Chapter 10

Targeted In Vivo Delivery of siRNA and an Endosome-Releasing Agent to Hepatocytes

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Abstract

The discoveries of RNA interference (RNAi) and short interfering RNAs (siRNAs) have provided the opportunity to treat diseases in a fundamentally new way: by co-opting a natural process to inhibit gene expression at the mRNA level. Given that siRNAs must interact with the cells' natural RNAi machinery in order to exert their silencing effect, one of the most fundamental requirements for their use is efficient delivery to the desired cell type and, specifically, into the cytoplasm of those cells. Numerous research efforts involving the testing of a large number of delivery approaches using various carrier molecules and inventing several distinct formulation technologies during the past decade illustrate the difficulty and complexity of this task. We have developed synthetic polymer formulations for in vivo siRNA delivery named Dynamic PolyConjugates™ (DPCs) that are designed to mimic the features viruses possess for efficient delivery of their nucleic acids. These include small size, long half-life in circulation, capability of displaying distinct host cell tropism, efficient receptor binding and cell entry, disassembly in the endosome and subsequent release of the nucleic acid cargo to the cytoplasm. Here we present an example of this delivery platform composed of a hepatocyte-targeted endosome-releasing agent and a cholesterol-conjugated siRNA (chol-siRNA). This delivery platform forms the basis of ARC-520, an siRNA-based therapeutic for the treatment of chronic hepatitis B virus (HBV) infection. In this chapter, we provide a general overview of the steps in developing ARC-520 and detailed protocols for two critical stages of the discovery process: (1) verifying targeted in vivo delivery to hepatocytes and (2) evaluating in vivo drug efficacy using a mouse model of chronic HBV infection.

Key words siRNA delivery, Nonviral, Dynamic PolyConjugates, Amphipathic polymers, Membrane-active peptides, Melittin, Endosomal release, *N*-Acetylgalactosamine, NAG, Asialoglycoprotein receptor, ASGPr, Hepatocyte, Hepatitis B virus, Mouse model

1 Introduction

1.1 Dynamic PolyConjugates for Targeted siRNA Delivery to Hepatocytes

We have developed a synthetic, polymer-based siRNA delivery platform named Dynamic PolyConjugates (DPCs) that enables efficient and targetable siRNA delivery to liver hepatocytes after intravenous injection [1]. The platform is based on the reversible modification of amphipathic endosomolytic polymers [1, 2].

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Modification of positively charged amino groups with carboxy dimethylmaleic anhydride (CDM) linked to low molecular weight polyethylene glycol (CDM-PEG) or *N*-acetylgalactosamine (CDM-NAG) provides for surface charge masking and hepatocyte targeting via the highly expressed asialoglycoprotein receptor (ASGPr), respectively [1]. After receptor-mediated endocytosis, the acid-labile CDM bonds are hydrolyzed in the acidic environment of endosomes, restoring the amphipathic and membrane-active nature of the polymer, which destabilizes the endosomal membrane. In the prototypical DPC configuration, the siRNA is covalently attached to the polymer via a disulfide bond. Once exposed to the reducing environment of the cytoplasm, the disulfide bond is cleaved and siRNA is released from the polymer, allowing the siRNA to engage the cellular RNAi machinery [1, 3].

Cholesterol-conjugated siRNAs (chol-siRNA) and other lipophilic siRNA conjugates have been used previously for liver-targeted systemic delivery [4, 5]. However, the approach required repeated injections of very large chol-siRNA doses and, thus, was not practical for clinical applications. We hypothesized that the low efficiency was likely due to poor endosomal release and RNA degradation in lysosomes. When we tested the effect of co-injecting chol-siRNA with hepatocyte-targeted DPCs, we demonstrated greater than 500-fold improvement in knockdown efficiency compared to injection of chol-siRNA alone [6]. It is important to note that this increased efficacy is not dependent on interaction between the chol-siRNA and the masked polymer prior to injection or in the bloodstream prior to contact with the target cell. In fact, the two components can be injected separately, up to 2 h apart. Yet, microscopic studies show that within 2 h, more than 80 % of the chol-siRNAs co-localize with NAG-targeted DPCs inside the endosomes of hepatocytes [6]. The discovery that the siRNA and the endosomolytic agent do not have to be linked not only simplifies the manufacturing of a potential drug candidate but also opens up the possibility to explore the use of alternative endosomolytic agents.

The prototypical DPC contained an amphipathic and endosomolytic polyvinylether with butyl and amino side chains that was named PBAVE [1, 2]. The inclusion of both hydrophobic and hydrophilic side chains was intended to mimic the membrane-lytic properties of the naturally occurring peptide melittin. Subsequent screening of other classes of amphipathic polymers, including peptides, led to the identification of a highly active melittin-like peptide (MLP). MLP modified with CDM-NAG (NAG-MLP) and co-injected with coagulation factor VII (F7) specific chol-siRNA led to knockdown of F7 expression by up to 99 % without toxicity both in mice and in nonhuman primates (NHPs) [7]. These modifications of the original DPC delivery platform enabled the design, development, and manufacturing of

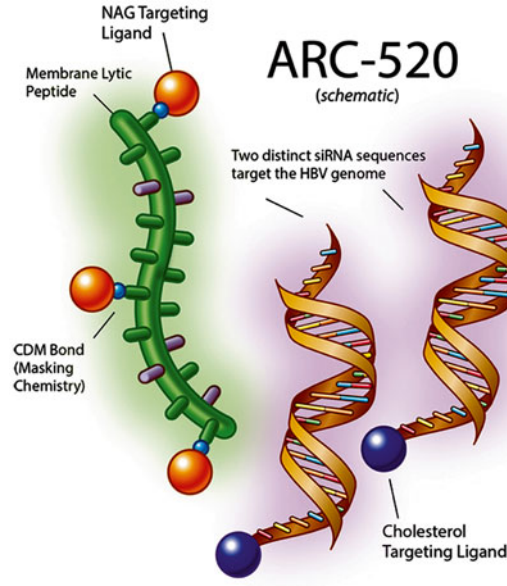

Fig. 1 Schematic illustration of drug-candidate ARC-520 for the treatment of chronic HBV infection. The drug's disease-specific component is an equimolar mixture of two distinct human HBV-specific siRNAs that are targeted to the liver by cholesterol (chol-siHBVs). The other component is a CDM-NAG labeled melittin-like peptide, NAG-MLP, which becomes an endosomal-releasing agent in the acidic environment of endosomes. The components reach hepatocytes separately, by independent pathways

an siRNA drug candidate—ARC-520—against chronic HBV infection [7]. The schematic illustration of drug components in ARC-520 is shown in Fig. 1.

1.2 Chronic Hepatitis B Virus Infection as a Clinical Target

According to the World Health Organization, 360 million people globally are chronically infected with HBV and between 500,000 and 1 million a year die as a result of the disease. One-third of the global population becomes infected with HBV at some point in their lives. Most who are infected as adults are able to mount a successful immune response that clears the infection and results in life-long immunity. However, those who are infected with HBV as neonates or young children usually become chronically infected. One hallmark of chronicity is the intricate and dynamic interplay between the virus and the host's immune system. It has been proposed that patients with chronic HBV infection mount a weak cytotoxic T-cell and a humoral B-cell response that is attenuated or "exhausted" by extremely high levels of circulating viral antigens, in particular the envelope protein called S antigen (HBsAg) [8, 9]. A significant drop in circulating antigen levels is expected to allow the expansion and activation of HBV-reactive

lymphocytes, such that the patient is able to mount an immune response and produce neutralizing HBV-specific antibodies—a process called HBsAg seroconversion. Treatment with nucleoside/nucleotide analogues or interferon has led to decreased serum HBsAg levels in some patients, and recent results demonstrated that a tenfold reduction in HBsAg levels was predictive of patients who achieve HBsAg seroconversion and a functional cure [10–13]. However, the percentage of patients with strong and sustained response after these treatment protocols is low (less than 10 %) [14], while the rest need active, life-long drug treatment in order to reduce the incidence and severity of liver cirrhosis, hepatocellular carcinoma, and liver failure.

