

Plasma PCSK9 preferentially reduces liver LDL receptors in mice

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Abstract Proprotein convertase subtilisin/kexin type 9 (PCSK9) is a secreted protein that regulates the expression of LDL receptor (LDLR) protein. Gain-of-function mutations in *PCSK9* cause hypercholesterolemia, and loss-of-function mutations result in lower plasma LDL-cholesterol. Here, we investigate the kinetics and metabolism of circulating PCSK9 relative to tissue levels of LDLRs. The administration of recombinant human PCSK9 (32 μ g) to mice by a single injection reduced hepatic LDLRs by $\sim 90\%$ within 60 min, and the receptor levels returned to normal within 6 h. The half-life of the PCSK9 was estimated to be ~ 5 min. Continuous infusion of PCSK9 (32 μ g/h) into wild-type mice caused a $\sim 90\%$ reduction in hepatic LDLRs within 2 h and no associated change in the level of LDLR in the adrenals. Parallel studies were performed using a catalytically inactive form of PCSK9, PCSK9(S386A), and similar results were obtained. Infusion of PCSK9(D374Y), a gain-of-function mutation, resulted in accelerated clearance of the mutant PCSK9 and a greater reduction in hepatic LDLRs. Combined, these data suggest that exogenously administered PCSK9 in plasma preferentially reduces LDLR protein levels in liver at concentrations found in human plasma and that PCSK9's action on the LDLR is not dependent on catalytic activity *in vivo*.—Grefhorst, A., M. C. McNutt, T. A. Lagace, and J. D. Horton. Plasma PCSK9 preferentially reduces liver LDL receptors in mice. *J. Lipid Res.* 2008. 49: 1303–1311.

Supplementary key words proprotein convertase subtilisin/kexin type 9 • LDL-cholesterol • sterol-regulatory element binding protein

Proprotein convertase subtilisin/kexin type 9 (PCSK9) is a member of the proteinase K subfamily of enzymes (1–4). In mice, overexpression of human PCSK9 results in decreased hepatic LDL receptors (LDLRs) and hypercholesterolemia (2–5), whereas *Pcsk9*^{−/−} mice have increased hepatic LDLRs and reduced plasma cholesterol concentrations (1). Similarly, humans carrying gain-of-function

alleles for *PCSK9* have increased plasma LDL-cholesterol (LDL-C) levels and premature coronary artery disease (6), whereas individuals with loss-of-function alleles have significantly reduced plasma LDL-C and are protected from atherosclerosis (7–9).

PCSK9 is rapidly secreted from cultured hepatocytes and is found in the plasma of humans (10–12). The addition of recombinant human PCSK9 to the medium of HepG2 cells leads to the degradation of LDLRs in a time- and concentration-dependent manner (10). PCSK9 directly binds selectively to the epidermal growth factor-like repeat A domain of the LDLR (13, 14), and this binding and subsequent internalization are required for exogenous PCSK9 to reduce LDLR levels (10, 13). Several studies suggest that PCSK9 also functions intracellularly to degrade LDLRs (2, 15, 16). Currently, the relative contribution of the intracellular pathway versus the exogenous pathway of PCSK9-mediated LDLR degradation is not known.

In parabiosis experiments, human PCSK9 secreted from livers of transgenic mice decreased hepatic LDLR protein levels when it was transferred via shared circulation to recipient wild-type mice (10). These studies demonstrated that human PCSK9 in plasma is capable of reducing hepatic LDLRs, although the plasma levels of PCSK9 (17–246 μ g/ml) in the transgenic mice were ~ 100 - to 1,000-fold higher than those measured in human plasma (~ 0.25 μ g/ml) (10). Here, we have probed the function of circulating PCSK9 and relevant mutants *in vivo* and investigated the effect of physiological levels of plasma PCSK9 on LDLR protein levels in both the liver and the adrenals.

MATERIALS AND METHODS

PCSK9 production and purification

Recombinant human wild-type PCSK9 and the gain-of-function PCSK9(D374Y) were produced in HEK 293S cells (17)

Abbreviations: LDL-C, LDL-cholesterol; LDLR, LDL receptor; PCSK9, proprotein convertase subtilisin/kexin type 9; SREBP, sterol-regulatory element binding protein; TFR, transferrin receptor.

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This work was supported by grants from the Perot Family Foundation and the National Institutes of Health (Grants HL-38049 and HL-20948 to A.G.), the Ter Meulen Fund, Royal Netherlands Academy of Arts and Science, The Netherlands (M.C.M.), Medical Scientist Training Grant GM08014 (T.A.L.), and by a fellowship from the Canadian Institutes of Health Research.

Manuscript received 15 January 2008 and in revised form 27 February 2008.

Published, JLR Papers in Press, March 19, 2008.

DOI 10.1194/jlr.M800027-JLR200

and purified as described (10). To produce catalytically inactive PCSK9(S386A), HEK 293S cells were stably transfected with two plasmids, one encoding the prodomain and the second encoding the catalytically inactive protease domains as described (18), except that the prodomain lacked a C-terminal V5 epitope tag. The prodomain vector used was constructed exactly as described (18), except that the reverse primer was 5'-CTATTACTGGGCAAGA-CAGAGGAGTCCTCC-3'.

Animals

Experiments were performed in wild-type, *Ldlr*^{-/-} (19), and *Pcsk9*^{-/-} (1) mice on a mixed C57BL/6J;129S6/SvEv background. All studies were approved by the Institutional Animal Care and Research Advisory Committee at the University of Texas Southwestern Medical Center.

Plasma analysis

Plasma human PCSK9 concentrations were determined by ELISA (10). Plasma cholesterol concentrations were measured as described (20).

PCSK9 time course and dose response

Male wild-type mice were anesthetized with sodium pentobarbital, and the indicated doses of PCSK9 were administered intravenously into the right jugular vein. At the indicated time points after injection, the animals were euthanized by isoflurane anesthesia, and blood, liver, and adrenals were removed for analysis.

PCSK9 continuous infusion

Male wild-type and *Pcsk9*^{-/-} mice were anesthetized with sodium pentobarbital, and a small lobe of the liver was removed through an abdominal incision. The tissue was snap frozen in liquid nitrogen, and the wound closed with a suture. A blood sample was obtained immediately prior to initiating a continuous infusion of PCSK9 in a saline solution for 3 or 6 h via the jugular vein under anesthesia. Blood samples were collected during infusion at the indicated time points via the tail vein, and the concentration of human PCSK9 was determined by ELISA (10). Animals were euthanized by cardiac puncture prior to harvesting the liver and the adrenals.

PCSK9 serum half-life

Wild-type or mutant (D374Y) PCSK9 was labeled with sodium ¹²⁵I (21), and 4 μg was injected into the right jugular vein of male wild-type and *Ldlr*^{-/-} mice. The specific activity of the ¹²⁵I-labeled PCSK9 ranged from 900–1,000 cpm/ng protein. Aliquots of blood were obtained from the left jugular vein 30 s after the injection (time 0) and at the indicated time points. At the end of the experiment, the animals were euthanized by cardiac puncture. For estimations of tissue PCSK9 uptake, mice were euthanized 15 min after the ¹²⁵I-labeled PCSK9 injection, and organs were harvested after whole-body perfusion with saline for 30 min.

