Internal Medicine Grand Rounds

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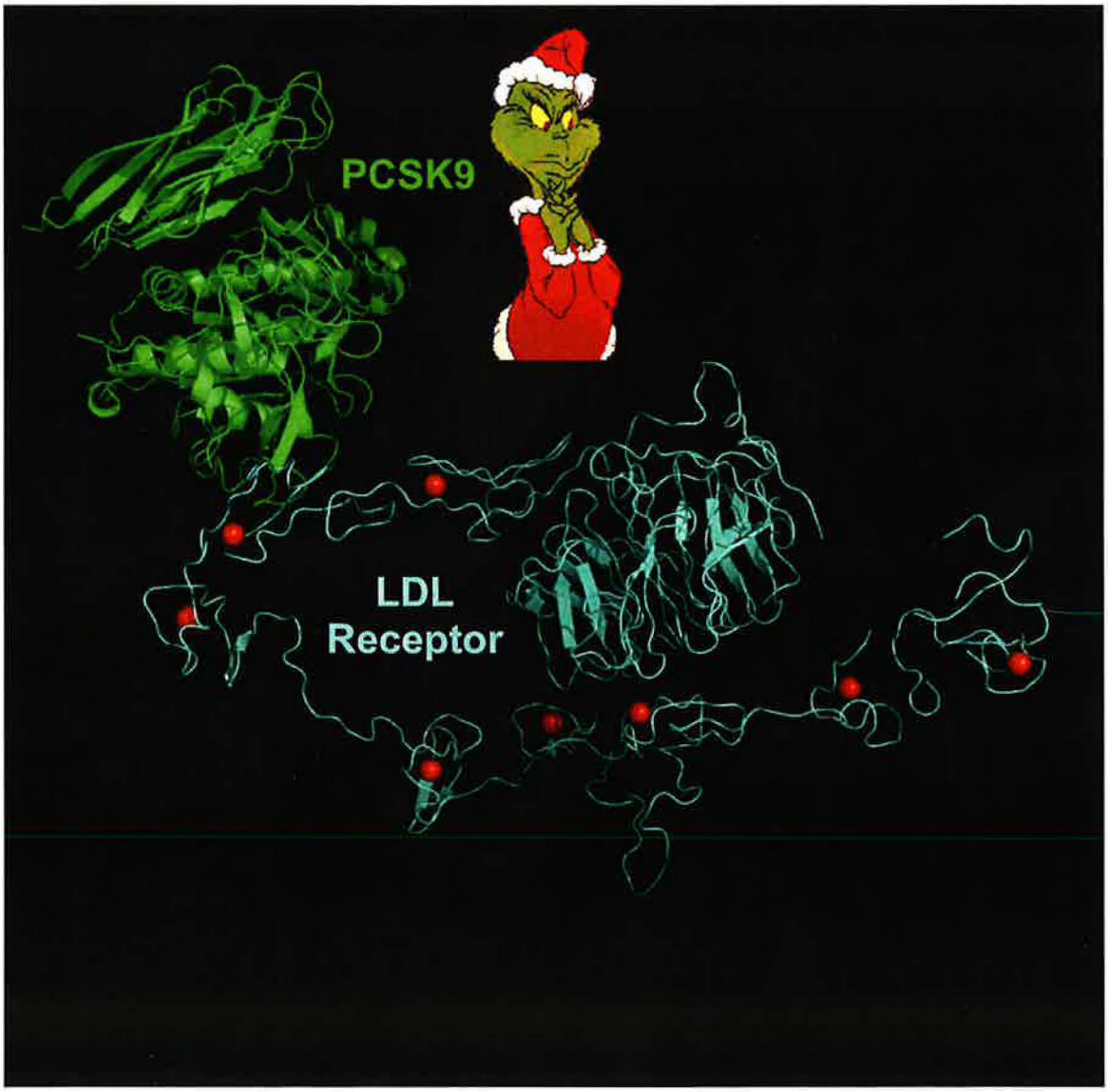
PCSK9: A New Regulator of Plasma LDL Cholesterol

[Jay D. Horton]



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Molecular Biology of PCSK9: Role in LDL Metabolism

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Abstract

Proprotein convertase subtilisin-like kexin type 9 (PCSK9) is a newly discovered serine protease that destroys low density lipoprotein (LDL) receptors in liver and thereby controls the level of LDL in plasma. Mutations that increase PCSK9 activity cause hypercholesterolemia and coronary heart disease (CHD), whereas mutations that inactivate PCSK9 have the opposite effect, lowering LDL and reducing CHD. Although the mechanism of action of PCSK9 is not yet clear, the protease is an ideal therapeutic target to lower plasma levels of LDL and prevent CHD.

Introduction

A new regulator of cholesterol trafficking

Just when we thought all of the major molecular players in low density lipoprotein (LDL) metabolism had been identified, another actor burst onto the scene. In 2003, four groups reported the characterization of a new member of the proprotein convertase gene family. First, Seidah and his colleagues (4) characterized a transcript encoding a novel proprotein convertase previously shown to be up-regulated during apoptosis in neuronal cells. The link between PCSK9 and cholesterol metabolism rapidly followed with the discovery that selected mutations in the gene cause autosomal dominant hypercholesterolemia (5), and the observation that PCSK9 was regulated by cholesterol (6, 7).

The discovery of PCSK9 has provided new insights into the metabolism of low density lipoproteins (LDL), and into the determinants of plasma LDL-cholesterol (LDL-C) levels. Here we review the current understanding of the cell biology, physiology and genetics of PCSK9 and its implication for the treatment of hypercholesterolemia and coronary heart disease.

