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ORIGINAL ARTICLE

Long-term inhibition of hepatitis B virus in transgenic mice by double-stranded adeno-associated virus 8-delivered short hairpin RNA

C-C Chen[1,2], T-M Ko[1], H-I Ma[3], H-L Wu[4], X Xiao[5], J Li[5], C-M Chang[1,6], P-Y Wu[1], C-H Chen[1], J-M Han[3], C-P Yu[7], K-S Jeng[8], C-P Hu[9] and M-H Tao[1]

[1]Institute of Biomedical Sciences, Academia Sinica, Taipei, Taiwan; [2]Graduate Institute of Microbiology, National Taiwan University, Taipei, Taiwan; [3]Department of Neurological Surgery, Tri-Service General Hospital, and the National Defense Medical Center, Taipei, Taiwan; [4]Hepatitis Research Center, National Taiwan University Hospital, Taipei, Taiwan; [5]Molecular Therapy Laboratory, Department of Orthopedic Surgery, University of Pittsburgh, Pittsburgh, PA, USA; [6]Graduate Institute of Life Sciences, National Defense Medical Center, Taipei, Taiwan; [7]Department of Pathology, Tri-Service General Hospital, and the National Defense Medical Center, Taipei, Taiwan; [8]Institute of Molecular Biology, Academia Sinica, Taipei, Taiwan and [9]Department of Medical Research and Education, Taipei Veterans General Hospital, Taipei, Taiwan

RNA interference (RNAi) was reported to block hepatitis B virus (HBV) gene expression and replication in vitro and in vivo*. However, it remains a technical challenge for RNAi-based therapy to achieve long-term and complete inhibition effects in chronic HBV infection, which presumably requires more extensive and uniform transduction of the whole infected hepatocytes. To increase the* in vivo *transfection efficiency in liver, we used a double-stranded adeno-associated virus 8-pseudotyped vector (dsAAV2/8) to deliver shRNA. HBV transgenic mice were used as an animal model to evaluate the inhibition effects of the RNAi-based gene therapy. A single administration of dsAAV2/8 vector, carrying HBV-specific shRNA, effectively suppressed the steady level of HBV protein, mRNA and replicative DNA in liver of HBV transgenic mice, leading to up to 2–3 log_{10} decrease in HBV load in the circulation. Significant HBV suppression sustained for at least 120 days after vector administration. The therapeutic effect of shRNA was target sequence dependent and did not involve activation of interferon. These results underscore the potential for developing RNAi-based therapy by dsAAV2/8 vector to treat HBV chronic infection, and possibly other persistent liver infections as well.*
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Introduction

Hepatitis B virus (HBV) is a major human pathogen that chronically infected over 350 million people worldwide, rendering these patients at high risk of developing liver failure, cirrhosis and hepatocellular carcinoma.[1] Current treatments for chronic HBV are suboptimal. The nucleoside or nucleotide analogs, such as lamivudine and adefovir dipivoxil, suppress HBV replication effectively,[2,3] but suffer from selection of drug resistant mutations and the high rate of relapse when treatment is discontinued.[4] Interferon (IFN)-α and pegylated IFN-α, although having both immune modulatory and antiviral effects, achieve a sustained response in only a small percentage of patients and are usually associated with a wide array of side effects.[5,6] Thus, development of new treatment strategies for chronic HBV remains a major medical challenge.

Correspondence: Dr M-H Tao, Institute of Biomedical Sciences, Academia Sinica, 128 Yen-Chiu-Yuan Rd., Sec. 2, Taipie 11529, Taiwan.
E-mail: bmtao@ibms.sinica.edu.tw
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RNA interference (RNAi) is a process by which small interfering RNA (siRNA) consisting of 19–23 nucleotide duplex directs sequence-specific degradation of mRNA.[7,8] In plants and *Caenorhabditis elegans*, RNAi serves as a natural host-defense mechanism against viruses.[9–11] In cultured mammalian cells, RNAi has also been proven effective in attenuating many human pathogenic viral infection and replication, including HBV.[12–14] As a result of its efficient and sequence-specific manner of gene silencing, RNAi has been considered as a potentially powerful approach to treat viral infections in humans. In HBV studies, several investigators demonstrated that HBV gene expression and replication by HBV plasmids, which were delivered to the mouse liver by hydrodynamic injection, were suppressed by co-injection of synthetic siRNAs or plasmid vectors that express small hairpin RNAs (shRNAs).[15–17] Furthermore, the *in vivo* anti-HBV effect of siRNAs can be further enhanced by chemical modification and lipid-encapsulation of siRNAs.[18,19] However, due to their short half-life and low *in vivo* transfection efficiency, synthetic siRNAs and plasmid vector-based shRNAs are unlikely to be effective in treating chronic HBV infection, because virtually all hepatocytes in these patients are infected.