Immunoblot analysis

Liver and adrenal membranes were prepared (22), and the protein concentrations were determined using the BCA Protein Assay kit (Pierce, Rockford, IL). For immunoblot analyses, individual or pooled samples were mixed with loading buffer and heated for 5 min at 96°C prior to being subjected to SDS-PAGE as described (2). Immunoblot analyses were performed with rabbit polyclonal LDLR antiserum (2) and a horseradish peroxidase-conjugated anti-rabbit IgG from donkey (Amersham Pharmacia Bioscience, GE Health Care Life Sciences, Piscataway, NJ). The

invariant control protein, transferrin receptor (TFR), was detected using monoclonal anti-TFR IgG raised in mice (Zymed; San Francisco, CA) and horseradish peroxidase-conjugated rabbit anti-mouse IgG as the secondary. Immunocomplexes were detected using the SuperSignal West Pico Chemiluminescent Substrate System (Pierce).

Quantitative immunoblots of hepatic and adrenal membrane proteins

A total of 5 μg protein from pooled hepatic and adrenal membrane protein of 18 male wild-type and 18 male *Pcsk9*^{-/-} mice was subjected to SDS-PAGE and transferred to nitrocellulose membranes (2). Following a 30 min blocking reaction in Odyssey blocking buffer (LI-COR Biosciences; Lincoln, NE), blots were incubated with primary antibodies recognizing the LDLR and the TFR, followed by IRDye680-conjugated secondary anti-rabbit and anti-mouse antibodies, respectively. Primary antibodies were diluted in TBS containing 5% bovine serum albumin, and secondary antibodies were diluted in Odyssey blocking buffer. The treated membrane was then scanned with a LI-COR Odyssey infrared imaging system on the 700 nm channel, and signal intensity was quantified with Odyssey v2.0 software (LI-COR).

RESULTS

To characterize the properties and function of PCSK9 in plasma, we first determined whether a single bolus injection of purified recombinant human PCSK9 into the circulation of wild-type mice would reduce hepatic LDLRs. Male wild-type mice were injected with saline vehicle or with 2, 4, 8, 16, or 32 μg PCSK9 and then euthanized 1, 2, or 4 h after the injection. Hepatic LDLR protein levels were determined by immunoblot analysis. LDLR levels did not change in liver after a single bolus injection of 2 or 4 μg of human PCSK9 (Fig. 1A, lanes 1–3, 7–9, and 13–15). When the dose was increased to 8 μg of PCSK9, hepatic LDLRs fell by ~50% within 1 h of the injection (Fig. 1A, lane 4). Higher doses of PCSK9 (16 or 32 μg) resulted in a more marked reduction (~80–90%) of hepatic LDLRs (Fig. 1A, lanes 5 and 6), and LDLR levels remained low for 2–4 h (Fig. 1A, lanes 11, 12, 17, and 18).

To define the kinetics of the response to PCSK9, mice were injected with recombinant human PCSK9 (32 μg) and euthanized at various times afterwards (Fig. 1B). No changes in hepatic LDLR levels were detected until the 30 min time point, when the LDLR protein was reduced by ~75%. Maximal reductions in LDLR levels were seen at 60 min (~90%). Hepatic LDLR protein levels returned to baseline 360 min after injection, which suggested that PCSK9 was rapidly cleared from the plasma.

To measure the half-life of PCSK9 in plasma and to determine whether LDLRs influenced PCSK9 clearance, ¹²⁵I-PCSK9 was injected into wild-type and *Ldlr*^{-/-} mice, and the protein's disappearance from plasma was determined. As shown in Fig. 1C, the clearance of labeled PCSK9 in mice occurred in two phases. The first phase was rapid and dependent on functional LDLRs, and the second phase was largely LDLR independent. The half-life of PCSK9 in wild-type mice was ~5 min, and this interval was increased to ~15 min in the absence of LDLRs. Recovery