We recently demonstrated that co-injection of NAG-MLP and chol-siHBVs in mouse models of chronic HBV infection results in substantial elimination of HBV mRNAs in hepatocytes (Fig. 2a, b), and profound and sustained reduction in the levels of circulating HBV proteins (Fig. 2c, d), and viral DNA (Fig 2e) [7]. Two of these, chol-siHBV-74 and chol-siHBV-77, are included in

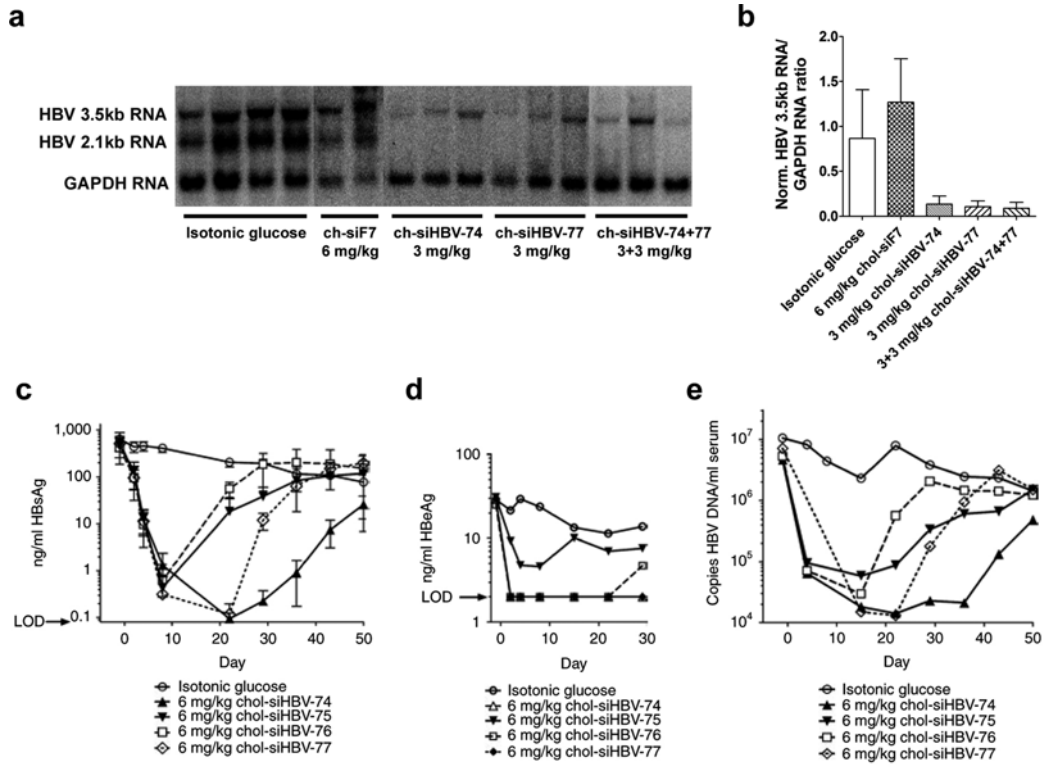

Fig. 2 In vivo efficacy of a single dose of HBV-specific chol-siRNAs co-injected with NAG-MLP in transgenic and pHBV mouse models of chronic HBV infection. HBV1.3.32 transgenic mice were injected once (**a**, **b**) with 6 mg/kg NAG-MLP and 3 mg/kg of the indicated chol-siRNA or a combination of 3 mg/kg chol-siHBV-74 plus 3 mg/kg chol-siHBV-77 ($n=2$–4). (**a**) RNA filter hybridization (Northern blot) analysis of 3.5 and 2.1 kb HBV

ARC-520. The duration of effect after a single dose of NAG-MLP and chol-siHBV suggests that a monthly dosing regimen of ARC-520 would be sufficient for use in clinical settings to maintain a significant reduction of serum antigen levels, and thus to provide an opportunity for de-repression of the host's immune response, HBsAg seroconversion, and a functional cure.

1.3 Major Phases of Developing HBV-Specific siRNA Drug Candidate ARC-520

1.3.1 Identification of Potent siRNA Sequences

The selection of siRNA sequences targeting the HBV genome began with in silico selection of 17-mer target sequences that are highly conserved among 2,754 human HBV genomes contained in GenBank. Mismatches at positions 1 and 19 of an siRNA are well tolerated; thus the initial sequence selection was for 17-mers. An in silico specificity filter was used to deselect those with close sequence similarity to off-target mRNAs of the human and mouse transcriptomes. This selection resulted in 140 candidate 17-mer siRNA sequences that tended to be in regions of overlapping reading frames (Fig. 3). Due to the structure of the HBV genome with overlapping mRNAs that all contain the same 3′ terminus, a single siRNA may be able to target multiple viral RNAs. The 140 candidate siRNAs were synthesized as 19-mers and screened in Cos-7 green monkey kidney cells using a plasmid (psiCHECK-HBV) containing *Renilla* and firefly luciferase genes. HBV target sites were cloned into the 3′ untranslated region of the *Renilla* luciferase gene. At 10 nM, 46 siRNAs gave ≥50 % knockdown and 14 gave ≥75 % knockdown (Fig. 4). Heavy chemical modification of siRNAs protects them from rapid degradation in serum and reduces cytokine induction and off-target effects [15–17]. Therefore, the 23 most effective siRNAs were synthesized with 2′-*O*-methyl/2′-fluoro-phosphoramidites and screened again using the same in vitro assay as above. The four most potent siRNAs performed well in both in vitro screens. Bases 2–18 of the four most potent siHBV lead sequences were analyzed for their conservation in genotypes A–H as annotated in the NCBI GenBank database. They were highly conserved across all genotypes.

Fig. 2 (continued) RNA from livers of mice injected once and sacrificed 7 days later for evaluation. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal control for RNA loading per lane. (**b**) RT-qPCR analysis of the 3.5 kb HBV RNA is shown relative to the GAPDH mRNA in mice that received one NAG-MLP plus chol-siRNA injection, and then normalized to the control groups. Mean + SD. NOD-SCID mice were given a hydrodynamic tail vein injection with 10 μg plasmid DNA containing an overlength HBV genome (pHBV1.3). Three weeks later mice were given one 200 μl IV co-injection of 6 mg/kg NAG-MLP and 6 mg/kg chol-siHBV-74, -75, -76, or -77 ($n = 3–4$). (**c**) HBsAg and (**d**) HBV e antigen (HBeAg) in serum were measured by enzyme-linked immunosorbent assays at the indicated times relative to injection on day 1; *LOD* limit of detection. (**e**) DNA was isolated from serum and the concentration of HBV genomes was quantitated by qPCR. The HBV antigen or DNA levels in chol-siRNA injected mice were compared with isotonic glucose-injected mice with similar initial HBsAg levels (isotonic glucose groups). Standard deviation bars are shown for HBsAg. Serum HBV DNA and HBeAg levels for each group were determined by combining equal proportions of serum from each mouse within the group to obtain sufficient pooled sample at each time point ($n = 3–4$). (Figure is reproduced from [7])

