HHS Public Access

Author manuscript

Atherosclerosis. Author manuscript; available in PMC 2016 February 01.

Published in final edited form as:

Atherosclerosis. 2015 February ; 238(2): 264–270. doi:10.1016/j.atherosclerosis.2014.12.017.

On the Function and Homeostasis of PCSK9: Reciprocal Interaction with LDLR and Additional Lipid Effects

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Abstract

Proprotein convertase subtilisin kexin type 9 (PCSK9) is a circulatory ligand that terminates the lifecycle of the low-density lipoprotein (LDL) receptor (LDLR) thus affecting plasma LDL-cholesterol (LDL-C) levels. Recent evidence shows that in addition to the straightforward mechanism of action, there are more complex interactions between PCSK9, LDLR and plasma lipoprotein levels, including: (a) the presence of both parallel and reciprocal regulation of surface LDLR and plasma PCSK9; (b) a correlation between PCSK9 and LDL-C levels dependent not only on the fact that PCSK9 removes hepatic LDLR, but also due to the fact that up to 40% of plasma PCSK9 is physically associated with LDL; and (c) an association between plasma PCSK9 production and the assembly and secretion of triglyceride-rich lipoproteins.

The effect of PCSK9 on LDLR is being succesfuly utilized toward the development of anti-PCSK9 therapies to reduce plasma LDL-C levels. Current biochemical research has uncovered additional mechanisms of action and interacting partners for PCSK9, and this opens the way for a more thourough understanding of the regulation, metabolism, and effects of this interesting protein.

Introduction

Proprotein convertase subtilisin/kexin 9 (PCSK9) is a circulating serine protease that efficiently binds low-density lipoprotein (LDL) receptor (LDLR) leading to its intracellular degradation, thus increasing plasma LDL-cholesterol (LDL-C) levels (1). Gain-of-function mutations in PCSK9 are a cause of autosomal dominant hypercholesterolemia (2) while loss-of-function mutations are associated with low LDL-C and low lifetime risk of cardiovascular disease (CVD) (3). Inhibiting PCSK9 production with genetic approaches (4) or the interaction of PCSK9 with LDLR using monoclonal antibodies (5, 6) significantly lowers LDL-C levels, and is an active area of clinical investigation. Recent comprehensive

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reviews have summarized the history of PCSK9 and the classical mechanism of action with relation to cardiovascular health (7, 8). This paper is a part of a review series on PCSK9 covering clinical studies and physiology of the protein. In this review, we will summarize the most recent findings on PCSK9 regulation and function based on its reciprocal interaction with LDLR and on LDLR-independent effects on plasma lipid metabolism. These novel finding are expected to help uncover the full physiological role of PCSK9.

The Unexpected Complexity of the PCSK9-LDLR Axis

PCSK9 and LDLR are both under the regulation of sterol regulatory element binding proteins (SREBPs), being over-expressed under conditions of cellular cholesterol deficiency (9). The most common cause of cellular cholesterol deficiency is treatment with a statin agent (10). Thus, although those taking statins experience a large LDL-C reduction due to the over-expression of LDLR, it is likely that this effect is diminished by the concomitant increase in PCSK9 (11, 12). The parallel expression pattern of PCSK9 and LDLR is represented in Figure 1A. In addition, PCSK9 and LDLR also share a common clearance pattern, as PCSK9 is a ligand for LDLR, and the interaction terminates the lifecycle of both proteins through targeting and degradation of the ligand-receptor pair in the lysosome (Figure 1B).

