Lipids and Lipoproteins: Post-transcriptional Regulation of Low Density Lipoprotein Receptor Protein by Proprotein Convertase Subtilisin/Kexin Type 9a in Mouse Liver

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Post-transcriptional Regulation of Low Density Lipoprotein Receptor Protein by Proprotein Convertase Subtilisin/Kexin Type 9a in Mouse Liver*

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Lipid homeostasis is transcriptionally regulated by three DNA-binding proteins, designated sterol regulatory element-binding protein (SREBP)-1a, -1c, and -2. Oligonucleotide arrays hybridized with RNA made from livers of transgenic SREBP-1a, transgenic SREBP-2, and SREBP cleavage-activating protein knockout mice recently identified 33 genes regulated by SREBPs in liver, four of which had no known connection to lipid metabolism. One of the four genes was PCSK9, which encodes proprotein convertase subtilisin/kexin type 9a, a protein that belongs to the proteinase K subfamily of subtilases. Mutations in PCSK9 are associated with an autosomal dominant form of hypercholesterolemia. Here, we demonstrate that hepatic overexpression of either wild-type or mutant PCSK9 in mice results in hypercholesterolemia. The hypercholesterolemia is due to a post-transcriptional event causing a reduction in low density lipoprotein (LDL) receptor protein prior to the internalization and recycling of the receptor. Overexpression of PCSK9 in primary hepatocytes and in mice lacking the LDL receptor does not alter apolipoprotein B secretion. These data are consistent with PCSK9 affecting plasma LDL cholesterol levels by altering LDL receptor protein levels via a post-transcriptional mechanism.

Plasma LDL, cholesterol concentrations are determined by the relative rates of VLDL and LDL production by the liver and the rate of LDL uptake via hepatic LDL receptors (LDLRs) (1, 2). VLDL secretion from hepatocytes is positively correlated with rates of hepatic lipid synthesis (3). Genes required for cholesterol and triglyceride biosynthesis and, thus, VLDL production are regulated by three sterol regulatory element-binding proteins (SREBPs), SREBP-1a, SREBP-1c, and SREBP-2 (4, 5). SREBPs also are the principal transcriptional regulators of the LDL receptor gene, which clears apoB-containing lipoproteins, such as VLDL and LDL, from the plasma (5).

To identify genes regulated by SREBPs, we used oligonucleotide arrays hybridized with RNA from livers of mice that overexpressed SREBPs (transgenic for SREBP-1a or transgenic for SREBP-2) and that lacked all SREBPs as a result of deleting SCAP, an escort protein required for SREBP activation (5). With this physiologic filter, 33 genes were identified that were increased in the transgenic livers and decreased in the SCAP-deficient livers. Four of these 33 genes had no known function. One of these four genes was Pcsk9, which encodes the proprotein convertase subtilisin/kexin type 9a, also designated NARC-1 (neural apoptosis-regulated convertase 1). Seidah et al. (6) showed that PCSK9 belongs to the proteinase K subfamily of subtilases. PCSK9 is synthesized first as a soluble zymogen that undergoes autocatalytic intramolecular processing in the ER to produce a prosegment that remains associated with the secreted enzyme.

A link between PCSK9 and cholesterol metabolism was established by Abifadel et al. (7), who showed that two missense mutations in PCSK9 were associated with an autosomal dominant form of hypercholesterolemia. The first mutation results in the substitution of an arginine for serine (S127R) in the catalytic domain of the enzyme. Subsequently, two additional missense mutations in the catalytic domain of PCSK9 (D374Y and N157K) were shown to segregate in families with elevated plasma LDL cholesterol concentrations (8, 9).

The clinical phenotype of subjects with these missense mutations in PCSK9 is indistinguishable from two other autosomal dominant forms of hypercholesterolemia, familial hypercholesterolemia, which is caused by mutations in the LDLR and familial defective apoB, due to mutations that interfere with LDL binding to the LDLR and clearance from the plasma (10). We hypothesized that mutations in PCSK9 cause hypercholesterolemia by altering SREBP expression, apoB synthesis/secretion, and/or LDLR expression. To distinguish between these mechanisms, a series of in vitro and in vivo studies with wild-type and mutant PCSK9 was performed.

