# Wild-type PCSK9 inhibits LDL clearance but does not affect apoB-containing lipoprotein production in mouse and cultured cells

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**Abstract** **Mutations in Proprotein Convertase Subtilisin Kexin 9 (PCSK9) have been associated with autosomal dominant hypercholesterolemia. In vivo kinetic studies indicate that LDL catabolism was impaired and apolipoprotein B (apoB)-containing lipoprotein synthesis was enhanced in two patients presenting with the S127R mutation on PCSK9. To understand the physiological role of PCSK9, we overexpressed human PCSK9 in mouse and cellular models as well as attenuated the endogenous expression of PCSK9 in HuH7 hepatoma cells using RNA interference. Here, we show that PCSK9 dramatically impairs the expression of the low density lipoprotein receptor (LDLr) and, in turn, LDL cellular binding as well as LDL clearance from the plasma compartment in C57BL6/J mice but not in LDLr-deficient mice, establishing a definitive role for PCSK9 in the modulation of the LDLr metabolic pathway. In contrast to data obtained in S127R-PCSK9 patients presenting with increased apoB production, our study indicates that wild-type PCSK9 does not significantly alter the production and/or secretion of VLDL apoB in either cultured cells or mice. Finally, we show that unlike PCSK9 overexpression in mice, the S127R mutation in patients led to increased VLDL apoB levels, suggesting a potential gain of function for S127R-PCSK9 in humans.—** Lalanne, F., G. Lambert, M. J. A. Amar, M. Chétiveaux, Y. Zaïr, A-L. Jarnoux, K. Ouguerram, J. Friburg, N. G. Seidah, H. B. Brewer, Jr., M. Krempf, and P. Costet. **Wild-type PCSK9 inhibits LDL clearance but does not affect apoB-containing lipoprotein production in mouse and cultured cells. *J. Lipid Res.* 2005.** 46: **1312–1319.**

**Supplementary key words** Proprotein Convertase Subtilisin Kexin 9 • low density lipoprotein • apolipoprotein B

Autosomal dominant hypercholesterolemia is associated with mutations in genes involved in the regulation of LDL homeostasis. The most common and severe form of monogenic hypercholesterolemia is familial hypercholesterolemia (FH), caused by mutations in the low density lipoprotein receptor (LDLr) (1). FH is characterized by increased plasma LDL-cholesterol levels and premature cardiovascular disease (2). Another form of this disease, familial defective apoB-100, is caused by mutations in the LDLr binding domain of apolipoprotein B-100 (apoB-100) (3). ApoB-100 is synthesized by the liver and is the major protein component of VLDL and LDL (4). Recently, Proprotein Convertase Subtilisin Kexin 9 (PCSK9) has been identified as the third gene involved in autosomal dominant hypercholesterolemia (5). PCSK9 encodes a proprotein convertase known as Neural Apoptosis-Regulated Convertase-1 (6). Proprotein convertases are proteolytic enzymes involved in the regulation of biological activities of a wide variety of proteins synthesized as inactive precursors, such as matrix metalloproteases, adhesion molecules, prohormones, and growth factors (7–9). PCSK9 is the ninth member of the mammalian subtilisin serine protease family (6, 10).

The negative regulation of PCSK9 expression in mice fed a high-fat/high-cholesterol diet suggests a direct role for PCSK9 in cholesterol metabolism (11). It has been shown that adenovirus-mediated expression of murine

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Abbreviations: apoB, apolipoprotein B; DiI, 3,3'-dioctadecylindocarbocyanine iodide; FCR, fractional catabolic rate; FH, familial hypercholesterolemia; FPLC, fast-protein liquid chromatography; HDL-C, high density lipoprotein-cholesterol; IDL, intermediate density lipoprotein; KO, knockout; LDLr, low density lipoprotein receptor; MTP, microsomal transfer protein; PCSK9, Proprotein Convertase Subtilisin Kexin 9; RIPA, radioimmunoprecipitation buffer; siRNA, short, interfering RNA; SR-BI, scavenger receptor class B type I; TC, total cholesterol; TG, triglyceride.

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PCSK9 in control but not in LDLr-knockout (KO) mice results in increased plasma LDL-cholesterol, which is associated with decreased hepatic LDLr expression. Furthermore, PCSK9 overexpression decreases the expression of the LDLr and, in turn, LDL uptake in cultured cells (12, 13). Our laboratory recently published a series of kinetic studies of apoB-100 in patients carrying the S127R mutation in PCSK9. These studies indicate that the S127R mutation in PCSK9 is associated with higher production rates of VLDL apoB-100 as well as decreased LDL apoB-100 fractional catabolic rate (FCR) (14), suggesting that PCSK9 may act on both apoB synthesis and catabolism in vivo.

To gain further insight into the role of PCSK9, we overexpressed human PCSK9 in mice as well as transiently overexpressed or attenuated PCSK9 expression in cultured HuH7 hepatoma cells and measured the catabolism of LDL particles and the endogenous synthesis of VLDL and/or apoB in both experimental models. We show that PCSK9 overexpression does not significantly alter apoB production and that PCSK9 directly inhibits LDLr expression and activity in mice and cultured cells.