In this regard, a viral vector-based delivery system, which can achieve efficient and uniform transduction of all liver cells, should be a prerequisite for developing an effective gene therapy for chronic HBV infection. Two recent reports demonstrated that shRNAs delivered by recombinant adenoviral vector could suppress HBV replication and gene expression in transgenic mice, an animal model more clinically relevant to chronic HBV in terms of containing heavily ongoing HBV replication in all hepatocytes.[20,21] However, as a strong stimulator for both innate and adaptive immune responses, recombinant adenoviral vector by itself could interrupt HBV replication and gene expression in liver of transgenic mice through induction of several inflammatory cytokines,[22] thereby complicating the analysis of RNAi-mediated repression effects carried by this vector. In clinical application, adenoviral vector is also not an ideal vehicle for chronic HBV gene therapy, because this vector sustains only transient gene expression and might cause severe toxicities at high doses.[23,24]

Adeno-associated virus (AAV)-based vectors are nonpathogenic and less immunogenic compared with other gene therapy vectors. The ability of AAV to infect both dividing and nondividing cells, to transduce a broad range of tissues *in vivo*, and to direct long-term gene expression in these tissues made it an ideal vehicle for gene therapy.[25] AAV2 is the most widely studied AAV vector for gene transfer and has been applied in several clinical studies.[26] However, its application in liver-directed gene therapy is limited because of the poor transduction rate of hepatocytes[27] and existence of neutralizing antibodies in a large human population.[28,29] Using other serotype capsids to pseudotype AAV2 genome provides the opportunities to overcome these issues. Among several recently isolated serotypes, AAV8 possesses high transduction rate in liver and low pre-existing immunity in human population, therefore, is an attractive vector for liver gene delivery.[29–33] Another major advancement in AAV vector development was the discovery that AAV with self-complementary double-stranded genome possessed 10- to 100-fold higher transduction rate in liver than conventional single-stranded AAV.[34,35] We, therefore, decided to construct double-stranded AAV2/8 pseudotyped vector (dsAAV2/8) to deliver shRNAs and to investigate its inhibition effects in HBV transgenic mice. Our results provide the first evidence that dsAAV2/8 vector could very efficiently deliver shRNA to the liver and effectively inhibit HBV replication and gene expression for a long period without causing significant side effects. Accordingly, dsAAV2/8-delivered RNAi-based therapy has a great potential to be developed as a treatment to control chronic HBV infection.

Results

HBV inhibition by shRNA in cultured cells

HBV contains a small DNA genome (3.2 kb) producing four extensively overlapping viral RNAs (3.5-, 2.4-, 2.1- and 0.7-kb transcripts).[36] In our previous studies (reference[37] and unpublished data), we examined a number of siRNAs and shRNAs that target various HBV transcripts (Figure 1a) for their abilities to suppress HBV replication and gene expression in cultured cells and in mice that were co-transfected with the plasmid pHBV1.3 containing a 1.3-fold HBV genome.[38] Three of the shRNAs (HBV-B4, -C, -S1) showed better HBV inhibition effect than the others. To examine whether the three shRNAs are effective in intervening more clinically relevant hepatitis B infection, we investigated their effects in HBV transgenic mice, which produce 1–10×10^8 HBV virions/ml of serum, a level comparable to or higher than that found in the serum of patients with chronic active hepatitis B infection. To achieve a high *in vivo* liver transduction rate, we decided to use pseudotyped dsAAV2/8 vector to express the HBV-specific shRNAs. The HBV-C, -B4 and -S1 shRNA-expressing cassettes, which are driven by the H1 promoter, were cloned into the pAAVEMBL plasmid between a truncated and a wild-type AAV ITR (Figure 1b). The pAAVEMBL plasmid was designed to produce double-stranded AAV because the D-sequence (the packaging signal) and the adjacent terminal resolution site were removed from one of its ITRs, thus the dimer AAV genomes fail to be resolved into monomers during viral replication.[35] The green fluorescent protein sequence in pAAVEMBL was preserved but deleted of its original cytomegalovirus promoter to maintain the size of dsAAV genome of $\sim$1.5 kb for vector packaging. The predicted hairpin structures and sequences of HBV-B4, -C and -S1 shRNA transcripts are shown in Figure 1b.

We then examined the gene-silencing effects of the three HBV-specific pAAVEMBL plasmids by co-transfecting HepG2 cells with pHBV1.3 and measuring the level of HBsAg and HBeAg, which are encoded, respectively, by the 2.1- and 3.5-kb transcripts. As shown in Figure 1c, upper panel, transfection of pAAVEMBL-HBV-S1 significantly reduced secretion of HBsAg and HBeAg (85 ± 5 and 77 ± 3% reduction, respectively) (lane 5) as compared with cells transfected with pHBV1.3 alone (lane 1), consistent with its ability to target both the 3.5- and 2.1-kb HBV transcripts. HBV-B4 was as effective as HBV-S1 in suppressing HBeAg (83 ± 3% reduction), but was less potent in inhibiting HBsAg (54 ± 9% reduction) (lane 4). HBV-C, which exclusively targets the 3.5-kb transcript, only marginally decreased HBeAg (36 ± 9% reduction), and as expected had no effect on HBsAg (lane 3). The unrelated pAAVEMBL-GL2 plasmid, which produces shRNA targeting firefly luciferase transcript, had no effect on either HBsAg or HBeAg expression (lane 2). We also used RNase protection assay to examine the transcription of small RNA molecules in transfected cells. Significant levels of shRNAs were detected in cells transfected with each of the pAAVEMBL plasmids (Figure 1c, lanes 2–5, lower panel). Detection of the microRNA miR-16 served as a loading control for each samples. Taken together, these results indicate that the three HBV-specific shRNAs inhibited HBV gene expression through a sequence-specific manner, but their inhibition potency was variable. The best two shRNA constructs, HBV-B4 and HBV-S1, were selected for further transgenic mice experiments.

dsAAV2/8/HBV-S1 clears HBV from most hepatocytes of transgenic mice

We next generated pseudotyped dsAAV2/8 vectors by packaging the various pAAVEMBL plasmids, which contain the AAV2 ITR, with the AAV8 capsid. HBV

