Dynamic PolyConjugates for targeted *in vivo* delivery of siRNA to hepatocytes

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Achieving efficient *in vivo* delivery of siRNA to the appropriate target cell would be a major advance in the use of RNAi in gene function studies and as a therapeutic modality. Hepatocytes, the key parenchymal cells of the liver, are a particularly attractive target cell type for siRNA delivery given their central role in several infectious and metabolic disorders. We have developed a vehicle for the delivery of siRNA to hepatocytes both *in vitro* and *in vivo*, which we have named siRNA Dynamic PolyConjugates. Key features of the Dynamic PolyConjugate technology include a membrane-active polymer, the ability to reversibly mask the activity of this polymer until it reaches the acidic environment of endosomes, and the ability to target this modified polymer and its siRNA cargo specifically to hepatocytes *in vivo* after simple, low-pressure i.v. injection. Using this delivery technology, we demonstrate effective knockdown of two endogenous genes in mouse liver: apolipoprotein B (*apoB*) and peroxisome proliferator-activated receptor alpha (*ppara*). Knockdown of *apoB* resulted in clear phenotypic changes that included a significant reduction in serum cholesterol and increased fat accumulation in the liver, consistent with the known functions of *apoB*. Knockdown of *ppara* also resulted in a phenotype consistent with its known function, although with less penetrance than observed in *apoB* knockdown mice. Analyses of serum liver enzyme and cytokine levels in treated mice indicated that the siRNA Dynamic PolyConjugate was nontoxic and well tolerated.

pH labile bonds | nonviral siRNA delivery | siRNA–polymer conjugates | endosomolytic polymers

The ability of siRNA to silence specific genes has generated great interest in its use as a research tool and therapeutic agent for a wide spectrum of disorders that include cancer, infectious disease, and metabolic conditions (1–3). Effective *in vivo* delivery of siRNA to the appropriate target cell is an essential component of these siRNA-based applications. Accordingly, a variety of nonviral (4–14) and viral (15–17) systems are being developed for delivery of siRNA to liver, tumors, and other tissues *in vivo*.

In addition to their importance in many infectious and metabolic disorders (18), hepatocytes are a particularly attractive target cell type for siRNA delivery given their ability to be accessed directly by nanoparticle-sized constructs after simple intravascular injection. Initial hepatocyte delivery efforts used hydrodynamic delivery of naked siRNA to the liver (19, 20). More recent work has used viral vectors, such as AAV or lentivirus (16, 17), or synthetic systems such as cholesterol–siRNA conjugates or stable nucleic acid lipid particles (SNALPs) (21, 22). Among the nonviral approaches, SNALP technology represents a significant advance, enabling target mRNA knockdown in liver after i.v. injection of clinically relevant doses of siRNA (21). More recently, another lipid-based system termed interfering nanoparticles (iNOPs) has also demonstrated the ability to deliver siRNA *in vivo* (23). A key drawback of the SNALP and iNOP systems, however, is that the siRNA complexes are only passively targeted to liver. As a result, siRNAs are delivered to a significant number of nontarget cells in the liver, potentially contributing to toxicity.

Hepatocyte targeting after administration into a peripheral vein requires that the delivery vehicle avoid nonspecific interactions *en route* to the target cell, which is commonly accomplished by the attachment of polyethylene glycol (PEG) (24) or other hydrophilic, noninteractive agents. Upon reaching the liver, the vehicle must then exit the intravascular space to access hepatocytes. Because of the open, fenestrated nature of the hepatic vasculature, particles <100 nm in diameter can readily exit hepatic vessels and interact with liver parenchymal cells (25). However, avoiding uptake and subsequent activation of Kupffer cells, the resident immune cells of the liver, are likely essential to avoid toxicity (26). As an example, Kupffer cell uptake of adenoviral vectors is the main cause of liver toxicity observed when these vectors are used for delivery (27). Galactose-derived ligands, which are recognized by the asialoglycoprotein receptor (ASGPr), can be used to specifically target hepatocytes (28). Certain galactose-containing ligands enable hepatocyte uptake and avoidance of Kupffer cells if properly displayed on the delivery vehicle (29, 30).

Once attached to the surface of hepatocytes, siRNA-containing complexes can enter the cells via receptor-mediated endocytosis. The siRNAs must then escape from endosomes to elicit RNAi. To accomplish efficient endosomal escape, we developed a strategy that relies on the selective activation of a latent endosomolytic agent in the acidic environment of the endosome (31). Selective activation ensures that deleterious interactions with other membranes the agent encounters before endocytosis are prevented. In our strategy, amine groups on the endosomolytic agent are modified with a maleic anhydride, creating acid-labile maleamate bonds (32). These bonds are cleaved within the acidic environment of the endosome, unmasking the agent's amines and activating its endosomolytic capabilities (31). The endosomolytic agent used in the present study is an amphipathic poly(vinyl ether) we previously developed termed PBAVE, which is composed of butyl and amino vinyl ethers (33).

