Self-Association of Human PCSK9 Correlates with Its LDLR-Degrading Activity†

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ABSTRACT: Genetic studies have demonstrated an important role for proprotein convertase subtilisin/kexin type 9 (PCSK9) as a determinant of plasma cholesterol levels. However, the underlying molecular mechanism is not completely understood. To this end, we have generated a mammalian cell expression system for human PCSK9 and its mutants and produced transgenic mice expressing human PCSK9. HEK293T cells transfected with the human PCSK9 DNA construct expressed and secreted PCSK9 and displayed decreased LDLR levels; functional PCSK9 protein was purified from the conditioned medium. *In vitro* studies showed that PCSK9 self-associated in a concentration-, temperature-, and pH-dependent manner. A mixture of PCSK9 monomers, dimers, and trimers displayed an enhanced LDLR degrading activity compared to monomeric PCSK9. A gain-of-function mutant, D374Y, displayed greatly increased self-association compared to wild-type PCSK9. Moreover, we demonstrated that the catalytic domain of PCSK9 is responsible for the self-association. Self-association of PCSK9 was enhanced by incubation with mouse apoE$^{-/-}$ VLDL and inhibited by incubation with both human and mouse HDL. When PCSK9 protein was incubated with total serum, it partially associated with LDL and HDL but not with VLDL. In transgenic mice, PCSK9 also associated with LDL and HDL but not with VLDL. We conclude that self-association is an intrinsic property of PCSK9, correlated to its LDLR-degrading activity and affected by plasma lipoproteins. These results provide a basis for developing strategies to manipulate PCSK9 activity in the circulation for the treatment of hypercholesterolemia.

Human proprotein convertase subtilisin/kexin type 9 (PCSK9[1]) was originally defined as neural apoptosis regulated convertase 1 (or NARC-1), because its mRNA was found to be up-regulated following induction of neural apoptosis by serum withdrawal (*1*). Domain analyses indicated that NARC-1 bears structural homology to the subtilisin-like proprotein convertase family (*2*); these proteases, including furin, are implicated in the limited proteolysis of precursors of secretory proteins that regulate a variety of cellular functions (*3*−*5*). NARC-1/PCSK9 is the ninth member of this family, following PC1/3, PC2, furin, PC4, PACE4, PC5/6, PC7/LPC, and SKI-1/S1P (*1*). PCSK9 is synthesized as a 72 kDa zymogen (pro-PCSK9) in the endoplasmic reticulum, where a prosegment is cleaved autocatalytically at the $FAQ_{152}\downarrow SIP$ to yield the mature form of PCSK9(*2*, *6*). The mature PCSK9 is secreted from the cell with the prosegment still associated with it. PCSK9 has been proposed to be inactivated by cleavage at arginine 218 within the mature protein by furin and/or PC5/6A (*7*).

Human genetic studies have confirmed that PCSK9 is a determinant of human cholesterol metabolism (*1*, *8*). A series of gain-of-function mutations of PCSK9, such as N157K, F216L, R218S, R357H, D374Y, and E670G were reported as an additional cause of autosomal dominant hypercholesterolemia (ADH), which is linked to increased risk of coronary heart disease (CHD) (*9*−*17*). On the other hand, some loss-of-function mutations, including R46L, Y142X, L253E, A443T, and C679X, result in hypocholesterolemia and longevity (*18*−*22*). These results are compatible with the notion that PCSK9 regulates plasma cholesterol levels through regulation of LDLR levels on the plasma membrane, as catalytically inactive mutations in PCSK9 abrogate its ability to degrade LDLR (*18*, *23*). However, it remains to be determined whether the LDLR is directly cleaved by PCSK9 and where the effect of PCSK9 takes place. Overexpression experiments suggest that PCSK9 may reduce LDLR either in a postendoplasmic reticulum (ER) compartment, while the LDLR is transported to the cell membrane, or on the cell surface (*23*, *24*). The cellular location of PCSK9 in rat hepatocytes is compatible with several possible sites of action, including the ER, early endosome, ER/Golgi

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[1] Abbreviations: PCSK9, proprotein convertase subtilisin/kexin type 9; LDL, low-density lipoprotein; SREBP, sterol regulatory element-binding protein; LDLR, LDL receptor; HDL, high-density lipoprotein; ADH, autosomal dominant hypercholesterolemia; ARH, autosomal recessive hypercholesterolemia; apoE, apolipoprotein E.

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intermediate compartment (ERGIC), and plasma membrane but not in Golgi cisternae, late endosomes, or lysosomes (*25*).

PCSK9 is most abundantly expressed in the liver and small intestine but is also expressed in the brain, skin, and kidney. Its expression in liver is transcriptionally regulated by cellular cholesterol in the same direction as that of LDLR through the sterol regulatory element-binding proteins (SREBPs) (*26*, *27*). For example, HMG-CoA reductase inhibitors are potent cholesterol-lowering drugs that up-regulate the expression of LDLR as well as PCSK9 through increased SREBP-2 levels (*28*, *29*). PCSK9 can be glycosylated at residue N533 (*30*) and can also undergo tyrosine sulfation before secretion (*6*, *30*). The functional implications of these modifications are unknown. It has recently been reported that PCSK9 is detectable in human plasma at concentrations of 50−600 ng/mL. Within this range, PCSK9 has been shown to effectively reduce LDLR expression in cultured cells (*31*), suggesting the possibility that circulating PCSK9, which is mainly secreted by the liver, may regulate the amount of LDLR expressed in peripheral tissues. Very recently, the crystal structure of PCSK9 has been solved (*32*, *33*), and the binding region in the LDLR has been identified (*34*). It appears that LDLR-mediated endocytosis of PCSK9, rather than intact catalytic activity, is required for effective PCSK9-mediated LDLR degradation (*35*−*37*).

Even considering the latest breakthroughs, little is known about the molecular mechanism underlying the role of PCSK9 in the pathophysiology of hypercholesterolemia. In this study, we determined that (1) PCSK9 *in vitro* can self-associate in a temperature, concentration and pH-dependent manner, (2) the catalytic domain is responsible for the self-association, (3) the self-association of PCSK9 is correlated with enhanced LDLR degrading activities, and (4) plasma lipoproteins bind and influence self-association of PCSK9.