Structural features of PCSK9

PCSK9 is a member of the family of subtilisin-like proconvertases

Secretory proprotein convertase (PC) enzymes are structurally related to the bacterial subtilisin-like serine protease kexin found in yeast. There are nine subtilisin-like serine proteinases in mammals designated PC1/3, PC2, furin, PC4, PC5/6, PACE4, PC7, S1P (site-1 protease) and PCSK9 (proprotein convertase subtilisin/kexin type 9) (8). Like other family members, the signal sequence (aa 1-30) in PCSK9 is followed by the prodomain (aa 31-152) and catalytic domain (4) (**Figure 1**). PCSK9 lacks a classical P domain (9), which is required for folding and regulation of protease activity in the other proprotein convertases (10); rather, the catalytic domain is followed by a 279-amino acid cysteine- and histidine-rich C-terminal region. PCSK9 is synthesized as a ~72 kDa precursor that undergoes autocatalytic cleavage between the prodomain and catalytic domain (4, 9). The prodomain (~14 kDa) remains bound to the mature protein (63kDa) as it traverses the secretory pathway. The site of intramolecular cleavage in PCSK9 (VFAQ↓SIP) differs from most other proconvertases, where cleavage occurs after a basic

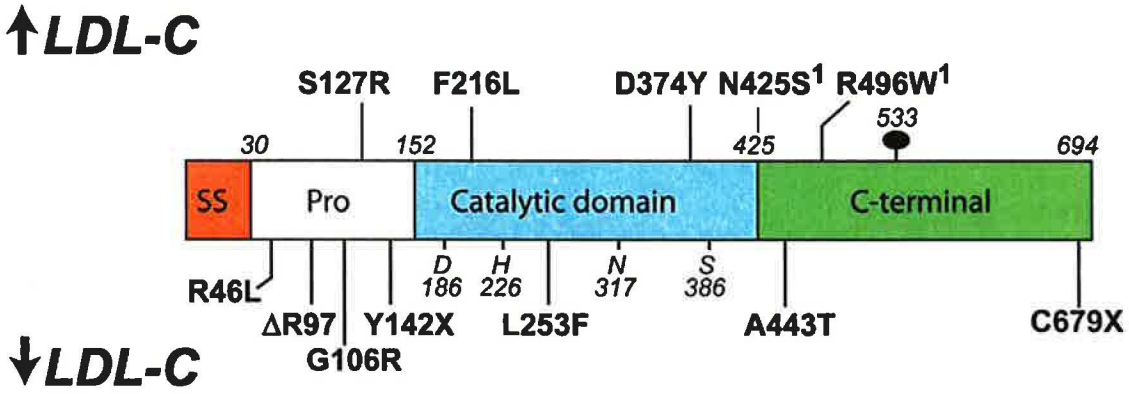

Figure 1. A schematic of PCSK9 with the location of naturally-occurring mutations associated with elevated (top) or reduced (bottom) plasma levels of LDL-C. The major domains of PCSK9 are delineated using different colors. The mutations included are limited to those associated with significant differences in plasma levels of LDL-C in at least two independent populations or those that co-segregate consistently with hypercholesterolemia in families. Mutations associated with elevated plasma cholesterol levels found only in families with familial hypercholesterolemia are indicated by [1] (2). The mutations for which there is functional information are referenced in **Table 1**. The location of the aspartate (D), histidine (H) and serine (S) that comprise the catalytic triad and the site of binding of the single N-linked sugar (N533) are shown (4). Abbreviations used: SS, signal sequence; Pro, prodomain.

residue (11). Obtaining a robust *in vitro* assay for PCSK9 activity has proved difficult and little is known about the requirements for catalytic activity. In contrast to other proprotein convertases, autocatalytic cleavage of PCSK9 does not require calcium (12). Mutagenesis studies have revealed that the sequence required for autocatalytic cleavage is degenerate, which has further complicated efforts to identify the natural substrate(s) of PCSK9 (9). The mature PCSK9 and the associated prodomain both undergo tyrosine sulfation in the late Golgi complex prior to secretion (12, 13). Sulfation of tyrosine resides in other proteins enhances protein-protein interactions, but the role of this post-translational modification in PCSK9 has not been defined (14).

The crystal structure of PCSK9 has been solved by three groups (1, 15, 16) and revealed a tightly bound prodomain that is predicted to render the active site inaccessible to exogenous substrates (**Figure 2**). The structure of the PCSK9 prodomain and catalytic domain is similar to that of other subtilisin-like serine proteases. The C-terminal domain of PCSK9 contains three six-stranded β-sheet subdomains arranged with quasi-three fold symmetry. This domain shares structural homology to the adipokine resistin, and has been speculated to mediate protein-protein interactions (1, 15, 16).

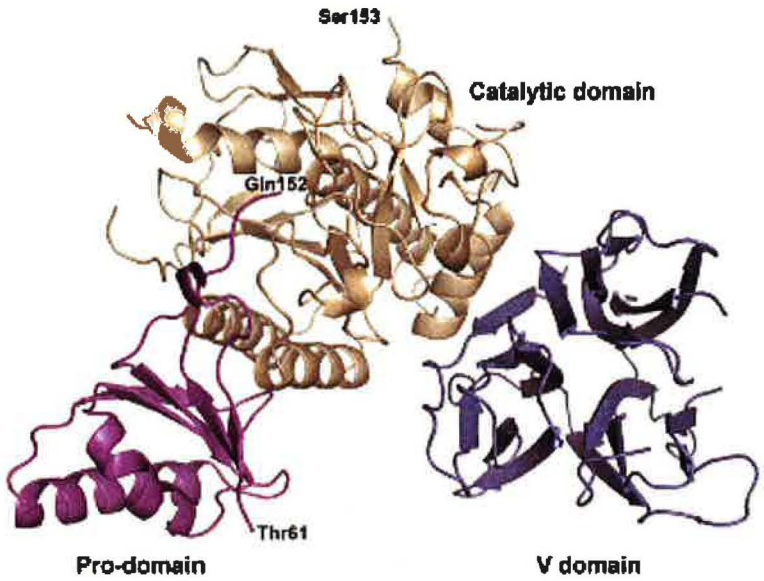

Figure 2. Overall structure of the PCSK9 protein. Ribbons diagram of the structure with the prodomain in magenta, the catalytic domain in wheat, and the V domain in blue. Thr61 marks the first observed residue, and Gln152 marks the C terminus of the prodomain. Ser153 marks the N terminus of the catalytic domain (1).

