nature biotechnology

Mechanisms and optimization of *in vivo* delivery of lipophilic siRNAs

Christian Wolfrum[1–3], Shuanping Shi[3], K Narayanannair Jayaprakash[4], Muthusamy Jayaraman[4], Gang Wang[4], Rajendra K Pandey[4], Kallanthottathil G Rajeev[4], Tomoko Nakayama[4], Klaus Charrise[4], Esther M Ndungo[3], Tracy Zimmermann[4], Victor Koteliansky[4], Muthiah Manoharan[4] & Markus Stoffel[1–3]

Cholesterol-conjugated siRNAs can silence gene expression *in vivo*. Here we synthesize a variety of lipophilic siRNAs and use them to elucidate the requirements for siRNA delivery *in vivo*. We show that conjugation to bile acids and long-chain fatty acids, in addition to cholesterol, mediates siRNA uptake into cells and gene silencing *in vivo*. Efficient and selective uptake of these siRNA conjugates depends on interactions with lipoprotein particles, lipoprotein receptors and transmembrane proteins. High-density lipoprotein (HDL) directs siRNA delivery into liver, gut, kidney and steroidogenic organs, whereas low-density lipoprotein (LDL) targets siRNA primarily to the liver. LDL-receptor expression is essential for siRNA delivery by LDL particles, and SR-BI receptor expression is required for uptake of HDL-bound siRNAs. Cellular uptake also requires the mammalian homolog of the *Caenorhabditis elegans* transmembrane protein Sid1. Our results demonstrate that conjugation to lipophilic molecules enables effective siRNA uptake through a common mechanism that can be exploited to optimize therapeutic siRNA delivery.

RNA interference (RNAi) is an evolutionarily conserved biological process for specific silencing of gene expression. Synthetic small-interfering (si)RNAs have emerged as an important tool for post-transcriptional gene silencing in mammalian cells and live animals owing to their unique properties, such as potency, specificity and lack of an interferon response[1]. Delivery remains the largest obstacle for *in vivo* applications of siRNAs, including their use as therapeutics following systemic administration. Delivery of siRNAs across plasma membranes *in vivo* has been achieved using vector-based delivery systems[2], high-pressure intravenous injections of siRNA[3] and chemically modified siRNAs, including cholesterol-conjugated[4], lipid-encapsulated[5] and antibody-linked siRNAs[6]. Although these delivery approaches have been shown to be effective in mice, no functional studies elucidating the mechanism of siRNA uptake have yet been reported.

Covalent conjugation of cholesterol to siRNAs facilitates cellular import and elicits RNAi, which results in silencing of endogenous genes *in vivo*[4]. Cholesterol in circulation is transported via lipoprotein particles, which contain amphiphilic phospholipids, thus maintaining the solubility of cholesterol in circulation[7]. Both high-density lipoprotein (HDL) and low-density lipoprotein (LDL) play a critical role in cholesterol transport. HDL is responsible for the reverse cholesterol transport from peripheral tissues to other lipoproteins and tissues[8–10]. Endocytosis-dependent and endocytosis-independent selective lipid-uptake mechanisms are responsible for the delivery of HDL cholesterol to cells. The most prominent HDL receptor, scavenger receptor class B, type I (SR-BI), mediates the selective uptake of cholesterol esters and other lipids from HDL particles by cells and facilitates the transfer of cholesterol from cells to HDL or to other acceptors present in the extracellular environment[8,9]. In contrast, LDL functions as an extracellular carrier for cholesterol to different tissues. LDL cholesterol is taken up by hepatocytes through LDL receptor–mediated endocytosis in which LDL particles are internalized after binding to the LDL receptor by interaction with apolipoprotein B100 (apoB-100). After endocytosis, lysosomal enzymes release free cholesterol into the cytosol and recycle the LDL receptor to the cell surface[7].

In a recent study, cholesterol-conjugated siRNA targeting apolipoprotein B (apoB) was injected in mice to modulate apoB expression[4]. Systemic administration of cholesterol-siRNA was shown to suppress apoB mRNA levels and decrease the levels of plasma apoB and serum cholesterol. In this study we analyzed the delivery mechanism of siRNAs conjugated to cholesterol and to other hydrophobic lipid molecules. We show that cholesterol-siRNA preassembled with HDL is 8 to 15 times more effective at silencing apoB protein expression *in vivo* compared with equal amounts of cholesterol-siRNA.

RESULTS

In vivo delivery of siRNAs linked to lipophilic conjugates

We synthesized and evaluated lipophilic conjugates, including several fatty acids and bile acids, to determine whether different lipophilic modifications facilitate uptake of chemically modified siRNA and silencing of gene expression *in vivo*. Chemically modified siRNAs with a partial phosphorothioate backbone and 2′-*O*-methyl sugar modifications on the sense and antisense strands targeting the gene encoding

[1]Institute of Molecular Systems Biology, Swiss Federal Institute of Technology, ETH Zürich, HPT E73, [2]Competence Center of Systems Biology and Metabolic Diseases, ETH Zürich, CH-8093 Zürich, Switzerland. [3]The Rockefeller University, 1230 York Avenue, New York, New York 10021, USA. [4]Alnylam Pharmaceuticals Inc., 300 3rd Street, Cambridge, Massachusetts 02142, USA. Correspondence should be addressed to M.S. (stoffel@imsb.biol.ethz.ch).

Received 10 July; accepted 27 August; published online 16 September 2007; doi:10.1038/nbt1339

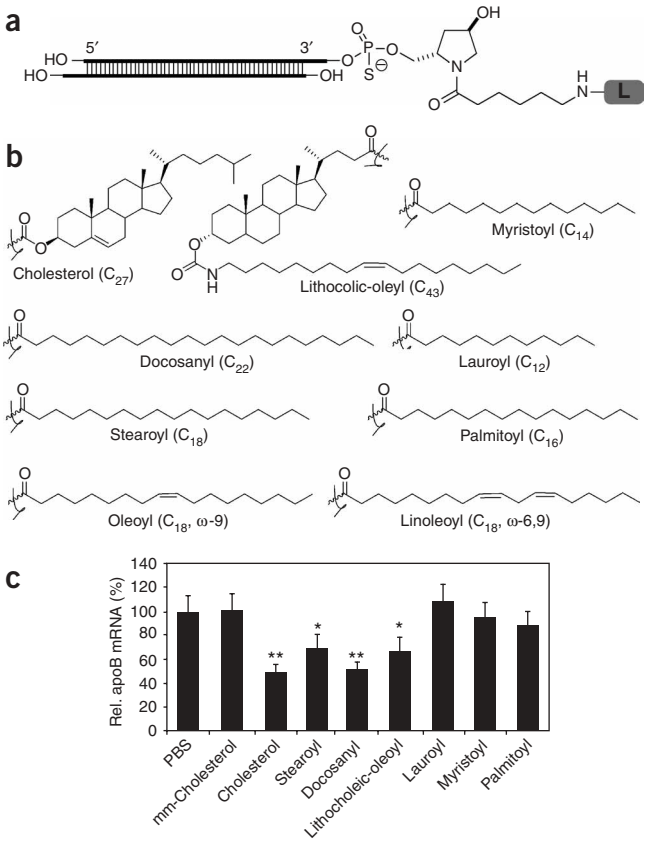

Figure 1 Lipophilic siRNA conjugates have different *in vivo* activities. (**a**) Structure of lipid conjugated siRNAs. The desired lipophile (L) is conjugated to the 3′-end of the sense strand of the apoB siRNA[4] (siRNA-apoB1) via a *trans*-4-hydroxyprolinol linker. (**b**) Structure of lipophile-siRNA conjugates. The desired lipophile (L) is conjugated to the 3′-end of sense strand of the siRNA-apoB1[4] via a *trans*-4-hydroxyprolinol linker (Hyp): sense 5′-GUCAUCACACUGAAUACCAAU*Hyp-L -3′ and antisense 5′-AUUGGUAU UCA GUGUGAUGAc*a*C -3′. Lower case letters represent 2′-*O*-methyl-sugar-modified nucleotides and asterisks stand for phosphorothioate backbone. (**c**) *In vivo* silencing of apoB mRNA by lipid-conjugated siRNAs. Liver apoB mRNA levels were normalized to GAPDH mRNA 24 h after three daily intravenous injections of saline or 50 mg/kg stearoyl-siRNA-apoB1, dodecyl-siRNA-apoB1, lithocholic-oleyl-siRNA-apoB1- or docosanyl-siRNA-apoB1 ($n = 5$ per group). Data show apoB mRNA levels as a percentage of the saline treatment group and is expressed as the mean ± the s.d. Data marked with asterisks are statistically significant relative to the saline treatment group as calculated by ANOVA without replication, alpha value 0.05 (*, $P < 0.05$; **, $P < 0.005$).

apoB were used. The chemical modifications enhance resistance toward degradation by exo- and endonucleases[4] (**Fig. 1a,b**). Newly synthesized lipophilic conjugates were injected intravenously, and apoB mRNA levels were measured by quantitative PCR (qPCR). The shorter chain fatty-acid conjugates, such as lauroyl (C_{12}), myristoyl (C_{14}) and palmitoyl (C_{16}) did not reduce apoB transcript levels in mouse livers. In contrast, fatty-acid conjugates with a longer, saturated, alkyl chain, such as stearoyl (C_{18}) and docosanyl (C_{22}), significantly lowered apoB mRNA levels (**Fig. 1c**). Similarly, a hybrid of lithocholic acid and oleylamine (lithocholic-oleyl, C_{43}) silenced apoB expression *in vivo* (**Fig. 1c**). These data demonstrate that diverse lipophilic conjugates can mediate cellular siRNA uptake and elicit endogenous gene silencing *in vivo*.

