Review Article

Management of chronic hepatitis B virus infection: A promising approach using small interfering RNA (siRNA)

MANISHA DWIVEDI, S. P. MISRA, VATSALA MISRA, R. K. WAIKHOM, MUKTI BHATNAGAR

ABSTRACT

Hepatitis B virus infection continues to be a major global health problem with an estimated 350 million carriers. The response to available treatment modalities is not impressive. The advent of RNA interference—a phenomenon of sequence-specific degradation of RNAs mediated by double-stranded RNA—holds promise as a potential therapy for chronic hepatitis B virus infection. Synthetic preparations of short RNA (21–23 bp long) can be used to mediate this process of gene silencing with a lower immune response. The duration of suppression can be further increased by using a vector delivery system. Small interfering RNA (siRNA) has several advantages over conventional therapy, which include fewer side-effects, a lower chance of developing escape mutants and non-requirement of viral replication for its action. A potent knockdown of the gene of interest with high sequence specificity makes RNA interference a powerful tool that has shown antiviral effect against hepatitis B virus. However, the 'off-target effect', i.e. suppression of genes other than the intended target, poor siRNA stability, inefficient cellular uptake, widespread biodistribution and non-specific effects need to be overcome. The problem of long term toxicity of siRNA should be addressed and an ideal vector delivery system needs to be designed before it can be put to clinical use.

Natl Med J India 2008;21:123–7

INTRODUCTION

Hepatitis B virus (HBV) infection is a major global health problem, particularly in Asia. Worldwide, there are approximately 350 million chronic carriers of HBV.[1] Though an effective vaccine has been available for the past 2 decades, the number of carriers continues to be fairly high. Treatment options for chronic HBV infection include interferons (IFNs) and nucleoside/nucleotide analogues such as lamivudine, adefovir, tenofovir and entecavir. These agents reduce viral loads to undetectable levels, but their use is associated with problems. IFNs cause serious side-effects while lamivudine may lead to recurrence of viraemia after cessation of therapy and there is a risk of developing 'escape mutants' after prolonged treatment with lamivudine.[2–4] The response with adefovir and entecavir too is not impressive, and these limitations have led to the search for an effective alternative strategy.[5,6]

RNA interference (RNAi) is a process of sequence-specific post-transcriptional gene silencing mediated by double-stranded RNA (dsRNA). This phenomenon was first observed in the nematode *Caenorhabditis elegans*.[7] After delivery of long dsRNA to *C. elegans*, it is processed into 21–25 bp si (small interfering) RNA by an enzyme called Dicer.[8] Each strand of this RNA has a 5' phosphate and a 3' hydroxyl end with 2–3 nucleotide overhangs in the 3' end. siRNA is incorporated into the RNA-induced silencing complex (RISC) which upon activation unwinds the siRNA to make it functional. The unwound siRNA is used by RISC to select the target by base pairing which is later degraded in regions of homology detected by the original siRNA.[9] The viral replication and gene expression of a number of human infectious viruses including HIV,[10,11] polio virus,[12] human papilloma virus,[13] human delta virus,[14] respiratory syncytial virus,[15] influenza virus[16] and dengue virus[17] is also affected by RNAi. This phenomenon can possibly be developed in the future to treat viral infections.

MECHANISM OF ACTION

A potent knockdown of the gene of interest, with high sequence specificity, can be achieved by using a short (21–23 nucleotides) interfering RNA. The siRNA duplex directs sequence-specific post-transcriptional silencing of homologous genes by binding to its complementary sequence in mRNA and triggering its elimination. siRNAs are the processing products originating from dsRNA that are cleaved by RNAse III-like ribonuclease. In the phenomenon of RNA interference, double-stranded RNA induces sequence-specific degradation of homologous mRNA. The delivery of siRNA into mammalian cells often induces innate interfering IFN responses and sequence-independent suppression of both endogenous and exogenous gene expression as an 'off-target' effect. The use of siRNA with an immunostimulatory RNA motif or siRNA with a length of 30 bp or more may induce a non-specific IFN response in mammalian cells through RNA-activated protein kinase (PKR) and interaction with endosomal Toll-like receptors (TLRs), in particular TLR 7/8.[17,18] The efficacy of siRNA in suppressing viral gene

