

Serum Proprotein Convertase Subtilisin/Kexin Type 9 and Cell Surface Low-Density Lipoprotein Receptor Evidence for a Reciprocal Regulation

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Background—Proprotein convertase subtilisin/kexin type 9 (PCSK9) modulates low-density lipoprotein (LDL) receptor (LDLR) degradation, thus influencing serum cholesterol levels. However, dysfunctional LDLR causes hypercholesterolemia without affecting PCSK9 clearance from the circulation.

Methods and Results—To study the reciprocal effects of PCSK9 and LDLR and the resultant effects on serum cholesterol, we produced transgenic mice expressing human (h) PCSK9. Although hPCSK9 was expressed mainly in the kidney, LDLR degradation was more evident in the liver. Adrenal LDLR levels were not affected, likely because of the impaired PCSK9 retention in this tissue. In addition, hPCSK9 expression increased hepatic secretion of apolipoprotein B–containing lipoproteins in an LDLR-independent fashion. Expression of hPCSK9 raised serum murine PCSK9 levels by 4.3-fold in wild-type mice and not at all in LDLR^{−/−} mice, in which murine PCSK9 levels were already 10-fold higher than in wild-type mice. In addition, LDLR^{+/−} mice had a 2.7-fold elevation in murine PCSK9 levels and no elevation in cholesterol levels. Conversely, acute expression of human LDLR in transgenic mice caused a 70% decrease in serum murine PCSK9 levels. Turnover studies using physiological levels of hPCSK9 showed rapid clearance in wild-type mice (half-life, 5.2 minutes), faster clearance in human LDLR transgenics (2.9 minutes), and much slower clearance in LDLR^{−/−} recipients (50.5 minutes). Supportive results were obtained with an in vitro system. Finally, up to 30% of serum hPCSK9 was associated with LDL regardless of LDLR expression.

Conclusions—Our results support a scenario in which LDLR represents the main route of elimination of PCSK9 and a reciprocal regulation between these 2 proteins controls serum PCSK9 levels, hepatic LDLR expression, and serum LDL levels. (*Circulation*. 2013;127:2403-2413.)

Key Words: cholesterol ■ lipoproteins ■ mouse model ■ PCSK9 protein ■ LDL receptor

Lowering low-density lipoprotein (LDL) cholesterol is the single most effective approach for cardiovascular risk reduction, and statins are the drug of choice to achieve target LDL cholesterol levels.¹ Statins inhibit 3-hydroxy-3-methylglutaryl-coenzyme A reductase, the rate-limiting enzyme for cholesterol biosynthesis, and consequently cause upregulation of LDL receptor (LDLR) levels via sterol regulatory element binding protein 2 activation (SREBP-2).² However, many patients cannot achieve LDL cholesterol goals even on a high dose of statins.³ Human (h) proprotein convertase subtilisin/kexin 9 (PCSK9) is a secreted 692-aa protein that promotes the reduction of LDLR protein levels, mainly in the liver,^{4,5} without affecting LDLR mRNA levels.⁶ Gain-of-function mutations in PCSK9 are associated with a rare form of autosomal-dominant hypercholesterolemia,⁷ whereas loss-of-function mutations cause low cholesterol levels and

are linked to lower coronary artery disease rates.⁸ The human PCSK9 gene is found on human chromosome 1 (chromosome 4 in mice).⁹ The human protein (hPCSK9) is synthesized mainly in the liver, kidney, and small intestine,¹⁰ under the regulation of SREBP-2,¹¹ and has 76.6% identity with murine (m) PCSK9.¹² By activating the SREBP-2 pathway, statins increase circulating PCSK9 levels, an effect that likely limits the LDL-lowering efficacy of these drugs.¹³

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Clinical Perspective on p 2413

The secreted form of PCSK9 binds directly to LDLR, and the complex is internalized and targeted to the lysosomes for destruction.^{14–16} Although LDLR-dependent uptake is the only known pathway for serum PCSK9 clearance, recent evidence suggests that the converse is not true, as reduced LDLR function

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does not appear to affect clearance of serum PCSK9,^{17–19} either as a result of LDLR-independent clearance routes²⁰ or because a receptor that is dysfunctional in binding LDL may normally bind PCSK9. The LDLR^{-/-} mouse provides a unique system in which to study the metabolism of both LDL and PCSK9 in the complete absence of LDLR.

It is not clear whether PCSK9 has a biological effect other than degrading LDLR or if this effect becomes more obvious or relevant in the absence of LDLR. Some studies reported no effect of PCSK9 overexpression on serum cholesterol levels in the absence of LDLR.^{21,22} More recent studies have shown increases in serum cholesterol levels in an LDLR-independent manner.^{23,24} Consensus is also lacking on how PCSK9 is transported in the bloodstream and whether this relates to PCSK9 function. We were the first to show a significant association of hPCSK9 with LDL in vivo and in vitro²⁵; other laboratories reported that serum PCSK9 was either free or in macromolecular complexes more or less associated with lipoproteins.^{24–27}

As a secretory protein that regulates LDLR posttranscriptionally, PCSK9 has revolutionized our knowledge of cell-based control of cholesterol homeostasis.^{28,29,29a} Unlike the well-defined intracellular pathways that regulate cholesterol metabolism, the function and regulation of serum PCSK9 are not well understood. To characterize PCSK9 levels, distribution, and activity as a function of LDLR expression, we developed a transgenic line of mice that express hPCSK9 in addition to their normal expression of mPCSK9. We also performed in vivo and in vitro experiments to determine the reciprocal effects of PCSK9 and LDLR under different conditions, including the absence and overexpression of either protein. Our data show that LDLR is the primary route for serum PCSK9 clearance.

Methods

Materials and Reagents

The pcDNA3.1 Directional TOPO Expression Kit, ProBond Purification System, and precast gels were purchased from Invitrogen (Grand Island, NY). Aml-12, Y-1 (CCL-79), HEK293T, and HeLa cell lines were purchased from ATCC (Manassas, VA). The bicistronic lentiviral vector pWPI, the lentiviral envelope plasmid pMD2.G, and the packaging plasmid pCMV ΔR8.91 were kindly provided by Dr Dider Trono (Lausanne, Switzerland). Chicken polyclonal antibody to LDLR and rabbit polyclonal antibody to chicken horseradish peroxidase were purchased from Abcam Inc (Cambridge, MA). Sheep polyclonal antibody toward hPCSK9 was obtained from R&D systems (Minneapolis, MN). Rabbit polyclonal antibody toward hPCSK9 was purchased from MBL International Corp (Woburn, MA). Mouse anti-histidine Tag was purchased from eBioscience (San Diego, CA). Horseradish peroxidase-conjugated donkey anti-sheep IgG antibody, goat anti-rabbit, Tyloxapol, and Optiprep Density Gradient Medium were purchased from Sigma (St. Louis, MO). The Mammalian Transfection System was purchased from Promega (Madison, WI). The cross-linking reagent dithiobis(succinimidylpropionate) (DSP) substrate was purchased from Thermo Scientific (Logan, UT). ELISA kits for hPCSK9 and mPCSK9 were purchased from MBL International Corp. Liver perfusion and digestion media were purchased from Gibco (Grand Island, NY). OptiSeal tubes were purchased from Beckman-Coulter (Brea, CA).

Human PCSK9 Expression Construct and Transfection

Methods for the generation of lentiviral PCSK9 construct, lentivirus preparation, and transduction were previously described.³⁰ Briefly,

bicistronic lentiviral expression plasmids for PCSK9–green fluorescent protein, along with the packaging plasmid pCMV8.91 and envelope plasmid pMD2.G, were cotransfected into HEK293T cells. The viral particle suspension was harvested over 2 days and then filtered and subjected to ultracentrifugation. The titer of resuspended concentrated viral particles was determined by a standard HeLa titer procedure. Green fluorescent protein–positive cells were sorted with the ARIAIII cell sorter.

Generation of Transgenic Mice

The generation of the hPCSK9 transgenic mice was done in our laboratory as previously described.²⁵ In short, the pcDNA-PCSK9 DNA was digested by *Bgl*III and *Pst*I, and this 3800-bp fragment was used for transgenic injection. The injection was performed by Vanderbilt Transgenic Mouse/Embryonic Stem Cell Shared Resource. The transgenic mice were confirmed by Southern blotting and polymerase chain reaction.

Primary Hepatocyte Isolation

Primary hepatocytes were isolated as previously described.³¹ In short, ligatures were made around the portal vein and the inferior vena cava. Livers were perfused through the portal vein with Liver Perfusion Medium containing 0.167 mmol/L insulin and 0.5 mmol/L EGTA for 5 minutes and then digested with Liver Digestion Medium containing 0.167 mmol/L insulin and 62.7 Units/mL collagenase for 15 minutes. Livers were dissected, homogenized, and filtered through a 70-μm filter. Hepatocytes were cultured in 6-well collagen-coated plates at 1.8×10⁶ cells per well in DMEM with 17% FBS and 0.167 mmol/L insulin. Hepatocytes were cultured for 2 hours; then the media was changed to 17% FBS without insulin for 4 to 6 hours before the addition of purified human PCSK9.

