Proprotein Convertase Subtilisin/ Kexin-Type 9 and Lipid Metabolism

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Shoudong Guo, Xiao-dan Xia, Hong-mei Gu, and Da-wei Zhang

Abstract

Plasma levels of cholesterol, especially low-density lipoprotein cholesterol (LDL-C), are positively correlated with the risk of cardiovascular disease. Buildup of LDL in the intima promotes the formation of foam cells and consequently initiates atherosclerosis, one of the main underlying causes of cardiovascular disease. Hepatic LDL receptor (LDLR) is mainly responsible for the clearance of plasma LDL. Mutations in LDLR cause familiar hypercholesterolemia and increase the risk of premature coronary heart disease. Proprotein convertase subtilisin/kexin-type 9 (PCSK9) promotes LDLR degradation and thereby plays a critical role in the regulation of plasma cholesterol metabolism. PCSK9 can bind to LDLR and reroute the receptor to lysosomes for degradation, increasing both circulating LDL-C levels and the risk of cardiovascular disease. PCSK9 is mainly regulated by sterol response element binding protein 2 (SREBP2) at the transcriptional level. Furthermore, many proteins have been identified as interacting with PCSK9, regulating plasma cholesterol levels. Pharmacotherapeutic inhibition of PCSK9 dramatically reduces plasma levels of LDL cholesterol and significantly reduces cardiovascular events. In this article, we summarize the latest advances in PCSK9, mainly focusing on the structure, function, and regulation of the protein, the underlying molecular mechanisms, and its pharmacotherapeutic applications.

Keywords

Hypercholesterolemia · Low-density lipoprotein receptor · Statin · Atherosclerosis · Proprotein convertase subtilisin/kexin-type 9

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Abbreviations

ADH	autosomal dominant hypercholesterolemia
Apo	apolipoprotein
ARH	autosomal recessive hypercholesterolemia
BACE1	β-site amyloid precursor protein-cleaving enzyme 1
bHLH	basic helix-loop-helix
CAP1	cyclase-associated protein 1
CAT	catalytic domain
COPII	the coat protein complex II
CSF	cerebrospinal fluid
CM	C-terminal module
CTD	C-terminal domain
CVD	cardiovascular disease
EGF-A	the epidermal growth factor precursor homology domain A
Epac2	exchange protein activated by cAMP-2
ER	endoplasmic reticulum
ERGIC	ER-Golgi intermediate compartment
FH	familial hypercholesterolemia
GPC3	glypican-3
HDL	high-density lipoprotein
HINFP	histone nuclear factor P
HMGCR	3-hydroxyl-3-methylglutaryl-CoA reductase
HNF1	hepatocyte nuclear factor 1
HSPG	heparan sulfate proteoglycan
INSIG	insulin-induced gene protein
LDL-C	low-density lipoprotein cholesterol
LDLR	LDL receptor
Lp(a)	lipoprotein (a)
miRNA	microRNA
PC	proprotein convertase
PCSK9	proprotein convertase subtilisin kexin-like 9
PLTP	phospholipid transfer protein
Rap1	ras-related protein-1
RISC	RNA-induced silencing complex
S1P	site-1 proteinase
S2P	site-2 proteinase
SCAP	SREBP cleavage activating protein
siRNA	small interfering RNA
SREBP-2	sterol regulatory element binding protein 2
Surf4	Surfeit 4
TLP	Toll-like receptor
UTR	untranslated region
VLDLR	very low-density lipoprotein receptor

9.1 Introduction

Cardiovascular disease (CVD) is the primary cause of morbidity and mortality worldwide. Atherosclerosis, characterized by the progressive accumulation of lipid and fibrous deposits in the vessel wall, is the most important underlying cause [1]. Plasma levels of cholesterol, especially low-density lipoprotein cholesterol (LDL-C), are positively correlated with the risk of atherosclerosis [2]. Cholesterol homeostasis in humans is regulated by well-balanced mechanisms of intestinal uptake, endogenous synthesis and metabolism, transport in lipoprotein particles, and biliary excretion. In humans, LDLs are the major cholesterol transport vesicle in the blood, carrying approximately 65–70% of plasma total cholesterol [2]. Elevated plasma LDL-C levels, such as those in patients with autosomal dominant hypercholesterolemia (ADH), lead to a progressive buildup of lipids in the inner walls of the arteries, promoting the formation of foam cells and consequently initiating atherosclerosis [3, 4].

LDL is produced as a metabolic by-product of very low-density lipoprotein (VLDL), a triglyceride-rich lipoprotein produced exclusively by the liver [5]. The LDL receptor (LDLR) in the liver is the protein primarily responsible for removal of LDL from circulation [2, 6]. Mutations in the LDLR cause familial hypercholesterolemia (FH), an inherited disorder associated with elevated circulating levels of LDL-C, which causes tendon and skin xanthomas, arcus cornea, and/or cardiovascular deposits and leads to increased risk in coronary heart disease and mortality [2, 6]. FH is the most common ADH and accounts for approximately 67% of case reports. The

second most common ADH is caused by mutations in apolipoprotein B100 (apoB100) (~14% of case reports), the ligand for LDLR. ApoB100 is synthesized and lipidated in the liver and then secreted in plasma as VLDL. It is the main structural protein on VLDL and LDL. Recently, a third form of ADH was identified, which is caused by selected missense mutations in proprotein convertase subtilisin/kexin type 9 (PCSK9) and accounts for 2.3% of ADH [7, 8]. Gain-of-function mutations of PCSK9 cause higher plasma LDL-C levels and lead to accelerated atherosclerosis and premature coronary heart disease [7–10]. Thus, genetic defects in these three genes contribute to approximately 83.3% of ADH. The causes for the remaining 26.7% have yet to be determined. Interestingly, unlike defects in LDLR and apoB, certain mutations in PCSK9 lead to loss of function, resulting in reduced plasma levels of LDL-C and enhanced protection from coronary heart disease [11–13].

