suited. It will be interesting to generate virions which combine these two advances, AAV-8 capsids and ds genomes; potentially, such vectors will yield complete and persistent liver transduction with antiviral shRNAs from minimal particle doses, making them exciting tools for clinical use.

Conclusion: prospects of RNAi as an anti-HBV/HCV therapeutic

The past 3 years have been a thrilling time for hepatitis research. Initiated in 2002/3 by two studies by McCaffrey *et al*.,[18,62] exemplifying the use of *in vivo* RNAi for HBV/HCV knockdown, the field is now exploding at incredible speed of exciting discovery. As documented by a plethora of recent reports, important advances are rapidly made in all aspects of this novel technology: better models become available to study the viral targets *in vivo*, the vectors for expression of antiviral shRNAs are being optimized, and our knowledge for rational shRNA design and target selection is growing daily. The door to a prolific new field of antiviral therapeutics is wide open – so should we take the step beyond proof-of-concept studies, and progress to evaluation and implementation of RNAi strategies in a therapeutic setting? The answer is perhaps, soon. At this current point, there are some essential issues that remain to be resolved, and daunting obstacles to be overcome, before the approach can live up to its potential in humans.

First and foremost, despite the overwhelming evidence that antiviral RNAi is transiently functional and efficient in cultured cells and mice, it is now mandatory to repeat and expand on long-term *in vivo* evaluation of these strategies in small and larger animals. In particular for HCV, these options were unavailable for the longest time, but the recent isolation of the first viral clone autonomously replicating in cultured cells, and secreting particles that are infectious for chimpanzees,[26–28] provides hope that testing of anti-HCV RNAi strategies in large animals will soon be possible.

Secondly, in the context of these studies, it will be essential to evaluate the long-term effects of *in vivo* shRNA expression in the livers of treated animals. An increasing number of reports question the specificity of exogenously induced RNAi, and find untoward effects such as induction of the interferon system, silencing of non-targeted genes, or dose-dependent, but otherwise poorly understood toxic consequences of high-level shRNA expression (e.g., Persengiev *et al*.,[87] Pebernard and Iggo,[88] Jackson and Linsley,[89] Fish and Kruithof,[90] Sledz *et al*.,[91] Saxena *et al*.[92] and Jackson *et al*.[93]). It is obvious that these unanticipated effects must be better characterized and defined in animal models, before the approach can be tested in humans.

Last but not least, further improvements also need to be made to the vectors for liver-directed shRNA delivery, as well as to the encoded shRNA cassettes. There are multiple levels where such advances are required, but they all must serve to increase efficacy and specificity of hepatocellular shRNA expression. One approach is to optimize the viral genome, as outlined above, to improve vector transduction and concurrently minimize required particle doses, as well as to eliminate potential tocixity from virally encoded proteins. For the purpose of achieving persistent *in vivo* RNAi, it will be particularly interesting to study the latest generation of integrating

adenoviruses,[94] or engineer AAV vector genomes with increased persistency as episomal forms.

Concurrently, more focus needs to be put on developing expression cassettes where the shRNA is under the control of an RNA polymerase II promoter, rather than polymerase III (e.g., U6 or H1) as in most current constructs. The reason is that the latter are usually constitutively active across many tissues, creating risks of uncontrolled and unwanted strong shRNA expression in non-liver organs which are susceptible to vector transduction. This concern is reasonable considering that the two most potent currently available viral vectors for liver gene transfer, AAV-8 and adenovirus 5, show a broad tropism and will thus deliver the shRNA to several tissues throughout the body, in particular when used at higher doses.[82] A solution to this problem is likely provided by the use of tissue-specific and conditionally active RNA polymerase II promoters, which could restrict shRNA expression to hepatocytes, and moreover allow exogenous control over the onset and level of intracellular shRNA production.[95,96] Recent pilot studies, using for instance the CMV promoter to drive shRNA expression from an adenoviral vector, demonstrate that this approach is feasible and worth pursuing.[97]

Finally, it is also crucial to expand on strategies for simultaneous expression of multiple shRNAs from a common viral vector backbone. This is particularly indicated with the HCV genome as a target, due to the above mentioned lack of proof-reading activity of the viral RNA polymerase and the resulting high mutation rate of the virus, allowing emergence of escape variants resistant to RNAi by one specific shRNA. Fortunately, even the before described ds, highly efficient AAV vector genomes with their limited DNA packaging capacity provide sufficient space to accommodate multiple shRNA expression cassettes, for instance three to four copies of a U6 promoter-driven shRNA (typically $\sim$500 bp). Together with efforts to strategically target highly conserved regions along the viral genome less prone to mutation, and/or to block host cell factors involved in virus uptake or replication, the problem of viral escape from RNAi-mediated repression should thus be surmountable. To combat escape mutants even more efficiently, it should also be beneficial to combine shRNAs with conventional antiviral drug therapies, assuming that this will result in synergistic effects.

In conclusion, it is striking to see how RNAi is rapidly exceeding expectations for its use in the study of basic biological processes, and the therapeutic potential of this novel technology for treatment of virally induced human liver disease is enormous. The current momentum to gain better understanding of RNAi-related mechanisms, and the constant improvement in their application and translation into a biomedical tool, raises considerable hope that we will see the clinical evaluation of efficient, safe and specific antiviral RNAi therapeutics in the not-too-distant future.

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