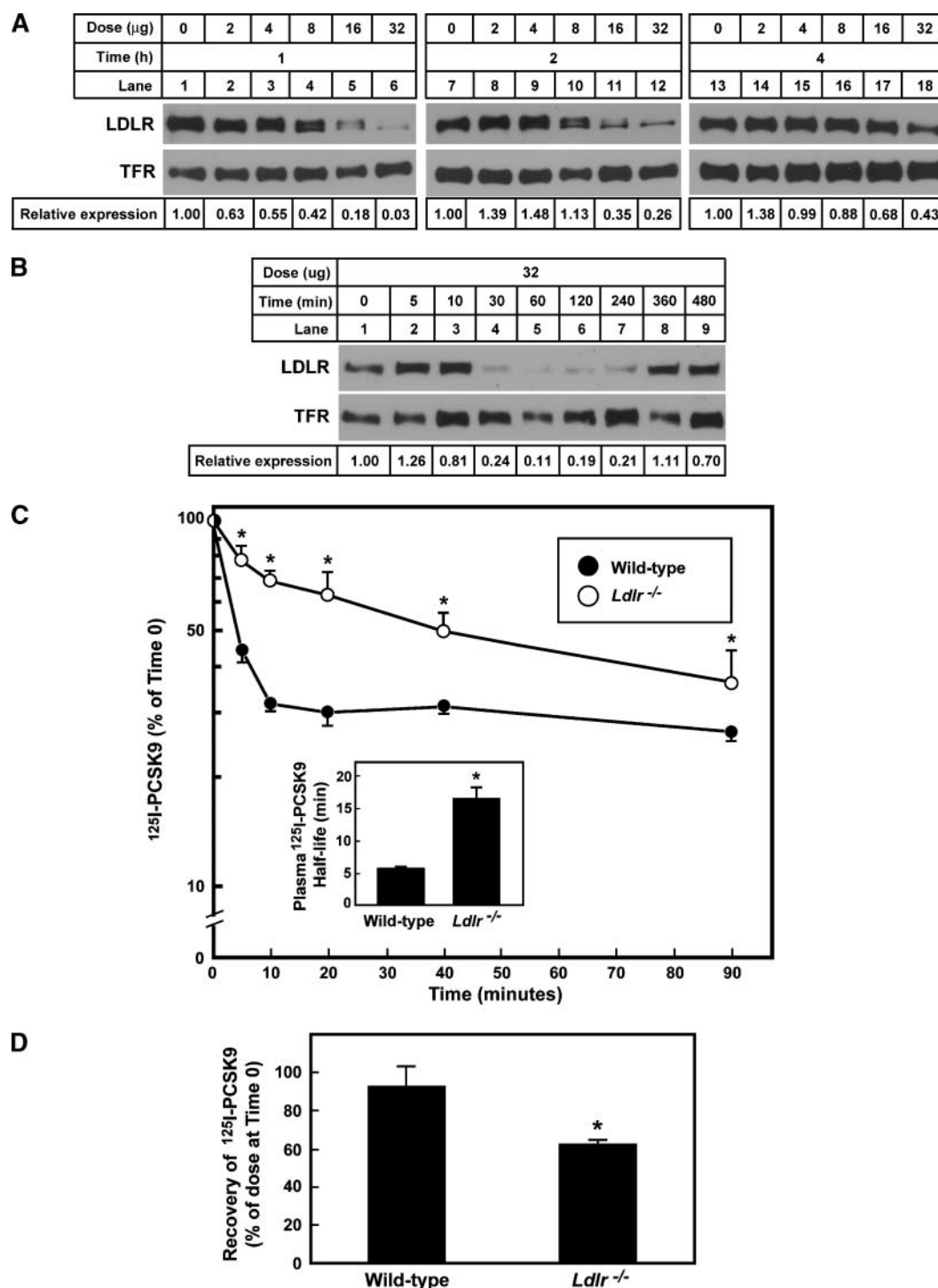


Fig. 1. Hepatic LDL receptor (LDLR) protein levels in mice injected with recombinant human proprotein convertase subtilisin/kexin type 9 (PCSK9). **A:** Immunoblot analysis of the LDLR and transferrin receptor (TFR) in liver membranes of male wild-type mice 1, 2, or 4 h following the injection of 0, 2, 4, 8, 16, or 32 μ g of recombinant human PCSK9 protein. Membrane proteins were isolated, equal aliquots from three mouse livers in each group were pooled, and a total of 5 μ g protein was subjected to 8% SDS-PAGE for immunoblot analysis of the LDLR and TFR as described in Materials and Methods. **B:** Immunoblot analysis of the LDLR and TFR protein in liver membranes of wild-type male mice 5, 10, 30, 60, 120, 240, 360, and 480 min after the injection of 32 μ g of recombinant human PCSK9. Membrane proteins were isolated, equal aliquots from three mouse livers at each time point were pooled, and immunoblots were performed as described in A. **C:** Clearance of 125 I-PCSK9 in wild-type and *Ldlr*^{-/-} mice. Recombinant human PCSK9 labeled with 125 I was produced as described in Materials and Methods. Six mice of each genotype were injected with 4 μ g of the 125 I-labeled PCSK9 protein, and blood samples were obtained at the indicated times following the injection. Values are averages \pm SD. Insert: Plasma half-life (min) of 125 I-PCSK9 calculated from the slope during the first 10 min. * $P < 0.05$ versus wild-type. **D:** 125 I-labeled PCSK9 uptake in livers of wild-type and *Ldlr*^{-/-} mice. Seven male mice of each genotype were administered 4 μ g of the 125 I-labeled PCSK9 protein, and the mice were euthanized 15 min after injection. Livers were harvested after whole-body perfusion with saline for 30 min, and radioactivity was counted. Values are averages \pm SD. * $P < 0.05$ versus wild-type.

of injected ^{125}I -PCSK9 in the liver after 15 min was $\sim 90\%$ in wild-type mice, but only $\sim 60\%$ in $Ldlr^{-/-}$ mice (Fig. 1D). Thus, PCSK9 acts rapidly, has a short half-life in plasma, and is predominantly cleared by the liver.

The efficacy of injected recombinant PCSK9 permitted the use of continuous infusions to study the effects of dif-

ferent plasma concentrations of PCSK9 on LDLR expression. To this end, various amounts of recombinant human PCSK9 were infused into wild-type mice for 3 h and plasma was obtained every 30 min to measure human PCSK9 concentrations (Fig. 2A). After 3 h of continuous infusion, PCSK9 concentrations reached a steady state in mice infused with 8, 16, or 24 $\mu\text{g}/\text{h}$. With the highest dose of PCSK9 (32 $\mu\text{g}/\text{h}$), plasma concentrations of PCSK9 reached 1.0 $\mu\text{g}/\text{ml}$ but were still increasing at the end of the 3 h infusion. Hepatic LDLR protein levels were measured by immunoblot analysis from liver samples taken prior to infusion (time 0) and from liver obtained at the end of the 3 h infusion (Fig. 2B). Infusion of saline or 8 $\mu\text{g}/\text{h}$ of PCSK9 did not alter LDL protein levels in liver;

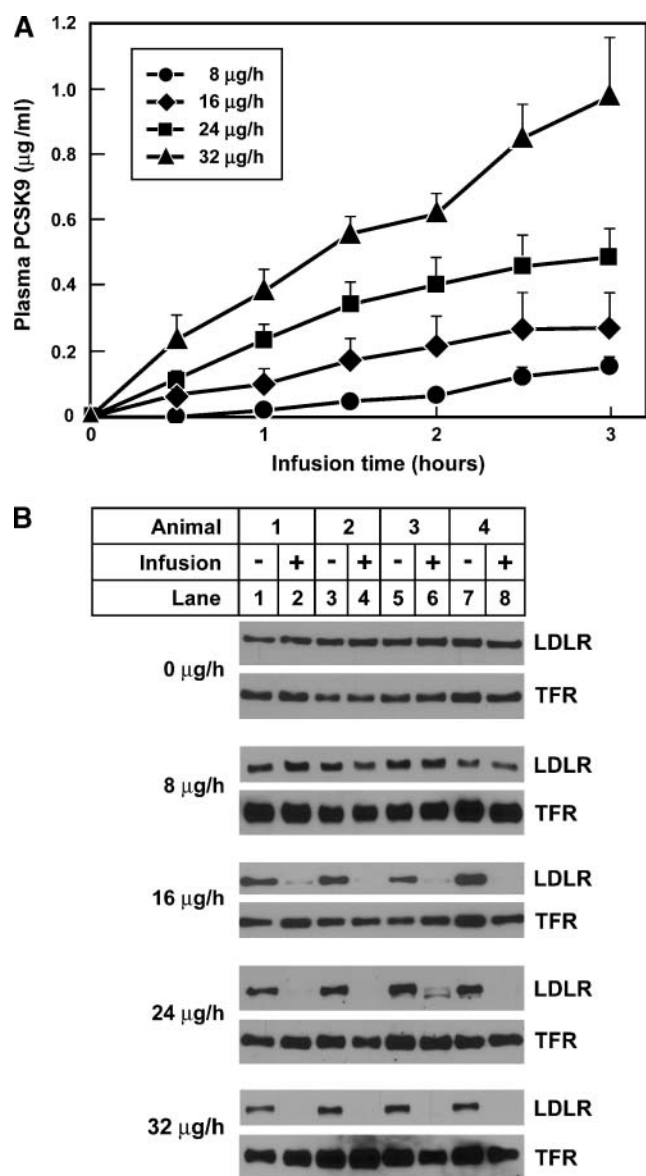


Fig. 2. Plasma PCSK9 concentrations and hepatic LDLR protein levels in wild-type mice infused with recombinant human PCSK9. **A:** Human PCSK9 plasma concentrations in male wild-type mice during the 3 h infusion of 8, 16, 24, or 32 $\mu\text{g}/\text{h}$ of recombinant human PCSK9. Plasma human PCSK9 concentrations were measured by ELISA (10). Data are averages \pm SEM, $n = 4$. **B:** Immunoblot analysis of the LDLR and TFR in liver membranes of male wild-type mice infused for 3 h with saline vehicle or 8, 16, 24, or 32 $\mu\text{g}/\text{h}$ of recombinant human PCSK9. A liver sample was obtained prior to infusion (–) and at the end of the infusion (+). Membrane proteins were isolated, and 5 μg protein was subjected to 8% SDS-PAGE for immunoblot analysis of the LDLR as described in Materials and Methods. TFR was used as a control for loading. Immunoblots show protein levels from individual mice before and after infusion.