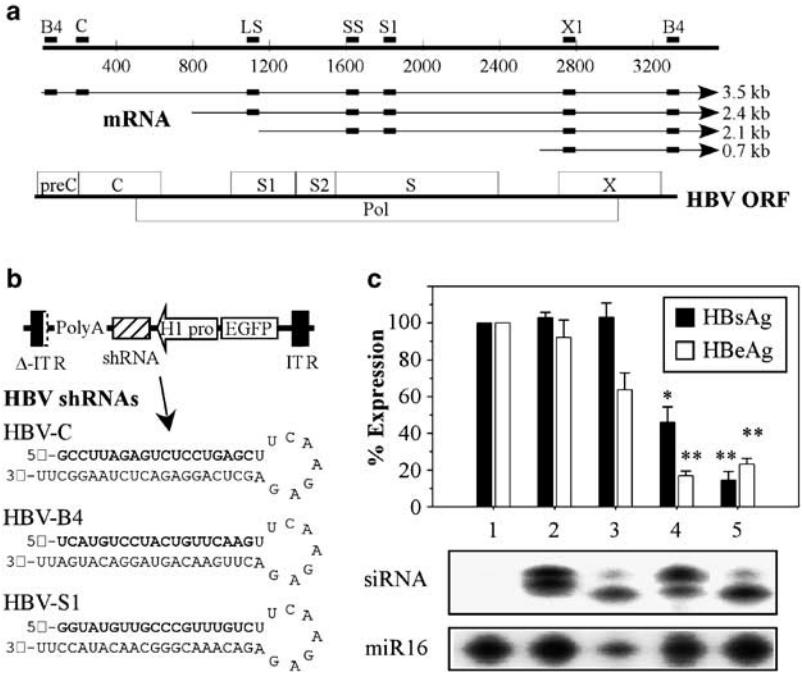

Figure 1 Inhibition of HBV gene expression by shRNAs *in vitro*. (**a**) Schematic representation of the HBV genome (heavy line), the four transcripts (thin lines) and the open reading frames (ORF, open box). The approximate locations of the shRNA target sequence used in this study are indicated. (**b**) Schematic representation of the pAAVEMBL plasmids. The predicted structures and sequences of the three HBV-specific shRNA transcripts are depicted with the sense strands shown in bold. (**c**) Inhibition of HBsAg and HBeAg expression by shRNAs in HepG2 cells. The amount of HBV proteins in shRNA-treated cells is presented as a percentage of that produced by cells transfected with the pHBV1.3 alone (mean ± s.d.). $^{*}P<0.05$; $^{**}P<0.005$. Small RNAs in the transfected HepG2 cells were detected by RNase protection assay using radiolabeled RNA probes identical in sequence to the corresponding HBV or luciferase shRNAs. The microRNA miR-16 was used as a loading control. Lane 1, mock; lane 2, pAAVEMBL-GL2; lane 3, pAAVEMBL-HBV-C; lane 4, pAAVEMBL-HBV-B4; lane 5, pAAVEMBL-HBV-S1.

transgenic mice with serum HBV titer $>1\times10^{8}$ genome copies/ml were intrasplenically injected with 1×10^{12} vector genomes per mouse of dsAAV2/8 vectors encoding HBV-B4 or HBV-S1 shRNAs. Mice injected with the same amount of dsAAV2/8/GL2 or saline were included as controls. Serum samples of each group were examined for HBsAg and HBV DNA 14 days after AAV administration. As shown in Figure 2a and b, treatment with dsAAV2/8/HBV-S1 profoundly reduced serum HBsAg and HBV DNA by an average of 99.6 and 99.96%, respectively, as compared with the pretreated titers of the same animals. Treatment with the other HBV-specific dsAAV2/8/HBV-B4, unrelated dsAAV2/8/GL2, or saline did not significantly reduce serum HBsAg and HBV DNA.

To determine which steps of the HBV replication cycle were affected by shRNA treatment, total cellular DNA and RNA were extracted from the liver tissues and analyzed, respectively, by Southern and Northern blotting. Treatment with dsAAV2/8/HBV-S1 reduced, respectively, an average of 93 and 81% of the steady level of the 2.4/2.1- and 3.5-kb HBV transcripts (Figure 2c, lower panel) when normalized to the albumin mRNA. dsAAV2/8/HBV-S1 treatment had an even more profound effect on HBV DNA replicative intermediates; the intrahepatic relaxed circular (RC) and single-stranded linear (SS) viral DNA was almost completely abolished in these animals (Figure 2c, upper panel). Treatment with dsAAV2/8/HBV-B4 partially suppressed liver HBV DNA (between 50 and 70% reduction versus the saline group), but did not cause significant reduction of HBV mRNA, while dsAAV2/8/GL2 had no effect on either the intrahepatic HBV DNA or RNA. We also examined the cellular distribution of HBV core by immunohistochemistry. In nontreated HBV transgenic mice, HBV core antigen (HBcAg) is present in the nucleus of almost all hepatocytes and in the cytoplasm of the centrilobular hepatocytes located around the central veins (data not shown). A single treatment of dsAAV2/8/HBV-S1 almost completely eliminated intracellular HBcAg, while dsAAV2/8/GL2 had little effect on HBcAg expression (Figure 2d). These results clearly show that the dsAAV2/8 vector can deliver shRNAs *in vivo* to almost all hepatocytes and effectively suppress HBV replication and gene expression, but its therapeutic efficacy is critically dependent on the shRNA target sequence.