Protein Purification

His-tagged full-length PCSK9 was purified with the ProBond protein purification system, as described previously.²⁵ Protein purification was measured with the use of gel electrophoresis with Coomassie blue staining, and the concentration was determined by Lowry assays and hPCSK9 ELISA (MBL International Corp).

Western Blotting

Samples were loaded onto NuPage 4% to 12% Bis-Tris or NativePage 4% to 16% precast gels (Invitrogen) for electrophoresis. The size-separated proteins were then transferred to nitrocellulose membranes. The indicated primary antibodies and horseradish peroxidase-conjugated secondary antibodies were used to detect target proteins. Signal was detected by use of a mixture of luminol, p-coumaric acid, and hydrogen peroxide in 100 mmol/L Tris (pH 8.5). Intensity quantification of the bands was obtained with ImageJ software and normalized to β-action or GAPDH.

Mice

Wild-type (WT) C57BL/6, LDLR^{-/-}, and hLDLR mice were purchased from the Jackson Laboratories (Bar Harbor, ME) and housed at the Vanderbilt University Medical Center. LDLR^{+/-} mice were bred in house using F1 from WT and LDLR^{-/-}. All animal experiments were carried out in compliance with National Institutes of Health guidelines and were approved by the Institutional Animal Care and Use Committee of Vanderbilt University. Blood and tissues were collected from 6- to 8-month-old mice.

Serum Analyses

The levels of hPCSK9 and mPCSK9 were measured in serum of nonfasting mice on chow diet and determined by ELISA kits (MBL International Corp) with a measured 5% cross-reactivity between mPCSK9 and hPCSK9 within the linear range of the kit (not shown). Serum cholesterol concentrations were measured with a colorimetric

method. Mouse lipoproteins were isolated by density (high-salt) ultracentrifugation as previously described³¹ or with natural gradient media (Optiprep) using TLN100 rotor and OptiSeal tubes at 90000 rpm for 2.5 hours. Mouse lipoprotein profile was determined by size exclusion chromatography (fast protein liquid chromatography [FPLC]).

PCSK9 Turnover

His-tagged hPCSK9 was purified from transduced HEK293T cells. Male WT, LDLR^{-/-} or hLDLR mice were anesthetized, and hPCSK9 (1.5 µg per mouse) was injected into the retro-orbital vein plexus. At the indicated time points, blood was collected from the tail vein. Animals were euthanized at the end of the experiment, and organs were collected for further analysis.

Cross-Linking Studies

For denaturing and nondenaturing gel analyses, FPLC fractions were incubated with DPS substrate (Thermo Scientific) at a final concentration of 0.5 mmol/L at ambient temperature for 30 minutes. Reaction was stopped by the use of 1 mol/L Tris (pH 7.5).

Statistical Analyses

GraphPad Prism 4 software was used to carry out statistical analyses. The Student paired *t* test was used to compare the means of 2 groups. ANOVA was used when >2 groups were compared. Results are presented as mean±SD or as percent±coefficient of variation.

Results

PCSK9 and LDLR Expression in WT and hPCSK9 Transgenic Mice

We generated a transgenic line of mice expressing hPCSK9, with a vector designed for expression in multiple tissues.²⁵ First, we measured the expression levels of endogenous mPCSK9 and LDLR in hPCSK9 transgenic mice compared with WT controls. As expected, endogenous mPCSK9 was expressed mainly in liver, small intestine, and kidney.^{10,32} Figure 1A and 1B shows that hPCSK9 expression did not alter the pattern of tissue mPCSK9 and LDLR expression. Expression of hPCSK9 was highest in the kidney (Figure 1C and 1D and Figure 1A in the online-only Data Supplement), whereas LDLR reduction was strongest in liver (−41%), followed by the kidney (−24%). There was no effect of hPCSK9 expression on LDLR levels in the adrenals (Figure 1D and Figure 1B in the online-only Data Supplement). These results support the notion that PCSK9 acts in a tissue-specific manner.^{26,33}

Cholesterol Levels and Distribution in hPCSK9 Transgenic Mice

Transgenic mice expressing hPCSK9 were backcrossed into LDLR^{-/-} background to generate hPCSK9/LDLR^{-/-} mice. Figure 2A shows that mice expressing hPCSK9 on WT background have a total cholesterol level of 168±18 mg/dL compared with 96±16 mg/dL in WT controls (*P*<0.01). This was due mainly to increased LDL cholesterol levels (Figure 2B). A more moderate but still significant (*P*<0.05) effect was observed in LDLR^{-/-} mice, in which expression of hPCSK9 increased total cholesterol to 377±98 mg/dL compared with 315±89 mg/dL in controls. Separation of serum lipoprotein by agarose gel electrophoresis shows that hPCSK9 expression increases the β-migrating band (LDL) both on WT and LDLR^{-/-} background mice compared with nontransgenic

controls (Figure 1IA in the online-only Data Supplement). Besides LDLR reduction, there was no effect of hPCSK9 on other hepatic lipoprotein receptors such as LDL receptor-related protein 1 (LRP1) (data not shown); therefore, we next investigated the influence of PCSK9 on lipoprotein production. Our results show that tyloxapol-induced blockade of lipolysis in fasted mice causes a significantly faster and larger serum triglyceride accumulation in hPCSK9 transgenic compared with nontransgenic controls on both WT and LDLR^{-/-} backgrounds (Figure 3C and 3D, respectively).

Serum mPCSK9 and hPCSK9 Levels

Although mRNA levels of mPCSK9 were not affected by hPCSK9 expression (Figure 1A), serum levels of mPCSK9 were increased by an average of 4.3-fold (682±105 versus 160±30 ng/mL) in transgenic mice compared with WT controls (Figure 3A). Conversely, serum levels of mPCSK9 were extremely elevated in LDLR^{-/-} controls compared with WT mice but did not increase further in hPCSK9/LDLR^{-/-} transgenic mice (Figure 3A). The increase in mPCSK9 levels in transgenic mice is probably attributable to hPCSK9-induced removal of hepatic LDLR, which in turn reduces mPCSK9 clearance. This mechanism likely also explains the difference in serum mPCSK9 levels between WT and LDLR^{-/-} mice. Figure 3A shows that the complete absence of LDLR resulted in a 9.5-fold increase in mPCSK9 levels (1514±245 ng/mL) compared with WT controls (160±30 ng/mL). Interestingly, heterozygote LDLR^{+/-} mice showed a 2.7-fold increase in serum mPCSK9 levels (to 429±89 ng/mL), whereas acute overexpression of hLDLR reduced serum levels of mPCSK9 by 67% (to 53±19 ng/mL; Figure 1IB in the online-only Data Supplement). Levels of hPCSK9 showed a similar trend, with a 2-fold increase in hPCSK9 levels in transgenic mice on LDLR^{-/-} background (4303±532 ng/mL) compared with transgenic mice on WT background (2181±423 ng/mL; Figure 3B). Additionally, in hPCSK9 transgenic mice on LDLR^{-/-} background, hepatic intracellular levels of hPCSK9 were lower than those in transgenic mice on WT background, suggesting that the absence of LDLR reduces the amount of circulating PCSK9 captured by the cell and that both newly synthesized and internalized hPCSK9 contribute to its cellular levels (Figure 1IC in the online-only Data Supplement).

To further study the effect of hPCSK9 on mPCSK9 accumulation, we transduced murine hepatocytes (Aml-12 cells), which naturally produce mPCSK9, with increasing multiplicities of infection of the bicistronic lentiviral DNA construct (pWPI-hPCSK9), as shown in Figure 3C. Transduced hepatocytes showed reduced LDLR levels with increasing multiplicities of infection of the pWPI-hPCSK9 lentivirus (Figure 3C). Interestingly, when levels of secreted hPCSK9 were low (below or equal to the levels of secreted mPCSK9), no effects were seen on media mPCSK9 accumulation. However, as the levels of hPCSK9 increased above those of mPCSK9, a linear increase in media mPCSK9 accumulation was observed (Figure 3D). This was the result of a nongenetic effect because pWPI-hPCSK9 transduction did not alter mRNA levels of mPCSK9 compared with control hepatocytes transduced with green fluorescent protein (Figure 1IIA in the online-only Data Supplement).