Lipophilic-siRNA conjugates bind to LDL and HDL particles

To investigate the mechanism of lipophile-conjugated siRNA uptake, we evaluated the binding of radiolabeled (^{32}P), chemically modified and cholesterol-conjugated siRNAs, targeting apoM and apoB mRNAs, to serum components. These compounds are stable in human and mouse serum for >48 h[4] (**Supplementary Fig. 1** online). ^{32}P-cholesterol-siRNAs were incubated with mouse blood, and the distribution of labeled material was analyzed by fast-performance liquid chromatography (FPLC) after separation from blood cells. In addition to being present in the unbound fraction (free), the ^{32}P-cholesterol-siRNA was found associated with fractions that contained either HDL particles or plasma proteins of ~60 kDa. In contrast, unconjugated siRNA was detected only in unbound fractions (**Fig. 2a,b**). Because albumin is the major component of the 60-kDa plasma fractions and is known to bind to lipophilic molecules, we hypothesized that albumin bound cholesterol-siRNA. To test this hypothesis, we evaluated the binding of albumin to cholesterol-siRNA or unconjugated siRNA in an albumin-containing solution. FPLC analysis showed that cholesterol-siRNA, but not unconjugated siRNA, localized to the same ~60-kDa complex found in plasma, indicating that cholesterol-siRNA binds to serum albumin (**Fig. 2c**). These data suggest that cholesterol-siRNA associates with HDL, albumin and also exists in an unbound form in mouse plasma. Because mice exhibit very low plasma LDL levels, we tested whether cholesterol-siRNA can also associate with LDL particles from hamster and human plasma. In addition to being present in its free form when incubated with either hamster or human plasma, cholesterol-siRNA associated with fractions containing HDL, LDL and albumin (**Fig. 2d,e**). As with mouse plasma, unconjugated siRNA did not elute in fractions containing lipoproteins or albumin (**Fig. 2e**).

To understand the nature of binding of cholesterol-siRNA to lipoproteins, we performed binding studies of all lipophilic-siRNA conjugates with lipoproteins and albumin (**Fig. 2f**). siRNA conjugated to cholesterol, stearoyl, docosanyl and lithocholic-oleyl associated with HDL particles. In contrast, short- and medium-chain fatty acids, such as lauroyl, myristoyl and palmitoyl siRNA, did not bind to lipoproteins but associated with either serum albumin or remained in an unbound form (**Fig. 2f**). The binding trend of the lipophilic conjugates to HDL particles was shown to correlate with the activity of the respective compound *in vivo* (**Fig. 1c**). Taken together, these data suggest that the ability of lipophile-conjugated siRNAs to efficiently bind to lipoprotein particles facilitates their intracellular uptake *in vivo*.

The binding properties of lipid-conjugated siRNA to lipoproteins and albumin, including their stoichiometry and binding affinity, were characterized in more detail for several lipid-siRNA conjugates. Saturation experiments using a fixed amount of lipoprotein and titration with varying concentrations of ^{32}P-siRNA were performed to determine the *in vitro* binding constants (K_d) (**Supplementary Fig. 2a,b** online). Binding constants were calculated from the ratio of unbound versus bound cholesterol-siRNA in binding equilibrium. The binding constants for HDL binding of cholesterol-, docosanyl- and lithocholic-oleyl–siRNA conjugates were ~120 μM, whereas the stearoyl siRNA conjugate with moderate hydrophobicity exhibited a K_d of ~430 μM for HDL. The lauroyl-siRNA conjugate, which exhibited the lowest hydrophobicity, bound to HDL with the lowest affinity of all conjugates tested (K_d ~ 1.2 mM). The binding constants for LDL were in a similar range to those for HDL, with cholesterol-, docosanyl- and lithocholic-oleyl–siRNAs having the highest affinity (K_d ~ 100 μM), stearoyl-siRNA with an intermediate

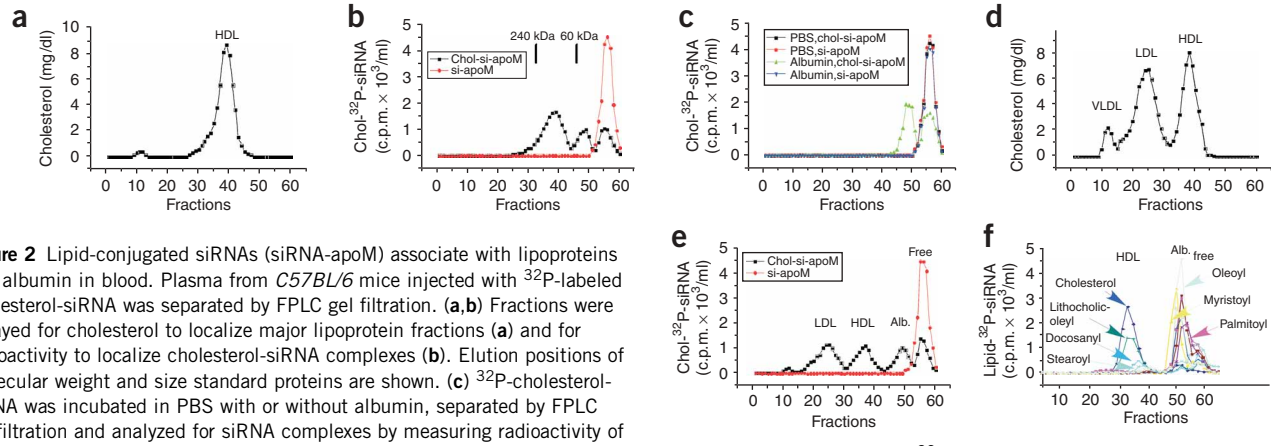

Figure 2 Lipid-conjugated siRNAs (siRNA-apoM) associate with lipoproteins and albumin in blood. Plasma from *C57BL/6* mice injected with ^{32}P-labeled cholesterol-siRNA was separated by FPLC gel filtration. (**a**,**b**) Fractions were assayed for cholesterol to localize major lipoprotein fractions (**a**) and for radioactivity to localize cholesterol-siRNA complexes (**b**). Elution positions of molecular weight and size standard proteins are shown. (**c**) ^{32}P-cholesterol-siRNA was incubated in PBS with or without albumin, separated by FPLC gel filtration and analyzed for siRNA complexes by measuring radioactivity of the different fractions. Chol, cholesterol; si, siRNA. (**d**,**e**) Plasma from Syrian hamsters that were injected with ^{32}P-cholesterol-siRNA was separated by FPLC gel filtration. Fractions were assayed for cholesterol to localize major lipoprotein fractions (**d**) and for radioactivity to localize siRNA complexes (**e**). (**f**) Gel permeation of different ^{32}P-labeled lipid-conjugated siRNAs. Cholesterol-, lithocholeic-oleyl-, docosanyl- and stearyl-conjugated siRNAs elute with HDL particles; myristoyl- (yellow), oleoyl- (light blue) and palmitoyl-linked siRNAs (pink) only elute with albumin or in "unbound" fractions.

affinity (K_d ~ 300 μM) and lauroyl-siRNA with the lowest affinity (K_d ~ 1.8 mM). HDL and LDL particles were saturated with cholesterol-, lithocholic-oleyl–, docosanyl- and stearoyl-siRNA conjugates at a stoichiometry of 0.94 and 1.26 mol/mol, respectively, whereas the lauryl-conjugated siRNA lipoprotein complex did not reach saturation under our experimental conditions.

