Improved efficacy for ezetimibe and rosuvastatin by attenuating the induction of PCSK9

Brandon Ason,†,‡,§ Samnang Tep,†,‡ Harry R. Davis Jr.,† Yiming Xu,†,‡ Glen Tetzloff,† Beverly Galinski,† Ferdie Soriano,§ Natalya Dubinina,† Lei Zhu,§ Alice Stefanni,† Kenny K. Wong,† Marija Tadin-Strapps,§ Steven R. Bartz,§ Brian Hubbard,† Mollie Ranalletta,† Alan B. Sachs,§ W. Michael Flanagan,§ Alison Strack,§ and Nelly A. Kuklin†

Sirna Therapeutics/Merck & Co. Inc.,§ San Francisco, CA 94158; and Cardiovascular and Metabolic Disease Research † and Genetically Engineered Models,§ Merck Research Laboratories, Rahway, NJ 07065

Abstract Reducing circulating LDL-cholesterol (LDL-c) reduces the risk of cardiovascular disease in people with hypercholesterolemia. Current approaches to reduce circulating LDL-c include statins, which inhibit cholesterol synthesis, and ezetimibe, which blocks cholesterol absorption. Both elevate serum PCSK9 protein levels in patients, which could attenuate their efficacy by reducing the amount of cholesterol cleared from circulation. To determine whether PCSK9 inhibition could enhance LDL-c lowering of both statins and ezetimibe, we utilized small interfering RNAs (siRNAs) to knock down Pcsk9, together with ezetimibe, rosuvastatin, and an ezetimibe/rosuvastatin combination in a mouse model with a human-like lipid profile. We found that ezetimibe, rosuvastatin, and ezetimibe/rosuvastatin combined lower serum cholesterol but induce the expression of Pcsk9 as well as the Srebp-2 hepatic cholesterol biosynthesis pathway. Pcsk9 knockdown in combination with either treatment led to greater reductions in serum non-HDL with a near-uniform reduction of all LDL-c subfractions. In addition to reducing serum cholesterol, the combined rosuvastatin/ezetimibe/Pcsk9 siRNA treatment exhibited a significant reduction in serum APOB protein and triglyceride levels. Taken together, these data provide evidence that PCSK9 inhibitors, in combination with current therapies, have the potential to achieve greater reductions in both serum cholesterol and triglycerides. Ason, B., S. Tep, H. R. Davis Jr., Y. Xu, G. Tetzloff, B. Galinski, F. Soriano, N. Dubinina, L. Zhu, A. Stefanni, K. K. Wong, M. Tadin-Strapps, S. R. Bartz, B. Hubbard, M. Ranalletta, A. B. Sachs, W. M. Flanagan, A. Strack, and N. A. Kuklin. Improved efficacy for ezetimibe and rosuvastatin by attenuating the induction of PCSK9. J. Lipid Res. 2011. 52: 679–687.

Supplementary key words cholesterol/metabolism • drug therapy • gene expression

Elevated circulating LDL cholesterol (LDL-c) is a risk factor for the development of atherosclerosis, and it is well documented that reducing the level of circulating LDL-c lowers the incidence of death from cardiovascular disease (as reviewed in Refs. 1–4). The level of circulating LDL-c is determined by the interplay between cholesterol synthesis, dietary cholesterol absorption, and cholesterol clearance (as reviewed in Refs. 5, 6). Current standard of care treatments to reduce serum LDL-c include statins, which inhibit cholesterol synthesis, and ezetimibe, which inhibits dietary and biliary cholesterol absorption (7–10). Both are effective means of reducing circulating LDL-c, although each inhibitor class induces a feedback mechanism leading to the induction of SREBP-2 (sterol-regulatory-element-binding protein-2). This leads to an increase in the expression of cholesterol biosynthesis genes, potentially blunting the efficacy of these treatments (11–17). In addition, induction of the SREBP-2 pathway potentially decreases LDL-c clearance through the increased expression of proprotein convertase subtilisin/kexin type 9 (Pcsk9) (18). PCSK9 increases circulating LDL-c by binding to and inducing the internalization and subsequent degradation of the LDL receptor (LDLr) (19–27). An increase in PCSK9 serum protein would thus reduce LDLr levels on the hepatic cell surface and consequently raise the amount of LDL-c in circulation (28–30). However, activation of the
SREBP-2 pathway also leads to increased \textit{Ldlr} expression. Therefore, the degree to which SREBP-2 pathway induction leads to decreased cholesterol clearance depends on the degree to which \textit{Pcsk9} expression is increased relative to \textit{Ldlr}.

PCSK9 inhibitors are currently being explored as a means to lower circulating LDL-c (31–38), and it is well-documented that both statin- and ezetimibe-treated patients exhibit elevated serum PCSK9 protein levels (7, 11–14, 39–43). A greater reduction in LDL-c is observed for \textit{Pcsk9} knockout mice administered statins, providing evidence that PCSK9 inhibitors, in combination with inhibitors of liver cholesterol synthesis, should result in a greater reduction in circulating LDL-c (20). Here, we evaluated whether the combined inhibition of cholesterol absorption, cholesterol synthesis, and increased LDL-c clearance could lead to greater reductions in circulating LDL-c by small interfering RNA (siRNA)-mediated knockdown of \textit{Pcsk9} in combination with ezetimibe, rosuvastatin, or an ezetimibe/rosuvastatin treatment.

**MATERIALS AND METHODS**

**siRNA design and synthesis**

siRNAs were designed and synthesized as described previously (44, 45). The complementary strands were annealed. The duplex was ultrafiltered and bophilized. Duplex purity was evaluated using LC/MS and tested for the presence of endotoxin by standard methods.

