Proprotein Convertase Subtilisin/Kexin Type 9 (PCSK9) Single Domain Antibodies Are Potent Inhibitors of Low Density Lipoprotein Receptor Degradation*

Received for publication, January 27, 2016, and in revised form, June 7, 2016 Published, JBC Papers in Press, June 8, 2016, DOI 10.1074/jbc.M116.717736

Elodie Weider 1, Delia Susan-Resiga 1, Rachid Essalmani 1, Josée Hamelin 1, Marie-Claude Asselin 1, Surendra Nimesh 1, Yahya Ashraf 1, Keith L. Wycoff 1, Jianbing Zhang 1, Annik Prat 1, and Nabil G. Seidah 1,2

From the 1Laboratory of Biochemical Neuroendocrinology, Institut de Recherches Cliniques de Montréal, University of Montreal, Montreal, Quebec H2W 1R7, Canada, 2Planet Biotechnology Inc., Hayward, California 94545-2740, and the 3Institute for Biological Sciences, National Research Council of Canada, Ottawa, Ontario K1A 0R6, Canada

Single domain antibodies (sdAbs) correspond to the antigen-binding domains of camelid antibodies. They have the same antigen-binding properties and specificity as monoclonal antibodies (mAbs) but are easier and cheaper to produce. We report here the development of sdAbs targeting human PCSK9 (proprotein convertase subtilisin/kexin type 9) as an alternative to anti-PCSK9 mAbs. After immunizing a llama with human PCSK9, we selected four sdAbs that bind PCSK9 with a high affinity and produced them as fusion proteins with a mouse Fc. All four sdAb-Fcs recognize the C-terminal Cys-His-rich domain of PCSK9. We performed multiple cellular assays and demonstrated that the selected sdAbs efficiently blocked PCSK9-mediated low density lipoprotein receptor (LDLR) degradation in cell lines, in human hepatocytes, and in mouse primary hepatocytes. We further showed that the sdAb-Fcs do not affect binding of PCSK9 to the LDLR but rather block its induced cellular LDLR degradation. Pcsk9 knock-out mice expressing a human bacterial artificial chromosome (BAC) transgene were generated, resulting in plasma levels of ~300 ng/ml human PCSK9. Mice were singly or doubly injected with the best sdAb-Fc and analyzed at day 4 or 11, respectively. After 4 days, mice exhibited a 32 and 44% decrease in the levels of total cholesterol and apolipoprotein B and 46% and 44% decrease in LDLR protein levels. At 11 days, the equivalent values were 24 and 44% decrease in the levels of total cholesterol and apolipoprotein B. Mice were singly or doubly injected with sdAbs and demonstrated that the selected sdAbs efficiently blocked PCSK9-mediated LDLR degradation.

For over 30 years, a large number of clinical trials have firmly consolidated the importance of lowering LDL-cholesterol (LDLc) in the prevention of cardiovascular diseases (CVD) and its associated devastating sequelae (1). Healthy diets and exercise are highly recommended to lower LDLc in patients with high baseline levels. However, many individuals, including those suffering from familial hypercholesterolemia (FH), cannot reach the recommended LDLc levels to prevent cardiovascular complications. With an overall incidence of ~1:200, FH is a common inherited disease that affects at least 30 million people, of whom ≤1% have been diagnosed. It is characterized by plasma LDLc levels greater than the 95th percentile, which result in tendon xanthomas, xanthelasmata, corneal arcus, and premature atherosclerosis, leading to premature ischemic vascular disease and mortality if left untreated. In most cases, FH subjects exhibit mutations in the LDL receptor (LDLR; 67%) and its ligand apolipoprotein B (apoB; 14%), hampering LDL clearance from the circulation (2). In 2003, merging biological studies with human genetics led to the discovery of PCSK9, the 9th and last member of the family of proprotein convertases related to subtilisin and kexin (3), and the demonstration that the PCSK9 gene represents the 3rd locus of autosomal dominant hypercholesterolemia (4).

PCSK9 is a serine protease first synthesized as a zymogen that autocatalytically cleaves itself in the endoplasmic reticulum (ER) to excise its N-terminal prodomain (3), which acts as a chaperone and a potent inhibitor. However, different from all other convertases (5), PCSK9 is secreted as an enzymatically inactive non-covalent complex with its inhibitory prodomain tightly bound to the catalytic subunit of mature PCSK9 (6). Thus, PCSK9 has no substrate other than itself. Rather, it binds to specific cell-surface receptors and escorts them toward intracellular acidic endosome/lysosome degradation compartments (7, 8). A schematic diagram of PCSK9’s primary structure and its domains (prodomain; catalytic domain; hinge; Cys- and His-rich domain (CHRD)) is shown in Fig. 1A. The crystal structure of PCSK9 revealed that the CHRD is composed of three distinct Cys-His-rich modules, denoted M1, M2, and M3 (6).

* This work was supported by Canadian Institutes of Health Research Grants P2-125775 and MOP 102741, Pfizer cardiovascular research awards, a grant from Amorbchem, Canada Research Chair 216684, and Fondation Leducq Grant 13CVD03.
1 Present address: Xiangxue Pharma, Jinfengyuan Road 2, Guangzhou 510663, China.
2 To whom correspondence should be addressed: Laboratory of Biochemical Neuroendocrinology, Institut de Recherches Cliniques de Montréal, 110 Pine Ave. West, Montréal, Québec H2W 1R7, Canada. Tel.: 514-987-5609; E-mail: seidahn@ircm.qc.ca.
3 The abbreviations used are: LDLc, LDL-cholesterol; apoB, apolipoprotein B; CHRD, cysteine- and histidine-rich domain; CVD, cardiovascular disease; ER, endoplasmic reticulum; FH, familial hypercholesterolemia; GOF, gain-of-function; LDLR, low density lipoprotein receptor; sdAb, single domain antibody; SFM, serum-free media; Tg, transgene; endo, endoglycosidase; WB, Western blotting; IHH, immortalized human primary hepatocyte; Dil, 1,1’-dioctadecyl 3,3,3’,3’-tetramethylindocarbocyanine perchlorate fluorescent carboxyanine dye; BAC, bacterial artificial chromosome.
**Inhibitory PCSK9 Nanobodies**

The first PCSK9 target that was identified is the LDLR at the surface of hepatocytes (9–11). The catalytic subunit of PCSK9 was shown to bind the LDLR through its EGF-A domain (8, 12), as well as the LDLR superfamily members VLDLR, ApoER2 (13, 14), and LRP1 (15). Upon LDL binding to cell surface LDLR, the complex is internalized into the cell within heavy chain clathrin-coated vesicles that fuse with early endosomes. Herein, the acidic pH causes conformational changes driving LDL release, the subsequent recycling of the LDLR to the cell surface, and the sorting of LDL to lysosomes for cholesterol recovery and distribution in the cell (16). The PCSK9-LDLR complex also enters the cell via clathrin-coated vesicles (7, 17). However, the acidic pH enhances the affinity of PCSK9 for the LDLR (6) and, through some unknown mechanism requiring the CHRD (7, 13, 18), favors its ability to escort the LDLR to late endosomes/lysosomes for degradation by as yet undefined proteases (7, 10).

The rare PCSK9 mutations identified in FH patients result in a gain-of-function (GOF), i.e., an increased potency of PCSK9 to promote LDLR degradation, with ensuing higher circulating LDLc levels (4, 10). The most dramatic GOF D374Y mutation increases ~10-fold the affinity of PCSK9 for the LDLR (6) and results in ~4-fold higher LDLc levels (~10 mmol/liter), as well as early death due to CVD (19). Loss-of-function PCSK9 mutations were also identified. Two non-sense mutations Y142X and C679X found in ~2% of black Africans were associated with an ~40% decrease in LDLc and an ~88% reduction in the risk of CVD (20, 21). This provided a proof-of-principle that PCSK9 inhibition may be safe and represents a promising approach to treat hypercholesterolemia and prevent CVD (22, 23).

Accordingly, PCSK9 monoclonal antibodies (mAbs) blocking its interaction with the LDLR were developed and are presently prescribed in clinics to patients suffering from severe hypercholesterolemia, who are statin-resistant and/or cannot reach target LDLc using available drugs. Such subcutaneously injected mAbs every 2 or 4 weeks result in a sustained ~60% reduction in LDLc (5, 22, 24) and thus represent a powerful drug against heart disease that is superior to statins. Over the past 30 years, mAbs became established as effective medicines for several serious diseases (25–27). However, their high molecular mass (~150 kDa) require large amounts to be injected to reach efficacy (e.g., 150 mg/14 days), and their high cost limits their wide applicability. The mAbs targeting PCSK9 cost ~$14,000/year/patient (28), likely making them the most costly class of medications marketed so far. This definitely restricts their use to high risk patients not reaching LDLc target levels despite maximal doses of statins (29). Thus, there is an unmet need for cheaper and more accessible inhibitory molecules.