Moti Lal Nehru Medical College, University of Allahabad, Allahabad, Uttar Pradesh, India

MANISHA DWIVEDI, S. P. MISRA, R. K. WAIKHOM, MUKTI BHATNAGAR Department of Gastroenterology

VATSALA MISRA Department of Pathology

Correspondence to MANISHA DWIVEDI; *manisha_dwivedi@hotmail.com*

expression has been proven in both cell culture studies[19–24] and mouse models[25–28] (Table I).

Klein *et al*.[25] transferred naked DNA of the HBV replication-competent vector into NMRI mice through the tail vein. HBV replication was studied in the serum and liver of animals. Tail vein transfer of an HBV replication-competent construct led to the expression of HBV-specific transcripts in the liver resulting in 10% of hepatocytes becoming positive for hepatitis B e antigen (HBeAg) and hepatitis B surface antigen (HBsAg). Intrahepatic HBV-specific mRNA transcripts were analysed by multiplex reverse transcriptase–polymerase chain reaction (RT–PCR) with a primer pair. At least 3 animals were treated in parallel. After 3 and 4 days of injection of the replication-competent vector in mice, the HBsAg and HBeAg levels were elevated in the sera and 10% of hepatocytes showed a positive stain for HBV by immunohistochemistry. Hepatocytes were also positive for HBeAg. After administration of siRNA, HBeAg suppression lasted longer than HBsAg suppression.[23] The RNAi activity is sequence-specific and results in post-transcriptional gene-silencing. The dsRNA triggers degradation of homologous mRNA in the cytoplasm and hence siRNA activity does not depend upon viral replication.

TARGET SEQUENCE

The HBV genome, which is a partial, double-stranded 3.2 kb DNA molecule, has a special genomic organization and its 4 viral transcripts overlap. They also utilize a common polyadenylation signal located within the core protein coding region. Targeting this overlapping sequence can result in suppression of multiple viral transcripts.[21] Various target sequences have been used in different studies (Table II).

Uprichard *et al*.[28] demonstrated that most pre-existing viral transcripts in the liver during ongoing viral replication are susceptible to inhibition by RNAi (RNA inhibitor). Clearance of these transcripts is sufficient to abolish HBV DNA replication in infected hepatocytes. The magnitude and suppression attained emphasizes the potential of this treatment for chronic hepatitis B and C virus infection. However, after treatment with siRNAs a stable pool of viral 3.5 kb RNA persisted in the liver in spite of the presence of HBV-specific shRNAs (short hairpin RNAs; have effects similar to siRNAs). This indicates that a population of HBV RNA is protected from RNAi-mediated degradation.

Two distinct 3.5 kb RNAs are transcribed from the HBV genome—the pre-genomic RNA (pg RNA) and the 31-nucleotide-longer 4 HBeAg mRNA. Following administration of siRNA, the decrease of HBsAg in the serum is more dramatic than that of HBeAg suggesting global protection of a subset of the HBeAg mRNA within the RNA–protein complexes; perhaps, polyribosome complexes of rough endoplasmic reticulum.[28]

TABLE I. Efficacy of small interfering RNA silencing on hepatitis B virus (HBV) in mouse models and cell culture studies

Author (year)	Type of study	Result
Shlomai *et al*.[19] (2003)	Cell culture; co-transfection of Huh 7 cells with whole HBV with super vectors against X-ORF, CORE-ORF	pSUPER-X: 59% reduction of core protein, 60% reduction of HBsAg, 68% reduction of all viral transcripts, 95% reduction of replication intermediates
		pSUPER CORE: 63% reduction of core protein, no effect on HBsAg, 13% reduction in 3.5 kb transcript and 40% reduction in replication intermediates
Klein *et al*.[25] (2003)	Animal model	Target S-ORF: HBsAg reduction by 70%, effect lasts 11 days, HBeAg reduction by 80%, suppression longer, decrease in viral RNA
		Target CORE-ORF: Reduction by 60% of HBeAg, effect transient (3–5 days)
Chen *et al*.[20] (2003)	Cell culture	HBV RNA expression decreased by 90%, HBV DNA concentration decreased by 90%–97%
Uprichard *et al*.[28] (2005)	Animal model study: Recombinant adenovirus used as vector	Ongoing HBV replication *in vivo* in HBV transgenic mice can be cleared by RNA targeted suppression of viral RNA for at least 26 days
Ren *et al*.[21] (2006)	Cell culture study: Stable transfection using vector	96% reduction of HBsAg level, 88.3% reduction of HBeAg, HBV DNA dropped 9.6–12.8-fold