To study the regulatory mechanism and physiology of PCSK9, several mouse models were developed, including: (1) PCSK9-deficient mice, which show lower cholesterol because of over-abundance of LDLR (13); (2) mice over-expressing PCSK9 through adenoviral infection, which show increased cholesterol levels (14, 15); and (3) transgenic models expressing human PCSK9 or some of its gain-of-function mutants (such as D374Y), which also show increase cholesterol levels (16, 17). These models have confirmed that the overall impact of PCSK9 on LDLR and cholesterol metabolism in mice is similar to that observed in humans, and they have validated the use of the mouse to study the physiology of PCSK9. However, the extreme circumstances of PCSK9's absence or its tremendous over-expression have limited applicability to the physiologic regulation, metabolism, and mechanism of action of this protein in humans (17-19). We developed transgenic lines of mice expressing normal human PCSK9 (20) that accumulates in the circulation within the physiologic range (21). In this model, the co-expression of both murine and human PCSK9 at near normal levels served as tool to study the regulation of plasma levels of PCSK9 vis-a-vis its interaction with LDLR. For example, we observed that LDLR-deficient mice had high levels of murine PCSK9 and that expression of the human PCSK9 transgene increases murine PCSK9 in wild type mice to the levels seen in LDLR-deficient mice (21). These results allow the visualization of a homeostatic pathway where the primary absence of LDLR leads to accumulation of PCSK9 in plasma (no receptor, ligand accumulates). LDLR depletion caused by expression of human PCSK9 also leads to accumulation of PCSK9. In this scenario, the excess human PCSK9 engages LDLR and reduces its abundance, which in turn causes accumulation of murine PCSK9. Similar results were recently reported using over-expression of the inducible degrader of LDLR (IDOL), a ubiquitin ligase involved in the terminal disposition of aged LDLR protein (22). IDOL expression in normal mice caused a drastic reduction in the levels of surface LDLR, due not only to direct degradation of LDLR but also to increased accumulation of PCSK9 in plasma caused by the primary loss of

LDLR. In support of this interpretation, no effect on PCSK9 levels was seen when IDOL was expressed in mice lacking LDLR (22) (Figure 1C). These findings support a scenario where the partial reduction of hepatic LDLR results in the lack of clearance of plasma PCSK9, leading to PCSK9 accumulation and further reduction in LDLR caused by elevated PCSK9 levels. In contrast, low levels of functional PCSK9 (naturally for loss-of-function mutations; therapeutically because of PCSK9 inhibition (23-25)) are followed by increased concentrations of surface LDLR, which in turn further removes plasma PCSK9, thus setting the stage for chronic low cholesterol levels (21) (Figure 1D). These data clearly show that the relationship between PCSK9 and LDLR goes beyond the expected linear pathway (PCSK9 leads to LDLR degradation) and into the domain of full reciprocal regulation (LDLR is the receptor through which the ligand PCSK9 leaves the circulation).

Support for a reciprocal regulation mechanism is also evident in humans. Subjects with familial hypercholesterolemia (FH), heterozygotes and to a greater extent homozygotes, show significantly higher levels of plasma PCSK9 compared with normo-cholesterolemic controls (26), suggesting that the loss of the receptor LDL-binding function may also impair the clearance of PCSK9 from the circulation. In addition, a common polymorphism (rs688) at exon 12 of the LDLR is known to cause low LDLR levels and impaired response to PCSK9 antibody treatment (27). Given the totality of murine and human data, it seem reasonable to hypothesize the possibility of a heterozygous LDLR mutation with impaired PCSK9 binding. This would cause or aggravate hypercholesterolemia via PCSK9 accumulation in plasma and enhanced degradation of the normal LDLR allele product.