Camelid single domain antibodies (sdAbs), also known as nanobodies, were first discovered in 1993 (30). Different from conventional antibodies, up to 75% of camelid antibodies are devoid of light chains. They are made of two heavy chains (hcAbs), each comprising an antigen-binding domain (VH or sdAb), followed by two constant domains CH2 and CH3 (31). Although 10-fold smaller (~13 kDa) than conventional IgGs, sdAbs bind antigen targets with equivalent specificity, affinity, and low toxicity (32) and show enhanced tissue penetration. Importantly, sdAbs can be produced from recombinant bacte-ria and are thus expected to be cheaper and easier to manufacture. They can also easily be engineered to achieve high potency by affinity maturation and can be humanized for pre-clinical studies in non-human primates and in humans (31, 33). Therefore, they constitute an attractive alternative to mAbs (34–36).

In this study, following llama injections of full-length human PCSK9, we isolated a number of sdAbs that inhibit the function of PCSK9 on LDLR degradation in various cell lines and primary hepatocytes. The validation of a selected sdAb called P1.40 was performed in Pcsk9 knock-out (KO) mice carrying a 67.5-kb human bacterial artificial chromosome (BAC) transgene that results in the expression of human PCSK9 under its own promoter.

**Results**

**Inhibitory PCSK9 sdAbs**—We obtained sdAbs directed against human PCSK9 by llama immunization and screening of an immune phage display library (Fig. 1B). The cDNAs of 10 selected sdAbs that bind PCSK9 efficiently were sequenced. Alignment of their corresponding protein sequences revealed that they belong to distinct sub-families (Fig. 1C). A member of each family was selected to favor sdAb diversity. P1.40, PK9, PKF8, and P2.57 sdAb cDNAs were then subcloned in an expression vector for production in *Escherichia coli* and purified by His-affinity chromatography.

To define the recognition domains within PCSK9, each sdAb carrying the N-terminal V5 and the C-terminal hexa-His tags was separately expressed in HEK293 cells. The conditioned media were then mixed with those containing different forms of PCSK9 as follows: the full-length protein harboring a C-terminal V5 tag (PCSK9); the CHRD alone; and the C-terminally truncated L455X mutant lacking the CHRD (Fig. 1D) (7, 10). The sdAb-PCSK9 complexes were then immunoprecipitated using a hexa-His-binding resin, resolved by SDS-PAGE, and analyzed by Western blotting (WB) using mAb-V5. As expected, all sdAbs interacted with full-length PCSK9, but surprisingly, all four sdAbs best recognized the CHRD as indicated by the very efficient immunoprecipitation of this domain (Fig. 1D). In comparison, only traces of the L455X mutant (Fig. 1D) were observed. Here, we only show the results for P1.40, as all other sdAbs gave similar data (data not shown). To define which module(s) in the CHRD is/are recognized by the sdAbs, we performed similar experiments with PCSK9 variants lacking the CHRD modules M2 alone (∆M2), M2 and M1 (∆M1M2) or M2 and M3 (∆M2M3). We could only use the above deletants as PCSK9 ∆M1, ∆M3, or ∆M1M3 are not secreted (37). The data showed that P1.40 does not recognize the M2 module (45% of the ∆M2 input bound by P1.40), but the presence of both M1 and M3 is needed for optimal recognition, as only 18% of ∆M1M2 and 14% of ∆M2M3 inputs were immunoprecipitated with P1.40 (Fig. 1E). The three other sdAbs also predominantly recognize ∆M2 (data not shown).

Chimeric sdAbs were then fused to a mouse IgG2a Fc domain, known to increase the half-life of sdAbs in plasma (38), followed by a C-terminal V5 tag (Fig. 1F). Each chimeric protein was produced in HEK293 cells and purified from 600 ml of spent media. The purity of each preparation was assessed by Coomassie staining of an SDS gel run under reducing (+DTT)
or non-reducing (−DTT) conditions. All sdAb-Fcs have an apparent molecular mass of ~42 kDa as monomers under reducing conditions (+DTT) and of ~85 kDa as disulfide-linked dimers under non-reducing conditions (−DTT) (Fig. 1G). The estimated molecular masses of the dimeric chimeras using MALDI mass spectral analysis were as expected from theoretical calculations, i.e. ~89 kDa for P1.40; ~85 kDa for PKE9 and PKF8; and ~87 kDa for P2.57.

We next used enhanced surface plasmon resonance to estimate the binding constants ($K_d$) of each sdAb-Fc to PCSK9. The rank orders of binding constants were PKF8 (5.6 nM) > P2.57 (9.8 nM) > PKE9 (37 nM) > P1.40 (49 nM). However, as will be shown later, the above in vitro binding affinities do not necessarily correlate with the most active sdAb-Fc to inhibit the PCSK9-enhanced cellular degradation of the LDLR.

Although these four sdAb-Fcs interact with the CHRD M1/M3 modules (Fig. 1, D and E), we assessed their ability to disrupt the PCSK9 interaction to the EGF-AB domains of the LDLR in an in vitro binding assay (Fig. 1H). The data show that the sdAb-Fcs did not decrease PCSK9 binding to LDLR, demonstrating that none of the sdAbs interferes with the ability of PCSK9 to bind the EGF-AB domain of the LDLR. Surprisingly, almost 2-fold higher PCSK9 binding was observed with all sdAb-Fcs except for P2.57. The doubling of PCSK9 levels bound to EGF-AB is likely due to the fact that sdAb-Fcs are homodimers (Fig. 1G) and hence could conceptually bind up to two PCSK9 molecules.

Effect of sdAb-Fcs on DiI-LDL Uptake and Cell Surface LDLR Levels in HepG2 Cells—To assess the ability of each sdAb-Fc to inhibit the PCSK9-mediated degradation of the LDLR, we pre-incubated purified PCSK9 (0.08 μM) with increasing concentrations of sdAb-Fcs (0–1 μM) for 1 h and applied these media onto HepG2 cells for a further 5 h incubation. PCSK9 alone resulted in an ~60% decrease of the DiI-LDL uptake, reflecting the reduced levels of functional LDLR at the cell surface (Fig. 2A). All sdAb-Fcs except P2.57 exhibited a dose-dependent inhibitory effect, with the P1.40 being the most potent, giving an ~80% inhibition at 0.1 μM and 100% at 1 μM (Fig. 2A). In a separate experiment, we also showed that the mAb evolocumab gave a similar ~80% inhibition between 0.04 and 0.1 μM and a 100% inhibition at 1 μM (Fig. 2B).

We also tested by immunofluorescence the ability of each sdAb-Fc to rescue the LDLR cell surface labeling of HepG2 cells. PCSK9 (0.05 μM) and the sdAb-Fcs (1.2 μM) were pre-

FIGURE 1. Generation of llama PCSK9-specific sdAbs, PCSK9 binding to sdAb, effect of the sdAb on LDLR binding. The primary structure of PCSK9 and its deletants are shown: aa, amino acid; sp, signal peptide; pro, prodomain; h, hinge domain; CHRD, Cys/His-rich domain and its modules M1, M2, and M3 (A). Flow chart of the steps for llama sdAb selection (B). Phylogenetic tree deduced from the alignment of the full protein sequences of 10 selected sdAbs (C). The media of HEK293 transiently expressing PCSK9 or its deletants lacking either the pro- and catalytic domains (CHRD) or the CHRD (L455X) and the sdAb P1.40 were mixed and immunoprecipitated with TALON metal affinity resin. The input (conditioned media containing the different PCSK9 variants) and pellets were analyzed by WB with mAb-V5 antibody after SDS-PAGE on 12% Tris-glycine gels (D). Similar experiments were performed on PCSK9 variants lacking the CHRD modules M2 alone (ΔM2), M2 and M1 (ΔM1M2) or M2 and M3 (ΔM2M3) (E). D and E are representative of three independent experiments. Schematic of the representative fusion of P1.40 with a mouse Fc comprising the hinge (h), CH2, and CH3 domains (F). Purified sdAb-Fcs (2 μg) were separated by SDS-PAGE in the presence (+) or absence (−) of DTT and revealed by Coomassie staining (G). The four sdAb-Fcs were incubated with PCSK9 and tested for their ability to prevent binding of PCSK9 to the LDLR EGF-AB coated on a binding assay plate (H). This assay is an average of three technical replicates.
incubated as above and applied onto cells for 4 h (Fig. 2C). LDLR labeling was quantified by confocal microscopy and expressed as a percentage of that in control cells not exposed to PCSK9. All four sdAb-Fcs were able to enhance the levels of immunoreactive LDLR, proving their inhibitory potential, but could not completely rescue the LDLR signal in this assay. Again, P1.40 seemed the most potent sdAb-Fc with ~50% rescue of immunoreactive cell surface LDLR.

