Decreased plasma cholesterol and hypersensitivity to statins in mice lacking Pcsk9


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PCSK9 encodes proprotein convertase subtilisin/kexin type 9a (PCSK9), a member of the proteinase K subfamily of subtilases. Missense mutations in PCSK9 cause an autosomal dominant form of hypercholesterolemia in humans, likely due to a gain-of-function mechanism because overexpression of either WT or mutant PCSK9 reduces hepatic LDL receptor protein (LDLR) in mice. Here, we show that livers of knockout mice lacking PCSK9 manifest increased LDLR protein but not mRNA. Increased LDLR protein led to increased clearance of circulating lipoproteins and decreased plasma cholesterol levels (46 mg/dl in Pcsk9−/− mice versus 96 mg/dl in WT mice). Statins, a class of drugs that inhibit cholesterol synthesis, increase expression of sterol regulatory element-binding protein-2 (SREBP-2), a transcription factor that activates both the Ldlr and Pcsk9 genes. Statin administration to Pcsk9−/− mice produced an exaggerated increase in LDLRs in liver and enhanced LDL clearance from plasma. These data demonstrate that PCSK9 regulates the amount of LDLR protein in liver and suggest that inhibitors of PCSK9 may act synergistically with statins to enhance LDLRs and reduce plasma cholesterol.

The activity of the low-density lipoprotein receptor (LDLR) in liver is the major determinant of plasma LDL cholesterol concentrations (1, 2). Transcription of the LDLR is regulated by sterol regulatory element-binding protein-2 (SREBP-2), one of three SREBP family members that regulate the expression of many enzymes involved in cholesterol and fatty acid synthesis (3, 4). When hepatocellular sterols are low, SREBP-2 is activated, which restores cholesterol to normal levels by simultaneously activating enzymes required for de novo cholesterol synthesis and by increasing cholesterol uptake from the plasma through enhanced expression of the LDLR (3).

Recent studies suggest that hepatic LDLRs also may be posttranscriptionally regulated by proprotein convertase subtilisin/kexin type 9a (PCSK9) (5–7). PCSK9 belongs to the proteinase K subfamily of subtilases, which are proteinases synthesized as soluble proproteins that subsequently undergo autocatalytic cleavage to active enzymes (8). Pcsk9 was identified as an SREBP-regulated gene in liver by using oligonucleotide arrays hybridized with RNA from livers of mice that either overexpressed or lacked SREBPs (4, 9). Pcsk9 was regulated in a manner similar to other SREBP-responsive genes involved in lipid homeostasis, suggesting that Pcsk9 might also participate in lipid metabolism.

This suggestion was confirmed by the finding that missense mutations in Pcsk9 are associated with an autosomal dominant form of hypercholesterolemia (10–12). The clinical phenotype of these subjects is indistinguishable from two other autosomal dominant forms of hypercholesterolemia, both of which are caused by defective receptor-mediated clearance of LDL: (i) familial hypercholesterolemia, which is caused by mutations in the LDLR; and (ii) familial defective apolipoprotein B (apoB), caused by mutations in the ligand for the LDLR (13). This similarity raised the possibility that Pcsk9 somehow lowers the amount or activity of LDLRs in liver.

This hypothesis was supported by the finding that overexpression of mutant forms of PCSK9 in mice significantly reduced LDLR protein in liver and raised plasma LDL (5–7). Overexpression of WT PCSK9 reduced hepatic LDLRs to a similar extent as expression of PCSK9 mutant forms (7). These studies suggested that PCSK9 might function normally to reduce LDLR expression levels in liver. If this hypothesis is correct, then the elimination of PCSK9 through targeted disruption of its gene should lead to an increase in LDLRs and a decrease in plasma LDL. To test this hypothesis, we deleted Pcsk9 in mice and characterized the effects on cholesterol metabolism.

Materials and Methods

DNA manipulations were performed by using standard molecular biology techniques (14). Cholesterol and triglyceride concentrations in plasma and liver were measured as described (15). Plasma lipoprotein fractions were separated by FPLC gel filtration by using a Superose 6 column. Measurements of cholesterol concentrations eluted from the FPLC fractions and Coomassie staining of plasma lipoproteins were performed as described (16). Protein concentrations were determined by using the BCA Protein Assay Reagent (Pierce). Other reagents were obtained from Sigma–Aldrich.