**Preparation of siRNA-lipid nanoparticle complex**

siRNA-lipid nanoparticles (LNPs) were made as described previously, except a 60:38:2 molar ratio of the cationic lipid C12MA (2-[4-[3-(3b)-cholest-5-en-3-yl oxy)-butoxy]-N,N,N,N-dimethyl-3-[(9Z,12Z)-octadeca-9,12-dien-1-yl oxy]propan-l-amine; Merck and Co), cholesterol (Northern Lipids), and PEG-DMG (monomethoxy(polyethyleneglycol)-1,2-dimyristoylglycerol; NOF Corporation) was used (46).

**In vivo**

C57Bl/6 mice engineered to be hemizygous for \textit{Ldlr} and the overexpression of \textit{hCetp} driven by the endogenous \textit{Apoa1} promoter (B6-Ldlr<tm1>Tg (APOA1-CETP, Taconic) were used for these studies. Female mice ~16–20 weeks of age were individually housed several days prior to the start of the study. At the start of the study, animals were switched to a low-fat Western diet (Lab Diets 5020 9F) containing 9% crude fat from breeder chow (Lab Diets 5001*) containing 4.5% crude fat. siRNAs were administered by intravenous injection. Both ezetimibe and rosuvastatin were formulated in 0.5% methyl cellulose and administered by oral gavage. Animals were euthanized by CO$_2$ inhalation. Immediately after euthanasia, serum was collected using serum separator tubes and allowed to clot at room temperature for 30 min. Liver sections were excised, placed in either RNA Later (right medial lobe), 10% neutral buffered formalin (NBF, left medial lobe), or flash frozen (the remainder) and stored until further use.

**RNA isolation, qRT-PCR, and ELISA**

RNA isolation and quantitative real-time PCR were performed using Qiagen’s RNeasy96 Universal Tissue Kit together with TaqMan Gene Expression reagents according to the supplied product protocol. An on-column DNase I treatment was performed, and samples were washed three times prior to elution in 100 µl RNase-free water. Reverse transcription was performed using the Cells to Ct kit (Ambion) in a 20 µl volume with 350 ng of RNA in 1× reverse transcriptase and buffer incubated at 37°C for 1 h. Changes in the expression of 361 genes (see supplementary Table I) involved in hepatic lipid metabolism were analyzed using a custom RT$^2$ Profiler PCR Array (SA Biosciences) according to the supplied product protocol.

PCSK9 serum protein levels were measured by a dissociation-enhanced lanthanide fluorescence immunoassay (DELFIA) as described previously (47). Briefly, Immulon 4HBX clear 96-well polystyrene high binding plates (ThermoLabsystems) were incubated with a monoclonal antibody reactive to mouse PCSK9. The antibody solution was removed, and 200 µl of 1× blocking buffer [1% BSA (Sigma) in 1× TBST (Sigma)] was added. Serum samples (20 µl) were incubated at 37°C for 1 h, and wells were subsequently washed three times with 300 µl of TBST. A second monoclonal antibody reactive to mouse PCSK9 and biotinylated (biotinylated anti-mPCSK9 Fab B) was added, and samples were incubated for 1 h to detect PCSK9. Wells were subsequently washed three times with 300 µl TBST. Samples were next incubated with a 1:1000 dilution of streptavidin-europium solution (PerkinElmer) at room temperature for 20 min with gentle agitation. Wells were washed three times with 300 µl TBST, 100 µl/well of DELFIA Enhancer (Perkin Elmer) solution was added, and samples were incubated for 30 min at room temperature with gentle agitation prior to measurement with a europium plate reader.

**Cholesterol and triglyceride analysis**

Liver samples of approximately 250 mg were frozen and stored at ~80°C until analysis. Individual samples were extracted according to the procedure of Folch, Lees, and Sloane-Stanley (48). Chromatography was performed as described by Burrier et al. (49) using an isocratic mobile phase containing 98.8% hexane and 1.2% isopropanol at a flow rate of 2 ml/min through a Zorbax Sil (4.6 × 25 cm) silica column (Agilent Technologies # 880952-701). Lipids in a 5 µl injection were detected by absorbance at 206 nm and quantitated by computer integration (System Gold, Beckman) of the area under the curve (AUC). Cholesterol, cholesterol ester (CE), and triglyceride concentrations were determined by comparison to standard curves using Nonpolar Lipid Mix-B (Matreya, Inc.; Pleasant Gap, PA), C/N 1130.

For serum lipase analysis, lipase inhibitor (Sigma-Aldrich) was added at a 1:100 (v/v) ratio of inhibitor to serum, followed by mixing (600 rpm at 4°C for 1.5 min) and centrifugation (2,000 rpm at 4°C for 2 min). Serum total and HDL levels were measured using Wako’s total and HDL kits according to the supplied product protocol. Non-HDL was calculated by subtracting HDL from total cholesterol measurements.

For LDL subfraction analysis, the lipoprotein profile of the mouse plasma was analyzed using gradient gel electrophoresis (Lipoprint LDL Subfraction System, Quantimetrix) according to the recommended protocol. The resolved lipoprotein bands were quantified based on lipid content using the software provided by the vendor.

**Histology and hematology**

Mouse liver samples (in 10% NBF) were processed to paraffin, sectioned, and stained with hematoxylin and eosin (H and E). The H and E-stained slides were reviewed by a board-certified veterinary pathologist for inflammation. Liver toxicity was measured by analyzing serum ALT (alanine aminotransferase), AST (aspartate aminotransferase), and LDH (lactate dehydrogenase) levels from 80 µl of serum using ACE Alera® Clinical Chemistry System (Alfa Wassermann, Inc.).
RESULTS

siRNA mediated knockdown of Pcsk9 reduced serum non-HDL in combination with a maximal efficacious dose of ezetimibe

Wild-type mice typically have very low LDL-c levels, and a strain with elevated LDL-c levels was specifically chosen to provide a better window to assess reductions in LDL-c. This strain contains hemizygous mutations resulting in the partial knockdown of Ldlr (Ldlr<sup>+/−</sup>) and overexpression of the human CE transferase protein (Cetp) gene driven by the mouse Apoa1 promoter (Apoa1<sup>hCetp<sub>+/−</sub></sup>). These two mutations increased circulating LDL-c and led to a lipid profile that resembled the lipid profile observed in humans. (Tadin-Strapps et al., unpublished observations).