9.2 PCSK9 Structure

PCSK9, first known as neural apoptosis regulated convertase 1 (NARC-1), is a member of the subtilisin-like serine protease family that includes seven basic amino acid-specific proprotein convertases (PC): PC1, PC2, furin, PC4, PC5/6, PACE4, and PC7; it also includes two members, site-1 protease and PCSK9, that cleave at the carboxyl terminus of non-basic residues [14]. The human PCSK9 gene is located in chromosome 1p32.3 and covers 39.91 kb with 13 exons. PCSK9 is highly conserved among different species including human, mouse, rat, hamster, monkey, chimpanzee, *S. cerevisiae*, chicken, zebrafish, and frog. It is a 692-amino acid secretory glycoprotein that consists of a signal sequence (amino acids 1–30), followed by a prodomain (amino acids 31–152), a catalytic domain (CAT, amino acids 153–425), and a cysteine- and histidine-rich C-terminal domain (CTD). The CTD domain contains an exposed hinge region (residues 422–439) and three repeat modules: module 1 (CM1: amino acids 457–528), module 2 (CM2: amino acids 534–601), and module 3 (CM3: amino acids 608–692) (Fig. 9.1) [15–17]. PCSK9 is synthesized as a zymogen (~75 kDa) and undergoes autocatalytic cleavage in the endoplasmic reticulum (ER) at the carboxy terminus of FAQ152↓SIPK site to form the mature form (~62 kDa) (Fig. 9.1). After autocleavage, the prodomain is tightly associated with the rest of the protein.

The crystal structures of PCSK9 reveal that the overall domain structure of PCSK9 is similar to other subtilisin-like serine proteases in a wide range of pH conditions (from pH 5–10) [15, 18–20]. The prodomain of PCSK9 consists of one four- to five-stranded antiparallel β-sheet flanked by two α helices. The catalytic domain contains a classical serine protease catalytic triad of Asp186, His226, and Ser386 and shows a similar structure as other subtilisin-like family members such as yeast Kexin and mouse furin [21, 22]. It is composed of a seven-stranded parallel β-sheet core with α helices on each side. However, unlike other convertases that contain the negatively charged substrate-binding groove [21], the substrate-binding pocket in PCSK9 is mostly neutral. The β-sheet of the C-terminal prodomain associates with the catalytic site tightly through hydrophobic and electrostatic interactions, which blocks further substrate accessibility and thereby shields further catalytic activity [19]. The C-terminal domain of PCSK9 is connected to the catalytic domain through a flexible liner region as well as through hydrogen bonds and hydrophobicity interaction. The C-terminal domain is made up of three subdomains, each containing six antiparallel β-domains without helices in a similar cylindrical shape secured through three structurally conserved disulfide bonds. The C-terminal domain is unique among the subtilisin-like serine protease family and displays structural homology to resistin that is related to type II diabetes [23]. Furthermore, the C-terminal domain is enriched in cysteine and histidine residues and contains multiple potential protein-protein interaction motifs [15].

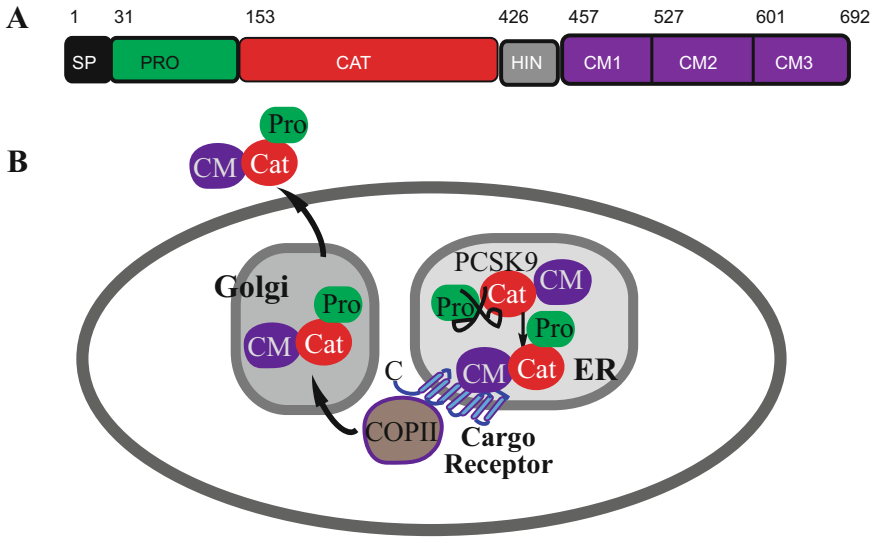

Fig. 9.1 *PCSK9 structure and secretion.* (**a**) Schematic of PCSK9. SP, signal peptide; PRO, prodomain; CAT, catalytic domain; HIN, hinge region; CM1, CM2, and CM3: three modules of C-terminal domain. Numbers on the top of the wild-type PCSK9 indicate the amino acid sequence of each domain. (**b**) PCSK9 secretion. PCSK9 undergoes autocleavage in the ER between the prodomain and the catalytic domain. The cleaved prodomain is tightly associated with the catalytic domain and is secreted together from cells

9.3 PCSK9 Secretion

PCSK9 is mainly expressed in the liver, intestine, and, to a lesser extent, in the kidneys, skin, and brain [24]. PCSK9 is secreted in plasma. However, the protein is undetectable in plasma of mice lacking hepatic PCSK9 [25], suggesting that circulating PCSK9 is mainly secreted from the liver. The autocatalytic cleavage of PCSK9 is required for its secretion. The cleaved N-terminal prodomain is tightly associated with the catalytic domain, and they are secreted together from cells [14, 26]. Structurally, it has been reported that the autocleavage of PCSK9 in the ER triggers a conformational change of the N-terminal α helix of the catalytic domain to permit PCSK9 secretion [15]. Further, the C-terminal PCSK9 has been implicated in its secretion. Loss-of-function mutations such as E498K and S462P located in the C-terminus of PCSK9 damage its secretion [27–29]. Biochemical studies reveal that removal of the whole C-terminus of PCSK9 (amino acids 456 to 692), the CM2 (amino acid 534 to 601), or the CM2 and CM3 (amino acids 534 to 692) does not significantly affect PCSK9 secretion. However, deletion of either CM1 (amino acids 457–528) or CM3 (amino acids 608–692) markedly impairs PCSK9 secretion [16, 17]. In addition, we found that the hinge region that connects the C-terminal domain to the catalytic domain played an important role in PCSK9 secretion. Deletion of the hinge region, in whole or in part, dramatically reduced PCSK9 secretion [30].