Construction of Targeting Vector for Disruption of Pcsk9. Mouse Pcsk9 was disrupted by using a gene-replacement vector that deleted the 3′ half of exon 2 through intron 4. Details of the gene-targeting vector construction are available upon request.

ES Cell Culture for Disruption of Pcsk9. Passage 11 SM-1 ES cells were electroporated with the Pcsk9 targeting vector as described (17). Recombined clones were identified by PCR using primers P1 (5′-GCT TCT GAG GGC GAA AGA ACC AGC-3′) from the 5′ coding region of the neo gene and P2 (5′-TCA TCA TCC AAT GGG TGG GCC TGA AG-3′) from the promoter of Pcsk9 located outside of the targeting vector. The targeted allele produced a 1.1-kb PCR product. Targeted clones were confirmed by Southern blot analysis using a 0.35-kb DNA probe from the Pcsk9 promoter region (Fig. 8, which is published as supporting information on the PNAS web site).

Generation of Pcsk9 Knockout Mice. Two targeted ES clones with a disrupted Pcsk9 allele were injected separately into C57BL/6J blastocysts, yielding chimeric males whose coat color (agouti) indicated a contribution of ES cells from 75% to 100%. Three chimeric males derived from each clone subsequently produced offspring that harbored the disrupted Pcsk9 allele. Mice carrying

Abbreviations: ARH, autosomal recessive hypercholesterolemia; Apo, apolipoprotein; LDLR, low-density lipoprotein receptor; LRP, LDL-related protein; PCSK9, proprotein convertase subtilisin/kexin type 9a; SREBP, sterol regulatory element-binding protein; RAP, receptor-associated protein; VLDL, very low-density lipoprotein; HDL, high-density lipoprotein.

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the disrupted allele were identified by Southern blotting or by PCR (Fig. 8).

Mice were housed in colony cages and maintained on a 12-h light/12-h dark cycle, fed Teklad Mouse/Rat Diet 7002 from Harlan Teklad Premier Laboratory Diets, and killed at the end of the dark cycle. All animal experiments were performed with the approval of the Institutional Animal Care and Research Advisory Committee at the University of Texas Southwestern Medical Center.

**Real-Time RT-PCR.** Total RNA was prepared from mouse livers by using an RNA STAT-60 kit (Tel-Test, Friendswood, TX). The primers for real-time PCR and details of PCR conditions were as described (17–19).

**Antibodies and Immunoblot Analysis.** The murine PCSK9 amino acid sequence was analyzed by using PROTEAN software (DNASTAR, Madison, WI), and two segments (amino acids 163–188 and amino acids 220–240) predicted to be immunogenic were synthesized by the Protein Chemistry Technology Center at the University of Texas Southwestern Medical Center. Peptides were conjugated to KLH by using the Inj ect Maleimide Activated mCKLH Kit (Pierce), and rabbits were immunized with a mixture of the peptides (20 μg each) as described (15). IgG fractions from preimmune and immune sera were purified by using the Immunopure (A/G) IgG purification kit (Pierce). The resulting antibody detected two proteins corresponding to proprotein (∼76 kDa) and cleaved (∼62 kDa) forms of PCSK9. Polyclonal antibodies directed against the mouse LDLR, LDL receptor-related protein (LRP), receptor-associated protein (RAP), autosomal recessive hypercholesterolemia (ARH), SREBP-1, and SREBP-2 were as described (7).

For immunoblot analysis, membranes (105 × g pellet) and nuclear extracts were prepared from mouse liver as described (15, 19). For whole-cell lysates, equal portions of liver were homogenized in buffer A (20 mM Tris–HCl, pH 7.4, 0.25 M sucrose/20 mM KCl/2 mM MgCl2/1 mM sodium EDTA supplemented with 1 mM phenylmethylsulfonyl fluoride/1 mM 1,10-phenanthroline/50 μg/ml leupeptin/1 μg/ml pepstatin A/0.5 μg/ml aprotinin/25 μg/ml N-acetylleucylleucynorleucinal). The homogenate was centrifuged at 105,000 × g for 15 s to pellet cellular debris. Protein concentrations were measured in the supernatant, and equal aliquots of protein were mixed with an equal volume of buffer B (62.5 mM Tris–HCl, pH 6.8/8 M urea/15% SDS [wt/vol]/10% glyceral [vol/vol]/100 mM DTT) and heated for 5 min at 95°C. Samples were subjected to SDS–PAGE as described (15, 19). Immunoblot analyses were performed by using either a horseradish peroxidase-conjugated anti-rabbit secondary antibody from donkey (Amersham Pharmacia Biosciences) and SuperSignal West Pico Chemiluminescent Substrate System (Pierce), or a125I-labeled secondary anti-rabbit antibody from donkey (Amersham Pharmacia Biosciences) (15).