We first evaluated the efficacy of ezetimibe in this model by administering a maximum efficacious dose of ezetimibe (10 mg/kg/day), daily, for 7 days. Following 7 days of treatment, serum non-HDL, which serves as a close approximation of LDL-c in this model, decreased by 21% (P < 0.0001), whereas Pcsk9 liver mRNA and serum PCSK9 protein levels increased by 2- and 3-fold (P < 0.0001 and P < 0.0001), respectively (see supplementary Figs. I, II; one-way ANOVA, Tukey posttest).

To investigate whether Pcsk9 knockdown could further reduce serum cholesterol when administered with ezetimibe, we utilized siRNAs designed against the mouse Pcsk9 mRNA transcript and formulated in a lipid nanoparticle (LNP) to achieve liver-targeted Pcsk9 knockdown. Mice were administered a maximum efficacious dose of ezetimibe (10 mg/kg/day) for 7 days, and on day 4, animals were administered a maximum tolerated dose (6 mg/kg) of a Pcsk9 siRNA [Pcsk9(1035)]. PCSK9 (liver mRNA and serum protein) levels were elevated 2-fold (P < 0.0001, respectively) following ezetimibe treatment. Both were reduced 4-fold (P < 0.0001) following the administration of the Pcsk9 siRNA (see supplementary Fig. IIA, B; one-way ANOVA, Tukey posttest). The combination of ezetimibe with Pcsk9(1035) led to a 50% (P < 0.0001) reduction in serum non-HDL relative to the vehicle/control siRNA treatment group and a 36% (P < 0.0001) and 33% (P < 0.0001) reduction relative to the ezetimibe and Pcsk9 siRNA individual treatments, respectively (see supplementary Fig. IIC; one-way ANOVA, Tukey posttest).

Both liver enzymes and pathological analysis of hematoxylin and eosin stained liver sections were evaluated to determine the impact that these treatments had on liver function. Liver enzyme levels (ALT, AST, and LDH) were within a range consistent with normal hepatic function, with no significant variation observed across groups (see supplementary Fig. IIA–C). Additionally, hematoxylin and eosin stained liver sections were reviewed by a board-certified pathologist and scored utilizing a subjective scoring system for the degree of inflammation. In all cases, inflammation was scored as minimal or mild and would not be expected to impede hepatic function (see supplementary Fig. IIID–E).

siRNA mediated knockdown of Pcsk9 led to greater reductions in both serum non-HDL and serum APOB protein levels in combination with ezetimibe, rosuvastatin, and ezetimibe/rosuvastatin treatments

In addition to ezetimibe, statins have been reported to elevate serum PCSK9 protein levels, either alone or in combination with ezetimibe (39–41, 43). To determine whether Pcsk9 knockdown can further reduce serum cholesterol following ezetimibe, statin, or an ezetimibe/statin combination, we treated mice with ezetimibe (10 mg/kg/day), rosuvastatin (20 mg/kg/day), or the combination for 14 days, and on day 11, administered a 6 mg/kg dose of another Pcsk9 targeting siRNA [Pcsk9(1076)]. Both Pcsk9 liver mRNA and serum PCSK9 protein levels were significantly elevated, 2-fold (P < 0.0001) for the rosuvastatin and 4-fold (P < 0.0001) for the ezetimibe/rosuvastatin combination. In this experiment, only a slight but not significant elevation was observed for ezetimibe relative to the vehicle/control siRNA treatment (Fig. 1A, B; one-way ANOVA, Tukey posttest). Administration of the Pcsk9 siRNA reduced both PCSK9 liver mRNA and serum protein levels, and this was associated with an increase in hepatic LDLr (see supplementary Fig. IV). Administration of the Pcsk9 siRNA with either ezetimibe or rosuvastatin reduced PCSK9 below the levels observed for the vehicle/control siRNA treatment (Fig. 1A, B; RSV/EZ/cntrl vs. RSV/EZ/Pcsk9).

Ezetimibe, rosuvastatin, and Pcsk9(1076) significantly reduced serum non-HDL levels by ~25% (P < 0.0001) relative to the vehicle/control siRNA treatment (Fig. 1C; one-way ANOVA, Tukey posttest). For both ezetimibe and Pcsk9(1076), this was associated with a significant reduction in serum apolipoprotein B (APOB), 18% (P < 0.001) and 33% (P < 0.0001) for ezetimibe and Pcsk9(1076), respectively (Fig. 2D; one-way ANOVA, Tukey posttest). Pcsk9(1076), in combination with either ezetimibe or rosuvastatin, led to even greater reductions in both serum non-HDL (43–65%), and APOB (38–48%) relative to the individual treatments (Fig. 1C, D). The combination of Pcsk9(1076) with the ezetimibe/rosuvastatin treatment led to significantly lower serum non-HDL cholesterol levels relative to the other combination groups (Fig. 1C; one-way ANOVA, Tukey post test) [compare RSV/EZ/Pcsk9(1076) to EZ/Pcsk9(1076) where differences of 53% (P < 0.0001) were observed, RSV/EZ/Pcsk9(1076) to RSV/Pcsk9(1076) where differences of 68% (P < 0.0001) were observed, and RSV/EZ/Pcsk9(1076) to EZ/RSV where differences of 48% (P < 0.0001) were observed]. In addition, significant reductions in serum APOB were observed for the RSV/EZ/Pcsk9(1076) group relative to EZ/Pcsk9(1076) (32%, P < 0.05), RSV/Pcsk9(1076) (33%, P < 0.001), and EZ/RSV (43%, P < 0.0001) groups (Fig. 1D; one-way ANOVA, Tukey posttest). The observed changes to PCSK9, LDLr, non-HDL, and APOB were confirmed with the Pcsk9(1035) sequence for the triple combination relative to EZ/Pcsk9(1035),
be more prone to uptake by macrophages in the atheroma (50). Here, we analyzed the size distribution of LDL particles using a gradient gel electrophoresis system (LDL Lipoprint) along with a human serum control (Liposure). The gel system can resolve serum lipoproteins to discrete bands consisting of VLDL, IDL bands C, B, A, LDL subfractions 1 to 7, and an HDL band. For normal human serum, LDL is distributed among subfractions 1 to 4, peaking at subfraction 1 (51). In our mouse model with a human-like serum lipid profile, LDL has a broader distribution centered at subfraction 2 (Fig. 2A, B). All combinations of rosuvastatin, ezetimibe, and the Pcsk9 siRNA that were evaluated resulted in a near-uniform reduction in LDL subfractions, suggesting that the combination treatments do not disproportionately lower a particular LDL subfraction (Fig. 2).