Secretion of certain abundant proteins such as N-acylglycotripeptides and amylase chymotrypsinogen is regulated by a default bulk flow pathway [31, 32]. Even so, there is emerging evidence suggesting that cargo receptors can facilitate ER-to-Golgi transport of secretory proteins in mammalian cells [33–40]. Cargo receptors are transmembrane proteins containing an ER lumen-exposed domain that binds cargo proteins within the lumen and a cytoplasmic domain that interacts with coat protein II complex (COPII) components, thereby sorting cargos into COPII vesicles. Several potential cargo receptors function as cargo-sorters for transport between the ER and Golgi: the ER-Golgi intermediate compartment (ERGIC)-53 family, the p24 family, and the

Erv family [33, 34]. It has been documented that the COPII adaptor proteins SEC24A and SEC24B facilitate the ER-to-Golgi transport of PCSK9 [41]. However, PCSK9 is located in the lumen of the ER and is unable to interact directly with SEC24, which is located in the cytosol. Thus, a cargo receptor is required. Recently, Emmer et al. reported that Surfeit locus protein 4 (SURF4) was implicated in PCSK9 secretion [42]. They found that SURF4 co-immunoprecipitated with PCSK9 and knockout of the cargo receptor significantly reduced secretion of PCSK9 overexpressed in HEK293 cells. SURF4 is a polytopic transmembrane protein containing seven putative transmembrane domains with a lumen-exposed N-terminus and a cytosolic C-terminus [43]. The protein is ubiquitously expressed and mainly localized in the ER [43]. However, we found that knockdown of SURF4 expression in cultured human-hepatoma-derived cell lines, HepG2 and Huh7, increased endogenous PCSK9 expression and secretion, indicating a negligible role for Surf4 in PCSK9 secretion in cultured hepatocytes [44]. This discrepancy might be caused by different cell lines used in the two studies. We investigated the secretion of PCSK9 endogenously expressed in HepG2 and Huh7 cells, while Emmer et al. studied the effect of Surf4 on the secretion of PCSK9 overexpressed in HEK293 cells that do not express endogenous PCSK9. In addition, conflicting data on the role of sortilin in PCSK9 secretion has been reported. Gustafsen et al. observed that plasma levels of PCSK9 were reduced in sortilin$^{-/-}$ mice but increased in sortilin-overexpressing mice. Circulating PCSK9 levels were also positively correlated with plasma levels of sortilin. Thus, the authors argued that sortilin interacted with PCSK9 in the trans-Golgi network and then facilitated its secretion [45]. Conversely, studies from Butkinaree et al. showed that knockdown of sortilin in cultured human hepatocytes or knockout of sortilin in mice had no detectable effect on PCSK9 secretion [46]. Nevertheless, these conflicting findings reveal the complexity of the molecular mechanisms of PCSK9 secretion.

9.4 PCSK9 Function

PCSK9 plays a central role in maintaining cholesterol homeostasis. Gain-of-function mutations lead to higher plasma LDL-C levels and accelerate premature coronary heart disease [7–9, 47, 48]. On the other hand, loss-of-function mutations result in low concentrations of LDL-C and protection from coronary heart disease [11–13, 49–53]. Overexpression of recombinant PCSK9 in mouse liver causes a significant reduction in hepatic LDLR protein levels without any effect on its mRNA levels, producing severe hypercholesterolemia [26, 54, 55]. On the other hand, knockdown or knockout of *PCSK9* expression in mice leads to increased levels of LDLR protein in the liver and accelerated LDL clearance [56, 57]. The natural gain-of-function mutation, D374Y, has a significantly increased binding affinity for LDLR and promotes LDLR degradation much more efficiently than the wild-type protein [15, 58], leading to a severe form of hypercholesterolemia [7]. Consistently, the FH mutation LDLR-H306Y binds PCSK9 with a higher affinity and exhibits enhanced sensitivity to PCSK9 as compared to the wild-type receptor [59]. Taken together, these findings demonstrate that the role of PCSK9 in homeostatic control of plasma LDL-C levels depends upon PCSK9-promoted degradation of LDLR, preventing clearance of LDL-C by the cells [26, 54–58, 60–64].

Studies in cultured cells and parabiotic mice demonstrate that PCSK9 promotes degradation of LDLR in an adaptor protein autosomal recessive hypercholesterolemia (ARH)-dependent manner in hepatocytes and lymphocytes [58, 60, 61, 65]. However, ARH is not required for PCSK9-promoted LDLR degradation in fibroblasts [65, 66]. McNutt et al. [59] showed that PCSK9 caused LDLR degradation primarily through interaction with the receptor on the cell surface. However, overexpression of PCSK9 in cultured cells and mouse liver also induces LDLR degradation intracellularly [55, 67]. For instance, the gain-of-function mutation R499H enhances PCSK9-promoted LDLR degradation intracellularly [68]. Similarly,

mutations D129G and A168E impair PCSK9 secretion but enhance the ability of PCSK9 to induce LDLR degradation intracellularly, thereby causing hypercholesterolemia [69]. Poirier et al. [70] observed that, upon dose and incubation period, PCSK9 could act both intracellularly and extracellularly to promote LDLR degradation in cultured cells and mouse primary hepatocytes.