In this study, we use a bifunctional maleamate linkage to reversibly attach the shielding agent PEG and the hepatocyte targeting ligand *N*-acetylgalactosamine (NAG) to PBAVE. The siRNA cargo itself is attached to PBAVE through a reversible disulfide linkage, which prevents displacement of the siRNA from the polymer *en route* to the target cell. We have named this delivery vehicle an siRNA Dynamic PolyConjugate, to indicate the fact that the siRNA, shielding agents, and targeting ligands are reversibly con-

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Abbreviations: CDM, carboxy dimethylmaleic anhydride; iNOP, interfering nanoparticle; NAG, *N*-acetylgalactosamine; SNALP, stable nucleic acid lipid particle.

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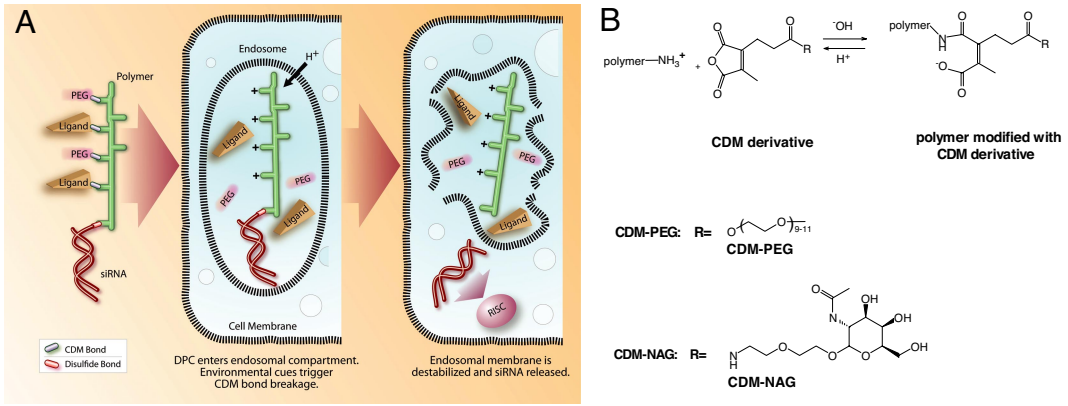

Fig. 1. Critical components of the siRNA polyconjugate and the proposed mechanism of siRNA delivery. (*A*) Schematic showing the siRNA Dynamic PolyConjugate, its cellular uptake, disassembly in the low pH environment of the endosome, and release of the siRNA into the cytoplasm of the target cell. CDM, carboxylated dimethyl maleic acid. (*B*) Mechanism of pH-sensitive CDM chemistry and the structures of the CDM derivatives used in this study. Depicted is the reaction of CDM with free tertiary amines on the polymer, which is reversible under acidic conditions.

jugated to a polymer whose endosomolytic properties are triggered by its chemical environment.

Results

Formulation of the siRNA Polyconjugate and Cellular Delivery. The formulation of the siRNA polyconjugate and the principles of polyconjugate-mediated siRNA delivery are shown in Fig. 1. The polyconjugate itself is constructed by first linking the siRNA payload to the PBAVE polymer through a disulfide linkage (Fig. 1*A*). The amount of conjugated siRNA from this reaction is typically 70–90% of the input. The siRNA–polymer conjugate is then reversibly modified with maleic anhydride derivatives synthesized from carboxy dimethylmaleic anhydride (CDM) (31) containing PEG or NAG groups (Fig. 1*B*). Modification with PEG reduces nonspecific interactions and allows hepatocyte targeting via the NAG ligand. The resulting siRNA polyconjugate is negatively charged, soluble, and nonaggregating under physiological conditions. The size of the siRNA polyconjugate is 10 ± 2 nm as measured by particle sizing, making it substantially smaller than the SNALP or iNOP siRNA complexes (21, 34).

After simple i.v. injection, the siRNA polyconjugate is designed to engage the ASGPr on hepatocytes and be taken into the cell via endocytosis (Fig. 1*A*). As the endosome matures, the decrease in pH induces release of the CDM-PEG and CDM-NAG groups, unmasking the positively charged amine groups on the PBAVE polymer. This release results in the activation of the endosomolytic capability of PBAVE and release of the siRNA into the reducing environment of the cytoplasm. Once there, the siRNA cargo is cleaved from the polymer, allowing the siRNA to engage RISC and induce RNAi.

Activity of the siRNA Polyconjugate in Tissue Culture. It would appear obvious that a delivery agent designed for *in vivo* use should also have transfection activity in culture. Therefore, we tested the ability of the polyconjugate to deliver siRNA and knock down target gene expression in mouse primary hepatocytes. We chose to target the mouse *apolipoprotein B* (*apoB*) gene, a hepatocyte-expressed gene involved in cholesterol transport. All siRNAs used in this report contained modifications designed to increase resistance to nucleases and suppress off-target effects (22, 35). We found that transfection of primary hepatocytes with *apoB-1* siRNA polyconjugate was highly effective, resulting in nearly 80% knockdown of apoB mRNA (Fig. 2). The level of target gene knockdown was comparable to that in cells transfected with *apoB-1* siRNA by using siQUEST, a commercially available *in vitro* siRNA transfection agent. As expected, decreasing the amount of siRNA polyconjugate added to the cells led to progressively decreased *apoB* knockdown.