The rosuvastatin/ezetimibe/Pcsk9 siRNA treatment reduced serum triglycerides

In addition to cholesterol, elevated serum triglyceride levels have been associated with atherosclerosis (52, 53).

RSV/Pcsk9(1035), and EZ/RSV treatments (see supplementary Figs. V–VII; compare RSV/EZ/Pcsk9(1035) to EZ/Pcsk9(1035) where differences of 65% for non-HDL (P < 0.0001) and 27% for APOB (P < 0.05) were observed, RSV/EZ/Pcsk9(1035) to RSV/Pcsk9(1035) where differences of 74% for non-HDL (P < 0.0001) and 45% for APOB (P < 0.0001) were observed, RSV/EZ/Pcsk9(1035) to RSV/EZ where differences of 55% for non-HDL (P < 0.0001) and 47% for APOB (P < 0.0001) were observed). Taken together, these data demonstrate that siRNA-mediated knockdown of Pcsk9 negates the ezetimibe/rosuvastatin-induced increase in PCSK9 (liver mRNA and serum protein) leading to greater cholesterol lowering.

A near-uniform reduction in all LDL-c subfractions was observed following combination treatments

Having a disproportionate number of small LDL particles has been demonstrated to be an independent risk factor for coronary heart disease, and it has been proposed that small LDL particles may reside longer within circulation and may

![Diagram of Pcsk9 mRNA and Serum PCSK9 levels](image1)

**Fig. 1.** Small interfering RNA (siRNA)-mediated knockdown of Pcsk9 led to greater reductions in both serum cholesterol and serum apolipoprotein B (APOB) protein levels in combination with ezetimibe, rosuvastatin, and ezetimibe/rosuvastatin treatments. (A) Pcsk9 mRNA expression and (B) serum PCSK9 protein levels following treatment. (C) siRNA-mediated knockdown of Pcsk9 resulted in a significant decrease in serum non-HDL and (D) serum APOB protein levels in combination with ezetimibe, rosuvastatin, and ezetimibe/rosuvastatin treatments. Vehicle/PBS and Vehicle/cntrl siRNA groups serve as negative controls. Individual animals (circles) and group means (bars) are shown. P values and the percent difference were calculated relative to the Vehicle/cntrl siRNA group unless otherwise indicated by a bar. * P < 0.05, ** P < 0.001, *** P < 0.0001.
Pcsk9 knockdown improves sensitivity to cholesterol lowering drugs

Table 1, and see supplementary Table I). In contrast to the ezetimibe and ezetimibe/rosuvastatin treatments, Pcsk9 knockdown did not result in a significant increase in the expression of Srebp-2 pathway genes (see supplementary Table I), even though serum PCSK9 protein levels were significantly ($P < 0.0001$) reduced (Fig. 1B, see supplementary Fig. IIB; Vehicle/Pcsk9 siRNA vs. Vehicle/cntrl siRNA), and there was a significant decrease (21–25%, $P < 0.0001$) in serum non-HDL (Fig. 1C, and see supplementary Fig. IIC; Veh/Pcsk9 siRNA vs. Veh/Cntrl). Consistent with Pcsk9 knockdown resulting in only minor changes to the hepatic gene signature, the level of Srebp-2 pathway induction was similar for the ezetimibe and the ezetimibe/Pcsk9 siRNA combination treatments. This was also found for the ezetimibe/rosuvastatin treatment relative to ezetimibe/rosuvastatin/Pcsk9 siRNA treatment (Table 1).

Both ezetimibe and statins have been reported to influence the expression of numerous genes involved in hepatic lipid metabolism (12, 16, 17). We analyzed the expression of 361 genes involved in hepatic lipid metabolism using qRT-PCR and found that many genes within the Srebp-2 pathway were induced following ezetimibe treatment (2.5-fold average induction relative to control); these were induced further (11-fold average induction relative to control) following the ezetimibe/rosuvastatin combination treatment (Table 1, and see supplementary Table I). In contrast to the ezetimibe and ezetimibe/rosuvastatin treatments, Pcsk9 knockdown did not result in a significant increase in the expression of Srebp-2 pathway genes (see supplementary Table I), even though serum PCSK9 protein levels were significantly ($P < 0.0001$) reduced (Fig. 1B, see supplementary Fig. IIB; Vehicle/Pcsk9 siRNA vs. Vehicle/cntrl siRNA), and there was a significant decrease (21–25%, $P < 0.0001$) in serum non-HDL (Fig. 1C, and see supplementary Fig. IIC; Veh/Pcsk9 siRNA vs. Veh/Cntrl). Consistent with Pcsk9 knockdown resulting in only minor changes to the hepatic gene signature, the level of Srebp-2 pathway induction was similar for the ezetimibe and the ezetimibe/Pcsk9 siRNA combination treatments. This was also found for the ezetimibe/rosuvastatin treatment relative to ezetimibe/rosuvastatin/Pcsk9 siRNA treatment (Table 1).