PCSK9's action on the LDLR is also cell-type specific. Increased plasma levels of PCSK9 in mice through infusion of purified PCSK9 or transgenic overexpression in the kidneys preferentially promoted LDLR degradation in the liver but not in the adrenal glands [71–73]. Consistently, the adrenal function of a human subject with no detectable plasma PCSK9 is normal [74]. Gustafsen et al. [75] recently reported that the prodomain of PCSK9 bound to the trisulfated heparan sulfate disaccharide repeats in heparan sulfate proteoglycans (HSPG) of the liver. Heparin mimetics such as sulfated oligosaccharides dextran sulfate and pentosan sulfate can suppress PCSK9-mediated LDLR degradation in HepG2 cells. The authors proposed that HSPG functioned as a coreceptor for PCSK9, capturing plasma PCSK9 and then presenting it to hepatic LDLR for the following degradation process. In cultured cells, the expression of PCSK9 in some cell types, such as human hepatoma cells (HepG2 and HuH7), dramatically reduces LDLR levels [55, 58, 60, 61]. On the other hand, PCSK9 appears to have no effect on LDLR expression in Chinese hamster ovarian cells (CHO-K1), monkey kidney cells (COS-7), and rat liver cells (McArdle RH7777) [55, 58, 60, 76]. The molecular mechanism of the cell type specific action of PCSK9 on LDLR is unknown. The dissociation of PCSK9 from LDLR after endocytosis may be responsible for the inability of PCSK9 to promote LDLR degradation in human skin fibroblasts SV-589 [77].

PCSK9-promoted degradation of LDLR requires binding of PCSK9 to LDLR and internalization of the receptor but does not require the proteolytic activity of PCSK9 [58, 60, 78]. Normally, the extracellular domain of the cell surface LDLR (neutral pH) adopts an extended linear open conformation that favors interactions between the receptor and LDL [79]. Upon ligand binding to the ligand binding repeats of LDLR, the receptors are internalized via clathrin-coated pits and delivered to endosomes [80, 81]. In the low pH environment of the endosome, LDLR undergoes a conformational change to form a close conformation that promotes the release of the bound LDL that is delivered to lysosomes for degradation and signals recycling of LDLR to the cell surface [79].

PCSK9 interacts with the EGF-A of LDLR at the cell surface, which is different from the LDL binding site on the receptor. Thus, the binding sites of PCSK9 and LDL on the receptor are not in proximity, and the binding of one ligand is unlikely to block the accessibility of another one to LDLR. We found that replacement of Leu at position 318 in the EGF-A of LDLR with Asp as it is in VLDLR markedly reduced PCSK9 binding to the receptor, indicating the important role of this residue in PCSK9 binding. Further, we observed that mutations G293H, D299V, L318D, and L318H in EGF-A reduced PCSK9 binding to LDLR at a neutral pH, while mutations R329P and E332G reduced PCSK9 binding at both neutral pH and acidic pH 6.0. Thus, EGF-A of the LDLR is critical for PCSK9 binding at the cell surface (neutral pH) and at the acidic endosomal environment (pH 6.0), but different determinants contribute to efficient PCSK9 binding in different pH environments [82].

Several lines of evidence demonstrate that PCSK9/LDLR complex enters cells via clathrin-coated pits. Knockdown of clathrin heavy chain markedly reduces PCSK9-promoted LDLR degradation in human hepatoma-derived cell lines, Huh7 and HepG2 cell [65, 83, 84]. Conversely, Jang et al. reported that knockdown of clathrin heavy chain did not affect PCSK9-promoted LDLR degradation in HepG2 cells; instead, the authors found that the PCSK9/LDLR complex entered cells via caveolae-dependent endocytosis [85]. The reasons for this discrepancy are unclear. It is of note that different approaches were used in the two studies. Romagnuolo et al. [83] overexpressed PCSK9 in HepG2 cells and then knocked down the expression of clathrin heavy chain, while Jang et al. silenced the expression of

clathrin heavy chain and then supplied cells with various doses of recombinant flag-tagged PCSK9 purified from HEK293 cells overexpressing PCSK9 [85].

While LDL binds to the receptor that is much weaker at the acidic endosome compared to that at the neutral cell surface, PCSK9 binds the receptor with a much higher affinity at the endosomal pH value than at the neutral pH. Consequently, the receptor is transported from the endosome to the lysosome for degradation, rather than being recycled (Fig. 9.2) [60]. The binding of PCSK9 to LDLR interferes with the acid-dependent conformational change of the receptor, but disrupting the pH-dependent conformational change in the LDLR is not sufficient to trigger LDLR degradation [61]. We also demonstrated that YWTD repeats, and a minimum of three ligand-binding repeats in the LDLR that were not required for PCSK9 binding at neutral pH were essential for efficient LDLR degradation induced by PCSK9 [61, 82]. Furthermore, we reported that the C-terminal domain of PCSK9 was essential for PCSK9-promoted degradation of LDLR, but was not required for binding to LDLR at the neutral pH value [61]. The X-ray crystallographic structure of PCSK9-LDLR complex shows that YWTD repeats of LDLR interact with the prodomain of PCSK9 [86]. Several biochemical studies indicate that the negatively charged ligand binding repeats (LR) of LDLR may interact with the positively charged C-terminal domain of PCSK9 in the acidic endosomal environment to enhance PCSK9 binding [87–89]. Consistently, we found that mutation of Asp at position 172 in the linker between the LR4 and LR5 of LDLR to Asn and replacement of Asp at position 203 in the LR5 to Asn significantly reduced PCSK9 binding