HBsAg hepatitis B surface antigen HBeAg hepatitis B e antigen

TABLE II. Target sequences of hepatitis B virus (HBV) used in various studies of small interfering RNA silencing

Study (Year)	Sequence	Region
Konishi *et al*.[22] (2003)	5'ACCCTTAUAAAGAATTTGG3'	Polyadenylation
	5'GCTGTGCCTTGGGTGGCTT3'	Pre C
	5'TACCGCAGAGTCTAGACTC3'	Surface
Hamasaki *et al*.[23] (2003)	5'CATTGTTCACCTCACCATA3'	Core
Ying *et al*.[24] (2003)	5'AAGACCTAGTCAGTTAT3'	Core
Giladi *et al*.[27] (2003)	5'CATCACATCAGGATTTCC3'	Surface
	5'CATCACATCAGGATTCCTA3'	
	5'CCAGTACGGGACCATGCAA3'	
	5'GTCTGTACAGCATCGTGAG3'	
McCaffrey *et al*.[26] (2003)	5'CTCAGTTACTAGTGCCATTTGTTC3'	Surface
	5'CCTAGAAGAAGAACTCCCTCGCCTC3'	Core
Shlomai *et al*.[19] (2003)	5'GATCAGGCAACTATTGTGG3'	Core X
	5'GGTCTTACATAAGAGGACT3'	
Klein *et al*.[25] (2003)	5'AAGCCTTAGAGTCTCCTGAGC3'	Core
	5'AATTTGTTCAGTGCCATTTGTTC3'	Surface

Further studies are needed to confirm if the HBeAg 3.5 kb transcript is less sensitive to RNAi-mediated clearance.

Zhe *et al*.[29] used increasing doses of siRNA to transfect HEP2.2.15 cells and found a higher degree of inhibition of viral expression molecules with increasing doses. Similar results were seen for siRNA targeting the pre-C region.

Another concept to prevent ongoing hepatocyte damage in chronic hepatitis B infection is the fact that these patients maintain an HBV-specific cytotoxic T lymphocyte (CTL) response throughout the infection. Though the response is insufficient to clear the virus, it continues to destroy cells expressing HBV antigens resulting in hepatocyte turnover. Consequently, cells in which HBV expression has been silenced, after administration of siRNA, would have a definite survival advantage and would be selected over time to repopulate the liver. This hypothesis can be tested by adoptive transfer of HBV-specific CTLs to transgenic mice after infection with siRNA-expressing vectors.

Drugs used to treat chronic HBV infection such as lamivudine and adefovir do not reduce the high viral antigenaemia which may blunt the T cell response and so avoid immune clearance. In contrast, a reduction in viral antigen levels attained after RNAi-mediated degradation of viral RNA may relieve the negative impact of chronic antigen stimulation on the T-cell response which leads to a clearance of the virus. This study conclusively demonstrated that adenovirus-mediated delivery of siRNA to the liver of mice is effective in checking HBV replication *in vivo*.

COMBINATION THERAPY

Cell culture studies have shown that a combination of different siRNAs results in more robust inhibition of viral antigen expression.[29] It has been assumed that akin to DNA, RNA molecules might have a repairing mechanism when they are cleaved by physical, biological or chemical forces. A combination of different siRNAs will lead to cleavage at multiple sites and make it difficult for the target to be repaired, thereby leading to greater efficacy.