Effective siRNA delivery using Dynamic PolyConjugate technology required that the PEG shielding agent be linked to the polymer through a reversible linkage. Attachment of PEG to the polymer backbone through a nonhydrolysable amide linkage, in place of a reversible CDM linkage, completely abolished knockdown of *apoB* expression [supporting information (SI) Fig. 7]. These results are consistent with previous studies of irreversible modifications of amphipathic polycations (31) and highlight the necessity for the use of reversible modifications to achieve endosomolysis.

Targeting of the siRNA Polyconjugate to Hepatocytes *in Vivo*. Previous studies have shown that attachment of galactose or NAG facilitates hepatocyte targeting of a variety of uncharged, water-soluble polymers (36–38). We attached NAG to the polymer–siRNA backbone through a CDM linkage to determine whether we could target the siRNA polyconjugate to liver hepatocytes after simple i.v. injection into the tail vein of mice.

Confocal micrographs of liver sections taken from mice 1 h after injection with polyconjugate containing a Cy3-labeled, 21-mer

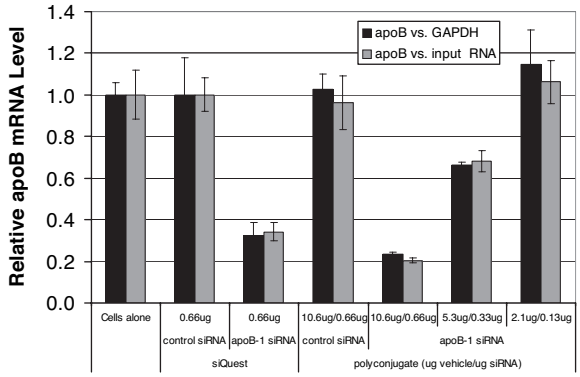

Fig. 2. siRNA polyconjugates can be used to transfect siRNA in mouse primary hepatocytes. Shown is RT-qPCR analysis of *apoB* mRNA knockdown in primary hepatocytes. Cells were transfected with the indicated amounts of siRNA by using a commercially available transfection reagent (siQUEST) or with serial dilutions of *apoB-1* siRNA polyconjugate. Twenty-four hours after transfection, relative *apoB* mRNA levels were measured versus GAPDH mRNA levels or versus the amount of input RNA in the RT-qPCR and then normalized to the values in untreated cells (cells alone). Data are shown as mean ± SD.

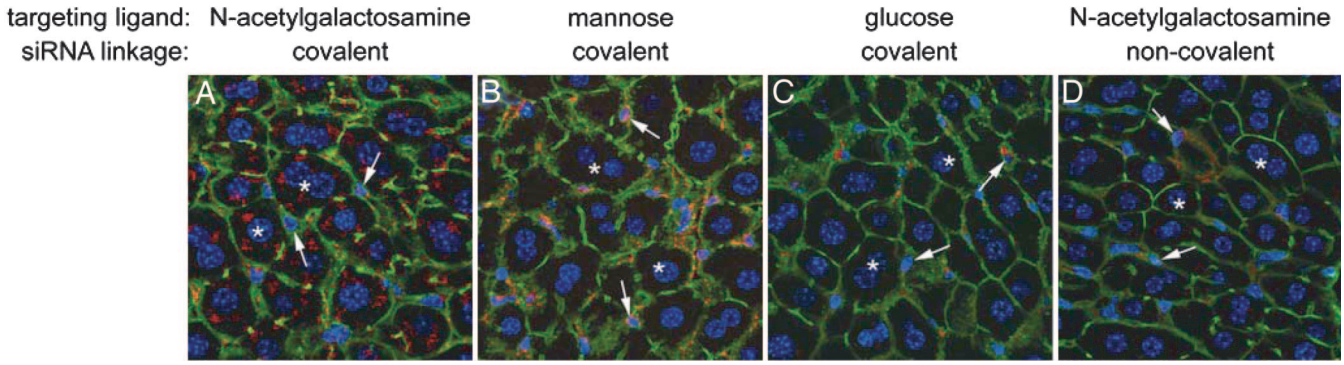

Fig. 3. Targeted delivery of oligonucleotide polyconjugates to liver hepatocytes in mice. Shown are confocal images of liver sections from mice injected i.v. with polyconjugate covalently linked through a CDM linkage to the targeting ligand NAG (*A* and *D*) or to mannose (*B*) or glucose (*C*) as controls. Cy3-labeled 21-mer dsDNA (red) was covalently attached to polyconjugate through a disulfide linkage (*A–C*) or was present in a noncovalent complex with polyconjugate (*D*). Livers were harvested 1 h after injection, fixed, and counterstained with ToPro-3 to visualize nuclei (blue) and Alexa 488 phalloidin to visualize cell outlines (green). 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. Asterisks mark representative hepatocytes, and arrows indicate representative nonparenchymal cells.