EXPERIMENTAL PROCEDURES

General Methods and Supplies—DNA manipulations were performed using standard molecular biology techniques (11). The concentrations of cholesterol and triglycerides in plasma were measured as described previously (12). Plasma lipoprotein fractions were separated by fast performance liquid chromatography (FPLC) gel filtration using...
a Superose 6 column, and cholesterol concentrations were measured as described (13, 14). Protein concentrations were determined using a BCA kit (Pierce). Newborn calf lipoprotein-deficient serum (NCLFDS) (d > 1.215 mg/ml) was prepared as described (15). Other reagents otherwise not specified were obtained from Sigma.

Expression plasmids pTK-HSV-BP1 and pTK-HSV-BP2, encoding wild-type HSV-tagged full-length human SREBP-1c or SREBP-2 (16), and pCMV-S1P-Myc, encoding hamster S1P (17), were harvested and processed as described in the indicated references.

**Antibodies and Immunoblot Analyses**—The following monoclonal antibodies were used in the current studies: anti-HSV IgG1 (from EMD Biosciences (Novagen Brand, Madison, WI), anti-Myc (clone 9E10) from Roche Applied Science, anti-FLAG (M2) from Sigma, anti-human transferrin receptor from Zymed Laboratories (South San Francisco, CA), polyclonal anti-human cAMP-responsive element-binding protein from Cell Signaling Technology, Inc. (Beverly, MA), horseradish peroxidase-conjugated donkey anti-rabbit IgG (affinity-purified) from Amersham Biosciences.

Polyclonal antibodies against the mouse LDLR, LDL receptor-related protein (LRP), receptor-associated protein (RAP), ARH, SREBP-1, and SREBP-2 were described previously (12, 18–23). Immunoblot analyses were performed using the SuperSignal West Pico Chemiluminescent Substrate System from Pierce.

**Construction of Wild-type and Mutant PCSK9 Expression Vectors**—An expression vector that encodes amino acids 1–692 of human PCSK9 followed by a FLAG epitope tag (DYKDDDDK) under the control of the CMV promoter-enhancer (pCMV-PCS9-FLAG) was constructed as follows. The human PCSK9 cDNA was amplified using nested PCR. First-strand cDNA was synthesized from total RNA prepared from HepG2 cells (ATCC number HB-8065) using mRNA-PrePACs from Applied Biosystems (Austin, TX). cDNA was synthesized from 2 μg of total RNA using random primers (Life Technologies, Inc.) and stored at -20 °C.

To generate plasmids expressing mutant forms of human PCSK9, the QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA) was used to change the nucleotide sequences of pCMV-PCS9-FLAG using the following oligonucleotides: 5'-CTTCCCTGTGAGATAAGAGAGGAGGAGGAGGG-3' for the primary amplification and 5'-CTTCCCTGTGAGATAAGAGAGGAGGAGGAGGG-3' for the secondary amplification. The 5' primer sequence for the secondary amplification contained an EcoRI restriction site, a Kozak sequence, and the sequence encoding amino acids 1–7 of human PCSK9. The sequence of 3' primer used for the secondary amplification consisted of nucleotides that encoded the 5' sequences of the plasmids pCMV-PCSK91-FLAG, pCMV-S127R, pCMV-F216L, and pCMV-S386A for the secondary amplification. The plasmids containing an EcoRI restriction site, a Kozak sequence, and the sequence encoding amino acids 1–7 of human PCSK9 were isolated from agarose gels and purified using the Qiaquick PCR Purification Kit from Qiagen. The resulting plasmids were used to transform E. coli DH5α and plasmid DNA was isolated using the QIAprep Miniprep Kit from Qiagen.

Vector plasmid sequences were confirmed by sequencing, and plasmids were used for the subsequent experiments.

**PCSK9 Proteins**—Wild-type and mutant forms of PCSK9 were selected for further studies and maintained in medium A containing 500 μg of G418 supplemented with 5% FCS.