Long-term HBV inhibition by dsAAV2/8/HBV-S1

We then examined the duration of the dsAAV2/8/HBV-S1-mediated silencing effect. Groups of HBV transgenic mice were treated with 1×10^{12} vector genomes per mouse of dsAAV2/8 vectors (HBV-S1, GL2) or saline. Sera samples were collected at different times to determine the amount of HBsAg and HBV DNA. We found that HBV viral protein and DNA decreased rapidly after injection of dsAAV2/8/HBV-S1. The inhibition began 3 days after treatment, peaked at 21 days (97.6 and 99.8% reduction for HBsAg and HBV DNA, respectively), and then recovered slowly over the next 3 months (Figure 3a and b). At the end of the observation

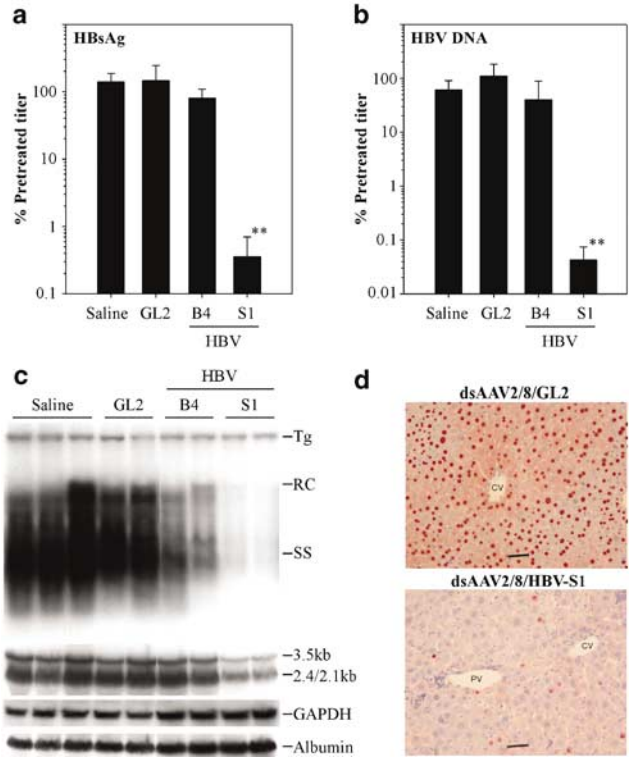

Figure 2 Inhibition of HBV replication and gene expression by dsAAV2/8 vectors in transgenic mice. Groups of HBV transgenic mice ($n = 3$–5) were intrasplenically injected with 1×10^{12} vector genomes of various dsAAV2/8 vectors or saline. At 14 days after injection, the amounts of serum HBsAg (**a**) and HBV DNA (**b**) were determined and are displayed as a percentage of the pretreated titer of the group (mean $\pm$ s.d.). $^{**}P < 0.005$. (**c**) (Upper) Total liver DNA was analyzed by Southern blotting. Bands corresponding to the integrated transgene (Tg), relaxed circular (RC) and single-stranded (SS) linear HBV DNA replicative forms are indicated. The integrated transgene was used to normalize the amount of DNA loaded to the gel. (Lower) Total liver RNA from the same mice was analyzed by Northern blotting for the 3.5 kb and 2.4/2.1 kb HBV transcripts. The same blot was hybridized with albumin and glyseraldehyde-3-phosphate dehydrogenase (GAPDH) probes as internal controls. (**d**) Immunohistochemical staining for HBcAg in liver sections. The portal vein (PV) and central vein (CV) are indicated. Original magnification, $\times 200$; bar, 5 μm.

period (120 days after treatment), significant suppression of HBsAg and HBV DNA were still observed in all animals treated with dsAAV2/8/HBV-S1 (an average of 66.1 and 77.1% reduction, respectively). This long-term suppressive effect of dsAAV2/8/HBV-S1 was confirmed by Southern blot analysis of the liver tissues removed at 120 days after treatment, which showed an average of 75.8% reduction of HBV DNA replicative intermediates in the dsAAV2/8/HBV-S1 group as compared with the saline group (Figure 3c). Treatment with dsAAV2/8/GL2 vector did not reduce serum HBsAg, but did cause an up to 10-fold reduction of serum HBV DNA in some animals (two of six mice). However, unlike the long-term HBV inhibition achieved by dsAAV2/8/HBV-S1, dsAAV2/8/GL2-mediated suppression was only observed within the first 3 weeks after treatment (Figure 3b).

To determine whether the decline of inhibition by dsAAV2/8/HBV-S1 was due to loss of vector DNA in liver, Southern blot analysis was performed on liver tissues of treated animals at different time points. At 3 days after injection, the input dsAAV2/8 vector in both the HBV-S1 and GL2 groups were primarily present in the form of linear dsDNA (LM, for linear monomer) of 1.5 kb (Figure 3d, lanes 10 and 11 and diagram below), which was confirmed by *Bam*HI digestion that cut once in the AAV genome to release a tail of 1.1 kb (lanes 4 and 5). At this time, more than 100 copies of AAV genome per cell were detected in each group. The AAV genome was decreased to 15–23 copies per cell 14 days after injection, with more than half of the genome now present as the circular monomer (CM) form, which migrated faster on the agarose gel than the linear DNA (Figure 3d, lanes 12 and 13). The circular DNA could be converted to 1.5 kb unit-length linear dsDNA genome by *Bam*HI digestion (lanes 6 and 7). By 120 days, the amount of AAV genome was further decreased to two of five copies per cell, with almost all remaining AAV DNA present in the circular monomer form (lanes 14 and 15). These results suggest that in the dsAAV2/8/HBV-S1-treated animals the decrease in HBV inhibition at later time points at least in part is due to the gradual loss of the AAV genome in liver.

dsAAV2/8 vector did not induce cytokine expression in liver

As IFN-α/β, IFN-γ and TNF-α were reported to inhibit HBV DNA replication,[39] these cytokine mRNAs in the liver tissues of each group at day 3, 14 and 120 were determined by quantitative reverse transcription-PCR. We measured 2′–5′-oligoadenylate synthetase 1 (OAS1) because it is a major target gene induced by IFN-α/β. As shown in Figure 4, only negligible amounts of OAS1 and TNF-α and no IFN-γ mRNAs were present in the liver samples of mice treated with saline or the dsAAV2/8 vectors (HBV-S1, GL2). In contrast, the same transgenic mice injected with a recombinant adenovirus carrying the mouse interleukin 12 gene produced high levels of IFN-γ and OAS1 but not TNF-α. These results suggest that HBV inhibition by dsAAV2/8/HBV-S1 treatment was unrelated to IFN or other cytokines.