A reverse trend in binding affinity for the lipophile-siRNA conjugate was observed with albumin. Lipophilic conjugates with lower hydrophobicity preferentially bound to albumin in a competition experiment (data not shown). Cholesterol and lithocholic-oleyl-siRNA conjugates showed the lowest binding to albumin, whereas docosanyl- and stearoyl-siRNA conjugates exhibited intermediate binding affinity to albumin and lipoproteins (**Supplementary Fig. 2c** online). Of all conjugates tested, stearoyl- and dodecyl-siRNA conjugates bound albumin with the highest affinity (K_d ~ 200 μM) (**Supplementary Fig. 2c**). Interestingly, albumin was saturated with

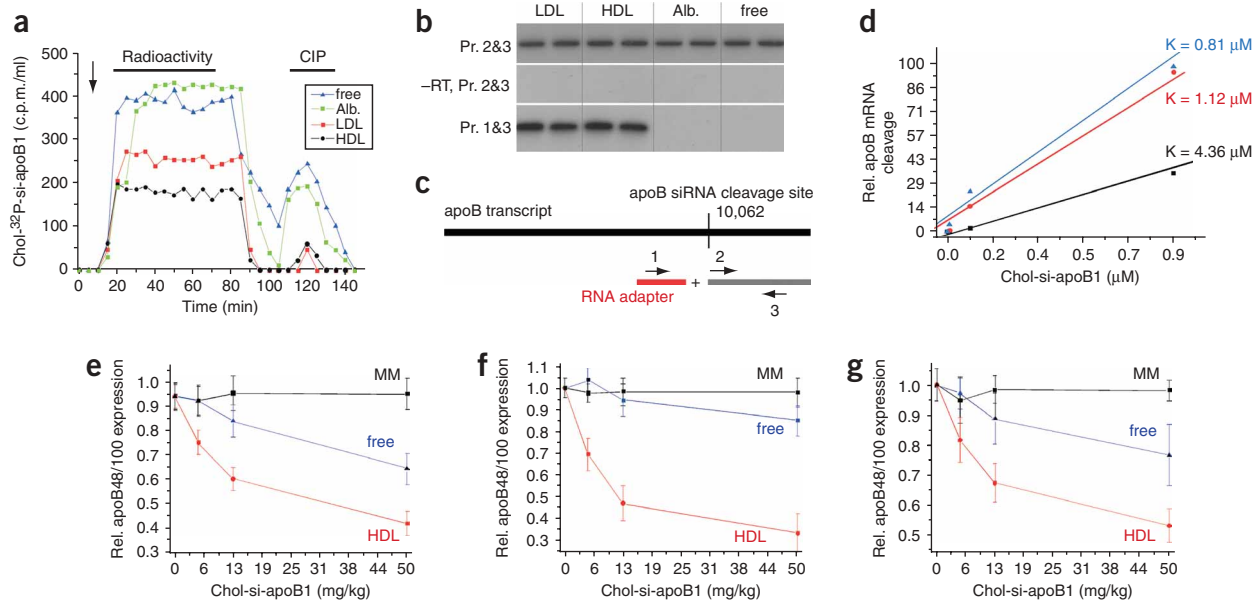

Figure 3 Cholesterol-siRNAs associated with lipoproteins are taken up by the liver more efficiently than in free or albumin-bound cholesterol-siRNAs. (**a**–**c**) ^{32}P-labeled cholesterol-siRNA-apoB1 was complexed with HDL, LDL or albumin. (**a**) Radioactivity was measured in the effluent perfusate to calculate the amount of cholesterol-siRNA taken up by the liver. The input (460 counts/ml) is shown as a bar. (**b**,**c**) The cleavage of hepatic apoB mRNA in livers perfused with precomplexed LDL, HDL, albumin or free cholesterol-siRNA-apoB1 was measured by 5′-RACE (**b**) by selective apoB-cDNA synthesis of RNA pools that were ligated to a 5′ RNA adaptor (**c**). The position of the siRNA-apoB1 cleavage site is shown. Pr., primer; -RT, without reverse transcriptase. (**d**) Comparison of functional activity of free cholesterol-siRNA-apoB1 (black) or preassembled HDL (blue) and LDL (red) cholesterol-siRNA-apoB1. Cleavage of apoB mRNA was measured 24 h after injection. The relative cleavage of apoB mRNA was plotted against the amount of injected label to calculate cleavage activity of different cholesterol-siRNA-apoB1 applications. (**e**–**g**) Mice were injected with different concentrations of free cholesterol-siRNA-apoB1 (blue), preassembled HDL/mismatch cholesterol-siRNA-apoB1 (MM) (black), or HDL/cholesterol-siRNA-apoB1 (red). After 48 h, apoB protein levels were measured in the jejunum (**e**), liver (**f**) and plasma (**g**). The relative apoB48/100 expression levels were plotted against the amount of injected cholesterol-siRNA-apoB1.

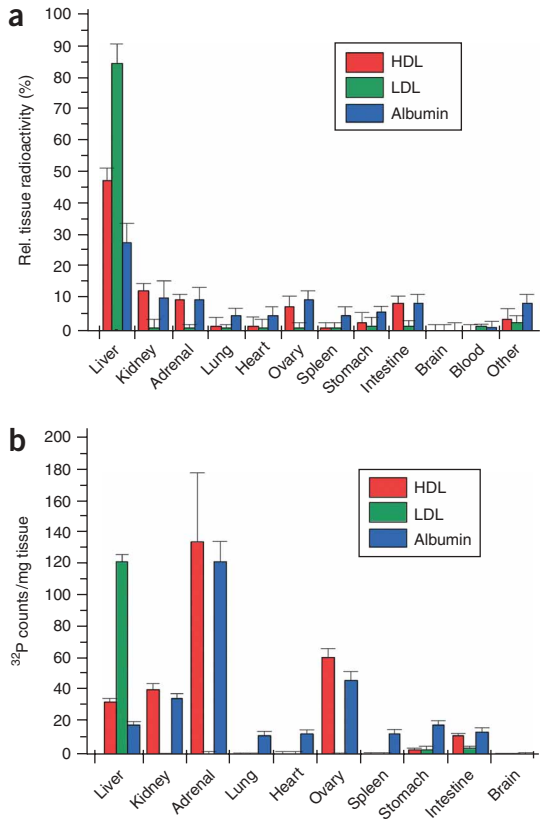

Figure 4 Cholesterol-siRNA is taken up by different tissues depending on its association with specific lipoproteins. *C57BL/6* mice were injected into the tail vein with ^{32}P-labeled cholesterol-siRNAs that were bound to HDL, LDL or albumin. (**a**,**b**) Total tissue uptake of ^{32}P-labeled cholesterol-siRNAs, relative to total tissue weight was measured by liquid scintillation counting.

stearoyl- and dodecyl-siRNA conjugates at a 1:3.6 molar ratio, whereas cholesterol-, lithocholic-oleyl- and docosanyl-conjugated siRNAs bound to albumin with a ~1.9 mol/mol stoichiometry (**Supplementary Fig. 2c**).

To determine whether the binding affinity of cholesterol-siRNA to HDL, LDL and albumin *in vitro* reflects their relative affinity *in vivo*, we determined the plasma distribution of ^{32}P-cholesterol-siRNA complexed with purified hamster LDL, HDL and albumin in hamsters at 1, 2, 3 and 4 h after systemic administration. FPLC analysis of hamster serum revealed that cholesterol-siRNA bound to LDL remained intact in circulation, whereas a modest redistribution of cholesterol-siRNA into LDL and very low density lipoprotein (VLDL) particles occurred when preassembled with HDL (**Supplementary Fig. 3a**,**b** online). Cholesterol-siRNA was bound even more loosely with albumin as evidenced by redistribution of ^{32}P-cholesterol-siRNA into HDL and LDL particles, consistent with the low affinity of cholesterol-siRNA for albumin (**Supplementary Fig. 3c** online).

Lipoproteins are required for cholesterol-siRNA uptake *in vivo*

Next, we investigated whether association of cholesterol-siRNA with lipoprotein particles facilitates *in vivo* uptake of cholesterol-siRNA by the liver. Hamster livers were perfused for 4 h with serum-free buffer containing LDL-, HDL-, albumin-bound or free ^{32}P-cholesterol-siRNAs. Uptake of cholesterol-siRNA was monitored by measuring the amount of ^{32}P-cholesterol-siRNA in the afferent (input) versus the efferent (output) over 80 min. The difference in radioactivity between the afferent and efferent perfusate reflects the uptake of ^{32}P-cholesterol-siRNA by the liver. Uptake of free and albumin-bound ^{32}P-cholesterol-siRNA was low (~8% ± 4 and 14% ± 4, respectively), whereas LDL- and HDL-bound ^{32}P-cholesterol-siRNA was removed more efficiently from the perfusate (~42% ± 6 and 58% ± 3, respectively) (**Fig. 3a**). Because removal of ^{32}P-labeled cholesterol-siRNA from the perfusate could be due to either cellular uptake or extracellular binding, the liver was perfused with calf intestinal alkaline phosphatase (CIP) to remove any ^{32}P exposed to the extracellular space. Perfusion with CIP led to a significant release of ^{32}P from livers perfused with free and albumin-bound ^{32}P-cholesterol-siRNA. However, only small quantities of ^{32}P were released from livers perfused with lipoprotein-bound cholesterol-siRNA (**Fig. 3a**). Liver uptake of cholesterol-siRNA (input radioactivity minus efferent radioactivity after CIP perfusion) was highest in mice treated with LDL- and HDL-associated cholesterol-siRNAs (38% ± 5 and 47% ± 6 of total perfused radioactivity, respectively). Conversely, only small amounts of cholesterol-siRNAs were taken up by the liver when administered as free- or albumin-bound forms (5% ± 3 and 8% ± 4 of total perfused radioactivity, respectively).