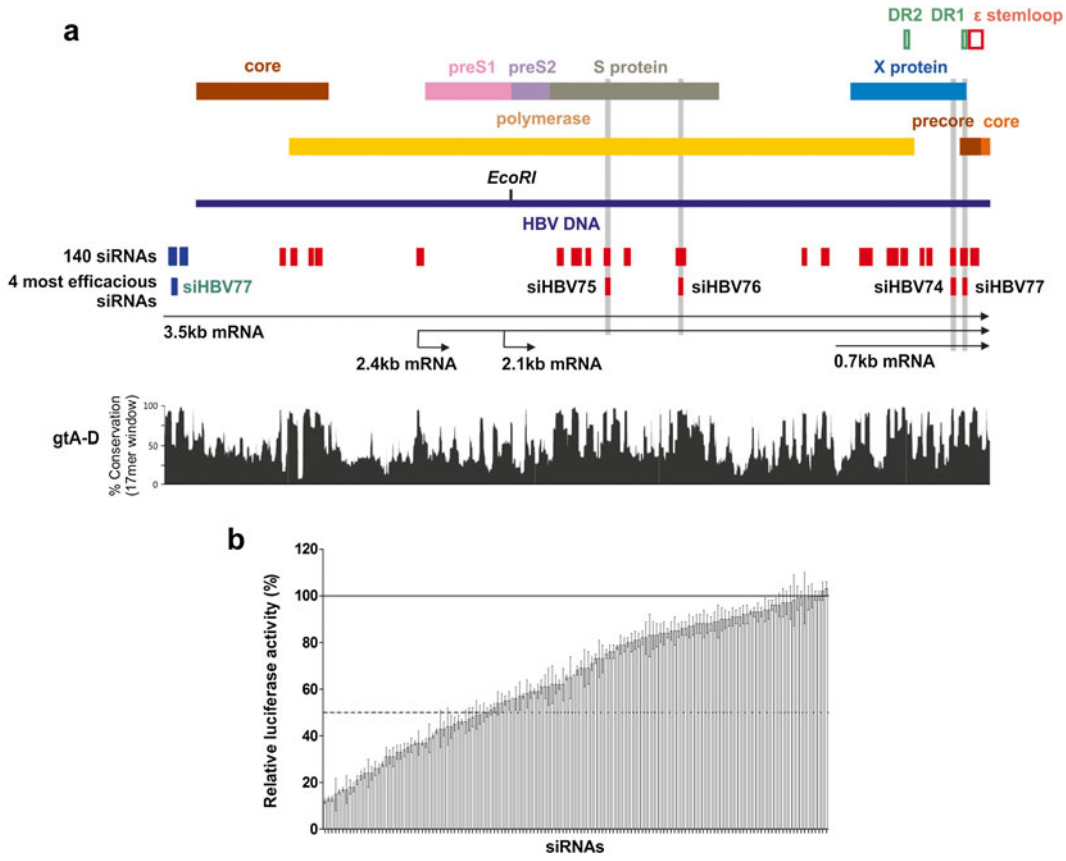

Fig. 3 Selection of siRNA sequences targeting human hepatitis B virus (HBV). (**a**) Locations of the open reading frames within the human HBV genome are shown above the *purple line* that represents the genomic DNA and locations of the mRNAs are shown below this. Numbering of HBV bases begins with position 1 at the EcoR I site. The direct repeats (DR1 and DR2) and the epsilon stem-loop are features of the pregenomic RNA important for replication. The conserved target sites of all screened siRNAs (140) and the four most efficacious siRNAs are indicated by *red bars. Blue bars* indicate siRNA sequences that target the 3.5 kb RNA in the terminally redundant region. The *graph* indicates percentage conservation of 17-mers in 2,754 full-length HBV sequences (genotypes A–D). Schematic was adapted from [24]. (**b**) 140 siRNAs directed against conserved target sites were selected in silico, synthesized and screened at 10 nmol/l in Cos-7 green monkey kidney cells that had been transfected with firefly and *Renilla* luciferase-expressing psiCHECK-HBV. The *Renilla*/firefly luciferase expression ratio was normalized to that in cells transfected with a control siRNA. (Figure is reproduced from [7])

Fig 4 (continued) counterstained with To-Pro-3 to visualize nuclei (*blue*) and with Alexa-633 phalloidin to visualize cell outlines (*blue*). At each time point, the channel containing signal from the Dy547-labeled cholsiF7 (*red*) and the channel containing signal from NAG-PBAVE (*green*) are shown separately or as a merged image with the channel containing signal from the counterstains. *White arrows* indicate representative sinusoidal areas. Each image comprised a flattened projection of 11 optical images (0.4 μm each) to represent combined fluorescence signals from a 4-μm-thick section. (Figure is reproduced from [6])

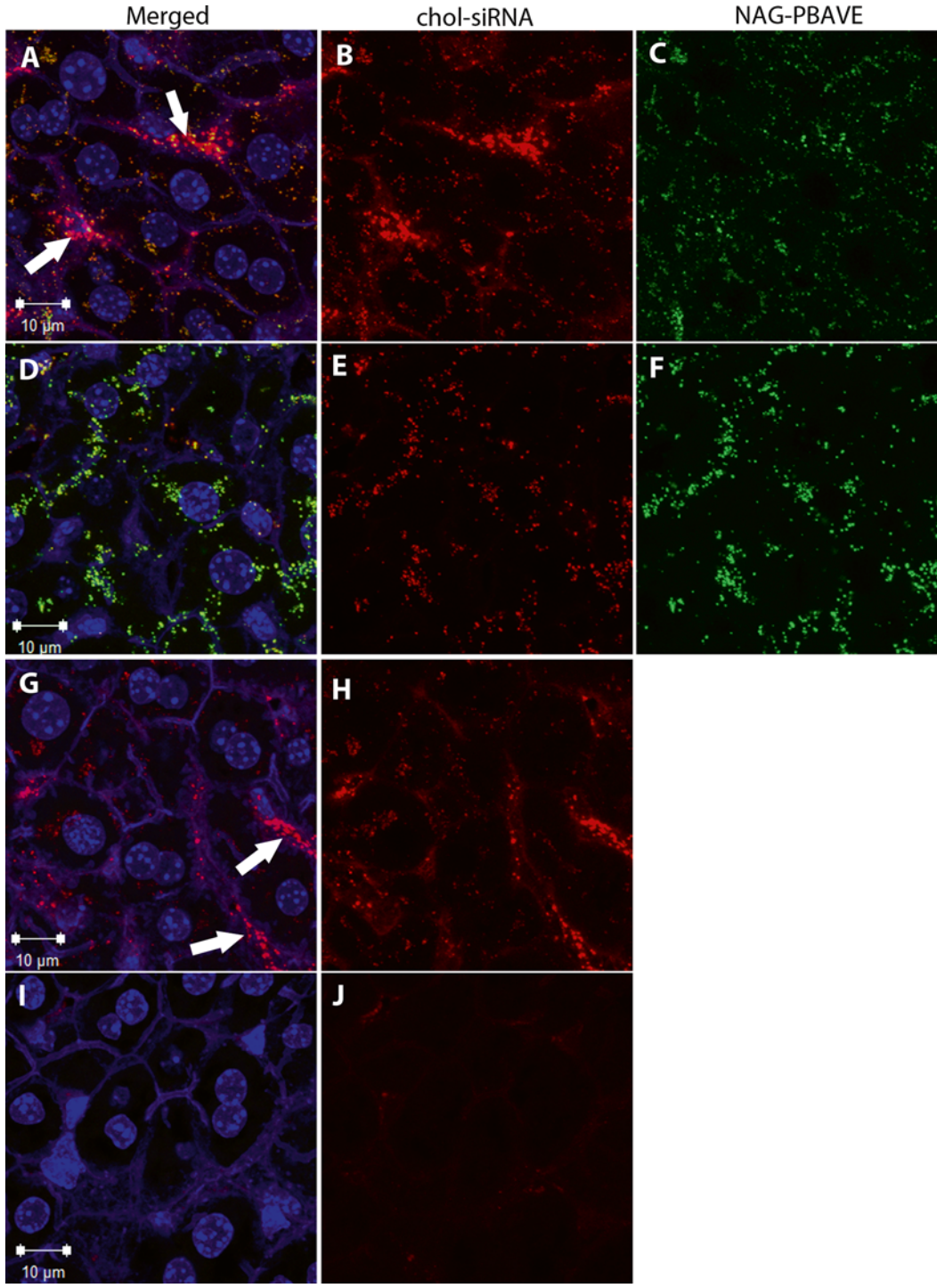

Fig. 4 Confocal micrographs of chol-siRNA and NAG-PBAVE in mouse liver sections demonstrate their co-localization in hepatocytes. Confocal images of co-injected Dy547-labeled chol-siRNA (2 mg/kg) and Oregon Green-labeled NAG-PBAVE (10 mg/kg) at 15 min (A–C) or 2 h (D–F), or Dy547-labeled chol-siRNA (2 mg/kg) alone at 15 min (G–H) or 2 h (I–J) after injection. Livers were harvested at the indicated times, fixed, and

Table 1
Target sites for HBV siRNAs

	Target site in HBV (accession #V01460)		Viral mRNAs			
siRNA ID	Sequence (5′–3′)	Position	3.5 kb	2.4 kb	2.1 kb	0.7 kb
siHBV-75	UCUGCGGCGUUUUAUCAUC	380–398	+	+	+	–
siHBV-76	UUUACUAGUGCCAUUUGUU	674–692	+	+	+	–
siHBV-74	CUGUAGGCAUAAAUUGGUC	1,779–1,797	+	+	+	+
siHBV-77	ACCUCUGCCUAAUCAUCUC	1,825–1,843	+	+	+	+

Figure 3 summarizes the siRNA screening strategy. Table 1 presents the sequence and mRNA specificity of the four most potent siRNAs. These four siRNAs were then further evaluated for in vivo efficacy in mouse models of HBV.