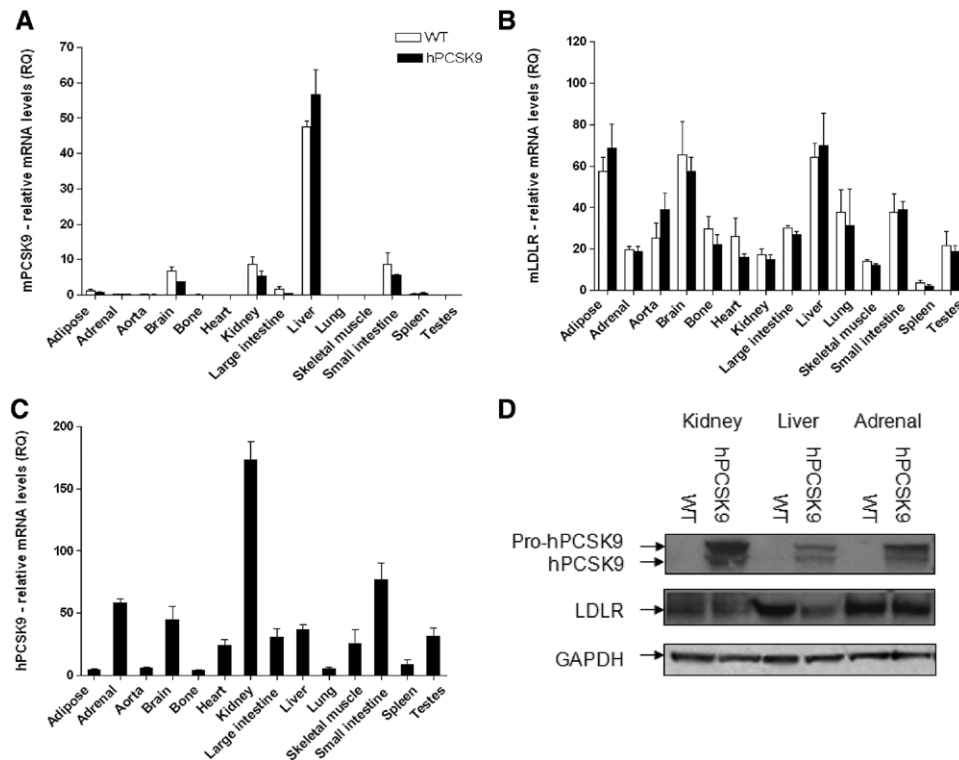


Figure 1. Proprotein convertase subtilisin/kexin type 9 (PCSK9) and low-density lipoprotein receptor (LDLR) levels in wild-type (WT) and human (h) PCSK9 transgenic mice tissues. Relative mRNA expression of (A) murine (m) PCSK9, (B) mLDLR, and (C) hPCSK9. Expression levels were calculated with the real-time polymerase chain reaction $\Delta\Delta$ CT method. Each tissue was measured in duplicate using 3 mice in each group ($n=3$). D, Representative immunoblot of hPCSK9, LDLR, and β -actin in WT and hPCSK9 transgenic mice (quantitative analysis is shown in Figure 1A and 1B in the online-only Data Supplement).

Turnover of Recombinant hPCSK9 in Mice

To characterize the effects of acute hPCSK9 exposure on LDLR levels in vivo, we performed a series of turnover studies using unmodified (nonradiolabeled) protein. The hPCSK9 was produced in HEK293T and purified (Figure IIIB in the online-only Data Supplement) as previously described.²⁵ In contrast to previous studies that have used PCSK9 at supraphysiological levels (4 to 32 μ g per mouse),^{33,34} we injected hPCSK9 at a dose of 1.5 μ g per mouse into WT,

LDLR^{-/-}, and hLDLR transgenic mice. These injections resulted in an average serum hPCSK9 peak level of 714 \pm 218 ng/mL, squarely in the physiological range (50–1000 ng/mL).^{35,36} Serum levels of injected hPCSK9 were then tracked over time (Figure 4A).

Rapid removal of hPCSK9 was observed in WT and hLDLR transgenic mice, with a calculated serum half-life of 5.2 and 2.9 minutes, respectively, and a nearly complete disappearance of the protein from serum after 1 hour. Conversely,

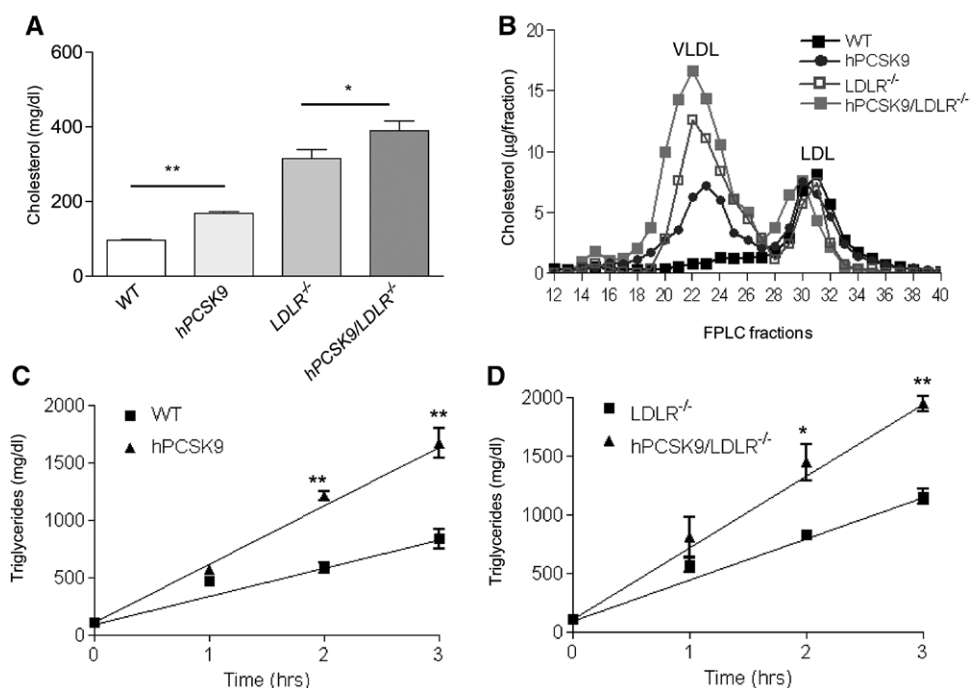


Figure 2. Effect of human proprotein convertase subtilisin/kexin type 9 (hPCSK9) on serum lipids in wild-type (WT) and low-density lipoprotein receptor-deficient (LDLR^{-/-}) background mice. A, Total serum cholesterol levels (in mg/dL; $n=9-16$). B, Serum fast protein liquid chromatography lipoprotein profiles (in μ g per fraction). Each profile represents pooled serum from 3 mice. Fractions: very-low-density lipoprotein, 12 to 17; low-density lipoprotein, 18 to 27; high-density lipoprotein, 28 to 34; and lipoprotein-deficient serum, 35 to 40. Changes in triglyceride levels after intravenous injection of 0.5 mg/g tyloxapol: (C) hPCSK9tg mice vs WT and (D) hPCSK9tg/LDLR^{-/-} vs LDLR^{-/-}. The slope indicates the secretion rate of triglyceride-rich lipoproteins. FPLC indicates fast protein liquid chromatography.