Analysis of cleavage products from apoB mRNA by rapid amplification of cDNA ends (5′RACE) was performed to determine whether the observed uptake after perfusion resulted in functional RNAi-mediated gene silencing. Total RNA was isolated from livers of mice treated with LDL-, HDL-, albumin-bound or free cholesterol-siRNA-apoB1 for our analysis. Cleavage fragments were detected in livers perfused with either LDL- or HDL-bound cholesterol-siRNA-apoB (**Fig. 3b**,**c**), but not in livers perfused with albumin-bound or free cholesterol-siRNA-apoB1 (**Fig. 3a**). The identity of the predicted cleavage site was confirmed by direct sequencing of 5′ RACE fragments, demonstrating RNAi-mediated cleavage upon uptake of cholesterol-siRNA-apoB1 by the liver.

The above data suggested that siRNA binding to lipoproteins is critical for delivery to the liver and that the uptake of cholesterol-siRNA-apoB1 preassembled with lipoprotein may be more efficient than systemic delivery of free cholesterol-siRNA-apoB1 alone. Therefore, we assessed the activity of cholesterol-siRNA-apoB1 versus HDL-bound cholesterol-siRNA-apoB1 by quantitative cleavage assays of liver apoB transcripts. No cleavage products were detected in mice that received PBS or HDL particles alone (data not shown). Injection of varying doses of cholesterol-siRNA-apoB1 allowed us to determine the equilibrium-relative cleavage constant for apoB mRNA (K_{rel}) expression, which was calculated by dividing the amount of circulating cholesterol-siRNA by the relative amount of cleavage observed for all time points used. This constant provides a description of the cleavage potential for each cholesterol-siRNA conjugate used. The K_{rel} for cleavage of apoB mRNA in response to injection of free cholesterol-siRNA-apoB1 was 4.83 μM, whereas injection of HDL-bound cholesterol-siRNA-apoB1 from saturated HDL fractions and nonsaturated HDL fractions resulted in a K_{rel} of 1.13 and 0.86 μM, respectively (**Fig. 3d**). Thus, RNAi-mediated cleavage is increased by a factor of ~5 in mice that received HDL-bound cholesterol-siRNA-apoB1 compared to mice receiving injections of the same amounts of free cholesterol-siRNA-apoB1. To test the effect of lipoprotein-bound or free cholesterol-siRNA on apoB protein levels directly, we injected mice with varying doses of cholesterol-siRNA-apoB1 or with equal doses of cholesterol-siRNA-apoB1 or control siRNAs (mismatch-cholesterol-siRNA-apoB1) bound at saturating or

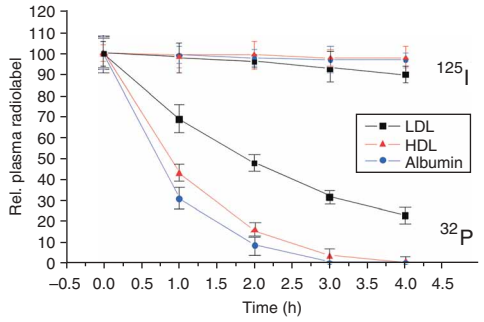

Figure 5 Cholesterol-siRNA associated with lipoproteins is taken up independently of lipoprotein particle endocytosis. *C57BL/6* mice were injected through the tail vein with ^{32}P-labeled cholesterol-siRNAs complexed with ^{125}I-tyramine cellobiose-labeled HDL, LDL or albumin. Blood was taken at indicated time points and cholesterol-siRNA as well as lipoprotein clearance was measured by dual label scintillation counting.

nonsaturating concentrations to purified mouse HDL particles. Silencing of apoB expression in the liver and jejunum was ~8- to 15-fold more effective in mice treated with HDL-bound cholesterol-siRNA compared to mice that were injected with cholesterol-siRNA only (**Fig. 3e**,**f**). The reduction of plasma apoB levels was proportional to the decrease in apoB tissue expression (**Fig. 3g**).

Differential delivery of lipoprotein-bound siRNAs

Lipoprotein particles deliver lipids differentially to specific cells by apolipoprotein or receptor-mediated processes. We investigated the distribution of HDL- and LDL-bound ^{32}P-cholesterol-siRNA uptake in tissues after intravenous application. Purified HDL and LDL particles were saturated with cholesterol-siRNA-apoB1 and injected into Syrian hamsters. Animals were killed 4 h after injection and thoroughly perfused with saline to avoid any radioactive signal from circulating in blood. Radioactivity in several tissues, including liver, kidney, adrenal, lung, heart, testis, ovary, spleen, stomach, intestine and brain were measured 4 h after injection. The majority of radioactivity for the HDL- and LDL-bound ^{32}P-cholesterol-siRNAs was found in the liver (**Fig. 4a**); however, low levels were also detected in other organs. Normalization of ^{32}P counts/mg tissue revealed that LDL particles mediated uptake of ^{32}P-cholesterol-siRNAs mainly in the liver, whereas HDL-bound ^{32}P-cholesterol-siRNAs were mainly found in the adrenals, ovary, kidney, liver and small intestine (**Fig. 4b**). To assess whether the protected cholesterol-siRNA is degraded during injection, we performed northern blots of RNA extracted from tissue, which showed that the radioactive phosphate was still associated with the cholesterol-siRNA (data not shown).

Uptake of cholesterol-siRNAs is mediated by lipoprotein receptors

Our data support the hypothesis that LDL and HDL particles mediate differential uptake of cholesterol-siRNA in tissues. One mechanism by which LDL and HDL particles could mediate cholesterol-siRNA uptake is by receptor-mediated internalization. The liver takes up the majority of LDL by LDL receptor–mediated endocytosis[11], whereas HDL particles can be either internalized by selective transcytosis or depleted of cholesterol by selective uptake through SR-BI[9,12]. If cholesterol-siRNA uptake occurs as a result of being internalized as a complex with LDL or HDL, its clearance from the circulation should follow the kinetics of LDL or HDL particles, respectively. To analyze the clearance of both lipoprotein particles and siRNA, we labeled LDL and HDL fractions with ^{125}I-tyramine cellobiose (TC) and loaded them with ^{32}P-cholesterol-siRNA[13]. Double-labeled particles were injected into hamsters and clearance of ^{32}P-cholesterol-siRNA relative

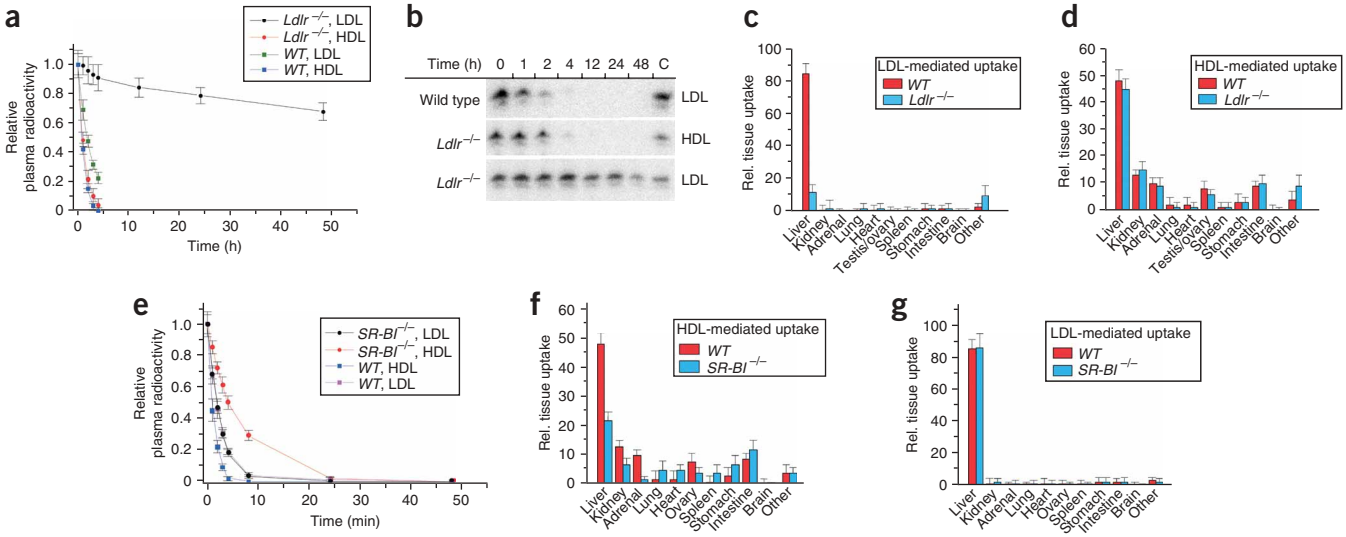

Figure 6 LDL receptor (Ldlr) mediates LDL-associated cholesterol-siRNA delivery *in vivo*. (**a**–**b**) *Ldlr*$^{-/-}$ mice were injected through the tail vein with ^{32}P-labeled cholesterol-siRNAs that were preassembled with HDL or LDL, respectively. Blood was taken at indicated time points and relative radioactivity was measured. (**b**) Autoradiograph of PAGE northern blot of 20 μl plasma harvested from wild-type or *Ldlr*$^{-/-}$ mice at the indicated time points that were injected trough the tail vein with preassembled LDL or HDL/^{32}P-cholesterol-siRNA-apoB1. (**c**,**d**) Relative tissue uptake of ^{32}P-cholesterol-siRNAs complexed to LDL (**c**) or HDL particles (**d**) in *Ldlr*$^{-/-}$ (blue) and wild-type littermates (*WT*) (red) was measured in indicated organs 4 h after injection by liquid scintillation counting. (**e**–**g**) Scavenger receptor SR-BI mediates HDL-associated cholesterol-siRNA tissue uptake *in vivo*. (**e**) Wild-type and *SR-BI*$^{-/-}$ mice were injected through the tail vein with ^{32}P-cholesterol-siRNAs complexed to HDL or LDL, respectively. Blood was taken at indicated time points and plasma cholesterol-siRNA clearance was measured. The relative tissue uptake of HDL (**f**) and LDL-associated cholesterol-siRNA-apoB1 (**g**) in *WT* (red) and *SR-BI*$^{-/-}$ mice (blue) was measured 4 h after injection in the indicated organs by liquid scintillation counting.