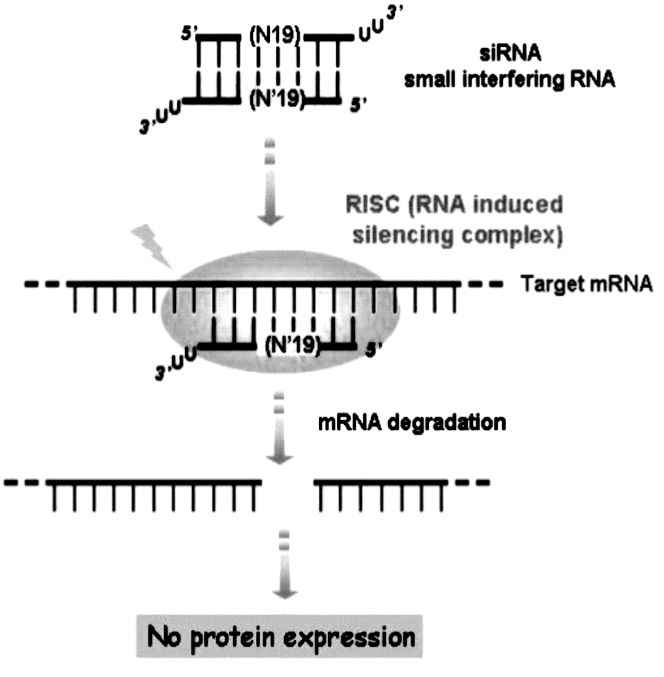

FIG 1. Model for RNA interference/post-transcriptional gene silencing mechanism

Combination with lamivudine

In HepG2.2.15 cells treated with a combination of siRNA and lamivudine, the secretion of HBeAg and HBsAg in the supernatant was inhibited at 96 hours by 91.8% and 82.4%, respectively. The number of HBV DNA copies within the culture medium was also significantly decreased at 96 hours. Moreover, mRNA concentration in HepG2.2.15 cells treated with a combination of siRNA and lamivudine was lower as compared with those treated either with siRNA or lamivudine. A combination of siRNA and lamivudine exhibited a stronger anti-HBV effect than the use of siRNA or lamivudine alone.[30]

VECTOR SYSTEM IN siRNA

In several organisms, introduction of dsRNA has proven to be a powerful tool to suppress gene expression through a process known as RNA interference. However, in most mammalian cells this provokes a strong cytotoxic response. This non-specific effect can be circumvented by the use of synthetic short (21- to 22-nucleotide) interfering RNAs (siRNAs), which can mediate strong and specific suppression of gene expression. However, this reduction in gene expression is transient, which severely restricts its applications.

To overcome this limitation, the pSUPER RNAi system provides a mammalian expression vector that directs intracellular synthesis of siRNA-like transcripts. The vector uses the polymerase-III H1-RNA gene promoter, as it produces a small RNA transcript lacking a polyadenosine tail and has a well-defined start of transcription and a termination signal consisting of 5 thymidines in a row (T5). Most important, the cleavage of the transcript at the termination site is after the second uridine, yielding a transcript resembling the ends of synthetic siRNAs, which also contain two 3' overhanging T or U nucleotides.

The pSUPER RNAi system has been used for efficient and specific downregulation of gene expression resulting in functional inactivation of the targeted genes. Stable expression of siRNAs using this vector mediates persistent suppression of gene expression, allowing the analysis of loss-of-function phenotypes that develop over longer periods of time.[31]

VECTOR DELIVERY SYSTEM

The first step in the delivery system includes annealing of DNA (bonding or linkage of bases/nucleotides) corresponding to that of the target genome sequence. The next step involves linearization of the circular pSUPER vector by using restriction enzyme Bgl II and either HIND III or Xho I which cut the circular pSUPER vector at specific recognition sites. The annealed oligos are inserted between specific sequences formed by restriction enzymes and ligated (joining of two sequence fragments) into the pSUPER vector by a ligase enzyme.

