Therapeutic RNAi targeting PCSK9 acutely lowers plasma cholesterol in rodents and LDL cholesterol in nonhuman primates

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Proprotein convertase subtilisin/kexin type 9 (PCSK9) regulates low density lipoprotein receptor (LDLR) protein levels and function. Loss of PCSK9 increases LDLR levels in liver and reduces plasma LDL cholesterol (LDLc), whereas excess PCSK9 activity decreases liver LDLR levels and increases plasma LDLc. Here, we have developed active, cross-species, small interfering RNAs (siRNAs) capable of targeting murine, rat, nonhuman primate (NHP), and human PCSK9. For *in vivo* studies, PCSK9 and control siRNAs were formulated in a lipidoid nanoparticle (LNP). Liver-specific siRNA silencing of PCSK9 in mice and rats reduced PCSK9 mRNA levels by 50–70%. The reduction in PCSK9 transcript was associated with up to a 60% reduction in plasma cholesterol concentrations. These effects were shown to be mediated by an RNAi mechanism, using 5′-RACE. In transgenic mice expressing human PCSK9, siRNAs silenced the human PCSK9 transcript by >70% and significantly reduced PCSK9 plasma protein levels. In NHP, a single dose of siRNA targeting PCSK9 resulted in a rapid, durable, and reversible lowering of plasma PCSK9, apolipoprotein B, and LDLc, without measurable effects on either HDL cholesterol (HDLc) or triglycerides (TGs). The effects of PCSK9 silencing lasted for 3 weeks after a single bolus i.v. administration. These results validate PCSK9 targeting with RNAi therapeutics as an approach to specifically lower LDLc, paving the way for the development of PCSK9-lowering agents as a future strategy for treatment of hypercholesterolemia.

plasma PCSK9 | tissue LDLR levels

Proprotein convertase subtilisin/kexin type 9 (PCSK9) is a member of the mammalian serine proprotein convertase family that typically functions in the proteolytic processing and maturation of secretory proteins (1, 2). PCSK9 was the first family member to be implicated in a dominantly inherited form of hypercholesterolemia (3). Mechanistic studies addressing the function of PCSK9 in mice and humans have demonstrated that overexpression or gain-of-function mutations in *PCSK9* reduced low density lipoprotein receptor (LDLR) protein levels in liver, which significantly increased circulating plasma cholesterol both in mice and humans (4). Additional studies showed that the deletion of *Pcsk9* in mice resulted in increased LDLR levels, accelerated the clearance of low density lipoprotein cholesterol (LDLc), and reduced circulating cholesterol levels (5). Recently, studies in mice have also shown that lowering PCSK9 transcript levels by antisense oligonucleotides resulted in reduced total cholesterol, LDLc, and HDL cholesterol (HDLc) in blood and increased LDLR levels in liver after 6 weeks of treatment (6). This effect was very similar to that observed in the $Pcsk9^{-/-}$ mice (5). Collectively, these studies have clearly established a role for PCSK9 in cholesterol homeostasis.

Validation of PCSK9 as an attractive therapeutic target for the treatment of hypercholesterolemia has come from genetic studies in humans. Cohen *et al.* (7) first identified loss-of-function mutations in *PCSK9* that lowered plasma LDLc in the Dallas Heart Study. In a larger 15-year prospective study, they demonstrated that nonsense mutations in *PCSK9* reduced LDLc levels by 28% and decreased the frequency of CHD by 88% in African Americans (8). Despite this genetic validation, several physiological aspects of potential PCSK9-modifying agents must be further defined to assess therapeutic potential and benefit. For instance, will the acute lowering of PCSK9 (e.g., over 48–72 h) result in LDLc lowering, and if so, will this reduction be associated with other potentially adverse consequences, such as increased liver lipids? Rodents lack cholesterol ester transferase protein (CETP) and carry the majority of their plasma cholesterol in HDL. Thus, they are not ideal models in which to determine whether PCSK9 silencing will only decrease LDLc and not HDLc. Studies in a more relevant model, such as nonhuman primates (NHPs), are required.