## METHODS

### Recombinant adenovirus and animal procedures

Human PCSK9 cDNA with a C-terminal V5 tag (6) was subcloned into pAdTrack-CMV (15). The adenovirus vectors coding human PCSK9 cDNA and a sham control adenovirus (Ad-PCSK9 and Ad-Null, respectively) were generated by the Vector Core of the University Hospital of Nantes (16). Male controls and LDLr-KO mice (Charles River, l'Arbresle, France) on a pure C57BL6/J background (8–12 weeks old) were housed in a pathogen-free facility under a standard 12 h light/12 h dark cycle and fed standard rodent chow and water ad libitum. Mice were anesthetized with isoflurane (Abbott, Rungis, France) and injected with $5 \times 10^8$ plaque-forming units via the penis vein. Blood samples were collected from the retro-orbital plexus and centrifuged at 2,500 *g* for 20 min at 4°C for plasma isolation. Five days after infusion, a subset of mice was killed and their livers harvested, frozen in liquid nitrogen, and stored at −80°C.

LDL ($1.019 < d < 1.006$) was isolated from 2 ml of pooled LDLr-KO mouse plasma by sequential ultracentrifugation in KBr at 5°C and 95,000 rpm for 4 h. The $^{125}$I-labeled apoB-LDL was prepared by a modification of the iodine monochloride method (17), then reisolated by ultracentrifugation and dialyzed overnight against 1× PBS and 0.01% EDTA. The preparation was analyzed by fast-protein liquid chromatography (FPLC) and agarose gel electrophoresis to ensure the integrity and purity of the particle. Mice were injected with $^{125}$I-labeled apoB-LDL ($10^6$ dpm) and sequentially bled over 2 days. The $^{125}$I-labeled apoB remaining in the plasma compartment was measured in plasma using a γ counter (Packard Instruments, Downers Grove, IL). We ascertained that more than 98% of the radioactivity remained associated with apoB by SDS-PAGE. The FCR was determined from the area under the apoB radioactivity curves using a multiexponential curve-fitting technique with the WinSAAM program (version 3.0.1) (18). Alternatively, mice were injected with tyloxapol (Sigma; 500 μg/g body weight). Plasma VLDL/chylomicron-triglyceride (TG) clearance in mice is completely inhibited under these conditions. Blood samples were taken from each mouse at 0, 45, 90, and 180 min after injection (19). The accumulation of newly synthesized VLDL/chylomicron-TG was measured in plasma aliquots. The accumulation of apoB was quantified by Western blot.

### Plasma collection, chemistry, and lipase activity

Fasting plasma from a normolipemic individual, from a representative heterozygous FH subject (20), and from two patients with the S127R PCSK9 mutation (14) was collected for apoB FPLC analysis. Plasma total cholesterol (TC), TGs, cholesteryl esters, and high density lipoprotein-cholesterol (HDL-C) were measured using commercial kits, and plasma lipoproteins from either pooled mouse samples (150 μl) or from patients (200 μl) were resolved by FPLC as described elsewhere (21). Human plasma apoB was quantified in the FPLC fractions as described previously (14). Mouse postheparin plasma lipase activity was assayed as described previously (22).

### Western blots and quantitative RT-PCR

Liver pieces were homogenized in 1× PBS containing 0.25% Na-deoxycholate and 1% Triton X-100. Cultured cells were lysed in a buffer containing 50 mM Tris, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, and 0.25% sodium deoxycholate. Both buffers contained a protease inhibitor cocktail (Roche Diagnostics, Mannheim, Germany). The supernatant was collected, and proteins (50 μg of liver protein, 25–30 μg of cell proteins) were resolved on Nu-PAGE 4–12% Bis-Tris gels in MES-SDS buffer (Invitrogen, Cergy Pontoise, France) under reducing conditions. Cell lysates were spun at 14,000 rpm for 15 min at 4°C. Protein concentration was determined using the bicinchoninic acid protein assay kit (Interchim, Montluçon, France). Proteins were transferred onto a Protran nitrocellulose membrane (Schleicher and Shuell, Dassel, Germany), probed with polyclonal chicken anti-LDLr antibody (RDI, Flanders, NJ) or scavenger receptor class B type I (SR-BI; Novus, Littleton, CO) for mouse proteins and anti-LDLr antibody (Progen Biotechnik, Heidelberg, Germany) for cellular proteins using Vectastain PK6101 (AbCys, Paris, France). The monoclonal anti-V5 antibody (Invitrogen), targeting only the human transgene, as well as rabbit IgG directed against the ERTARRLQAQAARRGY peptide (Neosystem, Strasbourg, France), referred to as E16Y, within the prodomain of PCSK9 were used to probe mouse liver proteins as described above using the ECL plus kit (Amersham, Little Shalfont, UK). Mouse apoA-I, apoA-II, apoE, and apoB within FPLC fractions (10 μl) were analyzed by Western blot using antibodies raised against the purified apolipoproteins (Biodesign, Saco, ME) (23).

Cellular RNA was isolated using the RNeasy kit and Qiashredder mini columns as well as RNase-free DNase I (Qiagen, Courtaboeuf, France). First-strand cDNA was synthesized with random primers using a Superscript II RNaseH reverse transcriptase reagent kit (Invitrogen). Quantification of human PCSK9 mRNA was performed using Assays-on-Demand and the Taqman universal PCR Master Mix (Applied Biosystems, Courtaboeuf, France). All samples were normalized to ribosomal protein L13a expression.