PCSK9 Overexpression Reduces Hepatic LDL Receptor Protein

On day 0, CHO-K1 cells (ATCC number CCL-61) were plated at a density of 5 × 10⁵ cells/100-mm dish in medium A supplemented with 5% (v/v) FCS. On day 1, cells were transfected with 5 μg of pCMV-PCS9-FLAG, pCMV-S127R, or pCMV-F216L per dish using the FuGene 6 reagent in a final volume of 0.2 ml. To generate control stable cell lines, 1 μg of pDNA3 was used to transfect CHO-K1 cells. On day 2, the medium was changed to medium A containing 700 μg/ml G418 supplemented with 5% FCS. The medium was changed daily for 12 days until individual colonies were visible. Single-cell clones that stably expressed wild-type or mutant PCSK9 protein were isolated by limiting dilution and screened for PCSK9 expression using immunoblot analysis with anti-FLAG monoclonal antibody. Cell lines expressing equivalent levels of wild-type and mutant forms of PCSK9 were selected for further studies and maintained in medium A containing 500 μg of G418 supplemented with 5% FCS.

**Construction of Adenoviral Vectors Expressing Wild-type or Mutant PCSK9 Proteins**—Adenoviruses that express wild-type, S127R, F216L, S386A, or S386E forms of human PCSK9 were generated using AdEasy (Qbiogene, Carlsbad, CA) according to the manufacturer's protocol. HindIII-XbaI fragments of pCMV-PCS9-K FLAG, pCMV-S127R, pCMV-F216L, and pCMV-S386A were ligated to a HindIII-XbaI-digested pShuttle-CMV vector. The resulting pShuttle constructs were co-transformed with the pAdEasy-1 vector into BJ5183 cells to produce recombinant adeno-associated virus particles for wild-type and mutant forms of human PCSK9. Recombinant adeno-associated virus constructs were linearized with PacI and transfected into QBI-293A cells (Qbiogene) cultured in DMEM supplemented with 5% FCS. Cells were overlaid with 1.25% agarose/DMEM 20 h after transfection and further cultured for 14 days until discrete plaques were identified. The resulting viral plaques were assayed for PCSK9 expression by immunoblot analysis with using anti-FLAG antibody (Sigma).

**PCSK9 Overexpression Reducing Hepatic LDL Receptor Protein**—Wild-type and mutant human PCSK9 proteins were subjected to four rounds of amplification before purification by CaCl₂ ultracentrifugation (28). All viruses were dialyzed against 10 mm Tris, pH 8.0, 2 mM MgCl₂, 4% sucrose buffer (29) and stored at -80 °C. Virus titers were determined using a plaque-forming unit (PFU) assay in QBI-293A cells (28). For administration to mice, the indicated amounts of each recombinant adeno-associated virus were injected as a single dose into tail veins of mice. Mice that lack the LDLR were previously described (32). All mice were on a low-cholesterol diet (1% cholesterol) and 10 μl of 2× SYBR Green PCR Master Mix in a final volume of 20 μl. The PCR reactions were carried out using the Applied Biosystems Prism 7700 Sequence Detection System. All reactions were done in triplicate, and the relative amounts of all mRNAs were calculated by using the comparative CT method (46). Cyclophilin mRNA was used as the invariant control.

**Studies using wild-type mice**—Mice were maintained on 12-h light/12-h dark cycles and fed a chow diet that contained 4% (w/w) animal fat and <0.04% (w/w) cholesterol (Teklad 4% mouse/rat diet 7001 from Harlan Teklad Premier Laboratory Diets, Madison, WI).

**ApoB Synthesis and Secretion in Mouse Primary Hepatocytes**—C57BL/6J male mice (8–10 weeks of age) were injected with the indicated adenovirus described below in 1 ml of medium D. After a 2-h incubation, 1 ml of medium D supplemented with 20% NCLFDS was added and incubated overnight at 37 °C. After an overnight incubation, the cells were harvested and processed as described above.