double-stranded DNA (dsDNA) mimic of siRNA are shown in Fig. 3. When NAG was used as the targeting ligand on the polyconjugate, we observed preferential accumulation of the Cy3-labeled dsDNA in hepatocytes and only minimal association with nonparenchymal cells in liver sinusoids (Fig. 3*A*). Distribution was nearly homogenous throughout the different zones and of the liver acinus (SI Fig. 8). Inspection of other organs revealed minor Cy3-fluorescence in spleen and kidney, with levels estimated to be at least 20-fold lower than in liver (data not shown). Replacement of CDM-NAG on the polyconjugate with CDM-glucose resulted in markedly reduced hepatocyte uptake (Fig. 3*C*) (data not shown), which is consistent with the lower affinity of the ASGPr on hepatocytes for glucose (39). Significantly, attachment of mannose to the polyconjugate instead of NAG redirected the polyconjugate to nonparenchymal cells in the liver including sinusoidal endothelial and Kupffer cells, which possess mannose receptors, and away from hepatocytes (Fig. 4*B*) (40). These results are evidence that active, hepatocyte-specific targeting of the polyconjugate to hepatocytes is afforded by attachment of NAG. They also suggest that siRNA polyconjugates can be directed to other cell types simply by attaching the appropriate ligand.

We also tested whether covalent attachment of the siRNA to the polyconjugate is required for efficient siRNA uptake in hepatocytes in mice. Our preliminary *in vitro* studies indicated that noncovalently complexed siRNA is rapidly displaced from all but the most charge-dense polycations in the presence of serum or physiological concentrations of salt (data not shown). Injection of a polyconjugate formulation in which the polymer and oligonucleotide were electrostatically complexed, but not covalently linked, resulted in much reduced hepatocyte accumulation (Fig. 3*D*). These results suggest that 21-mer double-stranded oligonucleotides such as siRNA are rapidly displaced from the delivery vehicle and that covalent attachment of the siRNA to the delivery vehicle is necessary for efficient delivery to the target organ.

Knockdown of Target Genes in Liver of Mice by Using siRNA Polyconjugates. To assess the ability of the polyconjugate to deliver siRNA and knockdown target gene expression *in vivo*, polyconjugate containing *apoB-1* siRNA (800 μg of polymer, 50 μg of siRNA) was delivered to C57BL/6 mice by using a single simple i.v. injection. Livers from injected mice were harvested 2 days after injection and assayed for *apoB* mRNA levels by using reverse transcriptase quantitative PCR (RT-qPCR). The *apoB* mRNA levels were measured relative to the level of the housekeeping GAPDH mRNA and micrograms of total input RNA, to reduce the possibility that any differences observed in relative *apoB* mRNA levels were due to nonspecific effects on housekeeping-gene expression. As shown in Fig. 4*A*, mice treated with *apoB-1* siRNA polyconjugate had significantly reduced *apoB* mRNA levels compared with mice receiving polyconjugate containing a non-*apoB* control siRNA or mice injected with saline only ($n = 5, P < 0.00001$). Specifically, the mean *apoB* mRNA level in mice receiving *apoB-1* siRNA polyconjugate was reduced by 76 $\pm$ 14% compared with the saline treated group relative to GAPDH mRNA levels, whereas *apoB* mRNA levels in mice injected with the control siRNA were unaffected. Similar results were obtained if *apoB* mRNA levels were measured relative to total RNA.

To confirm the specificity of the *apoB* knockdown, a separate group of mice was treated with an siRNA targeting a different region of the *apoB* mRNA. Mice receiving *apoB-2* siRNA polyconjugate also exhibited a significant reduction in *apoB* mRNA levels (60 $\pm$ 6% reduction, $n = 5$, $P < 0.00001$). Western blot analysis of *apoB-100* protein levels in serum reflected the reduction in liver *apoB* mRNA levels in mice receiving either apoB-1 or apoB-2 siRNAs (Fig. 4*B*). *ApoB* mRNA expression was not reduced in the jejunum, another tissue that expresses the apoB gene, suggesting that the polyconjugate does not target this tissue (data not shown).

We also prepared and tested polyconjugates containing siRNAs targeting peroxisome proliferator-activated receptor alpha (*ppara*), a gene important in controlling fatty acid metabolism in liver where it is expressed solely in hepatocytes (41, 42). Polyconjugate-mediated delivery of two different siRNAs targeting *ppara* resulted in significant knockdown of *ppara* mRNA levels in liver (Fig. 4*C*). Relative to mice receiving a control siRNA, *ppara* mRNA levels in mice receiving *ppara-1* siRNA were reduced by between 40 $\pm$ 9% and 64 $\pm$ 9%, as determined by Invader or RT-qPCR assays, respectively. A similar reduction in *ppara* mRNA levels was observed in mice injected with polyconjugate containing *ppara-2* siRNA, which targets a separate region of the *ppara* mRNA sequence. Injection of polyconjugates prepared without the hepatocyte-targeting ligand NAG resulted in no *ppara* knockdown (SI Fig. 9). This is consistent with the results obtained from siRNA tracking studies shown in Fig. 3, which revealed that the presence of NAG on the polyconjugate was necessary for hepatocyte uptake.