Discussion

Many investigators have shown that synthetic siRNAs or plasmid vector-based shRNAs suppressed several virus infection in mouse models when treatment began before or shortly after viral challenge.[40–43] In contrast, the therapeutic efficacy was greatly reduced if siRNAs/shRNAs were given a few days after viral challenge. The failure to treat more established viral infections was probably due to the low *in vivo* transduction rate of siRNAs and plasmid-based shRNAs. This raises a concern about whether RNAi-based therapy could be used in real medical practice, such as chronic HBV infection, because in these patients a large number of host cells are infected. In this study, we used a transgenic mouse model that produced large amounts of HBV DNA, mRNA and viral proteins in liver and stably maintained a high serum HBV titer ($> 1 \times 10^8$ genome copies/ml), and thus are more clinically relevant to chronic HBV infection than the commonly used HBV model generated by hydrodynamic injection of a plasmid containing a replication-competent HBV genome.[44] We

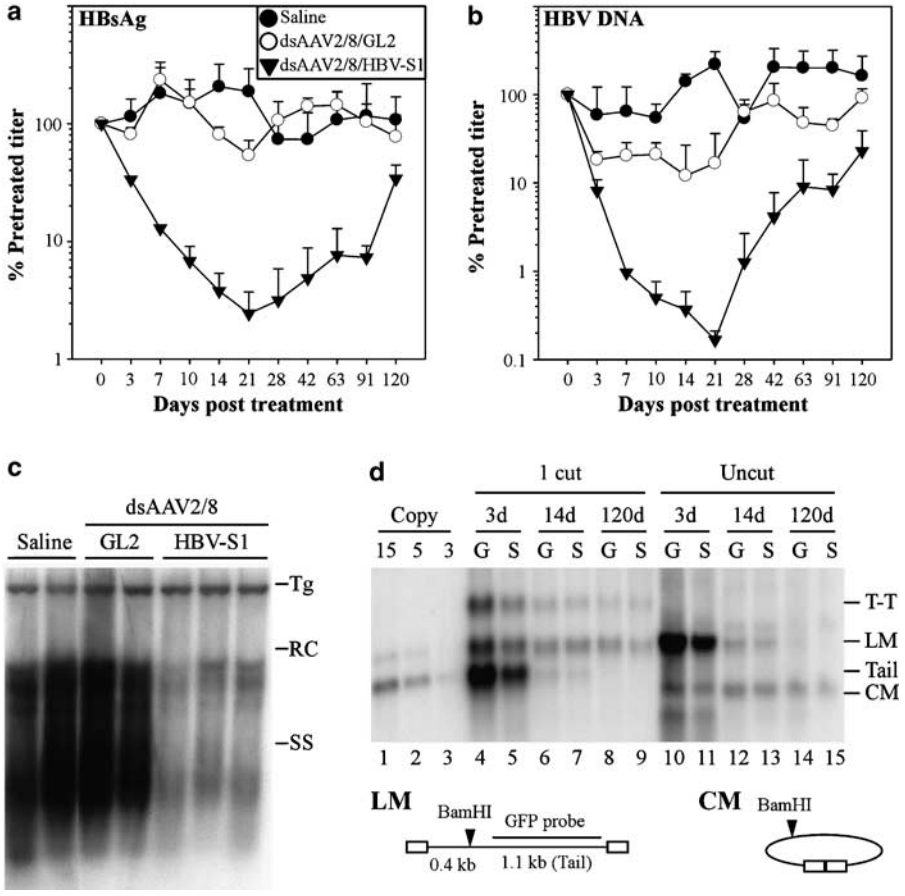

Figure 3 Time course effects of dsAAV2/8/HBV-S1 and the presence of AAV genome in the liver. Groups of HBV transgenic mice ($n=5$ or 6) were intrasplenically injected with 1×10^{12} vector genomes of dsAAV2/8/HBV-S1 or dsAAV2/8/GL2 or saline. Serum samples were collected at the indicated times for analysis of HBsAg (**a**) and HBV DNA (**b**) and are displayed as a percentage of the pretreated titer of each group (mean$\pm$s.d.). (**c**) Mice were sacrificed 120 days after treatment. Total liver DNA was extracted for analysis of HBV DNA by Southern blotting. (**d**) Analysis of dsAAV2/8 vectors in the liver 3, 14 and 120 days after injection. Total liver DNA (10 μg) from treated animals ($n=2\sim3$) were pooled and analyzed by Southern blotting with or without *Bam*HI digestion, which cut the 1.5 kb linear monomer (LM) into a 1.1 kb tail and a 0.4 kb head fragment. It also cut the circular monomer (CM) into a 1.5 kb linear monomer (LM), and the tail-to-tail dimer into a 2.2 kb fragment (T-T). The green fluorescent protein DNA probe used here only detected the full-length and the tail fragments.