### LDL binding, apoB synthesis, and microsomal transfer protein activity in cultured cells

HuH7 cells (24) were plated at a density of $2 \times 10^5$ cells on 12-well plates and transfected the next day with human PCSK9 short, interfering RNA (siRNA) (AAGGUCUGGAAUGCAAAGUCA) or a nonspecific SiCONTROL nontargeting siRNA#1 (Dharmacon, Lafayette, CO) and/or pIRES-hPCSK9 using the Lipofectamine 2000 reagent (Invitrogen). After 48 h in complete medium, cells were washed twice with PBS at 4°C and incubated with 4 μg/ml (3,3'-dioctadecylindocarbocyanine iodide)-LDL (DiI-LDL) (Invitrogen) in DMEM containing 10% lipoprotein-deficient serum for 30 min at 4°C. Cells were washed extensively

to remove unbound DiI-LDL and fixed for 25 min in 4% Paraformaldehyde at room temperature, washed twice with PBS, and maintained in PBS containing $NaN_3$ and analyzed for DiI fluorescence by microscopy. In a subset of studies, the cells were incubated in serum-free medium 1 day before LDL binding assay.

HuH7 cells plated on six-well plates were washed twice with PBS and incubated for 1 h in methionine/cysteine-free medium containing 0.5 mM oleic acid. Cells were then pulsed for 30 min with 200 μCi/ml [$^{35}$S]methionine/[$^{35}$S]cysteine mix (Express EasyTag; Perkin-Elmer Life Sciences, Boston, MA) and chased in DMEM containing 5 mM methionine and cysteine. At the end of the pulse-chase, medium was transferred into tubes containing a cocktail of protease inhibitors (Roche Diagnostics). Cells were washed twice with cold PBS and lysed directly in the wells by the addition of a 50 mM Tris, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, and 0.25% sodium deoxycholate buffer containing the protease inhibitor cocktail. Both media and cell lysates were homogenized on a rocking platform for 1 h at 4°C and centrifuged for 10 min at 10,000 *g* to pellet cell debris. Supernatants were combined with 3× Radioimmunoprecipitation (RIPA) buffer before immunoprecipitation. Cell extracts and medium were incubated with an excess of goat anti-human apoB antibody (Santa Cruz Biotechnology, Santa Cruz, CA) and incubated overnight at 4°C. Antigen-antibody complexes were isolated by centrifugation after incubation for 2 h at 4°C with protein A/G agarose plus (Santa Cruz Biotechnology). The complexes were subjected to three washes with RIPA and two with PBS. The tubes were incubated for 10 min at 4°C and centrifuged for 2 min at 4,000 rpm to pellet the complexes between each wash. The immunoprecipitated complexes were boiled for 5 min in 50 μl of lauryl dodecyl sulfate reducing buffer (Invitrogen) and centrifuged, and the supernatant was resolved by gel electrophoresis and scintillation counting. The $^{35}$S-labeled apoB protein bands were quantified using a phosphorimager.

For microsomal transfer protein (MTP) activity assay, the cells were washed twice with PBS, harvested in 200 μl of 10 mM Tris, 150 mM NaCl, and 1 mM EDTA buffer containing the protease inhibitor cocktail, and lysed by sonication. MTP activity was measured using 40 μg of the homogenate and a commercial kit (Calbiochem-Novabiochem Corp., San Diego, CA).