Although this reciprocal regulation mechanism is operational both in mice and humans, the extent to which LDLR simultaneously regulates plasma levels of PCSK9 and LDL seems to differ between species, with murine LDLR having a greater effect on PCSK9 than on LDL, and human LDLR showing the opposite. The absence of LDLR in the mouse leads to PCSK9 levels 10 times above normal and LDL-C levels barely twice normal (for a delta ratio PCSK9/LDL-C of about 5), whereas the absence or near absence of LDLR in humans leads to PCSK9 levels barely twice normal and LDL-C levels 4-5 times above normal (for a delta ratio PCSK9/LDL-C of about 0.5). Of course, part of this difference might be because in LDLR-deficient mice there is no remaining LDLR function whereas most homozygous FH patients not only have some residual LDLR function but the LDLR mutations they carry might not be impaired in binding PCSK9. This idea is supported by a two small trials of anti-PCSK9 antibody therapy in subjects with subjects with homozygous FH-causing LDLR mutations, most of whom responded with significant LDL-C lowering (28, 29). This suggests that circulating PCSK9 in these subjects is still active (as ligand) on an LDLR that is extremely dysfunctional in binding and internalizing LDL. Interestingly, in the few patients characterized to have true null homozygous FH (near complete absence of LDLR; less than 2% of normal function) anti-PCSK9 therapy did not affect plasma LDL-C levels in both trials (28, 29).

Due to the reciprocal regulation mechanism between PCSK9 and LDLR, it is reasonable to assume that anti-PCSK9 therapies will not only prevent the degradation of LDLR by PCSK9 but will also tip the homeostatic balance between PCSK9 and LDLR to create a new favorable steady state of high LDLR and low PCSK9 levels. This finely regulated balance

may help explain the unexpectedly low LDL-C levels in people with PCSK9 loss-of-function mutations, the ultra low LDL-C levels in subjects without circulating PCSK9 (3, 30), and the exaggeratedly high efficiency of PCSK9-blocking antibodies in reducing LDL-C levels (31).

PCSK9 and Friends

Protein interacting partners can influence versatility, concentration, and function of circulating and cellular proteins (32, 33). The modulating action of interacting partners on a given protein can involve a multitude of functional effects, including activation, inhibition, prolongation of function, and stabilization (34, 35). Some interactions may also be necessary for a protein to bind to its substrate or for directing it to its site of action or degradation (36). Annexin A2 (37), amyloid precursor like protein 2 (38) and intracellular apoB (39) have been shown to interact with PCSK9, and the potential physiologic roles of these interactions have been recently reviewed (7). As PCSK9 is a secreted protein with tremendous regulatory power over body cholesterol trafficking, the identification of protein partners that modulate its action may yield clues to its physiology and additional avenues for therapeutic modulation.

Interestingly, plasma lipoproteins act as important extracellular partners for PCSK9, and may impact its function. In 2008, we were the first to show that PCSK9 associates with LDL-sized particles both in vivo and in vitro (20). Later, several laboratories including ours (21, 39-41) proved the existence of PCSK9-LDL complexes both in mouse and human plasma. Quantification of PCSK9 binding shows that 20% to 40% of total PCSK9 in plasma associates with LDL, with a Kd of 325nM (40-42). However, the stoichiometry of the interaction also suggests that only one in 500 to 1000 LDL particles carries a PCSK9 molecule (42). It must be noted that earlier work from Alborn *et al*. (43) suggested that PCSK9 does not associate with lipoproteins. This was later shown to be an artifact caused by the dissociation of PCSK9 from LDL in the high-salt conditions (KBr) of density gradient ultracentrifuge (UC) (21). PCSK9 interaction with LDL suggests that the well-documented correlation between PCSK9 and LDL-C levels in plasma (26, 44) is not solely due to PCSK9 action on the LDLR, but is also a consequence of the direct interaction between PCSK9 and LDL. Thus, not only it is true that PCSK9 and LDLR interact to modulate plasma LDL-C levels, but it is also likely that primary, non-LDLR-dependent LDL-C changes (eg, hypobetalipoproteinemia) will modulate plasma PCSK9 levels and maintain a low cholesterol state through this additional mechanism.

The clinical significance of the PCSK9 association with LDL was demonstrated in FH patients undergoing lipoprotein apheresis (LA) treatment using dextran-sulfate cellulose beads column, a method to remove apoB-containing lipoproteins from plasma (42). We, and others, have found that more than 50% of plasma PCSK9 is removed after one standard LA session (41, 42, 45). The loss of PCSK9 during LA was mainly due to the removal of LDL-bound PCSK9. The removal of PCSK9 during LA not only provides additional proof that PCSK9 binds to LDL, but it also suggests that PCSK9 lowering by apheresis likely synergizes with LDL removal to keep LDL levels down over time. It remains to be

determined whether regular anti-PCSK9 therapy may benefit patients undergoing routine LA by reducing the frequency of sessions or the need for LA altogether.