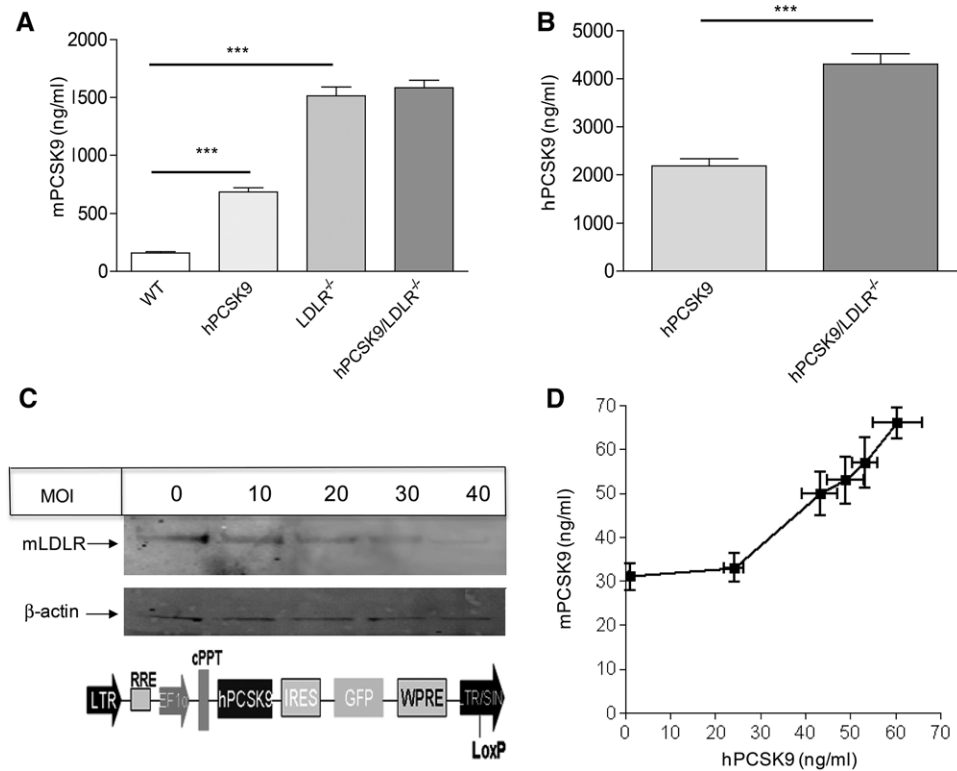


Figure 3. Effect of human proprotein convertase subtilisin/kexin type 9 (hPCSK9) on murine (m) PCSK9 level in wild-type (WT) and low-density lipoprotein receptor-deficient (LDLR^{-/-}) background mice. **A**, Serum mPCSK9 levels. Results for mPCSK9 levels in hPCSK9 transgenic mice take into account 5% cross-reactivity with hPCSK9. **B**, Serum hPCSK9 levels. Notice the different scales in **A** and **B**. Mice (n=9–16) were 6 to 8 months old, nonfasted, and on a chow diet. **C**, Immunoblot of LDLR and β-actin in transduced mouse Aml-12 hepatocyte and the bicistronic lentiviral DNA construct pWPI-hPCSK9. **D**, Secretion of mPCSK9 as a function of hPCSK9 levels in transduced Aml-12 cells. Transduction was performed in triplicate in each virus concentration (multiplicity of infection); the vertical standard deviation is for measured mPCSK9 in the media, and the horizontal standard deviation is for hPCSK9 measured in the same media. LTR indicates long terminal repeat; RRE, rev response element; EF1a, Elongation Factor 1a; hPCSK9, human Proprotein Convertase Subtilisin/Kexin 9; IRES, internal ribosome entry site; GFP, green fluorescent protein; WPRE, Woodchuck Hepatitis Virus (WHP) posttranscriptional regulatory element; and LTR/SIN, long terminal repeat/self inactivating. **P*<0.05; ***P*<0.01; ****P*<0.001.

hPCSK9 lingered in the serum of LDLR^{-/-} mice, with a calculated serum half-life of 50.5 minutes. The clearance pattern of hPCSK9 in WT and LDLR^{-/-} mice was similar to that of intravenously injected LDL (Figure IVA in the online-only Data Supplement), suggesting a dominant role for the LDLR in PCSK9 removal. Clearance of serum albumin served as a negative control for LDLR-dependent clearance and was similar in WT and LDLR^{-/-} mice (Figure IVB in the online-only Data Supplement).

In addition to tracking hPCSK9 in the serum, we monitored hPCSK9 accumulation and LDLR reduction in liver, kidney, and adrenal glands. Four hours after injection, the strongest effect of hPCSK9 in WT mice was seen in the liver, with a 44.8% reduction in LDLR levels, whereas the effect in the kidney was a 35% reduction in LDLR levels (Figure 4B and Figure VA in the online-only Data Supplement). Accumulation of hPCSK9 mirrored that of LDLR reduction, with the highest accumulation in the liver of WT animals. In contrast, livers from LDLR^{-/-} mice accumulated significantly lower levels of hPCSK9 (19.3±4.3%) compared with WT mice (Figure 4C). Despite the changes in LDLR levels after the injection of hPCSK9 in WT mice, no effects were seen on serum cholesterol levels, possibly owing to the transient effect and the low amount of PCSK9 used. To confirm the role of LDLR in

hPCSK9 uptake directly, we incubated primary hepatocytes from WT or LDLR^{-/-} mice with purified hPCSK9 and found that the uptake of hPCSK9 (500 ng/mL) by LDLR^{-/-} hepatocytes was 72.2% lower than that seen in WT hepatocytes (Figure 4D). As expected, exposure of WT hepatocytes to hPCSK9 reduced LDLR levels (Figure VB in the online-only Data Supplement).

In agreement with our findings in the hPCSK9 transgenic mice, LDLR levels in the adrenals were not affected by the injected hPCSK9, likely a consequence of the fact that exogenous hPCSK9 did not accumulate in this tissue (Figure 4C). Coimmunoprecipitation of hPCSK9 showed a weaker interaction between LDLR and PCSK9 in the adrenals compared with the liver of hPCSK9 transgenic mice (Figure VIA in the online-only Data Supplement). Such results suggest that tissue-specific penetration or retention of PCSK9 may be involved in its functional regulation. To test this hypothesis in vitro, we incubated mouse adrenal cells (Y-1) and mouse hepatocytes (Aml-12) with purified hPCSK9 (500 ng/mL) and found a greater LDLR reduction and more cellular hPCSK9 uptake by mouse hepatocytes compared with mouse adrenal cells (Figure VIB in the online-only Data Supplement). To determine whether PCSK9 can influence adrenal cell LDLR levels at all, we studied the effects of overexpression of hPCSK9 or

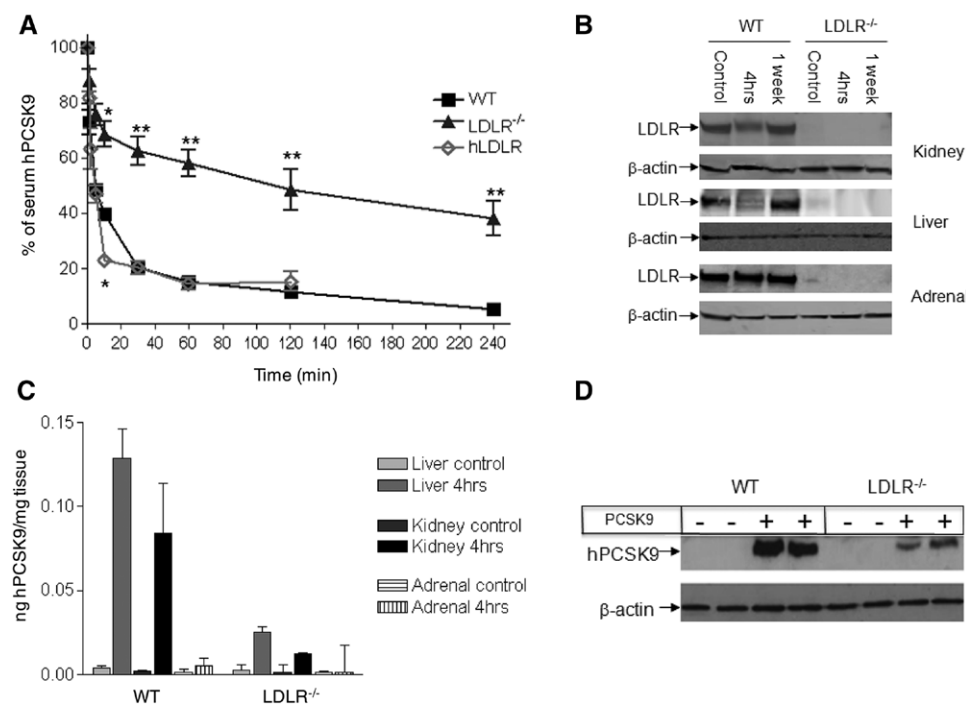


Figure 4. Turnover of recombinant human pro-protein convertase subtilisin/kexin type 9 (hPCSK9) in mice.

A, Serum levels of purified hPCSK9 injected in the retro-orbital vein plexus of wild-type (WT), low-density lipoprotein receptor-deficient (LDLR^{-/-}), or human (h) LDLR mice (n=3–4); results are presented as percent±coefficient of variation of hPCSK9 relative to time 0. Half-life of hPCSK9 was calculated from the exponential decay equation. **B**, Representative immunoblot of LDLR and β-actin levels in different tissues of WT or LDLR^{-/-} mice 4 hours after hPCSK9 injection (quantitative analysis is shown in Figure VA and VB in the online-only Data Supplement). **C**, Accumulation of hPCSK9 in tissues of WT or LDLR^{-/-} mice 4 hours after hPCSK9 injection (n=3). hPCSK9 levels were calculated by ELISA assay. **D**, Representative immunoblot for hPCSK9 in hepatocytes after a 4-hour incubation with or without 500 ng hPCSK9. *P<0.05; **P<0.01.

its gain-of-function mutation D374Y in Y-1 cells. Our results show that transduction of hPCSK9, particularly the D374Y mutant, results in reduced LDLR levels in Y-1 cells (47% and 77% reduction, respectively), showing that adrenal LDLR can be a target of hPCSK9 action in some circumstances (Figure VIC in the online-only Data Supplement).

Serum Distribution of hPCSK9

FPLC analysis of transgenic mouse serum shows that 75.2±6.1% of hPCSK9 coelutes with the apolipoprotein (apo) B-deficient fraction, and the rest (24.8±2.8%) coelutes with the LDL fraction, regardless of LDLR expression (Figure 5A). Similar results were obtained with ultracentrifugation of hPCSK9 transgenic mouse serum using neutral density gradient (Figure 5B), with 28.9±4% of total serum hPCSK9 found in the LDL fraction and the rest in the apoB-deficient fraction. Analyses of serum from 2 normolipidemic subjects confirmed a similar distribution pattern, with 35% and 39% of PCSK9 associated with the LDL fraction (not shown). To analyze the nature of the hPCSK9 association with LDL, we treated the FPLC fractions with a cross-linking reagent (DSP; 0.5 mmol/L for 30 minutes) and evaluated PCSK9 distribution after gel electrophoresis (Figure 5B, inset). The results show that very-low-density lipoprotein does not contain hPCSK9, LDL contains mainly monomeric hPCSK9, and the apoB-deficient fraction contains hPCSK9 that is mostly of higher molecular weight, likely dimers and trimers.²⁵ In agreement with our FPLC and ultracentrifugation results, direct immunoblotting of hPCSK9 transgenic mouse serum after agarose gel electrophoresis showed that ≈30% of hPCSK9 is associated with LDL (Figure 5C).