Gain-of-function mutations in PCSK9

Selected missense mutations in PCSK9 cause hypercholesterolemia

Plasma levels of LDL-cholesterol (LDL-C), the major cholesterol-carrying lipoprotein in humans, are determined by the relative rates of LDL production and clearance. Prior to 2003, only two autosomal dominant forms of hypercholesterolemia were known: familial hypercholesterolemia (FH), caused by mutations in the genes encoding the LDL receptor (*LDLR*), and familial defective Apo-B100 (FDB), caused by mutations in ApoB-100 (*APOB*) that disrupt binding of LDL to LDLR (17). Both of these disorders decrease LDLR-mediated endocytosis in the liver, the major route of clearance of circulating LDL. Chronic elevations in plasma LDL-C levels in FH and FDB result in the accumulation of cholesterol in tissues (xanthomas) and in arteries, especially the coronary arteries (coronary atherosclerosis).

Initially, three missense mutations in *PCSK9* were identified in families with a clinical phenotype resembling FH and FDB: S127R, F216L (5), and D374Y (18, 19). Subsequently, additional missense mutations were identified in hypercholesterolemic subjects (**Figure 1**). Mutations in *PCSK9* account for a much smaller percentage of dominant hypercholesterolemia than do mutations in *LDLR* and *APOB* (20-23). Probands heterozygous for mutations in both *LDLR* and *PCSK9* have plasma levels of LDL that are ~50% higher than relatives with either mutation alone (2). The only clinical findings that have been reported in subjects with hypercholesterolemia due to mutations in *PCSK9* are those related to lipoprotein metabolism suggesting that PCSK9 functions primarily in the cholesterol metabolic pathway.

PCSK9 expression and LDLR levels

Most enzyme defects cause recessive disorders. The observation that PCSK9 mutations cause dominant hypercholesterolemia suggested that the mutations confer a gain-of-function (5), either by increasing the normal activity of PCSK9 or by conferring a new activity to the protein. The first experimental evidence for a gain-of-function mechanism came from studies in which wild-type and mutant PCSK9 (S127R and F216L) were expressed at high levels in the livers of mice; hepatic LDLR protein levels fell

dramatically in the mice receiving either the wild-type or mutant PCSK9 (12, 24, 25). No associated reductions in LDLR mRNA levels were observed. Thus, overexpression of PCSK9, whether mutant or wild-type, reduces LDLRs through a post-transcriptional mechanism.

In contrast to the *in vivo* experiments, expression of PCSK9 in cultured cells has variable effects on LDLRs. In some cell types, such as human hepatoma cells (HepG2 and HuH7) or human embryonic kidney cells (HEK-293 cells), expression of PCSK9 dramatically reduces LDLR levels (12, 13, 25, 26). In other cells types, including fibroblasts, Chinese hamster ovarian (CHO-K1), monkey kidney cells (COS7), and rat liver (McArdle RH7777) cells (22, 25, 27, 28), PCSK9 appears not to affect LDLR expression. Cells unresponsive to PCSK9 might lack a factor required for PCSK9 function. Alternatively, either the kinetics or the pathway of LDLR internalization could differ in unresponsive and responsive cells.

PCSK9 directly binds the LDLR

Studies in cultured cells initially showed that the PCSK9 can be co-immunoprecipitated with the LDLR after PCSK9 is added exogenously to the medium of cells, implying a physical association between the two proteins (28). The presence of ARH, an endocytic adaptor protein required for LDLR internalization, is necessary for PCSK9-mediated degradation of LDLR. In the absence of the LDLR, PCSK9 fails to associate with the cell and cannot be internalized (28).

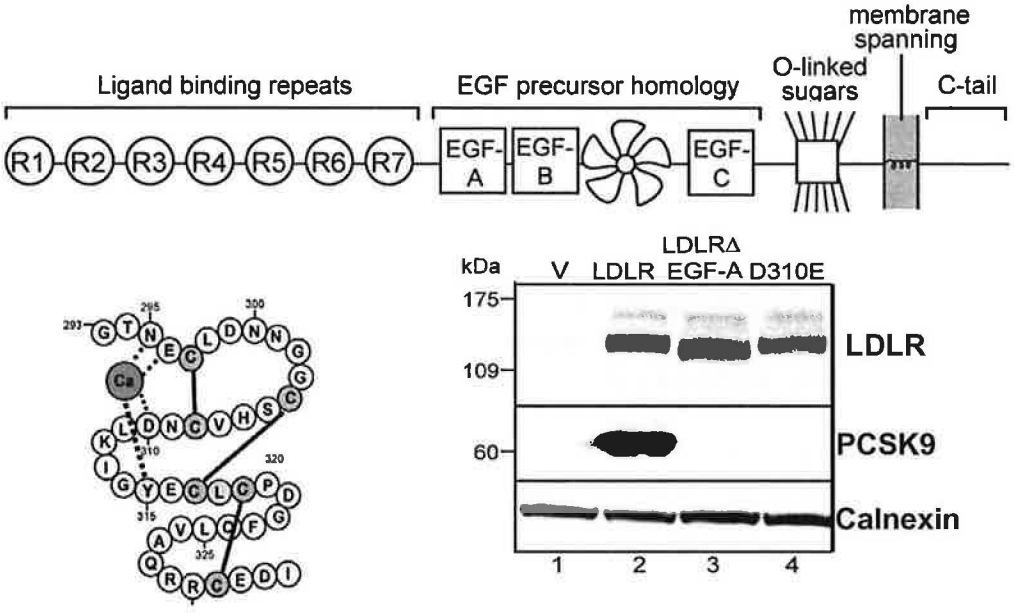

Figure 3. Binding of PCSK9 to the extracellular domain of the wild-type and mutant LDLR. Simian COS-M cells were transiently transfected with expression plasmids containing cDNAs for wild-type or mutant LDLRs with a deletion the EGF-A precursor homology domain of the LDLR or an amino acid substitution in the calcium coordinating residue D310 (D310E). After 48 h, the cells were incubated with purified PCSK9. After a 2 h incubation, cells were washed and lysed. Whole cell protein extracts from the cells were subjected to SDS-PAGE (8%) for immunoblot analysis for the indicated proteins (3).