A different line of evidence to support the clinical significance of PCSK9 binding to LDL comes from the study of the two main molecular forms of PCSK9 in plasma: (1) a 62-kDa band, representing the full-length (intact) plasma protein (only missing the pro-domain); and (2) a 55-kDa fragment, product of cleavage of the 62-kDa protein by the protease Furin. Furin-cleaved PCSK9 is generally considered less active than the intact form (46-48), although evidence can be found that Furin-cleaved PCSK9 maintains some residual ability to remove surface LDLR (49). We, and others, have shown that a significant amount of the intact PCSK9 is on the LDL particle (20, 21, 39, 41), whereas the Furin-cleaved form is found mainly in the apoB-free fraction (including HDL and lipoprotein-free serum) (41, 42). Therefore, it is tempting to speculate that the most active form of PCSK9 is carried by the canonical ligand for the LDLR, and that the termination of the lifecycle of LDLR is controlled by a stochastic system based on the rare LDL particle (1 in 500 or so) that carries PCSK9. To make the matter more complicated, one in vitro study suggested that LDL inhibits the action of PCSK9 on LDLR (40) but does not affect the binding of PCSK9 to the epidermal-growth factor repeat A (EGF-A) of the LDLR. Thus, the clinical significance of PCSK9 association to LDL remains to be determined. On a technical level, it must be kept in mind that current ELISA assays only measure total PCSK9 levels in plasma. However, we predict that concentrations of LDL-bound PCSK9 will become a better indicator of PCSK9 activity.

The interaction between PCSK9 and LDL is unique, as no association is evident with other apoB-containing lipoproteins such as VLDL (16, 21, 39) and chylomicrons (CM) [Fazio *et al*, unpublished]. As these lipoproteins differ from LDL both in lipids and proteins composition it remains to be determined whether the association of PCSK9 with the LDL is through protein-protein or protein-lipid interactions. On one hand, Sun *et al*. have shown that PCSK9 interacts with intracellular apoB (39) and Kosenko *et al*. showed that PCSK9 binds to the LDL at a single molecular site (40), suggesting a protein-protein interaction. On the other hand, the lipophilic nature of the pro-domain of PCSK9, a region previously shown to mediate PCSK9 binding to LDL (40), suggests that PCSK9 interaction with the LDL may be lipid-dependent. It is also important to mention that in theory PCSK9 may also bind to Lp(a), a lipoprotein particle compositionally similar to LDL and which responds with significant reductions to anti-PCSK9 therapy. However, no experimental evidence has thus far been provided that PCSK9 associates with Lp(a).

Aside from LDL, PCSK9 may have other interacting partners in plasma that can modulate its activity. Resistin is a small protein, secreted by human macrophages and murine adipocytes, that increases PCSK9 expression in liver cells (50). Resistin levels are increased in obesity, may contribute to insulin resistance and inflammation in patients with metabolic syndrome (51, 52), and are highly correlated with atherosclerotic cardiovascular diseases in humans (53). Interestingly, the plasma of obese subjects containing high levels of resistin induce LDLR degradation via PCSK9 up-regulation when tested in hepatic cell lines (50). The similarity between the C-terminal histidine-rich domains (CHRD) of PCSK9 and

resistin suggests the possibility of a resistin-PCSK9 association, although such an interaction has not yet been demonstrated.