**Figure 2.** Effect of sdAb-Fcs on DiI-LDL uptake by HepG2 cells and their LDLR cell surface levels. HepG2 cells were incubated for 5 h in the absence or presence of 0.08 μM (5 μg/ml) PCSK9 mixed with increasing concentrations of sdAb-Fcs. DiI-LDL (5 μg/ml final) was added during the last 2 h of incubation. The % DiI-LDL uptake shown here is normalized to the number of cells per well and calibrated to the % uptake in control cells (in absence of PCSK9) (A). A similar experiment was performed with evolocumab (B). In both A and B, the data represent averaged values ± S.E. for at least three independent experiments, with each comprising three independent samples per condition. HepG2 cells were incubated for 4 h without (no PCSK9) or with 0.05 μM (3 μg/ml) PCSK9 (−PCSK9) in the absence or presence of each sdAb-Fc (1.2 μM). LDLR immunocytochemistry with Alexa 555-labeled secondary antibodies was performed, and nuclei were stained with DAPI. Scale bar, 20 μm (C). In two separate experiments, LDLR labeling was quantified based on the average pixel values of 10 fields calibrated to the number of nuclei per field. The numbers (bottom left corner) are expressed as % of the value obtained in the absence of PCSK9 (no PCSK9) and represent averaged pixel values of the two independent experiments. *, p < 0.05; **, p < 0.005; ***, p < 0.0005. p values were obtained from Student’s t tests, except for some conditions in A, for which a two-way ANOVA test was more appropriate.

**Effect of sdAb-Fcs on Total LDLR Levels in Hepatocytes**—The inhibitory effect of sdAb-Fcs was then analyzed in mouse primary hepatocytes, which are closer to an in vivo model than HepG2 cells. The latter were isolated from PCSK9-deficient mice (39, 40) and used to test the sdAb activity toward human PCSK9 (in the absence of endogenous mouse Pcsk9) and to maximize LDLR levels, which were reported to increase by ~3-fold in KO versus WT mice (39, 40). Notably, human PCSK9 acts similarly on both human and mouse LDLR (41). HepatoZYME media control (Fig. 3A, upper panel) or containing 0.08 μM PCSK9 and 0–1.2 μM of each sdAb-Fc were preincubated for 1 h and then incubated with mouse primary hepatocytes for 24 h. Normalized Western blot analyses of mouse LDLR levels revealed that P1.40 did not affect the levels of endogenous LDLR in the absence of PCSK9 (Fig. 3A, upper panel), demonstrating the PCSK9 specificity for the inhibitory effect. In addition, in the presence of extracellular PCSK9 only P1.40 was able to totally rescue LDLR levels, whereas P2.57 had no effect (Fig. 3A, lower panels). PKF8 and PKE9 led to a partial LDLR rescue at 1.2 μM, with relative levels of 0.7 and 0.6, respectively, versus maximal 1.1 with P1.40. The above conclu-
Inhibitory PCSK9 Nanobodies

FIGURE 4. Inhibitory effect of P1.40 on WT and D374Y PCSK9. HepG2 cells were incubated 24 h with media lacking PCSK9 or containing 0.08 μM (5 μg/ml) WT PCSK9 or 0.01 μM (0.7 μg/ml) D374Y PCSK9 and 0–1.2 μM sdAb P1.40 (A). The LDLR signal (% LDLR) was calibrated to that of β-actin levels and expressed in percentage of the difference between the 1st lane (no PCSK9) and 2nd lane (+ PCSK9). Stably transfected HepG2 cells that expressed ∼300 ng/ml/24 h of WT or D374Y PCSK9 were incubated 24 h with increasing concentrations of the sdAb P1.40 (B). HepG2 cells were incubated 24 h with media lacking PCSK9 or containing 0.08 μM (5 μg/ml) WT PCSK9 and 0–1.2 μM sdAb P1.40 or evolocumab (C). Stably transfected HepG2 cells that expressed ∼300 ng/ml/24 h of D374Y PCSK9 were incubated 24 h with increasing concentrations of the sdAb P1.40 or evolocumab (D). The plots shown are representative of at least two independent experiments consisting of one (A), two (B), and three (C and D) biological samples per condition. In both C and D, we also quantified the total levels of cellular LDLR using an ELISA on pooled cell lysates. The calibrated LDLR signals were normalized to that of the 1st lane (no sdAb). The averaged normalized LDLR values are indicated below each blot. *, p < 0.05; **, p < 0.005; ***, p < 0.0005 (Student’s t test).

Inhibitory Effect of P1.40 on WT and D374Y PCSK9—The inhibitory properties of P1.40 toward WT PCSK9 or its GOF mutant D374Y, which has a 10-fold higher affinity for the LDLR (6), was examined in naive HepG2 cells (Fig. 4A) or HepG2 cells stably expressing PCSK9 (Fig. 4B) (42). Note that the LDLR appears as a doublet consisting of an LDLR that is not O-glycosylated (110 kDa) and that is fully mature and O-glycosylated (150 kDa) (43). Unexpectedly, in both cases, the strongest effect was obtained at 0.04 μM and a 1.7-fold increase obtained at all concentrations, revealing that the best effect was obtained at 0.04 μM P1.40 sdAb-Fc on cells stably expressing WT PCSK9 (Fig. 4B).

To compare the efficacies of P1.40 to the mAb evolocumab, we performed similar experiments with WT PCSK9 added extracellularly to HepG2 cells (Fig. 4C) and with HepG2 cells stably expressing D374Y PCSK9 (Fig. 4D). WB analysis of the lysates revealed an ∼90% recovery of the LDLR with 1.2 μM P1.40, whereas 150% was recovered with evolocumab at this concentration (Fig. 4C), suggesting that in this WB assay evolocumab was more active than P1.40. Similarly, in HepG2 cells expressing D374Y PCSK9, evolocumab was more active than P1.40, as evidenced by an ∼3-fold increase in LDLR with P1.40 versus ∼6-fold with evolocumab at 1.2 μM (Fig. 4D). Finally, these conclusions were supported by an LDLR ELISA performed on a pool of cell lysates obtained from experiments in Fig. 4, C and D, which also revealed the higher efficacy of evolocumab compared with P1.40, especially using the D374Y PCSK9 (Fig. 4, C and D, lower sections).

Effect of ER-localized sdAb-Fcs on PCSK9 Secretion and Activity—We next evaluated the ability of intracellular P1.40 to effectively retain PCSK9 within the ER, and hence block the PCSK9 activity. Accordingly, because addition of a C-terminal KDEL motif retains efficiently secretory proteins in the ER (44), we fused a KDEL retention signal to the C terminus of P1.40-Fc-V5. Thus, P1.40 ± KDEL forms were co-expressed in HEK293 cells with PCSK9 or its D374Y mutant, and the LDLR levels were assessed by WB analysis (Fig. 5, A and B). The data demonstrated that P1.40-KDEL was efficiently retained in the ER, as only traces of this protein were observed in the media (Fig. 5, A and B, lanes 3, 6, and 10). Neither P1.40 nor P1.40-KDEL had an impact on LDLR levels (Fig. 5A, lanes 2 and 3), likely due to the quasi-absence of PCSK9 expression in HEK293 cells (mRNAs are 10,000-fold lower than in HepG2 cells). As expected, LDLR levels were reduced significantly (70%) upon expression of D374Y or WT PCSK9, respectively (Fig. 5, A and B, lanes 4 and 8). The ER retention of P1.40-KDEL clearly increased LDLR levels in cells expressing WT or D374Y PCSK9 (Fig. 5, A and B, lanes 4–6 versus 8–10). Note that the inhibitory potency of P1.40 and P1.40-KDEL is almost similar (Fig. 5B, lanes 9 and 10), possibly related to the high luminal concentrations of P1.40 attained during its transit through the Golgi, such that most of the intracellular PCSK9 activity is inhibited. However, higher levels of ER-retained WT PCSK9 by P1.40-KDEL had no effect on LDLR recovery above that of P1.40 alone (0.8 and 0.9; Fig. 5B, lanes 9 and 10), although higher intracel-
Inhibitory PCSK9 Nanobodies

To assess the subcellular localization of the intracellular forms of PCSK9 and LDLR, the above cell lysates were digested with endoglycosidases H (endoH) and F (endoF). As reported previously, most of the overexpressed PCSK9 found in cells are endoH-resistant but endoF-sensitive, even the lower 110-kDa form (data not shown). This suggests that although P1.40 KDEL prevents the secretion of PCSK9, it does not result in the retention of the LDLR in the ER. This is very likely due to the recent discovery that LDLR does not bind efficiently to PCSK9 in the ER because of the presence of a competitive chaperone-like protein GRP94 that prevents such ER interaction (45).

It was shown previously that PCSK9 can induce the degradation of the LDLR by distinct intracellular and extracellular pathways and that cellular overexpression of WT PCSK9 mostly reflects the intracellular pathway, whereas expression of the ~10-fold more potent GOF D374Y PCSK9 affects both pathways (37, 46). Accordingly, the above data demonstrate that retention of PCSK9 in the ER by P1.40-KDEL has a bigger effect on the inhibition of the D374Y activity than that of WT PCSK9 (Fig. 5, A, lanes 4–6, versus B, lanes 8–10), likely due to the elimination of both pathways by P1.40-KDEL.