Both CEs and free cholesterol (FC) were measured by HPLC to determine whether the observed induction of the Srebp-2 pathway led to the accumulation of cholesterol within the liver. The hepatic levels of CE and FC were not elevated, despite increased expression of Srebp-2 pathway genes following ezetimibe and ezetimibe/rosuvastatin treatment, and in fact, a modest decrease in CE was observed for most groups relative to controls (see supplementary Fig. VIII).
It is worth noting that HDL was reduced in some experiments following \( \text{Pcsk9} \) siRNA, ezetimibe, rosuvastatin, or combination treatment. Reductions in HDL have been reported for siRNA-mediated knockdown (KD) of \( \text{ApoB} \), and it remains possible that the observed reductions in HDL are due to a reduction in serum \( \text{APOB} \) following \( \text{Pcsk9} \) siRNA, ezetimibe, rosuvastatin, or combination treatment. The mechanism behind \( \text{APOB} \)-mediated changes in HDL is unknown, although it has been proposed that \( \text{APOB} \) could influence mature HDL particles indirectly through the reduction of HDL components supplied by \( \text{APOB} \)-containing particles (54).

Both rosuvastatin and ezetimibe led to a decrease in serum cholesterol but induced the expression of the \( \text{Srebp-2} \) pathway in our model, which is also consistent with several previous reports (12, 16, 17, 55–57). Induction of the \( \text{Srebp-2} \) pathway most likely serves as a homeostatic response, probably blunting the efficacy of these treatments. Interestingly, siRNA-mediated knockdown of \( \text{Pcsk9} \) did not induce the \( \text{Srebp-2} \) pathway, although we cannot rule out the possibility that prolonged knockdown of \( \text{Pcsk9} \) (>3 days) would not have this effect.

In addition, ezetimibe and rosuvastatin induced PCSK9 (liver mRNA and serum protein), consistent with both

**DISCUSSION**

Here, we report that treatment with two distinct \( \text{Pcsk9} \)-targeting siRNAs results in a modest decrease in serum non-HDL and APOB protein levels in a mouse model with a human-like serum lipid profile (\( \text{Apoa1} \) promoter-driven \( \text{hCetp}+/- \) and \( \text{Ldlr}+/- \) mice). The results reported here are consistent with the reported decrease in serum cholesterol observed following monoclonal antibody-, siRNA-, and antisense-mediated inhibition of PCSK9 as well as for \( \text{Pcsk9}^{-/-} \) mice (20, 34–38, 47). Collectively, these data demonstrate the effectiveness of inhibiting PCSK9 in reducing serum cholesterol using multiple methods in several models, including wild-type mice, diet-induced hyperlipidemic mice, and mice engineered to exhibit a healthy human lipid profile. It has been proposed that serum cholesterol levels in \( \text{hCetp}+/- \text{Ldlr}+/- \) mice may be more sensitive to LDL-c-lowering treatments relative to wild-type mice, since the lipid profile is no longer dominated by HDL. \( \text{hCetp}+/- \text{Ldlr}+/- \) mice, therefore, represent an attractive alternative to mouse models of hyperlipidemia, and the fact that they are heterozygous for \( \text{Ldlr} \) expression may make this model more representative of the response expected from familial hypercholesterolemia heterozygotes.

**TABLE 1.** The \( \text{Srebp-2} \) pathway is induced following ezetimibe and ezetimibe/rosuvastatin combination treatments

| Gene involved in the \( \text{Srebp2} \) pathway | \( \text{Pcsk9} \) | \( \text{EZ} \) | \( \text{EZ}/\text{Pcsk9} \) | \( \text{RSV/EZ} \) | \( \text{RSV/EZ}/\text{Pcsk9} \) |
|-------------------------------|----------------|----------------|----------------|----------------|----------------|
| \( \text{Hmgcr} \) | 1.5 0.02 0.000078 | 2.4 0.000091 18.7 0.000008 | 13.0 0.000055 |
| \( \text{Hmgcs1} \) | 1.0 0.8 2.8 0.000006 | 2.4 0.000001 9.9 0 | 8.7 0 |
| \( \text{Mvk} \) | 1.3 0.02 1.8 0.000095 | 2.2 0.000004 9.5 0 | 8.9 0.000054 |
| \( \text{Pmvk} \) | 1.1 0.06 1.9 0 | 1.4 0.000023 6.0 0 | 4.6 0 |
| \( \text{Mvd} \) | 1.0 0.9 2.5 0.000013 | 2.3 0.000006 10.4 0 | 10.8 0.000004 |
| \( \text{Idil} \) | 1.2 0.05 3.0 0 | 2.7 0.000001 20.8 0 | 20.3 0.000001 |
| \( \text{Fdp1} \) | 1.2 0.2 2.2 0 | 1.7 0.000003 3.3 0 | 5.4 0 |
| \( \text{Cyp51a1} \) | 1.1 0.28 2.1 0 | 1.9 0 8.4 0 | 6.8 0 |
| \( \text{Dhcr7} \) | 1.2 0.01 2.0 0.000001 | 2.3 0 14.2 0 | 17.2 0 |

The expression of selected \( \text{Srebp2} \) pathway genes were analyzed using qRT-PCR. The fold regulation was calculated for the treatment group relative to the Veh/cntrl siRNA group. \( P \) values were calculated using a two-tailed t-test between control and treatment groups. The analysis software contains a 6-digit cut-off. Therefore, \( P \) values below 6-digits are represented with a 0.
been shown to shift the distribution in some instances. Our findings demonstrate that knocking-down \textit{Pcsk9} can counter this response leading to greater reductions in serum non-HDL and APOB protein levels. These data provide evidence that PCSK9 inhibitors could serve as a follow-on therapy to counter the feedback mechanism leading to the induction of \textit{Pcsk9}, which could be of value to patients who are unable to reduce their LDL-c to their target goal using current treatment strategies. The combined ezetimibe/rosuvastatin/\textit{Pcsk9} siRNA treatment additionally led to a reduction in serum triglycerides. Taken together, these data suggest that inhibiting PCSK9, in combination with these two cholesterol-lowering therapies, can improve the lipid profile for dislipidemic patients.