EXPERIMENTAL PROCEDURES

Materials and Reagents. pcDNA3.1 Directional TOPO Expression Kit and ProBond Purification System were purchased from Invitrogen (cat. no. K4900-01 and K850-01, respectively). HepG2 and HEK293T cell lines were from ATCC. Bicistronic lentiviral vector PWPI, lentiviral envelope plasmid pMD2.G, and packaging plasmid pCMVΔR8.91 were kindly provided by Dr. Dider Trono (Lausanne, Switzerland). Chicken polyclonal antibody to LDL receptor and rabbit polyclonal antibody to chicken IgY H&L (HRP) were purchased from Abcam Inc. (cat. no. ab14056 and ab6753, respectively). Rabbit polyclonal antibody to PCSK9 was obtained from Cayman Chemical (cat. no. 10007185). Rabbit polyclonal antibody to polyhistidine (6 x His) was from eBioscience (cat. no. 14−6757). HRP-conjugated goat anti-rabbit IgG antibody was from Sigma. Human liver total RNA was purchased from Stratagene (cat. no. 540017). ProFection Mammalian Transfection System was from Promega (cat. no. E1200). High capacity cDNA archive kit was obtained from Applied Biosystems (cat. no. 4322171).

Human PCSK9 Expression Construct and Transfection. Primers used for PCR human PCSK9 cDNA from a human liver cDNA library were forward primer, 5′ CAGTGAATTCATGGGCACCGTCAG-CTCCAGG 3′, and reverse primer, 5′ CGCCAAGCTTTCACTGGAGCTCCTGGGAGGC 3′. The PCR product was digested by HindIII and EcoR1 and

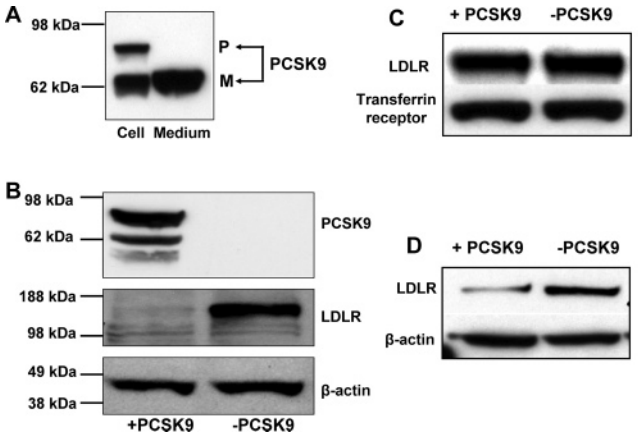

FIGURE 1: Expression of PCSK9 and its functional assessment. A. HEK293T cells were transfected with pcDNA-hPCSK9. Cell lysate and conditioned medium were subjected to 4−12% SDS-PAGE and western blot analysis using anti-HisTag Ab for hPCSK9. P, pro-PCSK9; M, mature PCSK9. B. Cell lysates from pcDNA-PCSK9 transfected (+PCSK9) or non-transfected (-PCSK9) HEK293T cells were blotted for PCSK9, LDLR, and β-actin. C. Purified PCSK9 was added to HEK293T cell membrane preparation at 6 μg/mL. After incubation at 37 °C for 16 h, the membrane preparation was subjected to western blot analyses for LDLR and transferrin receptor. D. Purified PCSK9 was added to HEK293T cell culture medium at 6 μg/mL. After incubation at 37 °C for 16 h, whole cell extracts were subjected to western blot analysis of LDLR and β-actin. Three experiments showed LDLR was reduced by 80% on average by incubation with PCSK9.

subcloned into pUC19 vector. Over 20 colonies were screened for correct hPCSK9 sequence. The one used for further subcloning is identical to published hPCSK9 cDNA sequence NM-174936 or gi31317306, specifically with encoded amino acids at the following positions: A28, D186, R218, H226, S386, N553, S127, F216, D374, I474, and G670. Primers for direct topo subcloning of human PCSK9 into the pcDNA3.1D/V5-His TOPO vector were forward primer, 5′ CACCATGGGCACCGTCAGCTCCAG 3′, and reverse primer, 5′ CTGGAGCTC-CTGGGAGGCCTG 3′; the resulting construct is designated pcDNA-hPCSK9 (Supporting Information Figure 1A). Methods for the generation of lentiviral PCSK9 construct, lentivirus preparation, and transduction are described in the Supporting Information, similar to that described previously (*38*).

Mutagenesis. Mutagenesis was carried out using the QuickChange II XL Site-Directed mutagenesis kit from Stratagene (Stratagene, La Jolla, CA). Briefly, primers containing the desired mutations were annealed to the denatured pcDNA-PCSK9 DNA which was then extended using PfuTurbo DNA polymerase to generate nicked, circular strands. The methylated, nonmutated parental DNA was digested using *Dpn*I. The circular, nicked, double-stranded DNA containing the mutation was then transformed into *E. coli* cells. The mutations were confirmed by DNA sequencing. The primers used for generation of PCSK9−D374Y were 5′ggtgcctccagctactgcagcacctgc3′ and 5′gcaggtgctgcagtagctggaggcacc3′. The primers used for generation of PCSK9-C679X were 5′ccgttgccatctgcaagg-gtcaagacaattc3′ and 5′gaattgtcttgacccttg cagatggcaacgg3′. The primers used for generation of PCSK9-RCD (with the C-terminal domain, residues 453−692, removed) were 5′cacccatggggcaggt-ggtcaagacaattctgc3′ and 5′gcagaattgtcttgaccacctgccccatgggtg3′. The primers for generation of PCSK9-RPSCA (with the prosegment and the

catalytic domain, residues 34−422, removed) were 5′ cgtgcgcaggaggacgtcatcaatgaggcctg 3′ and 5′ caggcctcattgatgacgtcctcctgcgcacg 3′. The method for the generation of pcDNA-PCSK9-GST DNA construct is described in the Supporting Information.

Protein Purification. The procedures for His-tagged and GST-tagged PCSK9 protein are described in the Supporting Information. The protein concentration was determined by Lowry assay (*39*).