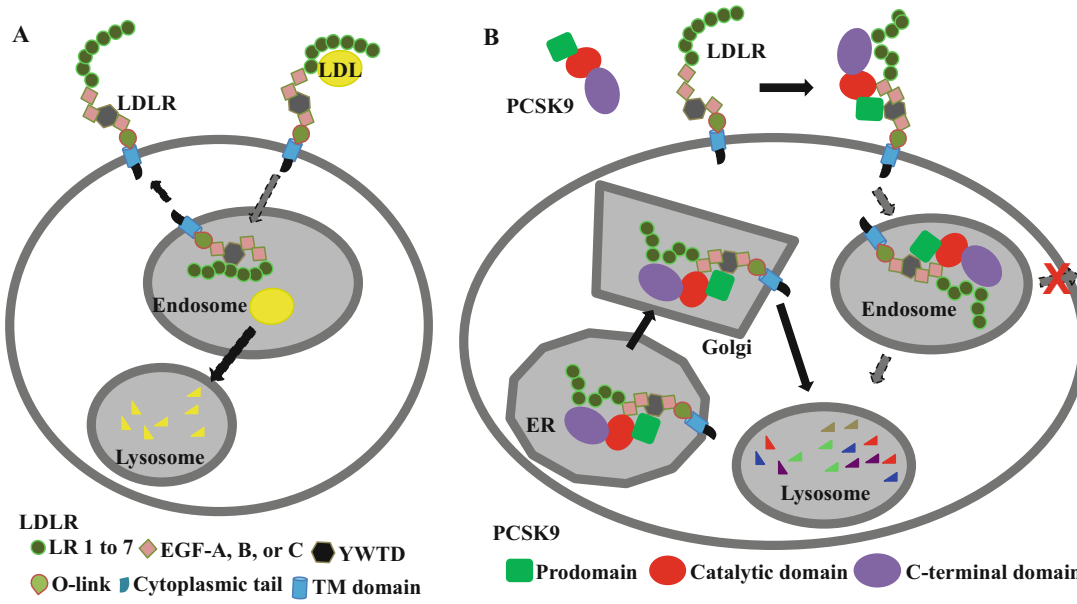

Fig. 9.2 *Recycling pathway of the LDLR.* (**a**) LDLR-mediated LDL uptake. Upon binding LDL, LDLR is internalized via clathrin-coated pits and delivered to endosomes, where the bound LDL is released from the receptor and delivered to lysosomes for degradation. LDLR recycles to the cell surface. (**b**) *PCSK9-mediated LDLR degradation.* Extracellularly, PCSK9 interacts with EGF-A of LDLR at the cell surface and enters cells via endocytosis. The LDLR/PCSK9 complex is then delivered to the endosome, where PCSK9 binds the receptor with a much higher affinity in the acidic environment. Consequently, the receptor-PCSK9 complex traffics from the endosome to the lysosome for degradation, rather than being recycled. For the intracellular pathway, PCSK9 binds to LDLR in the ER or during the secretory pathway and then reroutes the receptor to the lysosome for degradation

[90]. This further confirms the important role of the negatively charged amino acid residues within the LR in PCSK9 binding to LDLR.

Neither PCSK9 nor LDLR contains a lysosomal targeting signal. Removal of the C-terminal cytoplasmic tail of LDLR does not damage PCSK9-promoted LDLR degradation [82, 91, 92]. Thus, it is believed that co-factor(s) might be required for this process. Recently, DeVay et al. [93] reported that both amyloid precursor protein (APP) and amyloid precursor-like protein 2 (APLP2) co-immunoprecipitated with the full length but not the C-terminal deletion mutation PCSK9 at pH6.0 (endosomal environment), but not at pH7.4 (cytosol environment). The authors further showed that knockdown of APLP2 but not APP suppressed PCSK9-promoted LDLR degradation in HepG2 cells. This finding indicates that APLP2 binds to the C-terminus of PCSK9 and thereby targets the PCSK9/LDLR complex to lysosomes for degradation [93]. However, two independent groups reported that PCSK9 efficiently promoted LDLR degradation in *Aplp2*$^{-/-}$ mice [46, 94]. Butkinaree et al. [46] further showed that knockdown of APLP2 in both HepG2 and Huh7 cells had no significant effect on the ability of PCSK9 to enhance LDLR degradation. These studies suggest that APLP2 is not required for PCSK9-promoted LDLR degradation. In addition, glypican-3 (GPC3) and phospholipid transfer protein (PLTP) have been shown to interact with PCSK9 by co-immunoprecipitation and an unbiased mass spectrometry. Silence of either GPC3 or PLTP using their specific short hairpin RNAs increased LDLR levels in HepG2 cells [95]. Further, Jang et al. found that the Src homology 3 binding domain of adenylyl cyclase-associated protein 1 (CAP1) interacted with the C-terminal domain of PCSK9. The knockdown of expression of CAP1 increased LDLR levels in HepG2 cells, and haploid deficiency of Cap1 in mice led to increased hepatic LDLR levels and reduced plasma LDL-C. More interestingly, the author reported that CAP1 mediated endocytosis of the PCSK9/LDLR complex in a caveolae-dependent manner since addition of PCSK9 could not induce LDLR degradation in caveolin-deficient cells [85]. This finding contradicts several previous findings that clathrin is required for PCSK9-induced endocytosis and subsequent lysosomal degradation of the receptor [65, 84]. Nevertheless, presently, the mechanism by which binding of PCSK9 to LDLR reroutes the receptor to the lysosome for degradation is not well understood and is believed to be complex.

In addition to its regulatory role in plasma LDL-C levels via the LDLR pathway, PCSK9 regulates apoB secretion. The lack of PCSK9 in the liver of *Ldlr*$^{-/-}$/*Apobec1*$^{-/-}$ mice significantly reduces apoB100 secretion [96]. Gain-of-function PCSK9 mutation D374Y markedly increases the secretion of apoB-containing lipoprotein in transgenic mice expressing physiological levels of PCSK9 [97]. In human PCSK9 transgenic mice, the expression of microsomal triglyceride transfer protein *(MTP)* and lipogenic genes is significantly increased. Consequently, secretion of apoB48 and production of chylomicrons are increased in both LDLR-dependent and -independent manners [73, 98]. On the other hand, lymphatic apoB secretion is markedly reduced in *Pcsk9*$^{-/-}$ mice [99]. Consistently, plasma levels of PCSK9 and apoB-48 containing lipoproteins are positively correlated in men with insulin resistance [100]. Together, these findings suggest an important role of PCSK9 in the development of postprandial dyslipidemia. PCSK9 has also been shown to regulate plasma lipoprotein(a) (Lp(a)) levels in a LDLR-dependent manner. Lp(a) is an LDL-like particle that contains Apo(a) covalently linked to apoB by a disulfide bond. Several epidemiological studies show that (Lp(a)) is an independent risk factor for cardiovascular disease [101–104]. LDLR can mediate but is not required for the clearance of Lp(a) [105, 106]. PCSK9 is associated with Lp(a) in human plasma [107], and inhibition of PCSK9 reduces plasma Lp(a) levels and cardiovascular events [83, 108, 109].