It is also worth noting that although the HDL-to-LDL ratio in our model is comparable to the ratio observed for healthy humans, the distribution of the LDL subfraction is broader, with a greater percentage of LDL consisting of large and small LDL subfractions. In the clinic, pravastatin is broader, with a greater percentage of LDL consisting of smaller LDL subfractions. Following ezetimibe, rosuvastatin, \textit{Pcsk9} siRNA, or combination treatments, we observed a near-uniform reduction in both large and small LDL subfractions. In the clinic, pravastatin and simvastatin have generally been reported to only moderately impact the distribution of the LDL subfraction, whereas fluvastatin, atorvastatin, and rosuvastatin have all been shown to shift the distribution in some instances (58–61). Here, we observe no appreciable shift following rosuvastatin treatment, which could be due either to differences between our model and humans or differences in the duration of treatment (14 days in our study compared with 8 weeks in the clinic). The impact of ezetimibe on LDL subparticle distribution varies depending on the clinical study (59, 60, 62). Such variability between studies makes meaningful comparisons to our data set in mice difficult.

Finally, it is important to note that these data demonstrate the utility of siRNAs as tools for target validation. Given the speed with which siRNAs can be designed, synthesized, and validated against a target of interest, we believe that siRNAs will become a frequently used tool to help identify potential product combinations at an earlier phase of drug development.

The authors would like to thank Duncan Brown for siRNA design, Dipali Ruhela and her group for providing the LNP formulations, Michael Krimm for liver enzyme analysis, and Premier Laboratory for histological services and support with pathology review.

REFERENCES

1. Steinberg, D. 2006. Thematic review series: The Pathogenesis of Atherosclerosis. An interpretive history of the cholesterol controversy, part V: The discovery of the statins and the end of the controversy. \textit{J. Lipid Res.} 47: 1359–1351.

2. Steinberg, D. 2005. Thematic review series: the pathogenesis of atherosclerosis. An interpretive history of the cholesterol controversy, part II: the early evidence linking hypercholesterolemia to coronary disease in humans. \textit{J. Lipid Res.} 46: 179–190.

3. Steinberg, D. 2005. Thematic review series: the pathogenesis of atherosclerosis: an interpretive history of the cholesterol controversy, part III: Mechanistically defining the role of hyperlipidemia. \textit{J. Lipid Res.} 46: 2037–2051.

4. Steinberg, D. 2004. Thematic review series: the pathogenesis of atherosclerosis. An interpretive history of the cholesterol controversy: part I. \textit{J. Lipid Res.} 45: 1583–1595.

5. Maxfield, F. R., and I. Tabas. 2005. Role of cholesterol and lipid organization in disease. \textit{Nature} 438: 612–621.

6. Gilling, H. 2004. Cholesterol metabolism and its implications for therapeutic interventions in patients with hypercholesterolaemia. \textit{Int. J. Clin. Pract.} 58: 859–866.

7. Altmann, S. W., H. R. Davis, Jr., L. J. Zhu, X. Yao, L. M. Hoos, G. Tetzloff, S. P. Iyer, M. Maguire, A. Golovko, M. Zeng, et al. 2004. Niemann-Pick CI Like 1 protein is critical for intestinal cholesterol absorption. \textit{Science} 303: 1201–1204.

8. Endo, A., M. Kuroda, and Y. Tsujita. 1976. ML-236A, ML-236B, and ML-256C, new inhibitors of cholesterologenesis produced by Penicillium citrinum. \textit{J. Antibiot.} (Tokyo) 29: 1346–1348.

9. Endo, A., M. Kuroda, and K. Tanazawa. 1976. Competitive inhibition of 3-hydroxy-3-methylglutaryl coenzyme A reductase by ML-236A and ML-256B fungal metabolites, having hypercholesterolemic activity. \textit{FEBS Lett.} 72: 323–326.

10. Tsujita, Y., M. Kuroda, K. Tanazawa, N. Kitano, and A. Endo. 1979. Hypolipidemic effects in dogs of ML-236B, a competitive inhibitor of 3-hydroxy-3-methylglutaryl coenzyme A reductase. \textit{Atherosclerosis} 32: 307–313.

11. Davis, H. R., Jr., L. J. Zhu, L. M. Hoos, G. Tetzloff, M. Maguire, J. Liu, X. Yao, S. P. Iyer, M. H. Lam, E. G. Lund, et al. 2004. Niemann-Pick CI Like 1 (NPC1L1) is the intestinal phytosterol and cholesterol transporter and a key modulator of whole-body cholesterol homeostasis. \textit{J. Biol. Chem.} 279: 33586–33592.

12. Halleck, M., H. R. Davis, P. Kirschmeier, D. Levitan, R. D. Snyder, K. Treinen, and J. S. Macdonald. 2009. An assessment of the carni-nogenic potential of ezetimibe using nonclinical data in a weight-of-evidence approach. \textit{Toxicology} 258: 116–130.

13. Sudhop, T., M. Reber, D. Tribble, A. Sapare, W. Taggart, P. Gibbons, T. Mühlen, K. von Bergmann, and D. Lütjohann. 2009. Changes in cholesterol absorption and cholesterol synthesis caused by ezetimibe and/or simvastatin in men. \textit{J. Lipid Res.} 50: 2117–2123.