**Stable Transfection of Chinese Hamster Ovarian (CHO-K1) Cells**—On day 0, CHO-K1 cells (ATCC number CCL-61) were plated at a density of 5 × 10⁶ cells/100-mm dish in medium A supplemented with 5% (v/v) FCS. On day 1, cells were transfected with 5 μg of pCMV-PCS9-FLAG, pCMV-S127R, or pCMV-F216L per dish using the FuGene 6 reagent in a final volume of 0.2 ml. To generate control stable cell lines, 1 μg of pDNA3 was used to transfect CHO-K1 cells. On day 2, the medium was changed to medium A containing 700 μg/ml G418 supplemented with 5% FCS. The medium was changed daily for 12 days until individual colonies were visible. Single-cell clones that stably expressed wild-type or mutant PCSK9 protein were isolated by limiting dilution and screened for PCSK9 expression using immunoblot analysis with anti-FLAG monoclonal antibody. Cell lines expressing equivalent levels of wild-type and mutant forms of PCSK9 were selected for further studies and maintained in medium A containing 500 μg of G418 supplemented with 5% FCS.
**RESULTS**

To study the mechanism by which mutations in PCSK9 cause elevated plasma LDL cholesterol concentrations, we first determined whether wild-type or mutant PCSK9 could proteolytically activate SREBPs in an aberrant fashion. SREBPs are synthesized as inactive precursors in the endoplasmic reticulum (ER). To be active, the NH₂-terminal segment of SREBP must be released from the membrane to enter the nucleus (4). SREBP activation requires SCAP, an escort protein that functions as a sterol sensor and transports SREBPs from the ER to the Golgi apparatus, and two proteases, designated site-1 protease (S1P) and site-2 protease (S2P), located in the Golgi (35). When ER membranes become depleted of cholesterol, SCAP escorts SREBP to the Golgi, where it undergoes two sequential proteolytic cleavage events mediated by S1P, a membrane-bound subtilase-like serine proteinase, and S2P, a membrane-bound zinc metalloproteinase. S1P belongs to the same family of subtilase-like serine proteinase as does PCSK9; therefore, mutations in PCSK9 could result in a gain of function that results in unregulated or aberrant cleavage of SREBPs, which in turn would increase lipid biosynthesis and VLDL production.

To test this hypothesis, SRD-12B cells that harbor a genetic deletion of S1P and are thus incapable of cleaving SREBPs were transiently transfected with full-length SREBP-1a (Fig. 1A) or SREBP-2 (Fig. 1B) and either an empty vector, human S1P, wild-type PCSK9, or mutant PCSK9 (S127R). Cells were cultured either in the presence of sterols, conditions that normally suppress SREBP cleavage, or in the absence of sterols, conditions that induce SREBP cleavage. Cellular membranes and nuclear proteins were isolated, aliquots were separated by SDS-PAGE, and immunoblot analyses were performed to determine whether wild-type or mutant PCSK9 proteolytically cleaved the membrane-bound SREBP-1a or SREBP-2 precursor proteins.

As shown in Fig. 1, S1P restores normal sterol-regulated cleavage of SREBP-1a (A) and SREBP-2 (B). The transfection of wild-type PCSK9 results in equal expression of the proprotein (P) and cleaved (C) PCSK9 (compare P and C in Fig. 1, A and B, lanes 6 and 7). Transfection of the S127R mutant PCSK9 resulted in significantly less cleaved PCSK9. However, neither wild-type PCSK9 nor mutant PCSK9 restored cleavage of the transgenically expressed SREBPs in transfected SRD-12B cells.
of SREBP-1a (Fig. 1A) or SREBP-2 (Fig. 1B) in the presence or absence of sterols. These results demonstrate that mutations in PCSK9 do not increase plasma LDL cholesterol levels by bypassing the role of S1P in processing SREBPs.

To determine whether wild-type and mutant PCSK9 proteins alter LDLR expression or function, wild-type CHO-K1 cell lines were stably transfected with CMV-driven wild-type PCSK9 or mutant PCSK9 cDNAs encoding either the S127R mutation or the F216L mutation. The PCSK9 proteins contained a FLAG epitope tag at the COOH terminus. Three clones with equivalent levels of PCSK9 expression were identified, and the expression and function of the LDLR were assessed. As shown in Fig. 2A, the amount of wild-type and mutant PCSK9 proteins expressed was similar in the three cell lines, although the S127R mutation resulted in a reduction in the relative proportion of cleaved PCSK9 (A, lower band). The amounts of secreted wild-type and F216L mutant were also equivalent, whereas the amount of secreted S127R appeared to be slightly lower in amount. The LDLR protein immunoblots showed two bands. The lower band represents the precursor form that is present in the ER. The upper band represents the mature receptor that has undergone O-linked glycosylation in the Golgi (36). The amount of mature LDLR protein was unaffected by wild-type or mutant PCSK9 overexpression. The slight reduction in the amount of the precursor form of the LDLR observed in the transfected cells was not a consistent finding. In addition, assays of LDLR function that measured LDL binding and uptake were also not consistently different among the four immortalized hamster ovarian cell lines (data not shown).