Currently, a number of individuals with hypercholesterolemia are unable to reach target LDLc levels with available therapies. To address the efficacy of inhibiting PCSK9 via an siRNA mechanism, we designed and synthesized several siRNA therapeutic molecules to silence PCSK9 mRNA in mice, rats, NHPs, and humans. These siRNAs were administered by using a lipidoid nanoparticle (LNP) to achieve efficient hepatocyte delivery *in vivo*. This approach enabled us to study the effect of *PCSK9* silencing on the levels of PCSK9 mRNA, plasma PCSK9 protein, hepatic LDLR protein,

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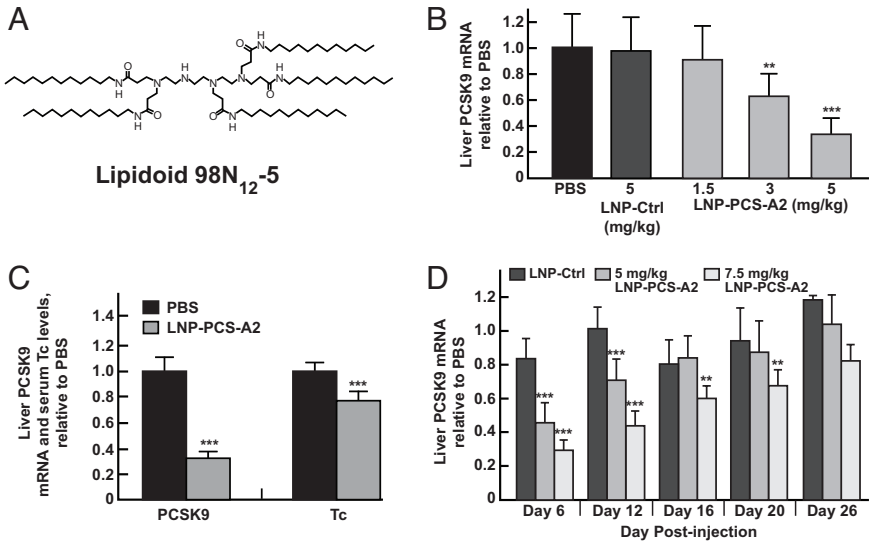

Fig. 1. Lipidoid formulation and effects of PCSK9 silencing in wild-type mice. (*A*) Cationic lipidoid component structure of the formulation. (*B*) Dose-dependent decrease in hepatic PCSK9 mRNA (relative to controls) 2 days after dose ($n = 6$ per group). (*C*) Liver PCSK9 mRNA and total serum cholesterol levels in mice ($n = 5$ per group) 3 days after a dose of 5 mg/kg LNP-PCS-A2 or PBS. (*D*) Duration of hepatic PCSK9 transcript silencing in mice ($n = 5$ per group) after a single injection of 5 mg/kg or 7.5 mg/kg LNP-PCS-A2. (*B*) one-way ANOVA with Student's *t* test; (*C* and *D*) two-way ANOVA with Bonferroni test. (*B–D*) Each value is the group mean ± STDEV. Asterisks represent statistical difference between PBS and PCSK9 siRNA treated groups. **, $P \leq 0.01$; ***, $P \leq 0.001$

total serum cholesterol, LDLc, and HDLc concentrations in multiple species. These *in vivo* studies demonstrate that PCSK9 lowering by siRNA has an acute effect on plasma LDLc, but not HDLc, in NHPs. Our data validate PCSK9 as a target for therapeutic intervention by siRNA and provide a strategy for treatment of hypercholesterolemia.

Results

Selection and Formulation of Active siRNA Molecules Targeting PCSK9. A series of approximately 150 siRNAs were designed to be cross-species reactive through an initial bioinformatics analysis and screened *in vitro* for activity in cultured HepG2 cells. Active molecules PCS-A1, PCS-A2, PCS-B2, and PCS-C2 were chosen for further studies based on their pM IC_{50} values as measured in primary cynomolgus monkey hepatocytes [supporting information (SI) Table S1].

Certain siRNAs can induce immune responses via interferons and proinflammatory cytokines (9, 10). The siRNAs studied here were designed to avoid immune stimulatory sequence motifs. The siRNAs selected for further study contained two nucleotide 3′ overhangs to prevent activation of the RIG-1 pathway (11, 12). Nevertheless, the selected siRNAs were also tested for activation of the immune system in primary human blood monocytes (hPBMCs). Specifically, IFN-α and TNF-α were measured in hPBMCs transfected with each molecule listed in Table S1. The parental compound PCS-A1 was found to induce both IFN-α and TNF-α. However, its chemically modified version, PCS-A2, and chemically modified duplexes PCS-B2 and PCS-C2, were negative for both IFN-α and TNF-α induction in these assays (Table S1 and Fig. S1 for a PCS-A1/PCS-A2 paired example). These results demonstrate that chemical modifications are capable of attenuating both IFN-α and TNF-α responses to siRNA molecules.