1.3.2 Establishment of Suitable Mouse Models of Chronic HBV Infection to Assess Efficacy

For in vivo evaluation of the four lead siHBVs identified in the in vitro screen, cholesterol-conjugated versions of chemically modified siHBVs (chol-siHBVs) were synthesized and co-injected with NAG-MLP. We used two murine models of chronic HBV infection in these studies. Both models harbored a terminally redundant full-length human HBV genome (HBV1.3) [18]. One of the models is the transgenic HBV1.3.32 mouse that has one chromosomally integrated copy of the HBV1.3 construct and allows HBV gene expression and replication [18]. In this case, the HBV genome is present in every hepatocyte, a scenario that resembles a chronically infected patient. However, measurement of HBsAg in serum is not meaningful in this mouse model because most of the HBV1.3.32 mice in the SV129 background develop neutralizing antibodies to HBsAg.

In the second mouse model, the HBV1.3 sequence is contained in a plasmid (pHBV1.3), which is delivered to the liver of the mouse by hydrodynamic tail vein (HTV) injection [19]. This technique delivers the plasmid DNA almost exclusively to hepatocytes in the liver, typically transfecting 5–20 % of hepatocytes [20]. Expression of the HBV genome from the plasmid results in long-term production of viral mRNAs and pregenomic RNA, which are used to support viral replication and the production of virions and viral proteins. We used the immune-deficient NOD-SCID mouse for these studies because, unlike wild-type mice, this strain does not mount an immune response to HBV [20]. Some acute damage to the liver can result from the hydrodynamic injection procedure. Therefore, mice are allowed at least 3 weeks following HTV injection prior to treatment with test materials. This also allows expression from the pHBV1.3 to stabilize, ensuring the establishment of

an accurate baseline of viral load measurements. We refer to this model as the pHBV model, and a detailed protocol for generating pHBV-carrying mice is described below in Subheadings 2.1 and 3.1 (*see* **Note** 1).

1.3.3 Establishment of Analytical Methods to Monitor In Vivo Anti-HBV Drug Effect

There are several viral markers that can be measured to assess the activity and efficacy of an anti-HBV agent. These include the viral antigens HBsAg and HBeAg, viral RNA and viral DNA (Subheading 3.1). Recombinant, purified HBsAg is less likely to have native conformation than HBsAg produced in the liver, which can affect its reactivity with antibodies. HBsAg standards with a conformation more similar to that of HBsAg antigen in serum can be obtained from mouse serum after hydrodynamic delivery of S antigen expression plasmid. Methods to generate standards by this procedure are in Subheading 3.1, **step 3**. Another important marker to monitor is the amount of HBV DNA in serum. We provide a detailed protocol for this step in Subheading 3.1, **steps 6** and **7**.

If liver tissue samples are also available for analysis, the effect of RNAi can be demonstrated by isolating total RNA, followed by either Northern blotting and/or RT-qPCR (Fig. 2a, b). Direct verification of the RNAi mechanism is performed by rapid amplification of the cDNA at the 5′ end (5′RACE), which shows that cleavage occurs at the expected site in the target mRNA. Protocols for these methods have long been established, and can be found in laboratory manuals.

1.3.4 Verification of Liver Targeting and Delivery into Hepatocytes In Vivo

One of the most important steps for establishing a targeted drug-delivery system is to demonstrate that drug components preferentially accumulate in the desired tissue and cell type. We used several methods to verify receptor-mediated and cell-specific uptake, and to verify that the two drug components co-localize in the target cells. We used fluorescently labeled drug components delivered by IV injection, followed by the harvest of various organs and tissues that were then used to generate cryosections. Sections were counterstained and observed by confocal microscopy to directly visualize the location of fluorescent compounds. One of the detailed protocols we provide below (Subheadings 2.2 and 3.2) describes this approach. In a recent publication, we showed confocal microscopy analyses of liver tissue sections from mice co-injected with Dy547-labeled chol-siRNA and Oregon Green (OG)-labeled PBAVE modified with CDM-PEG and CDM-NAG (NAG-PBAVE). The quantitative image analysis revealed that 2 h after co-injection, 87 % of Dy547-chol-siRNA signal overlapped with the OG-NAG-PBAVE signal, mostly within liver hepatocytes ([6], and Fig. 4). We also examined the liver accumulation of OG-labeled NAG-MLP, which is the endosome-releasing agent of ARC-520, and found that it was indistinguishable from OG-labeled NAG-PBAVE (unpublished data).

Another approach that we used to verify ligand-mediated targeting was injecting DPCs into ASGPr-deficient mice. The lack of gene silencing in these mice verified that NAG-targeted endosomolytic agents enter hepatocytes through NAG's cognate receptor, ASGPr [6]. In addition, no gene silencing is detected when MLP is modified with CDM-PEG instead of CDM-NAG [7]. Together, these results provided evidence that active, hepatocyte-specific targeting of DPCs is afforded by attachment of NAG.

Evaluation of the tissue distribution, and kinetics of accumulation and clearance are important components of drug development. Using ^{124}I-labeled NAG-MLP and positron emission tomography/computed tomography (PET/CT) scan, the NAG-MLP can be visualized in the liver within 3 h of IV injection (Fig. 5). By 7 h, very little NAG-MLP was seen in the liver and the vast majority of the ^{124}I label was in the bladder or intestines. This result suggested that NAG-MLP was significantly degraded by 7 h. MLP is a peptide composed of naturally occurring L-enantiomer amino acids. In order to confirm that the loss of label in the liver was due to degradation of the naturally occurring enantiomer, MLP was

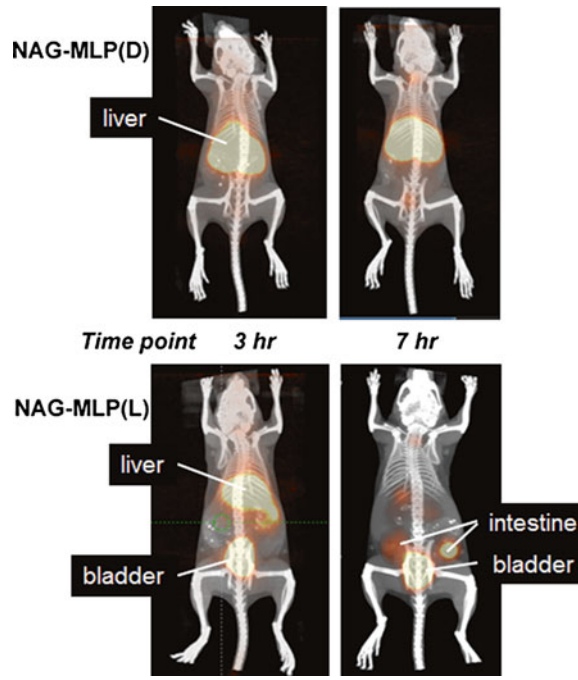

Fig. 5 PET/CT images of BALB/c mice intravenously injected with ^{124}I-labeled D and L enantiomers of CDM-NAG-modified MLP. The peptides were modified with Bolton-Hunter reagent (Pierce), labeled with 124-iodine and then reacted with CDM-NAG. The animals were injected at the dose of 5 mg/kg (approximately 100 μCi/animal) and imaged at the designated time points using Siemens Inveon PET/CT imager

also synthesized with D-enantiomer amino acids, which is expected to result in an MLP that is resistant to degradation. In this case, nearly all of the label accumulated in the liver by 3 h and was still present at 7 h. Pharmacokinetics and tissue distribution of the siRNA were also evaluated.