To effect the silencing of a specific gene, the pSUPER vector (plasmid) is used along with a pair of custom oligonucleotides that contain, among other features, a unique 19-nucleotide sequence derived from the mRNA transcript of the HBV gene targeted for suppression (the 'N-19 target sequence'). Various studies have shown that co-transfection of siRNA-producing vectors targeted against a specific sequence in the HBV genome results in significant reduction in the corresponding viral transcripts and proteins.

The N-19 target sequence corresponds to the sense strand of the pSUPER-generated siRNA, which in turn corresponds to a 19-nucleotide sequence within the mRNA. In the mechanism of RNAi, the antisense strand of the siRNA duplex hybridizes

to this region of the mRNA to mediate cleavage of the molecule.

These forward and reverse oligos are annealed (bonding between bases) and cloned by the user into the vector, between the unique Bgl II and either Hind III or Xho I enzyme sites. This positions the forward oligo at the correct site downstream from the H1 promoter's TATA box to generate the desired siRNA duplex.

The sequence of this forward oligo includes the unique N-19 target in both sense and antisense orientation, separated by a 9-nucleotide spacer sequence. The 5' end corresponds to the Bgl II site, while the 3' end contains the T5 sequence and any Hind III corresponding nucleotides.

The resulting transcript of the recombinant vector is predicted to fold back on itself to form a 19 bp stem-loop structure. Analysis indicates that the stem-loop precursor transcript is quickly cleaved in the cell to produce a functional siRNA.[31]

POTENTIAL LIMITATIONS

Many studies have shown that siRNA and shRNA do not induce an IFN response, but there are reports in which the IFN pathway seems to be activated.[32,33] In the study by Bridge *et al*.[32] there was a 500-fold induction of 2'5' oligoadenylate synthetase (OAS1) which is a well-known target of the IFN system. The data suggest that the ability to activate the IFN system depends upon the siRNA sequence and the DNA vector used. An acceptable level of the IFN activation system should be determined in therapeutic settings and the lowest dose of siRNA should be used because it seems plausible that the IFN response is dose-related. Another potential danger is that of an 'off-target effect'.[34] It has been noted that siRNA may suppress genes other than the intended target gene which are independent of the IFN effect. Further studies are needed before applying this modality in humans. The probability of development of an escape virus is also present. RNAi is extremely sensitive to mismatches in the targeted region. Even a small mismatch can result in abrogation of the gene action, so even a single mutation developed by the virus in the target sequence can lead to development of resistant strains. This problem has been seen in HIV and polio viruses.[12,35] This may be avoided by using a mixture of different siRNAs acting against different sequences. Another problem is an effective method of vector delivery. The apparent instability of siRNA in the vascular system should be kept in mind. Chemical modifications need to be done to increase the half-life. Methyl-modified siRNAs were found to be more stable inside cells and had longer lasting inhibitory effects than natural siRNAs. The hydrodynamic injection technique in the easily accessible tail vein of mouse is used to efficiently deliver HBV expression plasmids and siRNAs targeting various HBV gene regions with enhanced HBV gene silencing. A high volume injection can be given in the tail vein. One potential mode of delivery can be by combining the siRNA with liposomes and a small peptide which can bind to specific liver receptors.

ADVANTAGES OF RNA INTERFERENCE

siRNA specifically targets the viral transcripts and proteins. This in turn impairs the replication of the virus and promotes its eradication without activating a non-specific cellular response, hence minimizing undesirable effects. Second, by targeting specific conserved regions of the HBV genome, the ability of the virus to create mutants can also be limited because that particular sequence will be occupied by an siRNA sequence at post-transcriptional levels. Third, different sequences can be targeted simultaneously by using different siRNAs. This limits escape mutants and makes it possible to treat chronically infected people with a variation in genomes of the infective agent. Fourth, the ability of siRNAs to reduce the level of viral transcript and proteins even in the absence of active viral replication makes it a good candidate as an adjuvant with lamivudine which acts only on replication-competent HBV.[36]

CONCLUSIONS

siRNA seems to be an attractive potential therapy for the management of HBV infection. The response rate seen in cell culture and mice studies is impressive and with the introduction of a super vector system the problem of transient suppression seems to have been circumvented. However, further studies are required before this method can be used in clinical settings. The problem of long term toxicity needs to be addressed and an improved vector delivery system needs to be developed.