The studies described above were performed in immortalized hamster ovarian cells. Mutations in ARH, an adaptor protein that binds to the cytoplasmic domain of the LDLR and is required for the internalization of the LDLR, cause hypercholesterolemia by reducing LDL clearance predominantly in the liver and lymphocytes (37–39). Therefore, we hypothesized that PCSK9 could have a greater function in hepatocytes than in other cell types. To test this hypothesis, adenoviral constructs that express the wild-type or mutant PCSK9 proteins were produced and used to infect HepG2 cells, a human hepatoma cell line. As shown in Fig. 2B, adenovirus-mediated overexpression of wild-type PCSK9 or either mutant PCSK9 resulted in the near absence of detectable LDLR protein, suggesting that PCSK9 may have cell type-specific activity.
PCSK9 Overexpression Reduces Hepatic LDL Receptor Protein

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**Fig. 3.** LDLR levels in livers of mice injected with an adenovirus expressing β-galactosidase, wild-type PCSK9, or mutant PCSK9. 10-week-old male C57BL/6J mice were injected with adenovirus (2 × 10^9 PFU in 200 µl) expressing β-galactosidase (β-Gal), wild-type (WT-PCSK9), S127R mutant (S127R), or F216L mutant (F216L) PCSK9. Four days after injection, mice were sacrificed, and plasma and livers were collected. Each lane represents an individual mouse. A, immunoblot analyses of PCSK9, LDLR, LRP, ARH, transferrin receptor (membrane fraction), SREBP-1, SREBP-2 (membrane and nuclear fractions), and cAMP-responsive element-binding protein (CREB; nuclear fraction). An aliquot (30 µg) of protein derived from individual mouse livers was subjected to 8% SDS-PAGE and transferred to nitrocellulose membrane for immunoblot analysis. An anti-FLAG primary antibody was used to detect PCSK9 protein, and all other proteins were detected using the primary antibodies as described under "Experimental Procedures." P and N denote the precursor and cleaved nuclear forms of SREBP-1 and SREBP-2, P and C for PCSK9 denote the proproteins and cleaved forms of PCSK9, respectively. B, relative amounts of LDLR mRNA in livers of mice injected with the indicated adenoviruses. Total RNA from individual mouse livers was prepared and subjected to reverse transcription-PCR as described under "Experimental Procedures." Each value represents the amount of mRNA relative to that in the first mouse (lane 1) injected with β-galactosidase virus, which is arbitrarily defined as 1. Cyclophilin was used as an invariant control (data not shown). Similar results were obtained in four independent experiments.

To determine whether PCSK9 functioned in a similar manner in vivo in the liver, wild-type mice were injected with equivalent amounts of adenovirus expressing β-galactosidase, wild-type PCSK9, or the two mutant versions of PCSK9 (Fig. 3A). The level of total PCSK9 protein expressed was similar in all mice; however, the cleaved form of PCSK9 for the S127R mutation was consistently less plentiful than that seen for the wild-type PCSK9 and the F216L mutant forms of PCSK9. LDLR protein levels were markedly reduced in the livers of mice overexpressing either the wild-type or one of the mutant forms of PCSK9 (compare lanes 5–16 with lanes 1–4). The protein levels of LDL receptor-related protein (LRP), a member of the LDLR family (40), and ARH, an adaptor protein involved in hepatic LDLR internalization, were not altered by PCSK9 overexpression. Similarly, no consistent effects were observed in the expression of the precursor and nuclear forms of SREBP-1 and SREBP-2 in the mice expressing recombinant PCSK9. These results demonstrated that wild-type or mutant PCSK9 overexpression was associated with a dramatic reduction in the amount of LDLR protein in livers of mice.