PCSK9 Inhibition by P1.40 Versus EGF-AB Domains from the LDLR—Because PCSK9 and LDLR interact essentially through the EGF-A domain of the LDLR (8), we compared the potency of P1.40 (sdAb-Fc) and the chimera consisting of the EGF-AB domains, mimicking the LDLR-binding site to PCSK9, fused to a human Fc (EGFs-Fc). HepG2 cells were incubated for 4 h in the absence or presence of PCSK9 preincubated with the Fc alone as a control or its binding proteins P1.40 and EGFs-Fc (Fig. 6). LDLR levels at the cell surface were then quantified by FACS. At 4 h post-incubation, cell surface LDLR levels were reduced by ~60% in the presence of 0.2 μM PCSK9. A high concentration of Fc alone (7 μM) had no effect, whereas 0.04 μM P1.40 doubled the LDLR levels. In contrast, a 100-fold higher concentration of EGFs-Fc (4 μM) was required to obtain a similar effect. This suggests that P1.40 is between 10- and 100-fold more potent on a molar basis than the EGFs-Fc protein.

In Vivo Efficacy of sdAb-Fc P1.40—We next validated the activity of P1.40 in vivo. Pcsk9 KO transgenic mice carrying a 67.5-kb fragment of the human chromosome 1p32 containing the PCSK9 gene (Tg +) were obtained. These transgenic mice (Tg +) express exclusively human PCSK9 under the control of its own promoter, resulting in circulating PCSK9 levels of ~300 ng/ml, which are ~2-fold higher than endogenous levels of WT mice (47, 48). These Tg + mice exhibited ~1.7- and ~2.9-fold higher levels of circulating total cholesterol and apoB, respectively, as compared with transgene-negative (Tg −) WT PCSK9 mice (Fig. 7). Tg + and Tg − male mice were injected with equivalent volumes (~150 μl) of PBS or PBS containing P1.40 (10 mg/kg). Mice were bled 10 days pre-injection, and 1 and 2 and 4 days post-injection. After the last bleed, livers were collected and homogenized, and total LDLR protein levels were estimated by WB (Fig. 7, lower panel). As expected, Tg + mice exhibited lower LDLR levels than Tg − mice (0.4 versus 1.0). P1.40-Fc had no effect in Tg − mice but led to ~50% recovery of LDLR levels in Tg + mice (0.7 versus 0.4). Moreover, 32 and 44% reductions were observed in circulating total cholesterol and apoB levels 4 days post-injection (Fig. 7, upper panels). In conclusion, these data indicate that P1.40 neutralized 66 and 71% of the total cholesterol, and apoB increases resulting from the expression of human PCSK9 in KO mice.

We next performed a similar experiment to test the possible advantage of a double injection at days 0 and 7 (Fig. 8). Mice were bled 10 days pre-injection, and 1 h and 2, 4, 7, 9, and 11 days following the first injection. After the last bleed, LDLR protein levels were estimated by WB (Fig. 8, lower panel). The data showed that P1.40 led to ~80% recovery of LDLR levels in Tg + mice (~0.9 versus 0.4). In addition, 24 and 46% reductions were observed in circulating total cholesterol and apoB levels at 11 days after the first injection (Fig. 8, upper panels). These data
indicate that two injections of P1.40 resulted in the neutralization of 44 and 100% of the total cholesterol and apoB increases resulting from the expression of human PCSK9 in KO mice.

In conclusion, compared with a single injection, two injections of P1.40 spaced by 7 days resulted in a more efficacious increase in hepatic LDLR and reduction in apoB levels, close to the ones seen in absence of PCSK9.

Discussion

In this work, we report the generation of four PCSK9 sdAbs with nanomolar affinity against the CHRD (Fig. 1). As sdAb-Fc fusion proteins, they efficiently inhibited PCSK9-induced LDLR degradation, although they did not prevent the binding of PCSK9 to LDLR (Fig. 1). In HepG2 cells, IHH cells, and/or primary hepatocytes, they restored LDL uptake, as well as cell surface and total LDLR levels (Figs. 2–4). Even though P1.40 proved to be the most active sdAb candidate in our cell-based assays, it was surprising to find that in vitro it bound the CHRD of PCSK9 with a 5-fold lower $K_d$ value than P2.57, which has minimal inhibitory activity. Although this may not be the case in all functional sdAbs, it emphasizes the notion that a lack of correlation may exist between the binding affinity and functional inhibitory effect of PCSK9 sdAbs recognizing the CHRD.

Herein, we focused on the most promising sdAb-Fc, P1.40, and showed that its retention in the ER blocked PCSK9 secretion (Fig. 5). We also showed that P1.40 was more potent than an EGF-AB-Fc chimera (Fig. 6). Finally, 4 and 11 days following a single/double injection, respectively, in transgenic mice expressing exclusively human PCSK9, P1.40 led to 32/24% and 44/46% lower circulating cholesterol and apoB levels,
**Inhibitory PCSK9 Nanobodies**

respectively, and to 1.8-/2.3-fold higher levels of hepatic LDLR (Figs. 7 and 8).

The mAbs presently used in the clinic, e.g. evolocumab, bind the catalytic domain of PCSK9 and sterically hinder its binding to the LDLR (49). Although the four sdAbs analyzed in this study differed in their primary sequences (Fig. 1C), they all bind the CHRD modules M1/M3 of PCSK9 (Fig. 1, D and E). It is important to note that all the other proprotein convertases (5) seem poorly immunogenic, as only for PC1 and PC2 do the available antibodies allow the detection of endogenous proteins in tissue extracts or by immunohistochemistry (50). Thus, the CHRD may be the most antigenic domain of PCSK9. Although this domain is not involved in LDLR binding (6), it is required for the PCSK9-induced LDLR degradation. Human loss-of-function mutations are found in the CHRD, and PCSK9 lacking this domain cannot target the LDLR for degradation (7, 13, 18, 22, 46). In agreement, CHRD mAbs that do not block the LDLR interaction can reduce PCSK9 activity, albeit only partially (50–75% versus 100% for clinical mAbs) (51, 52). This likely occurs through prevention of the cellular internalization and/or lysosomal targeting of the PCSK9-LDLR complex (53). Similarly, the four tested sdAb-Fcs did not sterically hinder PCSK9 binding to the LDLR (Fig. 1H), suggesting a similar mode of action as the CHRD-specific mAbs. Future analysis of sdAb-PCSK9 complexes by x-ray crystallography should reveal their respective epitopes. Their degree of proximity to the catalytic domain will allow us to define the inhibitory mechanism of the corresponding sdAbs, and will possibly shed light on the better inhibition of D374Y than WT PCSK9 by P1.40 (Fig. 4A). Whether any antibody targeting the CHRD may achieve a total PCSK9 inhibition, for example by interfering in PCSK9 binding to the LDLR, remains unknown. Comparison of the potency of P1.40 to the mAb evolocumab that recognizes the catalytic domain of PCSK9 (49) revealed that at 1.2 μM the latter more efficiently inhibits LDLR degradation by WT and especially D374Y PCSK9 (Fig. 4, C and D).

Upon its ER localization via the addition of a C-terminal KDEL motif, P1.40-KDEL reduced PCSK9 secretion (by ~90%) and restored LDLR levels in cells expressing WT PCSK9 and more so D374Y PCSK9 (Fig. 5). The potency of P1.40 was compared with that of a similar construct comprising the LDLR EGF-like domains A and B (EGF5-Fc) and was found to be between 10- and 100-fold higher with an EC50 ~0.04 μM versus 0.4–4 μM (Fig. 6). In agreement with the above values, fusions containing EGF-A only or its improved form EGF66 exhibited EC50 of 11 and 1.6 μM, respectively, in the same HepG2 cells (54).

Mice lacking endogenous Pcsk9 and expressing human PCSK9 gene under its own promoter exhibited higher total cholesterol and apoB levels than Pcsk9 KO Tg− mice. Single or double injections of P1.40 neutralized the increase in apoB levels after 4 days by ~70% and after 11 days by 100%, respectively (Figs. 7 and 8). In a “pioneer” experiment, the total cholesterol of WT mice was reduced by a PCSK9 mAb evolocumab injection by 26 and 28% after 3 and 6 days, respectively (49). In our study, we found that P1.40 can reduce total cholesterol by 32 and 24% at 4 or 11 days following P1.40 injections (Figs. 7 and 8), which is similar to the results obtained with evolocumab.
Inhibitory PCSK9 Nanobodies

Purification of sdAb-Fc Fusion Proteins—Four selected sdAbs (P1.40, PKF8, PKE9, and P2.57) were fused through their C terminus to a mouse IgG2a Fc domain carrying a C-terminal V5 tag. Chimeric proteins were expressed in modified proprietary HEK293 cells and purified to homogeneity with a yield of 80–100 mg per 600 ml of medium (National Research Council Canada, Biotechnology Research Institute).

**sdAb-Fcs Affinity Measurements to PCSK9**—Affinity measurements were performed by the XTAL Biostructures, Inc. Briefly, PCSK9 carrying a C-terminal His tag was captured on a NiIIHCl1000M chip non-covalently at various low densities to determine kinetic binding parameters of the four different sdAb-Fcs. All binding experiments were performed at 25 °C on a BiOptix 404pi enhanced surface plasmon resonance instrument. A 7-point, 3-fold dilution of sdAbs starting at 200 nM was injected over each of three PCSK9 densities, and the sensorgrams were globally fitted with a floating Rmax using Scrubber2 software. Fits across all three ligand densities matched well, suggesting little influence from avidity due to bivalency of the antibodies to the calculated binding parameters.