REERENCES

1 Kao JH, Chen DS. Global control of hepatitis B virus infection. *Lancet Infect Dis* 2002;**2:**395–403.

2 Cooksley WG. Treatment with interferons (including pegylated interferons) in patients with hepatitis B. *Semin Liver Dis* 2004;**24** (Suppl 1):45–53.

3 Liaw YF, Leung NW, Chang TT, Guan R, Tai DI, Ng KY, *et al.* Effects of extended lamivudine therapy in Asian patients with chronic hepatitis B: Asia Hepatitis Lamivudine Study Group. *Gastroenterology* 2000;**119:**172–80.

4 Suzuki F, Suzuki Y, Tsubota A, Akuta N, Someya T, Kobayashi M, *et al.* Mutations of polymerase, precore and core promoter gene in hepatitis B virus during 5-year lamivudine therapy. *J Hepatol* 2002;**37:**824–30.

5 Marcellin P. Advances in therapy for chronic hepatitis B. *Semin Liver Dis* 2002;**22** (Suppl 1):33–6.

6 Lai CL, Rosmawati M, Lao J, Van Vlierberghe H, Anderson FH, Thomas N, *et al.* Entecavir is superior to lamivudine in reducing hepatitis B virus DNA in patients with chronic hepatitis B infection. *Gastroenterology* 2002;**123:**1831–8.

7 Fire A, Xu S, Montgomery MK, Kostas SA, Driver SE , Mello CC. Potent and specific genetic interference by double-stranded RNA in *Caenorhabditis elegans*. *Nature* 1998;**391:**806–11.

8 Bernstein E, Caudy AA, Hammond SM, Hannon GJ. Role for a bidentate ribonuclease in the initiation step of RNA interference. *Nature* 2001;**409:**363–6.

9 Elbashir SM, Harborth J, Lendeckel W, Yalcin A, Weber K, Tuschl T. Duplexes of 21-nucleotide RNAs mediate RNA interference in cultured mammalian cells. *Nature* 2001;**411:**494–8.

10 Coburn GA, Cullen BR. Potent and specific inhibition of human immunodeficiency virus type 1 replication by RNA interference. *J Virol* 2002;**76:**9225–31.

11 Jacque JM, Triques K, Stevenson M. Modulation of HIV-1 replication by RNA interference. *Nature* 2002;**418:**435–8.

12 Gitlin L, Karelsky S, Andino R. Short interfering RNA confers intracellular antiviral immunity in human cells. *Nature* 2002;**418:**430–4.

13 Jiang M, Milner J. Selective silencing of viral gene expression in HPV-positive human cervical carcinoma cells treated with siRNA. A primer of RNA interference. *Oncogene* 2002;**21:**6041–8.

14 Bitko V, Barik S. Phenotypic silencing of cytoplasmic genes using sequence-specific double-stranded short interfering RNA and its application in the reverse genetics of wild type negative-strand RNA viruses. *BMC Microbiol* 2001;**1:**34.

15 Ge Q, McManus MT, Nguyen T, Shen CH, Sharp PA, Eisen HN, *et al.* RNA interference of influenza virus production by directly targeting mRNA for degradation and indirectly inhibiting all viral RNA transcription. *Proc Natl Acad Sci USA* 2003;**100:**2718–23.

16 Adelman ZN, Sanchez-Vargas I, Travanty EA, Carlson JO, Beaty BJ, Blair CD, *et al.* RNA silencing of dengue virus type 2 replication in transformed C6/36 mosquito cells transcribing an inverted-repeat RNA derived from the virus genome. *J Virol* 2002;**76:**12925–33.

17 Balachandran S, Roberts PC, Brown LE, Truong H, Pattnaik AK, Archer DR, *et al.* Essential role for the dsRNA-dependent protein kinase PKR in innate immunity, to viral infection. *Immunity* 2000;**13:**129–141.

18 Hunter T, Hunt T, Jackson RJ, Robertson HD. The characteristics of inhibition of protein synthesis by double-stranded ribonucleic acid in reticulocyte lysates. *J Biol Chem* 1975;**250:**409–17.