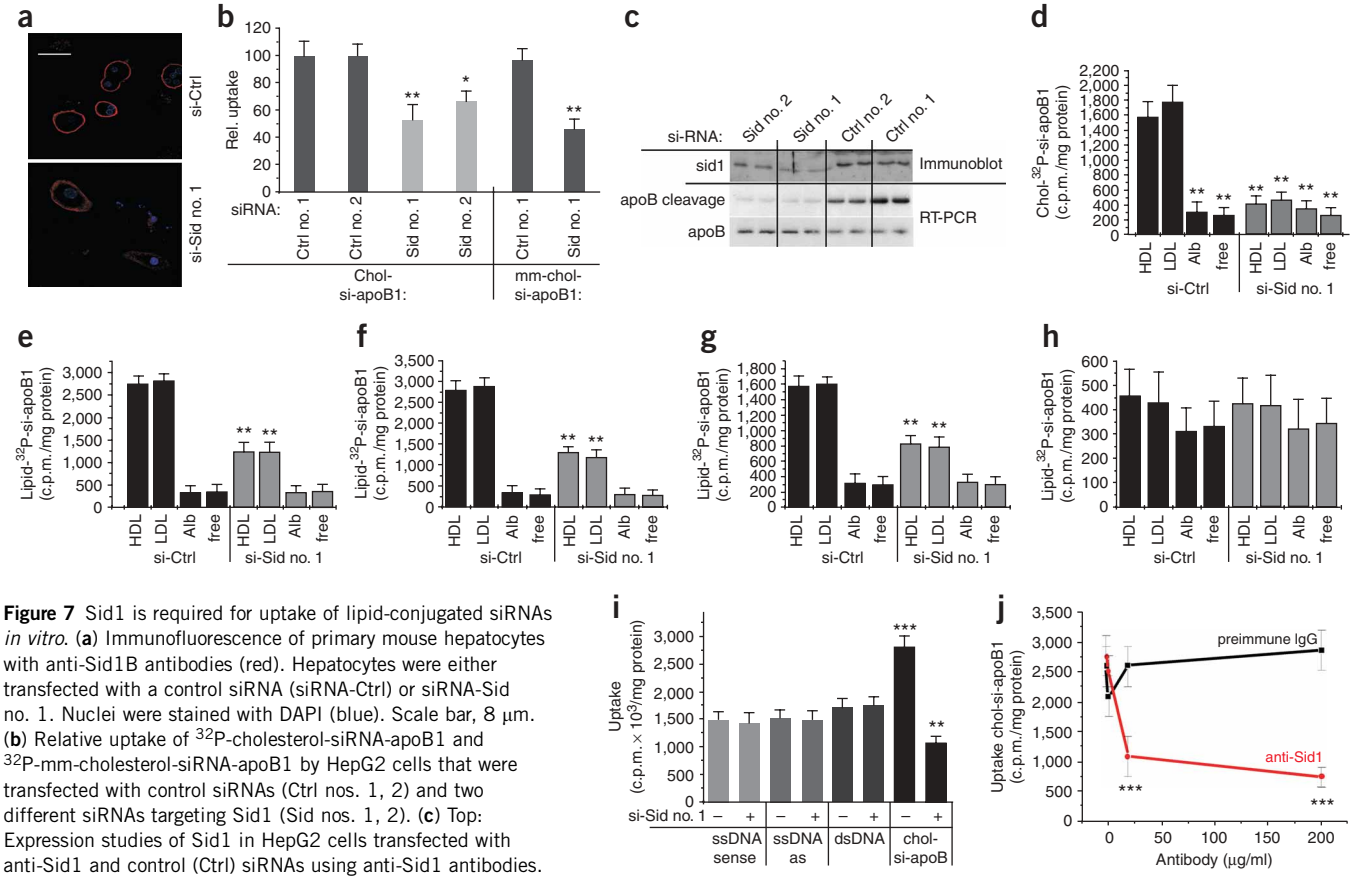

Figure 7 Sid1 is required for uptake of lipid-conjugated siRNAs *in vitro*. (**a**) Immunofluorescence of primary mouse hepatocytes with anti-Sid1B antibodies (red). Hepatocytes were either transfected with a control siRNA (siRNA-Ctrl) or siRNA-Sid no. 1. Nuclei were stained with DAPI (blue). Scale bar, 8 μm. (**b**) Relative uptake of ^{32}P-cholesterol-siRNA-apoB1 and ^{32}P-mm-cholesterol-siRNA-apoB1 by HepG2 cells that were transfected with control siRNAs (Ctrl nos. 1, 2) and two different siRNAs targeting Sid1 (Sid nos. 1, 2). (**c**) Top: Expression studies of Sid1 in HepG2 cells transfected with anti-Sid1 and control (Ctrl) siRNAs using anti-Sid1 antibodies. Bottom: RT-PCR analysis of specific apoB cleavage product in HepG2 cells transfected with control and anti-Sid1 siRNAs. (**d**) Uptake of ^{32}P-labeled cholesterol-siRNA by HepG2 cells transfected with control siRNA (siRNA-Ctrl) and siRNA-Sid no. 1 and treated with either cholesterol-siRNA-apoB1 that was preassembled to either HDL, LDL, albumin or added to the medium in an unbound form (free) ($n = 5$). (**e**–**h**) Uptake of ^{32}P-labeled lipophile-siRNA conjugates (lithocholic-oleyl- (**e**), docosanyl- (**f**), stearoyl- (**g**) and lauroyl-siRNA-apoB1 (**h**)) by HepG2 cells transfected with control siRNA no. 1 (Ctrl no. 1) or siRNA-Sid no. 1 (siRNA-Sid no. 1) and treated with preassembled lipoprotein/albumin–lipid-conjugated-siRNAs or unbound forms 24 h after transfection ($n = 5$). (**i**) ^{32}P-cholesterol-siRNA-DNA uptake in HepG2 cells in the presence or absence if Sid1 silencing by RNAi (siRNA-Sid no. 1). The cholesterol-conjugated DNA sequences are equivalent to cholesterol-siRNA-apoB1. "ssDNA sense" indicates the cholesterol-conjugates single-strand sense DNA, "as" indicates the antisense strand. "Ds" denotes the cholesterol-conjugated double-strand DNA. (**j**) ^{32}P-cholesterol-siRNA uptake assay in HepG2 cells in the presence of different concentrations of anti-Sid1 antibody (anti-Sid1B), or control preimmune serum (pre-bleed). Values represent means s.d. of six experiments. *, $P \leq 0.05$; **, $P \leq 0.01$.

to the clearance of ^{125}I-TC-HDL and ^{125}I-TC-LDL in plasma was measured. After uptake of ^{125}I-TC-LDL or ^{125}I-TC-HDL and intracellular degradation of the LDL protein, the nondegradable TC ligand remains trapped and thus demarcates the cells participating in the degradation of LDL. Surprisingly, plasma clearance of cholesterol-siRNA was markedly faster than clearance of either ^{125}I-TC-LDL or ^{125}I-TC-HDL. The calculated plasma half-life, $t_{1/2}$, of LDL was ~2.6 d whereas that of HDL was ~4.8 d. In contrast, $t_{1/2}$ of LDL and HDL-bound cholesterol-siRNA was ~2 h and 1 h, respectively (**Fig. 5**). The increased rate of clearance of the ^{32}P-cholesterol-siRNA was not due to serum nuclease degradation as the cholesterol-siRNA used in these studies is stable in plasma[4] (**Supplementary Fig. 1**). The differential clearance of the lipoprotein particles and cholesterol-siRNA suggests that lipoprotein-bound cholesterol-siRNA is carried by LDL and HDL particles but taken up by cells through a mechanism independent of endocytosis of whole lipoprotein particles.