To determine whether the reduction of LDLR protein was due to reduced LDLR transcription, we measured LDLR mRNA levels in the livers of the adenovirus-infected mice by real time PCR. No significant differences in LDLR mRNA levels were found in the mice expressing wild-type or mutant PCSK9 protein (Fig. 3B). The mRNA levels for apoB and several genes involved in cholesterol and fatty acid biosynthesis, including 3-hydroxy-3-methylglutaryl-CoA synthase, 3-hydroxy-3-methylglutaryl-CoA reductase, squalene synthase, acetyl-CoA carboxylase, and fatty acid synthase, also were not significantly different across groups (data not shown).

The marked reduction in hepatic LDLR expression associated with either wild-type or mutant PCSK9 overexpression resulted in a ∼1.5–2-fold increase in total plasma cholesterol concentrations (Fig. 4A). Separation of plasma lipoproteins by FPLC demonstrated that overexpression of all PCSK9 proteins resulted in a selective increase in plasma LDL cholesterol (Fig. 4B). A slightly greater increase in plasma levels of LDL cholesterol was associated with the expression of wild-type PCSK9 and the F216L mutant protein than with the S127R mutant. This difference may be related to less cleaved form of the PCSK9 protein produced in the mice expressing the S127R mutant form of the enzyme (Fig. 3A).

To determine whether the ability of PCSK9 to reduce the LDLR protein was dependent on the catalytic activity of PCSK9, an amino acid in the catalytic triad of human PCSK9 that is required for its proteolytic activity was mutated. A conserved serine at position 386 was changed to alanine (S386A), and the resulting cDNA was inserted into an adenoviral vector for injection into mice. The alanine substitution eliminated the autocatalytic cleavage activity of PCSK9 protein, as evidenced by the absence of the cleaved form of the protein (Fig. 5, lanes 5–8 and 9–12). The level of LDLR protein in livers of mice injected with the S386A mutant was essentially the same as that of the control mice injected with the β-galactosidase virus. These results suggested that the ability of PCSK9 to reduce the amount of LDLR protein was dependent on a functional catalytic domain.

The above experiments demonstrated that PCSK9, when overexpressed, reduced the amount of LDLR protein in liver. The normal function of the LDLR is dependent on an adaptor protein, ARH, for internalization (41, 42). In the absence of ARH, LDLRs accumulate on the cell surface of the hepatocyte due to a failure to undergo internalization (21, 42). To determine whether the ability of PCSK9 to reduce LDLR protein in liver required functional ARH, mice lacking ARH were injected with control or wild-type PCSK9 virus, and the amount of LDLR protein was measured. The immunoblots in Fig. 6 show that overexpression of PCSK9 in livers of Arh^-/- mice resulted in a reduction of hepatic LDLR protein. These findings suggested that PCSK9-mediated reduction in LDLR protein was not dependent on functional ARH and thus occurred either en route from the synthesis of the receptor in the ER to the cell surface or on the cell surface prior to internalization of the LDLR.
A recent report describing the phenotype of human subjects harboring the S127R mutation suggested that the principal metabolic defect responsible for hypercholesterolemia in these individuals is increased apoB secretion (43). To test this possibility, apoB synthesis and secretion were measured in primary hepatocytes derived from mice injected with adenoviruses expressing β-galactosidase, wild-type PCSK9, or mutant PCSK9. A, the concentration of total cholesterol in plasma from each mouse described in the legend to Fig. 3 was measured as described under “Experimental Procedures.” B, plasma from mice described in the legend to Fig. 3 was pooled and subjected to gel filtration by FPLC. The concentration of total cholesterol in each fraction was measured as described under “Experimental Procedures.”

Fig. 4. Concentrations of total plasma cholesterol and FPLC profiles of plasma cholesterol from mice injected with an adenovirus expressing β-galactosidase, wild-type PCSK9, or mutant PCSK9. A, the concentration of total cholesterol in plasma from each mouse described in the legend to Fig. 3 was measured as described under “Experimental Procedures.” B, plasma from mice described in the legend to Fig. 3 was pooled and subjected to gel filtration by FPLC. The concentration of total cholesterol in each fraction was measured as described under “Experimental Procedures.”