**Immunohistochemistry.** Indirect immunofluorescence using antibodies against LDLR in livers of WT, Ldlr−/−, and Pcsk9−/− mice was carried out as described (20).

**Plasma Clearance of [125I]LDL.** Mouse LDL (density 1.019–1.063 g/ml) was prepared from the pooled plasma of 70 Ldlr−/− mice by sequential ultracentrifugation and radiolabeled with sodium [125I]I (16). Clearance of [125I]-labeled apoB (apoB48 plus apoB100) from plasma of WT and Pcsk9−/− mice was measured at the indicated times by isopropanol precipitation (16, 17).

**ApoB Secretion from Primary Hepatocytes.** Hepatocytes were isolated as described (7) and cultured in Methionine/Cysteine-free DMEM (Sigma) supplemented with 5% lipoprotein-deficient newborn calf serum, 4 mM L-glutamine, 100 units/ml penicillin G sodium, and 100 μg/ml streptomycin sulfate (medium A) for 2 h. The hepatocytes were pulse-labeled with 250 μCi/ml (1 Ci = 37 GBq) [15S]Methionine/Cysteine (Redivue PRO-MIX [15S], Amersham Pharmacia Biosciences) in medium A without serum for 30 min. Thereafter, hepatocytes were washed twice with PBS, and the medium was changed to DMEM supplemented with 10 mM L-methionine, 10 mM L-cysteine, 5% lipoprotein-deficient newborn calf serum, 100 units/ml penicillin G sodium, and 100 μg/ml streptomycin sulfate. The cells were incubated for the indicated times, after which the medium and cells were harvested as described (7).

ApoB48 and apoB100 were immunoprecipitated and separated on a 4% SDS–PAGE gel (7). The gels were dried and exposed to a PhosphorImager plate, and the resulting signals were quantified by using a PhosphorImager Molecular Dynamics Storm 820 system (Amersham Pharmacia Biosciences). ApoB secreted from the hepatocytes was calculated as the percentage of apoB initially synthesized during the pulse-labeling period.

**Diet Studies.** Mice were fed ad libitum a cereal-based powdered diet (Teklad Mouse/Rat Diet 7001) or the powdered diet supplemented with 0.2% (wt/wt) lovastatin (Merck & Co.) for 7 days.

**Results**

The strategy used to disrupt Pcsk9 in mice is shown in Fig. 8. A targeting vector containing the neomycin-resistance gene was used to replace the C-terminal half of exon 2, exon 3, and exon 4. Northern blotting and immunoblot analysis confirmed that this deletion eliminated detectable PCSK9 mRNA and protein (Fig. 8). Matings between Pcsk9−/− mice produced offspring in the expected Mendelian 1:2:1 ratio. Pcsk9−/− mice were normal in appearance. Body and liver weights were not significantly different from littermate WT mice (Table 1). No significant differences were found when the hepatic cholesterol and triglyceride concentrations were compared with WT littersmates. Plasma cholesterol levels in the Pcsk9−/− mice were 48% lower than those of WT mice, despite similar levels of plasma triglycerides.

Lipoproteins from pooled plasma of 16 WT and 16 Pcsk9−/− male mice were isolated by ultracentrifugation and fractionated by FPLC (Fig. 1A). Normal mouse plasma contains very little apoB-containing lipoproteins [very low-density lipoprotein (VLDL) or LDL]; the majority of circulating cholesterol in mice is associated with high-density lipoprotein (HDL). In plasma from Pcsk9−/− mice, the level of LDL cholesterol was even further reduced so that it was nearly undetectable. The level of HDL cholesterol was reduced by ∼30%. Mouse HDL contains apoE, which allows it to bind to LDLRs. Fig. 1B shows the protein composition of the lipoprotein fractions resolved by FPLC. As expected from the reduction in LDL cholesterol, the plasma level of apoB100 was clearly lower in the Pcsk9−/− mice and the level of apoB48 was also diminished. There also was a marked reduction in the apoE content of VLDL and HDL fractions.