The residues of the LDLR that are required for binding to PCSK9 have been recently identified and characterized (3). As schematically shown in **Figure 3**, the LDLR

is a multidomain protein whose extracellular domain (ECD) consists of an N-terminal ligand binding domain (7 cysteine-rich repeats (R1-R7) that mediate binding to LDL and β-VLDL), followed by the epidermal growth factor (EGF)-precursor homology domain (a pair of EGF-like repeats (EGF-A and EGF-B) separated from a third EGF-like repeat (EGF-C) by a β-propeller domain), and an 'O-linked sugar' domain (29, 30). Following endocytosis of the receptor:ligand complex, bound lipoproteins are released in the acidic environment of the endosome and the LDLR recycles to the cell surface. PCSK9 binds in a calcium-dependent manner to the first EGF-like repeat (EGF-A) of the EGF-precursor homology domain of the LDLR. The integrity of the EGF-precursor homology domain is essential for normal LDLR turnover.

Zhang *et al.* (3) showed that recombinant human PCSK9 interacted in a sequence-specific manner with the first epidermal growth factor-like repeat (EGF-A) in the EGF homology domain of the human LDLR (**Figure 3**). Similar binding specificity was observed between PCSK9 and purified EGF-A. Binding to EGF-A was calcium-dependent and increased dramatically with reduction in pH from 7 to 5.2.

To determine the residues of PCSK9 that interact with the LDLR, the crystal structure of PCSK9 in complex with the EGF-A domain of the LDLR was solved by Kwon *et al.* (31) (**Figure 4**). EGF-A binds a surface of PCSK9 that is formed primarily by residues 367-381. The interface between PCSK9 and EGF-A is largely hydrophobic with a number of specific polar interactions surrounding the interface. The binding region is over 20Å from the catalytic site of PCSK9 and the prodomain and the C-terminal domain of PCSK9 do not contact the EGF-A domain.

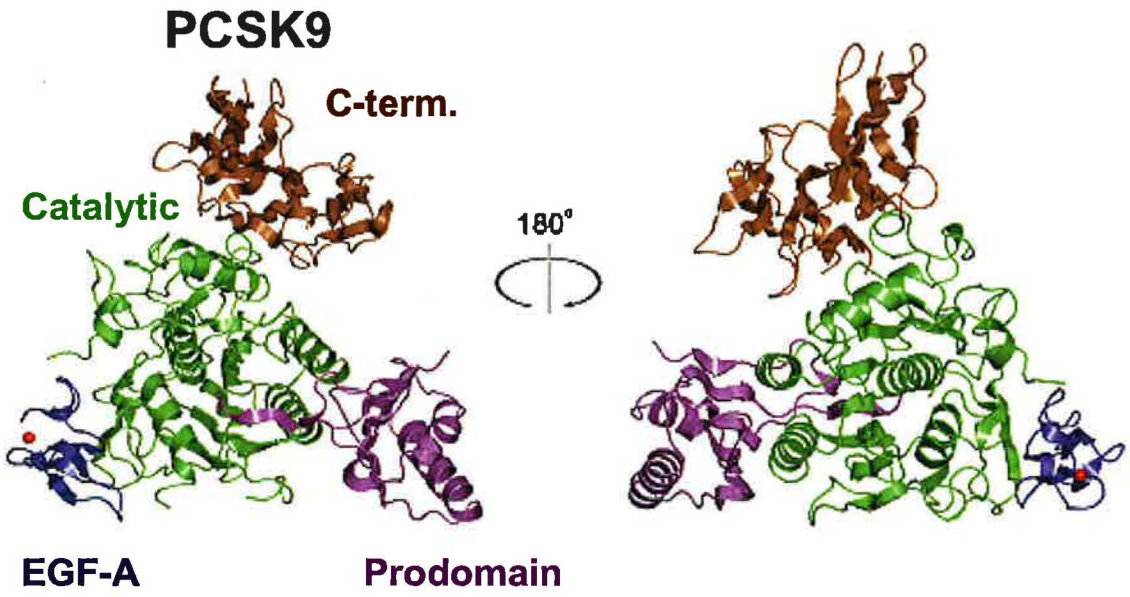

Figure 4. The PCSK9:EGF-A complex. PCSK9, with the prodomain (magenta), the subtilisin-like catalytic domain (green), and the C-terminal domain (brown), and the EGF-A domain of LDLR (blue) are represented as a ribbon diagram. The bound calcium ion within the EGF-A domain is shown as a red sphere.

Site of action of PCSK9 in cells

The intracellular itineraries of PCSK9 and the LDLR are similar, but their paths diverge at the cell surface (**Figure 5**). The LDLR remains associated with the cell membrane whereas PCSK9 is rapidly and efficiently secreted into the medium (4). PCSK9 is also secreted *in vivo*, presumably by the liver and is present in human plasma (13, 28, 32). The major cellular site at which PCSK9 acts has not been established. Stable overexpression of PCSK9 in HepG2 cells does not affect the synthesis or trafficking of LDLR out of the ER, and proteosome inhibitors do not interfere with PCSK9-mediated reduction in LDLR (33). In the same series of experiments, PCSK9 overexpression was associated with increased degradation predominantly of the mature, glycosylated from of the LDLR (33). Addition of brefeldin A prevented the PCSK9-induced degradation of the LDLR, suggesting PCSK9 might promote degradation of the LDLR as it migrates from the ER to the cell membrane (33).