14. Sudhop, T., D. Lütjohann, A. Kodal, M. Igel, D. L. Tribble, S. Shah, I. Perevozskaya, and K. von Bergmann. 2002. Inhibition of intestinal cholesterol absorption by ezetimibe in humans. \textit{Circulation} 106: 1943–1948.

15. Goldstein, J. L., R. A. DeBoe-Bovd, and M. S. Brown. 2006. Protein sensors for membrane sterols. \textit{Cell} 124: 35–46.

16. Roglans, N., J. C. Verd, C. Peris, M. Alegret, M. Vázquez, T. Adzet, C. Díaz, G. Hernández, J. C. Laguna, and R. M. Sánchez. 2002. High doses of atorvastatin and simvastatin induce key enzymes involved in VLDL production. \textit{Lipids} 37: 445–454.

17. Scharnagl, H., R. Schinker, H. Giersen, M. Nauck, H. Wieland, and W. Marz. 2001. Effect of atorvastatin, simvastatin, and lovastatin on the metabolism of cholesterol and triacylglycerides in HepG2 cells. \textit{Biochem. Pharmacol.} 62: 1545–1555.

18. Horton, J. D., N. A. Shah, J. A. Warrington, N. N. Anderson, S. W. Park, M. S. Brown, and J. L. Goldstein. 2003. Combined analysis of oligonucleotide microarray data from transgenic and knockout mice identifies direct SREBP target genes. \textit{Proc. Natl. Acad. Sci. USA} 100: 12027–12032.

19. Maxwell, K. N., and J. L. Breslow. 2004. Adenoviral-mediated expression of Pcsk9 in mice results in a low-density lipoprotein receptor knockout phenotype. \textit{Proc. Natl. Acad. Sci. USA} 101: 7100–7105.

20. Rashid, S., D. E. Curtis, R. Garuti, N. N. Anderson, Y. Bashmakov, Y. K. Ho, R. E. Hammer, Y. A. Moon, and J. D. Horton. 2005. Decreased plasma cholesterol and hypersensitivity to statins in mice lacking Pcsk9. \textit{Proc. Natl. Acad. Sci. USA} 102: 5374–5379.

21. Lagace, T. A., D. E. Curtis, R. Garuti, M. C. McNutt, S. W. Park, H. B. Prather, N. N. Anderson, Y. K. Ho, R. E. Hammer, and J. D. Horton. 2006. Secreted PCSK9 decreases the number of LDL receptors in hepatocytes and in livers of parabiotic mice. \textit{J. Clin. Invest.} 116: 2995–3005.

22. Cohen, J. C., E. Boerwinkle, T. H. Mosley, Jr., and H. H. Hobbs. 2006. Sequence variations in PCSK9, low LDL, and protection against coronary heart disease. \textit{N. Engl. J. Med.} 354: 1294–1272.

23. Hallman, D. M., S. R. Steinurs, W. Chen, E. Boerwinkle, and G. S. Berenson. 2007. Relation of PCSK9 mutations to serum low-density
lipoprotein cholesterol in childhood and adulthood (from The Bogalusa Heart Study). Am. J. Cardiol. 100: 69–72.

24. Hooper, A. J., A. D. Marais, D. M. Tanyanyiwa, and J. R. Burnett. 2007. The C670X mutation in PCSK9 is present and lowers blood cholesterol in a Southern African population. Atherosclerosis. 193: 445–448.

25. Zhao, Z., Y. Tsaklil-Wosornu, T. A. Lagace, L. Kinch, N. V. Grishin, J. D. Horton, J. C. Cohen, and H. H. Hobbs. 2006. Molecular characterization of loss-of-function mutations in PCSK9 and identification of a compound heterozygote. Am. J. Hum. Genet. 79: 514–523.

26. Kwon, D. H., A. D. Marais, D. M. Tanyanyiwa, and J. R. Burnett. 2008. A proprotein convertase subtilisin/kexin type 9 (PCSK9) C-terminal domain antibody antigen-binding fragment inhibits PCSK9 internalization and restores low density lipoprotein uptake. J. Biol. Chem. 283: 12882–12891.

27. Li, Y. G., J. H. Condra, L. Orsatti, X. Shen, D. Di Marco, S. Pandit, M. J. Bottomley, L. Ruggeri, R. T. Cummings, R. M. Cubbon, et al. 2010. A proprotein convertase subtilisin/kexin type 9 neutralizing antibody reduces serum cholesterol in mice and nonhuman primates. Proc. Natl. Acad. Sci. USA. 106: 9820–9825.

28. Shao, L., L. Pang, R. Zhang, N. J. Majerus, H. Lan, and J. A. Hedrick. 2009. PCSK9 binds to multiple receptors and can be functionally inhibited by an EGF-A peptide. Biochem. Biophys. Res. Commun. 375: 69–73.

29. Ni, Y. G., J. H. Condra, L. Orsatti, X. Shen, D. Di Marco, S. Pandit, M. J. Bottomley, L. Ruggeri, R. T. Cummings, R. M. Cubbon, et al. 2010. A proprotein convertase subtilisin/kexin type 9 neutralizing antibody reduces serum cholesterol in mice and nonhuman primates. Proc. Natl. Acad. Sci. USA. 106: 9820–9825.

30. Dong, B., D. W. Zhang, T. A. Lagace, R. Garuti, Z. Zhao, M. McDonald, J. D. Horton, J. C. Cohen, and H. H. Hobbs. 2006. Binding of proprotein convertase subtilisin/kexin type 9 to epidermal growth factor-like repeat A of low density lipoprotein receptor decreases receptor recycling and increases degradation. J. Biol. Chem. 281: 18602–18612.

31. Davis, H. R., K. K. Pula, K. B. Alton, R. E. Burrier, and R. W. Watkins. 2001. The synergistic hypcholesterolemic activity of the potent cholesterol absorption inhibitor, ezetimibe, in combination with 3-hydroxy-3-methylglutaryl coenzyme a reductase inhibitors in dogs. Metabolism. 50: 1234–1241.