19 Shlomai A, Shaul Y. Inhibition of hepatitis B virus expression and replication by RNA interference. *Hepatology* 2003;**37:**764–70.

20 Chen Y, Du D, Wu J, Chan CP, Tan Y, Kung HF, *et al.* Inhibition of hepatitis B virus replication by stably expressed shRNA. *Biochem Biophys Res Commun* 2003;**311:**398–404.

21 Ren X, Luo G, Xie Z, Zhou L, Kong X, Xu A. Inhibition of multiple gene expression and virus replication of HBV by stable RNA interference in 2.2.15 cells. *J Hepatol* 2006;**44:**663–70.
22 Konishi M, Wu CH, Wu GY. Inhibition of HBV replication by siRNA in a stable HBV-producing cell line. *Hepatology* 2003;**38:**842–50.
23 Hamasaki K, Nakao K, Matsumoto K, Ichikawa T, Ishikawa H, Eguchi K. Short interfering RNA-directed inhibition of hepatitis B virus replication. *FEBS Lett* 2003;**543:**51–4.
24 Ying C, De Clercq E, Neyts J. Selective inhibition of hepatitis B virus replication by RNA interference. *Biochem Biophys Res Commun* 2003;**309:**482–4.
25 Klein C, Bock CT, Wedemeyer H, Wustefeld T, Locarnini S, Dienes HP, *et al.* Inhibition of hepatitis B virus replication *in vivo* by nucleoside analogues and siRNA. *Gastroenterology* 2003;**125:**9–18.
26 McCaffrey AP, Nakai H, Pandey K, Huang Z, Salazar FH, Xu H, *et al.* Inhibition of hepatitis B virus in mice by RNA interference. *Nat Biotechnol* 2003;**21:**639–44.
27 Giladi H, Ketzinel-Gilad M, Rivkin L, Felig Y, Nussbaum O, Galun E. Small interfering RNA inhibits hepatitis B virus replication in mice. *Mol Ther* 2003;**8:**769–76.
28 Uprichard SL, Boyd B, Althage A, Chisari FV. Clearance of hepatitis B virus from the liver of transgenic mice by short hairpin RNAs. *Proc Natl Acad Sci USA* 2005;**102:**773–8.
29 Zhe C, Zex X, Feng, Jia YJ, Yao Ping YH, Shu Z, *et al.* Combination of small interfering RNAs mediate greater inhibition on human hepatitis B replication and antigen expression. *J Zhejiang University Science* 2005;**4:**236–41.
30 Li GQ, Xu WZ, Wang JX, Deng WW, Li D, Gu HX. Combination of small interfering RNA and lamivudine on inhibition of human B virus replication in HepG2.2.15 cells. *World J Gastroenterol* 2007;**13:**2324–7.
31 Brummelkamp TR, Bernards R, Agami R. A system for stable expression of short interfering RNAs in mammalian cells. *Science* 2002;**296:**550–3.
32 Bridge AJ, Pebernard S, Ducraux A, Nicoulaz AL, Iggo R. Induction of an interferon response by RNAi vectors in mammalian cells. *Nat Genet* 2003;**34:**263–4.
33 Sledz CA, Holko M, de Veer MJ, Silverman RH, Williams BR. Activation of the interferon system by short-interfering RNAs. *Nat Cell Biol* 2003;**5:**834–9.
34 Jackson AL, Bartz SR, Schelter J, Kobayashi SV, Burchard J, Mao M, *et al.* Expression profiling reveals off-target gene regulation by RNAi. *Nat Biotechnol* 2003;**21:**635–7.
35 Haasnoot PC, Bol JF, Olsthoorn RC. A plant virus replication system to assay the formation of RNA pseudotriloop motifs in RNA–protein interactions. *Proc Natl Acad Sci USA* 2003;**100:**12596–600.
36 Shin D, Kim SI, Kim M, Park M. Efficient inhibition of hepatitis B virus replication by small interfering RNAs targeted to the viral X gene in mice. *Virus Res* 2006;**119:**146–53.