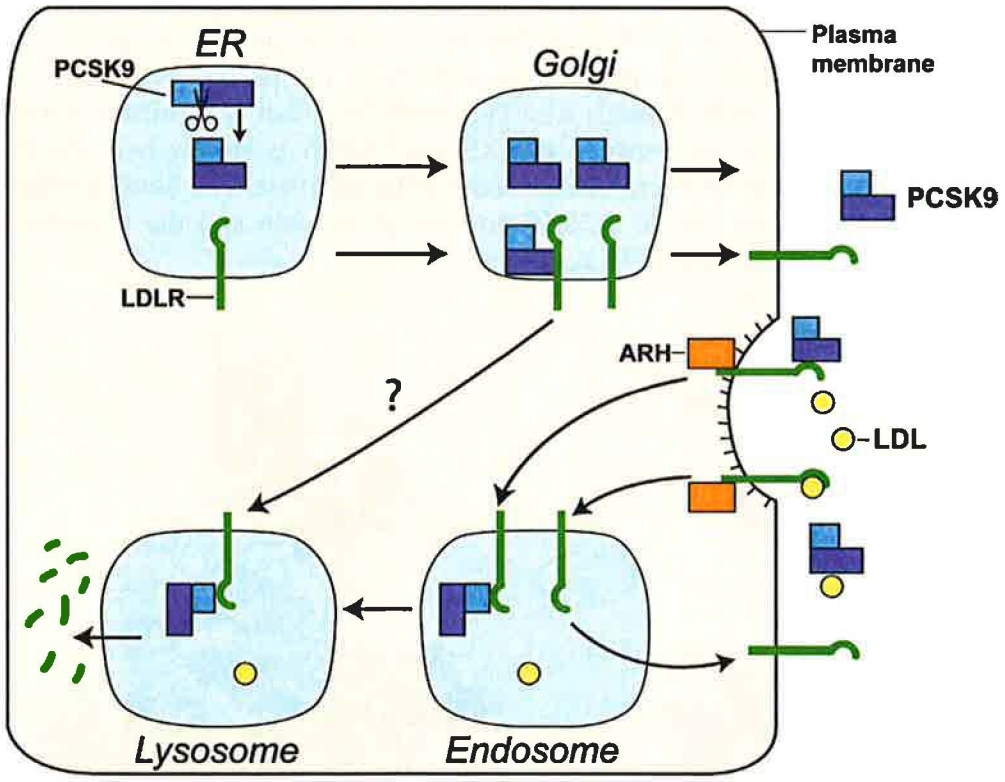

Figure 5. Cellular trafficking and potential sites of action of PCSK9. PCSK9 undergoes autocatalytic cleavage in the ER. The cleaved prodomain (light blue) associates with the catalytic fragment (dark blue) and acts as a chaperone permitting the mature protein to move from the ER into the secretory pathway. Current evidence suggests that PCSK9 may work at two cellular sites. The first potential location is in a post-ER compartment, depicted here as the Golgi apparatus, where PCSK9 may target the LDLRs (green) for degradation in an acidic compartment such as the lysosome. In the second possible pathway, the PCSK9 that is secreted binds to LDLRs on the cell surface. The LDLR/PCSK9 complex is internalized via the adapter protein ARH (orange). PCSK9 might prevent the recycling of the LDLR from the endosome back to the cell surface and/or direct the LDLR to the lysosome where it is degraded.

Alternatively, PCSK9 could remain inactive while it migrates through the secretory pathway and act on the LDLR only after it is secreted. The prodomain of PCSK9 remains tightly attached to the mature protein during its secretion, presumably inhibiting catalytic activity (4, 12, 28). In other proprotein convertases, the prosegment undergoes a secondary proteolytic processing event either in the Golgi or after secretion that relieves the inhibition and unmasks enzymatic activity (11). The first experimental evidence that PCSK9 could function extracellularly came from the finding that addition of conditioned medium containing PCSK9 (34) or of purified PCSK9 (28) to the medium of HepG2 cells reduces the number of cell surface LDLRs. The presence of ARH, an endocytic adaptor protein required for LDLR internalization, is necessary for PCSK9-mediated degradation of LDLR. In the absence of ARH, the LDLR and PCSK9 fail to be internalized and no change in LDLR number is observed (28).

The behavior of LDLRs in cultured cells might not accurately reflect conditions *in vivo*. To address this possibility, parabiosis experiments were carried out in mice (28). The circulations of PCSK9 transgenic mice were connected to those of wild-type mice. Comparison of liver biopsies before and after parabiosis revealed a dramatic reduction in LDLR protein in livers of the recipient wild-type mice (28). Thus, exogenous PCSK9 can reduce LDLR number *in vivo* as well as in cultured cells.

A potential artifact of the cell culture studies, adenoviral studies in the liver, and the parabiosis studies relate to the superphysiological amounts of PCSK9 used to promote LDLR degradation. Overexpression could promote an interaction between PCSK9 and the LDLR in a cellular compartment that does not usually occur. To address this possibility, we developed an enzyme linked immunoabsorbent assay (ELISA) to measure circulating levels PCSK9 in human plasma (28). The levels of PCSK9 were measured and found to range from ~50 to ~600 ng/ml in 40 subjects. The concentration of purified PCSK9 needed to promote LDLR degradation (~500 ng/ml) falls in cultured cells fall within this range (28). In addition, recently completed studies using recombinant human PCSK9 continuously infused into mice revealed that PCSK9 can reduce hepatic LDLRs at concentrations found in human plasma.

The results of these studies suggest several possible mechanisms by which PCSK9 might promote LDLR degradation. PCSK9 could bind to the LDLR in a catalytically inactive state on the cell surface and then become active in the acidic environment of the endosome, resulting in LDLR degradation. Alternatively, by binding to the LDLR, PCSK9 might interfere with the normal recycling of the LDLR after internalization, redirecting the LDLR to lysosomes rather than back to the cell surface (**Figure 3**). If the latter hypothesis is correct, the action of PCSK9 on LDLR might not involve catalytic activity.