LNP is a lipidoid formulation comprised of a novel cationic component $98N_{12}$-5 (1)·4HCl (Fig. 1*A*), cholesterol, and a poly(ethylene glycol)-lipid (13). We have shown that LNP-formulated siRNAs that target apoB or Factor VII mediated *in vivo* silencing in liver at doses of 5 mg/kg with minimal toxicity and without perturbation of the endogenous miRNA biogenesis pathway (12). Here, we use LNP to formulate and test our RNAi therapeutics against PCSK9 in mice, rats, and NHPs.

Silencing of Hepatic PCSK9 mRNA in Rodents Results in Rapid and Reversible Lowering of Serum Cholesterol. *Pcsk9*$^{-/-}$ mice have ≈50% reduction in total serum cholesterol concentrations (5). To test whether acute silencing of the PCSK9 transcript by a PCSK9-specific siRNA would result in acutely lower serum cholesterol levels, we formulated a cross-species siRNA molecule PCS-A2 in LNP for study *in vivo*. Various doses of the lipidoid-formulated PCS-A2 (LNP-PCS-A2) were injected via the tail vein into mice and rats.

In mice, livers were harvested to measure PCSK9 mRNA levels and blood was collected for total cholesterol analysis. As shown in Fig. 1*B*, LNP-PCS-A2 displayed a dose response with maximal PCSK9 mRNA silencing of ≈60–70% at a dose of 5 mg/kg. The decrease of mRNA transcript levels (at the highest dose) translated into ≈30% lowering of total plasma cholesterol (Fig. 1*C*). The reduction in serum cholesterol was similar to that measured in mice heterozygous for a disrupted *Pcsk9* allele (5 and J.D.H., unpublished observations). Moreover, the effect on the PCSK9 transcript persisted for ≈20 days, with higher doses displaying greater initial transcript level reduction and subsequently more prolonged effects (Fig. 1*D*).

Next, we studied rats, which are resistant to cholesterol lowering by high doses of HMG-CoA reductase inhibitors (statins) (14, 15). In rats, LNP-PCS-A2 was dosed 1–5 mg/kg, which resulted in a dose-dependent reduction in the PCSK9 transcript with 50–60% silencing at the highest dose (Fig. 2*A*). The mRNA silencing was associated with an acute 50–60% decrease of serum total cholesterol (Fig. 2 *A* and *B*) lasting 10 days, with a gradual return to predose levels by ≈3 weeks (Fig. 2*B*)

Lowering of proteins involved in very-low-density lipoprotein assembly and secretion (microsomal triglyceride transfer protein; MTP or apoB) by genetic deletion, small molecule inhibitors, or siRNA, results in increased liver TGs (16, 17) (T.Z., unpublished data). To determine whether cholesterol lowering via PCSK9 inhibition alters liver lipid content, hepatic cholesterol and TG concentrations in livers of treated and control animals were quantified. As shown in Fig. 2*C*, there was no