1.3.5 Confirmation That In Vivo Target Gene Knockdown Is DPC-Dependent, Ligand-Dependent, Dose-Dependent, and Sequence-Specific

When developing a drug candidate it is expected that drug components truly have the function ascribed to them. Below we show two experiments that reflect critical controls used to analyze the function of various elements in our DPC formulations. Figure 6a shows the in vivo knockdown effect of a F7-specific siRNA in mice. Chol-siF7 doses ranged between 0.003 and 1.0 mg/kg, and all doses were co-injected with 6 mg/kg NAG-MLP. To demonstrate sequence-specific effect, a firefly luciferase-specific siRNA (chol-siLuc) was also used at the highest 1 mg/kg dose. To confirm that NAG-MLP endosomal-releasing agent was indeed required to achieve knockdown, the chol-siF7 RNA was also delivered alone, without mixing it with NAG-MLP, at a tenfold higher dose. An additional control verified the importance of targeting: for one group of animals the MLP was masked with CDM-PEG instead of CDM-NAG and, thus, was not targeted to hepatocytes. A group of animals received only isotonic glucose (IG) vehicle—F7 levels of this group served as baseline. Results clearly showed that only chol-siF7 mixed with NAG-MLP could lead to significant target gene knockdown, and the effect was siRNA dose-dependent, endosomal release-dependent, hepatocyte-targeting-dependent, and sequence-specific (Fig. 6a). Figure 6b shows a similarly controlled HBV study, without testing such a wide dose range. This experiment included the empty vehicle (IG) control group to obtain the baseline, but then also had a control that the previous study did not include: 6 mg/kg NAG-MLP endosome-releasing agent injected alone, without any co-delivered chol-siRNA. The 6 mg/kg chol-siHBVs alone, the 6 mg/kg NAG-MLP alone, and the 6 mg/kg off-target (in this case primate F7-specific) chol-siRNAs delivered with NAG-MLP all had no significant knockdown effect on HBsAg expression. In contrast, 3 or 6 mg/kg doses of the two most potent chol-siHBVs, co-injected with 6 mg/kg NAG-MLP, resulted in dramatic and dose-dependent drops in circulating HBsAg levels (Fig. 6b). The last group was injected with an equimolar mixture of the two most potent chol-siHBVs (as in ARC-520), and the results reflect an intermediate activity between an equal total dose of one or the other [7]. By designing and performing these types of experiments, the role of each component in our delivery platform could be verified.

1.3.6 Verification That the Drug Candidate's Mechanism of Action Is RNA Interference

Although siRNA-mediated knockdown of genes is generally considered highly sequence-specific, siRNA delivery is not necessarily without unexpected nonspecific effects. SiRNAs have been shown to activate the immune system through toll-like receptors (TLRs),

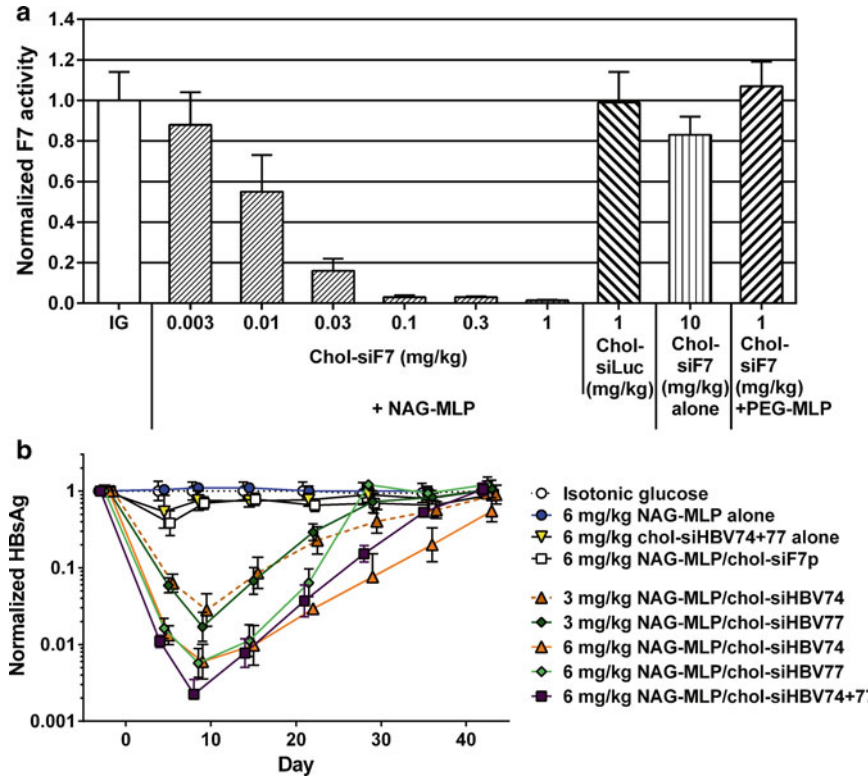

Fig. 6 The function and activity of drug components were verified by in vivo tests. (**a**) Cholesterol-siRNA dose titration: ICR mice were injected intravenously with 6 mg/kg NAG-MLP or with 6 mg/kg of MLP with polyethylene glycol (PEG) attached instead of NAG (PEG-MLP); $n = 4$. Chol-siRNAs targeting either mouse F7 (0.003–1 mg/kg) or luciferase (1 mg/kg) were co-injected with NAG-MLP. One group received 10 mg/kg chol-siF7 alone, with no endosomal-releasing agent. F7 activity in serum was measured 48 h later and was normalized to that in mice injected with isotonic glucose. (**b**) Properly controlled in vivo efficacy study in the pHBV mouse model. NOD SCID mice were given a hydrodynamic tail vein injection with 10 μg minicircle HBV1.3 (MC-HBV1.3). Three weeks later, mice were given one 200 μl IV injection of isotonic glucose alone ($n = 8$) or 6 mg/kg NAG-MLP alone ($n = 7$); an injection of 6 mg/kg chol-siHBV-74 plus chol-siHBV-77 (3 mg/kg each) without NAG-MLP ($n = 4$); a co-injection of 6 mg/kg NAG-MLP with 6 mg/kg chol-siF7p (primate F7) ($n = 4$); or a co-injection of the indicated doses of NAG-MLP with equal doses of chol-siHBV-74, chol-siHBV-77, or chol-siHBV-74 plus chol-siHBV-77 ($n = 4$–11). HBsAg was measured in serum at the indicated time points before and after injection, mean ± SD. Figures are reproduced from [7]

and they can trigger interferon, tumor necrosis factor, and other cytokine signaling cascades, which can modify the drug's effect. Also, the endosomal-releasing agent may cause toxicity, and may skew siRNA-mediated gene silencing effects. To properly assess the mechanism of drug action, toxicity and cytokine activation must be closely monitored. Toxicity is typically evaluated based on clinical chemistry: elevated serum alanine aminotransferase (ALT), aspartate aminotransferase (AST), and glutamate dehydrogenase (GLDH) levels indicate liver toxicity, while serum blood urea

nitrogen (BUN) and creatinine levels reflect kidney toxicity. Depending on the specific delivery technology, other toxic effects (e.g., muscle toxicity) may also have to be monitored. There are commercially available protocols for the measurement of these parameters, or clinical laboratories can perform the tests for a fee. For analyzing signs of liver and kidney toxicity in mouse serum samples we used a COBAS Integra 400 machine (Roche Diagnostics, Indianapolis, IN) according to the manufacturer's instructions. This analyzer requires only a few microliters of serum to test for each analyte, a very important consideration when working with small animals such as mice. Some analytical methods require much more serum than can be safely collected from a mouse.

Cytokine activation can also be assessed using various commercial kits and a Luminex instrument. We measured cytokine levels in mouse serum samples using the MILLIPLEX MAP mouse cytokine/chemokine magnetic bead panel with premixed 25-plex kit and in nonhuman primate serum samples using a cytokine 23-plex kit [7].