Is the catalytic activity of PCSK9 required for its function?

Protein substrates are known for eight of the nine subtilisin-like serine proteinases. Cleavage of the substrates generally results in the production of mature bioactive proteins as well as processing intermediates, or occasionally, the inactivation of the cleaved protein (35). The only subtilisin-like serine proteinase without an identified protein substrate is PCSK9.

Determining whether PCSK9 degrades the LDLR by catalytic cleavage has proved to be more challenging than expected. PCSK9 in which the catalytic histidine has been substituted to an alanine does not undergo autocatalytic cleavage and fails to exit the ER (4). LDLR levels do not change when a catalytically dead enzyme is expressed in liver (25) or in cultured liver cells (22, 33). Thus, autocatalytic activity appears to be required for PCSK9 to leave the ER, but is it required for PCSK9-stimulated LDLR degradation? To address this question, the prodomain and the catalytic domain were expressed *in trans* in cells and the resultant recombinant protein complex was purified from the medium (36). The success of this approach permitted the introduction of a mutation in PCSK9 that abolishes catalytic activity but did not interfere with the secretion of the protein. As shown in **Figure 6**, the catalytically inactive protein could mediate the destruction of LDLRs when added to the medium of cultured HepG2 cells in a manner that was nearly identical to that of the wild-type protein. The infusion of this catalytically inactive protein into mice also led to the reduction in hepatic LDLRs. These results support a model in which exogenous PCSK9 binds to the LDLR, which then either targets the LDLR to the lysosome for degradation or prevents the recycling of the receptor in a manner that is independent of inherent catalytic activity of the protein. These data also indicate that unlike other PCs, PCSK9 is unique as a subtilisin-like serine protease in that the protein carries out a biological function that is independent of its proteolytic activity.

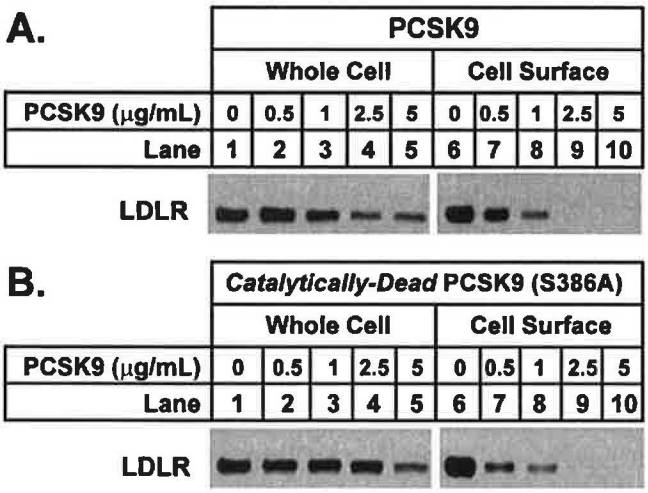

Figure 6. Catalytically inactive PCSK9 degrades the LDLR when added to HepG2 cells. Cells were cultured with the indicated concentrations of: *A,* PCSK9; *B, Catalytically-Dead* PCSK9(S386A) for 4 h. Cell surface proteins and whole cell extracts were resolved by SDS-PAGE and immunoblot analysis was performed for LDLR

Loss-of-function mutations in PCSK9

Nonsense and missense mutations in PCSK9 causing hypocholesterolemia

To test the hypothesis that loss-of-function mutations in PCSK9 would cause hypocholesterolemia by increasing LDL clearance, Cohen *et al.* (37) sequenced the coding region of *PCSK9* in individuals with the lowest plasma levels of LDL-C ($<5^{th}$ percentile) in a population-based sample. Surprisingly, one out of every 50 African-Americans in the population had a nonsense mutation in *PCSK9* (either Y142X or C679X, **Figure 1**) that lowered LDL-C levels by ~40% (**Figure 7**) (37). Subsequently, additional *PCSK9* mutations associated with a reduction in plasma levels of LDL-C have been found, including in-frame deletions and missense mutations (34, 38, 39) (**Figure 1**).

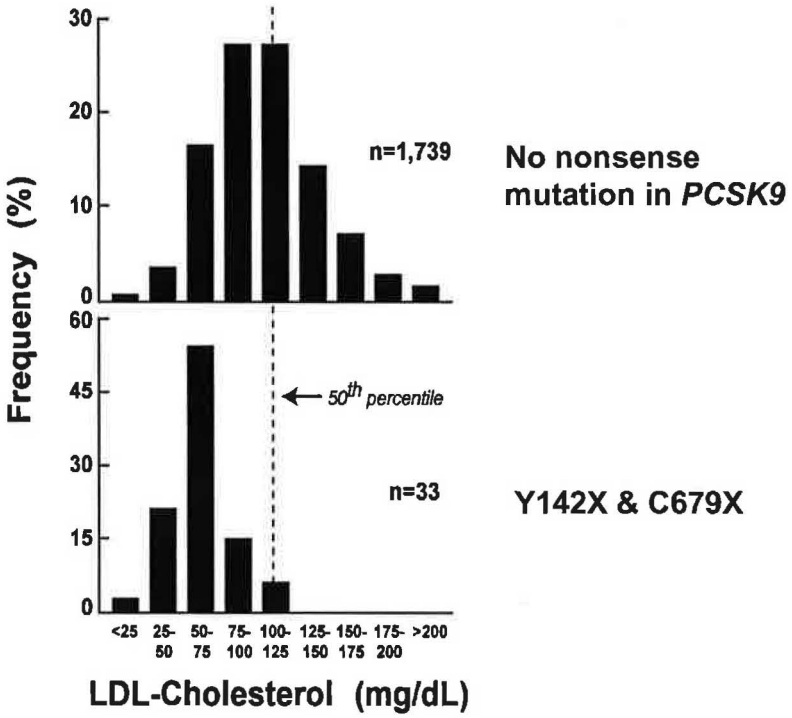

Figure 7. Distribution of plasma LDL-C levels in African American subjects in the Dallas Heart Study without (top) and with (bottom) a nonsense mutation in *PCSK9*. 5'-nucleotidase assays were performed to identify individuals who had a *PCSK9* allele with either of the two nonsense mutations identified in this study (426C →G and 2037C →A). Fasting plasma levels of LDL-C were measured as described and adjusted for the effects of age and gender by linear regression.