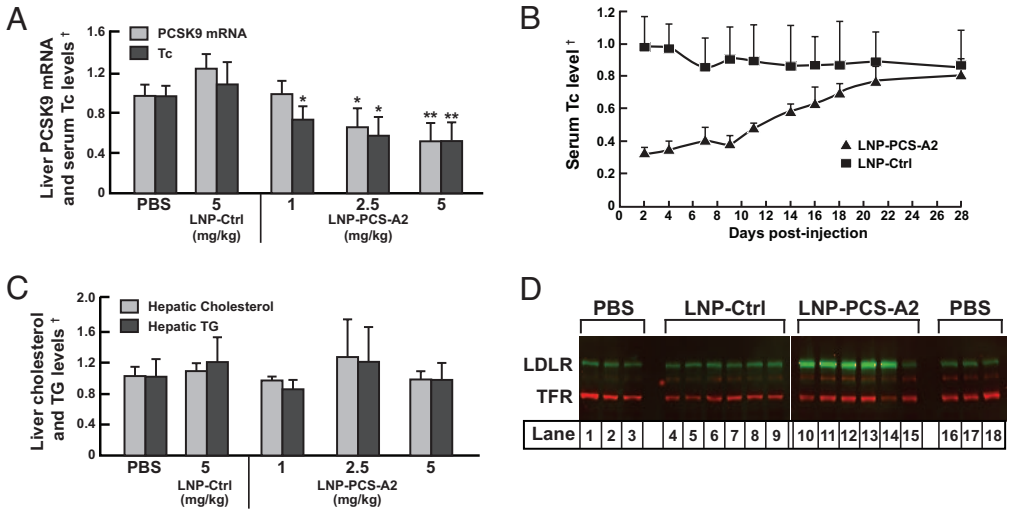

Fig. 2. Hepatic PCSK9 silencing, hepatic TGs, and LDLR levels in rats. (*A*) LNP-PCS-A2 mediated dose-dependent lowering of hepatic PCKS9 mRNA and total serum cholesterol 3 days after dose ($n = 6$ per group). Each value is the group mean ± STDEV. One-way ANOVA with Student's *t* test. Asterisks represent statistical difference between PBS- and PCSK9 siRNA-treated groups *, $P \leq 0.05$; **, $P \leq 0.01$. (*B*) Total serum cholesterol lowering of LNP-PCS-A2 treated rats ($n = 6$ per group) is maximal (≈60%) by 2 days after dose and returns to baseline over ≈21 days. PCSK9-treated groups are statistically significant (until approximately day 16) compared with PBS and LNP-Crtl groups (one-way ANOVA, Student's *t* test, with *P* values of ≤ 0.05). (*C*) Liver TGs and cholesterol contents from treated and control animals (same as in *A*). There were no significant differences in the liver TGs (ANOVA: $P = 0.824$ for cholesterol content ANOVA on ranks; $P = 0.935$ for LNP-PCS-A2-treated animals vs. the LNP-Crtl- or PBS-treated control groups for TG levels). (*D*) Immunoblot of liver extracts from LNP-PCS-A2-, LNP-Crtl-, and PBS-treated rats (same as in *A*). Transferrin receptor (TFR) levels were used to normalize for protein loading. (Note that treated animal lane 15 was a noticeable outlier that did not up-regulate LDLR and on close examination also did not lower PCSK9 levels, possibly because of a misinjection). †, relative to PBS.

statistically significant difference in liver TG or cholesterol concentrations among animals administered PCSK9 siRNAs compared with control rats.

The mechanism by which PCSK9 impacts plasma cholesterol levels has been linked to the density of LDLRs on the hepatocyte cell surface (5, 18–20). *Pcsk9*$^{-/-}$ mice have 2- to 3-fold higher levels of liver LDLR protein compared with wild-type mice, and this effect is magnified by statin treatment (5). Similarly, reduction of PCSK9 (using antisense oligos) over a 6-week period in high-fat-fed mice resulted in an up-regulation of LDLR levels (6). To investigate whether regulation of hepatic LDLRs occurred upon siRNA silencing of PCSK9 in rats, liver LDLR levels were quantified by immunoblot analysis after their treatment with 5 mg/kg LNP-PCS-A2. As shown in Fig. 2*D*, LNP-PCS-A2-treated animals had a significant 3- to 5-fold induction of LDLR levels compared with PBS- or LNP-Crtl-treated animals. Together, the rodent studies demonstrate that lowering of PCSK9 mRNA levels with siRNAs targeting PCSK9 in the liver results in an acute and durable decrease of serum cholesterol as a result of increased hepatic LDLR expression, and the acute change in LDLR expression is not associated with excess lipid accumulation in the liver.

***In Vivo* Mechanism of PCSK9 Silencing Is siRNA Mediated.** To confirm that the reduction in PCSK9 transcript observed in rodents was because of a siRNA mechanism, liver extracts from either treated or control rats were subjected to rapid amplification of cDNA ends (5′-RACE), a method previously used to demonstrate that siRNA-mediated cleavage occurs (21, 22). 5′-RACE analysis of liver mRNA from animals treated with LNP-PCS-A2 revealed a product of the expected size (Fig. 3). Sequence analysis of cloned PCR products demonstrated that 73 of 84 of these products were derived from the predicted cleavage event at position (*GAGT*/*TTAT*). No specific bands were amplified in the 5′-RACE experiments from PBS- or LNP-Crtl-treated animals. These results demonstrate that the effect of LNP-PCS-A2 observed on hepatic PCSK9 mRNA levels is consistent with cleavage of the PCSK9 transcript via a targeted RNAi-specific mechanism.