An RNAi-mediated drug effect should result in reduced mRNA levels in the targeted tissue, in this case hepatocytes. While recovering liver tissue from human patients is an invasive procedure, in mouse models of chronic HBV infection we have demonstrated significant reduction in HBV transcript levels both by Northern blotting and by RT-qPCR (Fig. 2a, b and [7]). These results confirmed that the multi-log drop in serum antigen levels and viral titers is indeed a result of silencing HBV gene expression.

1.3.7 Evaluation of Efficacy in Nonhuman Primates

Since there is no chronic HBV model in the commonly used nonhuman primate species of Macaca and Cynomolgus monkeys, we assessed the efficacy of DPC delivery in nonhuman primates using chol-siRNA against the endogenous F7. Co-injections of 1 or 3 mg/kg NAG-MLP and 2 mg/kg chol-siF7 siRNAs into cynomolgus monkeys resulted in sequence-specific and dose-dependent knockdown of F7 expression, without significant liver or kidney toxicity [7]. These and other unpublished data verified that our hepatocyte-targeted siRNA delivery platform also works in nonhuman primates with efficacy comparable to that in rodent models.

1.3.8 Summary of Protocols

One of the protocols below describes in detail how we generated the pHBV model and demonstrated the in vivo efficacy of chol-siHBVs as shown in Fig. 2c–e of Subheading 1.2. Co-delivering 6 mg/kg NAG-MLP with 6 mg/kg of the four most potent chol-siHBV siRNAs resulted in rapid decrease in major parameters reflecting viral activity: circulating HBsAg, HBeAg, and viral particles in serum samples. Chol-siHBV-75 and -76 were somewhat less efficient with a shorter duration of knockdown while the other two chol-siHBVs, chol-siHBV-74 and -77, resulted in remarkably long-lasting multi-$\log_{10}$ reduction in both circulating antigens and

also circulating viral particles (Fig. 2c–e). Note that all four chol-siRNAs appear to have equal activity at time points through Day 8. It was only by monitoring long-term effect that potency of these siRNAs was distinguished. Based on these and other tests, chol-siHBV-74 and -77 were chosen and are included in ARC-520 (*see* Fig. 1 for schematic illustration). These two siRNAs together supply broad genotype coverage, and thus would be suitable for the treatment of patients in any part of the world.

The second protocol below provides details for one of the key steps to demonstrate delivery of the siRNA and the endosomolytic agent into hepatocytes in vivo. Bringing an RNAi therapeutic to the clinic naturally involves many steps and this chapter focuses only on the mouse efficacy studies.

2 Materials

2.1 Generating a Mouse Model of Chronic HBV Infection for Evaluating In Vivo Drug Efficacy

Due to HBV being a human pathogen, all HBV-related work must be performed in a dedicated Biosafety Level 2 facility. Virions produced in mice are infectious to humans and the blood must be handled with great care. Also, employees working with HBV should be immunized against the virus and have a confirmed positive antibody titer.

2.1.1 Facility

2.1.2 Equipment

1. Desk or reptile heat lamp to warm mice.
2. Microcentrifuge.
3. 7500 Fast or StepOne Plus Real-Time PCR system (Life Technologies, Grand Island, NY).
4. Microplate reader for HBsAg ELISA assays (SpectraMax 190, Molecular Devices).
5. Microplate strip washer (ELx50, BioTek, Winooski, VT).

2.1.3 Animals

1. Immune-deficient NOD.CB17-*Prkd*scid/NcrCrl (NOD-SCID) mice, 6–8 weeks old (Charles River Laboratories, Wilmington, MA).
2. ICR mice, 6–8 weeks old (Harlan Sprague-Dawley, Indianapolis, IN), 18–21 g.

2.1.4 Plasmid Constructs

1. Plasmid pHBV1.3 contains a terminally redundant HBV1.3 sequence (GenBank accession #V01460) that includes bases 1,068–3,182 followed by 1–1,982 as shown in Fig. 1 of Guidotti et al. [18]. HBV1.3 was synthetically constructed (DNA2.0, Menlo Park, CA) and inserted into a pUC-based cloning vector.

2. MC-HBV1.3 is a minicircle derived from pHBV1.3. The HBV1.3 sequence was subcloned from pHBV1.3 into a minicircle cloning vector by PlasmidFactory (Bielefeld, Germany). The minicircle construct included sites that allow recombination to remove all the bacterial backbone sequence and leave only a short (approximately 400 base pairs) synthetic sequence between the 5′ and 3′ ends of HBV1.3 [21]. It was prepared by PlasmidFactory (Bielefeld, Germany). HBsAg expression from this construct is higher and more stable than from pHBV1.3.
3. Plasmid pRc/CMV-HBs expresses HBsAg and is used in mice to generate the HBsAg protein standard for the HBsAg ELISA.
4. Plasmid pHCR/UbC-SEAP contains the human ApoE hepatic control region, the human ubiquitin C promoter, and human placental secreted alkaline phosphatase gene [22]. It was spiked into mouse serum samples as a recovery control for the purification of HBV DNA from serum. Any plasmid could be used for this purpose as long as it contains a sequence that can be quantitated by qPCR and the primers to do so do not cross-react with mouse genomic sequence nor with the pHBV1.3.
5. Plasmid pSEAP-HBV353–777 was used to generate a standard curve for the quantitative real-time PCR (qPCR) assay to determine the number of copies of HBV in serum samples. This plasmid contained a segment of HBV sequence within the *S* gene, bases 353–777 of GenBank accession #V01460 [7]. This HBV sequence could be cloned into any plasmid for the purpose of generating a standard curve as long as the qPCR probes do not cross-react with any other sequence within the plasmid.

2.1.5 Reagents and Materials

1. Ringer's solution: 0.85 % w/v NaCl, 0.03 % w/v KCl, 0.03 % $CaCl_2$ in ultrapure H_2O; filter sterilized.
2. Sterile 50 ml conical polypropylene tubes.
3. 3 ml syringes with Luer lock.
4. 27-G syringe needles that are 0.5 in. long.
5. Mouse restraining device for tail vein injection (home-made): cut a small opening in the tip of a 50 ml conical tube to create a breathing hole. Create a slit opening in the cap end of the tube to allow tail exposure.
6. Gauze squares or tissues.
7. 70 % ethanol.
8. Blood collection tubes (Capiject, Gel Barrier/Clot Activator, Cherry Red, 500 μl, Cat # TETMG; VWR International, Batavia, IL).
9. GS HBsAg EIA 3.0 Kit (Cat # 32591; Bio-Rad, Redmond, WA).

10. Recombinant HBsAg protein standard of the ayw subtype (Aldevron).
11. HBsAg in serum to generate a standard curve (*see* Subheading 3.1).
12. QIAamp MinElute Virus Spin Kit (Cat # 57704; Qiagen, Valencia, CA).
13. TaqMan chemistry-based primer pairs and probes: primers 5′-GCCGGACCTGCATGACTA-3′ and 5′-GGTACAGCAA CAGGAGGGATACATA-3′, and 6-carboxyfluorescein (FAM)-labeled reporter 5′-CTGCTCAAGGAACCTC-3′ for HBV S gene (Life Technologies, Gran Island, NY); primers 5′-CATGC CACCTCCAACATCCACTC-3′ and 5′-GGCATAGCCA CTTACTGACGACTC-3′, and reporter 5′-FAM/TTGTC CTGGC/ZEN/GTGGTTTAGGTAGTGTGA/IBFQ-3′ (Integrated DNA Technologies, Coralville, IA) for human ApoE HCR encoded on pHCR/UbC-SEAP.

2.2 Verifying Targeted In Vivo Delivery to Hepatocytes in Normal Mice by Fluorescent Confocal Microscopy

2.2.1 Equipment

1. Microm HM 505N cryostat (Zeiss, Thornwood, NY).
2. Zeiss LSM710 confocal microscope (Carl Zeiss Microimaging, Inc., Thornwood, NY).

2.2.2 Animals

ICR mice, 6–8 weeks old (Harlan Sprague-Dawley, Indianapolis, IN).