Cell Membrane Preparation. Total cell membranes were isolated by the method of Nagamatsu et al. (*40*) with minor modifications. The detailed procedure is described in the Supporting Information.

Cross-Linking. Proteins were diluted with storage buffer (50 mM sodium phosphate, pH 7.0, 50 mM sodium chloride) to 0.5 μg/mL and incubated with or without bis(sulfosuccinmidyl) suberate (BS3; Pierce, final concentration 0.1 mM) at 37 °C for 10 min. The cross-linking reactions were quenched by adding 1/3 volume 1 M Tris-HCl, and then the samples were analyzed by western blot analysis using anti-HisTag antibody.

GST Pull-Down Experiment. PCSK9-GST and PCSK9-HT at the indicated concentration in 50 μL of PBS were mixed and incubated at 37 °C for 10 min before adding to 10 μL of prewashed glutathione beads at 4 °C. After incubation at 4 °C for 1 h with continuous shaking, the beads were extensively washed with 1 mL PBS for four times. Then 30 μL of 2X SDS buffer was added to the beads, the beads were boiled for 5 min before centrifugation, and the supernatant was loaded to SDS-PAGE for western blot analysis. Samples with PCSK9-GST alone or PCSK9-HT alone were included as controls.

Immunocytochemistry and Confocal Microscopy. HEK293T cells were plated to each well of a 12-well plate with a piece of thin 12-mm circular glass at the bottom, the proper amount of viral concentrate was added to each well and mixed, and the plate was spun at 1000*g* for 1 h at 25 °C. Sixteen to twenty-four hours later, the medium was changed to DMEM with 1% FBS. Another 24 h later, the circular glass with the cells were used for immunostaining and confocal microscopy. The cells were fixed in 4% paraformaldehyde in 0.01M PBS for 20 min at 4 °C and blocked with 10% normal donkey serum and 1% bovine serum albumin in PBS for 2 h. The rabbit primary antibody for LDLR (USBiological, cat. no. L2601) was diluted (1:200) in the blocking solution and applied to the cells overnight at 4 °C under non-permeablized conditions. The cells were then washed three times with PBS followed by the application of an Alexa Fluor 488-conjugated donkey anti-rabbit secondary antibody (Invitrogen, Carlsbad, CA) for 2 h. The slides were washed three times and mounted in the imaging chambers for confocal analysis. Digital images were acquired with a Zeiss LSM510 confocal microscope using a 40× oil immersion lens (numerical aperture 1.3). To compare the fluorescent intensity of cell surface labeling, all images from the same experiment were acquired with identical settings for laser power, photomultiplier gain, offset, and pinhole sizes. Both single images and stacks of images were taken across the focal planes that showed maximal fluorescent intensity for each sample. The digital images were examined visually, and differences in intensity were also confirmed by analysis with MetaMorph software (Molecular Devices, Sunnyvale, CA).

Lipoprotein Isolation. Mouse lipoproteins were isolated by density ultracentrifugation as previously described (*41*).

Generation of Transgenic Mice. The pcDNA-PCSK9 DNA was digested by BglII and PsiI, and the yielded 3800-bp fragment was used for transgenic injection (Supporting Information Figure 1C). The injection was performed by Vanderbilt Transgenic Mouse/Embryonic Stem Cell Shared Resource. The transgenic mice were confirmed by Southern blot and PCR.

Western Blot. Samples were separated by NuPAGE 4−12% Bis-Tris gel (Invitrogen). After electrophoresis, proteins were transferred to nitrocellulose membranes (Amersham Bioscience). Rabbit anti-polyhistidine or rabbit anti-human PCSK9 and goat anti-rabbit IgG (HRP) antibodies were used to detect PCSK9. Chicken anti-LDLR and rabbit anti-chicken IgG (HRP) antibodies were used to detect the LDLR. The signal was detected using ECL kit (Amersham). To quantify the immunoreactivity of Western blot bands, integrated optical density (IOD) was calculated using Quantity One software (Bio-rad, Hercules, CA).

RESULTS

Expression, Purification, and Functional Assessment of Human PCSK9. We started with the generation of a high-yield mammalian cell expression system for human PCSK9. First we reverse transcribed human total liver RNA to cDNA and subcloned the human PCSK9 coding sequence into a pUC19 vector. We screened 20 colonies, and found numerous variations from the published sequence. This was likely due to the high degree of polymorphism of the human PCSK gene, as the hepatic RNA was derived from multiple donors. Some of the variations were reported previously by others as mutations, such as I474V, R237W, and E670G; some of them have not been reported, such as H87N, A103D, G308R, S376G, D480G, and R499C. One of the colonies had the identical sequence to the published human PCSK9 cDNA sequence (NM-174936 or gi31317306). We further subcloned this sequence into pcDNA3.1 vector by direct TOPO cloning. In this construct, human PCSK9 is followed by a C-terminal tag including a V5 epitope and a 6-His tag (total 47 residues) to facilitate its purification and detection. We transfected HEK293T cells with this construct and were able to achieve a very high level of expression, with the mature form of PCSK9 efficiently secreted from the cells into the medium. Figure 1A shows a western blot of human PCSK9 from the cell lysate and medium of the transfected HEK293T cells.

To measure the functionality of the PCSK9 protein expressed by transfected cells, and to check whether the C-terminal tag impairs its function, we measured the LDLR levels of transfected HEK293T cells. We found that overexpression of human PCSK9 almost completely deleted LDLR protein from HEK293T cells (Figure 1B), proving that the C-terminally tagged PCSK9 is functional.

