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Review

PCSK9: A new participant in lipophagy in regulating atherosclerosis?

Jun Xiao[a], Yi-Min Deng[a], Xiang-Rui Liu[a], Jian-Ping Cao[b], Min Zhou[a], Ya-Ling Tang[a], Wen-Hao Xiong[a], Zhi-Sheng Jiang[a], Zhi-Han Tang[a,*], Lu-Shan Liu[a,*]

[a] Institute of Cardiovascular Disease, Key Laboratory for Arteriosclerology of Hunan Province, Hunan International Scientific and Technological Cooperation Base of Arteriosclerotic Disease, University of South China, Hengyang, Hunan 421001, PR China
[b] Hunan Environmental Biology Vocational and Technical College, Hengyang, Hunan 421001, PR China

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ABSTRACT

Proprotein convertase subtilisin kexin 9 (PCSK9) regulates lipid metabolism by degrading low-density lipoprotein receptor on the surface of hepatocytes. PCSK9-mediated lipid degradation is associated with lipophagy. Lipophagy is a process by which autophagosomes selectively sequester lipid-droplet-stored lipids and are delivered to lysosomes for degradation. Lipophagy was first discovered in hepatocytes, and its occurrence provides important fundamental insights into how lipid metabolism regulates cellular physiology and pathophysiology. Furthermore, PCSK9 may regulate lipid levels by affecting lipophagy. This review will discuss recent advances by which PCSK9 mediates lipid degradation via the lipophagy pathway and present lipophagy as a potential therapeutic target for atherosclerosis.

1. Introduction

Proprotein convertase subtilisin kexin 9 (PCSK9) is a gene that was discovered in 2003, and it is important in lipid metabolism and atherosclerosis [1]. It is the ninth member of the proprotein convertase family, which consists of an N-terminal pro-domain followed by a subtilisin-like catalytic domain, a cysteine domain, and histidine-rich C-terminal domain [2,3]. The catalytic domain of PCSK9 can specifically bind to the extracellular epidermal growth factor-like repeat A (EGF-A) domain of low-density lipoprotein receptor (LDLR) [4]. LDLR, as the major apolipoprotein B (apoB)–lipoprotein metabolic receptor, plays a crucial role in cholesterol metabolism by mediating cholesterol-rich LDL uptake and utilization by various cells. After LDL binds to LDLR, the LDL–LDLR complex is internalized into clathrin-coated pits. In the low-pH environment of the endosome, LDLR dissociates from LDL. The LDLR is then recirculated back to the cell membrane, and LDL is delivered to the lysosome for degradation [5]. PCSK9 can mediate the internalization of LDLR after it binds with the LDL–LDLR complex [6]. Compared with LDL, PCSK9 has a higher affinity for LDLR in the low-pH environment of the endosome [7], and the PCSK9–LDLR complex is more closely combined than the LDL–LDLR complex. Subsequently, the PCSK9-LDLR complex is transported to the lysosome for degradation, thus preventing LDLR from being recycled to the cell membrane, but its precise mechanism needs to be elaborated.

Lipophagy, as an important pathway for intracellular lipid metabolism, is a selective form of autophagy [8]. Autophagy refers to the degradation of intracellular lipids, proteins or damaged organelles in the lysosomes [9], while lipophagy refers to the selective recognition and degradation of intracellular lipids by the macroautophagic pathway [10]. Intracellular lipids are mainly composed of triglycerides (TG) and cholesterol esters (CE), and exist as lipid droplets (LDs), whose catabolism occurs via lipophagy. During lipophagy, intracellular lipids are first engulfed by autophagosomes and transported to the lysosomes. Then, within the lysosomes, lysosomal acid lipase (LAL) hydrolyzes TG and CE into free fatty acids (FFA) and free cholesterol (FC) to provide energy for mitochondrial β oxidation [11]. Thus, lipophagy can regulate intracellular lipid content to prevent lipid deposition and maintain a high level of β oxidation to provide energy and prevent cell death. Notably, lipophagy is not exactly equivalent to autophagy. First, autophagy is a non-selective degradation process to degrade intracellular lipids and proteins, whereas lipophagy can selectively degrade intracellular lipids. Second, autophagy is primarily involved in an array of lysosomal hydrolases, whereas lipophagy is mainly involved in LAL.