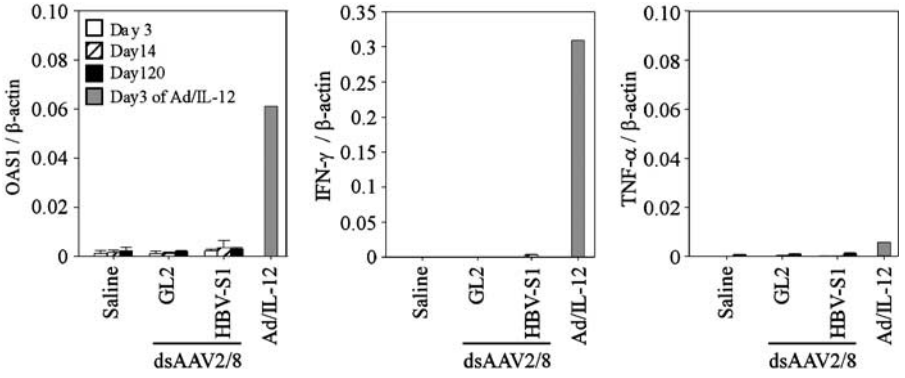

Figure 4 Analysis of cytokine mRNAs in dsAAV2/8 vector-treated animals. Total liver RNA from HBV transgenic mice ($n=2\sim3$) collected 3, 14 and 120 days after treatment was assayed by real time reverse transcription-PCR. Liver RNA samples from HBV transgenic mice collected 3 days after injection of 1×10^{9} PFU of adenovirus carrying the mouse IL-12 gene (Ad/IL-12) were included as controls. Results are expressed as ratios of the RNA copy number to that of β-actin (mean$\pm$s.d.).

showed that a single treatment of dsAAV2/8 vector carrying HBV-S1 shRNA led to a near complete and long-term suppression of HBV replication in liver of these transgenic animals. Interestingly, we found that the shRNA-mediated virus inhibition effect in the transgenic mouse model was target sequence dependent, because only HBV-S1 but not other HBV-specific shRNAs could effectively suppress HBV replication and gene expres-

sion. dsAAV2/8/HBV-B4 only partially suppressed liver HBV DNA but had little or no effect on the steady level of liver HBV mRNA and serum HBsAg or HBV virions (Figure 2a, b and c). The same results were observed in two independent experiments using different preparations of dsAAV2/8 vectors. The failure of dsAAV2/8/HBV-B4 to suppress HBV in transgenic mice was not due to its lower *in vivo* transduction rate, because Southern blot analysis showed that at day 14 after injection about equal amount of AAV genome was present in the liver tissues of either dsAAV2/8/HBV-B4- or dsAAV2/8/HBV-S1-treated animals (data not shown). However, in the *in vitro* co-transfection study, we did observe HBV-S1 shRNA was more effective than HBV-B4 in inhibiting HBsAg expression (Figure 1c). Whether the *in vitro* difference in shRNA potency can explain the great difference of the *in vivo* therapeutic effect of the two dsAAV2/8 vectors requires further investigation.

The sustained virus inhibition achieved by dsAAV2/8HBV-S1 is particularly remarkable when compared with similar HBV transgenic mice treated with conventional chemotherapeutic drugs. In those studies, daily treatment of transgenic mice with lamivudine or adefovir for 7–14 days could result in 2–3 $\log_{10}$ reduction of serum HBV DNA,[45,46] a level similar to that achieved by dsAAV2/8/HBV-S1 (Figures 2b and 3b). However, after termination of nucleoside/nucleotide drug treatment, serum HBV rebounded to the pretreatment titer in only a few days (reference[45] and our unpublished data). This was not a surprising result since in this transgenic mice model HBV transcriptional templates were continuously produced from the integrated transgene, and the serum half-life of the antiviral drugs was short. Similar quick viral rebound was also observed in chronic HBV patients after withdraw of nucleoside/nucleotide drug treatment,[3] a phenomenon mainly due to the HBV covalently closed circular DNA (cccDNA), which is difficult to be cleared by the antiviral drug therapy. In our study, a single injection of dsAAV2/8/HBV-S1 in HBV transgenic mice not only achieved a near complete clearance of liver HBV DNA and RNA at early time, more significantly, a substantial level of HBV inhibition was maintained for at least 120 days (Figure 3b and c). However, it should be noted that in the transgenic mouse model, HBV is constitutively expressed from the integrated transgene as compared with reinfection and generation from HBV cccDNA in naturally occurring infection. Our preliminary data showed that the HBV transgenic mice used in this study produced only negligible amount of HBV cccDNA in the liver, and thus prevented us from investigating whether long-term RNAi activity could decrease the clinically important HBV cccDNA. This question might be addressed using HBV transgenic mice in hepatocyte nuclear factor 1α null background that was reported to significantly amplify the liver cccDNA pool.[47]