PCSK9 effects on triglyceride metabolism

One area of study that is gaining traction is the effect of PCSK9 on lipoprotein assembly and secretion, with emerging evidence having identified effects of PCSK9 on triglyceride-rich lipoproteins (TRL), an additional lipid risk factor for coronary heart disease (39, 54, 55). TRL remnants are directly involved in the pathogenesis of atherosclerosis as they are taken up by arterial macrophages and cause massive cholesterol loading (56, 57). Recent clinical studies show that PCSK9 levels associate with levels of TRL markers (58, 59) in humans. Studies in large populations showed that plasma PCSK9 levels associate with plasma triglycerides in both genders (60-62). Consistent with this observation, subjects carrying a unique PCSK9 gain-of-function mutation showed a three-fold elevation in apoB100 production rates compared with normal individuals (54). Several mouse studies have also shown a direct causal role of PCSK9 on TRL metabolism based on liver-specific effects: (1) acute adenoviral PCSK9 overexpression in fasted mice causes hypertriglyceridemia due to dramatically increased hepatic VLDL-triglyceride and apoB production rates (63); (2) second-generation adenoviruses, which induce moderate chronic PCSK9 overexpression, showed significant stimulatory effects of PCSK9 on hepatic VLDL production (39); and (3) transgenic expression of human PCSK9 in mice increased secretion of hepatic TRL (21). Additionally, changes in hepatic VLDL production parallel those of PCSK9 production in hepatocytes incubated with different lipoproteins (64)

The intestine is a another major source of TRL, accounting for a significant portion of plasma lipids in the form of postprandial CM with apoB48 as an integral component required for CM assembly and secretion (65, 66). PCSK9 expression in the intestine is quantitatively second only to its hepatic expression (67), and knockdown of PCSK9 was shown to reduce apoB48 secretion (13) and protect mice from postprandial hypertriglyceridemia (68). This is an indication of the therapeutic potential of PCSK9 inhibition in reducing intestinal TRL production, postprandial hypertriglyceridemia, and plasma triglyceride levels. We, and others, have shown that PCSK9 increases levels of both cellular and secreted apoB48- and apoB100-TRL in human cultured enterocytes (Caco-2) (68-70). Mechanisms to explain PCSK9 stimulation of apoB-TRL by enterocytes include significant transcriptional influences (69, 70), somewhat contrary to the established knowledge that apoB is regulated mostly at the post-transcriptional level (71). It has been previously shown that LDLR can directly affect apoB stability, as it promotes post-translational degradation of apoB in hepatocytes, consequently reducing TRL particle secretion (72). As expected, PCSK9 reduces LDLR levels in enterocytes thus, directly increasing apoBTRL secretion (68-70). PCSK9 also increases intracellular neutral lipids via de-novo cellular triglyceride biosynthesis mediated by affecting cholesterol transporters (e.g. CD36 and NPC1L1), SRE gene activation, and activity of MTP (69, 70). MTP is an endoplasmic reticulum protein with a critical role in transferring the lipid droplet to apoB, thus ensuring intracellular stability of apoB (73, 74). The underlying mechanism leading to these effects of PCSK9 are unknown. We, and others, have shown that a portion of PCSK9 entering the cell with LDLR remains intact and does not undergo lysosomal degradation for

several hours (21, 75). A mechanism to explain how PCSK9 is able to alter intracellular processes is presented in Figure 2. It is also important to remember that the S127R mutation of PCSK9 results in a non-secreted protein that can accumulate in the cell (76) and acts as a gain-of–function mutation through increasing apoB production (54), suggesting an important role for intracellular PCSK9 in apoB-TRL secretion.

It is possible that PCSK9 may have additional targets, such as VLDL-receptor (77), ApoE receptor 2 (77) and LDLR-related protein 1 (78), possibly through mechanisms similar to LDLR degradation, since all these receptors contain an EGF-A domain (79). PCSK9 was also shown to affect surface scavenger receptor CD36 levels (70) and the tetraspanin receptor CD81 (80). In addition, PCSK9 also reduced the expression of beta-secretase 1 (BACE1) (81) and the Endothelial Sodium Channel (ENaC) (82) through ER/Golgi interactions. The above observations indicate that a deeper investigation of non-canonical targets of PCSK9 is needed to uncover the full physiological role, especially with the likely approval of anti-PCSK9 therapies in the near future (83).