Intracellular LDL can be degraded by lipophagy [12]. LDL is mainly consumed by cells through LDLR-mediated endocytosis on the cell surface in which LDL induces lipophagy by binding to LDLR. In addition, PCSK9 binds to LDLR and mediates the degradation of LDLR. Hence, PCSK9 may participate in the LDL–LDLR-induced lipophagy by degrading LDLR, thereby regulating lipid metabolism. Notably, PCSK9

* Corresponding authors.
E-mail addresses: Tangzhihan98@163.com (Z.-H. Tang), liuls2000@163.com (L.-S. Liu).

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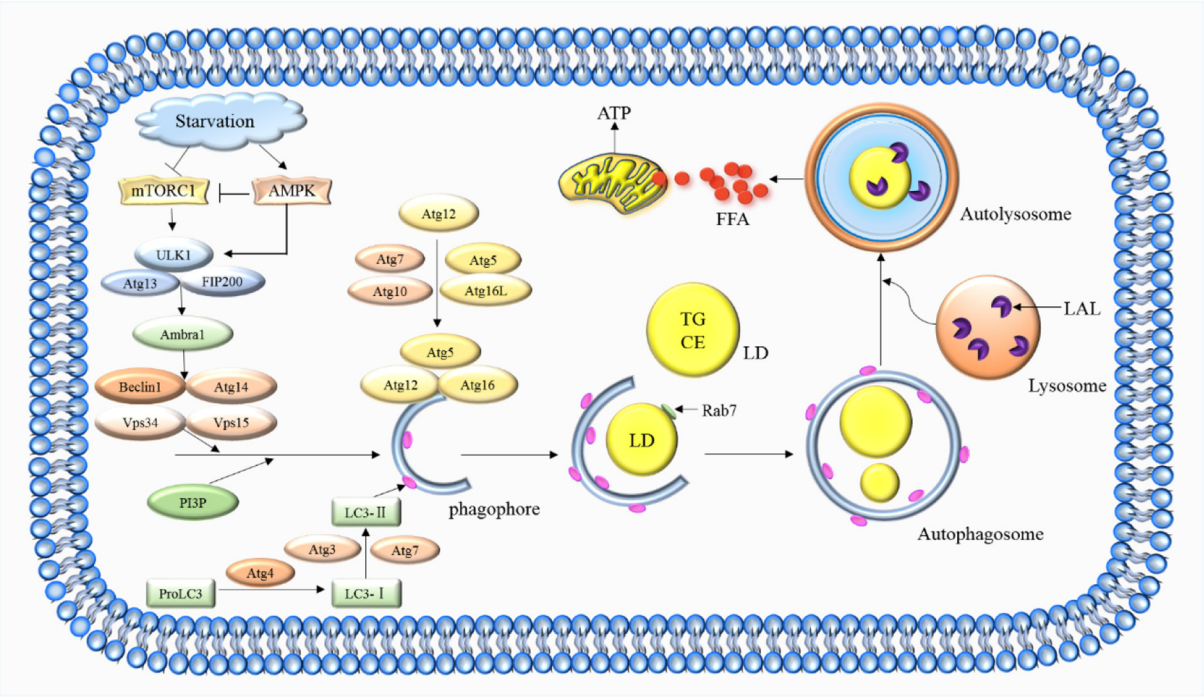