It has been reported that PCSK9 binds to and stimulates degradation of several LDLR family members such as VLDLR and ApoER2 [110], as well as CD36 [111], but to a far lesser extent when compared to its binding to LDLR [60, 82]. PCSK9 can act on CD36 in HepG2 and 3T3-L1 cells, but not on HL-1 or THP-1

cells. In vivo, the levels of CD36 in the small intestine and the heart which highly express CD36 are comparable between wild-type and *Pcsk9*$^{-/-}$ mice, while the expression of CD36 in the liver and mouse adipose tissue is significantly increased [111]. The lack of PCSK9 in mice also markedly increases the expression of VLDLR in perigonadal depots and promotes accumulation of visceral fat [112]. On the other hand, Liu et al. reported that PCSK9 cannot promote degradation of VLDLR and apoER2 in the adult mouse brain [113]. Additionally, PCSK9 has been shown to promote LDLR-related protein 1 (LRP-1) degradation in mouse B16F1 melanoma cells [92] but not in mouse hepatocytes [58]. The expression of LDLR in CHO cells can suppress PCSK9's effect on LRP-1 [92]. Thus, it is possible that the relative high expression of hepatic LDLR competitively suppresses the effect of PCSK9 on LRP1 in the liver. Controversial data on the role of PCSK9 in neuron function and pathogenesis of Alzheimer's disease has also been reported in the literature. Knockdown of PCSK9 expression increases levels of ApoER2 and protects against apoptosis in cerebellar granule neurons [114], and silence of PCSK9 alleviates middle cerebral artery occlusion-induced cerebral histological injury and neuronal apoptosis in mice fed a high fat diet, probably through the regulation of apoER2 expression [115]. These findings indicate a pro-apoptosis role of PCSK9 in neurons. Consistently, inhibition of PCSK9 reduces Aβ aggregation and neuroinflammation, alleviating dendritic spine loss in a cardiac ischemic/reperfusion injury rat model [116]. On the other hand, Jonas et al. showed that PCSK9 promoted degradation of unacetylated β-site amyloid precursor protein-cleaving enzyme 1 (BACE1). The authors observed an increase in the levels of Aβ and BACE1 in the brain of *Pcsk9*$^{-/-}$ mice [117]. Thus, further studies are needed to elucidate these potential functions of PCSK9.

9.5 Regulation of PCSK9

Transcription of *PCSK9* is mainly controlled by the sterol regulatory element binding protein 2 (SREBP2) that regulates expression of genes involved in cholesterol metabolism such as *LDLR* and 3-hydroxy-3-methylglutaryl-CoA reductase (*HMGCR*), the rate-limiting enzyme in the cholesterol biosynthesis pathway [118, 119]. SREBP2 is a master regulator of cellular cholesterol homeostasis. It contains an NH_2-terminal transcriptionally active domain that belongs to the basic helix-loop-helix (bHLH) class, two transmembrane alpha-helixes, and a COOH-terminal regulatory domain [120]. The transcriptional activity of SREBP2 is strictly regulated by cellular cholesterol levels [121]. The COOH-terminal regulatory domain of newly synthesized SREBP2 immediately forms a complex with SREBP cleavage-activating protein (SCAP) in the ER. When ER cholesterol content is lower than 5% of total ER lipids, SCAP is separated from an ER-resident protein, insulin-induced gene protein (INSIG), and escorts SREBP2 to the Golgi, where SREBP2 is cleaved by S1P and site-2 protease (S2P) sequentially to liberate the transcriptionally active form. The active form then travels to the nucleus, where it recognizes and binds to sterol regulatory element (SRE) located in the promoter region of its target genes, promoting their transcription. Conversely, higher ER cholesterol content (more than the 5% threshold values) promotes formation of SCAP/INSIG complex, blocking transport of SREBP2 to the Golgi and the subsequent processing of SREBP2. Consequently, transcription of SREBP2 target genes is suppressed [121]. SREBP2 binds to an SRE motif in the promoter of *PCSK9,* stimulating its transcription. mRNA levels of *Pcsk9* are increased six- to ninefold in mice transgenic expressing the active form of SREBP2 but reduced in *Scap*$^{-/-}$ mice [118]. Additionally, Li et al. [122] identified a histone nuclear factor P (HINFP) recognition motif present within 20 bp upstream of the SRE motif and found that HINFP functioned as a co-activator for the transcriptional activity of SREBP2 through promoting the histone H4 acetylation of *PCSK9* promoter. Resistin, a small cysteine-rich protein secreted from macrophages and adipose tissue, increases mRNA levels of PCSK9 via SREBP2

[123]. Fibroblast growth factor 21 can suppress expression and activity of SREBP2 in mouse liver and reduce expression of PCSK9 [124].