To further investigate the possible involvement of lipoprotein receptors in cholesterol-siRNA uptake, we evaluated clearance of LDL- or HDL-bound ^{32}P-cholesterol-siRNA in mice lacking the LDL receptor (*Ldlr*$^{-/-}$) or SR-BI (*SR-BI*$^{-/-}$) and compared it to clearance in their respective wild-type littermates. Strikingly, only ~20% of LDL-bound cholesterol-siRNA was cleared from the circulation of *Ldlr*$^{-/-}$ mice after 50 h compared to complete elimination of cholesterol-siRNA in wild-type mice after ~5h (**Fig. 6a**,**b**). In contrast, no difference was observed in plasma clearance of HDL-bound cholesterol-siRNA from *Ldlr*$^{-/-}$ versus wild-type mice (**Fig. 6a**,**b**). Consistent with the effects observed on plasma clearance, there was a significant reduction in hepatic uptake of LDL-bound cholesterol-siRNAs and very little effect on uptake of HDL-bound cholesterol-siRNAs in *Ldlr*$^{-/-}$ mice (**Fig. 6c**,**d**). The dependence of HDL-bound ^{32}P-cholesterol-siRNA on expression of SR-BI was studied in *SR-BI*$^{-/-}$ mice. The half-life of HDL-bound cholesterol-siRNA in plasma of *SR-BI*$^{-/-}$ mice was increased approximately twofold compared to controls, and uptake into liver, kidneys and steroidogenic organs was reduced (**Fig. 6e**). No difference in clearance or liver uptake was noted for LDL-bound cholesterol-siRNA (**Fig. 6f**,**g**). These data demonstrate that the LDL and SR-BI receptors are required for cholesterol-siRNA uptake by the liver and other tissues.

Sid1 is required for cellular uptake of lipophile-siRNA conjugates

Our data suggested that lipoprotein-associated cholesterol-siRNAs were taken up by hepatocytes through a mechanism that is dependent on lipoprotein receptors but independent of internalization of lipoprotein particles. Therefore we hypothesized that HDL and LDL particles, complexed with cholesterol-siRNA, bind to their respective receptors on hepatocytes and that cellular uptake of cholesterol-siRNA is mediated by a separate transmembrane transporter. One possible candidate is the mammalian homolog of Sid1 in *C. elegans*, a multispan protein essential for systemic RNAi[14–16]. The expression of Sid1 was evaluated by immunohistochemistry staining using specific antibodies and in a multiple tissue RT-PCR panel. A plasma membrane staining was observed in primary hepatocytes and this staining was markedly reduced when Sid1 expression was silenced using RNAi (**Fig. 7a**). RT-PCR analysis revealed that Sid1 expression is highest in liver, spleen and thymus and lowest in kidney, adrenal, lung and buffy coat (data not shown). To test whether Sid1 plays a role in siRNA transport, we measured ^{32}P-cholesterol-siRNA-apoB1 or ^{32}P-mm-cholesterol-siRNA-apoB1 uptake in primary hepatocytes 24 h after they were transfected with siRNAs targeting Sid1. Interestingly, knockdown of Sid1 using specific siRNAs resulted in decreased ^{32}P-cholesterol-siRNA-apoB1 and ^{32}P-mm-cholesterol-siRNA-apoB1 uptake (**Fig. 7b**). Furthermore, cleavage of the apoB transcript was markedly reduced in hepatocytes in which Sid1 expression was silenced (**Figs. 3c** and **7c**). Silencing of Sid1 in HepG2 cells also reduced the uptake of ^{32}P-cholesterol-siRNA-apoB1 after incubation with precomplexed HDL/LDL particles (**Fig. 7d**). The dependence on Sid1 for cholesterol-siRNA uptake was not unique to cholesterol. A reduction in uptake was also observed for other lipophilic-siRNA conjugates, such as lithocholic-oleyl, docosanyl- and stearoyl-siRNA when preassembled with HDL and LDL (**Fig. 7e–g**). In contrast, lauroyl-conjugated siRNA, which only poorly associates with lipoproteins, was only taken up at low levels by HepG2 cells and uptake was independent of Sid1 expression (**Fig. 7h**). We also studied whether Sid1 can facilitate uptake of single (ss-) and double (ds-) stranded DNA that is conjugated to cholesterol. Cholesterol-DNA uptake in HepG2 cells was lower compared to cholesterol-siRNAs, and Sid1 silencing by RNAi had no effect on uptake (**Fig. 7i**). Lastly, to confirm Sid1 involvement in lipophile-siRNA conjugate uptake, Sid1 function was blocked using an anti-Sid1 antibody that targets an extracellular epitope of Sid1. Pretreatment of HepG2 cells with anti-Sid1 antibodies resulted in reduced uptake of ^{32}P-cholesterol-siRNA compared to cells treated with preimmune sera (**Fig. 7j**). Taken together, these results demonstrate that siRNAs conjugated to different lipophilic moieties are taken up by hepatocytes through a Sid1-dependent mechanism.

DISCUSSION

Our findings demonstrate that cholesterol-conjugated siRNAs are taken up *in vivo* by a lipoprotein-dependent mechanism. These data are supported by binding studies of cholesterol-siRNAs and lipoprotein particles, selective organ perfusion and uptake measurements with purified HDL and LDL particles that bind cholesterol-siRNAs, and functional assays monitoring cholesterol-siRNA-apoB activity *in vivo*. Cholesterol is not unique in its ability to bind siRNAs to lipoprotein particles: other highly lipophilic conjugates, such as long-chain fatty acids and bile acids, are also effective in binding to lipoproteins and mediating siRNA uptake into cells. A critical factor determining the affinity of fatty acid–conjugated siRNAs to lipoprotein particles is the length of the alkyl chain, a major determinant of lipophilicity. In the series of fatty acid siRNA conjugates, docosanyl (C_{22}) and stearoyl (C_{18}) conjugates show stronger binding to HDL and efficiently silence gene expression *in vivo*, whereas lauroyl (C_{12}) and myristoyl (C_{14}) conjugates and other medium and small-chain fatty-acids exhibit weak interactions with lipoprotein particles and ineffectively silence the targeted apoB gene *in vivo*. Interestingly, cholesterol and other lipid-conjugated siRNAs selectively bind to lipoproteins that are rich in phospholipids and cholesterol (that is, HDL and LDL), whereas the binding to triglyceride-rich lipoprotein particles (VLDL) is weak. The affinity of lipid-conjugated siRNA binding to HDL and LDL is most likely determined by the lipid component, because different siRNAs (siRNA-apoM and siRNA-apoB) have similar binding properties to lipoproteins. It also seems unlikely that apolipoprotein composition is the determining factor as LDL and HDL have different protein compositions[17]. Thus, the presence of phospholipids is likely to facilitate binding of lipophile-siRNA conjugates to the respective lipoprotein particles.

The importance of lipoproteins in the delivery of cholesterol-siRNAs *in vivo* was examined by comparing the *in vivo* silencing activity of HDL-bound cholesterol-siRNA-apoB1 with equal amounts of unbound cholesterol-siRNA-apoB1. We found that HDL-bound cholesterol-siRNA-apoB1 was about fivefold more efficient in cleaving the apoB transcript in mice compared to free cholesterol-siRNA-apoB1. Furthermore, the enhanced cleavage of the apoB transcript resulted in a ~8- to 15-fold improvement of lipoprotein-bound cholesterol-siRNA-apoB versus unbound cholesterol-siRNA-apoB in reducing apoB protein levels in the liver, gut and blood. These data indicate that significant quantities of cholesterol-siRNA-apoB1 do not readily associate with lipoprotein particles and therefore cannot be taken up by cells (and are secreted in the urine). Therefore, preassembly strategies of lipoprotein-bound lipophile-conjugated siRNAs or identification of lipophilic conjugates with more favorable association kinetics to lipoproteins may be important for future drug development.

HDL and LDL particles mediate delivery of cholesterol-siRNAs into different tissues. Whereas LDL-bound cholesterol-siRNAs are mainly taken up by the liver, HDL-bound cholesterol-siRNAs are taken up by various tissues, including adrenal, ovary, kidney and liver. The mechanism for this uptake can be explained by the high binding affinity of HDL to scavenger receptor SR-BI, which facilitates cholesterol uptake from HDL to cells[18]. The SR-BI receptor is highly expressed in the liver and steroidogenic tissues[18,19], the main sites of selective HDL cholesterol uptake *in vivo*, and expression of the SR-BI receptor correlates well with tissues that were identified by us to take up cholesterol-siRNA bound to HDL. Our data also indicate that uptake of cholesterol-siRNA can be facilitated by SR-BI, as SR-BI null mice exhibit substantially reduced uptake of cholesterol-siRNA-apoB1 into cells.