Fig. 1. Overview of the lipophagy process. The signals that activate the lipophagy process are often derived from stress conditions such as starvation and lipid stimulation. In the state of starvation, mTOR complex 1 (mTORC1) is inactive and AMP-activated protein kinase (AMPK) is active, leading to the activation of Unc-51-like kinase 1 (ULK1). Activated ULK1 phosphorylates ATG13 and RB1-inducible coiled-coil protein 1 (FIP200) in the ULK1-ATG13-FIP200 complex, which then activates Beclin1-Vps34 (PI3KC3) complex by Ambra1 phosphorylation. The Beclin1-Vps34 complex generates PI-3,4,5-phosphate (PI3P), resulting in the formation of autophagosomal membranes (phagophore). Further expansion of autophagosomal membranes involves two protein conjugation systems, including the formation of the ATG5-ATG12-ATG16 complex and the lipidation of LC3-I to LC3-II. The LC3-II positive membrane engulfing Lipid droplets (LDs) to form mature autophagosomes. Subsequently, autophagosomes fuse with lysosomes to form autolysosomes, where Lysosomal acid lipase (LAL) degrades LDs into free fatty acid (FFA) to provide energy.

may mediate lipid degradation through a lipophagic mechanism [13], irrespective of LDLR. This review focuses on the role of lipophagy in lipid metabolism and provides new insight on the effect of PCSK9 on lipid metabolism through lipophagic pathways. Simultaneously, the relative contribution of lipophagy to atherosclerosis is discussed.

2. Process, regulation, and biomarkers of lipophagy

2.1. Lipophagy is a complex lipid metabolism process involving multiple genes

Lipophagy relies on the same process as macroautophagy, which proceeds via several distinct steps by over 30 autophagy (ATG)-related proteins, including nucleation, elongation of autophagosomes or its subsequent fusion with lysosomes [14]. During long-term fasting, lipophagy is activated primarily through the mammalian target of rapamycin (mTOR) and AMP-dependent protein kinase (AMPK) signaling pathways (Fig. 1) [15–17]. In starvation, mTOR complex 1 (mTORC1) in lysosomes is inactivated, thereby activating downstream autophagy targets, including ULK1, Ambra1, Beclin1 and transcription factor EB (TFEB), which trigger lipophagy [18]. Moreover, nutritional starvation activates AMPK, which promotes lipophagy by phosphorylating ULK1 and inhibiting mTOR [19]. The ULK1 complex (ULK1/ 2-ATG13-FIP200) activates the Beclin1 complex (Beclin1-Atg14-VPS34) to promote the formation of LD-containing autophagosomal membranes [20]. The elongation to maturation of autophagosomes involves two ubiquitin-like binding systems: the first system is mediated by the E1 enzyme ATG7 and the E2 enzyme ATG10 [21], and the ATG5-ATG12 conjugate forms a complex with ATG16 [22]. The second system is mediated by cysteine protease ATG4, E1 enzyme ATG7, and E2 enzyme ATG3 to form lipidated microtubule-associated protein 1 light chain 3 (LC3-II), which is exposed on the external side of the mature autophagosome [23]. Mature autophagosomes fuse with lysosomes to form autolysosomes, which cause LD triglycerides and cholesterol to be degraded by acid lipases into FFAs to provide energy for nutrient deficiencies. In addition, the process of lipophagy also requires the involvement of complex transcriptional regulators, including TFEB [24], TFE3 [25], FOXO1 [26], CREB [27] and FXR [28] (Table 1).

Table 1
Effect of transcriptional regulators on lipophagy.

Transcription factor	Conditions of activation	Function	Effect on lipophagy	References
TFEB	Fasting	Upregulates PPAR-α and PGC-1α, autophagy gene and lysosomal gene	Promote	[24]
TFE3	Fasting	Promotes ATG5 and induces PGC1α-mediated fatty acid β-oxidation	Promote	[25]
FOXO1	Fasting	Promotes ATG14 and LAL	Promote	[26]
CREB	Fasting	Promotes ATG7, ULK1 and TFEB by recruiting CRTC2	Promote	[27]
FXR	Feeding	Disrupts the formation of functional CREB-CRTC2 complex	Suppress	[28]

TFEB, transcription factor EB; TFE3, transcription factor E3; FOXO1, forkhead box O1; CREB, cAMP response element-binding protein; FXR, farnesoid X receptor; PPAR-α, peroxisome proliferator-activated receptor (PPAR)-α; PGC-1α, PPAR-γ coactivator 1α; LAL, lysosomal acid lipase; ULK1, unc-51-like kinase 1; CRTC2, CREB regulated transcription coactivator 2.