The potential toxicity of the siRNA polyconjugate was assessed by measuring serum levels of liver enzymes and cytokines (SI Table 1). Slight elevations of ALT and AST levels were detected in mice receiving control siRNA or *apoB-1* siRNA polyconjugates as compared with saline-treated mice, 48 h after injection. However, the increased levels were not significant ($P > 0.05$), and histological examination of liver sections did not reveal signs of liver toxicity

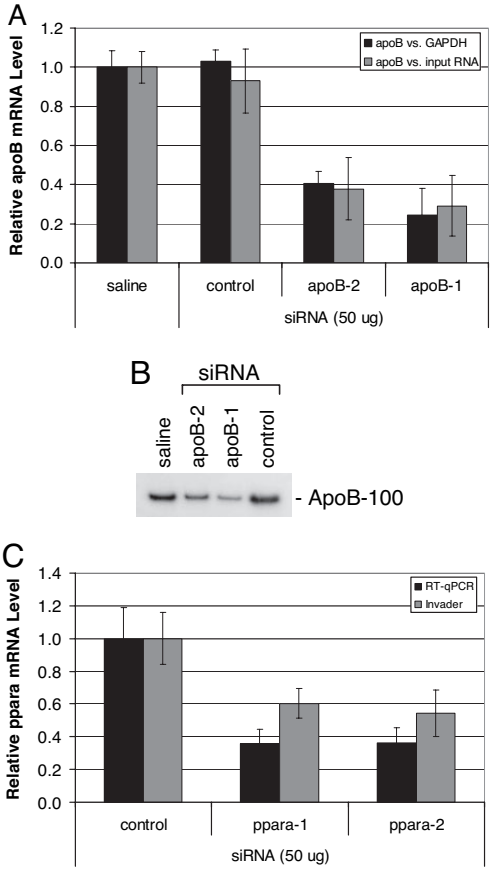

Fig. 4. Knockdown of target gene expression in livers of mice after i.v. injection of siRNA polyconjugates. (*A*) Reduction of *apoB* mRNA levels in liver after treatment with *apoB* siRNA polyconjugates. RT-qPCR analyses of liver *apoB* levels relative to GAPDH mRNA or total input RNA were performed 2 days after injection of *apoB-1*, *apoB-2*, or control siRNA polyconjugate (800 μg of polymer, 50 μg of siRNA). Shown are the data normalized to mice receiving saline alone. $n = 5$, data are shown as mean $\pm$ SD. (*B*) Serum levels of *apoB-100* protein are reduced in *apoB* siRNA polyconjugate-treated mice 2 days after injection. An equal volume of serum from individual mice was pooled for each group ($n = 5$) and subjected to Western blot analysis. (*C*) Reduction of *ppara* mRNA levels in liver after i.v. injection of *ppara* siRNA polyconjugates. RT-qPCR and Invader analyses of liver *ppara* mRNA levels relative to GAPDH or ubiquitin mRNA, respectively, 2 days after injection of *ppara-1*, *ppara-2*, or control siRNA polyconjugate (800 μg of polymer, 50 μg of siRNA). Shown are the data normalized to mice receiving saline alone. $n = 5$, data are shown as mean $\pm$ SD.

(data not shown). Similarly, analysis of TNF-α and IL-6 levels in serum by using ELISA revealed that both were slightly elevated 6 h after injection of polyconjugate but returned to baseline by 48 h. The increases observed at 6 h would not be expected to cause significant immune stimulation and are at least four orders of magnitude lower than those observed upon stimulation with lipopolysaccharide (43, 44) and one to three orders of magnitude lower than after injection of adenovirus (27, 45). No significant differences in serum levels of INF-α were detected at any of the time points, except for a slight increase at 6 h after injection of *apoB-1* siRNA polyconjugate. These results indicate that the targeted siRNA polyconjugate is well tolerated.

Dose–Response and Phenotypic Analyses of Mice Receiving *apoB* siRNA Polyconjugate. We investigated dose–response using two different experimental strategies: by decreasing the amount of siRNA polyconjugate delivered to the mice by serial dilutions of the formulation and by holding the amount of polymer constant but decreasing the amount of siRNA conjugated to it. Injection of simple serial dilutions of the *apoB-1* siRNA polyconjugate into mice led to a progressive decrease in the amount of knockdown of liver *apoB* mRNA (Fig. 5*A*). At the highest injected dose (800 μg of polymer, 50 μg of siRNA, i.e., 2.5 mg/kg), *apoB* mRNA levels in the liver were reduced 84 $\pm$ 5% relative to GAPDH mRNA on day 2 after injection compared with mice injected with saline only. Similar results were obtained when *apoB* mRNA levels were measured relative to total RNA. Injection of 2-fold less siRNA polyconjugate (400 μg of polymer, 25 of μg siRNA) resulted in a 50 $\pm$ 8% reduction in relative *apoB* mRNA levels. Injection of 4-fold less resulted in no *apoB* knockdown as compared with the saline control group. Holding the amount of polymer constant but decreasing the amount of *apoB-1* siRNA conjugated to it led to quantitatively similar results at each siRNA dose (Fig. 5*B*). This finding suggests that the amount of endosomolytic polymer present in the delivery vehicle is not the limiting factor for the knockdown observed, but rather it is the amount, or potency, of the siRNA conjugated to it.