Interestingly, ultracentrifugal lipoprotein separation with a high-salt solution (KBr) resulted in >95% of PCSK9 being found in the lipoprotein-deficient serum (Table I in the online-only Data Supplement), as was previously shown.²⁷ Further analysis of transgenic mouse serum using coimmunoprecipitation of hPCSK9 (with either anti-hPCSK9 or anti-histidine-tag antibodies) showed that PCSK9 is associated with apoB100, with a weak association with apoB48 and no association with apoAI, or apoE (Figure 5D). Native gel immunoblot of the cross-linked FPLC fractions confirmed that PCSK9 is indeed part of the LDL particle (Figure VII in the online-only Data Supplement).

Discussion

We used transgenic expression and turnover studies of human PCSK9 to investigate its serum levels, tissue distribution, and activity. Our goal was to investigate the existence and degree of a reciprocal regulation between PCSK9 and LDLR and its influence on lipid levels in the mouse. Using multiple approaches, we show that clearance of serum PCSK9 is due predominantly to LDLR-mediated uptake. We also found that PCSK9 increases serum cholesterol levels via both LDLR-dependent and LDLR-independent pathways and that serum PCSK9 associates with LDL in a way that can affect peripheral or hepatic PCSK9 action. Finally, we investigated the basis for the lack of PCSK9 effect on LDLR levels in adrenals and provide results suggesting that higher levels of PCSK9 are needed to reduce LDLR levels in adrenals compared with liver, a phenomenon that is likely aggravated by the limited retention of circulating PCSK9 in the adrenal tissue.

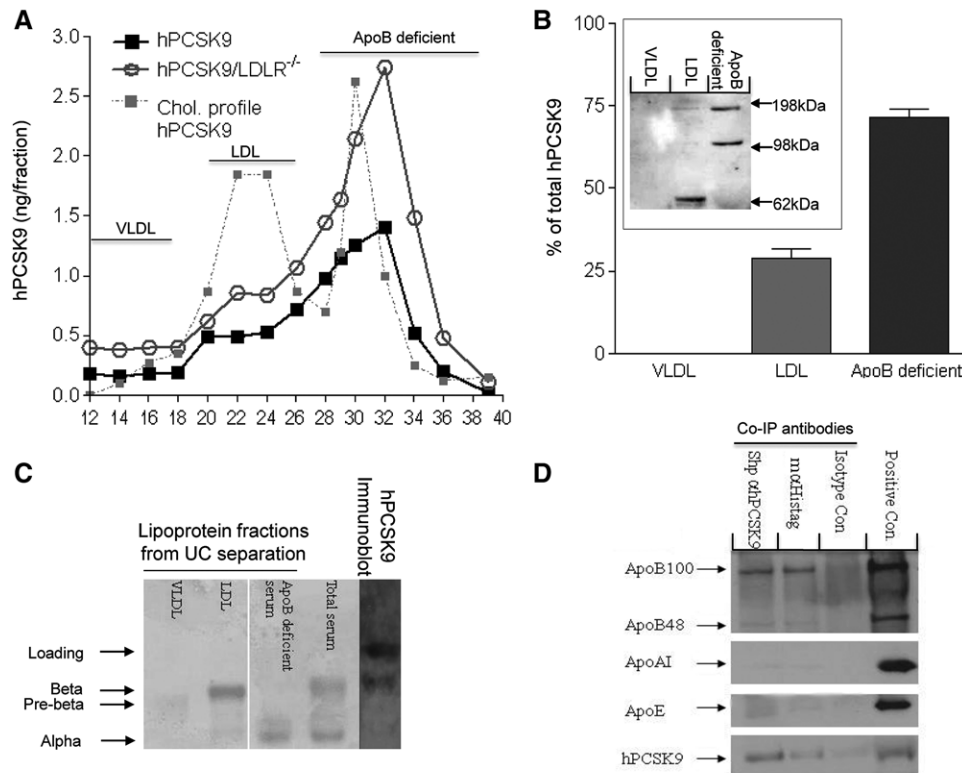


Figure 5. Lipoprotein association of serum proprotein convertase subtilisin/kexin type 9 (PCSK9). **A**, Serum fast protein liquid chromatography (FPLC) profile from human (h) PCSK9 transgenic mice on either wild-type (WT) or low-density lipoprotein receptor-deficient (LDLR^{-/-}) backgrounds. Each profile represents pooled serum from 3 to 4 mice. Fractions: very-low-density lipoprotein (VLDL), 12 to 17; LDL, 18 to 27; and apolipoprotein (Apo) B-deficient serum (including high-density lipoprotein), 28 to 39. PCSK9 in each fraction was calculated as the area under the curve (AUC). **B**, Relative hPCSK9 levels in transgenic mouse serum after ultracentrifugation (UC) using neutral density gradient medium (Optiprep). A pool of 21 mice was used for 3 different separations. **Inset**, hPCSK9 immunoblot from concentrated FPLC fractions of transgenic mouse serum. **C**, Lipid staining of serum from transgenic mice after Optiprep UC (**left**) or direct immunoblot (**right**). **D**, Apolipoprotein detection via immunoblot of hPCSK9 transgenic mouse serum after immunoprecipitation with anti-PCSK9 antibodies. Serum from hPCSK9 transgenic mice served as positive control. Co-IP indicates coimmunoprecipitation; m, mouse; and Shp, sheep (isotype control was Shp IgG).

A race to use the inhibition of PCSK9 as a treatment for hypercholesterolemia is underway. Current drug development efforts are directed either at reducing the production of PCSK9^{37,38} or at blocking circulating PCSK9 via neutralizing antibodies^{39–41} because the only known effect of PCSK9 is the binding and degradation of LDLR.⁴² PCSK9 is expressed in liver, small intestine, and kidney, but its main target is hepatic LDLR.^{10,32} In our transgenic mouse, hPCSK9 was produced mainly in the kidney and affected mostly LDLR levels in the liver (Figure 1). The LDLR-reducing effect in the kidney was more modest, and there was no effect in the adrenals, as previously reported.^{26,33} We show that in addition to LDLR reduction, hPCSK9 expression increases the secretion of triglyceride-rich lipoproteins in an LDLR-independent fashion, thus increasing cholesterol levels also in LDLR^{-/-} mice (Figure 2). Recent reports showing that PCSK9 increases apoB secretion in hepatocytes²⁴ and intestinal cells⁴³ and decreases very-low-density lipoprotein receptor levels in adipocytes²³ provide a mechanistic basis for an LDLR-independent effect of PCSK9 on cholesterol levels.

Current evidence does not suggest the presence of a correlation between the degree of LDLR function loss and

serum PCSK9 levels in humans.²⁰ Similar PCSK9 levels were found among hypercholesterolemia patients with or without LDLR mutations.¹⁷ Additionally, PCSK9 levels similar to those in control subjects were found in subjects with familial hypercholesterolemia and cholesterol levels above the 90th percentile, whereas PCSK9 levels even lower than those in control subjects were found in subjects with familial hypercholesterolemia and cholesterol levels below the 75th percentile.¹⁹ Although a recent study reported modestly increased PCSK9 levels in familial combined hyperlipidemia patients compared with control subjects, LDLR mutations did not have an influence on this small effect.¹⁸ Others have reported a minimal (11%) increase in PCSK9 levels of untreated familial hypercholesterolemia patients compared with control subjects.⁴⁴ It has to be kept in mind that most familial hypercholesterolemia presentations in humans are attributable to LDLR mutations that cause dysfunctional interaction with LDL while the receptor protein is intact and appropriately located on the cell surface. Because PCSK9 binds a different domain of the LDLR,¹⁶ one would expect normal PCSK9-LDLR interactions in these situations. In contrast, our approach allowed us to study a different scenario in which both LDL and PCSK9 are affected by the

absence of LDLR. This scenario is repeated clinically in familial hypercholesterolemia subjects with receptor-negative mutations of the LDLR.