<table>
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<th>Parameter</th>
<th>WT</th>
<th>Pcsk9−/−</th>
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<td>No. of mice</td>
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<td>4</td>
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<tr>
<td>Body weight, g</td>
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<td>Liver cholesterol, mg/g</td>
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<td>2.00 ± 0.02</td>
</tr>
<tr>
<td>Liver TG, mg/g</td>
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<td>7.2 ± 0.7</td>
</tr>
<tr>
<td>Plasma cholesterol, mg/dl</td>
<td>95.7 ± 9.4</td>
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<tr>
<td>Plasma TG, mg/dl</td>
<td>70.0 ± 11</td>
<td>85.8 ± 7.5</td>
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</table>

Male mice (12–13 weeks of age) were fed a rodent chow and killed nonfasted. Each value represents the mean ± SEM. *A level of statistical significance (Student’s t test) of P < 0.01 between WT and Pcsk9−/− mice.

TG, triglycerides. Similar results were obtained from four independent experiments.

**Table 1. Phenotypic comparison of wild-type and Pcsk9−/− mice**

*Phone numbers are not meaningful in this context.*
The reduction in apoE- and apoB-containing lipoproteins suggested that less VLDL was secreted from liver or else these particles were cleared faster from the circulation in the Pcsk9−/− mice. The major route of clearance of apoE- and apoB-containing lipoproteins is by means of LDLR-mediated endocytosis in the liver (2). Fig. 2 shows the levels of LDLR and other proteins in pooled livers from four Pcsk9−/− and four WT littermate controls as determined by immunoblotting. As expected, no PCSK9 protein was detected in the livers of Pcsk9−/− mice when a polyclonal antibody directed against the mouse protein was used for immunoblotting (Fig. 2A). The level of hepatic LDLR protein was ∼2.8-fold higher in the Pcsk9−/− mice than in the WT mice as determined by densitometric scanning of the autoradiogram. No changes were found in the amount of LRP, a member of the LDLR family (21), and ARH, an adaptor protein involved in hepatic LDLR internalization (20), or in SREBP-1 or SREBP-2, the transcriptional regulators of the Ldlr gene, leading to increased LDLRs and other proteins involved in cholesterol and fatty acid biosynthesis (see Table 3, which is published as supporting information on the PNAS web site).

To determine whether the increase in LDLR was secondary to an increase in the level of mRNA encoding the LDLR, we measured the level by quantitative RT-PCR. We found no changes in the mRNAs encoding the LDLR or several other proteins involved in cholesterol and fatty acid biosynthesis (Table 3). Indirect immunofluorescence confocal microscopy was used to confirm the increase in LDLRs on the surface of the hepatocytes from Pcsk9−/− mice (Fig. 3). The specificity of the polyclonal anti-LDLR antibody was confirmed by the absence of staining in a liver of an Ldlr−/− mouse (Fig. 3A) (22). A marked increase in staining for the LDLR was observed in hepatocytes from Pcsk9−/− mice compared with those from WT mice (compare Fig. 3B and C).

To measure the rate of LDL clearance from plasma, we isolated LDL from the blood of Ldlr−/− mice and labeled its apoB with 125I. Radiolabeled LDL was injected into two different groups of WT and Pcsk9−/− mice (six mice in each group). The first group was used to measure the rate of disappearance of 125I-labeled LDL during the first 30 min after injection (Fig. 4A), and the second group was used to measure the rate of LDL clearance at longer time points (Fig. 4B). The time required for clearance of 50% of the 125I-LDL was 12 min in Pcsk9−/− mice and >60 min in WT mice. We attribute this 5-fold increase in 125I-LDL clearance to the ∼3-fold increase in hepatic LDLRs in Pcsk9−/− mice.