Efficacy of siRNA-Mediated Inhibition of Human PCSK9 in Transgenic Mice. Next, we tested the ability of LNP-PCS-A2 and LNP-PCS-C2 (PCS-C2 targets only human and NHP PCSK9 mRNA) (see Table S1) to silence human *PCSK9 in vivo*. To this end, we used a line of transgenic mice that express human PCSK9 cDNA under the apoE promoter (23). Specific PCR reagents and antibodies were designed that detect human, but not mouse, transcripts and protein, respectively. Cohorts of the humanized transgenic mice were injected with a single 5 mg/kg dose of LNP-PCS-A2 or LNP-PCS-C2, and both livers and blood were collected 72 h later. As shown in Fig. 4*A*, a single dose of LNP-PCS-A2 or LNP-PCS-C2 was able to decrease the human PCSK9 transcript levels by >70%, and this resulted in >500-fold reduction in the levels of circulating human PCSK9 protein as measured by ELISA (Fig. 4*B*). These results demonstrated that both siRNAs were capable of silencing the human transcript and, subsequently, reducing the amount of circulating plasma human

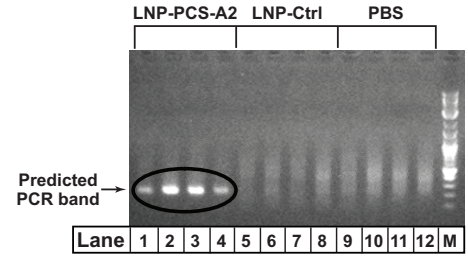

Fig. 3. siRNA-mediated cleavage of PCSK9 mRNA in rats (5′-RACE). Rats ($n =$ 4 per group) were administered with 4 mg/kg LNP-PCS-A2, LNP-Crtl, or PBS and killed 4 days later. 5′-RACE detects the predicted mRNA cleavage product in LNP-PCS-A2- and not in LNP-Crtl- or PBS-treated animals. Eighty-seven percent of clones from the circled bands mapped to the predicted siRNA specific cleavage site.

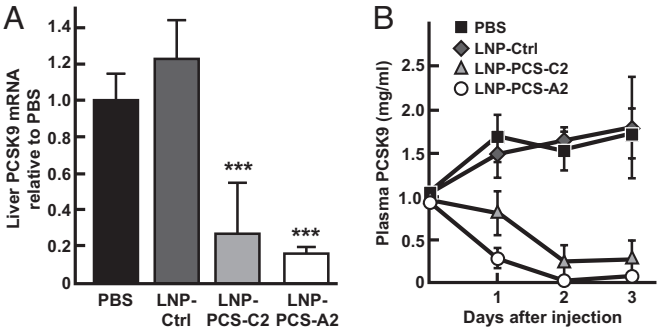

Fig. 4. Silencing of human PCSK9 mRNA and protein reduction in PCSK9 humanized mice. (*A*) Transgenic mice ($n = 4$ per group) expressing the human PCSK9 full-length cDNA under the apoE promoter were dosed with LNP-PCS-A2 and LNP-PCS-C2, and LNP-Crtl or PBS. Both LNP-PCS-C2 and LNP-PCS-A2 significantly lowered the human transcript as measured by quantitative PCR 3 days after dose. Each value is the group mean ± STDEV. (One-way ANOVA, Student's *t* test, $P \leq 0.001$ between PBS- and PCSK9 siRNA-treated groups). (*B*) Circulating human PCSK9 protein levels were reduced in treated vs. control transgenic mice ($n = 4$ per group) as measured by ELISA. Each value represents group mean ± STDEV. All time points for the PCSK9 siRNA treated groups are statistically significant compared with PBS (Two-way ANOVA, Bonferroni test, $P \leq 0.01$).

PCSK9 protein. Interestingly, the human/NHP selective PCS-C2 showed efficacy comparable to that of the cross-species PCS-A2. This result validates an approach where cross-species active siRNAs can be used in the development of RNAi therapeutic drugs.

RNAi Silencing of PCSK9 Acutely Reduces PCSK9 Protein and Plasma LDLc, but Not Plasma HDLc, in NHPs. The results above demonstrated that siRNAs targeting PCSK9 acutely lower both plasma PCSK9 protein and total cholesterol levels with an effect that lasts ≈3 weeks after a single dose in mice and rats. To extend these findings to a more relevant species with lipoprotein profiles that more closely resemble humans, we carried out similar experiments in cynomolgus monkeys.

Animals were randomized based on day 3 LDLc levels. On the day of dosing (designated day 1), PBS and 1 mg/kg or 5 mg/kg of LNP-PCS-A2, -B2, and -Crtl were administered as a single infusion over 30 min. As the experiment progressed, it became apparent that the 1 mg/kg dose was not efficacious in reducing plasma LDLc (Fig. S2). We therefore dosed the PBS group animals on day 14 with 5 mg/kg LNP-Crtl so that they could serve as controls for animals that received the 5 mg/kg LNP-PCS-A2 and LNP-PCS-B2. The PBS- and LNP-Crtl-treated animals behaved similarly for all measured endpoints (data not shown and Figs. S2 and S3*A*).