Summary

The discovery of PCSK9 has changed our understanding of body cholesterol metabolism from a system exclusively controlled by intracellular processes, to one eminently regulated by a circulatory protein. Humans with loss-of-function of PCSK9 have extremely low levels of plasma LDL-C, and even small LDL-C reduction due to common mutations in PCSK9 have been shown to reduce lifetime CVD events by 80% (84). Similarly, anti-PCSK9 therapies are exceptionally effective in lowering LDL-C levels (8). Potential explanations of this pivotal role for PCSK9 in lipid metabolism includes: (1) a reciprocal regulation mechanism that leads to an extreme increase in LDLR levels upon removal of PCSK9, and vice-versa; (2) a direct role for LDL particles as they carry an active form of PCSK9 in plasma, and; (3) additional metabolic effects such as the impact of PCSK9 on TRL production rate. Although PCSK9 is being extensively studied at the clinical level, it is important to remember that this pathway is a newly discovered and unexpectedly over-arching regulator of cholesterol trafficking, with still plenty of gaps in our understanding of its full physiologic function.

Acknowledgements

This study was supported by the National Institutes of Health (National Heart, Lung, and Blood Institute) through grant R01-HL106845 to Dr. Fazio.

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Highlights

We review recent finding on PCSK9 biochemical properties focusing on:

- Physical association between PCSK9 and LDL.
- Effects of PCSK9 on intestinal lipogenesis and synthesis of triglyceride-rich lipoproteins
- Modulatory interactions between PCSK9 and circulating or cellular partners
- Regulatory influences on PCSK9 and LDLR that are simultaneously parallel and reciprocal mechanisms.