A 10-week-old male C57BL/6J mice were injected with adenovirus (2 × 10⁶ PFU in 200 μl) expressing β-galactosidase (β-Gal), wild-type (WT-PCSK9), or S386A mutant (S386A) PCSK9. Four days after injection, mice were sacrificed, and livers were harvested for immunoblot analysis as described in the legend to Fig. 3. P and C for PCSK9 denote the proprotein and cleaved forms of PCSK9, respectively. Each lane is the result from an individual mouse. Similar results were obtained in three independent experiments.

A recent report describing the phenotype of human subjects harboring the S127R mutation suggested that the principal metabolic defect responsible for hypercholesterolemia in these individuals is increased apoB secretion (43). To test this possibility, apoB synthesis and secretion were measured in primary hepatocytes derived from mice injected with adenoviruses ex-
pressing β-galactosidase, wild-type PCSK9, or one of the mutant forms of PCSK9 (S127R). As shown in Fig. 7A, the PCSK9 proteins were expressed in the primary hepatocytes, and the expression of the LDLR was significantly reduced when either the wild-type or mutant form of PCSK9 was expressed in these cells. No significant differences in the amount of apoB synthesized or secreted from the primary hepatocytes were found between the primary hepatocytes from the mice expressing the wild-type PCSK9 or the S127R mutant compared with the β-galactosidase control (Fig. 7B).

To confirm these findings in vivo, mice that lack the LDLR (Ldlr−/−) were injected with adenoviruses expressing wild-type PCSK9. If PCSK9 functions to increase apoB secretion, then plasma VLDL and/or LDL cholesterol levels should be increased in the Ldlr−/− mice expressing wild-type PCSK9, since the LDLR is the major route of clearance of VLDL and LDL from plasma. Plasma VLDL and LDL cholesterol levels were not increased in Ldlr−/− mice injected with the wild-type PCSK9 adenovirus (Fig. 8A), despite documented expression of both the precursor and cleaved forms of the protein in the livers of the mice (Fig. 8B). Thus, no evidence was found to indicate that the increased plasma levels of LDL cholesterol associated with hepatic PCSK9 expression were due to increased apoB secretion in mice.

**DISCUSSION**

The current studies suggest that PCSK9 acts through a post-transcriptional mechanism that acts prior to internalization and recycling of the LDLR. This was demonstrated by determining whether PCSK9 overexpression altered the expression of the LDLR protein in livers of mice that lack ARH (Fig. 7). ARH is an adaptor protein that binds to the cytoplasmic domain of the LDLR and is required for endocytosis and subsequent recycling of the LDLR to the cell surface (37, 41, 42). PCSK9 overexpression in Ldlr−/− mice markedly reduced the amount of LDLR protein. The observed level of reduction was similar to that in mice with functional ARH, suggesting that PCSK9 mediates LDLR degradation at a point in the transit of the LDLR to the cell surface or when the LDLR is on the cell surface. Studies designed to elucidate the cellular site at which PCSK9 functions are currently in progress.

PCSK9 overexpression does not appear to alter apoB synthesis and secretion in mice. Primary hepatocytes derived from mice injected with adenoviruses expressing wild-type and mutant PCSK9 proteins exhibited no significant differences in apoB secretion (Fig. 7), and overexpression of PCSK9 in livers of Ldlr−/− animals did not alter VLDL or LDL cholesterol levels. These data support the conclusion that PCSK9 does not increase apoB secretion from liver but rather affects plasma levels of LDL cholesterol by reducing LDLR activity directly.

Maxwell and Breslow (44) reported studies using adenoviral overexpression of wild-type mouse PCSK9 in mice. The current studies confirm and extend these observations to normal human PCSK9 protein and demonstrate that two altered versions of PCSK9 with mutations found in families with hypercholesterolemia have similar activities when overexpressed in mouse liver. Furthermore, a catalytically inactive version of PCSK9 was shown not to alter LDLR expression (Fig. 5).