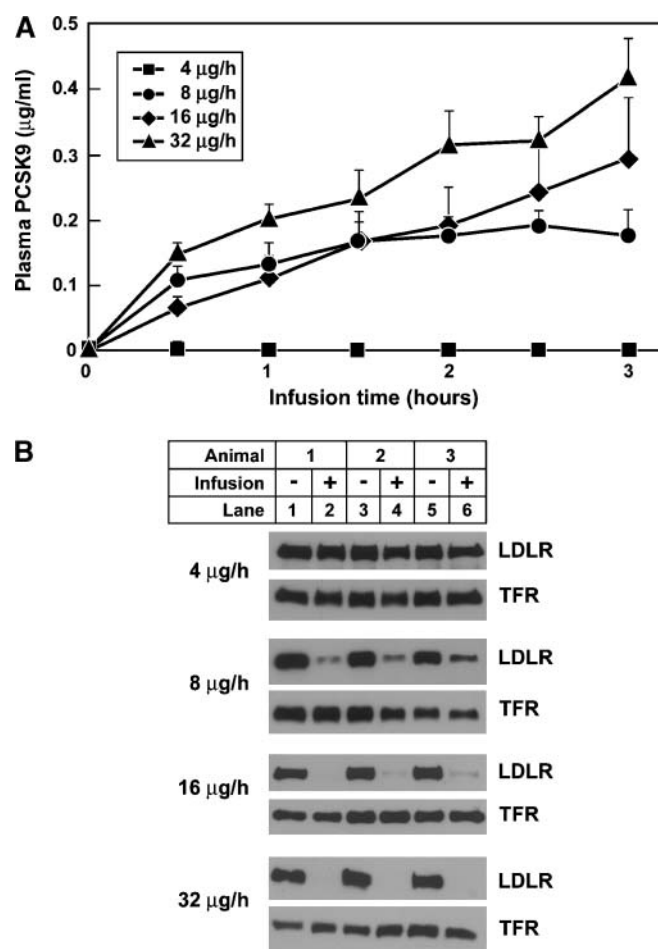


Fig. 3. Plasma PCSK9 concentrations and hepatic LDLR protein levels in $Pcsk9^{-/-}$ mice infused with recombinant human PCSK9. **A:** Human PCSK9 plasma concentrations in male $Pcsk9^{-/-}$ mice during a 3 h infusion of 4, 8, 16, or 32 $\mu\text{g}/\text{h}$ of recombinant human PCSK9. Plasma human PCSK9 concentrations were measured by ELISA (10). Data are averages \pm SEM, $n = 3$. **B:** Immunoblot analysis of the LDLR and TFR protein in liver membranes of male $Pcsk9^{-/-}$ mice infused for 3 h with 4, 8, 16, or 32 $\mu\text{g}/\text{h}$ of recombinant human PCSK9. A liver sample was obtained prior to infusion (–) and at the end of the infusion (+). Membrane proteins were isolated, and 5 μg protein was subjected to 8% SDS-PAGE for immunoblot analysis of the LDLR as described in Materials and Methods. TFR was used as a control for loading. Immunoblots show protein levels from individual mice before and after infusion.

however, infusion of 16, 24, or 32 $\mu\text{g}/\text{h}$ nearly abolished hepatic LDLRs. After infusion, plasma concentrations of human PCSK9 associated with reduced hepatic LDLRs were 0.15, 0.27, and 0.49 $\mu\text{g}/\text{ml}$, respectively. These concentrations are within the range (0.05–0.60 $\mu\text{g}/\text{ml}$) of those previously measured in human plasma using this ELISA (10). Infusions with up to 32 $\mu\text{g}/\text{h}$ of PCSK9 for 3 h did not affect plasma cholesterol levels (data not shown).

To confirm and extend these results, we repeated the infusion experiments in *Pcsk9*^{−/−} mice to eliminate the contribution of endogenous mouse PCSK9 to the observed effects on hepatic LDLRs (Fig. 3). At baseline, *Pcsk9*^{−/−} mice had ~3-fold more hepatic LDLRs than wild-type mice (1). Infusion of 4 $\mu\text{g}/\text{h}$ of PCSK9 into the *Pcsk9*^{−/−} mice did not produce measurable steady-state levels of PCSK9 protein (Fig. 3A). At the end of the 3 h infusion of PCSK9 at 8, 16, and 32 $\mu\text{g}/\text{h}$, the plasma concentrations of human PCSK9 were 0.18, 0.29, and 0.42 $\mu\text{g}/\text{ml}$, respectively (Fig. 3A). Liver samples obtained prior to infusion and at the end of the 3 h infusion were analyzed by immunoblot to determine the effects of PCSK9 on the levels of LDLRs (Fig. 3B). Infusion of PCSK9 at rates of 8, 16, and 32 $\mu\text{g}/\text{h}$ resulted in significant reductions in hepatic LDLR protein in a dose-dependent manner. These results confirmed that the presence of plasma PCSK9 at concentrations measured in human plasma causes degradation of hepatic LDLRs.

The adrenal gland has the highest levels of LDLRs per gram tissue (23) of any organ in the body. To determine whether PCSK9 in plasma affects LDLRs in the adrenals in a manner similar to that found in liver, wild-type mice were infused with the highest dose of recombinant human PCSK9 protein (32 $\mu\text{g}/\text{h}$) for 3 h and 6 h, and LDLRs were measured by immunoblot analysis (Fig. 4). Consistent with the data shown in Fig. 3, continuous infusion of the 32 $\mu\text{g}/\text{h}$ of PCSK9 for 3 h and 6 h nearly eliminated all LDLRs in liver (Fig. 4A). No change in the levels of LDLR was seen in the adrenal glands of these animals (Fig. 4B). Next, we examined the amount of LDLR protein in livers and adrenals from 18 wild-type and 18 *Pcsk9*^{−/−} mice. To more accurately measure the LDLR protein levels, the LI-COR Odyssey infrared imaging system was used. In this experiment, the LDLR protein level in *Pcsk9*^{−/−} livers was increased 2.8-fold (Fig. 4C), whereas the level in the adrenals of the *Pcsk9*^{−/−} mice was only 1.4-fold higher than in the wild-type mice. These data further support the conclusion that PCSK9 has a reduced activity on LDLRs in the adrenals.