We next determined whether the absence of Pcsk9 altered the rate of apoB secretion from primary hepatocytes derived from WT and Pcsk9−/− mice. For this purpose, freshly isolated hepatocytes were incubated with [35S]methionine/cysteine. After a 30-min labeling period, the medium was switched to medium containing cold methionine/cysteine. ApoB100 and apoB48 were immuno-precipitated from cells and medium after 1 or 2 h, and the amount of labeled apoB was quantified by SDS/PAGE autoradiography. The deletion of Pcsk9 was associated with a slight reduction in the secretion of apoB48 (Fig. 5). ApoB100 secretion was not significantly different in hepatocytes from WT or Pcsk9−/− mice. Whether this reduction is due to a presecretory mechanism that degrades apoB or to recapture of secreted apoB owing to higher levels of LDLR protein expression in hepatocytes from Pcsk9−/− mice could not be determined in these experiments.

The SREBP-mediated up-regulation of PCSK9 creates a potential problem for therapy with 3-hydroxy-3-methylglutaryl CoA (HMG-CoA) reductase inhibitors like lovastatin and other statins. These inhibitors block cholesterol synthesis and thereby cause an increase in cleaved SREBP-2. Nuclear SREBP-2 enhances the transcription of the LDLR gene, leading to increased LDLRs and a fall in plasma LDL. The elevated nuclear SREBP-2 also increases the mRNA for PCSK9, and this result would lead to a reduction in LDLR protein. If this scenario is correct, lovastatin should increase LDLRs to a greater extent in Pcsk9−/− mice than in WT mice.

To test this hypothesis, WT and knockout mice were fed normal chow or chow supplemented with 0.2% lovastatin. Table 2 contains...
LDLR and RAP, a125I-labeled secondary antibody was used to determine information on the PNAS web site). The fold-increase of cholesterol in WT mice (71 mg/dl versus 81 mg/dl). As observed previously, the Pcsk9−/− mice maintained on chow had a plasma cholesterol level that was significantly lower than WT mice (51 mg/dl versus 81 mg/dl). Addition of lovastatin to the diet of knockout mice resulted in the plasma cholesterol level falling to 41 mg/dl; this value was significantly less than those measured in Pcsk9−/− mice fed chow. Plasma triglycerides were significantly reduced in mice from both genotypes fed lovastatin.

Analysis of hepatic gene expression revealed that feeding the lovastatin-containing diet to WT and Pcsk9−/− mice resulted in increases in SREBP-2 mRNA levels and a corresponding increase in all measured SREBP target gene mRNA levels, including those encoding 3-hydroxy-3-methylglutaryl CoA (HMG-CoA) reductase, PCSK9, and LDLR (Table 4, which is published as supporting information on the PNAS web site). The fold-increase of cholesterol biosynthetic gene mRNAs in livers of lovastatin-treated Pcsk9−/− mice was similar to that measured in WT mice fed the same diet.

Lovastatin administration to WT mice led to an increase in PCSK9 protein in liver (Fig. 6A). Lovastatin also resulted in the predicted increase in nuclear SREBP-2 protein levels in WT and Pcsk9−/− mice. To more accurately quantify the changes in levels of LDLR and RAP, a 125I-labeled secondary antibody was used to generate the data shown in Fig. 6B. LDLR protein levels in livers of WT mice fed lovastatin were consistently lower than chow-fed mice despite the measured increase in LDLR mRNA expression. The expression of the LDLR was 2.8-fold higher in livers of chow-fed Pcsk9−/− mice compared with the WT mice and was further increased after administration of lovastatin to a level 4.6-fold higher than chow-fed WT mice. Fig. 6C shows the relative amount of LDLR protein in livers of WT and Pcsk9−/− mice administered lovastatin from three independent experiments. In all experiments, lovastatin administration resulted in a significantly greater increase in LDLR expression in the absence of PCSK9.

To determine whether lovastatin increased LDL clearance, radiolabeled LDL was injected into WT and Pcsk9−/− mice fed chow or lovastatin (eight mice per group) (Fig. 7). Lovastatin administration to WT mice did not significantly alter LDL clearance from the plasma after only 30 min. Pcsk9−/− mice again displayed a marked increase in the rate of LDL clearance from plasma compared with that of WT mice. Lovastatin administration to Pcsk9−/− mice resulted in an additional ~2-fold increase in the rate of labeled LDL clearance from the plasma compared with chow-fed Pcsk9−/− mice. This change was similar in magnitude to the change measured in LDLR protein levels in chow versus lovastatin-fed Pcsk9−/− mice (Fig. 6B).