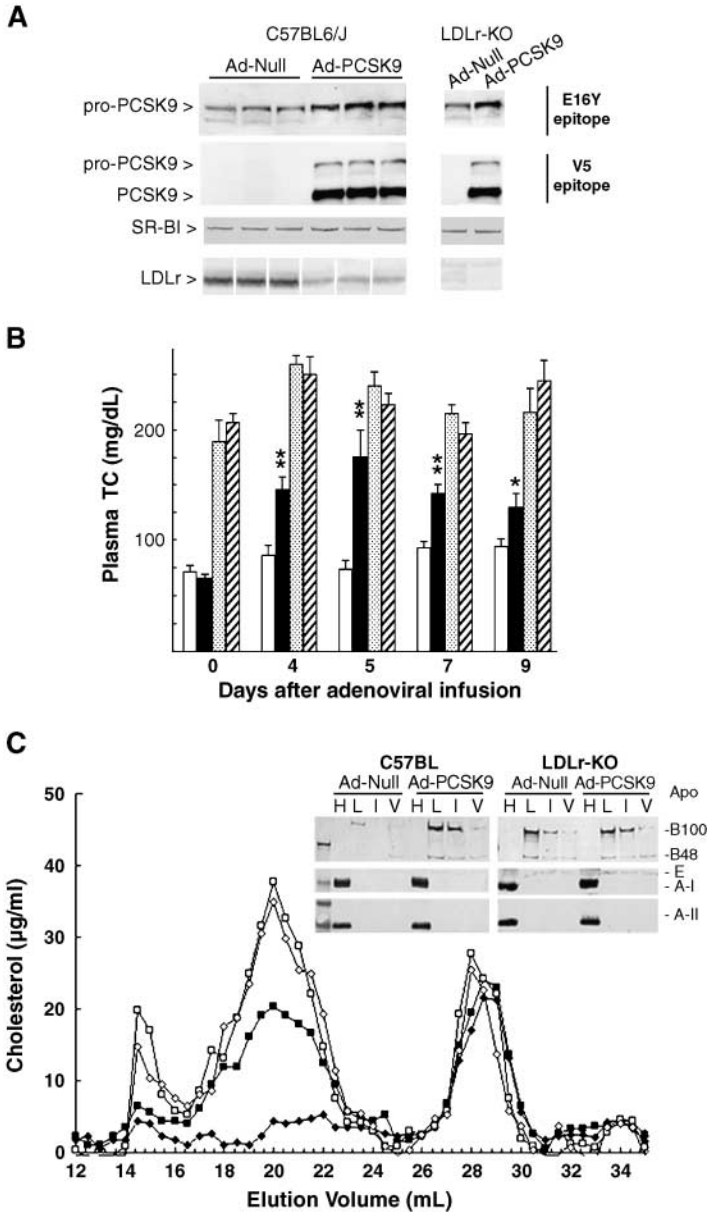


**Fig. 1.** A: Immunoblot analysis of Proprotein Convertase Subtilisin Kexin 9 (PCSK9), scavenger receptor class B type I (SR-BI), and low density lipoprotein receptor (LDLr) hepatic expression in C57BL6/J and LDLr-knockout (KO) mice at 5 days after adenoviral infusion of Ad-PCSK9 or Ad-Null. PCSK9 expression was assessed using a monoclonal anti-V5 antibody targeting the human transgene or a polyclonal anti-E16Y antibody targeting the prodomains of both human and murine PCSK9. B: Plasma total cholesterol (TC) levels of C57BL6/J mice injected with either Ad-Null (open bars) or Ad-PCSK9 (closed bars) and of LDLr-KO mice injected with either Ad-Null (dotted bars) or Ad-PCSK9 (diagonal bars) at days 0, 4, 5, 7, and 9 after adenoviral infusion. Values are expressed as means ± SEM. * $P < 0.05$ and ** $P < 0.01$ versus Ad-Null controls. C: Fast-protein liquid chromatography (FPLC) profile of pooled plasma (150 μl) samples from C57BL6/J (closed symbols) and LDLr-KO (open symbols) mice (n = 4 for all) at 5 days after infusion with either Ad-Null (diamonds) or Ad-PCSK9 (squares). Cholesterol concentrations in the eluted fractions are indicated on the *y* axis. Inset: Immunoblot analysis of apolipoprotein B (apoB), apoE, apoA-I, and apoA-II in the VLDL (V), intermediate density lipoprotein (IDL; I), LDL (L), and HDL (H) fractions (eluting at 15, 18, 20, and 29 ml, respectively) from C57BL6/J and LDLr-KO mice infused with either Ad-PCSK9 or Ad-Null.

### Statistical analysis

Values are expressed as means ± SEM. Comparisons between groups were made using Student's *t*-test for independent samples (one-tailed).

## RESULTS

### Human PCSK9 expression in mice after infusion of recombinant adenovirus

The expression of PCSK9 in C57BL6/J mice after injection of either Ad-PCSK9 or Ad-Null was quantified by immunoblot analysis of liver extracts using a polyclonal anti-PCSK9 antibody that recognizes both murine and human pro-PCSK9. Peak expression of pro-PCSK9 occurred 4–5 days after infusion (data not shown). **Figure 1A** shows representative data from day 5 C57BL6/J mouse liver extracts of both Ad-PCSK9- and Ad-Null-infused groups. Mice injected with Ad-PCSK9 had two to three times more hepatic pro-PCSK9 protein than sham-injected mice. Immunoblot analysis of the same liver extracts using a monoclonal V5 antibody that recognizes only the human PCSK9 transgene indicated that in mouse liver human pro-PCSK9 is processed into mature PCSK9 (Fig. 1A).

### Plasma lipids and LDL catabolism in mice overexpressing PCSK9

The plasma lipids of C57BL6/J mice after Ad-PCSK9 infusion were determined at day 5 after infusion (**Table 1**). The plasma levels of TC and cholesteryl ester but not those of HDL-C and TG were significantly increased in mice overexpressing PCSK9 versus controls, with the maximal plasma TC level reached 5 days after adenoviral infusion (i.e., upon peak expression of the transgene) (Fig. 1B). To assess the distribution of cholesterol within the plasma lipoproteins, pooled plasma samples from both Ad-PCSK9- and Ad-Null-infused mice were fractionated by FPLC (Fig. 1C). The cholesterol distribution in the Ad-PCSK9-infused group exhibited a dramatic increase in cholesterol content within the LDL and intermediate density lipoprotein (IDL)-sized lipoproteins compared with controls. To explore the mechanisms responsible for increased LDL/IDL-C in mice infused with Ad-PCSK9, we measured the hepatic expression of two major lipoprotein receptors (i.e., LDLr and SR-BI) by immunoblot analysis. The expression of the LDLr was dramatically decreased in the livers of Ad-PCSK9-infused mice (Fig. 1A), whereas the expression of the HDL receptor SR-BI remained unchanged. To further explore the molecular pathway responsible for increased LDL-cholesterol in mice overexpressing PCSK9, we performed Ad-PCSK9 and Ad-Null infusions in LDLr-KO male mice. Representative data for the hepatic levels of pro-PCSK9, SR-BI, and LDLr of LDLr-KO mice injected with either Ad-Null or Ad-PCSK9 are reported in Fig. 1A. The plasma lipids (Table 1) as well as the FPLC profiles (Fig. 1C) of LDLr-KO mice infused with either Ad-Null or Ad-PCSK9 were similar. The plasma TC levels of LDLr-KO mice infused with either Ad-Null or Ad-PCSK9 were similar at days 4, 5, 7, and 9 after adenoviral infusion (Fig. 1B). Notably, the dramatic LDL/IDL-C increase observed in C57BL6/J mice overexpressing PCSK9 was associated with a concomitant increase in the levels of LDL/IDL-apoB-100 and, to a lesser extent, LDL/IDL-apoB-48, similar to those of LDLr-KO mice (Fig. 1C, inset). No changes in apolipoprotein levels were detected after infusion of Ad-PCSK9 in LDLr-KO mice. Consistent with plasma HDL-C levels, the infusion of Ad-PCSK9 did not affect the apoA-I and apoA-II contents of the HDL particles. Thus, our data indicate that expression of PCSK9 in control mice results in a selective increase of LDL/IDL-C and LDL/IDL-apoB associated with a decrease in the hepatic expression of the LDLr.