We next examined the plasma kinetics and activities of a PCSK9 protein with a gain-of-function mutation (D374Y) associated with hypercholesterolemia in humans (6). Previously, it was shown that PCSK9(D374Y) added to the medium of HepG2 cells was ~10-fold more potent than

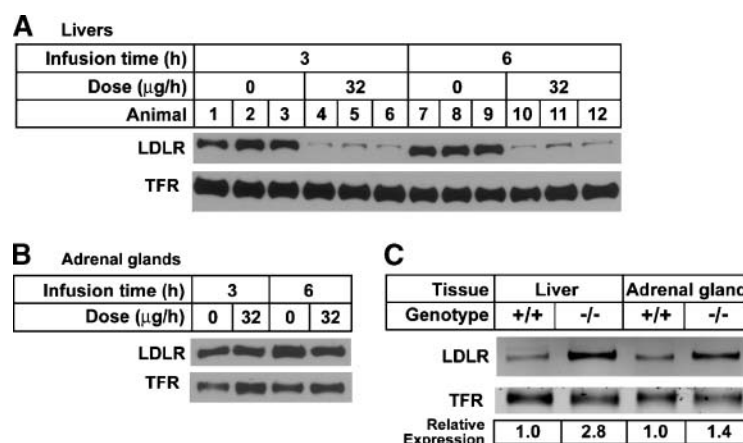


Fig. 4. Liver and adrenal LDLR protein levels in wild-type mice infused with recombinant human PCSK9 and in noninfused wild-type and *Pcsk9*^{−/−} mice. **A:** Immunoblot analysis of the LDLR and TFR in liver membranes of male wild-type mice infused for 3 h or 6 h with saline vehicle or 32 $\mu\text{g}/\text{h}$ of recombinant human PCSK9 protein. Membrane proteins were isolated, and 5 μg of protein was subjected to 8% SDS-PAGE for immunoblot analysis of the LDLR as described in Materials and Methods. TFR protein was used as a control for loading. Immunoblots show protein levels from individual mice after infusion. **B:** Immunoblot analysis of the LDLR and TFR in membranes from adrenal glands of male wild-type mice infused for 3 h or 6 h with saline vehicle or 32 $\mu\text{g}/\text{h}$ of recombinant human PCSK9 protein. Adrenal gland membrane proteins were isolated as described in Materials and Methods. Immunoblots show protein levels from pooled adrenal glands from three mice after infusion. **C:** Quantitative immunoblot analysis of the LDLR and TFR in liver and adrenal gland membranes of wild-type (+/+) and *Pcsk9*^{−/−} (−/−) mice. Membrane proteins were isolated, and 5 μg was subjected to 8% SDS-PAGE for immunoblot analysis of the LDLR as described in Materials and Methods. TFR protein was used as a control for loading. Secondary antibodies labeled with IRDye 680 were used for visualization. Detection and quantification of LDLR and TFR protein were performed with the LI-COR Odyssey Infrared Imaging System. Ratiometric analysis of wavelength intensities was used to quantify the LDLR and TFR membrane protein. Data are from pooled membrane fractions from 18 male wild-type and 18 male *Pcsk9*^{−/−} mice. Similar results were obtained from two smaller studies that used livers and adrenals pooled from six mice per genotype.

wild-type PCSK9 in reducing LDLRs (10). A potential mechanism for the increased activity of the mutant protein is that it binds the LDLR with ~5- to 30-fold greater affinity than the wild-type PCSK9 (24, 25). To determine whether the gain-of-function protein had an altered plasma half-life, PCSK9(D374Y) was radiolabeled with ^{125}I and injected into wild-type mice, and clearance from the plasma was determined (Fig. 5A). Clearance of ^{125}I -PCSK9(D374Y) from the blood was ~1.5 times faster than that of wild-type ^{125}I -PCSK9 protein [first-phase half-lives of 4.7 ± 0.1 min and 3.2 ± 0.3 min for wild-type-PCSK9 and PCSK9(D374Y), respectively]. This result is compatible with the higher affinity of the mutant protein for the LDLR.

To determine whether the gain-of-function mutant had increased activity in plasma, PCSK9(D374Y) was continuously infused for 3 h into *Pcsk9*^{-/-} mice at three different rates (4, 8, and 32 $\mu\text{g}/\text{h}$) and effects on hepatic LDLR protein levels were measured. As shown in Fig. 5B, infusion of 4 $\mu\text{g}/\text{h}$ of PCSK9(D374Y) did not result in measurable PCSK9 concentrations in blood, a result also found upon infusion of wild-type PCSK9 protein (Fig. 3A). Infusion of 8 $\mu\text{g}/\text{h}$ resulted in plasma concentrations of 0.02 $\mu\text{g}/\text{ml}$ at 3 h, which is significantly lower than was seen with the wild-type protein (0.18 $\mu\text{g}/\text{ml}$) in the *Pcsk9*^{-/-} mice (Fig. 3A). Despite the lower plasma concentration of PCSK9(D374Y), hepatic LDLR levels were reduced significantly (Fig. 5C). These results are consistent with PCSK9(D374Y) having a more potent effect on LDLR expression in vitro (10).

Next, we determined whether an intact catalytic site was required for PCSK9-mediated degradation of hepatic LDLRs in vivo. Previously, we showed that catalytically inactive PCSK9 reduced LDLRs as efficiently as wild-type PCSK9 when added to the medium of HepG2 cells (18). To determine whether this finding was also true in the intact animal, catalytically inactive PCSK9 protein was purified from the medium of cells by coexpression of the prodomain and a catalytically inactive protease domain containing an alanine in place of a conserved serine at position 386 (S386A) [designated *trans*-PCSK9(S386A)] (18). Wild-type PCSK9 or *trans*-PCSK9(S386A) was then infused into *Pcsk9*^{-/-} mice at a rate of 32 $\mu\text{g}/\text{h}$, and the effects on hepatic LDLRs were assessed by immunoblot analysis (Fig. 6). Similar reductions in hepatic LDLRs were seen with the catalytically inactive and wild-type protein, confirming that catalytic activity is not required for PCSK9 to mediate the degradation of hepatic LDLRs in vivo.

DISCUSSION

Although PCSK9 has emerged as a potent regulator of plasma LDL-C and cardiovascular risk, details regarding the protein's site and mode of action on LDLR number are only partially defined. In the current report, we characterized the kinetics and activities of plasma PCSK9 and a gain-of-function variant, PCSK9(D374Y), which causes hypercholesterolemia. Our primary purpose was to determine whether PCSK9 in mouse plasma affects LDLR num-