Hepatocyte nuclear factor 1 (HNF1) also regulates expression of *PCSK9* at the transcriptional level. The promoter region of *PCSK9* contains a highly conserved HNF1 binding site at the upstream of SRE. Berberine, a plant-derived cholesterol-lowering compound, inhibits PCSK9 expression mainly through interfering with the HNF1's action [125, 126]. Additionally, stimulation of mTORC1 by insulin reduces activity of HNF1α and consequently suppresses PCSK9 expression. An opposite phenotype is observed when mTORC1 is inhibited by rapamycin or knockdown of hepatic insulin receptor [127]. Further, E2F2, a transcription factor that regulates the G1/S transition during the cell cycle, binds to the *PCSK9* promoter region. Feeding and high cellular cholesterol levels can stimulate E2F2 and consequently increase *PCSK9* expression [128]. Tao et al. [129] reported that forkhead transcription factor FoxO3 can recruit deacetylase Sirt6 to the proximal promoter region of *PCSK9*, which deacetylates histone H3 and consequently suppresses *PCSK9* expression. These transcriptional factors can regulate the expression of *PCSK9* separately and/or cooperatively since mutation of the HNF1 site reduces the action of both HNF1 and SREBP2 on *PCSK9* transcription [125]

At the post-transcriptional level, the 3'--untranslated region (UTR) of PCSK9 contains putative microRNA (miRNA) binding sites for miR-191, miR-222, and miR-224. Expression of these miRNAs significantly reduces mRNA and protein levels of *PCSK9* in HepG2 cells [130]. Posttranslationally, PCSK9 is N-glycosylated at Asn533 in the C-terminal domain and sulfated in the prodomain and catalytic domain. However, inhibition of the glycosylation and sulfation has no effect on PCSK9 autocleavage, secretion, and activity [24, 26, 131]. PCSK9 is also partially phosphorylated at Ser47 and Ser688 in a cell-type-dependent manner [132]. This posttranslational modification, however, is not necessary for PCSK9 function since PCSK9 can be efficiently processed and secreted from HEK293 cells and CHOK1 cells, in which PCSK9 is either poorly or not phosphorylated [132]. On the other hand, PCSK9 is phosphorylated at Ser as positions 47, 666, 668, and 688 by Farm20C in hepatocytes, and this phosphorylation significantly increases PCSK9 secretion and its ability to stimulate LDLR degradation [133]. These findings indicate a cell-type specific effect of PCSK9 phosphorylation relating to its function. In addition, PCSK9 is cleaved by furin at RFHR218↓QA to generate a truncated form that can be secreted to an extracellular milieu such as a culture medium and serum but loses the ability to promote LDLR degradation [131, 134]. The lost-of-function mutation A443T shows an increased susceptibility to furin cleavage [131].

PCSK9-promoted LDLR degradation is regulated by different cofactors. Circulating PCSK9 binds to LDL, but not to HDL or VLDL, through its N-terminal region (amino acid residues 31 to 52). Kosenko et al. observed that approximately 40% of plasma PCSK9 stays in its LDL-bound form [135]. Plasma levels of LDL are much higher than those of PCSK9. The reason why more than half of plasma PCSK9 remains as the LDL-free form is unclear. Further, the physiological significance of this association is unknown, but the binding of LDL inhibits PCSK9's ability to bind and degrade LDLR [135, 136]. LDL also can suppress PCSK9-mediated LDLR degradation through a direct association with cell surface heparin-like molecules, interfering with HSPG-facilitated binding of PCSK9 to LDLR [75, 137]. In addition, GRP94 can bind to the C-terminus of PCSK9 and block its binding to LDLR in the ER, protecting the early degradation of LDLR. The lack of GRP94 in mouse liver leads to a significant reduction in hepatic LDLR levels and an increase in plasma LDL-C levels [138]. The C-terminal domain of PCSK9 also directly interacts with annexin A2, which subsequently inhibits the extracellular PCSK9-promoted LDLR degradation. The high expression of annexin 2 in fibroblasts and COS-7 cells may account for PCSK9-resistance in these cells

[139]. On the other hand, the progestin and adipoQ receptor 3 associate with the prodomain of PCSK9 and the YWTD domain of LDLR probably in the early endosome, enhancing their interaction and consequently promoting PCSK9-mediated LDLR degradation [97]. In addition, matrix metalloproteinase-2 can associate with and cleave PCSK9, inhibiting PCSK9-promoted LDLR degradation [140].

The half-life of circulating PCSK9 is very short. Approximately 90% of PCSK9 is cleared from the blood within 15 min in the wild-type mice with a half-life of five min [71, 73]. Conversely, the half-life of PCSK9 in *Ldlr*$^{-/-}$ mice is 15 min [71]. *Ldlr*$^{-/-}$ mice also show a tenfold increase in plasma levels of PCSK9, whereas LDLR transgenic mice clear PCSK9 much faster compared to the wild-type mice [73]. We have shown that the PCSK9/LDLR complex was delivered to the lysosome for degradation after endocytosis [61]. Thus, PCSK9 may be quickly removed from circulation and then delivered for lysosomal degradation via the hepatic LDLR pathway. The LDLR-independent mechanism of PCSK9 clearance is currently unclear. Spotlitu et al. reported that hepatic glucagon receptor signaling activated the exchange protein activated by cAMP-2 (Epac2) and the ras-related protein-1 (Rap1) pathway, and then enhanced the lysosomal degradation of PCSK9 in a LDLR-independent pathway [141]. It is also possible that the other LDLR family members, such as VLDLR, may mediate PCSK9 clearance when LDLR is absent.

9.6 Pharmacotherapeutic Inhibition of PCSK9 and Perspectives

Plasma levels of LDL-C are positively correlated with the risk of atherosclerosis [2]. Statins reduce cardiovascular events by 20% to 40%. Evidence is also mounting that people with severe dyslipidemia or who are at high cardiovascular risk fail to achieve LDL-C targets even with high-intensity statin treatment [142]. Further, 15% of statin-treated people show statin intolerance [142]. Thus, there is an urgent need for an alternative strategy to reduce plasma LDL-C.