Recently, the mechanisms by which autophagy selectively recognizes and targets LDs, namely the molecular mechanism of lipophagy have been confirmed. Proteomic analysis shows that Rab protein can affect the catabolism of LD [29–31]. Rab7, the first member of the Rab GTPase family, is associated with lipophagy as an important regulator of lipophagy. Rab7 regulates late endocytic pathway and participates in the maturation of autophagic vesicles [32]. The interaction of Rab7 with downstream effectors, such as FYVE and coiled-coil domain protein 1 (FYCO1) or Rab7 interacting-like protein, promotes the maturation and movement of autophagosomes [33,34]. Moreover, Rab7 mediates autophagosome-lysosomal fusion by regulating the interaction between numerous soluble N-ethylmaleimide-sensitive factor attachment protein receptor proteins and the members of the homotypic fusion and protein sorting complex [35,36]. These studies highlighted the central role of Rab7 in lipophagy and promoted lipophagy by directing LD docking and degradation.

Starvation activates Rab7 on LDs and vesicle structures [37]. Activated Rab7 mediates the binding of multivesicular bodies and lysosomes with LDs, thereby promoting LDs for targeted degradation via lipophagy. Conversely, the siRNA-mediated depletion of Rab7 or expression of a Rab7 dominant-negative mutant resulted in the accumulation of LDs in hepatocytes. Moreover, Rab10, the second member of the Rab GTPase family, is activated during starvation and is recruited to LDs and autophagosomes. Subsequently, Rab10 combines with the EHBP1–EHD2 complex to drive the extension of autophagosome membrane around LD and promote autophagosome to engulf LD [38]. These findings suggest that Rab7 and Rab10 play an important role in the initiation and progression of lipophagy.

2.2. Lipophagy is strictly regulated by multiple factors

The regulation of lipophagy mainly depends on nutritional status and lipid load. In the basal state, lipids and LD proteins are found in autophagosomes, indicating that lipophagy can occur randomly. During hunger and/or lipid stimulation, the level of lipophagy is enhanced to promote the breakdown of LDs, thereby providing FFA for β-oxidation or other pathways [10]. Moreover, fatty acids can promote LD growth and repair damaged LD, which is caused by the feedback inhibition of FFA released by lipid breakdown. However, if the excessive FFA is released by enhanced lipophagy and not removed in time, it will cause cell lipotoxicity. By culturing HepG2 cells and primary mouse hepatocytes, high concentrations or certain special types of lipids were found to regulate lipophagy levels. For example, unsaturated FFAs, such as oleic acid, promote the formation of triglyceride-rich LDs and induce lipophagy, whereas saturated FFAs, such as palmitoleic acid, rarely form LDs and inhibit lipophagy [39]. Therefore, the formation of LDs and the induction of lipophagy may be a protective mechanism against lipotoxicity. Yang et al. [40] found that the lipophagic level and autophagy gene Atg7 were decreased in obese mice, but the lipophagic level was enhanced and the mice body fat were remarkably reduced after overexpression of Atg7. Interestingly, in the long-term high-fat-diet-fed mice, lipophagic activity was enhanced in the first week; afterward, lipophagic activity was gradually reduced, which promoted the accumulation of LD and the occurrence of lipid metabolic disorders, such as obesity, fatty liver, and atherosclerosis [39,41]. Thus, although lipophagy can regulate the degradation of lipids, long-term exposure to high-fat environment causes cell damage and apoptosis to exceed the level of lipophagy, which will also decrease lipophagic activity.