TABLE 1. Plasma lipids of C57BL6/J controls and LDLr-KO male mice at 5 days after infusion of either Ad-PCSK9 or Ad-Null

| | C57BL6/J | | LDLr-KO | |
|---|---|---|---|---|
| Variable | Ad-Null (n = 6) | Ad-PCSK9 (n = 6) | Ad-Null (n = 4) | Ad-PCSK9 (n = 4) |
| | *mg/dl* | | | |
| Total cholesterol | 74 ± 8 | 176 ± 23[a] | 239 ± 13 | 222 ± 11 |
| High density lipoprotein-cholesterol | 59 ± 7 | 64 ± 5 | 71 ± 10 | 70 ± 12 |
| Cholesteryl ester | 64 ± 9 | 149 ± 18[a] | 203 ± 11 | 201 ± 12 |
| Triglyceride | 88 ± 15 | 86 ± 9 | 123 ± 14 | 120 ± 12 |

KO, knockout; LDLr, low density lipoprotein receptor; PCSK9, Proprotein Convertase Subtilisin Kexin 9.

[a] $P < 0.05$ versus Ad-Null injected C57B16/J.

To define the underlying mechanism by which PCSK9 reduces LDL cholesterol in C57BL6/J but not LDLr-KO mice, we performed a series of kinetic analyses of $^{125}$I-apoB-labeled LDL 5 days after injection of either Ad-Null or Ad-PCSK9 into both mouse lines. Plasma was collected at multiple time points after the initial injection of radiolabeled LDL, and the plasma decay of apoB was ascertained (**Fig. 2**). Compared with Ad-Null-injected controls, Ad-PCSK9-injected C57BL6/J mice had markedly delayed plasma clearance of $^{125}$I-apoB-LDL (FCR = 5.4 ± 0.2 vs. 4.5 ± 0.2 day$^{-1}$, respectively; $P < 0.03$). In contrast, the catabolism of $^{125}$I-apoB-LDL was similar in LDLr-KO mice infused with either Ad-Null or Ad-PCSK9 (FCR = 4.0 ± 0.3 vs. 4.1 ± 0.4 day$^{-1}$, respectively; $P > 0.9$) and delayed compared with C57BL6/J mice infused with Ad-Null ($P < 0.01$). Thus, PCSK9 attenuates the clearance of $^{125}$I-apoB-LDL in controls but not in LDLr-KO mice, establishing that PCSK9 promotes a decrease in hepatic LDLr expression and activity in vivo.

### PCSK9 gene silencing and overexpression in HuH7 hepatomas modulate LDLr-mediated DiI-LDL binding

To elucidate the role of PCSK9, we developed in parallel a transient knockdown model in the human hepatoma cell line HuH7 using RNA interference attenuation. As shown in **Fig. 3A**, transient transfection of siRNA duplexes against human PCSK9 in HuH7 decreases endogenous PCSK9 mRNA by ~80% and totally suppresses PCSK9 overexpression mediated by pIRES-hPCSK9 (Fig. 3B). Overexpression of human PCSK9 in HuH7 decreases LDLr protein expression, and cotransfection of human PCSK9 siRNA

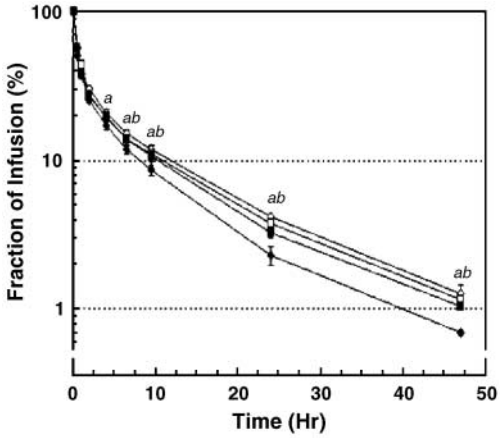


**Fig. 2.** Kinetic analysis of plasma $^{125}$I-apoB-labeled LDL in C57BL6/J controls (n = 6 per group; closed symbols) and LDLr-KO male mice (n = 4 per group; open symbols) infused with either Ad-PCSK9 (squares) or Ad-Null (diamonds). Five days after adenoviral infusion, the mice were injected with $^{125}$I-apoB-labeled LDL, and the plasma decay of the label was measured in plasma aliquots from sequential bleedings over 48 h. Values are expressed as means ± SEM. [a] $P < 0.05$ between LDLr-KO and C57BL6/J controls, both infused with Ad-Null. [b] $P < 0.05$ between C57BL6/J controls infused with Ad-PCSK9 and infused with Ad-Null.

virtually blocks the effects of the human PCSK9 overexpression (Fig. 3B). Transfection of human PCSK9 siRNA increased endogenous expression of the LDLr in HuH7 by 33% (Fig. 3C). We investigated the effect of PCSK9 gene silencing on LDLr function in HuH7 overexpressing or attenuated for PCSK9. The activity of the LDLr was determined by measuring the cellular binding of DiI-LDL by fluorescence microscopy at 4°C. PCSK9 gene silencing caused a 2.2-fold increase in DiI-LDL binding, whereas overexpression of human PCSK9 decreased DiI-LDL binding by 55% (Fig. 3D).