Among the viral vectors used nowadays, the AAV8 vector has been very promising for liver-directed gene therapy. This vector, at a dose of 7.2×10^{12} vector genomes per mouse, was able to transduce all the hepatocytes without toxicity.[48] Several recent studies showed that the AAV8 vector was very efficient in transducing genes to the liver of small and large animal models, resulting in long-term correction of several genetic diseases.[30–33] The high transduction rate of AAV8 appears to be at least partially due to rapid uncoating of the vector genome.[49] In addition, the pre-existing neutralizing antibodies to AAV8 in human populations are much lower than that of AAV2,[29] adding another appeal for using AAV8 for clinical therapy. To further improve the efficacy of *in vivo* liver transduction, our vector adopted a double-stranded AAV2 genome packaged within AAV8 capsid, because the self-complementary double-stranded AAV genome displayed a rapid onset and a high level of transgene expression in mouse hepatocytes *in vivo*.[34,35,50] Indeed, we showed that dsAAV2/8-expressed HBV-S1 shRNA reduced serum HBsAg and HBV DNA as early as 3 days after introduction of the vector, and reached its peak inhibition effect within 3 weeks (Figure 3a and b). At this time, the steady level of liver HBV DNA and mRNA were greatly reduced (Figure 2c), and the intracellular HBcAg was cleared from almost all hepatocytes of these transgenic mice (Figure 2d). In addition to the AAV vector used in this study, two recent studies used recombinant adenoviral vectors carrying shRNAs and examined their *in vivo* silencing effect in similar HBV transgenic mice model. Uprichard *et al*.[20] noted significant suppression of serum HBsAg and HBeAg as well as hepatic HBV transcripts and replicative DNA for at least 20–26 days after injection of $2–5 \times 10^9$ PFUs of recombinant adenoviruses expressing HBV-specific shRNAs. The HBV suppression effect was much less significant in another study using adenoviral vectors as shRNA-expressing vehicle, resulting in only 10-fold reduction of HBsAg and two to fivefold reduction of HBeAg and circulating HBV virion in transgenic mice.[21] For those studies using adenoviral vectors one should be cautious in evaluating how much HBV inhibition was indeed contributed by RNAi-mediated inhibition, because infection of several hepatotropic viruses, including adenovirus, could interfere HBV replication and gene expression in HBV transgenic mice through induction of IFN-α/β, IFN-γ and TNF-α and possibly other unknown mechanisms.[22,51] This concern was actually addressed in one study, which used HBV transgenic mice deficient in IFN-γ and IFN-α/β receptor expression to rule out the possibility that HBV suppression was due to the adenoviral vector-induced cytokine effect.[20] Nevertheless, we believe that dsAAV2/8 vector used in this study is a much more appropriate vehicle than adenoviral vector in conducting shRNA studies in HBV transgenic mice. Our result showed that injection of up to 1×10^{12} vector genomes of dsAAV2/8 vector did not induce expression of IFN-α/β, IFN-γ and TNF-α (Figure 4), indicating that these previously known HBV inhibitory cytokines were not involved in the RNAi-mediated viral inhibition in our study. In addition, our dsAAV2/8 vector-delivered HBV-S1 shRNA could lead to a much more complete and longer HBV inhibition than shRNAs delivered by adenoviral vectors.

We also noted a few problems associated with the dsAAV2/8-based RNAi therapy. First, dsAAV2/8/HBV-S1 quickly reached its peak suppression between 21 and 28 days after injection of the vector (Figure 3a and b), and then the inhibition effect gradually decreased with time. Similar drop-off in transgene expression was also observed in several studies using single-stranded AAV2/8 pseudotyped as delivery vehicle.[32,52] Our data suggest that reduction in transgene expression was likely

due to a gradual loss of the AAV2/8 genome from the liver (Figure 3d). The real mechanism that resulted in the gradual loss of AAV genomes from the transduced liver tissues is currently unknown. One possibility is that the presence of HBV genomes in transgenic mice might interfere with processing and stabilization of AAV genomes. However, this appears not the case because our unpublished data showed that after dsAAV treatment an about equal number of AAV genome was present in both the HBV transgenic mice and their transgene negative littermates. Another possible explanation for the decrease of AAV genomes in the liver is that dsAAV2/8 treatment might result in liver injury and subsequent liver regeneration, which might then dilute out the vector genome in hepatocytes. However, during the 120-day observation period, we did not see apparent liver regeneration or toxicity in the dsAAV-treated animals even at a high dose of 1×10^{12} vector genomes per mouse. While in preparing this revised manuscript, we were aware of a report by Grimm *et al*.[53] also demonstrated a persistent HBV inhibition in transgenic mice by dsAAV2/8-delivered shRNAs. But in their study severe dose-dependent liver injury and even animal death was found to be associated with overexpression of shRNAs, which interfere with endogenous microRNA processing and functionality. Whether similar shRNA-mediated toxicity was present in our dsAAV2/8/shRNA vector-treated animals will be more closely investigated in our future experiment. It should also be noted that in our experiments the AAV vectors were infused through intrasplenic injection, which is a less commonly used procedure for AAV delivery, while the injection route in Grimm's study was not clearly indicated. We are currently examining whether other injection routes, such as portal vein or tail vein injection, could produce a more sustained suppressive effect. In addition, we are also investigating whether a second injection of an alternative AAV vectors of other serotype, in particular AAV5, AAV7 and AAV9, which also showed high liver transduction efficiency,[54] might help to sustain the maximum inhibition effect of shRNA.

Another potential problem associated with RNAi-based therapy for chronic HBV is the selection of siRNA-resistant viral variants as those reported for poliovirus and human immunodeficiency viruses.[55,56] Indeed, a HBV-S1-resistant mutant virus carrying a silent mutation in the target sequence was isolated from blood samples of a chronic HBV patient.[37] This problem might be overcome by introducing a new shRNA against the mutant sequence or against another conserved sequence on HBV mRNAs. Indeed, our preliminary data showed that the HBV-S1 mutant virus could be effectively suppressed by a shRNA carrying a modified S1 sequence with the mutant nucleotide.