**Cell Culture**—Human hepatocellular carcinoma HepG2 cells from the American Type Culture Collection (ATCC) were grown in Eagle’s minimal essential medium (Wisent). Cells were stably transfected with either wild type (WT) or D374Y GOF PCSK9 using the FuGENE HD transfection reagent (Promega) and grown with 600 μg/ml G418 (Wisent) for selection of cells stably expressing the cDNA coding for PCSK9. IHH were generously provided by Dr. H. Moshage from the University Hospital Groningen, the Netherlands (71), and were grown in William’s E medium (Wisent). HEK293 cells were grown in DMEM. All cells were maintained at 37 °C under 5% CO2.

**sdAb-Fc Assays**—Purified PCSK9 (69) or conditioned media from HEK293 cells overexpressing either WT or D374Y PCSK9 were preincubated for 1 h at 37 °C with various amounts of purified sdAb-Fcs. The mixture was then applied onto cells for 4–24 h. For experiments with HepG2 cells stably overexpressing WT or D374Y PCSK9, sdAb-Fcs were diluted in the culture medium and directly added to cells. In separate experiments we also incubated the above cells with various concentrations of the inhibitory mAb evolocumab, originally denoted as Amgen’s AMG-145 (49), kindly supplied by Dr. Robert Dufour (Institut de Recherches Cliniques de Montréal).

**Dil-LDL Uptake Assay**—HepG2 cells were plated at a density of 25,000 cells/well in 96-well plates (Corning) in Eagle’s minimal essential medium containing 10% FBS. After 18–20 h, cells were washed and switched to serum-free medium (SFM) containing 5% lipoprotein-deficient serum for 24 h. Purified PCSK9 and sdAb-Fcs were pre-incubated at 37 °C in the same medium and added onto the cells (three wells/condition). After 2 h at 37 °C, 10 μl of Dil-LDL (Biomedical Technologies) were added to the cell medium (5 μg/ml), and cells were further incubated for 2 h. After three washes in D-PBS (without calcium and magnesium; Wisent), plates were scanned on a SpectraMax i3 Multi-Mode Detection Platform (Molecular Devices). Dil-LDL uptake was measured in each well as an average fluorescence intensity. Plates were then frozen overnight (−80 °C) and used the next day to perform a CyQuant cell assay.

**Experimental Procedures**

**Generation of Anti-PCSK9 Single Domain Antibodies**—Human PCSK9 carrying a C-terminal His tag was purified to homogeneity from the media of baculovirus-infected High Five cells (69). A male llama (Lama glana) was immunized with 80 μg of PCSK9 on day 1 and with 20 μg on days 21, 36, 50, and 64. Complete Freund’s adjuvant (Sigma) was used for the primary immunization, and incomplete Freund’s adjuvant was used for immunizations 2–5. Leukocytes were isolated from the last bleed at day 71 post-immunization. Total RNA was then isolated, reverse-transcribed, and used to produce an immune VHH library as described previously (70). Briefly, the cDNAs encoding VHH domains were amplified using specific sense (MJ1, 5'-GCCAGCCGCGGCATGGCCSMKGTGCAGCTGGTGGAGTCTGA-3'; MJ2, 5'-KGCGCCGCGGCATGGCCSMKGTGCAGCTGGTGGAGTCTGA-3'; MJ3, 5'-GCCAGCCGCGGCATGGCCSMKGTGCAGCTGGTGGAGTCTGA-3'; and MJ3, 5'-GCCAGCCGCGGCATGGCCSMKGTGCAGCTGGTGGAGTCTGA-3') primers for VHH and CH2 domains, respectively, and cloned into the phagemid pMED1 vector. The size of the library was measured as 2.3 × 1010 independent transformants, greatly exceeding the number of leukocytes used for library construction. Exponentially growing E. coli expressing the phagemid library were infected with helper phages to “rescue” the phage particle auto-assembly and grown overnight for phage production. Isolation of PCSK9-specific sdAbs was performed by phage display. Using PCSK9-coated 96-well plates, four rounds of panning were performed, with each round including steps of phage binding to PCSK9, elution, and amplification. In a last step, the phages produced by 45 individual clones were tested for their ability to bind PCSK9, and the 10 strongest binders were selected. Corresponding sdAbs were sequenced and subcloned in a pSJF2H vector comprising an OmpA signal peptide that targets the protein to the periplasmic space of E. coli, and a C-terminal hexa-His tag that allowed sdAb purification by an automated procedure using a KingFisher Flex (Thermo Scientific).

**Co-immunoprecipitation**—HEK293 cells were plated in 100-mm² plates and transiently transfected using JetPRIME (Polyplus-transfection®) with 10 μg of vectors (7, 10) coding for various PCSK9 constructs or each of the four sdAbs. The sdAb conditioned media were incubated with those containing PCSK9 variants (Fig. 1A) and immunoprecipitated with TALON Metal Affinity Resin (Clontech) overnight at 4 °C. The following day, the samples were washed four times with radio-immunoprecipitation assay buffer (50 mM Tris–HCl, pH 8.0, 1% (v/v) Nonidet P-40, 0.5% sodium deoxycholate, 150 mM NaCl, and 0.1% (v/v) SDS), and lastly with PBS. The immunoprecipitates were then resolved on a 12% Tris-glycine SDS-PAGE and revealed by a V5-HRP antibody (Invitrogen).

PCSK9-sdAb Complex Binding to the LDLR—The ability of sdAbs to prevent the interaction of PCSK9 with the EGF-A domain of LDLR was assessed using a PCSK9-LDLR in vitro binding assay kit (Circulex).
Inhibitory PCSK9 Nanobodies

(Invitrogen) for normalizing the DiI-LDL uptake to the number of cells in each well.

**Immunofluorescence**—About 70,000 HepG2 cells/well were seeded in 24-well plates containing coverslips (Fisher) coated with polylysine (Sigma) and cultured overnight in complete medium and in SFM containing 5% lipoprotein-deficient serum for the following 24 h. Following a 4-h incubation with PCSK9 (3 μg/ml) alone or in combination with each sdAb-Fc (1.2 μM), cells were rinsed with cold PBS, incubated 10 min on ice with LDLR antibody (1:200 in SFM; mouse, R&D Systems), rinsed twice with cold PBS, and finally fixed with 4% paraformaldehyde for 10 min on ice and 5 min at room temperature. After two washes in PBS at room temperature and a 30-min incubation with PBS containing 1% BSA, LDLR-antibody complexes were revealed by a 5-min incubation with anti-mouse Alexa Fluor 555-tagged antibody (1:200; goat, Molecular Probes). Cells were then rinsed twice with PBS, and nuclei were stained with DAPI during the final mounting step (ProLong Gold Antifade Reagent with DAPI, Life Technologies, Inc.). Immunofluorescence analyses were performed with a confocal microscope (Zeiss LSM-710) and the Volocity software (x64, PerkinElmer Life Sciences). Because the parameters remained unchanged throughout image acquisition, the values obtained were proportional to LDLR levels (47).

**Western Blotting**—Cells were washed twice with ice-cold PBS and lysed 40 min on ice with radiolmmunoprecipitation assay buffer supplemented with 1× complete protease inhibitor mixture (Roche Applied Science). The cell lysates were then subjected to 8% Tris-glycine SDS-PAGE. The gels were transferred overnight on PVDF membranes (Millipore) that were blocked for 1 h at room temperature in TBST (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.1% Tween 20) containing 5% nonfat dry milk. Membranes were then incubated overnight at 4 °C in 5% milk/TBST with human (goat, R&D Systems) or mouse (goat, Novus Biologicals) LDLR antibody (1:1,000) and human β-actin antibody (mouse, Sigma; 1:2,500) for normalization. After incubation with secondary antibodies coupled to horseradish peroxidase (HRP) at 1:10,000 in 5% milk/TBST, immunoreactive bands were revealed by chemiluminescence using the ECL Plus kit (GE Healthcare). Quantification was performed with ImageJ software or using ChemiDoc MP imaging system (Bio-Rad).

**Mouse Primary Hepatocytes**—Primary hepatocytes were isolated from the liver of 6–14-week-old mice using a two-step collagenase perfusion method as described previously (40). Fibronectin-coated (0.5 mg/ml, Sigma) plates (24-well) were seeded with ~10^5 cells/well in Williams’ medium E (Wisent) containing 10% FBS. After 2 h, the medium was replaced with HepatoZYME medium (Gibco) for 12 h prior to treatment.