32. Kadowaki, J. J., J. S. Zuideman, M. L. Yots, A. F. Stalenhoef, E. L. Visseren, E. J. Sibstands, M. D. Tipt, E. A. Stein, et al. 2008. Simvastatin with or without ezetimibe in familial hypercholesterolemia. N. Engl. J. Med. 358: 1431–1443.

33. Greffhorst, A., M. C. McNutt, T. A. Lagace, and J. D. Horton. 2008. Plasma PCSK9 preferentially reduces liver LDL receptors in mice. J. Lipid Res. 49: 1303–1311.

34. Caresch, J. C., D. E. Phiper, Q. Cao, D. Liu, C. King, W. Wang, J. Tang, Q. Liu, J. Higbee, Z. Xia, et al. 2009. A proprotein convertase subtilisin/kexin type 9 neutralizing antibody reduces serum cholesterol in mice and nonhuman primates. Proc. Natl. Acad. Sci. USA. 106: 9820–9825.

35. Ni, Y. G., J. H. Condra, L. Orsatti, X. Shen, D. Di Marco, S. Pandit, M. J. Bottomley, L. Ruggeri, R. T. Cummings, R. M. Cubbon, et al. 2010. Antibody-mediated disruption of the interaction between PCSK9 and the low-density lipoprotein receptor. J. Lipid Res. 52: 78–86.

36. Folch, J., M. Lees, and G. Sloane Stanley. 1957. A simple method for the isolation and purification of total lipides from animal tissues. J. Biol. Chem. 226: 497–509.

37. Burrier, R. E., A. A. Smith, D. G. McGregor, L. M. Hoos, D. L. Zilli, and H. R. Davis, Jr. 1995. The effect of acyl CoA cholesterol acyltransferase inhibition on the uptake, esterification and secretion of cholesterol by the hamster small intestine. J. Pharmacol. Exp. Ther. 272: 156–163.

38. Coresh, J., P. O. Keiterovich, J., H. H. Smith, and P. S. Bachorik. 1993. Association of plasma triglyceride concentration and LDL particle diameter, density, and chemical composition with premature coronary disease in men and women. J. Lipid Res. 34: 1667–1697.

39. Hoefner, D. M., S. D. Hodel, J. F. O’Brien, E. L. Branum, D. Sun, I. Rosenman, and P. M. Smith. 2004. Development of a rapid, quantitative method for LDL subclassification with use of the Quanimetrix Lipoprint LDL System. Clin. Chem. 47: 266–274.

40. Austin, M. A., J. E. Hokanson, and K. L. Edwards. 1998. Hypertriglyceridemia as a cardiovascular risk factor. Am. J. Cardiol. 81: 12B–128.

41. Hokanson, J. E., and M. A. Austin. 1996. Plasma triglyceride level is a risk factor for cardiovascular disease independent of high-density lipoprotein cholesterol level: a meta-analysis of population-based prospective studies. J. Cardiovasc. Risk. 3: 213–219.

42. Lieu, H. D., S. K. Withcombe, Q. Walker, J. X. Rong, R. L. Walzem, J. S. Wong, R. L. Hamilton, E. A. Fisher, and S. G. Young. 2003. Eliminating atherosogenesis in mice by switching off hepatic lipoprotein secretion. Circulation. 107: 1315–1321.

43. Griendling, H. K., A. H. Oliveira, E. R. Nakandakare, and E. C. Quintanilha. 2006. Regulation of hepatic cholesterol metabolism in CETP/LDL mice by cholesterol feeding and by drugs (cholestyramine and lovastatin) that lower plasma cholesterol. Clin. Exp. Pharmacol. Physiol. 33: 1209–1215.

44. Davis, H. R., L. Hoos, G. Tetzloff, M. Maguire, L. J. Zhu, M. P. Graziano, and S. W. Altmann. 2007. Deficiency of Niemann-Pick C1-like 1 prevents atherosclerosis in ApoE−/− mice. Arterioscler. Thromb. Vasc. Biol. 27: 841–849.

45. Davis, H. R., J. S. Compton, L. Hoos, and G. Tetzloff. 2001. Ezetimibe, a potent cholesterol absorption inhibitor, inhibits the development of atherosclerosis in ApoE knockout mice. Arterioscler. Thromb. Vasc. Biol. 21: 2032–2038.

46. Rizzo, M., and K. Berneis. 2006. The clinical relevance of low-density-lipoprotein size modulation by statins. Cardiovasc. Drugs Ther. 20: 205–217.
59. Berneis, K., M. Rizzo, H. K. Berthold, G. A. Spinas, W. Krone, and I. Gouni-Berthold. 2010. Ezetimibe alone or in combination with simvastatin increases small dense low-density lipoproteins in healthy men: a randomized trial. *Eur. Heart J.* 31: 1633–1639.

60. Mihalis Kalogirou, V. T., V. Saougos, K. Lagos, A. D. Tselepis, and M. Elisaf. 2007. Clinical research effect of ezetimibe on lipoprotein subfraction concentrations: the role of atorvastatin pretreatment. *Arch. Med. Sci.* 3: 344–350.

61. Caslake, M. J., G. Stewart, S. P. Day, E. Daly, F. McTaggart, M. J. Chapman, P. Durrington, P. Laggner, M. Mackness, J. Pears, et al. 2005. Phenotype-dependent and -independent actions of rosuvastatin on atherogenic lipoprotein subfractions in hyperlipidaemia. *Atherosclerosis.* 171: 245–253.

62. Farnier, M., M. W. Freeman, G. Macdonell, I. Perevozkaya, M. J. Davies, Y. B. Mitchel, and B. Gumbiner. 2005. Efficacy and safety of the coadministration of ezetimibe with fenofibrate in patients with mixed hyperlipidaemia. *Eur. Heart J.* 26: 897–905.