In summary, we show that by combining the high liver transduction efficiency of the dsAAV2/8 vector and the power of RNAi silencing effect, a rapid, profound, and durable HBV inhibition could be achieved in animal models with a heavily ongoing HBV replication. In the 4-month observation period, these mice showed no apparent toxicity. These results strongly suggest that dsAAV2/8-delivered shRNA might be further developed as an effective therapy for chronic HBV infection.

Materials and methods

Construction and production of pseudotyped AAV2/8 vectors

The AAV vectors that produce various HBV shRNAs were constructed by insertion of the *Bam*HI/*Cla*I fragment, which contains the H1 promoter and the shRNA coding sequence, released from the corresponding pSUPER plasmids into the pAAVEMBL-CMV-GFP plasmid.[35] This *Bam*H1/*Cla*I replacement resulted in deletion of the cytomegalovirus promoter of the original vector while the green fluorescent protein coding sequence was preserved to maintain the size of dsAAV genome of ~1.5 kb for dsAAV packaging. The entire expression cassette is flanked at both ends by the inverted terminal repeats (ITRs) of AAV2 with the D-sequence and the terminal resolution site deleted in the 5′ ITR (Figure 1b). The dsAAV2/8 vectors were produced by the triple transfection method as previously described[57] and purified by cesium chloride sedimentation.

Co-transfection in vitro

HepG2 cells were co-transfected with 2 μg of pHBV1.3 plasmid containing a 1.3-fold overlength HBV genome (ayw subtype)[38] and 2 μg of various pAAVEMBL plasmids using lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) in 24-well plates. The amount of hepatitis B surface antigen (HBsAg) and e antigen (HBeAg) in the supernatant was analyzed 3 days later by enzyme-linked immunosorbent assay.

RNase protection assay

Detection of shRNAs was carried out using RNase protection assay on cellular extracts enriched for small RNAs isolated from transfected HepG2 cells using a mirVana miRNA isolation kit (Ambion, Austin, TX, USA). ^{32}P-labeled RNA probes were generated using a mirVana miRNA Probe Construction Kit (Ambion) and hybridized to the enriched small RNAs with mirVana miRNA Detection Kit (Ambion). The protected RNA fragments were then fractionated on a 15% denaturing polyacrylamide gel and detected by autoradiography.

HBV transgenic mice and viral vector delivery

HBV transgenic mice were generated by microinjection of the *Hind*III/*Sac*I DNA fragment excised from pHBV1.3 into ICR embryo. The founder line Tg[HBV1.3]24-3 was selected to be used in this study, because it produced high levels of HBV replicative DNA and all forms of viral mRNAs and proteins in the liver. These animals also secreted high titers of HBsAg, HBeAg and HBV DNA in the circulation. The average serum HBV DNA concentration quantified by real-time PCR was 4.5×10^8 and 1.9×10^8 genome copies/ml in male and female mice, respectively. We used age (8–12 weeks), sex, and serum HBV titer ($>1 \times 10^8$ genome copies/ml)-matched transgenic mice in all the shRNA-treated experiments. Each mouse received a single dose of 1×10^{12} vector genomes of various dsAAV2/8 vectors by intrasplenic injection. Serum and liver samples were collected at different times for analysis.

HBV DNA, RNA and protein analysis

Serum HBsAg and HBeAg were quantified using commercial enzyme-linked immunosorbent assay kits SURASE and EASE, respectively, according to the manufacture's protocols (General Biologicals Corp, Hsin-Chu, Taiwan). The amount of HBsAg and HBeAg was calculated from standard curves generated using HBsAg and HBeAg provided in the kits. Serum HBV DNA was extracted with QIAmp Blood Mini kit (Qiagen, Sussex, UK) and quantified by HBV-specific LightCycler SYBR green real-time PCR (Roche Diagnostics, Mannheim, Germany). The forward primer, QHBVcF 5′-CGTTTTTGCCTTCTGACTTCTTTC-3′, and reverse primer, QHBVcR 5′-ATAGGATAGGGGCATTTGGTGGTC-3′, were used in the PCR reaction. Plasmid pHBV-Core, containing the HBV core sequence, was prepared at 10-fold dilutions (2×10^4–2×10^9 copies/ml) to generate a standard curve in parallel PCR reactions. Total DNA and RNA were extracted from liver tissues and examined for the presence of HBV DNA and RNA by Southern and Northern blotting, respectively.[38] The signals were quantified using ImageQuant software (Amersham Biosciences, Piscataway, NJ, USA). HBcAg was detected by immunohistochemical staining of paraffin-embedded tissues.

Detection of dsAAV2/8 DNA

The liver transduction efficiency of dsAAV2/8 vectors was evaluated by Southern blotting.[35] Representative liver extracts were from two to three animals at different time points after injection of dsAAV2/8 vectors.

Expression of cytokine and cytokine-induced genes

One microgram of total liver RNA from mice of each group was reverse transcribed (Epicentre, Madison, WI, USA) and applied in a quantitative real time PCR using commercially available primers and a 3′-fluorescein-labeled donor probe and a 5′-Red640-labeled acceptor probe (LightCycler FastStart, Roche Diagnostics). PCR was carried out by one cycle at 95°C for 10 min, followed by 40 cycles of 95°C 10 s, 53°C 10 s and 72°C 16 s. Genes assayed included (OAS1), IFN-γ, tumor necrosis factor (TNF)-α and β-actin. A serial dilution of known copy numbers of PCR fragments containing the respective gene was included in each assay to construct a threshold crossing cycle versus copy number standard curve.

Statistical analysis and ethical considerations

Results are expressed as mean $\pm$ s.d. Differences between groups were examined for statistical significance using Student's *t*-test. Experimental protocols were approved by the Academia Sinica Animal Care and Use Committee.

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