As shown in Fig. 5*A*, a single dose of 5 mg/kg LNP-PCS-A2 or LNP-PCS-B2 resulted in a statistically significant reduction of LDLc beginning at day 3 after the dose that returned to baseline over ≈14 days (for LNP-PCS-A2) and ≈21 days (LNP-PCS-B2). This effect was not observed in the PBS group, the LNP-Crtl group, or the 1 mg/kg treatment groups (Fig. 5*A* and Figs. S2 and S3*A*). LNP-PCS-B2 resulted in an average LDLc lowering of 56%, 72 h after the dose, with one of the four animals achieving nearly a 70% reduction in plasma LDLc compared with predose levels (see Fig. 5*A* and Fig. S3*A*). As expected, the lowering of LDLc in the treated animals correlated with a trend toward lower circulating apoB levels as measured by serum ELISA (Fig. S3*B*).

Neither LNP-PCS-A2 nor LNP-PCS-B2 treatments resulted in a lowering of HDLc in this study. In fact, both siRNAs resulted (on average) in a trend toward a decreased total cholesterol/HDLc ratio (Fig. 5*B*). In addition, plasma TG levels were not

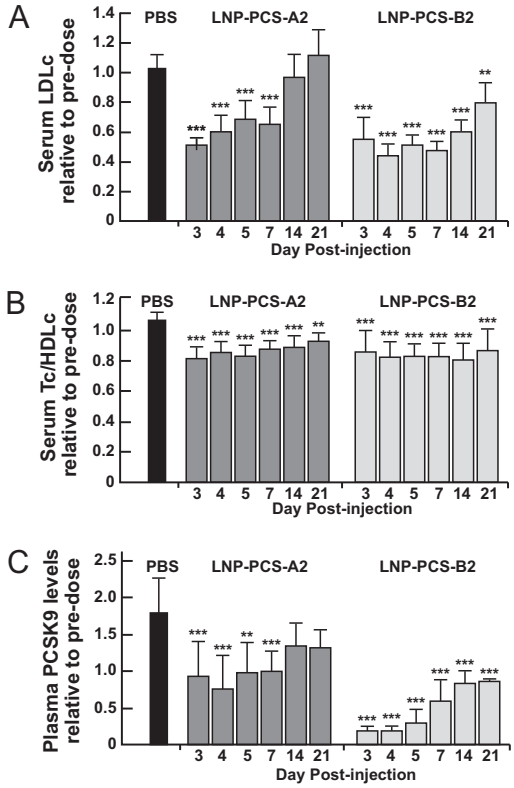

Fig. 5. Pharmacology of PCSK9 silencing in NHPs. (*A*) Direct LDLc measurements of serum from cynomolgus monkeys treated with 5 mg/kg LNP-PCS-A2 ($n = 5$ per group), LNP-PCS-B2 ($n = 4$ per group), or PBS ($n = 3$ per group). (*B*) Total cholesterol/HDL ratios as measured in the samples described in *A*. (*C*) Plasma samples from the treatment groups in *A* were analyzed for their levels of PCSK9 protein by ELISA. Values for LDLc, Tc/HDLc, and PCSK9 concentrations were graphed as a ratio of the average values of after dose compared with predose values within an animal. Those values were then combined into group averages. (*A–C*) LNP-Crtl behaved similarly on d4 and d7 as PBS (data not shown). PBS values are the mean of the groups ± STDEV averaged over days 3–14. Each value represents the mean of the group ± STDEV. Two-way ANOVA with Bonferroni test was used. Asterisks represent statistical difference between PBS- and PCSK9 siRNA-treated groups. **, $P \leq 0.01$; ***, $P \leq 0.001$.

altered (data not shown). ALT and AST levels were not significantly impacted (<3-fold induction over baseline in the same animal) with the exception of animal 6002, which had a 4- to 5-fold increased ALT and 4- to 9-fold increased AST (Table S2). We note that animals in this study were a mixture of naïve and nonnaïve animals (one naïve per group), with several animals having somewhat elevated ALT or AST at baseline (animal 6002 was nonnaïve). However, the cholesterol effects observed were independent of these measures as animals with no apparent increases in liver enzymes had similar LDLc reductions as animal 6002 (Table S2 and Fig. S3*A*).