Several important questions regarding the function of PCSK9 remain unresolved. First, does PCSK9 cleave the LDLR directly, or does PCSK9 cleave another unidentified protein involved in LDLR trafficking or stability? A computer search for sequences that correspond to those recognized and cleaved by PCSK9 did not reveal any potential cleavage sites in the LDLR (6). However, it remains possible that the LDLR is

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**Fig. 6.** Immunoblot analysis of LDLR receptor in livers of Arh−/− mice injected with adenovirus expressing wild-type PCSK9. 10-week-old male wild-type and Arh−/− mice were injected with adenovirus (2 × 10⁶ PFU in 200 μl) expressing β-galactosidase (β-Gal) and wild-type PCSK9 (WT-PCSK9). Four days after injection, mice are sacrificed, and livers were processed for immunoblot analysis as described in the legend of Fig. 3. The results of one wild-type mouse injected with the β-galactosidase included as a positive control for the ARH antibody. P and C for PCSK9 denote the proprotein and cleaved forms of PCSK9, respectively. Each lane represents results from an individual mouse.
directly cleaved by PCSK9 at an as yet unidentified sequence.

Studies in cultured cells indicate that PCSK9 is not equally active in all immortalized cell types (Fig. 2). In addition to HepG2 cells, PCSK9 overexpression reduces the expression of the LDLR in HEK 293 cells but not in cultured human fibroblasts or in the Huh7 human hepatoma cells. Since all of these cultured cells express the LDLR protein, these observations raise the possibility that PCSK9 requires an additional protein or proteins not present in all cell types to effectively reduce LDLR.

A second question is whether PCSK9 functions intracellularly or as a secreted protein. If the secreted form of PCSK9 cleaves the LDLR, it could reduce the expression of the receptor in tissues that do not express PCSK9 (i.e., act in trans). Along these lines, we measured LDLR protein expression in other tissues from mice overexpressing PCSK9 in liver and found a marked reduction in LDLR protein in the adrenal gland (data not shown). Real time PCR analysis revealed very low levels of human PCSK9 mRNA in this and other tissues, which precludes definitive conclusions regarding the possibility that PCSK9 may be active in plasma.

A third unanswered question is how missense mutations in PCSK9 cause hypercholesterolemia in humans. Our studies did not reveal measurable differences in the ability of mutant and wild-type PCSK9 protein to reduce LDLR expression in liver.

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S. W. Park and J. D. Horton, unpublished observations.
therefore, we cannot definitively explain why individuals who harbor a mutant form of PCSK9 develop hypercholesterolemia. The phenotypes observed in individuals with mutations in PCSK9 are inherited in an autosomal dominant manner; therefore, it is predicted that mutations in PCSK9 result in a gain of function that ultimately results in hypercholesterolemia. If this is the case, the most likely explanation for our results is that the mutated forms of PCSK9 result in a subtle increase in PCSK9 activity compared with the wild-type PCSK9 protein, but the increased activity is masked by the level of overexpression.

An alternative explanation for our results is that the high level of overexpression PCSK9 achieved with adenoviral infection elicits an activity that is not physiological. Although this possibility cannot be excluded in the current studies, it is less likely, since the mutations in PCSK9 result in increased plasma levels of LDL cholesterol, and all previously defined mutations that result in elevated plasma LDL are due to changes in the ability of the LDLR to clear apoB-containing lipoproteins (10). Definitive studies demonstrating a difference in activity between wild-type and mutant PCSK9 proteins will require studies in mice that lack PCSK9 as well as knockin mice that express mutant forms of PCSK9 expressed at physiological levels.

The current and previous studies (3, 5, 44) demonstrate that plasma LDL cholesterol levels are principally determined by the expression of LDLRs in liver, which is regulated both transcriptionally and post-transcriptionally. Both mechanisms, however, are regulated by a single transcription factor family, the SRBPs, since these control the expression of both the LDLR and PCSK9 genes. The biological reason why SRBPs transcriptionally activate both the LDLR and PCSK9, a protein that apparently reduces LDLR protein levels, cannot be easily reconciled with our current information. It has previously been shown that the half-life of the LDLR protein in fibroblasts is 25 h (45). Therefore, the simultaneous transcriptional activation of PCSK9 by SRBPs may provide a post-transcriptional mechanism to degrade the LDLR and shorten the protein half-life, which could protect the cell from excessive LDL uptake and cholesterol accumulation. If this is the case, then an inhibitor of PCSK9 function may increase LDLR protein levels and enhance LDL clearance from the plasma. Confirmation of the function of PCSK9 and its potential value as a therapeutic target for the treatment of hypercholesterolemia will require studies in mice that lack PCSK9.

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