Similarly, cholesterol has an indispensable effect on lipophagy. In macrophage-derived foam cells, accumulated cytoplasmic LDs contain large amounts of cholesterol esters. Lipophagy is activated when the uptake of LDL increases in macrophage-derived foam cells [12]. Moreover, lipophagic level is upregulated in smooth muscle cells (SMC) treated with FC [42]. The same effect has been observed during cell cholesterol and sterol depletion [43,44]. Hence, lipophagy not only removes excess cholesterol from cells but also breaks down LD cholesterol in the absence of nutrients. The effect of cholesterol on lipophagy occurs through the sterol regulatory element binding protein-2 (SREBP-2), a major regulator of cholesterol synthesis [45]. SREBP-2 can activate autophagic genes during cell sterol depletion to induce lipophagy. Additionally, the knockdown of SREBP-2 during nutrient depletion remarkably diminishes autophagosome formation and LD breakdown [44]. Subsequently, Kim et al. [46] demonstrated that SREBP-2 upregulates lipophagy by promoting the expression of PNPLA8. Hence, SREBP-2 can increase cholesterol by upregulating the expression of cholesterol-synthesizing genes and lipophagic function when the amount of intracellular cholesterol decreases.

2.3. Biomarkers are used to determine lipophagy

Several typical biomarkers are used for determining lipophagy. LC3 is a marker protein for autophagy detection, and is stable on the autophagosome membrane and can be retained to fuse with lysosomes. It is widely used in Western blot (WB) and fluorescence microscopy to detect the occurrence of autophagy [47]. Wang et al. [48] found that by using anti-LC3 immunostaining, Bodipy dye, and confocal microscopy, the co-localization of LC3 and LD could be used to detect autophagosomes in LDs, and LC3 signals could be found around LDs. Kimura et al. [49] developed a tandem GFP-RFP-LC3 sensor with monomeric red fluorescent protein (RFP) coupled to GFP-LC3 to trace autophagosome formation and degradation. The p62 protein, also known as sequestosome1 (SQSTM1), is the most important adaptor protein for selective autophagy, and acts as a bridge between autophagosomes by interacting with LC3 and LDs [48]. When the autophagic flux is inhibited, p62 will accumulate, whereas the p62 level will decrease when the autophagic flux is activated. Wang et al. [48] used anti-SQSTM1 staining and LD staining confocal imaging to determine if autophagosomes are recruited to LDs. LAMP1 (lysosomal markers) is commonly used for autolysosomes. In recent years, multispectral imaging flow cytometry has been used to display bright-detailed images through the co-localization of LC3, p62, and LAMP1 combined with LC3 punctae counting to measure autophagy accurately and stably [50]. Furthermore, lipophagy can be determined either by WB to analyze the internalization of GFP-tagged LD-resident proteins [51] or by measuring cellular TG content [52]. Sathyanarayan used a dual sensor vector targeting LD proteins to quantify LD turnover and lipophagy [53]. Consistent with this finding, double-labeled perilipin 2 (the maker of LDs) has been used to confirm lipophagic flux [37].

3. Role of lipophagy in atherosclerosis

Atherosclerosis is a chronic disease characterized by lipid accumulation and inflammatory cell infiltration. The accumulation of lipids and lipoproteins in the vascular intima are the initiating factors of atherosclerosis [54]. Animal experiments and human specimen studies have confirmed that hypercholesterolemia induces the abnormal accumulation of apoB-containing lipoprotein in the intima. The excessive accumulation of LDL and very-low-density lipoprotein (VLDL) can activate endothelial cells (EC) and trigger the secretion of inflammatory cytokines, thus leading to the recruitment of monocytes to differentiate into macrophages. Accumulated lipids and lipoproteins are engulfed by macrophages and exist in the cells as LDs to form foam cells, which contribute to the development of inflammatory and atherosclerotic lesions [55]. The primary LD in foam cells consists of free and esterified cholesterol. Accordingly, promoting cholesterol efflux and LD breakdown from macrophages is a potential therapeutic strategy to prevent foam cell formation and inhibit atherosclerosis progression [56,57]. Ouimet et al. [12] found that lipophagy can degrade LDs by LAL and mediate the efflux of cholesterol in macrophage-derived foam cells depending on ATP-binding cassette transporter A1, which has an important contribution to attenuate atherosclerotic lesions. Macrophage lipophagy was elevated in vitro and in vivo in response to lipid load, but

lipophagy was impaired after the knockdown of the autophagy gene Atg5, resulting in ineffective clearance of macrophages cholesterol, which eventually led to atherosclerotic lipid accumulation.