Inasmuch as these experiments were nonterminal, we were unable to measure the reduction of transcript levels in the liver. As a surrogate for effects of siRNA treatment on PCSK9 transcript silencing, we measured plasma PCSK9 protein levels in both treated and control NHPs by ELISA. As shown in Fig. 5*C*, compared with predose concentrations, both LNP-PCS-A2 and LNP-PCS-B2 treatments significantly reduced plasma PCSK9 concentrations. One caveat to measuring circulating PCSK9 levels in the context of LNP01 formulated siRNAs is that the delivery and silencing of PCSK9 is greatest in the liver as opposed to other tissues, such as the intestine, where PCSK9 is also expressed. It therefore remains possible that our measurements are an underestimate of liver PCSK9 protein lowering.

Overall, however, the reduction in blood PCSK9 protein levels detected here is consistent with the extent and duration of LDLc and circulating apoB reduction observed.

Discussion

The current standard of care for hypercholesterolemia is adequate for many patients, yet falls far short in others who are unable to reach target LDLc levels with currently available therapies. Although only recently described, PCSK9 represents one of the best validated targets for the reduction of LDLc. Humans carrying *PCSK9* loss-of-function mutations have significantly lower plasma LDLc and are remarkably protected from cardiovascular disease (8). Furthermore, human compound heterozygous *PCSK9*-null individuals have been identified with very low LDLc (<20 mg/dl), but with otherwise normal health (24). Finally, the effects of PCSK9 have been shown to be highly conserved in animal models including *PCSK9* transgenic and knockout mice.

Here, we have shown that pharmacologic and RNAi-mediated reduction of PCSK9 transcript levels in liver achieves acute lowering of total plasma cholesterol levels in mice, rats, and cynomolgus monkeys. Silencing of PCSK9 mRNA in mice and rats was specific, and the silencing effects were proven to be mediated by an RNAi mechanism in rats using 5′-RACE. Delivery of the PCSK9 siRNA to the liver was facilitated by a lipidoid nanoparticle formulation as described in ref. 13. The formulated siRNAs silenced hepatic PCSK9 mRNA, resulting in a marked increase in liver LDLR protein levels. The same siRNA demonstrated silencing in mouse, rat, transgenic mouse, and NHP models systems.

An unexpected finding in the rat studies was the robust total cholesterol reduction found with PCSK9 silencing compared with the well described lack of a statin effect (14). As opposed to other proposed LDLc-lowering targets such as MTP or apoB, this effect was achieved with no evidence of increased liver triglycerides or other untoward effects, thereby further validating PCSK9 as the target of choice for therapeutic intervention. The effects of statins on plasma cholesterol are secondary to their effects on LDLR levels in NHPs and humans. Our results indicate that at least with regard to the level of PCSK9 and its control of the LDLR, the pathways in mice, rats, NHPs, and humans are conserved.

Rodents lack CETP and carry most of their plasma cholesterol in HDL particles. In addition, HDL in rodents also has a significant amount of apoE, which facilitates clearance by LDLRs. Genetic data in humans also confirms that loss of PCSK9 reduces LDLc but has no effect on HDLc (7). We therefore sought to show an acute and specific effect on LDLc lowering in a model closer to humans, the cynomolgus monkey. In studies with cynomolgus monkeys, we demonstrated that a single 30-min infusion of two different formulated PCSK9 siRNAs resulted in a highly significant, acute, specific, and durable reduction of plasma LDLc, apoB, and PCSK9 protein levels, but not HDLc or TGs. Specifically, siRNA-mediated reduction of PCSK9 mRNA and protein resulted in lowering of LDLc by ≈50–60% within 48 h after administration; this reduction lasted for nearly 3 weeks. If the ≈50–60% LDLc reduction observed in NHP were translated into humans, this amount of lowering would compare favorably with the LDLc reductions observed with current cholesterol absorption inhibitors (≈20% LDLc lowering in humans with ezetimibe) or HMG-CoA reductase inhibitors (≈21% LDLc lowering with pravastatin and ≈51% LDLc decreases at highest 80 mg/day atorvastatin over 30 days in the PROVE-IT-TIMI trial) (25, 26). In addition, observations in mice suggest that PCSK9 lowering and statin activity may act synergistically (5). It will be interesting to test whether this observation holds true in other animal models, such as NHPs. Finally, the acute onset of LDLc lowering obtained with PCSK9 siRNAs could prove beneficial in a subset of patients who suffer an acute myocardial infarction where the rapid onset lowering of LDLc may be desirable (27).