We purified the mature form of human PCSK9 from the conditioned medium of the transfected HEK293T cells using a His-tag system, yielding 0.02 mg of highly purified PCSK9 per milliliter of conditioned media (Supporting Information Figure 2). SDS-PAGE analysis also showed an ∼14 kDa band derived from the prosegment of PCSK9. N-Terminal sequencing detected an eight-residue fragment (S-I-P-W-N-L-E-R) from the 64 kDa band, confirming that the mature

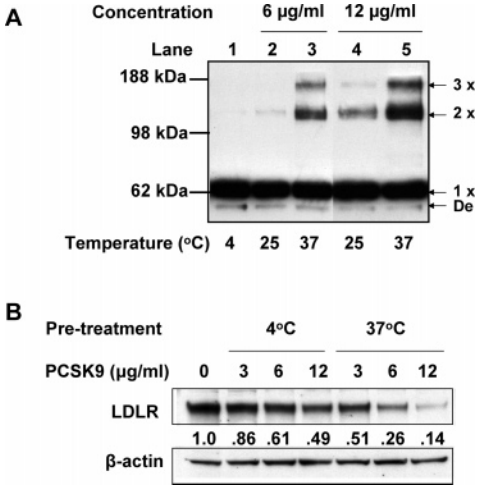

FIGURE 2: Self-association of PCSK9 and its impact on LDLR degrading activity. A. Purified hPCSK9 was incubated at indicated concentration and temperature for 24 h before subjected to western blot analysis. De, a degraded fragment. B. Before added to HEK293T cells, purified PCSK9 protein at indicated concentration was incubated in DMEM without FBS for 24 h at 4 °C or 37 °C. Then the media were added to HEK293T cells and the cells were incubated at 37 °C for an additional 4 h. The whole cell lysates were subjected to western blot analyses of LDLR and β-actin. Normalized ratios of LDLR to β-actin were measured by densitometry with Quantity One software in three separate experiments and the average numbers are shown.

form of PCSK9 starts with residue S153, consistent with reports in the literature (*2*, *6*). SDS-PAGE gel also showed a low concentration ~58 kDa band, which is compatible with the alternatively cleaved form of PCSK9 reported by Seidah et al. (*30*).

To verify the activity of purified PCSK9 protein, we carried out two types of experiments. When the purified PCSK9 protein (6 μg/mL) was added to a cell membrane preparation from HEK293T cells, it did not decrease LDLR levels, even after 16 h incubation (Figure 1C). But when the same amount of PCSK9 protein was added to cultured HEK293T cells, a significant decrease was detected in LDLR level after 16 h incubation (Figure 1D). These results indicate that PCSK9 either mediates the degradation of LDLR only intracellularly after endocytosis or needs the presence of other intracellular protein(s) to gain the LDLR-degrading competence.

Self-Association of hPCSK9 and the Impact on Its LDLR Degrading Activity. Since our data and those of others (*31*) suggest that PCSK9 needs to be endocytosed by the cells to function as an LDLR-degrading agent, we proposed that PCSK9 may undergo modifications or associate with other components extracellularly to facilitate its endocytosis. Interestingly, we found that incubation of purified PCSK9 protein in storage buffer (50 mM sodium phosphate, pH 7.0, 50 mM NaCl) caused it to self-associate and form SDS-stable dimers and trimers in a temperature and concentration-dependent manner (Figure 2A). Since the samples were analyzed under reducing condition, the dimers and trimers cannot be attributable to disulfide bonds. Densitometric analyses revealed that, after 24 h incubation at 25 °C and at a concentration of 6 μg/mL, PCSK9 formed trace amounts of dimers (~5%); in comparison, incubation at 37 °C for 24 h at a concentration of 12 μg/mL, approximately 30% of total PCSK9 was in dimeric form and 10% in trimeric form.

We further measured whether the self-association of PCSK9 affects its LDLR degrading activity. Figure 2B shows a representative experiment indicating that purified PCSK9 protein degraded LDLR in a dose-dependent manner, and preincubation of PCSK9 at 37 °C for 24 h before it was added to the cultured cells significantly enhanced the activity of PCSK9 in degrading LDLR.

To examine whether PCSK9 mutants self-associate differently from the wild-type protein and whether pH affects the self-association, we generated DNA constructs for one gain-of-function mutant, D374Y, and one loss-of-function mutant, C679X. Figure 3A shows that C679X was not secreted by the cells and did not degrade LDLR. On the other hand, D374Y was efficiently secreted and nearly abolished LDLR by 24 h after transfection. The D374Y mutant protein was purified from the medium using the same procedure for wild-type PCSK9. When the wild-type protein and D374Y mutant protein were incubated at 1 μg/mL and different pH for 24 h, we found that (1) at both pH5.2 and 7.0, the D374Y mutant displayed significant more dimer and higher multimer formation compared to the wild-type protein, and (2) at pH 5.2, both wild-type and D374 mutant protein displayed significantly more multimer formation than at pH 7.0 (Figure 3B).

To determine which structural domain mediates the self-association of PCSK9, we generated two domain-deletion mutants based on the recently determined crystal structure of PCSK9 (*32*, *33*). We called one RCD, harboring the deletion of the whole C-terminal domain (residues 453−692), and the other RPSCA, harboring the deletion of the prosegment and the catalytic domain (residues 34−422). Both RCD and RPSCA were secreted into the media of transfected HEK293T cells. The proteins were purified from the media using a standard procedure (Supporting Information Figure 2B). We compared the self-association of full-length PCSK9 and of the two domain deletion mutants by two methods. First, we incubated the proteins at 5 μg/mL and pH 7.0, at 4 °C or 37 °C for 24 h. Figure 3C shows that while full-length PCSK9 and RCD formed SDS-stable dimers and trimers, RPSCA was exclusively monomeric. Second, we performed BS3 cross-linking experiments using the proteins at much lower concentration (0.5 μg/mL). Figure 3D shows that, after incubation of the proteins with 0.1 mM BS3 at 37 °C for 10 min, full-length PCSK9 and RCD formed dimers, trimers, and higher multimers, whereas RPSCA was only in monomeric form.