LDL receptor expression is high in the liver but this receptor is also expressed in a variety of other tissues including adrenal and kidney and intestine. Interestingly, we only observed a very low uptake of cholesterol-siRNAs into these other tissues. Previous studies have suggested that the majority (>60%) of circulating LDL is taken up by the liver[20], which is significantly lower than the 80% uptake of cholesterol-siRNA bound to LDL. A possible explanation for this discrepancy is the rapid uptake of cholesterol-siRNAs compared to a much slower cellular LDL uptake by endocytosis. Thus it is possible that lipoprotein-associated cholesterol-siRNA is brought in proximity to the cell plasma membrane and then internalized by a transport mechanism independent of whole lipoprotein particle–mediated endocytosis. These data are supported by classical *in vitro* experiments showing that LDL is bound to and released from its cognate receptor on the cell surface with an average recirculation time of 6 min[21,22].

Sid1 is the mammalian homolog of Systemic RNAi Deficient-1 in *C. elegans,* a multi-span transmembrane protein that is necessary for systemic RNAi activity in this organism[14] and whose expression in *Drosophila melanogaster* and mammalian cells can potentiate RNAi by facilitating dsRNA uptake[15,16]. Our data indicate that Sid1 is at least in part responsible for lipophile-siRNA uptake after docking of lipoprotein/siRNA complexes. Silencing of Sid1 in HepG2 cells resulted in a marked reduction of HDL- and LDL-mediated cholesterol-siRNA uptake and silencing activity *in vitro*. Similar results were also obtained when siRNAs were conjugated to other lipophiles that are chemically distinct, suggesting that the oligoribonucleotides and not the lipophilic conjugates are recognized by this transporter. Furthermore, a Sid1 antibody that recognizes an extracellular epitope of Sid1 inhibited cholesterol-siRNA uptake. Future studies are warranted to address the role of Sid1 in lipid-conjugated siRNA uptake *in vivo* using Sid1 knockout mice and to study the involvement of Sid1-related proteins (e.g., the protein encoded by NM_001040455) in cholesterol-siRNA uptake.

In this report we demonstrate that lipoprotein particles are efficient delivery vehicles of lipophile-siRNA conjugates *in vivo*. Studies can now be designed to further improve LDL- and HDL-mediated delivery of lipid conjugated-siRNAs and optimize tissue delivery to treat a wide spectrum of diseases.

METHODS

Animals experiments. Animals experiments using animal models were performed in accordance with animal care ethics approval and guidelines, as per animal care certificate no. 05047 and 49/2007, 50/2007 of Rockefeller University and Kantonales Veterinäramt Zürich, respectively. Wild-type (*WT*) and *Ldlr*$^{-/-}$ mice[23] were maintained on a *C57Bl/6J* background, whereas *SR-BI*$^{-/-}$ animals[24] were maintained on an *FVB* background. Mice were 6–10 weeks of age at the time of studies, whereas Syrian hamsters were 4–6 weeks of age at the time of studies. All animals were kept on a 12-h light/dark cycle in a pathogen-free animal facility with free access to food and water.

Synthesis of sequences of lipophile-conjugated si-RNAs, The chemically modified, lipophile-conjugated siRNA-apoB1 constituted from the corresponding sense strand: 5′-GUCAUCACACUGAAUACCAAU$_s$Hyp-L-3′ and antisense strand: 5′-AUUGGUAUUCAGUGUGAUGAc$_s$a$_s$C-3′ was obtained as described earlier[4]. The cholesterol-siRNA-apoM was prepared from cholesterol-conjugated sense strand: 5′-AsGsfUfCAAfUfCGGfUAfUGfUfC fCAfC$_s$dT$_s$dT$_s$-cholesterol-3′ and 5′-phosphorylated antisense strand: 5′-P-GsfUGGAfCAfUAf CfCGAfUfUGAfCfU$_s$dT$_s$dT, where all pyrimidines except overhangs were substituted with corresponding 2′-deoxy-2′-fluoro nucleosides. The corresponding mismatched cholesterol-mm-siRNA-apoM was constituted sense strand: 5′-AsGsfUfCAGfUfCAGfUGfUAfUfCfCAfCsdTsdTs-cholesterol-3′ and 5′-phosphorylated antisense strand: 5′-P-GsfUG GAfU AfCA fCfUG AfCfU GAfC fUsdTsdT-3′. Abbreviations are as follows: L, the lipophile; P, phosphate group; fC and fU, 2′-deoxy-2′-fluoro cytidine and uridine respectively; lower case letters, 2′-*O*-methyl sugar modification; subscript 's', phosphorothioate linkages. The sequence of the mismatch control mm-cholesterol-siRNA-apoB1 has been described previously[4].

Sense and antisense strands of control and corresponding 3′-lipophile conjugated sense strands were individually synthesized and purified according to the standard oligonucleotide synthesis and deprotection protocols[4,25]. Commercially available 5′-*O*-(4,4'-dimethoxytrityl)-2′-*O*-*t*-butyldimethylsilyl-3′-*O*-(2-cyanoethyl-*N*,*N*-diisopropyl) RNA and the corresponding 2′-*O*-methyl phosphoramidite monomers of 6-*N*-benzoyladenosine (A^{Bz}), 4-*N*-acetylcytidine (C^{Ac}), 2-*N*-isobutyrylguanosine (G^{iBu}), and uridine (U) were used for unmodified and 2′-*O*-methyl sugar modified RNA synthesis. The 2′-deoxy-2′-fluoro sugar modified pyrimidines were introduced to the RNA by using 5′-*O*-(4,4′-dimethoxytrityl)-2′-deoxy-2′-fluoro-3′-*O*-(2-cyanoethyl-*N*,*N*-diisopropyl) phosphoramidite monomers of 4-*N*-acetylcytidine (C^{Ac}) and uridine (U).

All lipophile-bearing solid supports to conjugate the lipophiles to 3′-end of sense strand were synthesized at Alnylam. Each individual lipophile-conjugated sense strand was synthesized from the corresponding hydroxyprolinol-lipophile solid support. Lipophile was tethered to *trans*-4-hydroxyprolinol via a 6-aminohexanoate linkage to obtain a hydroxyprolinol-lipophile moiety that was subsequently attached to a functionalized control pore glass to obtain the solid support[26]. Beaucage reagent was used as an oxidant to obtain the phosphorothioate backbone modification[27]. The lipophile-conjugated sense strands were purified by reversed phase–high-performance liquid chromatography (RP-HPLC) on an in-house packed Source 15RPC resin reversed-phase column. The buffers were 20 mM sodium acetate in 10% CH_3CN (buffer A) and 20 mM sodium acetate in 70% CH_3CN (buffer B). The unconjugated RNA oligonucleotides were purified by anion-exchange HPLC. Fractions containing full-length oligonucleotides were pooled, desalted and lyophilized. Analytical HPLC, capillary gel electrophoresis and electrospray liquid chromatography–mass spectrometry established the integrity of the compounds. For duplex generation, equimolar amounts of sense and antisense strand were heated in 1× PBS at 95 °C for 5 min and slowly cooled to 20 °C. Annealing of equimolar mixture of sense and antisense strands in 1× PBS buffer afforded the desired siRNA.

Radiolabeling of siRNAs and lipoproteins. Different siRNAs were labeled using T4 polynucleotide kinase (New England Biolabs) and γ^{32}P-ATP. Unbound nucleotides were separated by gel filtration using G50 spin columns. Lipoproteins were isolated by FPLC (Amersham) and dialyzed twice against 2 liters of reaction buffer (1 M glycine pH 9.5, 100 mM NaCl). Lipoprotein-containing fractions were concentrated using Centripreps (10-kDa cutoff) to 10 mg/ml protein. Lipoproteins were labeled indirectly with tyramine cellobiose[13] using freshly prepared [^{125}I]Cl solution. Unbound free iodine or iodine-labeled tracer was separated by PD-10 columns (Pharmacia).

Annealing of siRNA to lipoproteins and albumin. Lipoproteins from hamster or mouse were isolated by ultracentrifugation. Labeled or unlabeled siRNAs were annealed with lipoproteins or albumin at a 4-M siRNA excess at 20 °C for 30 min, unbound siRNAs were separated from the complex by gel filtration using a Suprose6 column (Pharmacia). To assess binding constants and ratios for cholesterol-siRNA binding to albumin or lipoprotein particles, we incubated known amounts of cholesterol-siRNA with either binding partner. After annealing, bound and unbound fractions were separated and quantified. The binding constant was calculated from the ratio of bound versus unbound cholesterol-siRNA.

Injection of siRNA. Mice were injected with saline or different lipophile-siRNA conjugates through the tail vein, whereas hamsters were injected through the saphenous vein. For *in vivo* silencing, the apoB siRNA, cholesterol-siRNA-apoB1 (ref. 4) was administered at a dose of 50 mg kg^{-1} at a dosing volume of 10 μl/g. For uptake and transport studies, animals were injected with ~0.5–2 mg kg^{-1} of ^{32}P-labeled siRNA bound to lipoproteins/albumin or in a free form. Animals were killed at different time points and blood and tissue was taken for RNA analysis or to measure uptake of radiolabeled siRNAs. Circulating cholesterol-siRNA concentration was calculated from the specific activity of the radiolabeled compounds.