A hallmark of *apoB* deficiency is decreased serum cholesterol levels due to impairment of VLDL assembly and cholesterol transport from the liver (46). To determine whether the level of *apoB* knockdown shown in Fig. 5*B* was sufficient to elicit a physiological response in these mice, we measured their total serum cholesterol levels. At the highest delivered siRNA dose (800 μg of polymer, 50 μg), we observed a significant decrease in mean serum cholesterol levels (30 $\pm$ 7%, $n = 5$, $P < 0.001$) relative to mice receiving a control siRNA or saline only (Fig. 5*C*). Similar results were obtained in animals treated with *apoB-2* siRNA polyconjugate (data not shown). Decreasing the amount of siRNA attached to the polyconjugate led to a progressive decrease in the amount of cholesterol lowering observed, consistent with decreased *apoB* mRNA knockdown measured in these animals (Fig. 5*B*).

Impairment of VLDL assembly in the liver and the resultant decrease in VLDL export might also be expected to alter hepatic triglyceride levels because triglycerides are also incorporated into VLDL particles (47). Indeed, transgenic mice expressing a truncated form of *apoB* found in patients with familial hypobetalipoproteinemia, also display a reduced capacity to transport hepatic triglycerides (48). To assess the effects of *apoB* knockdown on triglyceride transport, we performed oil red staining of liver sections obtained from mice injected with *apoB-1* siRNA polyconjugate. Inspection of the liver sections revealed dramatically increased hepatic lipid content compared with control mice (Fig. 5*D*). Decreased serum triglyceride levels were also detected in these mice, providing further evidence for diminished hepatic triglyceride export capacity (data not shown). Together, these results indicate that simple i.v. injection of *apoB-1* siRNA polyconjugate results in a knockdown of expression of *apoB* in the liver with expected phenotypic effects.

Longevity and Phenotypic Effect of *apoB* Knockdown. We performed a time course experiment to determine the duration of *apoB* knockdown and cholesterol lowering in mice after injection of a single dose of *apoB-1* siRNA polyconjugate. Consistent with our results described in the previous sections, injection of *apoB-1* siRNA polyconjugate (800 μg of polymer, 50 μg of siRNA) resulted in a reduction of mean *apoB* mRNA levels by 87 $\pm$ 8% on day 2 relative to control mice (Fig. 6*A*). The reduction in *apoB* expression was accompanied by a 42 $\pm$ 5% reduction in total serum cholesterol levels (Fig. 6*B*). Decreases in *apoB* mRNA expression remained significant through day 10 and had returned to near control levels by day 15. Reduction in serum cholesterol remained significant through day 4 ($n = 5, P < 0.01$) and did not fully recover to control levels until day 10. These results indicate that sustained *apoB* knockdown and lowered serum cholesterol levels can be attained after a single i.v. injection of siRNA polyconjugate, and that the phenotype can be reversed as *apoB* expression returns to normal.