2.2.3 Reagents and Materials

1. Dy547-labeled cholesterol-conjugated siRNA was prepared as described in Subheading 3.2, **step 1**.
2. OG-labeled and CDM-NAG targeted PBAVE was prepared as described in Subheading 3.2, **step 2**.
3. Isotonic glucose solution: 5 % w/v glucose in 50 mM HEPES, pH 8.0; filter sterilized.
4. Mouse restraining device for tail vein IV injection (as in Subheading 2.1.5, **step 5**).
5. 1 cc syringe and 27-G needle.
6. Scissors and forceps for dissecting tissue specimens.
7. Phosphate buffered saline (PBS, pH 7.4): 8.0 g NaCl (FW 58.43), 0.2 g KCl (FW 74.55), 1.6 g Na_2HPO_4 (FW 358.14), 0.24 g KH_2PO_4 (FW 136.09) dissolved in 700 ml ultrapure H_2O, pH adjusted to 7.4 with NaOH or HCL as needed, and volume filled up to 1,000 ml with ultrapure H_2O.

8. 4 % paraformaldehyde (Cat No. 0380; EM Grade; Polysciences, Inc. Warrington, PA) freshly prepared in PBS, pH 7.4.
9. 30 % sucrose in PBS (pH 7.4); filter sterilized.
10. HistoPrep Embedding Medium (Cat # SH75-125D, Fisher Scientific, Pittsburgh, PA).
11. Peel-A-Way® Embedding Molds (Cat # 18646A, Polysciences, Inc. Warrington, PA), pre-labeled with study #, animal ID #s, and tissue type to be frozen.
12. Liquid nitrogen, a small Dewar container to hold it, and a Styrofoam box for floating samples on the liquid nitrogen.
13. Styrofoam box with dry ice pellets.
14. Ziploc® bags, pre-labeled with study #, animal ID #s, tissue types, etc.
15. Superfrost®-Plus microscope slides (Cat # 48311-703; VWR, Radnor, PA).
16. Alexa633-conjugated Phalloidin (Cat # A22284; Invitrogen, Carlsbad, CA) and ToPro-3 (Cat # T3605; Invitrogen) to counterstain actin filaments on the cells' periphery, and nuclear DNA, respectively.
17. VECTASHIELD mounting medium (Cat # H-1000; Vector Laboratories, Burlingame, CA).

3 Methods

3.1 Generating a Mouse Model of Chronic HBV Infection for Evaluating In Vivo Drug Efficacy

1. Inject 10–15 μg pHBV1.3 or 5–10 μg MC-HBV1.3 DNA in 2 ml Ringer's Solution into a 20 g NOD-SCID mouse by hydrodynamic (also called as "high pressure") tail vein injection [20] (*see* **Note 2**). To get ready for the injections, first prepare all samples, then label and fill the syringes. The optimal injection volume is 10 % of body weight, and an additional 100 μl is required for the dead volume in the needle hub and syringe. Calculate sample volumes for the number of mice that will be injected plus at least a 10 % overage that will accommodate the dead volume. In a sterile 50 ml conical propylene tube, dilute a sufficient quantity of the DNA in Ringer's Solution. Warm this solution to 37 °C. For each 20 g mouse to be injected, draw 2.1 ml solution into a 3 ml syringe. Attach the capped 27-G needle to the syringe. Warm the mouse for a few minutes under a heat lamp to dilate the blood vessels in the tail (*see* **Note 3**). Place mouse in the restraining tube with its nose near the hole at the bottom of the tube and its tail protruding through the slot at the end. Insert a piece of gauze or tissue behind the mouse and then screw the cap onto the tube. Wipe the tail with 70 % ethanol to further dilate the vein

and disinfect the skin. Carefully remove the cap from the needle. While holding the tail at its tip with thumb and index finger of one hand, with the other hand place the needle on the distal part of the tail pointing away from your hand and toward the body of the mouse. Insert the full length of the needle into the tail vein, beveled side down, keeping the needle parallel to the tail vein. The key to efficient hepatocyte transfection is the injection rate: the entire volume must be rapidly and continuously delivered within 4–6 s. After the injection, withdraw the needle, wipe off the blood, and release the mouse from the restraining tube. Place mice under a heating lamp for 10–20 min after the injection. Mice resume normal activity after this period.

2. Allow HBV expression and replication to stabilize over a period of 3 weeks.
3. Generate HBsAg standards for the ELISA assay by HTV injection of 10 μg plasmid pRc/CMV-HBs in 2 ml Ringer's Solution (as described in Subheading 3.1, **step 1**) into each of two ICR mice weighing 18–21 g. This plasmid expresses only the S gene and not the remainder of the viral genome. At 24 h post-injection, euthanize the mice and collect blood immediately by cardiac puncture. Isolate serum from each mouse and combine (*see* **Note 4**). Freeze in 50 μl aliquots at −20 °C. During the first use of each tube, thaw and sub-aliquot into smaller volumes as needed. Quantitate the amount of HBsAg in the serum using the HBsAg ELISA kit and 1 ng/ml recombinant HBsAg protein as a standard. The amount of HBsAg in the mouse serum is expected to be approximately 20 μg/ml, but this is dependent on successful hydrodynamic injections in both mice.
4. Collect blood from the animals to assess circulating HBsAg levels and viral load (HBV DNA copy number) in serum samples. Blood collection can be performed by using one of several standard protocols (e.g., retro-orbital or submandibular bleeding techniques) after mice are anesthetized with 2–3 % isoflurane. Allow blood to clot in the collection tube for 20 min at room temperature, then centrifuge at 9,000 × *g* for 3 min, and pipet clear serum into clean tubes. Store at 4 °C for up to 1 week (*see* **Note 5**).
5. Assess circulating HBsAg levels in serum samples using the Bio-Rad GS HBsAg EIA 3.0 Kit according to the manufacturer's instructions. It is important to properly dilute serum samples in 5 % nonfat dry milk prepared in PBS so that antigen concentrations fall in the linear range of the assay. Dilution of samples with naïve animal serum causes nonspecific binding that increases the background. Our experience is that 3 weeks after

successful transfection of typically ~10 % hepatocytes by hydrodynamic tail vein delivery, animals produce enough HBsAg to require 500- to 2,000-fold serum dilutions to bring concentrations into the linear range of the assay, depending on the HBV expression vector. Generate a standard curve using 5 ng/ml HBsAg from the mouse serum (as determined in Subheading 3.1, **step 4**) and twofold dilutions down to 0.078 ng/ml.

6. Isolate HBV genomic DNA from serum samples to assess viral load. Use either 75 μl serum per mouse or pool equal volumes of serum from mice in each group to a final volume of 100 μl and perform duplicate isolations from the pooled serum (*see* **Note 6**). Add sterile 0.9 % saline to each sample to a final volume of 200 μl. Isolate DNA using the QIAamp MinElute Virus Spin Kit following the manufacturer's instructions. Serum samples are added to tubes containing buffer and protease, followed by addition of carrier RNA from the kit to aid in the isolation of small amounts of DNA, and 1 ng of plasmid pHCR/UbC-SEAP DNA that serves as a recovery control. After incubating for 15 min at 56 °C, precipitate nucleic acids from the lysates with ethanol and apply the entire solution to a spin column. After washing steps, elute each sample with 50 μl Buffer AVE from the kit.
7. Determine absolute HBV DNA genome copy number in serum samples using qPCR. Generate a standard curve for HBV using pSEAP-HBV353–777 over a range of 7 – $\log_{10}$ (10^8–10^1 copies) and a standard curve in the same range for the recovery control plasmid pHCR/UbC-SEAP. Perform qPCR assays using HBV and HCR-specific primer pairs and probes (*see* **Note 7**).
8. Based on serum HBsAg levels and HBV genome copy numbers, assign animals to experimental groups so that each group has similar average expression level and viral load. Due to their impaired immune system, these animals can be expected to show stable HBV protein expression and replication for an extended time, similar to chronic HBV infection.