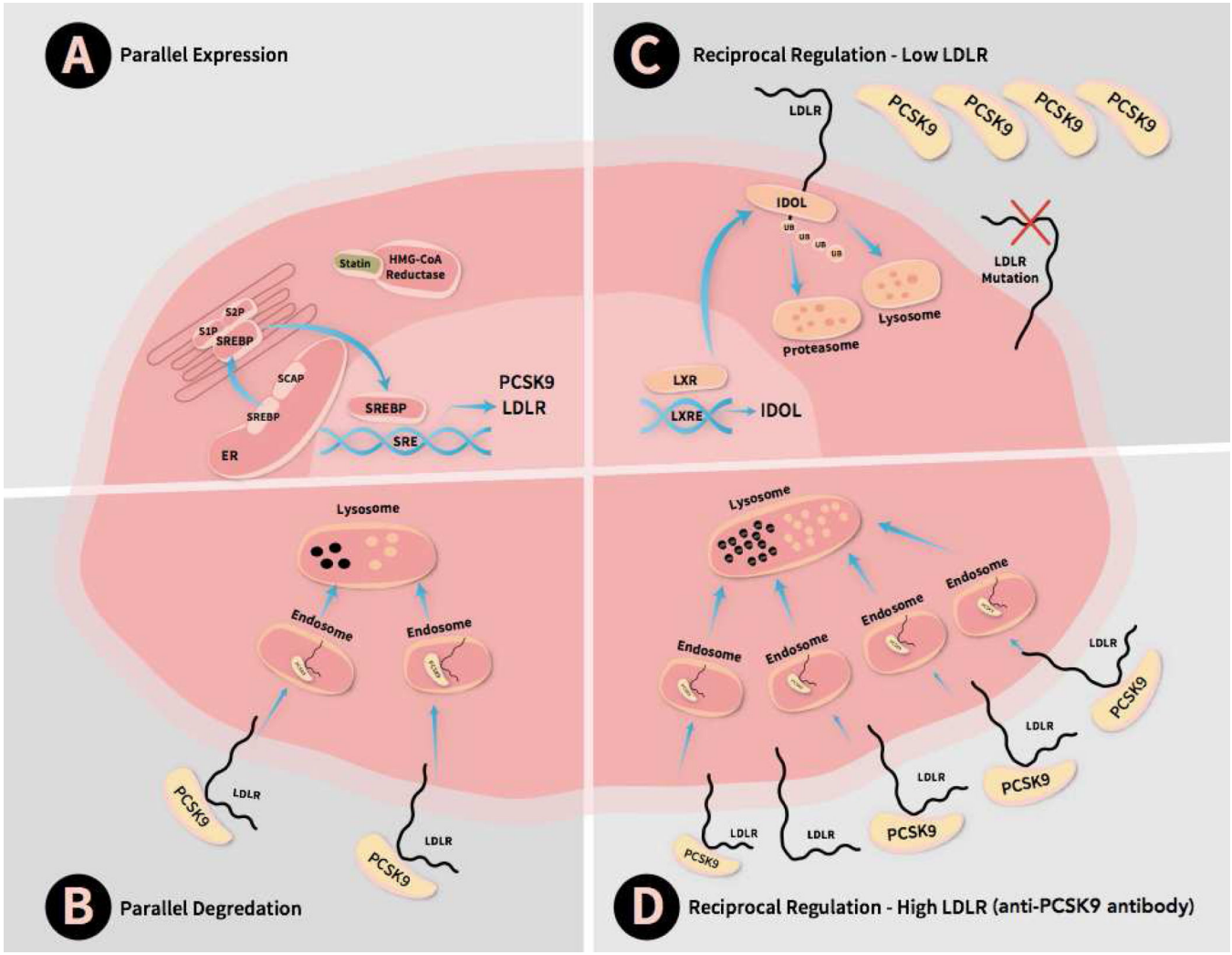

Figure 1.

Parallel and reciprocal regulation of PCSK9 and LDLR: **(A)** Parallel Expression -SREBP activation leads to increased transcription of both PCSK9 and LDLR. **(B)** Parallel Degradation - The interaction between PCSK9 and surface LDLR leads to the internalization of the LDLR-PCSK9 complex and targeting to the lysosome for degradation of both proteins. **(C)** Reciprocal Regulation, Low LDLR - Impaired PCSK9 clearance due to LDLR mutations. In addition, increased degradation of surface LDLR by IDOL can recreate this scenario. **(D)** Reciprocal Regulation, High LDLR - Blocking PCSK9 function leads to elevated levels of LDLR.

Abbreviations: 3-hydroxy-3-methyl-glutaryl-CoA, HMG-CoA; Low-Density-Lipoprotein Receptor, LDLR; Proprotein Convertase Subtilisin/Kexin 9,PCSK9; Inducible Degrader Of LDLR, IDOL; Sterol Regulator Element, SRE; SRE Binding Protein, SREBP; SREBP-Cleavage-Activating Protein, SCAP; site-1 protease, S1P; site-2 protease, S2P; Liver X Receptor, LXR; LXR Element, LXRE.