Cell lines and siRNA transfection. HepG2 cells were maintained in DMEM supplemented with 4.5 g/l glucose, 10% FCS, 2 mM glutamine; 50 μg/ml gentamycin/streptomycin in a humidified incubator at 5% CO_2. Cells were grown on collagen-treated plates and transfected with siRNAs using the Mirus transfection reagent (Mirus). For knockdown of human Sid1 (NM_017699) either Sid no. 1 siRNA (5′-GCAGCAACTGATATTTGTA) or Sid no. 2 siRNA (5′-AGCTGGTCATTACCTATCA) were used.

Liver perfusion. After anesthesia with pentobarbitone sodium (60 mg/kg intraperitoneally), the portal vein and the inferior vena cava were cannulated. The liver was perfused with oxygenated Krebs-Henseleit buffer[28] with varying amounts of lipoproteins and cholesterol-siRNAs at 37 °C in a single-pass mode with a total flow rate of 1.5–2 ml/min.

ApoB mRNA cleavage assay. Total RNA (5 μg) from liver and cells was ligated to an adaptor primer without prior treatment. Ligated RNA was

reverse-transcribed using a gene-specific primer (5′-CTCCTGTTGCAGTA GAGTGCAGCT-3′) (Ambion). To detect cleavage products a radiolabeled, we performed PCR using primers complementary to the RNA adaptor (GR5′: 5′-CTCTAGAGCGACTGGAGCACGAGGACACTA-3′) and apoB mRNA (Rev2: 5′-ACGCGTCGACGTGGGAGCATGGAGGTTGGCAGTTGTTC-3′). Products were resolved by gel electrophoresis and visualized by autoradiography. Band intensity was quantified using the LabImage software.

Quantitative RT-PCR. TheQuantiGene assay (Genospectra) was used to quantify the reduction of apoB mRNA in liver after siRNA treatment. Small uniform tissue samples were collected 24 h after the last injection. Lysates from three tissue samples per animal were directly used for apoB and GAPDH mRNA quantification, and the ratio of apoB and GAPDH mRNA was calculated and expressed as a group average relative to the saline control group. Specific probes for detection of apoB mRNA levels have been described earlier[4].

Antibodies. Peptide anti-Sid1B (EGTPAESREKNRECVLLDFF) against the human Sid1 homolog (NM_017699) was synthesized, processed to >90% purity, conjugated to KLH and used for immunization of rabbits (Bethyl Laboratories). Antisera were affinity purified and tested by western blotting and immunohistochemistry. Affinity-purified antisera were used for all studies. Secondary antibodies used for immunoblotting and immunohistochemistry were Alexa 488 donkey anti-rabbit (Molecular Probes).

Note: Supplementary information is available on the Nature Biotechnology website.

ACKNOWLEDGMENTS

We would like to thank J. Maraganore, H.-P. Vornlocher, M.A. Maier and P. Sharp for helpful discussion and suggestions. These studies were supported by National Institutes of Health grant 1 P01 GM073047-01 (M.S.).

COMPETING INTERESTS STATEMENT

The authors declare competing financial interests: details accompany the full-text HTML version of the paper at http://www.nature.com/naturebiotechnology.

Published online at http://www.nature.com/naturebiotechnology
Reprints and permissions information is available online at http://npg.nature.com/reprintsandpermissions

1. McManus, M.T. & Sharp, P.A. Gene silencing in mammals by small interfering RNAs. *Nat. Rev. Genet.* **3**, 737–747 (2002).
2. Li, M. & Rossi, J.J. Lentiviral vector delivery of siRNA and shRNA encoding genes into cultured and primary hematopoietic cells. *Methods Mol. Biol.* **309**, 261–272 (2005).
3. Song, E. *et al.* RNA interference targeting Fas protects mice from fulminant hepatitis. *Nat. Med.* **9**, 347–351 (2003).
4. Soutschek, J. *et al.* Therapeutic silencing of an endogenous gene by systemic administration of modified siRNAs. *Nature* **432**, 173–178 (2004).
5. Morrissey, D.V. *et al.* Potent and persistent in vivo anti-HBV activity of chemically modified siRNAs. *Nat. Biotechnol.* **23**, 1002–1007 (2005).
6. Song, E. *et al.* Antibody mediated in vivo delivery of small interfering RNAs via cell-surface receptors. *Nat. Biotechnol.* **23**, 709–717 (2005).
7. Brown, M.S. & Goldstein, J.L. The receptor model for transport of cholesterol in plasma. *Ann. NY Acad. Sci.* **454**, 178–182 (1985).
8. Assmann, G. & Nofer, J.R. Atheroprotective effects of high-density lipoproteins. *Annu. Rev. Med.* **54**, 321–341 (2003).
9. Rigotti, A., Miettinen, H.E. & Krieger, M. The role of the high-density lipoprotein receptor SR-BI in the lipid metabolism of endocrine and other tissues. *Endocr. Rev.* **24**, 357–387 (2003).
10. Stein, O. & Stein, Y. Atheroprotective mechanisms of HDL. *Atherosclerosis* **144**, 285–301 (1999).
11. Brown, M.S. & Goldstein, J.L. A receptor-mediated pathway for cholesterol homeostasis. *Science* **232**, 34–47 (1986).
12. Connelly, M.A. & Williams, D.L. Scavenger receptor BI: a scavenger receptor with a mission to transport high density lipoprotein lipids. *Curr. Opin. Lipidol.* **15**, 287–295 (2004).
13. Pittman, R.C. *et al.* A radioiodinated, intracellularly trapped ligand for determining the sites of plasma protein degradation in vivo. *Biochem. J.* **212**, 791–800 (1983).
14. Duxbury, M.S., Ashley, S.W. & Whang, E.E. RNA interference: a mammalian SID1 homologue enhances siRNA uptake and gene silencing efficacy in human cells. *Biochem. Biophys. Res. Commun.* **331**, 459–463 (2005).
15. Winston, W.M., Molodowitch, C. & Hunter, C.P. Systemic RNAi in *C. elegans* requires the putative transmembrane protein SID1. *Science* **295**, 2456–2459 (2002).
16. Breckenridge, W.C. The catabolism of very low density lipoproteins. *Can. J. Biochem. Cell Biol.* **63**, 890–897 (1985).
17. Acton, S. *et al.* Identification of scavenger receptor SR-BI as a high density lipoprotein receptor. *Science* **271**, 518–520 (1996).
18. Landschulz, K.T., Pathak, R.K., Rigotti, A., Krieger, M. & Hobbs, H.H. Regulation of scavenger receptor, class B, type I, a high density lipoprotein receptor, in liver and steroidogenic tissues of the rat. *J. Clin. Invest.* **98**, 984–995 (1996).
19. Rudling, M.J., Reihner, E., Einarsson, K., Ewerth, S. & Angelin, B. Low density lipoprotein receptor-binding activity in human tissues: quantitative importance of hepatic receptors and evidence for regulation of their expression in vivo. *Proc. Natl. Acad. Sci. USA* **87**, 3469–3473 (1990).
20. Brown, M.S., Ho, Y.K. & Goldstein, J.L. The low-density lipoprotein pathway in human fibroblasts: relation between cell surface receptor binding and endocytosis of low-density lipoprotein. *Ann. NY Acad. Sci.* **275**, 244–257 (1976).
21. Brown, M.S. & Goldstein, J.L. Receptor-mediated control of cholesterol metabolism. *Science* **191**, 150–154 (1976).
22. Feinberg, E.H. & Hunter, C.P. Transport of dsRNA into cells by the transmembrane protein SID1. *Science* **301**, 1545–1547 (2003).
23. Ishibashi, S. *et al.* Hypercholesterolemia in low density lipoprotein receptor knockout mice and its reversal by adenovirus-mediated gene delivery. *J. Clin. Invest.* **92**, 883–893 (1993).
24. Rigotti, A. *et al.* A targeted mutation in the murine gene encoding the high density lipoprotein (HDL) receptor scavenger receptor class B type I reveals its key role in HDL metabolism. *Proc. Natl. Acad. Sci. USA* **94**, 12610–12615 (1997).
25. Damha, M.J. & Ogilvie, K.K. Oligonucleotide synthesis. The silyl phosphoramidite method. *Methods Mol. Biol.* **20**, 81–114 (1993).
26. Manoharan, M., Kesavan, V. & Rajeev, K.G. SiRNA's containing ribose substitutes to which lipophilic moieties may be attached. US patent application 2,005,107,325 (2005).
27. Iyer, R.P., Eagan, W., Regan, J.B. & Beaucage, S.L. 3H–1,2 benzodithiole- 3-one 1,1-dioxide as an improved sulfurizing reagent in the solid-phase synthesis of oligodeoxyribonucleoside phosphorothioates. *J. Am. Chem. Soc.* **112**, 1253–1254 (1990).
28. Daugaard, H., Egfjord, M. & Olgaard, K. Isolated perfused rat kidney and liver combined. A new experimental model. *Pflugers Arch.* **409**, 220–222 (1987).