### Analysis of VLDL metabolism

To further explore the molecular mechanisms by which PCSK9 affects apoB-containing lipoprotein metabolism, we injected tyloxapol, which blocks the hydrolysis of TG by plasma lipases, in C57BL6/J mice infused with either Ad-PCSK9 or Ad-Null. There was no significant difference in the plasma accumulation of newly synthesized VLDL between groups (**Fig. 4A**). There was a trend ($P = 0.08$) toward increased apoB-100 (Fig. 4A, inset) but not apoB-48 (data not shown) plasma accumulation after tyloxapol injection in C57BL6/J mice overexpressing PCSK9 versus controls. In addition, the postheparin plasma lipolytic activity was similar in Ad-Null- and Ad-PCSK9-infused animals (206 ± 59 vs. 184 ± 38 nmol FFA/ml/h, respectively; $P > 0.8$).

To assess whether PCSK9 is able to modulate apoB synthesis and secretion in vitro, we performed in vitro metabolic studies in HuH7 cells overexpressing (Fig. 4B) or attenuated (data not shown) for PCSK9. PCSK9 does not change [$^{35}$S]apoB synthesis and secretion rates. Because of its central role in VLDL assembly, we also assessed the possibility that PCSK9 may act on MTP activity. We performed MTP activity measurements using protein extracts of HuH7 cells overexpressing or knocked down for PCSK9.

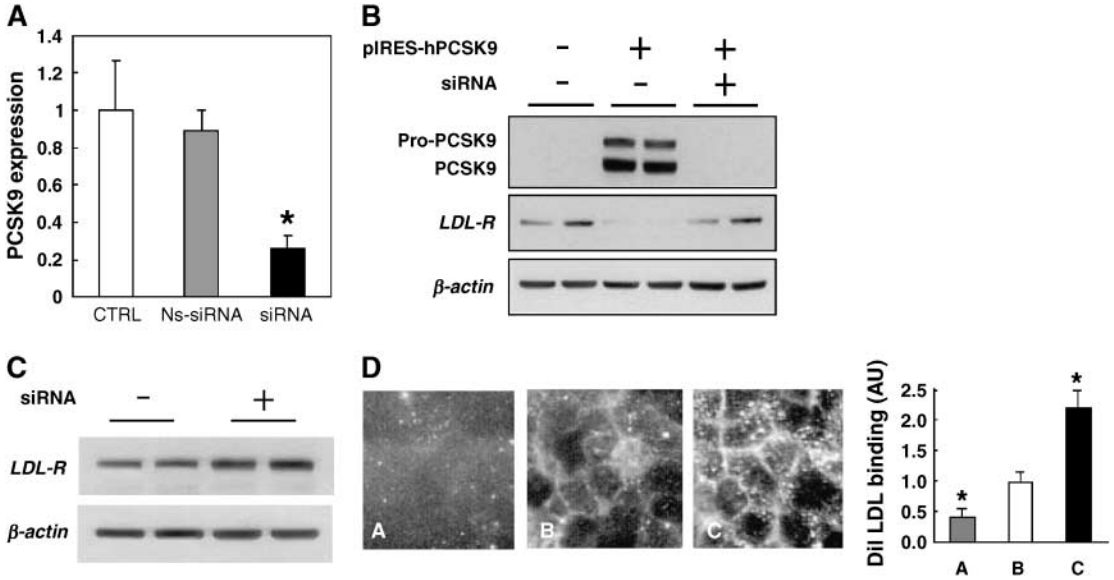


**Fig. 3.** A: Real-time PCR measurement of PCSK9 expression in HuH7 hepatoma cells transfected with short, interfering RNA (siRNA) targeting human PCSK9 (black bar). Controls were either not transfected (white bar; CTRL) or transfected with a nonspecific (Ns) siRNA (gray bar). The assay is standardized for human ribosomal protein L13a. Values are expressed as means ± SEM. B: Western blot analysis of V5-tagged PCSK9 and LDLr in HuH7 cells cotransfected with pIRES-hPCSK9 with or without anti-human PCSK9 siRNA. C: Western blot analysis of LDLr in HuH7 cells transfected or not with anti-human PCSK9 siRNA. D: DiI-LDL binding to HuH7 cells at 48 h after transfection with pIRES-hPCSK9 (A), nothing (B), or siRNA targeting human PCSK9 (C) (magnification ×50). The quantification of the DiI-LDL binding from three independent experiments performed in duplicate is presented. AU, arbitrary units; DiI, 3,3'-dioctadecylindocarbocyanine iodide. Values are expressed as means ± SEM. * $P < 0.05$ versus nontransfected cells.