**LDLR ELISA**—Cells were lysed for 40 min on ice at 4 °C with ice-cold non-denaturing cell lysis buffer (20 mM Tris-HCl, pH 8, 137 mM NaCl, 2 mM Na2EDTA, 1% Nonidet P-40, 10% glycerol, 4% protease inhibitor mixture without EDTA). Cell lysates were pooled for each experiment; total human LDLR was measured by ELISA (human LDLR DuoSet ELISA Development kit, R&D Systems) and protein levels by a protein assay (Bio-Rad DC). Briefly, a high binding 96-well plate was coated and incubated overnight at room temperature with mouse anti-human LDLR antibody (4 μg/ml) diluted in PBS. The next day, the plate was washed four times with PBS, 0.05% Tween 20, and blocking was carried out with PBS, 1% + BSA, 0.05% + Tween 20, 0.01% sodium azide for 1.5 h at room temperature. Following four washes, standards (7 points, 2-fold serial dilutions starting at 8 ng/ml, and a zero point) and pooled cell lysates (5-fold dilution in PBS/1% BSA) were added to the plate and incubated for 2 h at room temperature. After four washes, detection was carried out with biotinylated goat anti-human LDLR antibody (0.4 μg/ml, diluted in ELISA buffer: 20 mM Tris-HCl, pH 7.2–7.4, 137 mM NaCl, 0.05% Tween 20, 0.1% BSA) for 2 h at room temperature. Following four washes, streptavidin–HRP diluted in ELISA buffer (1:200) was added and incubated for 20 min at room temperature, protected from direct light. After four washes, substrate solution containing a 1:1 mixture of H2O2 and tetramethylbenzidine (R&D Systems) was added to the wells and incubated in the dark for 20 min at room temperature. The reaction was stopped with 2 N sulfuric acid (R&D Systems). The optical density of the yellow-colored product was determined using a SpectraMax i3 plate reader (Molecular Devices) set to 450 nm, from which readings at 540 nm were subtracted to correct for any plate imperfections. The total amount of LDLR was normalized to the quantity of total protein measured in each cell lysate.

**Expression of sdAb-Fc Fusion Proteins Carrying a KDEL C-terminal Tag**—The pIRE2-EGFP vector expressing the sdAb-Fc fusion protein carrying a KDEL ER-retention signal at the C terminus of the protein, downstream of the V5 tag, was obtained as described previously (7). HEK293 cells (~300,000 cells/well) were seeded in 12-well plates in complete media, transfected overnight with various constructs using FuGENE HD, and switched to SFM for another 24 h.

**Expression of EGFs-Fc Fusion Proteins**—A DNA sequence encoding amino acids 314–393 of GenBank™ accession number P01130 (the epidermal growth factor-like repeat AB domain (EGF-AB) of human LDLR) was synthesized by GeneArt (Thermo Fisher). Both sequences were ligated between a signal peptide and the sequence of human IgG1 Fc in the plant expression/transformation vector pTRAKc (72). The clones were transformed into Agrobacterium tumefaciens, which was then used for transient expression in Nicotiana benthamiana (73). The recombinant proteins were purified by protein A affinity chromatography.

**Fluorescence-associated Cell Sorting (FACS) Analysis**—About 500,000 HepG2 cells/well were seeded in 12-well plates in complete media overnight and in SFM for 24 h. Cells were then incubated with PCSK9 (15 μg/ml) for 4 h alone, with sdAb-Fc (0.04 μM), with increasing concentrations of EGF-AB-Fc (Planet Biotechnology Inc.), or their Fc control. Cells were rinsed in FACS buffer (D-PBS containing 0.5% BSA and 1 g/liter glucose (Wisent)), then in 2.5 mM EDTA-2Na-2H2O (Bioshop), and incubated for 20 min at 37 °C in 0.5 ml of EDTA-2Na-2H2O. Detached cells were collected, centrifuged for 5 min at 900 rpm at 4 °C, re-suspended in 0.5 ml of FACS buffer containing 1:250 of human LDLR antibody (R&D Systems), and incubated for 10 min on ice. Cells were then washed with 5 ml of FACS buffer, centrifuged, resuspended in 0.5 ml of FACS buffer containing 1:500 of goat Alexa Fluor 647-labeled anti-mouse antibody (Molecular Probes), and incubated for 5 min. Cells...
were washed again, re-suspended in 0.3 ml of FACS buffer containing 1.67 μg/ml propidium iodide (Sigma), and finally analyzed by FACS using a CyAn flow cytometer (Beckman Coulter).

Animal Studies—The RPCI human BAC library 11 was screened by PCR for the presence of the PCSK9 gene, and the clone 55M23 was identified. A 67.5-kb fragment of the BAC was injected into B6C3F1 fertilized eggs. Mice were then backcrossed to Pcsk9 KO mice in C57BL/6J background for five generations. Thus, these mice only express human PCSK9 and no mouse orthologue. Mice were injected in the tail vein with vehicle (PBS) or 10 mg/kg sdAb-Fc. For single injections mice were analyzed 4 days post-injection, and for double injections, mice were injected at day 0 and 7 and sacrificed at day 11. In both cases blood samples and livers were collected. Total cholesterol levels were determined by the colorimetric assay Infinity (Thermo Scientific) and apoB levels by ELISA (Cloud-Clone Corp.). Liver LDLR levels were measured by Western blot using a mouse LDLR antibody, as for primary hepatocytes.

Statistics—Two-tailed Student’s t test was performed to assess the statistical significance of the data sets. For the DiI-LDL uptake assay, a two-way ANOVA was performed when triplicates were repeated more than once.

Acknowledgments—We are grateful to all the members of the Seidah laboratory for technical support and discussions. We are grateful to Suzanne Benjannet, who helped us with some experiments. We thank Drs. Rex Parker and Franck Daclas (Bristol Myers Squibb) for the donation of pure human PCSK9; Manon Laprise, Anna Roubtsova, and Jadwiga Marcinkiewicz for animal experimentation; and Dr. Dominic Filion for help in confocal imaging analysis. We also thank Shenghua Li for sdAb production in Dr. Zhang’s laboratory, and Drs. Archana Belle and James Maclean from Planet Biotechnology who worked on the development of EGFs-Fc. Finally, we acknowledge the precious help of Dr. Robert Dufour from the Institut de Recherches Cliniques de Montréal for the generous gift of evolocumab.