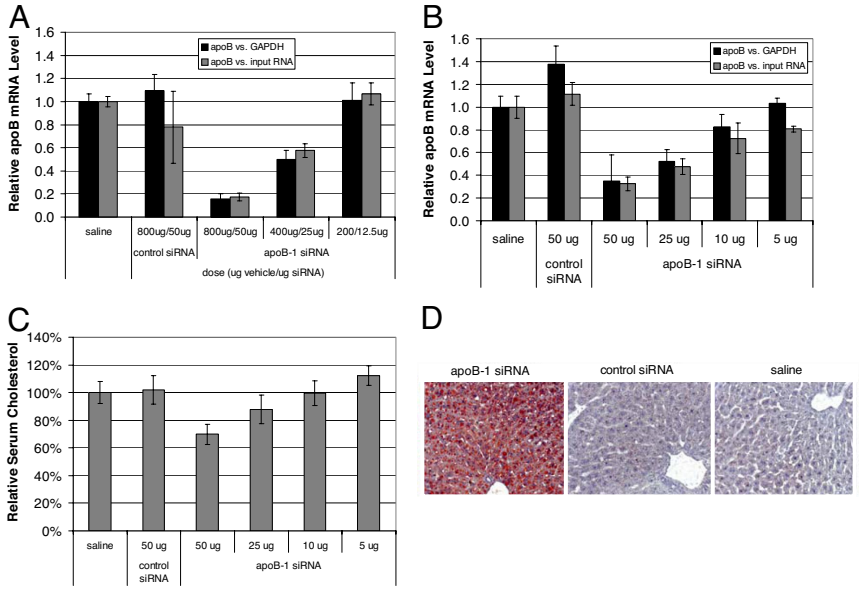

Fig. 5. *apoB-1* siRNA polyconjugate dose–response and phenotypes of treated mice. (*A*) Knockdown of *apoB* mRNA after injection of serial dilutions of the *apoB-1* siRNA polyconjugate. The indicated dose of *apoB-1* or control siRNA polyconjugate was injected intravenously in mice. The livers were harvested 2 days later, and the relative levels of *apoB* mRNA to those of GAPDH mRNA or total input RNA were measured by RT-qPCR. Data are normalized to mice receiving saline alone. $n = 5$, data are shown as mean ± SD. (*B*) Reducing the amount of *apoB-1* siRNA attached to the polyconjugate decreases *apoB* knockdown. Mice were injected with polyconjugate (800 μg of polymer) covalently attached to the indicated amount of *apoB-1* or control siRNA. The livers were harvested and relative *apoB* mRNA levels were determined as in *A*. $n = 5$, data are shown as mean ± SD. (*C*) Serum cholesterol is reduced in a siRNA dose-dependent manner in mice treated with *apoB-1* siRNA polyconjugate. Serum from mice in *B* was collected after a brief fast (4 h) and analyzed for total cholesterol. Values were normalized to mice receiving saline only. $n = 5$, data are shown as mean ± SD. (*D*) Knockdown of *apoB* results in increased hepatic lipid content. Liver sections were taken from briefly fasted mice 2 days after injection of *apoB-1* or control siRNA polyconjugate (800 μg of polymer, 50 μg of siRNA), or saline only. Sections were fixed, and lipids were detected by staining with oil red.

Discussion

In this study, we demonstrated the ability to use Dynamic PolyConjugate technology to deliver siRNA to hepatocytes in cells in culture and in mice. In mice, siRNA polyconjugates were used to elicit knockdown of two different genes, *apoB* and *ppara*. Maximal knockdown of 80–90% was achieved *in vivo* with a 2.5 mg/kg dose of *apoB* siRNA, a result that is similar to what has been achieved by using the SNALP delivery system (21, 22). Time-course studies also indicate a similar knockdown longevity, with an apparent half-life of between 7 and 10 days. On the basis of serum liver enzyme analyses, cytokine assays, and liver histology, liver toxicity was not observed. The ability of the siRNA polyconjugate to target hepatocytes and avoid Kupffer cell uptake is a likely explanation for the lack of toxicity.

Delivery of *apoB* siRNA caused a phenotypic effect that was manifested by lowered serum cholesterol and ApoB protein levels, and by a fatty liver. A single dose of siRNA polyconjugate resulted in decreased cholesterol levels for 1 week to 10 days. Once the serum cholesterol levels had recovered, we were able to re-administer the *apoB* siRNA and reduce the serum cholesterol levels a second time (data not shown). The presence of fatty liver was not commented upon in prior reports in which *apoB* siRNA was delivered *in vivo*, but a study in which antisense *apoB* oligonucleotides were used indicated that *apoB* knockdown did not cause fatty liver (10, 21, 22, 49). However, fatty livers are observed in transgenic mice possessing a mutant *apoB* gene associated with familial hypobetalipoproteinemia in humans (48). The possibility that *apoB* knockdown causes fatty liver should be taken into account if *apoB* is to be considered a therapeutic target in the treatment of hypercholesterolemia in humans. On the other hand, knockdown of *apoB* may provide a useful mouse model to study the acute effects of fatty liver, an important human clinical problem (50).

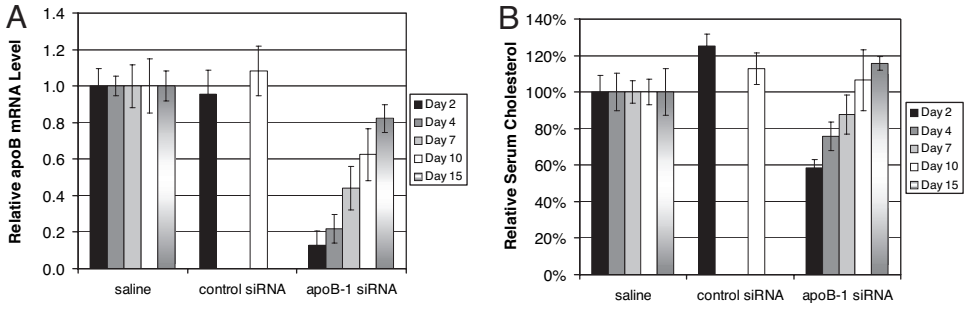

Fig. 6. Longevity of *apoB* knockdown and the phenotypic response in mice. (*A*) Duration of *apoB* mRNA knockdown in liver. RT-qPCR analyses of liver *apoB* levels relative to GAPDH mRNA or total input RNA were performed at the indicated time after injection of *apoB-1* or control siRNA polyconjugate (800 μg of polymer, 50 μg of siRNA). Shown are the data normalized to mice receiving saline alone. $n = 5$, data are shown as mean ± SD. (*B*) Duration of serum cholesterol reduction. Serum from mice in *A* was collected after a 4-h fast and analyzed for total cholesterol. Values were normalized to mice receiving saline only. Mice treated with control siRNA polyconjugate were only assayed on days 2 and 10 after injection. $n = 5$, data are shown as mean ± SD.