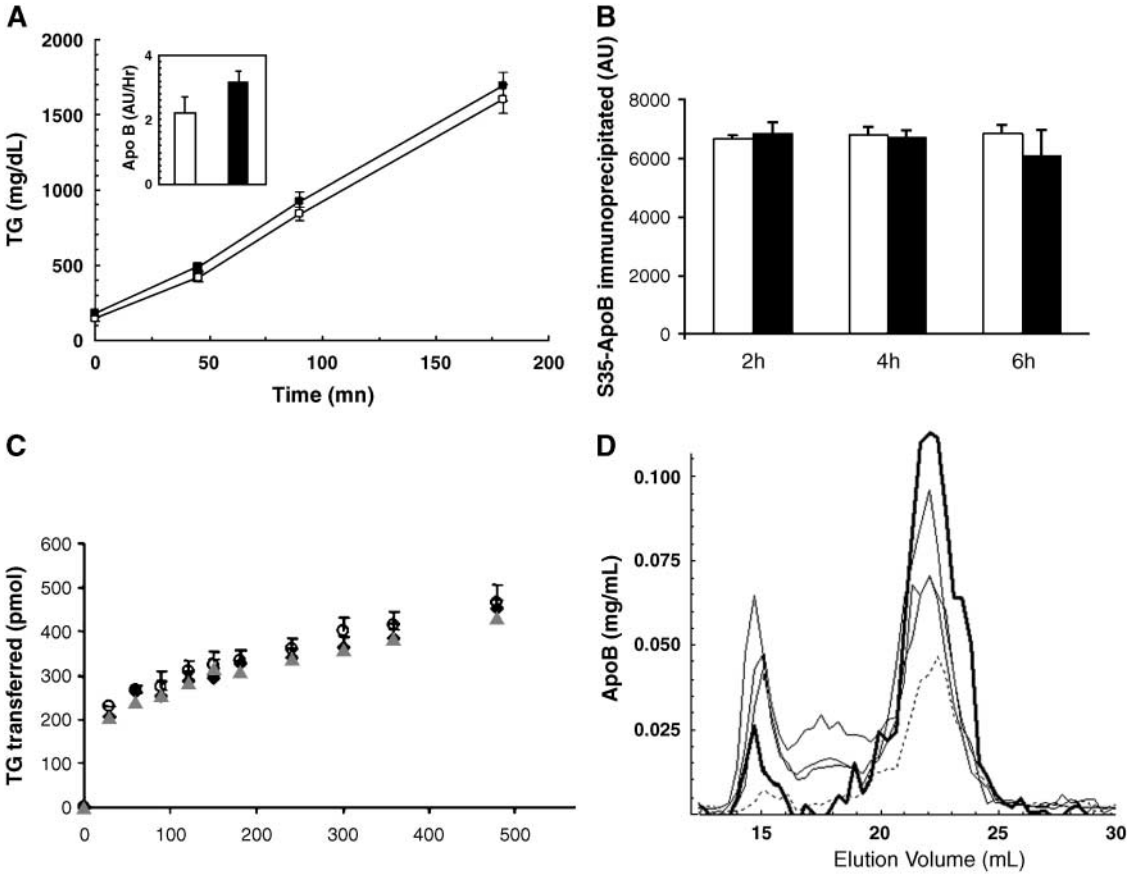


**Fig. 4.** A: VLDL-triglyceride (TG) production in C57BL6/J mice (n = 5 per group) infused with Ad-Null (open squares) or Ad-PCSK9 (closed squares) after transient inhibition of plasma lipases by tyloxapol injection. Inset: Plasma apoB-100 accumulation in C57BL6/J mice infused with Ad-Null (open bar) or Ad-PCSK9 (solid bar) at 90 min after tyloxapol injection measured by immunoblot and densitometric scanning. AU, arbitrary units. Values are expressed as means ± SEM. B: Pulse-chase of secreted apoB-100 in HuH7 cells transfected (black bars) or not (white bars) with pIRES-hPCSK9. C: Microsomal transfer protein kinetic activity in control HuH7 cells (open circles) or HuH7 cells transfected with either pIRES-hPCSK9 (shaded triangles) or anti-human PCSK9 siRNA (closed diamonds). In B and C, data are expressed as means ± SD of eight independent replicates of two independent experiments. D: FPLC apoB profile of fasting plasma samples (200 μl) from a normolipemic individual (dotted line), three patients presenting with the S127R mutation in PCSK9 (thin lines), and a representative heterozygous familial hypercholesterolemia patient (thick line). VLDL, IDL, and LDL elute at 14–16, 16–20, and 20–24 ml, respectively.

Neither PCSK9 overexpression nor silencing significantly alters MTP activity in our cellular model (Fig. 4C).

### Plasma from S127R-PCSK9 patients is enriched in apoB-containing VLDL and IDL

To address the apparent discrepancy between apoB kinetics data from S127R-PCSK9 patients and the apoB production results obtained in cultured cells and mice, we performed FPLC analysis of lipoprotein apoB content in a normolipemic individual, three patients presenting with the S127R mutation in PCSK9, and a representative heterozygous FH patient (Fig. 4D). Whereas less than 10% of plasma apoB eluted in the VLDL/IDL lipoprotein range in the normolipemic and heterozygous FH patients, more than 30% of plasma apoB from our three S127R-PCSK9 patients was recovered at these elution volumes. These data suggest that in addition to the modulation of LDL clearance from the plasma compartment, PCSK9 also plays a role in TG-rich lipoprotein metabolism in humans. Conversely, in mice and cultured cells, PCSK9 mostly seems to affect LDL catabolism without significantly altering plasma VLDL lipids and/or apoB metabolism.

## DISCUSSION

In the present study, we investigated the role of PCSK9 in modulating the metabolism of apoB-containing lipoproteins in vivo and in vitro. Adenovirus-mediated overexpression of human PCSK9 in C57BL6/J mice resulted in a 140% increase in plasma TC with no significant change in HDL-C levels, associated with a decrease in hepatic LDLr expression. Conversely, adenoviral infusion of PCSK9 had no apparent effect on serum lipids in LDLr-deficient mice. Kinetic analysis of $^{125}$I-apoB-LDL established for the first time that LDLr activity and LDL plasma clearance are impaired in vivo upon PCSK9 overexpression. In vitro overexpression and knockdown studies using human hepatoma cells showed that PCSK9 directly inhibits the LDLr pathway. The hepatic production of VLDL and the plasma li-

pase activities were not significantly altered in mice infused with Ad-PCSK9. In cells, PCSK9 did not alter apoB synthesis or MTP activity. In sharp contrast, we showed that the modulation of PCSK9 function in three patients resulted not only in altered LDL clearance but also in the accumulation of VLDL-apoB in the plasma compartment.