We show that expression of hPCSK9 increases serum mPCSK9 levels in WT but not in LDLR^{-/-} mice, an effect likely attributable to the reduction in hepatic LDLR caused by hPCSK9, in turn impairing LDLR-mediated clearance of mPCSK9. The massive accumulation of serum mPCSK9 caused by the absence of LDLR obviously cannot be modulated further by the expression of hPCSK9 (Figure 3). This phenomenon was also spontaneously evident in LDLR^{+/-} and to a greater extent in LDLR^{-/-} mice (Figure II in the online-only Data Supplement). A mirror image of this was seen with the acute induction of hepatic hLDLR in transgenic mice, which caused a drastic and prompt two-thirds reduction of circulating mPCSK9 levels. This novel observation also provides the first proof of interaction between hLDLR and mPCSK9, likely explained by the high degree of homology between murine and human PCSK9.¹² Additionally, serum hPCSK9 levels in transgenic mice on LDLR^{-/-} background were 2 times higher than those of transgenic mice on WT background (Figure 3), again showing that LDLR regulates serum levels of PCSK9 by acting as its primary clearance route. Additionally, hPCSK9/LDLR^{-/-} mice had lower levels of intrahepatic hPCSK9, suggesting that internalization of PCSK9 by liver cells depends mainly on LDLR.

Similarly, transduced mouse hepatocytes showed a temporal and causal relationship between hPCSK9 expression and levels of both medium mPCSK9 and membrane LDLR. In particular, we showed that mPCSK9 started to accumulate in the medium when the secreted levels of hPCSK9 exceeded basal mPCSK9 levels (>1:1 ratio; Figure 3). This observation shows that the ability of murine hepatocytes to take up PCSK9 is directly related to the levels of surface LDLR. The above observations support our hypothesis that the relationship between PCSK9 and LDLR is reciprocal rather than unidirectional. It is undisputed that PCSK9 regulates LDLR levels,^{4,6,21} but it is also clear from our studies that LDLR regulates PCSK9 levels. Because both effects are posttranscriptional, it is likely that steady-state serum cholesterol levels in different individuals are affected by the global balance of these reciprocal regulatory influences, in which a primary excess of LDLR would deplete the PCSK9 pool and maintain proper LDL clearance whereas a primary excess of PCSK9 would deplete the LDLR pool and cause serum LDL elevations. Thus, it is possible that some forms of hypercholesterolemia result from unbalanced homeostasis between levels of serum PCSK9 and those of membrane LDLR. An example of this was recently published, with the demonstration that resistin increases PCSK9 levels and causes hypercholesterolemia in obese subjects.⁴⁵

Because transgenic expression of hPCSK9 influences LDLR levels long term and allows counterregulatory mechanisms to help reach a steady state, we also investigated the short-term effects of hPCSK9 after intravenous injection in mice. Our results show that LDLR is the dominant clearance route for serum PCSK9. We injected a small amount of hPCSK9 to raise serum hPCSK9 levels to an average of 714±218 ng/mL, well within the physiological range, and showed a marked

difference in the half-life of hPCSK9 between WT (5.2 minutes)^{33,34} and LDLR^{-/-} mice (50.5 minutes). In contrast, the acute overexpression of hLDLR decreased the hPCSK9 serum half-life by almost half (2.9 minutes; Figure 4). The nearly 10-fold increase in the hPCSK9 half-life matches the 9.5-fold increase in mPCSK9 in the serum of LDLR^{-/-} mice compared with controls. The significant increase in hPCSK9 half-life we observed in LDLR^{-/-} mice is different from the slightly prolonged increase (15 minutes) previously reported.³³ In addition, our *in vivo* and *in vitro* data show a much larger reduction in hPCSK9 uptake by liver and primary hepatocytes of LDLR^{-/-} mice (19.3% and 27.8% of control, respectively) compared with the milder accumulation defect (≈50%) previously reported.³³ These differences may derive from the use of ¹²⁵I-labeled PCSK9, with its inherent drawbacks resulting from protein oxidation, or from the supraphysiological levels of PCSK9 used in previous studies, causing an artificial exaggeration of the LDLR-independent clearance pathway. This latter possibility is also suggested by our data with transgenic mice showing that mPCSK9 levels are 9.5-fold higher in LDLR^{-/-} compared with WT mice, whereas hPCSK9 levels are only 2-fold higher in hPCSK9/LDLR^{-/-} mice compared with hPCSK9 WT.

Although the clearance of LDL reflected the expected pattern in both WT mice (fast) and LDLR^{-/-} mice (slow; Figure IV in the online-only Data Supplement), the absence of LDLR had a stronger effect on the half-life of hPCSK9 (9.7-fold increase over WT) than on that of LDL (5.2-fold increase over WT), suggesting that LDLR-dependent clearance is actually more important to PCSK9 than to LDL. This observation was confirmed in heterozygous LDLR^{+/-} mice, in which no significant differences were seen in cholesterol levels (not shown) but mPCSK9 levels were increased 2.7-fold compared with controls (Figure II in the online-only Data Supplement).

Transgenic expression of hPCSK9 did not have an effect on adrenal LDLR levels, a finding that confirms previous reports.^{26,33} In addition, we show that PCSK9 injected in WT mice accumulates in the liver and kidney but not in the adrenals, and we found a weaker association between PCSK9 and LDLR in the adrenals compared with liver. *In vitro*, adrenal LDLR levels were not affected by the addition of hPCSK9, and these cells internalized much less hPCSK9 compared with hepatocytes. It has been suggested that annexin A2 may act as an extrahepatic inhibitor of PCSK9⁴⁶; however, the impaired adrenal retention observed in our study appears to be independent of annexin A2 (Figure VIB in the online-only Data Supplement). Moreover, a significant LDLR reduction in adrenal cells was seen after overexpression of hPCSK9 or its D374Y gain-of-function mutation. These data combined suggest that the lack of effect of PCSK9 in adrenals is attributable to its limited retention in this tissue (Figure VI in the online-only Data Supplement).

Finally, we have studied the association of PCSK9 with serum lipoproteins, a controversial issue. We were the first to report a PCSK9 association with LDL particles *in vivo* and *in vitro*,²⁵ whereas others reported that PCSK9 either simply coelutes with lipoproteins (using size exclusion chromatography)^{24–26} or does not bind at all to lipoproteins

(using high-salt ultracentrifugal separation).²⁷ We used multiple approaches to clarify this important issue and to explain the discrepancies in the literature. First, using FPLC separation of serum lipoproteins, we showed that 24.8% of serum hPCSK9 elutes with the LDL fraction as a result of either physical association or coincidental coelution. Second, by direct immunoblotting of serum separated on an agarose gel, we were able to show, for the first time, that $\approx 30\%$ of hPCSK9 is directly associated with the β -migrating LDL band. Third, using a natural gradient solution, we showed that 28.9% of serum hPCSK9 is found in the LDL fraction. Fourth, by coimmunoprecipitation of total serum, we showed an association between hPCSK9 and apoB-100. Finally, by native gel immunoblotting of FPLC fractions, we showed that PCSK9 is associated with LDL. Taken together, our data allow us to conclude that more than a quarter of serum hPCSK9 in mouse serum (more than a third in human serum) is associated with LDL, and the remainder is found on the apoB-deficient fraction. In addition, hPCSK9 associated with LDL is a monomer, whereas the rest of serum hPCSK9 is found in larger complexes. We have previously reported that self-associated PCSK9 is more active than its monomer,²⁵ thus suggesting that the LDL particle can directly influence PCSK9 activity in the serum and regulate LDLR levels in peripheral tissues. Of note, using a high-salt ultracentrifugation approach, we did not find PCSK9 to be associated with LDL, in line with results from a previous study²⁷ and suggesting that this harsh method causes PCSK9 dissociation from LDL. During the revision of this manuscript, a study by Kosenko et al⁴⁷ used a natural density gradient ultracentrifugation approach similar to the one we describe here and showed that in human serum, PCSK9 associates with LDL and modulates its function.

Conclusions

Modulating hPCSK9 and LDLR levels in mice enabled us to show that LDLR is the primary route for serum PCSK9 clearance. Our data present a tight, tissue-specific, reciprocal regulation between these interacting partners, which determines serum PCSK9 levels, hepatic LDLR levels, and serum LDL cholesterol levels. We conclude that the lack of correlation between PCSK9 and LDLR functionality in humans stems from the ability of dysfunctional LDLR (impaired interaction with LDL) to still internalize PCSK9, not from alternative LDLR-independent internalization pathways. Our results forecast a clinical scenario in which LDLR mutations that affect both LDL and PCSK9 clearance (eg, receptor-negative mutations) aggravate hypercholesterolemia via the effect of accumulated PCSK9 on the normal LDLR allele product. In this regard, it must be noted that carriers of LDLR-negative mutations have been reported to show significantly higher LDL levels compared with carriers of dysfunctional LDLR mutations.⁴⁸ Patients carrying such mutations would be particularly good targets for PCSK9 inhibition approaches. A paper published online⁴⁹ after the acceptance of our manuscript reports that serum PCSK9 levels are higher in untreated FH subjects than in normal controls, with the highest levels seen in homozygous patients (HoFH). The large variation in PCSK9 levels in HoFH was hypothesized to be a result of

varying degrees of LDLR expression (receptor negative versus receptor defective). Our results provide mechanistic support to this hypothesis.

