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 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, VLDDL 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 solublezymogen 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*
medium and Dulbecco's modified Eagle's medium (DMEM) (Invitrogen) supplemented with 5 % (v/v) FCS. On day 1, cells were transfected with 5 µg of pCMV-PCSK9-FLAG, pCMV-S127R, or pCMV-F216L per dish using the Fu- gene 6 reagent in a final volume of 0.2 ml. To generate control stable cell lines, 1 µg of pCDNA3 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. Recombinant adenoviral genomic constructs anti-FLAG monoclonal antibody. Cells line 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, or S386A forms of human PCSK9 were constructed using AdEasy system (Qiagen, Carlsbad, CA) according to the manufacturer's protocol. HindIII-Xbal fragments of pCMV-PCSK9-FLAG, pCMV-S127R, pCMV-F216L, and pCMV-S386A were ligated to a HindIII-Xbal-digested pShuttle-CMV vector. The resulting pShuttle constructs were co-transformed with the AdEasy-1 vector into BJS183 cells to produce recombinant adenoviral genomic constructs for wild-type and mutant PCSK9. Following transfection, adenoviral genomic constructs were linearized with PacI and transfected into QBI-293A cells (Qiagen) 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 using anti-FLAG antibody (Sigma).

HepG2 cells expressing wild-type human PCSK9 proteins were subjected to four rounds of amplification before purification by CsCl-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 adenovirus were injected as a single dose into tail vein in 0.1 ml. Mice were monitored for 14 days after infection, mice were treated as described above, and plasma and livers were harvested. Membrane fraction and nuclear extract from liver were prepared as described (30).

Real Time Reverse Transcription-PCR—Total RNA was prepared using an RNA STAT-60 kit (Tel-Test, Friendswood, TX). DNase I treatment of total RNA was performed using a DNA-free kit (Ambion, Austin, TX). cDNA was synthesized from 2 µg of total RNA using a TaqMan reverse transcription reagent kit and random hexamer primers (Applied Biosystems, Foster City, CA). Specific primers for each gene were designed using PRIMER EXPRESS software (Applied Biosystems) and previously published (31). The real time reverse transcription-PCR contained 20 ng of reverse-transcribed total RNA, a 167 ng concentration of the forward and reverse primers, and 10 µl of 2x SYBR Green PCR Master Mix in a final volume of 20 µl. The PCRs 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 with Animals and Diets—Studies using wild-type mice were performed in 10–12-week-old male C57BL/6 mice purchased from The Jackson Laboratory (Bar Harbor, ME). Mice with the genetic deletion of ARH (Arh−/−) (21) were generously provided by Drs. Joachim Herz and Helen Hobb (University of Texas Southwestern Medical Center). Knockout mice that lack the LDLR were previously described (32). All mice were maintained on 12-h dark/12-h light cycles and fed a chow diet that provided 10% calories from fat in 6,000 kcal/100 g diet. Life table studies were performed in wild-type and knockout mice on a diet containing 25% of the diet. Values are the mean ± SEM of three to six determinations.
and with the antibodies, 30 min antibody (Biodesign International, Saco, ME). After a 6-h incubation in medium containing 0.15 M NaCl, 5 mM EDTA, 10 mM HEPES, pH 8.0) were added. The cells were collected, and protease inhibitors (1 mM benzamidine, 0.5 mM phenylmethylsulfonyl fluoride, 2 mM EDTA, 50 mM Tris-Cl, pH 7.4, 62.5 mM sucrose, 0.5% Triton X-100, 0.5% sodium deoxycholate, 10 μg/ml leupeptin, 5 μg/ml pepstatin A, 0.5 mM phenylmethylsulfonyl fluoride, 2 μg/ml aprotinin, 25 μg/ml ALLN, and 1 mM benzamidine)

To immunoprecipitate apoB, 500 μg of cell extract or 0.5 ml of medium was incubated with 10 μg/ml of purified rabbit anti-mouse apoB antibody (provided by Dr. Helen Hobbs) in 0.5× NET (0.15 M NaCl, 5 mM EDTA, 50 mM Tris-Cl, pH 7.4, 0.5% Triton X-100, 0.1% SDS). To immunoprecipitate albumin, 50 μg of cell extract protein or 50 μl of medium was incubated with 10 μg/ml of rabbit anti-mouse albumin antibody (Biodesign International, Saco, ME). After a 6-h incubation with the antibodies, 30 μl of protein A/G-agarose (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) was added and incubated at 4 °C for 16 h. The protein-antibody-protein A/G-agarose complexes were washed five times with 500 μl of NET. The immunoprecipitated proteins were eluted in the loading buffer (0.125 M Tris-Cl, pH 6.8, 4% SDS, 20% glycerol, 10% 2-mercaptoethanol) by boiling for 5 min and then subjected to nitrocellulose filters for immunoblot analysis. The filters were incubated with the following primary antibodies: anti-HSV for SREBP-1 and SREBP-2, anti-Myc for S1P, and anti-FLAG for PCSK9. Bound primary antibodies were visualized using a peroxidase-conjugated affinity-purified donkey anti-mouse IgG secondary antibody.

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 protease, and S2P, a membrane-bound zinc metalloproteinase. S1P belongs to the same family of subtilase-like serine protease 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 PSCK9, 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 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 proprotein 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, adenovirus-mediated overexpression of wild-type PCSK9 or either 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.

![Fig. 2. LDLR expression in CHO-K1 and HepG2 cells transfected with wild-type or mutant forms of PCSK9. A, CHO-K1 cells stably transfected with pcDNA3 (CHO-K1), pCMV-PCSK9 (WT-PCSK9), pCMV-S127R (S127R), or pCMV-F216L (F216L) were grown in monolayer cultures as described under “Experimental Procedures.” For immunoblot analysis, 50 μg of cell lysate protein was subjected to 8% SDS-PAGE, transferred to a nitrocellulose membrane, and blotted with primary antibodies directed against FLAG, LDLR, and RAP. Aliquots of media from stably transfected cells in each group were trichloroacetic acid-precipitated, and the proteins were resuspended in SDS sample buffer as described under “Experimental Procedures.” Aliquots corresponding to 100 μl of pooled medium were subjected to 8% SDS-PAGE, and immunoblot analysis was performed using an anti-FLAG primary antibody and a peroxidase-conjugated affinity-purified donkey anti-mouse IgG secondary antibody. B, HepG2 cells were set up at 5 × 10⁵ cells/60-mm dish in medium D supplemented with 10% PCS on day 0. On day 1, cells were infected with adenovirus (5 × 10⁹ PFU) expressing β-galactosidase (β-Gal), wild-type (WT-PCSK9), S127R mutant (S127R), or F216L mutant (F216L) PCSK9 in 1 ml of medium D for 2 h. After a 2-h incubation, an additional 1 ml of medium D supplemented with 20% NCLPDS was added, and the cells were incubated overnight at 37 °C. After an overnight incubation, the cells were harvested and processed as described under “Experimental Procedures.” Aliquots (30 μg) of cell membrane and 5 μl of media were subjected to 8% SDS-PAGE and transferred to nitrocellulose filters 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 C for PCSK9 denote the proprotein and cleaved forms of PCSK9, respectively. These experiments were repeated twice with similar results.](50633)
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 Ar−/− 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.”
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 Arh−/− 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 × 10⁹ 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.
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.

2 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 physiologic levels.

The current and previous studies (3, 5, 44) demonstrate that plasma LDL cholesterol levels are principally determined by 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 physiologic levels.

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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.

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