3.2 Verifying Targeted In Vivo Delivery to Hepatocytes in Normal Mice by Fluorescent Microscopy

1. The siRNA sense strand with 5′-cholesterol modifier (cholesterol-CE phosphoramidite, Cat# 2814; Link Technologies Ltd., Bellshill, Scotland) and the antisense strand with 5′-Dy547 phosphoramidite (Cat# SY633204, Thermo Scientific, Pittsburgh, PA) were synthesized using conventional solid-phase phosphoramidite syntheses. The sense and antisense strands were then annealed to form double stranded Dy547-Chol-siRNA.
2. Thirty milligram of PBAVE polymer at 15 mg/ml concentration in 200 mM $NaHCO_3$ buffer was reacted with 1 mg Oregon

Green® 488 NHS ester (Cat # O-6147; Life Technologies, Grand Island, NY) overnight, with constant stirring. The labeled polymer was purified on a Sephadex G-25 size-exclusion column using 20 mM ammonium formate (Sigma-Aldrich) as running buffer, then was lyophilized to dry. Dry OG-PBAVE was brought back up at 10 mg/ml concentration in ultrapure H_2O.

3. A 2:1 w:w mixture of CDM-PEG and CDM-NAG was added to OG-PBAVE at a 7× weight excess, in 5 % (isotonic) glucose solution buffered with 50 mM HEPES, pH 8.0. The mixture was incubated for a minimum of 1 h at room temperature prior to injecting into a mouse. Samples were used without purification. For preparing untargeted control, only CDM-PEG was added to OG-PBAVE at a 7× weight excess (*see* **Note 8**).
4. Mix Dy547-Chol-siRNA and OG-NAG-MLP in 5 mM Hepes-buffered (pH 7.5) isotonic glucose at the desired dose. Typically, we deliver 2–6 mg/kg siRNA with 2–10 mg/kg endosomolytic agent, through a low pressure intravenous (IV) injection into the tail vein, shortly after mixing the components, in a volume of 200 μl per mouse. Calculate sample volumes to include an additional 100 μl per injection to allow for the dead volume in the needle hub and syringe. As untargeted control, OG-PEG-PBAVE can be used instead of OG-NAG-PBAVE, and Dy™547-Chol-siRNA can be delivered alone, without mixing it with the endosome-releasing agent.
5. To get ready for the injections, first prepare all samples, then label and fill the syringes. Place mouse in the restraining tube with its nose near the hole at the bottom of the tube and its tail protruding through the slot at the cap end. Insert a piece of gauze or tissue behind the mouse and then screw the cap onto the tube. Dilate the tail veins by soaking the tail in warm water, or wiping it with ethanol. While holding the tail in place, insert a 27-G needle into the tail vein at a shallow angle as distally as possible and slowly inject the solution (*see* **Note 9**).
6. At the desired time points (e.g., 15–20 min, 1 h, or 2 h post-injection), euthanize the mouse and harvest all tissues of interest (*see* **Note 10**).
7. Fix tissues in 4 % paraformaldehyde/PBS for 6 h at room temperature, or overnight at 4 °C. Do not expose samples to sunshine or direct light.
8. Transfer tissue samples into 30 % sucrose/PBS solution overnight at 4 °C, or until tissues sink to the bottom of the tube. This typically happens after 4–5 h. Again, avoid exposure to direct, strong light.
9. Using forceps, transfer tissue samples into pre-labeled plastic cryo-molds containing a small amount of HistoPrep Embedding

Medium. Sectioning will be started from the bottom of the mold, so orient tissue pieces accordingly. If multiple tissues are placed into a single block, try to arrange them at the same depth, and make a note about their location inside the block. This will make sectioning and analysis significantly easier.

10. Fill up the mold with HistoPrep until all tissue pieces are covered. Avoid air bubbles.
11. Transfer the mold into a Styrofoam box containing 1–2 in. of LN and float the mold on the liquid using a sheet of Styrofoam with holes large enough to hold the mold in place, surrounded by the liquid but not submerged in it. Wait until the blocks turn completely white (frozen), then using forceps transfer them into pre-labeled Ziploc® bags. Close the bags and temporarily store them on dry ice until all samples are processed. Long-term storage is at −80 °C.
12. Cryo-sections (8–10 μm) are prepared using the cryostat, and are placed onto Superfrost-Plus microscope slides. Sections can be stored in the dark at room temperature for a few days (*see* **Note 11**).
13. Briefly rehydrate sections in PBS, then counterstain them with Alexa633-Phalloidin (20 nM) and ToPro-3 (40 nM) in PBS, at room temperature, for 20–60 min.
14. Rinse slides 3× with PBS.
15. Mount sections in VECTASHIELD, and image tissue samples by confocal microscopy. We typically collect Z-stacks of 11 0.4 μm optical sections, and project the stack into a flattened image representing 4 μm tissue depth.

4 Notes

1. It is important to note that although using HTV delivery of expression plasmids into hepatocytes is a convenient and effective approach to deliver expression constructs, it is essential that the proper "expression cassette" be used to drive high levels of long-term expression. While the cytomegalovirus promoter is known to allow high levels of gene expression in almost any cell type, its inability to drive long-term expression in the liver is less well known. We have studied requirements for optimal and stable liver-specific expression vectors [23] for sustained transgene expression in hepatocytes. We suggest that features of an expression cassette are always carefully analyzed and optimized before attempting to establish an animal model that relies on stable transgene expression. In the case of HBV, the length of the HBV genome needed for full expression of viral sequences has been determined: it is 1.3 genome lengths [18].

2. The hydrodynamic procedure is most efficient in mice that weigh 18–21 g.
3. The tail vein is more easily seen in white compared to black mice. Instead of using a heat lamp, the tail may be soaked in warm water to dilate the tail vein and make it more visible. If the vein is still difficult to see, inserting the needle into the vein by feel requires some experience or practice. If the tail turns white at or proximal to the injection site or a bleb of fluid appears in the tissue, stop immediately. The needle is not in the vein and the solution is perivascular. Attempt the injection again proximal to the white area. Keep detailed notes to match injected samples with animal identification numbers, and record anything unusual during injection, such as a failed attempt, or lost volume. Mirus Bio LLC has a very helpful, detailed description of the hydrodynamic injection procedure on its website, including a short video clip to demonstrate proper injection technique: http://www.mirusbio.com/delivery/tailvein/.
4. Blood collection can be performed by using one of several standard protocols (e.g., retro-orbital or submandibular bleeding techniques) after mice are anesthetized with 2–3 % isoflurane. Cardiac puncture is a procedure that allows collection of a larger volume, but it is terminal. Allow blood to clot in the collection tube for 20 min at room temperature, then centrifuge at 9,000 × *g* for 3 min, and pipet clear serum into clean tubes.
5. On the average, mice have 79 ml blood per kg body weight. Thus, a 25 g mouse has approximately 2 ml blood. The guideline is that no more than 10 % of the total blood can be collected in 1 week (e.g., 200 μl for a 25 g animal). This means that assays must be designed to work with small serum volumes.
6. Due to the small serum volumes collected from individual mice, take care to have enough samples to be able to determine statistically significant results. One approach is to use 75 μl serum per mouse with a suitable number of mice in each group. The alternative is to pool equal volumes of serum from mice in each group to a final volume of 100 μl and perform duplicate or triplicate isolations from the pooled serum. The number of animals in a group must be adequate to allow for statistically significant results even if there are a few samples that are poorly recovered during isolation of DNA from serum.
7. If the recovery plasmid in any sample is present at less than one standard deviation from the average cycle threshold of all the samples, flag this sample as having poor recovery and omit from the analysis.
8. For preparing NAG-MLP instead of NAG-PBAVE, CDM-PEG was omitted from the reaction, and CDM-NAG was

added to MLP at a 5× weight excess. For preparing untargeted control MLP, a 5× weight excess of CDM-PEG was used instead of CDM-NAG.

9. If the tail turns white at or proximal to the injection site, stop immediately. The needle is not in the vein and the solution is perivascular. Attempt the injection again proximal to the white area. If multiple IV injections or attempts are needed to inject the sample, each injection should be more proximal than the last. Keep detailed notes to match injected samples with animal identification numbers, and record anything unusual during injection, such as a failed attempt, or lost volume.
10. In addition to the liver, in some experiments we also harvested other tissues, such as lung, kidney, spleen, brain, pancreas, and skeletal muscle, in order to assess fluorescent signal intensity in nontarget tissues.
11. Place two samples of each sectioned tissue block on the slides. Not all sections are equally pretty: folds and tears, or distorted structures do happen.

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