References
1. Cholesterol Treatment Trials’ (CTT) Collaboration, Baigent, C., Blackwell, L., Emberson, J., Holland, L. E., Reith, C., Bhatia, N., Peto, R., Barnes, E. H., Keech, A., Simes, J., and Collins, R. (2010) Efficacy and safety of more intensive lowering of LDL cholesterol: a meta-analysis of data from 170,000 participants in 26 randomised trials. Lancet 376, 1670–1681
2. Hobbs, H. H., Brown, M. S., and Goldstein, J. L. (1992) Molecular genetics of the LDL receptor gene in familial hypercholesterolemia. Hum. Mutat. 1, 445–466
3. Seidah, N. G., Benjannet, S., Wickham, L., Marcinkiewicz, J., Jasmin, S. B., Stifani, S., Basak, A., Prat, A., and Chretien, M. (2003) The secretory pro-protein convertase neural apoptosis-regulated convertase 1 (NARC-1): liver regeneration and neuronal differentiation. Proc. Natl. Acad. Sci. U.S.A. 100, 928–933
4. Abifadel, M., Varret, M., Rabi, J. P., Allard, D., Ouguerram, K., Devillers, M., Cruaud, C., Benjannet, S., Wickham, L., Erlich, D., Derré, A., Villegier, L., Farnier, M., Beucler, I., Bruckert, E., Chambaz, J., Chanu, B., Lecerf, J. M., Luc, G., Moulin, P., Weißenbach, J., Prat, A., Krempf, M., Junien, C., Seidah, N. G., and Boileau, C. (2003) Mutations in PCSK9 cause autosomal dominant hypercholesterolemia. Nat. Genet. 34, 154–156
5. Seidah, N. G., and Prat, A. (2012) The biology and therapeutic targeting of the proprotein convertases. Nat. Rev. Drug Discov. 11, 367–383
6. Cunningham, D., Danley, D. E., Geoghegan, K. F., Grillo, M. C., Hawkins, J. L., Subahi, T. A., Varghese, A. H., Ammirati, M. J., Culp, J. S., Hoith, L. R., Mansour, M. N., McGrath, K. M., Seddon, A. P., Shenolikar, S., Stutzmans-Engwall, K. J., et al. (2007) Structural and biophysical studies of PCSK9 and its mutants linked to familial hypercholesterolemia. Nat. Struct. Mol. Biol. 14, 413–419
7. Nassoury, N., Blaisle, D. A., Tebon Oler, A., Benjannet, S., Hamelin, I., Poupon, V., McPherson, P. S., Attie, A. D., Prat, A., and Seidah, N. G. (2007) The cellular trafficking of the secretory proprotein convertase PCSK9 and its dependence on the LDLR. Traffic 8, 718–732
8. Zhang, D. W., Lagace, T. A., Garutti, R., Zhao, Z., McDonald, M., Horton, J. D., Cohen, J. C., and Hobbs, H. H. (2007) Binding of proprotein convertase subtilisin/kexin type 9 to epidermal growth factor-like repeat of low density lipoprotein receptor decreases receptor recycling and increases degradation. J. Biol. Chem. 282, 18602–18612
9. Maxwell, K. N., and Breslow, L. J. (2004) Adenoviral-mediated expression of Pcsk9 in mice results in a low-density lipoprotein receptor knockout phenotype. Proc. Natl. Acad. Sci. U.S.A. 101, 7100–7105
10. Benjannet, S., Rahinds, D., Essalami, R., Mayne, J., Wickham, L., Jin, W., Asselin, M. C., Hamelin, J., Varret, M., Allard, D., Trillard, M., Abifadel, M., Tebon, A., Attie, A. D., Rader, D. J., 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
11. Park, S. W., Moon, Y. A., and Horton, J. D. (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
12. Lo Surdo, P., Bottomley, M. J., Calzetta, A., Settembre, E. C., Cirillo, A., Pandit, S., Ni, Y. G., Hubbard, B., Sitlani, A., and Carfi, A. (2011) Mechanistic implications for LDL receptor degradation from the PCSK9/LDLR structure at neutral pH. EMBO Rep. 12, 1300–1305
13. Poirier, S., Mayer, G., Benjannet, S., Bergeron, E., Marcinkiewicz, J., Nassoury, N., Mayer, H., Nimpf, J., Prat, A., and Seidah, N. G. (2008) The proprotein convertase PCSK9 induces the degradation of low density lipoprotein receptor (LDL-R) and its closest family members VLDL-R and ApoER2. J. Biol. Chem. 283, 2363–2372
14. Shan, L., Pang, L., Zhang, R., Murgo, N. J., Lan, H., and Hedrick, J. A. (2008) PCSK9 binds to multiple receptors and can be functionally inhibited by an EGF-A peptide. Biochem. Biophys. Res. Commun. 375, 69–73
15. Camuel, M., Sun, X., Asselin, M.-C., Paramithiotis, E., Prat, A., and Seidah, N. G. (2013) Proprotein convertase subtilisin/kexin type 9 binds to epidermal growth factor-like repeat of low density lipoprotein receptor (LDL-R) and induces its degradation. J. Biol. Chem. 288, 13004–13010
16. Huang, S., Henry, L., Ho, Y. K., Pownall, H. J., and Rudenko, G. (2010) Mechanism of LDL binding and release probed by structure-based mutagenesis of the LDL receptor. J. Lipid Res. 51, 297–308
17. Holla, A. L., Cameron, J., Berge, K. E., Ranheim, T., and Leren, T. P. (2007) Degradation of the LDL receptors by PCSK9 is not mediated by a secreted protein acting upon by PCSK9 extracellularly. BMC Cell Biol. 8, 9–20
18. Zhang, D. W., Garutti, R., Tang, W. J., Cohen, J. C., and Hobbs, H. H. (2008) Structural requirements for PCSK9-mediated degradation of the low-density lipoprotein receptor. Proc. Natl. Acad. Sci. U.S.A. 105, 13045–13050
19. Timms, K. M., Wagner, S., Samuels, M. E., Forbery, K., Goldfine, H., Jamulapati, S., Skolnick, M. H., Hopkins, P. N., Hunt, S. C., and Shattuck, D. M. (2004) A mutation in PCSK9 causing autosomal-dominant hyper-
30. Cohen, J., Pertsemlidis, A., Kotowski, I. K., Graham, R., Garcia, C. K., and Hobbs, H. H. (2005) Low LDL cholesterol in individuals of African descent resulting from frequent nonsense mutations in PCSK9. *Nat. Genet.* **37**, 161–165

31. Muyldermans, S. (2013) Nanobodies: natural single-domain antibodies.

32. van der Linden, R., de Geus, B., Stok, W., Bos, W., van Wassenaar, D., Gupta, M., Hegele, R. A., Lau, D. C., Leiter, L., Pentier, A., Couture, P., Dufour, R., Fodor, G., Francis, G. A., Grover, S., Hamelin, J., Tremblay, M., Jacques, H., Jin, W., Davignon, J., Seidah, N. G., and Prat, A. (2012) Calcium-independent inhibition of PCSK9 by affinity-purified anti-HER2 monoclonal antibody against the C-terminal domain of PCSK9 lowers LDL cholesterol with the M1 and M2 domains of PCSK9. *J. Biol. Chem.* **287**, 33745–33755

33. Stein, E. A., and Raal, F. (2014) Reduction of low-density lipoprotein cholesterol by monoclonal antibody inhibition of PCSK9. *Annu. Rev. Med.* **65**, 417–431

34. Bartunek, J., Barbato, E., Heyndrickx, G., Vanderheyden, M., Wijns, W., and Hobbs, H. H. (2006) A spectrum of PCSK9 alleles contributes to plasma levels of low-density lipoprotein cholesterol. *Am. J. Hum. Genet.* **78**, 410–422

35. Kratz, F., and Elsadek, B. (2012) Clinical impact of serum proteins on drug delivery. *J. Control Release* **161**, 429–445

36. Sarker, S. A., Jäkel, M., Sultana, S., Alam, N. H., Bardhan, P. K., Chisti, M. J., Guerriero, M., Tesh, W., Hammarström, L., and Frenken, L. G. (2013) A proprotein convertase subtilisin/kexin type 9 neutralizing antibody reduces serum cholesterol in mice and non-human primates. *Proc. Natl. Acad. Sci. U.S.A.* **106**, 9820–9825

37. Powers, D. B., Amersdorfer, P., Poul, M., Nielsen, U. B., Shalaby, M. R., Adams, G. P., Weiner, L. M., and Marks, J. D. (2001) Expression of single-chain Fv-Fc fusions in *Pichia pastoris*. *J. Immunol. Methods* **251**, 123–135

38. Zaid, A., Roubtsova, A., Essalmani, R., Marcinkiewicz, J., Chamberland, A., Hamelin, J., Tremblay, M., Jacques, H., Jin, W., Davignon, J., Seidah, N. G., and Prat, A. (2008) Proprotein convertase subtilisin/kexin type 9 (PCSK9): Hepatocyte-specific low-density lipoprotein receptor degradation and critical role in mouse liver regeneration. *Hepatology* **48**, 646–654

39. Essalmani, R., Susan-Resiga, D., Chamberland, A., Abifadel, M., Creemers, J. W., Boileau, C., Seidah, N. G., and Prat, A. (2011) In vivo evidence that furin from hepatocytes inactivates PCSK9. *J. Biol. Chem.* **286**, 4257–4263

40. Ly, K., Saavedra, Y. G., Canuel, M., Routhier, S., Desjardins, R., Hamelin, J., Mayne, J., Lazare, C., Seidah, N. G., and Day, R. (2014) Annexin A2 reduces PCSK9 protein levels via a translational mechanism and interacts with the M1 and M2 domains of PCSK9. *J. Biol. Chem.* **289**, 17732–17746

41. Benjannet, S., Hamelin, J., Chrétien, M., and Seidah, N. G. (2012) Loss- and gain-of-function PCSK9 variants: cleavage specificity, dominant negative effects, and low density lipoprotein receptor (LDLR) degradation. *J. Biol. Chem.* **287**, 33745–33755

42. Pedersen, N. B., Wang, S., Narimatsu, Y., Yang, Z., Halim, A., Scholdager, K. T., Madsen, T. D., Seidah, N. G., Bennett, E. P., Levery, S. B., and Clausen, H. (2014) Low density lipoprotein receptor class A repeats are O-glycosylated in liver regions. *J. Biol. Chem.* **289**, 17312–17324

43. Pelham, H. R. (1988) Evidence that luminal ER proteins are sorted from secreted proteins in a post-ER compartment. *EMBO J.* **7**, 913–918

44. Poirier, S., Mamarchi, M., Chen, W. T., Lee, A. S., and Mayer, G. (2015) GRP94 regulates circulating cholesterol levels through blockade of PCSK9-induced LDLR degradation. *Cell Rep.* **13**, 2064–2071

45. Poirier, S., Mayer, G., Poupon, V., McPherson, P. S., Desjardins, R., Ly, K., Asselin, M. C., Day, R., Duclos, F. J., Witmer, M., Parker, R., Prat, A., and Seidah, N. G. (2009) Dissection of the endogenous cellular pathways of PCSK9-induced LDLR degradation: evidence for an intracellular route. *J. Biol. Chem.* **288**, 28856–28864

46. Roubtsova, A., Munkonda, M. N., Awan, Z., Marcinkiewicz, J., Chamberland, A., Lazare, C., Gianflone, K., Seidah, N. G., and Prat, A. (2011) Circulating proprotein convertase subtilisin/kexin 9 (PCSK9) regulates VLDLR protein and triglyceride accumulation in visceral adipose tissue. *Arterioscler. Thromb. Vasc. Biol.* **31**, 785–791

47. Roubtsova, A., Chamberland, A., Marcinkiewicz, J., Essalmani, R., Fazel, A., Bergeron, J. J., Seidah, N. G., and Prat, A. (2015) PCSK9 deficiency unmasks a sex/tissue-specific subcellular distribution of the LDL and VLDL receptors in mice. *J. Lipid Res.* **56**, 2133–2142