To rule out the possibility that the 6 x His tag may have contributed to the aggregation of purified PCSK9, and to further test that PCSK9 can self-associate at more physiological conditions, we generated another PCSK9 DNA construct, replacing the V5 and 6 x His tag with a GST tag, and then purified the GST-tagged PCSK9 protein (PCSK9-GST, Supporting Information Figures 1B and 2A). Figure 4A showed that, after incubation at 12 μg/mL, pH 5.2, and 37 °C for 4 h, PCSK9-GST formed SDS-stable dimers and higher multimers similar to His-tagged PCSK9. More importantly, GST pull-down experiments showed that when His-tagged PCSK9 and PCSK9-GST were co-incubated at a concentration of 1 μg/mL at 37 °C for just 10 min, PCSK9-HT was pulled down by GST beads, and at pH 5.2, more PCSK9-HT was pulled down than at pH 7.0 (Figure 4B), indicating that even before the formation of SDS-stable

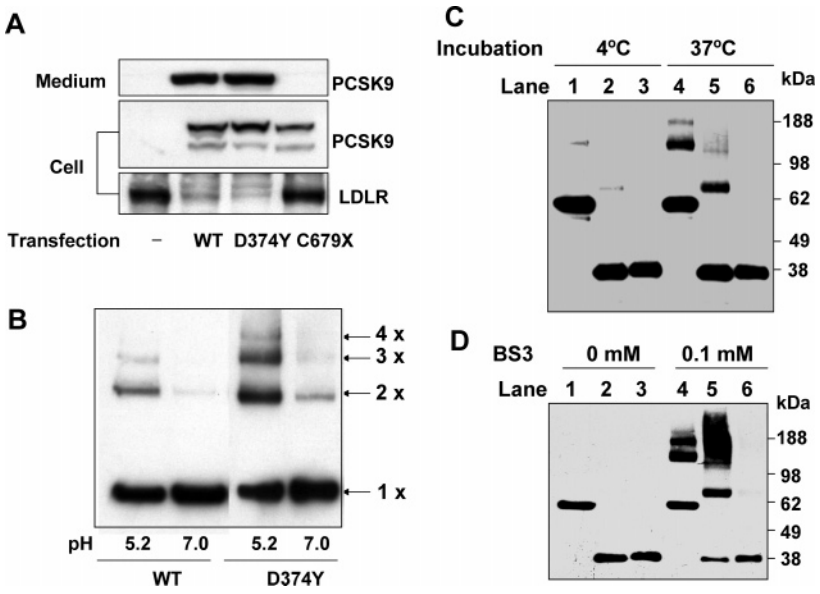

FIGURE 3: Self-association of PCSK9 mutants. A. HEK293T cells were transfected with wild-type (WT) PCSK9, a gain-of-function mutant D374Y and a loss-of-function mutant C679X; 24 h after transfection, the medium and cell lysate were subjected to western blot analysis of PCSK9 (rabbit anti-PCSK9 antibody) and LDLR. B. Purified Wild-type PCSK9 and D374Y mutant were incubated at 1 μg/mL, 37 °C and pH 5.2 or pH 7.0 for 24 h, and then subjected to western blot analysis using rabbit anti-PCSK9 antibody. C. Purified WT PCSK9 and its domain-deletion mutants were incubated at 4 °C or 37 °C for 24 h at 5 μg/mL concentration and pH 7.0 and then were subjected to western blot analysis. D. Cross-linking of WT PCSK9 and its domain-deletion mutants at a concentration of 0.5 μg/mL (see Experimental Procedures). In both panel C and panel D, Lanes 1 and 4: full-length PCSK9; Lanes 2 and 5: PCSK9-RCD; Lanes 3 and 6: PCSK9-RPSCA.

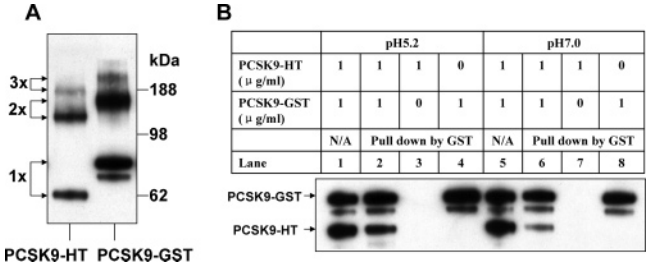

FIGURE 4: Self-association of differently tagged PCSK9. A. Purified PCSK9, either with HisTag or GST tag, formed SDS-stable dimers and trimers after incubation at 12 μg/mL concentration, pH 5.2 and 37 °C for 4 h. B. Purified His-tagged PCSK9 and GST-tagged PCSK9 were mixed and incubated at 37 °C for 10 min and then pulled down by GST-affinity beads, and the pulled-down protein was subjected to western blot using rabbit anti-PCSK9 antibody.

dimers and higher multimers, PCSK9 indeed self-associates in solution in a pH-dependent manner (lower pH favors the self-association).

To test whether the dimers and higher multimers of PCSK9 can be taken up by the cells faster than monomers, a mixture of PCSK9 monomers and multimers was added to cultured HEK293T cells. Figure 5A shows that the dimers and higher multimers almost disappeared by 1 h, much faster than did the monomers. However, we were unable to detect dimers and higher multimers in the cells after the uptake, and neither could we detect multimers in the culture media of transfected cells up to 72 h after transfection. We hypothesize that the dimers and higher multimers formed in the medium are quickly taken up by the cells and immediately degraded intracellularly. To test this hypothesis, we transfected HEK293T cells in a 10-cm dish and collected half of the medium with a protease inhibitor cocktail in a separate cell culture dish 24 h after the transfection. Both the dish with the transfected cells and the plate with only the PCSK9-containing medium were further incubated at 37 °C for 24 h before the media were subjected to western blot for PCSK9. Interestingly, the conditioned medium only displayed monomeric PCSK9, while the cell-free medium displayed the presence of PCSK9 multimers (Figure 5B). These results are compatible with a scenario where PCSK9 forms dimers and higher multimers in the medium which are rapidly and preferentially taken up by the cell. To detect the presence of PCSK9 multimers in the cells, we concentrated the multimer-containing medium to 1/10 of the original volume, used isotonic-K^+ buffer (0.15 M KCl solution) to bring the volume back, and added the PCSK9-containing isotonic K^+