Chen et al. indicated that toll-interacting protein (Tollip), a critical molecule involved in the fusion of lysosomes with autophagosomes, plays an important role in lipophagy and modulates lipid accumulation during the pathogenesis of atherosclerosis [58]. The fusion of lysosomes with LDs was disrupted aortic macrophages and hepatocytes and accelerated the development of atherosclerosis and hepatic steatosis in Tollip-knockout mice. Thus, Tollip deficiency disrupts the lipophagic pathway and leads to increased lipid deposition and enlarged yet stable atherosclerotic plaques. Hence, lipophagy contributes to the clearance of excess lipid from macrophages and has become a new target for the treatment of atherosclerosis.

Interestingly, the role of lipophagy in preventing atherosclerosis may be related not only to macrophages but also to SMC and EC. FC-overload-induced SMC death is a significant event in the progression of atherosclerosis. Xu et al. [42] reported that the activation of lipophagy induced by excess FC in SMC attenuates cell death and provides an anti-atherosclerotic effect. Grootaert et al. [59] demonstrated that SMC-specific Atg7 deficiency in apolipoprotein E (apoE) knockout mice promotes the formation of atherosclerotic lesions. Moreover, EC injury is a major step in the pathological progression of atherosclerosis. Natural and oxidized LDL activate autophagy to convey protection against endothelial damage [60]. This evidence underscores the importance of endothelial autophagy in attenuating lipid accumulation within the vessel wall.

4. PCSK9 regulates lipid metabolism by lipophagy-mediated lipid degradation

4.1. Effect of PCSK9-mediated LDLR degradation on lipophagy

PCSK9 is involved in the regulation of lipid metabolism by enhancing the LDLR degradation on the cell membrane [61]. LDL is mainly taken up into cells by LDLR and metabolized through the lipophagy pathway. Lipophagy is a major regulator of lipid metabolism. Lipophagy is a new process of LDL degradation [62]. Lipophagy degrades LDL in macrophages via the autophagosome pathway to generate FC for efflux [12]. Therefore, the combination of LDL and LDLR can induce the occurrence of lipophagy. However, PCSK9 binds to the LDLR on the cell surface and mediates LDLR internalization. The PCSK9–LDLR complex traffics through the endosome to the lysosome for degradation, inhibits the release of LDLR, and returns to the cell surface [63,64]. Wu et al. [65] reported that in hamsters, fasting reduced the expression of PCSK9 with concomitant promoted LDL-C uptake by increasing the expression of LDLR on the surface of hepatocytes, which may imply induced lipophagy. Lan et al. [66] found that the overexpression of PCSK9 promotes the degradation of LDLR to inhibit LDL uptake, which in turn hinders the removal of lipids via the lipophagy pathway. These findings support the inhibitory effect of PCSK9 on lipophagy that occurs at least partly through its targeted degradation of LDLR. Further studies are required to elucidate the relationship between PCSK9-mediated LDLR degradation and lipophagy, and PCSK9 and lipophagy play essential roles in lipid metabolism.

4.2. Effect of PCSK9-mediated VLDLR degradation on lipophagy

Interestingly, Poirier et al. [67] demonstrated that in the LDLR family, VLDLR, which is the closest receptor to LDLR, is also a target of PCSK9 in lipid metabolism. VLDLR sequences are similar to LDLR (60% identity), and VLDLR has a highly reserved EGF-A domain. VLDLR is the main endocytic receptor recognizing TG-rich VLDL, which is widely expressed in the heart, skeletal muscles, and adipose tissue [68]. Jo et al. [69] reported that the overexpression of VLDLR resulted in the accumulation of TG in hepatocytes. As mentioned earlier, under increased cellular lipid, lipophagy is upregulated to efficiently and rapidly remove excess TG and maintain cellular lipid homeostasis [10]. Poirier et al. [67] found that wild-type PCSK9 and its natural mutant D374Y can efficiently degrade VLDLR. Membrane-bound PCSK9 chimeras reinforce the intracellular targeting of PCSK9 to endosomes/lysosomes and enhance the degradation of VLDLR. In summary, PCSK9 is linked with VLDLR by binding to VLDL and promoting lipophagy. Hence, PCSK9 may partially block lipophagy.