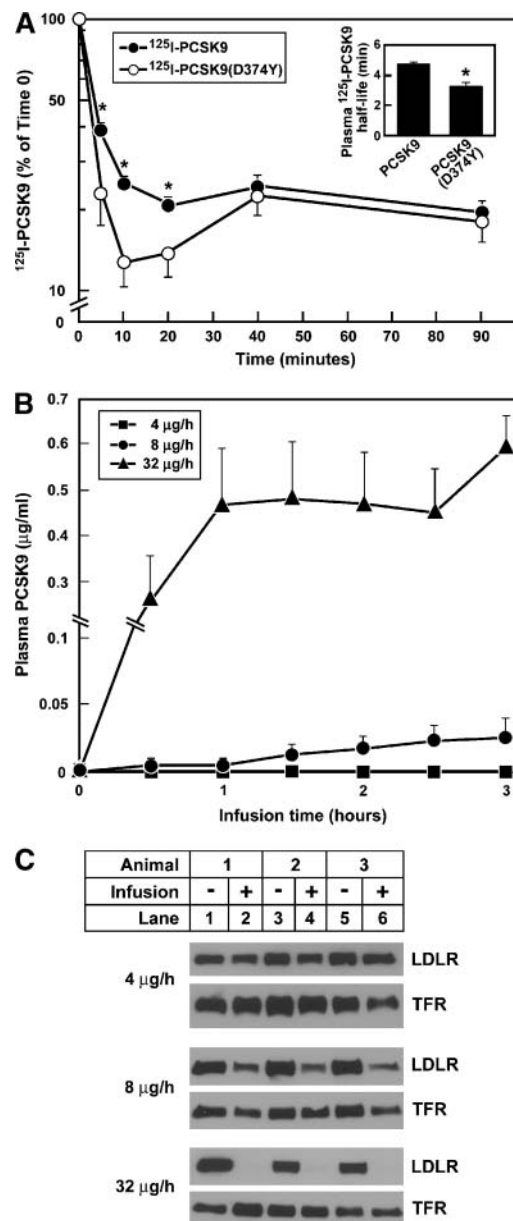


Fig. 5. Plasma half-life and activity of recombinant gain-of-function human PCSK9(D374Y) protein in mice. **A:** Clearance of ^{125}I -wild-type-PCSK9 and gain-of-function ^{125}I -PCSK9(D374Y) in wild-type mice. Recombinant human wild-type-PCSK9 or PCSK9(D374Y) labeled with ^{125}I was produced as described in Materials and Methods. Five mice of each genotype were injected with 4 μg of the ^{125}I -labeled protein, and blood samples were obtained at the indicated times following the injection. Values are averages \pm SD. Insert: Plasma half-life (min) of ^{125}I -wild-type-PCSK9 and ^{125}I -PCSK9(D374Y) calculated from the slope during the first 10 min. * $P < 0.05$, PCSK9(D374Y) versus wild-type-PCSK9. **B:** Human PCSK9 plasma levels during a 3 h infusion of 4, 8, or 32 $\mu\text{g}/\text{h}$ of recombinant human PCSK9(D374Y) in male *Pcsk9*^{-/-} mice. Plasma human PCSK9 concentrations were determined by ELISA (10). Data are averages \pm SEM, $n = 3$. **C:** Immunoblot analysis of the LDLR and TFR protein in liver membranes of male *Pcsk9*^{-/-} mice infused with 4, 8, or 32 $\mu\text{g}/\text{h}$ of recombinant human PCSK9(D374Y) protein for 3 h. Liver membrane proteins were isolated, and 5 μg protein was subjected to 8% SDS-PAGE for immunoblot analysis of the LDLR as described in Materials and Methods. TFR was used as a control for loading. Immunoblots show protein levels from individual mice before and after infusion.

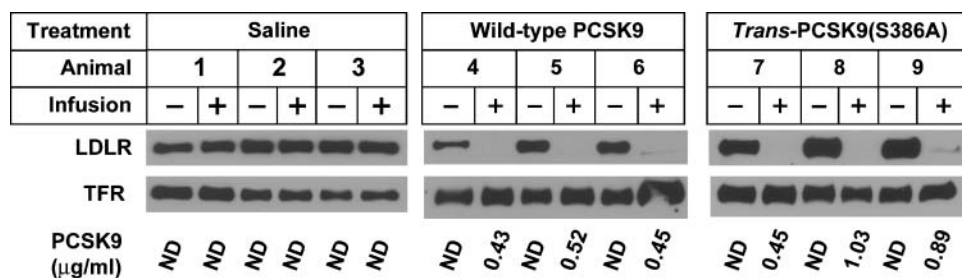


Fig. 6. Hepatic LDLR protein levels in mice infused with catalytically inactive recombinant *trans*-PCSK9(S386A) protein. Male *Pcsk9*^{-/-} mice were infused with saline, 32 μg/h of recombinant human wild-type-PCSK9, or 32 μg/h of *trans*-PCSK9(S386A) for 3 h. A liver sample was obtained prior to infusion (-) and at the end of the infusion (+). Liver membrane proteins were isolated, and 5 μg protein was subjected to 8% SDS-PAGE for immunoblot analysis of the LDLR as described in Materials and Methods. The TFR protein was used as a control for loading. Immunoblots show protein levels from individual mice before and after infusion. Plasma human PCSK9 concentrations were determined by ELISA from blood samples obtained at the end of the 3 h infusion (10). ND, not detected.

bers at concentrations typically found in human plasma. The ability to obtain large quantities of purified recombinant human PCSK9 from stably transfected HEK293S cells permitted the direct injection of the protein into mice to determine the levels of circulating human PCSK9 that effect changes in LDLR number in the liver and the adrenal gland. We found that plasma PCSK9 levels within the range of the concentrations found in humans (0.05–0.60 μg/ml) (10) were associated with reductions in hepatic LDLR protein. A plasma concentration of human PCSK9 of only 0.18 μg/ml in mice expressing no endogenous PCSK9 was sufficient to reduce hepatic LDLRs by more than 80% (Fig. 3). These findings are consistent with circulating PCSK9 having a functional effect on LDLR number in liver. Our studies cannot exclude the possibility that PCSK9 also acts intracellularly to promote LDLR degradation.

Unexpectedly, infusion of PCSK9 either did not affect or only modestly lowered LDLR number in adrenals. The mRNA for PCSK9 is not expressed in the adrenals of mice (Grefhorst and Horton, unpublished observations); thus, any effects measured in this tissue should result from the action of plasma PCSK9. Even when the highest infusion rate of PCSK9 was used (32 μg/h) for up to 6 h, resulting in plasma PCSK9 concentrations of 1.73 ± 0.03 μg/ml (data not shown), no reduction in LDLR protein was detected in the adrenal glands, despite the near ablation of LDLRs in liver (Fig. 4). In untreated *Pcsk9*^{-/-} mice, LDLRs in the adrenals were slightly increased (1.4-fold) compared with wild-type mice (Fig. 4C); however, this increase was significantly less than that measured in liver (2.8-fold) and supported the conclusion that PCSK9 is less but not completely inactive in the adrenals. The reasons for the reduced responsiveness of the adrenals to the effects of PCSK9 are not known. PCSK9 has varying effects on LDLR levels in different cell types in cultured cells (2, 10, 24). The reduced responsiveness of the adrenal glands to PCSK9 may be subject to cell type-specific modulators. We do not know the effect of PCSK9 on LDLR expression in other organs, because of the low levels of LDLR expression in

other tissues and the low sensitivity of the immunoblotting assay employed. Thus, future studies using LDL uptake as a measure of LDLR number will be necessary for examining the effect of PCSK9 in tissues other than the liver and the adrenal glands.