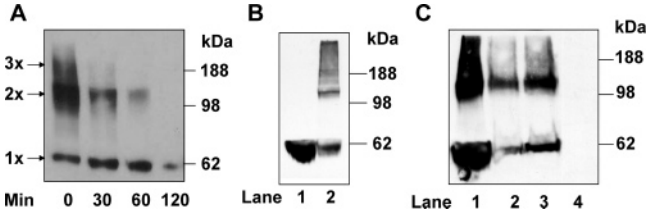

FIGURE 5: Uptake of PCSK9 multimers by cells. A. Purified PCSK9 was incubated in DMEM at 12 μg/mL and 37 °C for 24 h before being added to cultured HEK293T cells. The medium was then sampled at indicated time points for western blot analysis of PCSK9. B. Self-association of secreted PCSK9 in the culture medium of transfected HEK293T cells. After the cells were transfected in a 10-ch dish with PCSK9 for 24 h, half of the medium was transferred to an empty dish (no cells cultured) with protease inhibitor cocktail added. After further incubation at 37 °C for 24 h, the medium containing the cells (lane 1) and the cell-free medium (lane 2) were subjected to western blot. C. Intracellular presence of PCSK9 multimers. The cell-free medium containing PCSK9 was concentrated by Amicon Ultra centrifugal filter device to 1/10 of the original volume and added to cultured HEK293T cells along with 9 times the volume of 0.15 M KCl solution, and the cells were further cultured for 1 or 2 h. The cells were washed with 0.15 M KCl solution three times and lysed for western blot analysis. Lane 1: concentrated PCSK9-containing medium; lane 2: cell lysate at 1 h time point; lane 3: cell lysate at 2 h time point; lane 4: control cell lysate without addition of PCSK9-containing medium.

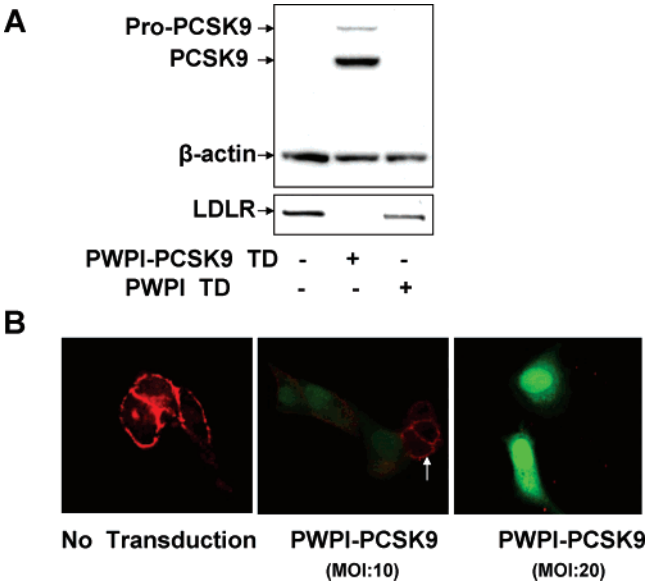

FIGURE 6: Autocrinal and paracrinal effects of secreted PCSK9. A. Transduction of HEK293T cells with PWPI-PCSK9 lentiviruses diminished cellular LDLR; non-transduced cells and cells transduced with PWPI control lentiviruses were included as controls. B. Confocal microscopy images of transduced HEK293T cells. The cells were transduced with indicated MOI of lentiviruses. Green fluorescence is from GFP, and LDLR was stained in red in a non-permeablized condition. Note: in the middle panel, the white arrow-pointed 3 cells were not transduced, LDLR staining is stronger than the transduced cells in the same image, but weaker than the non-transduced cells in the left image.

buffer to cultured HEK293T cells. The isotonic-K^+ buffer is reported to inhibit the late endosome−lysosome fusion, blocking the endocytic pathway in the early endosomes (*42*). Figure 5C shows that PCSK9 multimers accumulated in the cells 1 and 2 h after the addition of the PCSK9 multimer-containing isotonic-K^+ buffer.

We conceived that, at physiological conditions, PCSK9 may self-associate in the close proximity of the cell surface, and the multimers may be taken up quickly by the cells along with LDL/LDLR complex and degraded immediately in the late endosome or lysosome. PCSK9 self-association may be serving autocrine and paracrine functions to maximize LDLR degradation among the PCSK9-secreting cells and those cells in close proximity to them. To test this hypothesis, we transduced HEK293T cells with bicistronic lentiviral constructs that express PCSK9 as well as GFP (see Supporting Information Figure 1D). Lentiviral transduction effectively decreased LDLR level (Figure 6A). When the MOI was adjusted to ensure only incomplete transduction, LDLR immuno-staining demonstrates that the LDLR levels of non-transduced cells (Figure 6B, middle panel, white arrow) were higher than those of transduced cells in the same panel but lower than control (no transduction, left panel). The right panel shows the complete disappearance of LDLR immuno-staining with high-level expression of the PCSK9.

Lipoproteins Affect PCSK9 Self-Association, and PCSK9 Binds to Lipoproteins. In order to test the possibility that components in the circulation can regulate PCSK9 self-association, we investigated whether different lipoproteins affect the self-association of PCSK9 *in vitro*. We incubated PCSK9 with different lipoproteins (30 μg/mL) at 37 °C for 24 h. Interestingly, we found that while human and mouse HDL significantly inhibited the self-association of PCSK9,

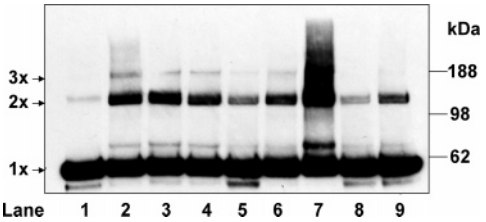

FIGURE 7: Lipoproteins affect self-association of PCSK9. Purified PCSK9 protein at 12 μg/mL was incubated at 37 °C for 24 h with different lipoproteins (30 μg protein/mL). At the end of incubation, the protein was subjected to western blot using anti 6 x His antibody for PCSK9. Lane 1: 4 °C control; lane 2: 37 °C control without addition of lipoprotein; lanes 3−9, with addition of human VLDL, human LDL, human HDL, wild-type mouse VLDL, apoE$^{-/-}$ mouse VLDL, wild-type mouse HDL, and apoE$^{-/-}$ mouse HDL, respectively.