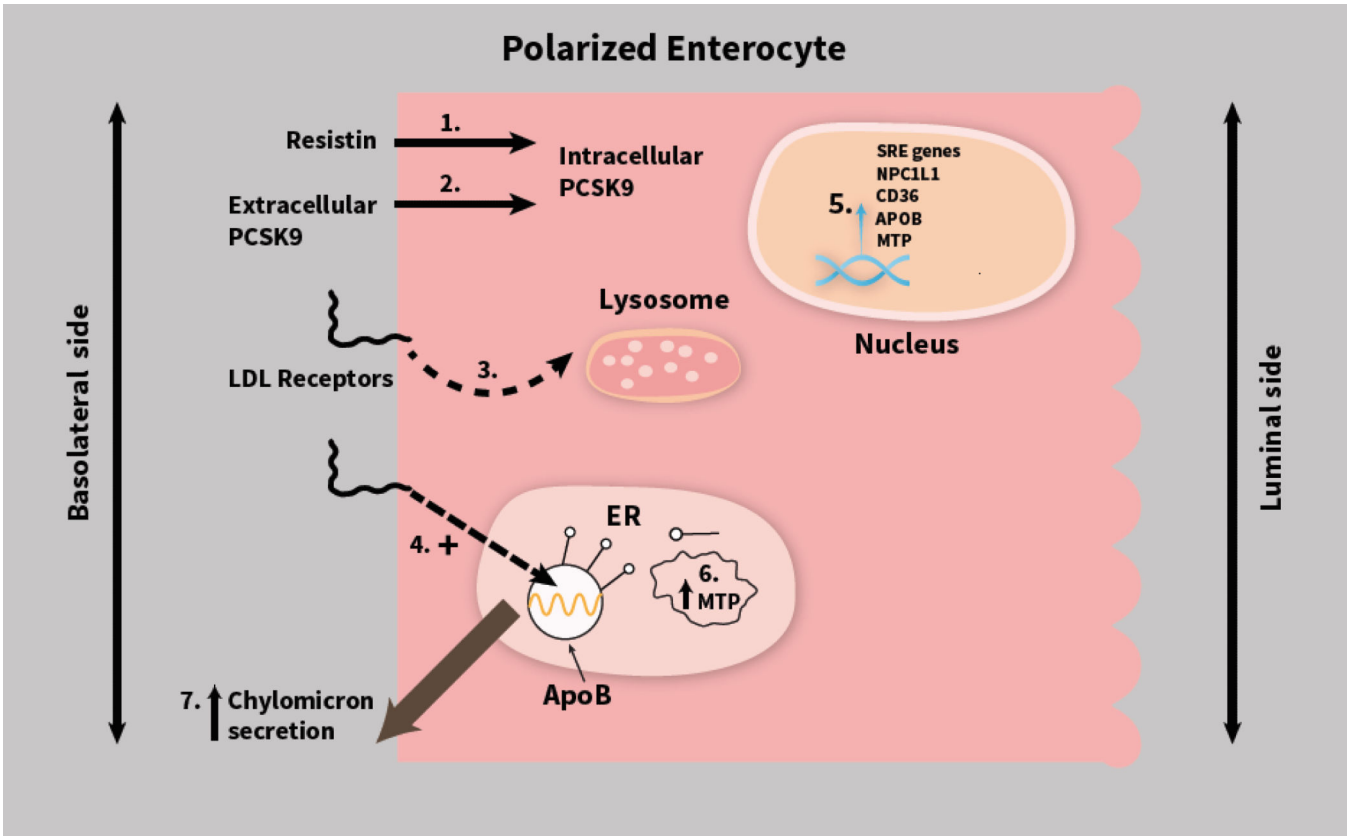

Figure 2.

The effect of PCSK9 on intestinal TRL production: **(1)** Resistin-mediated processes - Resistin stimulates the production of intracellular PCSK9. **(2)** PCSK9-mediated processes - A portion of extracellular PCSK9 protein that enters the cell is spared from degradation and alters transcriptional and post-transcriptional pathways shown in points 5, 6 and 7. **(3)** PCSK9 down-regulates cell surface LDLR by targeting it to lysosomal degradation. **(4)** Loss of LDLR increases intracellular apoB protein levels, presumably through inhibition of proteasome-mediated degradation. **(5)** PCSK9 stimulates the transcription of SRE genes, *APOB*, CD36, NPC1L1 and *MTP*. **(6)** PCSK9 increases intracellular MTP protein and enzyme activity in the intestine. **(7)** The overall effect of PCSK9 is to stimulate the levels of cellular and secreted TRL particles from enterocytes into the circulation.

Abbreviations: Chylomicrons, CM; Low-Density Lipoprotein Receptor, LDLR; Proprotein Convertase Subtilisin/Kexin 9, PCSK9; Triglyceride-Rich Lipoprotein, TRL; Apolipoprotein B, ApoB; Microsomal triglyceride transfer protein, MTP.