Three loss-of-function mutations in *PCSK9* – Y142X and C679X in African-Americans, and R46L in Caucasians – were sufficiently common to address a more general question regarding the relationship between plasma levels of LDL-C and coronary heart disease (CHD). Do life-long reductions in plasma levels of LDL-C confer greater protection from CHD than cholesterol-lowering therapies instituted later in life? In a large biracial 15-year prospective study, nonsense mutations in PCSK9 that reduced LDL-C levels by 28% decreased the frequency of CHD (defined as myocardial infarction, coronary death, or coronary revascularization) by 88% (40). In the same study, Caucasians with a R46L allele manifest a 50% reduction in CHD despite having a mean reduction in LDL-C levels of only 15%. The reductions in CHD associated with these mutations were significantly greater than those observed in more short-term (typically 5 year) clinical trials employing statins (41). These data suggest that earlier intervention might significantly magnify the clinical efficacy of cholesterol-lowering therapy by attenuating the development and progression of atherosclerosis.

The mechanisms by which loss-of-function mutations in PCSK9 reduce plasma cholesterol levels were investigated in mice in which *Pcsk9* was inactivated. These animals have increased hepatic LDLR protein levels, accelerated LDL clearance, and reduced plasma cholesterol levels (42). Thus, PCSK9 acts to tonically suppress LDLR levels, thereby limiting LDLR-mediated uptake of lipoproteins.

PCSK9 and lipoprotein synthesis

An alternative mechanism by which mutations in PCSK9 might alter plasma LDL levels is by influencing the rate of secretion of ApoB-100-containing lipoproteins from the liver. Ouguerram *et al.* (43) reported that two heterozygotes for a gain-of-function mutation in PCSK9 (S127R) had ApoB production rates that were 3-fold higher than

controls. LDL production rates in the PCSK9 heterozygotes were comparable to those observed in five FH heterozygotes studied concurrently. Whereas PCSK9 overexpression in *Ldlr*$^{-/-}$ mice does not increase plasma cholesterol levels (24, 25, 28), the combined data suggest that if PCSK9 does alter ApoB secretion it is likely a secondary event related to the reduction in LDLRs.

VLDL is the major vehicle for the secretion of triglycerides from the liver. If PCSK9 causes hypercholesterolemia by increasing VLDL production, it would be expected that loss-of-function mutations in PCSK9 would reduce VLDL secretion. Mutations that interfere with VLDL secretion and cause hypocholesterolemia, such as those in *MTP* and *APOB,* also result in the accumulation of triglycerides in the liver (44, 45). No increase in hepatic triglyceride content was observed in 20 African-Americans heterozygous for nonsense mutations in *PCSK9* (39). These results do not support the concept that changes in LDL levels associated with mutations in *PCSK9* are primarily due to effects on VLDL synthesis. It remains possible that *PCSK9* mutations do reduce VLDL secretion but that the magnitude of the reduction is insufficient to affect hepatic triglyceride content.

Structure-function relationship of PCSK9

Functional analysis of the naturally-occurring mutations in PCSK9 has provided insights into the mechanism of action of PCSK9. In the same manner in which mutations in the *LDLR* have been classified, we propose that the mutations in *PCSK9* can be separated into 5 groups according to their effects physiological alterations on synthesis and secretion of the PCSK9 protein (**Table 1**). Surprisingly, it is difficult to distinguish the phenotypic effects of some gain-of-function mutations from some loss-of-function mutations in cultured cells. For example, both S127R (gain-of-function) and L253F (loss-of-function) in *PCSK9* impair autocatalytic cleavage and secretion. Only one allele (Y142X) produces no detectable protein (***Class 1*** – null alleles), presumably due to nonsense-mediated mRNA decay (32). Three mutations in the prodomain (Δ97, G106R, and S127R) and one in the catalytic domain (L253F) interfere with the autocatalytic cleavage and are therefore ***Class 2*** mutations (processing-defective). The L253F resides near the catalytic triad and might disrupt the catalytic site (32). Inasmuch as autocatalytic cleavage of PCSK9 is required for export of the protein out of the ER, all ***Class 2*** mutations also delay transport of PCSK9 from the ER to the cell surface (***Class 3***). The most common nonsense mutation (C679X) in PCSK9, which truncates the protein by 14 amino acids, is also a ***Class 3*** mutation. The mutant protein is cleaved normally but is misfolded and retained in the ER (13, 32).

Some loss-of-function mutations might affect the stability of PCSK9 (***Class 4*** mutations). In cultured cells, a small fraction of the PCSK9 synthesized undergoes a second, membrane-bound furin-mediated cleavage event (13). Benjannet *et al.* (13) suggested that some loss-of-function mutations, such as A443T, might be more susceptible to furin cleavage. However, more careful studies to assess the half-life of the protein will be required to determine if furin cleavage is physiologically relevant.

Mutations in *PCSK9* could also affect the affinity of the protein for the LDLR, or other proteins that promote receptor degradation (***Class 5*** mutation). A gain-of-function mutation, D374Y, binds more avidly to the LDLR and is ~10-fold more active in reducing LDLR protein compared to the wild-type protein (28). Another gain-of-function

mutation (F216L) is predicted to reside very close to D374Y on the outer surface of the catalytic domain (32) and might also be a ***Class 5*** mutation.