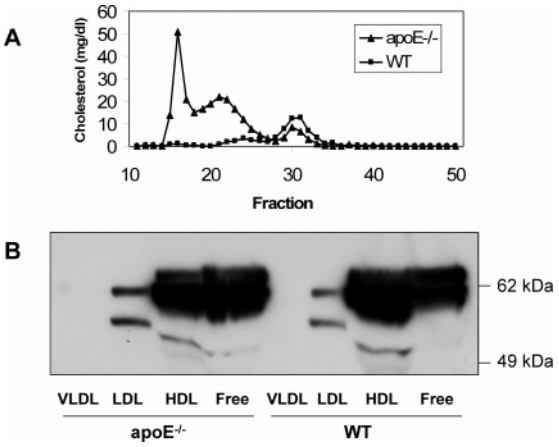

FIGURE 8: Purified PCSK9 associates with mouse lipoproteins. Purified PCSK9 (50 μg/mL) was incubated with mouse serum at 37 °C for 30 min. The serum (100 μL) was then subjected to FPLC. Cholesterol concentration was measured for each FPLC fraction (A). On the basis of cholesterol content, fractions were pooled to present VLDL (fractions 14−18), LDL (fractions 19−27), HDL (fractions 28−33), and cholesterol free (fractions 34−50) part. Each part was concentrated to 100 μL; 20 μL of each part was subjected to western blot analysis using anti 6 x His antibody (B).

apoE$^{-/-}$ mouse VLDL dramatically increased the PCSK9 self-association (Figure 7).

We next investigated whether PCSK9 binds to lipoproteins directly. We incubated purified PCSK9 (50 μg/mL) with mouse whole sera at 37 °C for 30 min, and then the sera were subjected to FPLC to separate the lipoprotein fractions (Figure 8A). In both wild-type and apoE$^{-/-}$ mouse sera, PCSK9 was associated with LDL and HDL but not with the VLDL fraction (Figure 8B). Notably apoE$^{-/-}$ LDL bound 2-fold more PCSK9 than did wild-type LDL, whereas wild-type HDL bound 1.5-fold more PCSK9 compared to apoE$^{-/-}$ HDL.

To further test whether PCSK9 binds to lipoproteins *in vivo*, we produced transgenic mice using the DNA construct shown in Supporting Information Figure 1C. The transgenic mice displayed similar plasma lipoprotein profile as that of LDLR$^{-/-}$ mice (Figure 9A), without other apparent phenotype. The sera from three transgenic mice were pooled and underwent FPLC separation of the lipoprotein fractions. Western blot analyses showed that, similarly to the *in vitro* incubation studies described above, PCSK9 was detected in the LDL, HDL, and lipoprotein-free fractions but not in the VLDL fraction (Figure 9B). However, in contrast to the *in*

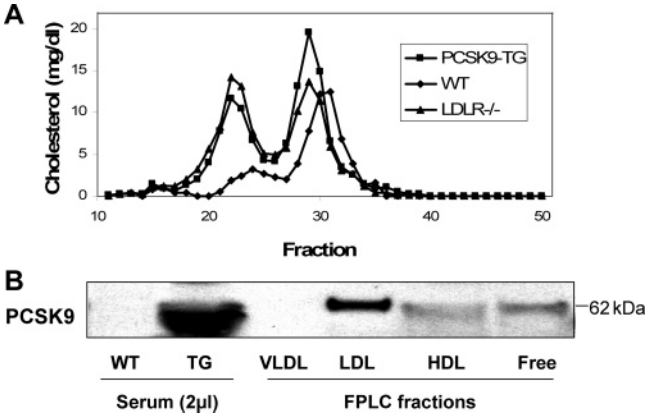

FIGURE 9: Association of PCSK9 with lipoproteins in PCSK9 transgenic mouse serum. A. A pooled serum sample from three human PCSK9 transgenic mice was subjected to FPLC separation of lipoprotein fractions. Cholesterol content of each fraction was shown. Fractions were pooled to present VLDL (fractions 14−17), LDL (fractions 18−25), HDL (fractions 26−34), and cholesterol free (fractions 35−50). B. The pooled FPLC parts were concentrated to 250 μL each, and 30 μL from each part was loaded to SDS-gel for western blot analysis for PCSK9 using rabbit anti-PCSK9 antibody, 2 μL wild-type mouse serum, and PCSK9 transgenic mouse serum were loaded as controls.

vitro incubation study with high concentration of PCSK9 in which majority of PCSK9 was associated with HDL or in lipoprotein-free state (Figure 8), the majority of the PCSK9 in transgenic sera was associated with LDL (Figure 9).

DISCUSSION

In this study we report a high-level mammalian cell expression system and a high-yield purification procedure for human PCSK9. The C-terminally tagged PCSK9 protein expressed by the transfected cells was functional in degrading cell surface LDLR. Mature PCSK9 protein purified from the conditioned medium was also functional, when added to cultured cells, in reducing cell LDLR levels. Upon incubation *in vitro*, purified PCSK9 self-associated to form dimers and trimers in a concentration, pH, and temperature-dependent manner. The catalytic domain of PCSK9 was found to be responsible for its self-association. The self-association of PCSK9 seemed to enhance its activity in degrading the LDLR through a selective faster uptake of multimers by the cells. ApoE$^{-/-}$ mouse VLDL increased, whereas human and wild-type mouse HDL decreased, the self-association of PCSK9. When incubated with mouse total serum, and in the serum of PCSK9 transgenic mice, PCSK9 partially associated with LDL and HDL but not VLDL.

PCSK9 has been suggested by genetic studies to be the third locus implicated in autosomal dominant hypercholesterolemia (ADH) (*8*). Its protein sequence variations play a significant role in determining plasma lipoprotein levels. Very recent structural and functional studies of PCSK9 have provided significant new insights into the mechanisms of PCSK9 function and dysfunction. The X-ray structure of human PCSK9 revealed a unique prodomain-catalytic site interaction which manipulates PCSK9 function (*32*, *33*). The binding site of PCSK9 in the LDLR was located to the EGF-A repeat (*34*). Two elegant studies demonstrated that catalytic activity is not required for secreted PCSK9 to reduce LDLR levels (*37*, *43*). However, the molecular basis of PCSK9's function and the regulation mechanism still remain largely unknown.