Previous experiments demonstrated that PCSK9 binds directly to the LDLR and that functional LDLRs were required for hepatocytes to internalize PCSK9 (10, 13). It was not known whether the number of functional LDLRs alters PCSK9 clearance from blood. The ¹²⁵I-PCSK9 clearance studies shown in Fig. 1 suggest that LDLRs themselves play an important role in mediating PCSK9 clearance. The route of clearance of the remainder of the protein, which is cleared in an LDLR-independent manner, is not known. The affinity of the LDLR for PCSK9 also appears to affect the half-life of the protein. Similar clearance studies using PCSK9(D374Y), which exhibits a 5- to 30-fold greater affinity for the LDLR (24, 25), showed that the clearance of PCSK9(D374Y) was significantly faster than that of the wild-type protein (Fig. 5A). The gain-of-function PCSK9 protein also was significantly more active than wild-type PCSK9 in plasma; steady-state plasma concentrations of PCSK9(D374Y) that were 10-fold less than those found in mice infused with wild-type protein resulted in equivalent levels of hepatic LDLR destruction (cf. Figs. 3B, 5C).

The rapid clearance of PCSK9 from plasma suggested that PCSK9 production rates must also be relatively high to maintain plasma PCSK9 levels. An ELISA to determine the concentration of mouse PCSK9 in blood is currently not available, but mouse PCSK9 is detectable in this compartment by immunoprecipitation (Lagace and Horton, unpublished observations). Here, the use of human PCSK9, for which an ELISA is available, provided an opportunity to estimate production rates of PCSK9 by infusing recombinant protein into *Pcsk9*^{-/-} mice. The data given in Fig. 3A show that an infusion rate of 4 μg/h of PCSK9 does not result in detectable plasma levels in PCSK9 knockout mice, yet an infusion rate of 8 μg/h of PCSK9 results in near steady-state levels (0.15 μg/ml) in blood. These data provide indirect evidence to suggest that the rate of whole-

body PCSK9 production is more than 4 $\mu\text{g}/\text{h}$ in mice. It is likely that the majority of the PCSK9 produced is from liver, because this organ has the highest mRNA expression of PCSK9 (26).

A recent surprising finding is that although catalytic activity of PCSK9 is required for self-cleavage, proper folding, and secretion from the cell (26), the catalytic activity of PCSK9 is not required for the degradation of LDLRs when added to the medium of cultured cells (18, 27). The data shown in Fig. 6 demonstrate that infusion of catalytically inactive protein led to the destruction of hepatic LDLRs in a manner that was virtually identical to that of the catalytically active protein. These *in vivo* data confirm that the LDLR degradation activity of PCSK9 is independent of the protein's proteolytic activity.

A major unresolved question is why PCSK9 has evolved and persisted in the genome, given the existence of the transcriptional regulatory mechanism controlling LDLR expression that is present in all cells. The current studies raise the possibility that the primary site of PCSK9 action is the liver and that the secreted form of PCSK9 preferentially downregulates LDLR expression in this tissue to divert apolipoprotein B- (apoB) and apoE-containing lipoproteins away from the liver to peripheral tissues. The *LDLR* and all genes required to synthesize cholesterol are transcriptionally activated by a single transcription factor, sterol-regulatory element binding protein-2 (SREBP-2) (28). This same transcription factor also regulates PCSK9, which ultimately degrades LDLRs (29, 30). In the short term, SREBP-2 activation increases lipid synthesis and VLDL secretion from liver. SREBP-2 also activates *PCSK9*, which preferentially reduces hepatic LDLRs, thereby preventing the immediate reuptake of the secreted lipoprotein particles and diverting the newly synthesized and secreted lipoproteins to peripheral tissues. Over the long term, high levels of PCSK9 would ultimately increase plasma LDL-C levels and the risk of cardiovascular disease.

Combined, the data of the current report suggest that exogenously administrated PCSK9 in plasma preferentially reduces LDLR protein levels in liver at concentrations found in human plasma. Furthermore, this effect was not dependent on PCSK9 catalytic activity, suggesting that inhibitors of PCSK9 catalytic activity that only function in plasma will not be successful in blocking PCSK9's action on hepatic LDLRs. However, the results of these studies do suggest that approaches that utilize small molecules or antibodies to block the interaction of PCSK9 and the LDLR at the cell surface may be a viable approach to inhibit PCSK9 function and provide an alternative therapy for hypercholesterolemia. 

The authors wish to thank Jonathan Cohen, Joseph L. Goldstein, Helen H. Hobbs, and David W. Russell for their critical reading of the manuscript and Michael S. Brown for helpful suggestions. Norma Anderson, Y. K. Ho, Scott Clark, Amy Smith, Lauren Koob, Tuyet Dang, Linda Donnelly, Richard Gibson, Anh Pho, and Judy Sanchez provided excellent technical assistance.