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Disclosures

None.

References

- Hunt KJ, Resendez RG, Williams K, Haffner SM, Stern MP; San Antonio Heart Study. National Cholesterol Education Program versus World Health Organization metabolic syndrome in relation to all-cause and cardiovascular mortality in the San Antonio Heart Study. *Circulation*. 2004;110:1251–1257.
- Horton JD, Goldstein JL, Brown MS. SREBPs: transcriptional mediators of lipid homeostasis. *Cold Spring Harb Symp Quant Biol*. 2002;67:491–498.
- Glassberg H, Rader DJ. Management of lipids in the prevention of cardiovascular events. *Annu Rev Med*. 2008;59:79–94.
- Benjannet S, Rhainds D, Essalmani R, Mayne J, Wickham L, Jin W, Asselin MC, Hamelin J, Varret M, Allard D, Trillard M, Abifadel M, Tebon A, Attie AD, Rader DJ, Boileau C, Brissette L, Chretien M, Prat A, Seidah NG. NARC-1/PCSK9 and its natural mutants: zymogen cleavage and effects on the low density lipoprotein (LDL) receptor and LDL cholesterol. *J Biol Chem*. 2004;279:48865–48875.
- Cohen JC, Boerwinkle E, Mosley TH Jr, Hobbs HH. Sequence variations in PCSK9, low LDL, and protection against coronary heart disease. *N Engl J Med*. 2006;354:1264–1272.
- Rashid S, Curtis DE, Garuti R, Anderson NN, Bashmakov Y, Ho YK, Hammer RE, Moon YA, Horton JD. Decreased plasma cholesterol and hypersensitivity to statins in mice lacking *Pcsk9*. *Proc Natl Acad Sci USA*. 2005;102:5374–5379.
- Abifadel M, Varret M, Rabès JP, Allard D, Ouguerram K, Devillers M, Cruaud C, Benjannet S, Wickham L, Erlich D, Derré A, Villéger L, Farnier M, Beucler I, Bruckert E, Chambaz J, Chanu B, Lecerf JM, Luc G, Moulin P, Weissenbach J, Prat A, Krempf M, Junien C, Seidah NG, Boileau C. Mutations in PCSK9 cause autosomal dominant hypercholesterolemia. *Nat Genet*. 2003;34:154–156.
- Cohen J, Pertsemlidis A, Kotowski IK, Graham R, Garcia CK, Hobbs HH. Low LDL cholesterol in individuals of African descent resulting from frequent nonsense mutations in PCSK9. *Nat Genet*. 2005;37:161–165.
- Seidah NG, Prat A. The biology and therapeutic targeting of the proprotein convertases. *Nat Rev Drug Discov*. 2012;11:367–383.
- Seidah NG, Benjannet S, Wickham L, Marcinkiewicz J, Jasmin SB, Stifani S, Basak A, Prat A, Chretien M. The secretory proprotein convertase neural apoptosis-regulated convertase-1 (NARC-1): liver regeneration and neuronal differentiation. *Proc Natl Acad Sci USA*. 2003;100:928–933.
- Dubuc G, Chamberland A, Wassef H, Davignon J, Seidah NG, Bernier L, Prat A. Statins upregulate PCSK9, the gene encoding the proprotein convertase neural apoptosis-regulated convertase-1 implicated in familial hypercholesterolemia. *Arterioscler Thromb Vasc Biol*. 2004;24:1454–1459.
- Pearson WR, Wood T, Zhang Z, Miller W. Comparison of DNA sequences with protein sequences. *Genomics*. 1997;46:24–36.
- Ason B, Tep S, Davis HR Jr, Xu Y, Tetzloff G, Galinski B, Soriano F, Dubinina N, Zhu L, Stefanni A, Wong KK, Tadin-Strapps M, Bartz SR, Hubbard B, Ranalletta M, Sachs AB, Flanagan WM, Strack A, Kuklin NA. Improved efficacy for ezetimibe and rosuvastatin by attenuating the induction of PCSK9. *J Lipid Res*. 2011;52:679–687.
- Mousavi SA, Berge KE, Leren TP. The unique role of proprotein convertase subtilisin/kexin 9 in cholesterol homeostasis. *J Intern Med*. 2009;266:507–519.