The ability to generate a knockdown phenotype after delivery of siRNA polyconjugates was not limited to treatment with *apoB* siRNA. Mice treated with siRNA polyconjugates targeting *ppara* also displayed the gene-appropriate phenotype, characterized by a significant increase in serum triglycerides after delivery of *ppara* siRNA polyconjugate, a phenotype that is consistent with known *ppara* function (data not shown) (41). However, this phenotype was less reproducible than the *apoB* knockdown phenotype. It is possible that the level of knockdown achieved against this target was not always sufficient to yield consistent phenotypic effects. The use of more potent siRNAs may help in this regard.

A key feature of Dynamic PolyConjugate technology is the use of targeting ligands to direct the polyconjugate to a specific cell type. We present evidence in this report that hepatocyte-specific targeting is achieved by using polyconjugates containing the NAG ligand and that substituting mannose for NAG results in redirection of the polyconjugate to nonparenchymal liver cells and away from hepatocytes. The targetability of the Dynamic PolyConjugates makes it unique among other recently developed nanotechnologies such as SNALP and iNOP for delivery of siRNA to hepatocytes in liver. Rather than using ligand-mediated targeting, these latter technologies instead rely on the inherent ability of the liver to clear foreign particles of a certain size. One drawback of this approach is that it can result in uptake by cell types in liver other than hepatocytes. Indeed, an intense signal from fluorescently labeled siRNA is observed in Kupffer cells when delivered by using SNALPs (51), a situation that would have the potential to induce Kupffer-cell-mediated liver toxicity. In addition, the size of the SNALP or iNOP siRNA complexes is much greater than the siRNA polyconjugate (21, 34), further increasing the risk of inducing Kupffer cell activation (52). The larger size of the SNALP and iNOP siRNA complexes would also restrict their use to vascular endothelial cells or tissues such as liver with blood vessel fenestrations of >50 nm. The small size of the particles prepared by using Dynamic PolyConjugate technology should allow more flexibility in targeting other cell types.

In summary, Dynamic PolyConjugate technology seamlessly incorporates several design features including an endosomolytic polymeric carrier that is shielded by reversible covalent modifications, reversibly attached cellular receptor ligands, and labile conjugation of siRNA. This is a modular platform system in which these different elements could be adapted to enable siRNA delivery for a wide variety of purposes. Selective targeting is an important characteristic of the Dynamic PolyConjugate technology, and we anticipate that other ligands could be easily incorporated into this system to enable siRNA-mediated knockdown of genes in other tissues and cell types.

Methods: Polyconjugate Synthesis and Formulation

SATA-modified siRNAs were synthesized by reaction of 5′ amine-modified siRNA with 1 weight equivalents (wt eq) of *N*-succinimidyl-*S*-acetylthioacetate (SATA) reagent (Pierce) and 0.36 wt eq of $NaHCO_3$ in water at 4°C for 16 h. The modified siRNAs were then precipitated by the addition of 9 vol of ethanol and incubation at −80°C for 2 h. The precipitate was resuspended in 1× siRNA buffer (Dharmacon) and quantified by measuring absorbance at the 260-nm wavelength.

PBAVE was synthesized according to published procedures (33). PBAVE (30 mg/ml in 5 mM TAPS, pH 9) was modified by addition of 1.5 wt % SMPT (Pierce). After a 1-h incubation, 0.8 mg of SMPT-PBAVE was added to 400 μl of isotonic glucose solution containing 5 mM TAPS (pH 9). To this solution was added 50 μg of SATA-modified siRNA. For the dose–response experiments where [PBAVE] was constant, different amounts of siRNA were added. The mixture was then incubated for 16 h. To the solution was then added 5.6 mg of Hepes free base followed by a mixture of 3.7 mg of CDM-NAG and 1.9 mg of CDM-PEG. The solution was then incubated for at least 1 h at room temperature before injection.

CDM-PEG and CDM-NAG were synthesized from the acid chloride generated by using oxalyl chloride. To the acid chloride was added 1.1 molar equivalents polyethylene glycol monomethyl ether (molecular weight average of 450) to generate CDM-PEG or (aminoethoxy)ethoxy-2-(acetylamino)-2-deoxy-β-D-glucopyranoside to generate CDM-NAG. The final product was purified by using reverse-phase HPLC with a 0.1% TFA water/acetonitrile gradient.

Additional methods are available in *SI Materials and Methods*.

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