First, we showed that overexpression of human PCSK9 in C57BL6/J controls but not in LDLr-KO mice results in decreased LDLr hepatic expression and a concomitant increase in plasma LDL-cholesterol levels. Similar results with adenoviral expression of murine PCSK9 have been reported, indicating a similar role for murine and human PCSK9 with respect to LDLr function in vivo (11). In addition, we performed a series of kinetic studies to provide direct evidence that LDL catabolism is impaired upon PCSK9 overexpression in C57BL6/J mice but not in LDLr-KO mice. The decay curves of $^{125}$I-apoB-labeled LDL clearly demonstrate in vivo that LDL clearance from the plasma compartment is impaired only in C57BL6/J animals infused with Ad-PCSK9. Thus, LDL clearance in mice overexpressing PCSK9 is delayed significantly compared with that in controls, in a pattern similar to that observed in LDLr-KO animals. Together with the results obtained in cells overexpressing or knocked down for PCSK9, our study clearly demonstrates a direct and definitive role for PCSK9 in modulating the LDLr pathway.

Next, we investigated the effects of PCSK9 overexpression on the production of TG-rich lipoproteins after transient inhibition of the major plasma lipases by tyloxapol injection. Our data indicate that the secretion rate of VLDL-TGs and -apoB is outwardly similar in Ad-PCSK9- and Ad-Null-infused C57BL6/J animals. This result is consistent with recent data establishing that LDLr-KO mice have similar VLDL production than controls (25). However, it has been shown that variations in LDLr expression in mouse primary hepatocytes result in significant changes in VLDL-apoB production (26). It has been suggested that the use of tyloxapol in mice may preclude the accurate measurement of VLDL-apoB and/or -TG secretion (27). In vivo tyloxapol infusion transiently blocks VLDL lipolysis and allows direct measurement of VLDL production (19). Thus, tyloxapol injection permits the demonstration of decreased VLDL production in mice after inhibition of MTP, the major enzyme involved in VLDL assembly (28). Similarly, VLDL production is shown to be decreased in mice upon adenoviral overexpression of the hypotriglyceridemic gene apoA-5 by the tyloxapol method (29), whereas, also using tyloxapol, others have shown that apoA-5 transgenic mice present with similar VLDL secretion rates than controls (30). Based on this controversy, one must consider tyloxapol experimental data with caution. It was tempting to speculate that MTP could be a physiological substrate for PCSK9. MTP activity assays performed in our study indicate that neither overexpressing nor silencing PCSK9 affects the MTP-mediated transfer of TGs in HuH7 hepatoma cells. Because neither plasma lipolytic activity, MTP activity, nor the levels of VLDL-apoB-100 and -TGs are significantly altered upon PCSK9 overexpression, we conclude that PCSK9 does not or only minimally affect the production and lipolysis of VLDL in mice. In agreement with our findings, Park, Moon, and Horton (31) recently reported that primary hepatocytes from control mice and from mice overexpressing human PCSK9 synthesize and secrete similar amounts of apoB.

While this paper was being written, it was reported that overexpression of human PCSK9 in LDLr-KO mice increases plasma LDL cholesterol levels 7 days after adenoviral infusion (12). We did not observe any change in plasma lipids between day 0 and day 9 after Ad-PCSK9 infusion in LDLr-KO mice. This discrepancy may result from different levels of PCSK9 expression, as suggested by the injected dose of adenoviral vectors ($10^{11}$ vs. $5 \times 10^{8}$ plaque-forming units per mouse, respectively). In our hands, LDLr-KO mice did not respond to a 2- to 3-fold increase in PCSK9 hepatic expression, whereas PCSK9 seems to have a significant effect on plasma lipids at higher doses in this animal model (12). Thus, only high expression levels of PCSK9 might have a significant effect on VLDL metabolism. Notably, the LDLr has been proposed to promote the presecretory degradation and the reuptake of apoB, in addition to its well-defined role in plasma LDL uptake (25). Furthermore, increased apoB production is observed in FH patients carrying mutations on the LDLr, resulting in a virtual absence of ligand (apoB-100) binding (32, 33). Based on these studies, a definitive explanation for the absence of modulation in apoB production despite a sharp decrease in LDLr expression and activity upon PCSK9 infusion in mice remains elusive.

Finally, our study indicates that patients presenting with the S127R mutation on PCSK9 have increased VLDL-apoB levels, in contrast to heterozygous FH patients and control subjects. The discrepancy between results related to apoB production obtained in mice or hepatomas and patients could be attributable to natural differences existing between mice and humans, although we tried to reduce this by overexpressing the human form of PCSK9. It might also be far-fetched to speculate on the function of PCSK9 by comparing the effects of a mutation on one allele of the gene in adult patients with the transient overexpression of the conserved protein in mice. Interestingly, among the families our laboratory is investigating, an 8 year old individual bearing the S127R mutation does not exhibit hypercholesterolemia (Y. Zaïr, personal communication). Generation of the Ad-PCSK9-S127R should prove useful in addressing a potential gain or loss of function resulting from the mutated allele in vivo.

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