REFERENCES

- Rashid, S., D. E. Curtis, R. Garuti, N. N. Anderson, Y. Bashmakov, Y. K. Ho, R. E. Hammer, Y.-A. Moon, and J. D. Horton. 2005. Decreased plasma cholesterol and hypersensitivity to statins in mice lacking *Pcsk9*. *Proc. Natl. Acad. Sci. USA*. **102**: 5374–5379.
- Park, S. W., Y.-A. Moon, and J. D. Horton. 2004. Post-transcriptional regulation of low density lipoprotein receptor protein by proprotein convertase subtilisin/kexin type 9a in mouse liver. *J. Biol. Chem.* **279**: 50630–50638.
- Maxwell, K. N., and J. L. Breslow. 2004. Adenoviral-mediated expression of *Pcsk9* in mice results in a low-density lipoprotein receptor knockout phenotype. *Proc. Natl. Acad. Sci. USA*. **101**: 7100–7105.
- Benjannet, S., D. Rhainds, R. Essalmani, J. Mayne, L. Wickham, W. Jin, M. C. Asselin, J. Hamelin, M. Varret, D. Allard, et al. 2004. NARC-1/PCSK9 and its natural mutants: zymogen cleavage and effects on the low density lipoprotein (LDL) receptor and LDL cholesterol. *J. Biol. Chem.* **279**: 48865–48875.
- Lalanne, F., G. Lambert, M. J. A. Amar, M. Chetiveaux, Y. Zair, A.-L. Jarnoux, K. Ouguerram, J. Friburg, N. G. Seidah, H. B. Brewer, Jr., et al. 2005. Wild-type PCSK9 inhibits LDL clearance but does not affect apoB-containing lipoprotein production in mouse and cultured cells. *J. Lipid Res.* **46**: 1312–1319.
- Abifadel, M., M. Varret, J.-P. Rabès, K. Ouguerram, M. Devillers, C. Cruaud, S. Benjannet, L. Wickham, D. Erlich, L. Villéger, et al. 2003. Mutations in *PCSK9* cause autosomal dominant hypercholesterolemia. *Nat. Genet.* **34**: 154–156.
- Cohen, J., A. Pertsemlidis, I. K. Kotowski, R. Graham, C. K. Garcia, and H. H. Hobbs. 2005. Low LDL cholesterol in individuals of African descent resulting from frequent nonsense mutations in *PCSK9*. *Nat. Genet.* **37**: 161–165.
- Cohen, J. C., E. Boerwinkle, T. H. Moseley, and H. H. Hobbs. 2006. Sequence variations in *PCSK9*, low LDL, and protection against coronary heart disease. *N. Engl. J. Med.* **354**: 1264–1272.
- Scartezini, M., C. Hubbart, R. A. Whittall, J. A. Cooper, A. H. Neil, and S. E. Humphries. 2007. The *PCSK9* gene R46L variant is associated with lower plasma lipid levels and cardiovascular risk in healthy UK men. *Clin. Sci.* **113**: 435–441.
- Lagace, T. A., D. E. Curtis, R. Garuti, M. C. McNutt, S. W. Park, H. B. Prather, N. N. Anderson, Y. K. Ho, R. E. Hammer, and J. D. Horton. 2006. Secreted PCSK9 decreases LDL receptors in hepatocytes and in livers of parabiotic mice. *J. Clin. Invest.* **116**: 2995–3005.
- Mayne, J., A. Raymond, A. Chaplin, M. Cousins, N. Kaefer, C. Gyamera-Acheampong, N. G. Seidah, M. Mbikay, M. Chretien, and T. C. Ooi. 2007. Plasma PCSK9 levels correlate with cholesterol in men but not in women. *Biochem. Biophys. Res. Commun.* **361**: 451–456.
- Alborn, W. E., G. Cao, H. E. Careskey, Y.-W. Qian, D. R. Subramaniam, J. Davies, E. M. Conner, and R. J. Konrad. 2007. Serum proprotein convertase subtilisin kexin type 9 is correlated directly with serum LDL cholesterol. *Clin. Chem.* **53**: 1814–1819.
- Zhang, D.-W., T. A. Lagace, R. Garuti, Z. Zhao, M. McDonald, J. D. Horton, J. C. Cohen, and H. H. Hobbs. 2007. 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.* **282**: 18602–18612.
- Kwon, H. J., T. A. Lagace, M. C. McNutt, J. D. Horton, and J. Deisenhofer. 2008. Molecular basis for LDL receptor recognition by PCSK9. *Proc. Natl. Acad. Sci. USA*. **105**: 1820–1825.
- Maxwell, K. N., E. A. Fisher, and J. L. Breslow. 2005. Overexpression of PCSK9 accelerates the degradation of the LDLR in a post-endoplasmic reticulum compartment. *Proc. Natl. Acad. Sci. USA*. **102**: 2069–2074.
- Nassoury, N., D. A. Blasiolo, A. Tebon Oler, S. Benjannet, J. Hamelin, V. Poupon, P. S. McPherson, A. D. Attie, A. Prat, and N. G. Seidah. 2007. The cellular trafficking of the secretory proprotein convertase PCSK9 and its dependence on the LDLR. *Traffic*. **8**: 718–732.
- Reeves, P. J., R. L. Thurmond, and H. G. Khorana. 1996. Structure and function in rhodopsin: high level expression of a synthetic bovine opsin gene and its mutants in stable mammalian cell lines. *Proc. Natl. Acad. Sci. USA*. **93**: 11487–11492.
- McNutt, M. C., T. A. Lagace, and J. D. Horton. 2007. Catalytic activity is not required for secreted PCSK9 to reduce low density lipoprotein receptors in HepG2 cells. *J. Biol. Chem.* **282**: 20799–20803.

19. Ishibashi, S., M. S. Brown, J. L. Goldstein, R. D. Gerard, R. E. Hammer, and J. Herz. 1993. Hypercholesterolemia in low density lipoprotein receptor knockout mice and its reversal by adenovirus-mediated gene delivery. *J. Clin. Invest.* **92**: 883–893.
20. Shimano, H., J. D. Horton, R. E. Hammer, I. Shimomura, M. S. Brown, and J. L. Goldstein. 1996. Overproduction of cholesterol and fatty acids causes massive liver enlargement in transgenic mice expressing truncated SREBP-1a. *J. Clin. Invest.* **98**: 1575–1584.
21. Horton, J. D., H. Shimano, R. L. Hamilton, M. S. Brown, and J. L. Goldstein. 1999. Disruption of LDL receptor gene in transgenic SREBP-1a mice unmasks hyperlipidemia resulting from production of lipid-rich VLDL. *J. Clin. Invest.* **103**: 1067–1076.
22. Engelking, L. J., H. Kuriyama, R. E. Hammer, J. D. Horton, M. S. Brown, J. L. Goldstein, and G. Liang. 2004. Overexpression of Insig-1 in the livers of transgenic mice inhibits SREBP processing and reduces insulin-stimulated lipogenesis. *J. Clin. Invest.* **113**: 1168–1175.
23. Kovanen, P. T., J. L. Goldstein, D. A. Chappell, and M. S. Brown. 1980. Regulation of low density lipoprotein receptors by adrenocorticotropin in the adrenal gland of mice and rats in vivo. *J. Biol. Chem.* **255**: 5591–5598.
24. Fisher, T. S., P. L. Surdo, S. Pandit, M. Mattu, J. C. Santoro, D. Wisniewski, R. T. Cummings, A. Calzetta, R. M. Cubbon, P. A. Fischer, et al. 2007. Effects of pH and low density lipoprotein (LDL) on PCSK9-dependent LDL receptor regulation. *J. Biol. Chem.* **282**: 20502–20512.
25. Cunningham, D., D. E. Danley, K. F. Geoghegan, M. C. Griffor, J. L. Hawkins, T. A. Subashi, A. H. Varghese, M. J. Ammirati, J. S. Culp, L. R. Hoth, et al. 2007. Structural and biophysical studies of PCSK9 and its mutants linked to familial hypercholesterolemia. *Nat. Struct. Mol. Biol.* **14**: 413–419.
26. Seidah, N. G., S. Benjannet, L. Wickham, J. Marcinkiewicz, S. B. Jasmin, S. Stifani, A. Basak, A. Prat, and M. Chretien. 2003. The secretory proprotein convertase neural apoptosis-regulated convertase 1 (NARC-1): liver regeneration and neuronal differentiation. *Proc. Natl. Acad. Sci. USA.* **100**: 928–933.
27. Li, J., C. Tumanut, J.-A. Gavigan, W.-J. Huang, E. N. Hampton, R. Tumanut, K. F. Suen, J. W. Trauger, G. Spraggon, S. A. Lesley, et al. 2007. Secreted PCSK9 promotes LDL receptor degradation independently of proteolytic activity. *Biochem. J.* **406**: 203–207.
28. Horton, J. D., J. L. Goldstein, and M. S. Brown. 2002. SREBPs: activators of the complete program of cholesterol and fatty acid synthesis in the liver. *J. Clin. Invest.* **109**: 1125–1131.
29. Horton, J. D., N. A. Shah, J. A. Warrington, N. N. Anderson, S. W. Park, M. S. Brown, and J. L. Goldstein. 2003. Combined analysis of oligonucleotide microarray data from transgenic and knockout mice identifies direct SREBP target genes. *Proc. Natl. Acad. Sci. USA.* **100**: 12027–12032.
30. Jeong, H. J., H.-S. Lee, K.-S. Kim, Y.-K. Kim, D. Yoon, and S. W. Park. 2008. Sterol-dependent regulation of proprotein convertase subtilisin/kexin type 9 expression by sterol-regulatory element binding protein-2. *J. Lipid Res.* **49**: 399–409.