Gain-of-function PCSK9 mutations such as S127R, F216L, and D374Y are associated with an increase in plasma levels of mean LDL-C and the incidence of coronary heart disease [7, 143]. Conversely, subjects carrying loss-of-function PCSK9 mutations Y142X or C679X display a 40% reduction in plasma levels of mean LDL-C and an 88% reduction in the risk of coronary heart disease. Loss-of-function mutation R46L reduces plasma levels of LDL-C and the incidence of coronary heart disease by 21% and 47%, respectively, as shown in the Atherosclerosis Risk in Communities study and the Dallas Heart Study [11, 49]. Data from the Copenhagen General Population Study and the Copenhagen City Heart Study also shows that loss-of-function PCSK9 mutations R46L, R237W, I474V, and E670G are associated with a significant reduction in mean LDL-C (18%) and cardiovascular mortality [144]. A 15-year follow-up study of 4232 subjects (2039 men and 2193 women, all 60 years old at recruitment) demonstrates that serum levels of PCSK9 are positively associated with the future risk of cardiovascular disease [145]. Knockout of PCSK9 increases, while overexpression of PCSK9 reduces the development of atherosclerosis in *apoE*$^{-/-}$ mice [146]. Further, statins increase expression of *LDLR* and *PCSK9*. Elevated circulating PCSK9 levels then promote LDLR degradation, attenuating the lipid-lowering effect of statins. Plasma PCSK9 levels are increased in patients treated with atorvastatin, and *Pcsk9*$^{-/-}$ mice display hypersensitivity to statin treatment [56]. Together, these findings strongly indicate the potential of PCSK9 inhibition as a lipid-lowering strategy.

Currently, two monoclonal anti-PCSK9 antibody therapies, Repatha (evolocumab) and Praluent (alirocumab), are approved in the USA, Canada, Europe, and China for patients who have hereditary high cholesterol such as heterozygous and homozygous FH patients and high-risk patients intolerant to statins or experiencing poor LDL-C-lowering response even with high-intensity statin therapy. Both antibodies are

against the catalytic domain of PCSK9 and block binding of plasma PCSK9 to LDLR, increasing hepatic clearance of LDL and reducing plasma levels of LDL-C. Subcutaneous administration of 150 mg alirocumab biweekly lowers plasma levels of LDL-C approximately 60% in patients and reduces the rate of main cardiovascular events from 3.3% to 1.7% [147]. Alirocumab at a dose of 75 mg once every two weeks also reduces the incidence of recurrent ischemic cardiovascular events in patients who have a previous acute coronary syndrome and are treated with maximally tolerated statin dosages [148]. Similarly, the FOURIER trial shows that evolocumab at a dose of 140 mg biweekly or 420 mg monthly leads to a 60% reduction in plasma levels of LDL-C and significantly reduces the risk of the primary end point (9.8% vs. 11.3%) and the main secondary end point (5.9% vs. 7.4%) as compared to the placebo group [149]. The two inhibitors do not show significant major side effects.

Inclisiran (ALN-PCSsc) is a chemically modified small interfering RNA (siRNA) inhibitor that targets PCSK9 mRNA and suppresses translation of PCSK9. It reduces hepatic PCSK9 production and plasma PCSK9 levels. A subcutaneous injection of 500 mg Inclisiran once every 6 months in patients with atherosclerotic cardiovascular disease on high-intensity statin therapy reduces plasma levels of LDL-C by approximately 50%. No major side effect has been reported in both Phase I and II trials [150, 151].

The monoclonal antibodies against PCSK9 show an impressive lipid-lowering effect in heterozygous and homozygous FH patients, high-risk patients intolerant to statins, and patients with poor LDL-C-lowering response even with maximally tolerated statin dosages [147, 152, 153]. Furthermore, when adding to the statin therapy, PCSK9 inhibitors can markedly reduce cardiovascular events, such as myocardial infarction and ischemic stroke, with no significant adverse side effects [147, 152]. However, this therapy requires injections of large amounts of antibodies to achieve clinical efficacy, with extremely high production costs. Given that the treatment of patients with hypercholesterolemia is lifelong, and it is predicted that PCSK9 inhibitors would cost approximately $592 billion but reduce cardiovascular care costs by only $29 billion on US health care spending over 5 years if used for all eligible patients at current pricing [154]; this treatment will place a high burden on the healthcare system. Inclisiran might reduce costs since it requires only two injections per year. However, siRNAs are small RNA duplexes that have 20–30 nucleotides. They interact with the RNA-induced silencing complex (RISC) in the cytosol. After cleavage of the sense strand by the endonuclease Argonaute 2 in the RISC, the antisense strand remains binding to RISC and guides the complex to the target mRNA for Argonaute 2-mediated cleavage. siRNAs silence target genes more specifically as compared to miRNA, since the antisense strand of siRNA duplexes theoretically only binds to mRNA that completely matches to it. However, it has been reported that siRNA can cause off-target translational inhibition [155]. In addition, duplex siRNA can trigger innate immune response in Toll-like receptor (TLR)-dependent and -independent mechanisms [156]. Considering the lifelong use of PCSK9 inhibitors, it is important to monitor the long-term safety of Inclisiran. Therefore, the need for more effective, more specific, and more cost-efficient therapies to lower LDL-C is urgent.

Crystallographic studies of PCSK9-EGF-AB complex show that the interaction face between the catalytic domain of PCSK9 and the EGF-A of LDLR is relatively flat and big, making it impossible to design a specific inhibitor to block the interaction between PCSK9 and LDLR [20]. Therefore, mechanistic studies of PCSK9 regulation, its secretion, and its ability to promote LDLR degradation are necessary. Questions need to be elucidated including, but not limited to the following:

1. PCSK9 is a serine proteinase. Currently, the only physiological substrate of PCSK9 is itself. Can PCSK9 cleave other proteins?
2. PCSK9 is expressed extrahepatically and most likely retained inside cells in the kidneys and the intestine. What are the physiological functions of PCSK9 in these tissues?

3. How does circulating PCSK9 preferentially stimulate degradation of hepatic LDLR?
4. Why is only hepatic PCSK9 efficiently secreted into circulation? What is the molecular machinery system that assists PCSK9 secretion?
5. It is believed that PCSK9 needs assistance from other proteins to efficiently redirect LDLR to the lysosome for degradation. What are these co-factors?
6. PCSK9 is expressed in the brain and present in cerebrospinal fluid (CSF). CSF PCSK9 levels are increased in patients with AD [157]. What are the physiological and pathophysiological roles of brain PCSK9?

Answering these questions will not only deepen and widen our understanding of the physiological and pathophysiological role of PCSK9 but also provide a foundation for the future development of PCSK9-specific small inhibitors that can lower plasma LDL cholesterol efficiently, specifically, and cost-effectively.

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