Discussion

The current study demonstrates that the inactivation of Pcsk9 reduces plasma cholesterol levels primarily by increasing LDLR protein expression in liver and accelerating the clearance of circulating cholesterol. These results, together with the previous observation that overexpression of WT PCSK9 in mice leads to elevated plasma LDL cholesterol levels by reducing the LDLR protein in liver (7), indicate that one function of PCSK9 is to negatively regulate LDLR protein levels. No changes were detected in

![Fig. 3](image-url) Indirect immunofluorescence in liver using antibodies against LDLR. Frozen sections of liver from Ldlr−/− (A), WT (B), and Pcsk9−/− (C) mice were incubated with a polyclonal antibody against the LDLR. Bound IgG was detected with 20 μg/ml Alexa Fluor 488-labeled goat anti-rabbit IgG (20).

![Fig. 4](image-url) Plasma clearance of 125I-labeled mouse LDL in WT and Pcsk9−/− mice. (A) Six male mice (10–12 weeks of age) of the indicated genotype were injected i.v. with 125I-labeled LDL (30 μg of protein, 294 cpm/ng apoB protein). Blood was obtained at 30 s (time 0) and at 5, 10, 15, and 30 min for quantification of plasma content of 125I-labeled total apoB (17). (B) Six male mice (10–12 weeks of age) of the indicated genotype were injected i.v. with the same 125I-labeled LDL used in A. Blood was obtained at 30 s (time 0) and at 0.5, 1, 2, and 4 h for quantification of plasma content of 125I-labeled total apoB. Data are plotted as the percentage of zero time value. Each value represents mean ± SEM of six mice.
SREBP-2 protein or in the mRNAs of genes encoding cholesterol synthesis enzymes in livers of Pcsk9−/− mice, which suggests that the reduction in plasma cholesterol levels was not due to decreased cholesterol synthesis. A small reduction in apoB48 secretion was detected in primary hepatocytes derived from mice of the indicated genotype, and apoB48 and apoB100 were immunoprecipitated and separated by SDS/PAGE gel electrophoresis as described under Materials and Methods. The data are expressed as the apoB content in the medium as a percentage of the 35S-labeled apoB in the cells at zero time. Each value is mean ± SEM of duplicate incubations from eight WT and eight Pcsk9−/− mice. * Statistical difference of P < 0.05 (Student’s t test).

Fig. 5. Rates of apoB secretion by primary hepatocytes from WT mice and Pcsk9−/− mice. Hepatocytes were prepared from mice of the indicated genotype, and apoB48 and apoB100 were immunoprecipitated and separated by SDS/PAGE gel electrophoresis as described under Materials and Methods. The data are expressed as the apoB content in the medium as a percentage of the 35S-labeled apoB in the cells at zero time. Each value is mean ± SEM of duplicate incubations from eight WT and eight Pcsk9−/− mice. * Statistical difference of P < 0.05 (Student’s t test).

Fig. 6. Levels of proteins in livers of WT and Pcsk9−/− mice fed chow (C) or chow supplemented with 0.2% lovastatin (L). Livers from four male mice in the groups of Table 2 were pooled, and aliquots of membrane protein (40 μg), whole cell lysate (30 μg), or nuclear protein (30 μg) were subjected to SDS/PAGE. (A) Immunoblot analysis of PCSK9, ARH (whole cell lysate), SREBP-2 (membrane and nuclear fractions), CAMP response element binding protein (CREB) (nuclear fraction), LRP, and RAP (membrane fraction). P and C for SREBP-2, P and N denote the precursor and cleaved forms of PCSK9. For SREBP-2, P and N denote the precursor and cleaved nuclear forms. (B) Immunoblot analyses of LDLR and RAP. A 125I-labeled secondary anti-rabbit antibody from donkey was used for the LDLR and RAP to quantify the expression of the LDLR by using a PhosphorImager. The relative expression of the LDLR protein is normalized to the amount of LDLR expressed in livers of WT mice fed chow. (C) Relative amount of hepatic LDLR protein in WT and Pcsk9−/− mice fed 0.2% lovastatin versus chow. Each symbol represents an independent experiment with four mice per group.