48. Chan, J. C., Piper, D. E., Cao, Q., Liu, D., King, C., Wang, W., Jiang, T., Liu, Q., Hibgee, I., Xia, Z., Di, Y., Shetty, S., Arimura, Z., Salomons, H., Romanow, W. G., et al. (2009) A proprotein convertase subtilisin/kexin type 9 neutralizing antibody reduces serum cholesterol in mice and non-human primates. *Proc. Natl. Acad. Sci. U.S.A.* **106**, 9820–9825

49. Marcinkiewicz, M., Ramla, D., Seidah, N. G., and Chrétien, M. (1994) Developmental expression of the prohormone convertases PC1 and PC2 in mouse pancreatic islets. *Endocrinology* **135**, 1651–1660

50. Ni, Y. G., Condra, J. H., Orcalli, L., Shen, X., Di Marco, S., Pandit, S., Bottomley, M. J., Ruggeri, L., Cummings, R. T., Cubbon, R. M., Santoro, J. C., Ehrhardt, A., Lewis, D., Fisher, T. S., Ha, S., et al. (2010) A proprotein convertase subtilisin-like/kexin type 9 (PCSK9)-C-terminal domain antibody antigen-binding fragment inhibits PCSK9 internalization and restores low density lipoprotein uptake. *J. Biol. Chem.* **285**, 12882–12891

51. Schiele, F., Park, J., Redemann, N., Luigold, G., and Nar, H. (2014) An antibody against the C-terminal domain of PCSK9 lowers LDL cholesterol levels in vivo. *J. Mol. Biol.* **426**, 834–852

52. Butkinaree, C., Canuel, M., Essalmani, R., Poirier, S., Benjannet, S., Asselin, M.-C., Roubtsova, A., Hamelin, J., Marcinkiewicz, J., Chamberland, A., Guillenrot, J., Mayer, G., Bisioda, S. S., Jacob, Y., Prat, A., and Seidah, N. G. (2015) Amyloid precursor-like protein 2 and sortilin do not regulate the PCSK9-mediated low density lipoprotein receptor degradation but interact with each other. *J. Biol. Chem.* **290**, 18609–18620

53. Zhang, Y., Zhou, L., Kong-Beltran, M., Li, W., Moran, P., Wang, J., Quan, C., Tom, J., Kolumam, G., Elliott, J. M., Skelton, N. J., Peterson, A. S., and Kirchhofer, D. (2012) Calcium-independent inhibition of PCSK9 by affin
ity-improved variants of the LDL receptor EGF(A) domain. J. Mol. Biol. 422, 685–696
55. Ni, Y. G., Di Marco, S., Conдра, J. H., Peterson, L. B., Wang, W., Wang, F., Pandit, S., Hammond, H. A., Rosa, R., Cummings, R. T., Wood, D. D., Liu, X., Bottomley, M. I., Shen, X., Cubbon, R. M., et al. (2011) A PCSK9-binding antibody that structurally mimics the EGF(A) domain of LDL-receptor reduces LDL cholesterol in vivo. J. Lipid Res. 52, 78–86
56. Mitchell, T., Chao, G., Sitkoff, D., Lo, F., Monshizadegan, H., Meyers, D., Low, S., Russo, K., DiBella, R., Denhez, F., Gao, M., Myers, J., Duke, G., Witmer, M., Miao, B., et al. (2014) Pharmacologic profile of the adnectin BMS-962476, a small protein biologic alternative to PCSK9 antibodies for low-density lipoprotein lowering. J. Pharmacol. Exp. Ther. 350, 412–424
57. Graham, M. J., Lemonidis, K. M., Whipple, C. P., Subramaniam, A., Mo-nia, B. P., Crooke, S. T., and Crooke, R. M. (2007) Antisense inhibition of proprotein convertase subtilisin/kexin type 9 reduces serum LDL in hyperlipidemic mice. J. Lipid Res. 48, 763–767
58. Gupta, N., Fisker, N., Asselin, M. C., Lindholm, M., Rosenbohm, C., Ørum, H., Elnén, J., Seidah, N. G., and Straarup, E. M. (2010) A locked nucleic acid antisense oligonucleotide (LNA) silences PCSK9 and enhances LDLR expression in vitro and in vivo. PLoS ONE 5, e10682
59. Yamamoto, T., Harada-Shiba, M., Nakatani, M., Wada, S., Yasuhara, H., Narukawa, K., Sasaki, K., Shibata, M. A., Torigoe, H., Yamaoka, T., Imanishi, T., and Obika, S. (2012) Cholesterol-lowering action of BNA-based antisense oligonucleotides targeting PCSK9 in atherogenic diet-induced hypercholesterolemic mice. Mol. Ther. Nucleic Acids 1, e22
60. Frank-Kamenetsky, M., Grefhorst, A., Anderson, N. N., Racie, T. S., Bram-lage, B., Akinc, A., Butler, D., Charisse, K., Dorkin, R., Fan, Y., Gamba-Vitalo, C., Hadwiger, P., Jayaraman, J., John, M., Jayaprakash, K. N., et al. (2008) Therapeutic RNAi targeting PCSK9 acutely lowers plasma cholesterol in rodents and LDL cholesterol in nonhuman primates. Proc. Natl. Acad. Sci. U.S.A. 105, 11915–11920
61. Galabova, G., Brunner, S., Winsauer, G., Juno, C., Wanko, B., Mairhofer, A., Lührs, P., Schneeberger, A., von Bonin, A., Mattner, F., Schmidt, W., and Staffler, G. (2014) Peptide-based anti-PCSK9 vaccines – an approach for long-term LDLc management. PLoS ONE 9, e114469
62. Crosse, E., Amar, M. J., Sampson, M., Peabody, J., Schiller, J. T., Chack-erian, B., and Remaley, A. T. (2015) A cholesterol-lowering VLP vaccine that targets PCSK9. Vaccine 33, 5747–5755
63. Cortez-Retamozo, V., Lauwereys, M., Hassanzadeh Gh, G., Gobert, M., Conrath, K., Muyldermans, S., De Baetselier, P., and Revets, H. (2002) Efficient tumor targeting by single-domain antibody fragments of camels. Int. J. Cancer 98, 456–462
64. Denis, M., Marcinkiewicz, J., Zaid, A., Gauthier, D., Poirier, S., Lazere, C., Seidah, N. G., and Prat, A. (2012) Gene inactivation of PCSK9 reduces atherosclerosis in mice. Circulation 125, 894–901
65. Guella, L., Asselta, R., Ardissino, D., Merlino, P. A., Peyvandi, F., Kathiresan, S., Mannucci, P. M., Tubaro, M., and Duga, S. (2010) Effects of PCSK9 genetic variants on plasma LDL cholesterol levels and risk of premature myocardial infarction in the Italian population. J. Lipid Res. 51, 3342–3349
66. Huang, Y., Ballinger, D. G., Stokowski, R., Belharz, E., Robinson, J. G., Liu, Robinson, R. D., Henderson, V. W., Rossouw, J. E., and Prentice, R. L. (2012) Exploring the interaction between SNP genotype and postmeno-spaual hormone therapy effects on stroke risk. Genome Med. 4, 57
67. dos Santos, C., and Marshall, J. C. (2014) Bridging lipid metabolism and innate host defense. Sci. Transl. Med. 6, 258fs41
68. Sun, X., Essalmani R, Day R, Khatib, A. M., Seidah, N. G., and Prat, A. (2012) Proprotein convertase subtilisin/kexin type 9 deficiency reduces melanoma metastasis in liver. Neoplasia 14, 1122–1131
69. Benjannet, S., Saavedra, Y. G., Hamelin, J., Asselin, M. C., Essalmani, R., Pasquato, A., Lemaire, P., Duke, G., Miao, B., Duclos, F., Parker, R., Mayer, G., and Seidah, N. G. (2010) Effects of the prosegment and pH on the activity of PCSK9: evidence for additional processing events. J. Biol. Chem. 285, 40965–40978
70. Hussack, G., Arbabi-Ghahroudi, M., van Faassen, H., Songer, J. G., Ng, K. K., MacKenzie, R., and Tanha, J. (2011) Neutralization of Clostridium difficile toxin A with single-domain antibodies targeting the cell receptor binding domain. J. Biol. Chem. 286, 8961–8976
71. Schippers, I. J., Moschate, H., Roelofsen, H., Müller, M., Heymans, H. S., Ruiters, M., and Kuipers, F. (1997) Immortalized human hepatocytes as a tool for the study of hepatocytic (de-)differentiation. Cell Biol. Toxicol. 13, 375–386
72. Maclean, J., Koekemoer, M., Olivier, A. J., Stewart, D., Hitzeroth, I. I., Rademacher, T., Fischer, R., Williamson, A. L., and Rybicki, E. P. (2007) Optimization of human papillomavirus type 16 (HPV-16) L1 expression in plants: comparison of the suitability of different HPV-16 L1 gene variants and different cell-compartment localization. J. Gen. Virol. 88, 1460–1469
73. Voinnet, O., Rivas, S., Mestre, P., and Baulcombe, D. (2003) An enhanced transient expression system in plants based on suppression of gene silencing by the p19 protein of tomato bushy stunt virus. Plant J. 33, 949–956