Table 1. *Functional defects associated with naturally-occurring loss- and gain-of-function mutations of PCSK9 in humans.*

Class of mutation	1 Null	2 ↓ Processing	3 ↓ Transport from ER	4 Alters stability	5 Alters affinity for LDLR	Reference
Gain-of-function						
S127R	---	X	X	?	?	(5, 12, 25)
F216L	---	----	---	?	?	(5, 12, 25)
D374Y	---	---	---	?	X	(18, 19, 28)
Loss-of-function						
R46L	---	---	---	?	?	(32, 38, 39)
Δ97R	---	X	X	---	---	(32)
G106R	---	X	X	---	---	(38)
Y142X	X	---	---	---	---	(32)(37)
L253F	---	X	X	---	---	(32, 39)
A443T	---	---	---	?	?	(13, 39)
C679X	----	---	X	---	---	(13, 32, 37)

Phenotypic classifications are based on expression of the recombinant protein in cultured cells.

---, no defect observed; X, defective; ?, not defined.

PCSK9 as a therapeutic target

PCSK9 and LDLR are coordinately regulated

The LDLR and PCSK9 are coordinately regulated by sterol regulatory element-binding protein-2 (SREBP-2), a transcription factor that regulates many genes involved in cholesterol metabolism (6, 7). The dual regulation of the LDLR and PCSK9 by SREBP-2 might permit the exploitation of this pathway for cholesterol-lowering therapies. Statin administration lowers LDL by inducing SREBP-2 expression, which increases expression of LDLR (46, 47). PCSK9 mRNA and protein levels are also increased in response to statins (42, 48). The increase in PCSK9 would attenuate the cholesterol-lowering effect of statins and might reduce their effectiveness, particularly at higher doses. Therefore, inhibition of PCSK9 activity would be predicted to augment statin-induced LDLR expression and accelerate LDL-C clearance, as was observed when statins were administered to PCSK9 knockout mice (42). The low plasma LDL-C levels associated with loss-of-function mutations in PCSK9 indicate that PCSK9 inhibitors either through small molecules, antibodies, or RNAi should be effective cholesterol lowering drugs independently of statins.

Safety issues associated with PCSK9 inhibition

Would pharmacological inhibition of PCSK9 be safe in humans? PCSK9 is expressed in the kidney and cerebellum of adult mice, in addition to the liver and small intestine (4). Although inactivation of PCSK9 in embryos of zebrafish results in disordered neuronal development and death (49), mice lacking PCSK9 develop normally and have no gross neurological defects (42). Humans heterozygous for loss-of-function mutations in PCSK9 appear to be healthy (37) and have a normal life-span (40). Moreover, a compound heterozygote with two inactivating mutations in PCSK9 (Y142X and ΔR97) and consistently low plasma levels of LDL-C (14-34 mg/dL) was recently found to have no circulating immunodetectable PCSK9 (32). This 31-year-old African-American has grossly normal renal, hepatic, and neuronal function (unpublished observations). Another individual homozygous for the C679X mutation was identified in Zimbabwe; she has a plasma LDL-C of 16 mg/dL (50). Careful clinical assessment of these individuals and other subjects with inactivating mutations in PCSK9 might reveal additional phenotypes, providing clues to substrates of PCSK9 other than the LDLR.

Physiological role of PCSK9

Role of PCSK9 in cholesterol metabolism

As stated above, both the *LDLR* and *PCSK9* are transcriptionally regulated by SREBP-2 (7). Thus, as more LDLR protein is produced, more PCSK9 is made, ultimately leading to the degradation of the LDLR protein in hepatocytes. An explanation for this seemingly futile regulatory cycle is that if PCSK9 primarily functions at the cell surface and as a secreted protease, it could act as a "brake" to slow the uptake of cholesterol by degrading LDLRs after they have internalized LDL. PCSK9 can potentially avert excessive cholesterol accumulation within the cell by preventing the recycling of LDLRs to the cell surface.

The liver is likely to be the primary site of synthesis of circulating PCSK9. By controlling the secretion of PCSK9, the liver might regulate the levels of LDLR expression in peripheral tissues. Tissues *in vivo* could vary in their responsiveness to PCSK9, as is seen *in vitro*. Thus, PCSK9 expression could direct LDL to specific tissues. For example, in times of stress, PCSK9 might shunt cholesterol away from the liver to steroidogenic tissues, such as the adrenal gland. In support of this idea, the LDLRs of the adrenals in mice appear to be less responsive to PCSK9-mediated degradation. The effect of PCSK9 expression on the levels of LDLR in extra-hepatic tissues has not been examined.

PCSK9 and human evolution

The high frequency of nonsense mutations in individuals of African descent begs the question as to whether inactivation of PCSK9 confers a selective advantage (37). We can only speculate on the nature of the selective pressure that maintained nonsense mutations in PCSK9 in Africans. The best evidence of positive selection in Africans is the accumulation of sequence variations in various red blood cell proteins in populations exposed to malaria (51). Inactivation of PCSK9 may interfere with the life cycle of the malaria parasite. The LDLR has been implicated as a portal for entry into the liver for several viruses, such as a minor group of rhinovirus and possibly hepatitis C (52, 53). Increased LDLR activity in the liver may reduce the exposure of peripheral tissues to

viruses or other infectious agents that circulate in association with lipoproteins. Reductions in PCSK9 activity might decrease the pathological sequelae of an infectious agent. Other members of the proprotein convertase family are hijacked by viruses and bacteria to process proteins required for infection (54). Alternatively, the high frequency of PCSK9 nonsense mutations in Africans and not Caucasians may simply result from genetic drift. Additional population genetic studies will be required to address this question.

Unresolved questions

Despite the rapid progress that has been made in the last four years regarding the biological importance of PCSK9 in LDL metabolism, a number of important mechanistic and clinical questions remain to be answered. First, the mechanism by which PCSK9 expression and the gain-of-function mutations promote the degradation of the LDLR remains to be defined. Does PCSK9 circulate in association with other proteins and can it act on receptors in tissues other than the liver? Finally, why has PCSK9 been retained through vertebrate evolution? Although genetic deficiency of PCSK9 does not appear to be associated with obvious phenotypes independent of LDL metabolism, it remains possible that PCSK9 acts on other pathways.

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