15. Zaid A, Roubtsova A, Essalmani R, Marcinkiewicz J, Chamberland A, Hamelin J, Tremblay M, Jacques H, Jin W, Davignon J, Seidah NG, Prat A. Proprotein convertase subtilisin/kexin type 9 (PCSK9): hepatocyte-specific low-density lipoprotein receptor degradation and critical role in mouse liver regeneration. *Hepatology*. 2008;48:646–654.
16. Zhang DW, Lagace TA, Garuti R, Zhao Z, McDonald M, Horton JD, Cohen JC, Hobbs HH. Binding of proprotein convertase subtilisin/kexin type 9 to epidermal growth factor-like repeat A of low density lipoprotein receptor decreases receptor recycling and increases degradation. *J Biol Chem*. 2007;282:18602–18612.
17. Ahmad Z, Adams-Huet B, Chen C, Garg A. Low prevalence of mutations in known loci for autosomal dominant hypercholesterolemia in a multi-ethnic patient cohort. *Circ Cardiovasc Genet*. 2012;5:666–675.
18. Brouwers MC, Konrad RJ, van Himbergen TM, Isaacs A, Otokoza S, Troutt JS, Schaefer EJ, van Greevenbroek MM, Stalenhoef AF, de Graaf J. Plasma proprotein convertase subtilisin kexin type 9 levels are related to markers of cholesterol synthesis in familial combined hyperlipidemia [published online ahead of print January 17, 2013]. *Nutr Metab Cardiovasc Dis*. doi:10.1016/j.numecd.2012.1011.1008.
19. Huijgen R, Fouchier SW, Denoun M, Hutten BA, Vissers MN, Lambert G, Kastelein JJ. Plasma levels of PCSK9 and phenotypic variability in familial hypercholesterolemia. *J Lipid Res*. 2012;53:979–983.
20. Cameron J, Bogsrud MP, Tveten K, Strøm TB, Holven K, Berge KE, Leren TP. Serum levels of proprotein convertase subtilisin/kexin type 9 in subjects with familial hypercholesterolemia indicate that proprotein convertase subtilisin/kexin type 9 is cleared from plasma by low-density lipoprotein receptor-independent pathways. *Transl Res*. 2012;160:125–130.
21. Maxwell KN, Breslow JL. Adenoviral-mediated expression of Pcsk9 in mice results in a low-density lipoprotein receptor knockout phenotype. *Proc Natl Acad Sci USA*. 2004;101:7100–7105.
22. Denis M, Marcinkiewicz J, Zaid A, Gauthier D, Poirier S, Lazure C, Seidah NG, Prat A. Gene inactivation of proprotein convertase subtilisin/kexin type 9 reduces atherosclerosis in mice. *Circulation*. 2012;125:894–901.
23. Roubtsova A, Munkonda MN, Awan Z, Marcinkiewicz J, Chamberland A, Lazure C, Cianflone K, Seidah NG, Prat A. Circulating proprotein convertase subtilisin/kexin 9 (PCSK9) regulates VLDLR protein and triglyceride accumulation in visceral adipose tissue. *Arterioscler Thromb Vasc Biol*. 2011;31:785–791.
24. Sun H, Samarghandi A, Zhang N, Yao Z, Xiong M, Teng BB. Proprotein convertase subtilisin/kexin type 9 interacts with apolipoprotein B and prevents its intracellular degradation, irrespective of the low-density lipoprotein receptor. *Arterioscler Thromb Vasc Biol*. 2012;32:1585–1595.
25. Fan D, Yancey PG, Qiu S, Ding L, Weeber EJ, Linton MF, Fazio S. Self-association of human PCSK9 correlates with its LDLR-degrading activity. *Biochemistry*. 2008;47:1631–1639.
26. Luo Y, Warren L, Xia D, Jensen H, Sand T, Petras S, Qin W, Miller KS, Hawkins J. Function and distribution of circulating human PCSK9 expressed extrahepatically in transgenic mice. *J Lipid Res*. 2009;50:1581–1588.
27. Alborn WE, Cao G, Careskey HE, Qian YW, Subramaniam DR, Davies J, Conner EM, Konrad RJ. Serum proprotein convertase subtilisin kexin type 9 is correlated directly with serum LDL cholesterol. *Clin Chem*. 2007;53:1814–1819.
28. Brown MS, Goldstein JL. The SREBP pathway: regulation of cholesterol metabolism by proteolysis of a membrane-bound transcription factor. *Cell*. 1997;89:331–340.
29. Poirier S, Mayer G, Benjannet S, Bergeron E, Marcinkiewicz J, Nassoury N, Mayer H, Nimpf J, Prat A, Seidah NG. The proprotein convertase PCSK9 induces the degradation of low density lipoprotein receptor (LDLR) and its closest family members VLDLR and ApoER2. *J Biol Chem*. 2008;283:2363–2372.
- 29a. Fazio S, Linton MF. Proprotein convertase subtilisin/kexin type 9 as transducer of physiologic influences on cellular cholesterol. A case for resistin. *J Am Coll Cardiol*. 2012;59:1706–1708.
30. Du F, Hui Y, Zhang M, Linton MF, Fazio D, Fan D. Novel domain interaction regulates secretion of proprotein convertase subtilisin/kexin type 9 (PCSK9) protein. *J Biol Chem*. 2011;286:43054–43061.
31. Farkas MH, Swift LL, Hasty AH, Linton MF, Fazio S. The recycling of apolipoprotein E in primary cultures of mouse hepatocytes: evidence for a physiologic connection to high density lipoprotein metabolism. *J Biol Chem*. 2003;278:9412–9417.
32. Poirier S, Prat A, Marcinkiewicz E, Paquin J, Chitramuthu BP, Baranowski D, Cadieux B, Bennett HP, Seidah NG. Implication of the proprotein convertase NARC-1/PCSK9 in the development of the nervous system. *J Neurochem*. 2006;98:838–850.
33. Grefhorst A, McNutt MC, Lagace TA, Horton JD. Plasma PCSK9 preferentially reduces liver LDL receptors in mice. *J Lipid Res*. 2008;49:1303–1311.
34. Schmidt RJ, Beyer TP, Bensch WR, Qian YW, Lin A, Kowala M, Alborn WE, Konrad RJ, Cao G. Secreted proprotein convertase subtilisin/kexin type 9 reduces both hepatic and extrahepatic low-density lipoprotein receptors in vivo. *Biochem Biophys Res Commun*. 2008;370:634–640.
35. Lagace TA, Curtis DE, Garuti R, McNutt MC, Park SW, Prather HB, Anderson NN, Ho YK, Hammer RE, Horton JD. Secreted PCSK9 decreases the number of LDL receptors in hepatocytes and in livers of parabiotic mice. *J Clin Invest*. 2006;116:2995–3005.
36. Konrad RJ, Troutt JS, Cao G. Effects of currently prescribed LDL-C-lowering drugs on PCSK9 and implications for the next generation of LDL-C-lowering agents. *Lipids Health Dis*. 2011;10:38.
37. Graham MJ, Lemonidis KM, Whipple CP, Subramaniam A, Monia BP, Crooke ST, Crooke RM. Antisense inhibition of proprotein convertase subtilisin/kexin type 9 reduces serum LDL in hyperlipidemic mice. *J Lipid Res*. 2007;48:763–767.
38. Frank-Kamenetsky M, Grefhorst A, Anderson NN, Racie TS, Bramlage B, Akinc A, Butler D, Charisse K, Dorkin R, Fan Y, Gamba-Vitalo C, Hadwiger P, Jayaraman M, John M, Jayaprakash KN, Maier M, Nechev L, Rajeev KG, Read T, Röhl I, Soutschek J, Tan P, Wong J, Wang G, Zimmermann T, de Fougerolles A, Vornlocher HP, Langer R, Anderson DG, Manoharan M, Kotliansky V, Horton JD, Fitzgerald K. Therapeutic RNAi targeting PCSK9 acutely lowers plasma cholesterol in rodents and LDL cholesterol in nonhuman primates. *Proc Natl Acad Sci USA*. 2008;105:11915–11920.
39. Stein EA, Gipe D, Bergeron J, Gaudet D, Weiss R, Dufour R, Wu R, Pordy R. Effect of a monoclonal antibody to PCSK9, REGN727/SAR236553, to reduce low-density lipoprotein cholesterol in patients with heterozygous familial hypercholesterolemia on stable statin dose with or without ezetimibe therapy: a phase 2 randomised controlled trial. *Lancet*. 2012;380:29–36.
40. Giugliano RP, Kohli P, Rogers WJ, Somaratne R, Huang F, Liu T, Mohanavelu S, Hoffman EB, McDonald ST, Abrahamsen TE, Wasserman SM, Scott R, Sabatine MS. Efficacy, safety, and tolerability of a monoclonal antibody to proprotein convertase subtilisin/kexin type 9 in combination with a statin in patients with hypercholesterolemia (LAPLACE-TIMI 57): a randomised, placebo-controlled, dose-ranging, phase 2 study. *Lancet*. 2012;380:2007–2017.
41. Raal F, Scott R, Somaratne R, Bridges I, Li G, Wasserman SM, Stein EA. Low-density lipoprotein cholesterol-lowering effects of AMG 145, a monoclonal antibody to proprotein convertase subtilisin/kexin type 9 serine protease in patients with heterozygous familial hypercholesterolemia: the Reduction of LDL-C With PCSK9 Inhibition in Heterozygous Familial Hypercholesterolemia Disorder (RUTHERFORD) randomized trial. *Circulation*. 2012;126:2408–2417.
42. Poirier S, Mayer G, Poupon V, McPherson PS, Desjardins R, Ly K, Asselin MC, Day R, Duclos FJ, Witmer M, Parker R, Prat A, Seidah NG. Dissection of the endogenous cellular pathways of PCSK9-induced low density lipoprotein receptor degradation: evidence for an intracellular route. *J Biol Chem*. 2009;284:28856–28864.
43. Levy E, Ben Djoudi Ouadda A, Spahis S, Sane AT, Garofalo C, Grenier É, Emonnot L, Yara S, Couture P, Beaulieu JF, Ménard D, Seidah NG, Elchebly M. PCSK9 plays a significant role in cholesterol homeostasis and lipid transport in intestinal epithelial cells. *Atherosclerosis*. 2013;227:297–306.
44. Dubuc G, Tremblay M, Paré G, Jacques H, Hamelin J, Benjannet S, Boulet L, Genest J, Bernier L, Seidah NG, Davignon J. A new method for measurement of total plasma PCSK9: clinical applications. *J Lipid Res*. 2010;51:140–149.
45. Melone M, Wilsie L, Palyha O, Strack A, Rashid S. Discovery of a new role of human resistin in hepatocyte low-density lipoprotein receptor suppression mediated in part by proprotein convertase subtilisin/kexin type 9. *J Am Coll Cardiol*. 2012;59:1697–1705.

46. Seidah NG, Poirier S, Denis M, Parker R, Miao B, Mapelli C, Prat A, Wassef H, Davignon J, Hajjar KA, Mayer G. Annexin A2 is a natural extrahepatic inhibitor of the PCSK9-induced LDL receptor degradation. *PLoS One*. 2012;7:e41865.
47. Kosenko T, Golder M, Leblond G, Weng W, Lagace TA. Low-density lipoprotein binds to proprotein convertase subtilisin/kexin type-9 (PCSK9) in human plasma and inhibits PCSK9-mediated LDL receptor degradation. *J Biol Chem*. 2013;288:8279–8288.
48. Guardamagna O, Restagno G, Rolfo E, Pederiva C, Martini S, Abello F, Baracco V, Pisciotto L, Pino E, Calandra S, Bertolini S. The type of LDLR gene mutation predicts cardiovascular risk in children with familial hypercholesterolemia. *J Pediatr*. 2009;155:199–204.e2.
49. Raal F, Panz V, Immelman A, Pilcher G. Elevated PCSK9 levels in untreated patients with heterozygous or homozygous familial hypercholesterolemia and the response to high-dose statin therapy. *J Am Heart Assoc*. 2013; doi: 10.1161/JAHA.112.000028.

CLINICAL PERSPECTIVE

We show novel posttranscriptional regulatory mechanisms determining serum levels and function of proprotein convertase subtilisin/kexin type 9 (PCSK9) : clearance via the low-density lipoprotein (LDL) receptor (LDLR) and transport on LDL in a quantitatively significant but less active form. Although PCSK9 is known to regulate LDLR levels, we report here that the reverse is also true and that the main exit route for serum PCSK9 is via LDLR. Thus, LDLR mutations may have larger effects on LDL metabolism if they affect not only LDL internalization but also PCSK9 clearance. We foresee a clinical scenario in which LDLR mutations that affect PCSK9 clearance in addition to LDL clearance (eg, receptor negative) can aggravate hypercholesterolemia via the effect of accumulated PCSK9, resulting from defective clearance, on the normal LDLR allele product. Along this line of thinking, it is also possible to forecast hypercholesterolemia resulting from an LDLR mutation that only causes defective PCSK9 clearance. It is interesting that carriers of *LDLR*-negative mutations have indeed been reported to show significantly higher LDL levels compared with carriers of dysfunctional LDLR mutations. Our finding that about one third of serum PCSK9 is part of LDL and is in a monomeric, less active form suggests that peripheral distribution and function of this regulator of LDLR levels are influenced by the lipoprotein that acts as a canonical ligand for LDLR. Maneuvers that increase the PCSK9 content of LDL in serum may allow us to broaden the search for inhibitory strategies of PCSK9 action, currently limited to blocking the interaction between PCSK9 and LDLR via antibodies.