By degrading hepatocyte LDLR, PCSK9 causes decreased hepatic uptake of LDL and an accumulation of LDL in the circulation. Paradoxically PCSK9 and LDLR are regulated in hepatocytes through SREBPs in the same direction (*26*, *27*), as inhibition of cholesterol synthesis in hepatocytes increases SREBP-dependent transcription of both LDLR and PCSK9 (*28*, *29*). It is therefore likely that inhibition of PCSK9 activity would complement the LDL lowering effect of statin therapy. Although it was suggested that PCSK9 may degrade the LDLR before the receptor is transported to the cell surface (*23*), this pathway may not be a major mechanism. Legace et al. demonstrated that PCSK9 is endocytosed via the LDLR with the assistance of the adaptor protein ARH, and that it gains LDLR degradation activity in the LDLR recycling pathway (*31*). This has been confirmed recently (*35*−*37*, *44*). Consistent with that, our data indicated that PCSK9 did not degrade LDLR from isolated cell membranes (Figure 1C) but effectively reduced LDLR levels from the cells when added to the cell culture medium (Figure 1D).

The mature form of PCSK9 can be secreted into the circulation, and the circulating PCSK9 may have the potential to distribute to peripheral cells and thus regulate LDL entry in extrahepatic tissues. Therefore, plasma PCSK9 levels may be an indicator of metabolic status with regard to peripheral uptake of cholesterol and may also serve as a regulator of cholesterol flux between liver and peripheral tissues. A previous report by Benjannet et al. indicated that PCSK9 can be processed and inactivated by furin and/or PC5/6A extracellularly, and that sequence variations in PCSK9 affect furin and/or PC5/6A-mediated processes and inactivation (*30*). We show here another potential mechanism for PCSK9 activity to be modulated in the circulation. Our data show that PCSK9 could self-associate to form dimers and higher multimers which may have increased LDLR degrading activity (Figure 2). The self-association of PCSK9 was concentration, temperature, and pH dependent (Figures 2 and 3). A gain-of-function mutant, D374Y, which has much higher affinity to LDLR and higher LDLR-degrading activity, displayed more self-association (Figure 3). PCSK9 was reported to bind more tightly to the LDLR at acidic pH (*32*, *45*); interestingly, at acidic pH, PCSK9 displayed significantly more self-association than at neutral pH (Figure 3). These results indicated that the self-association of PCSK9 is correlated to its LDLR binding and degrading activity. The self-association of PCSK9 did not result from artificially high protein concentrations, since multimerization was observed at 0.5−1 μg/mL (Figure 3D and Figure 4B), which is close to the PCSK9 concentration in human plasma (*31*). These results indicated that self-association is an intrinsic property of PCSK9.

While our experimental results point to the possibility that self-association may occur *in vivo* and is related to the LDLR-degrading activity, it was harder than expected to directly detect SDS-stable dimer and higher multimer formation in transfected cell culture medium and inside the cells. However, we did observe a quick uptake of multimers by the cells (Figure 5A) and managed to detect PCSK9 multimers formation in cell-free medium (Figure 5B) and in cells under conditions of blocked endosome−lysosome fusion (Figure 5C). These results suggest that PCSK9 may form multimers in the proximity of the cell surface which are selectively and efficiently taken up by the cells. PCSK9

may further self-associate while moving along the endocytic pathway and cross link multiple PCSK9-LDLR-LDL complexes, facilitating the rapid degradation of the complexes in lysosome. Grozdanov et al. previously reported that PCSK9 is not detected in the late endosome and lysosome (*25*), also indicating the fast degradation of PCSK9 in these components.

Our lentiviral transduction and confocal microscopy studies show that secreted PCSK9 can mediate LDLR degradation in cells not producing PCSK9 in co-culture systems. However, the LDLR degrading activity is stronger in cells expressing PCSK9 than in cells acted on by extracellular PCSK9 (Figure 6). On one hand, this data confirmed that PCSK9 in the extracellular environment can mediate LDLR degradation in cells through a paracrine function; on the other hand, this data clearly demonstrated that the autocrine function of PCSK9 is more effective than the paracrine function. A reasonable explanation is that PCSK9 may be enriched on the cell surface of the PCSK9-producing cells, where it self-associates and therefore more effectively mediates LDLR degradation. However, we do not exclude the possibility that PCSK9 may functionally mediate LDLR degradation intracellularly, before its secretion.

We further showed that different lipoproteins affect the self-association of PCSK9 differently (Figure 7). While apoE$^{-/-}$ VLDL enhance PCSK9 self-association, human and wild-type mouse HDLs inhibit it. As a proof of the physiologic relevance of the lipoprotein connection, we show that PCSK9 directly associates with LDL and HDL (Figures 8 and 9). These findings not only provide a novel insight into how intracellular and extracellular cholesterol homeostasis mechanisms control PCSK9 expression and function but also point to a direction for therapeutic manipulation of PCSK9 activity through a circulation-based approach. The inhibitory effect of HDL on PCSK9 self-association may add another potential mechanism to the list of HDL's anti-atherogenic functions. Although purely speculative at this stage, the possibility that HDL may regulate plasma LDL levels through functional sequestration of circulating PCSK9 is hard to ignore, given its therapeutic promise. In addition, we found that the absence of apoE demolished the anti-self-association effect of mouse HDL and conferred mouse VLDL the ability to enhance PCSK9 self-association. This may represent yet another mechanism by which apoE modulates plasma lipoprotein levels. Further studies are needed to unravel the mechanism through which lipoproteins affect PCSK9 self-association.

In summary, we report here an intrinsic self-association property of PCSK9, which is mediated by the catalytic domain, correlated to its LDLR binding and degrading activity, and may be modulated by plasma lipoproteins. The data from this study provide a rationale for inhibition of the activity of the circulating PCSK9 as a strategy for plasma cholesterol control. Inhibition of plasma PCSK9 may be achieved through reduction of its self-association, preventing it from binding to and degrading LDLR.

ACKNOWLEDGMENT

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SUPPORTING INFORMATION AVAILABLE

Detailed experimental procedures for the generation of pcDNA-PCSK9-GST construct, generation of lentiviral PCSK9 construct, lentivirus preparation and transduction, and protein purification. Schematic drawing of DNA constructs used in this study (supplemental Figure 1), Purification of human PCSK9 protein and its mutants (supplemental Figure 2). This material is available free of charge via the Internet at http://pubs.acs.org.

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