Ldlr, total hepatic LDLR protein was slightly lower than WT mice fed chow, and the plasma cholesterol levels were not statistically reduced (Table 2 and Fig. 6). One reason for the apparent paradoxical response seems to be the simultaneous induction of PCSK9, which posttranscriptionally reduces LDLR protein levels. This interpretation is supported by the findings that Pcsk9−/− mice administered lovastatin had higher LDLR protein levels, increased LDL clearance from plasma, and lower plasma cholesterol levels than Pcsk9−/− mice fed chow. These results suggest that inhibitors of PCSK9 may have beneficial effects on plasma cholesterol levels, especially when combined with statins.

Our findings in mice are consistent with those of Ness et al. (23), who previously showed that statins administered to rats increased the LDLR mRNA levels but not LDLR protein levels in liver. The lack of an increase in LDLR protein was attributed to increased LDLR degradation in these studies. It is likely that

Table 2. Phenotypic comparison of wild-type and Pcsk9−/− mice fed 0.2% lovastatin

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<td>12</td>
<td>12</td>
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</tr>
<tr>
<td>Body weight, g</td>
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<td>Plasma cholesterol, mg/dl</td>
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<td>71.1 ± 3.0</td>
<td>51.4 ± 2.3*</td>
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<tr>
<td>Plasma TG, mg/dl</td>
<td>94.4 ± 8.7</td>
<td>64.5 ± 4.6*</td>
<td>100 ± 5.9</td>
<td>66.2 ± 4.2**</td>
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</table>

Mean values of pooled data from three independent experiments. The eight male and four female mice (12–14 weeks of age) in each group were fed a standard rodent chow or the standard chow supplemented with 0.2% (wt/wt) lovastatin (lov.) for 7 days. The mice were killed nonfasted. Each value represents the mean ± SEM. * Statistical difference of P < 0.01 (Student’s t test) between the indicated group and WT mice fed chow. ** A statistical difference of P < 0.01 (Student’s t test) between Pcsk9−/− mice fed 0.2% lovastatin and chow.

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reason why SREBP-2 up-regulates both Ldlr and Pcsk9 still cannot be easily reconciled with the current information. However, it is possible that the simultaneous transcriptional activation of Pcsk9 and Ldlr by SREBP-2 may provide a posttranscriptional mechanism to degrade the LDLR and shorten the protein’s half-life, which could protect the cell from excessive LDL uptake and cholesterol accumulation (7).

Several important questions regarding the function of Pcsk9 remain unanswered. First, the substrate(s) of Pcsk9 has not been defined, and whether this protease directly cleaves the LDLR, or acts indirectly by destroying another protein, remains to be determined. Second, although recent studies indicate that Pcsk9 mediates the degradation of the LDLR in a post-ER compartment (25), it is not known whether this degradation occurs intracellularly or after the LDLR reaches the cell surface. Third, the importance of Pcsk9-mediated regulation of LDLR expression in tissues other than liver remains to be determined. Pcsk9 is expressed at low levels in all tissues tested (8). The liver normally accounts for 65–70% of all LDL-cholesterol clearance in mice (2). Most tissues other than liver and the adrenal gland have low LDLR activity and presumably low LDLR protein levels (2). In experiments not shown, levels of LDLR protein in the adrenal gland were higher in Pcsk9-/- mice, suggesting that Pcsk9 also may regulate the expression of LDLR in extrapatic tissues. Further delineation of each tissue’s contribution to plasma LDL-cholesterol clearance in Pcsk9-/- mice will require more quantitative LDL uptake studies.

The results reported here are consistent with the findings of Cohen et al. (26), who found that individuals heterozygous for a nonsense mutation in Pcsk9 had a 40% reduction in LDL cholesterol levels. Individuals with loss-of-function mutations in Pcsk9 were heterozygous for the mutant allele. Heterozygous Pcsk9-/- mice had a phenotype that was intermediate between homozygous Pcsk9-/- mice and WT mice in terms of plasma cholesterol concentrations and LDL clearance, which is consistent with a gene-dosage effect. The phenotypes observed in humans with mutations in Pcsk9, combined with results of the current studies in mice, suggest that variations in the levels of Pcsk9 significantly affect plasma cholesterol levels and that inhibitors of Pcsk9 may be useful for the treatment of hypercholesterolemia.

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