Together, the results presented here validate PCSK9 lowering by RNAi as a therapeutic approach with high potential for acutely reducing LDLc and pave the way for the development of novel PCSK9 lowering agents for use in the treatment of hypercholesterolemia.

Materials and Methods

Synthesis of siRNAs Targeting PCSK9. Single-stranded RNAs were produced at Alnylam Pharmaceuticals. Deprotection and purification of the crude oligoribonucleotides by anion exchange HPLC were carried out according to established procedures. siRNAs were generated by annealing equimolar amounts of complementary sense and antisense strands.

siRNA Formulation into LNP Lipidoid Nanoparticles. Stock solutions of lipidoid $98N_{12}$-5 (1)·4HCl, cholesterol, and $mPEG_{2000}$-DMG MW 2660 (synthesized by Alnylam) were prepared in ethanol and mixed to yield a molar ratio of 42:48:10 (13). siRNA was incorporated in the nanoparticles at 1:7.5 (wt:wt) siRNA:total lipids. Resulting particles had a mean particle diameter of ≈50 nm and siRNA entrapment efficiency of >95%.

***PCSK9 siRNA in Vitro* Screening in HepG2 Cells and Primary Cynomolgus Monkey Hepatocytes.** For siRNA transfection experiments, HepG2 or primary hepatocyte cells were seeded at 2.5×10^4 cells per well in 96-well plates. siRNA were transfected by using Lipofectamine 2000 according to the manufacturer's protocols. Cells were lysed 24 h after transfection, and PCSK9 mRNA levels were quantified by using the branched-DNA-technology-based QuantiGene Reagent System (Panomics), according to the manufacturer's protocols. PCSK9 mRNA levels were normalized to GAPDH mRNA.

5′-RACE was conducted as described in ref. 20 (see *SI Text*).

***In Vivo* Rodent Experiments.** All procedures used in animal studies conducted at Alnylam were approved by the Institutional Animal Care and Use Committee and were consistent with local, state, and federal regulations as applicable. Mice and rats were maintained on a 12-h light/12-h dark cycle and killed at the end of the dark cycle. C57BL/6 mice and Sprague–Dawley rats received either PBS or siRNA in lipidoid formulations via tail vein injection at a volume of 0.01 ml/g. After dosing, animals were anesthetized by isofluorane inhalation and blood was collected into serum separator tubes by retro-orbital bleed. Total cholesterol in mouse serum was measured by using the Wako Cholesterol E enzymatic colorimetric method (Wako Chemicals). In experiments where liver mRNA levels were assessed, livers were harvested and snap frozen in liquid nitrogen. Frozen liver tissue was ground and tissue lysates were prepared. PCSK9 mRNA levels relative to those of GAPDH mRNA were determined in the lysates by using a branched DNA assay (QuantiGene Reagent System, Panomics). LDLR protein was quantified by using 20 μg of liver membrane protein that was subjected to SDS/PAGE and transferred to nitrocellulose membranes as described in ref. 20, followed by immunoblotting and imaging by using LI-COR Odyssey infrared imaging system (28).

Studies in Transgenic Mice Expressing Human PCSK9. Transgenic mice that express human PCSK9 have been described in ref. 23. Human PCSK9 mRNA transcript was measured from liver extracts by using standard quantitative PCR methods as described in ref. 23. Human PCSK9 protein concentration in collected mouse plasma was carried out by using a sandwich ELISA method as described (20, 23).

NHP Study. Treatment of the animals was conducted by a certified contract research organization in accordance with the testing facility's standard operating procedure, which adheres to the regulations outlined in the United States Department of Agriculture Animal Welfare Act (9 CFR, Parts 1–3) and the conditions specified in the *Guide for the Care and Use of Laboratory Animals* (ILAR publication, 1996, National Academy Press). On the first day of dosing (day 1 or day 15 for PBS group redose), all monkeys were given a single 30-min i.v. infusion of LNP-formulated siRNAs. Blood samples were collected for pharmacodynamic analysis at various time points after dose administration. Serum chemistry experiments were carried out via direct measurement of LDLc, HDLc, TGs, and Tc.

PCSK9 ELISA. A quantitative assay for cynomolgus monkeys PCSK9 concentration in blood was carried out by using a sandwich ELISA method as previously reported for humans with minor modifications as described (see *SI Text*).

Other Materials and Methods. Detailed descriptions of experimental protocols and reagents are described in *SI Text*.

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