4.3. PCSK9 mediates lipid and apoB degradation via the lipophagy pathway

ApoB is a structural protein of LDL and VLDL, and is synthesized by hepatocytes [70]. Guo et al. [71] indicated that PCSK9 can regulate apoB lipoprotein degradation and lipid metabolism. The degradation of intracellular apoB is mainly involved in the ubiquitin-proteasome pathway and the autophagosome/lysosome pathway. In the endoplasmic reticulum, apoB interacts with LDLR and targets proteasomal degradation [72]. Moreover, apoB degradation can occur through lipophagy. Ohsaki et al. [73] found that apoB is highly concentrated in cytoplasmic lipid droplets as apoB-crescents. The activation of lipophagy increases the attachment of LC3 on the LD surface to the apoB-crescents and promotes its degradation by autophagosomes/lysosomes fusion [74]. Lipophagy is a dynamic regulation process for the degradation of apoB [75,76]. Sun et al. [13] found that the deletion of PCSK9 in mice without LDLR reduced atherosclerosis through a lipophagic mechanism. This study revealed that the deletion of PCSK9 promotes intracellular degradation of lipids and apoB by increasing autophagy signaling and autophagic flux in mouse hepatocytes, thereby reducing the synthesis and secretion of apoB. Moreover, PCSK9 can increase Akt and ULK1 protein expression, thereby affecting the lipophagic pathway. Thus, PCSK9 deletion can inhibit mTOR by inhibiting Akt expression, thereby resulting in the activation of lipophagy (Fig. 2). Conversely, the overexpression of PCSK9 showed that PCSK9 interacts with apoB to inhibit intracellular apoB degradation via the autophagosome/lysosome pathway, resulting in increased production and secretion of apoB and apoB-containing lipoproteins (LDL) [77]. These studies demonstrate that PCSK9 is associated with LDL. Intriguingly, Ouimet et al. [12] indicated that LDL was cleared by lipophagy in activated macrophages. Hence, the association of PCSK9 with LDL by apoB may suppress the uptake of LDL through macrophages, thereby hindering lipids from being removed by lipophagy-mediated efflux processes to promote atherosclerotic lipid accumulation. These findings highlight the exciting new role for PCSK9 in regulating lipid and apoB degradation by influencing the lipophagic pathway and may have a significant implication for lipid metabolism and atherosclerosis.

5. Conclusions and future perspectives

Lipophagy is significant in cell physiology, pathology, and common diseases related to lipid metabolism [58,78,79]. At present, the mechanism by which autophagy selectively acts on LDs has basically clarified through the identification of proteins such as Rab7 that mediate lipophagy, but this process may be far more complicated than our current understanding and requires further exploration. Lipophagy plays a pivotal role in the regulation of lipid metabolism, which can remove excess lipids and maintain cell lipid homeostasis, and it is a novel therapeutic target for atherosclerosis. Moreover, PCSK9 affects lipid metabolism and is an attractive target in the treatment of familial hypercholesterolemia and atherosclerosis [80]. PCSK9 mediates the degradation of LDLR, which binds to LDL and induces lipophagy. Hence, PCSK9 has a certain association with lipophagy. PCSK9 participates in lipid metabolism through lipophagy, and thus affects atherosclerosis. However, the specific mechanism for linking PCSK9 to lipophagy has not been fully confirmed. Therefore, further research is needed to elucidate the relationship between PCSK9 and lipophagy, which may be a very effective therapeutic target for cardiovascular

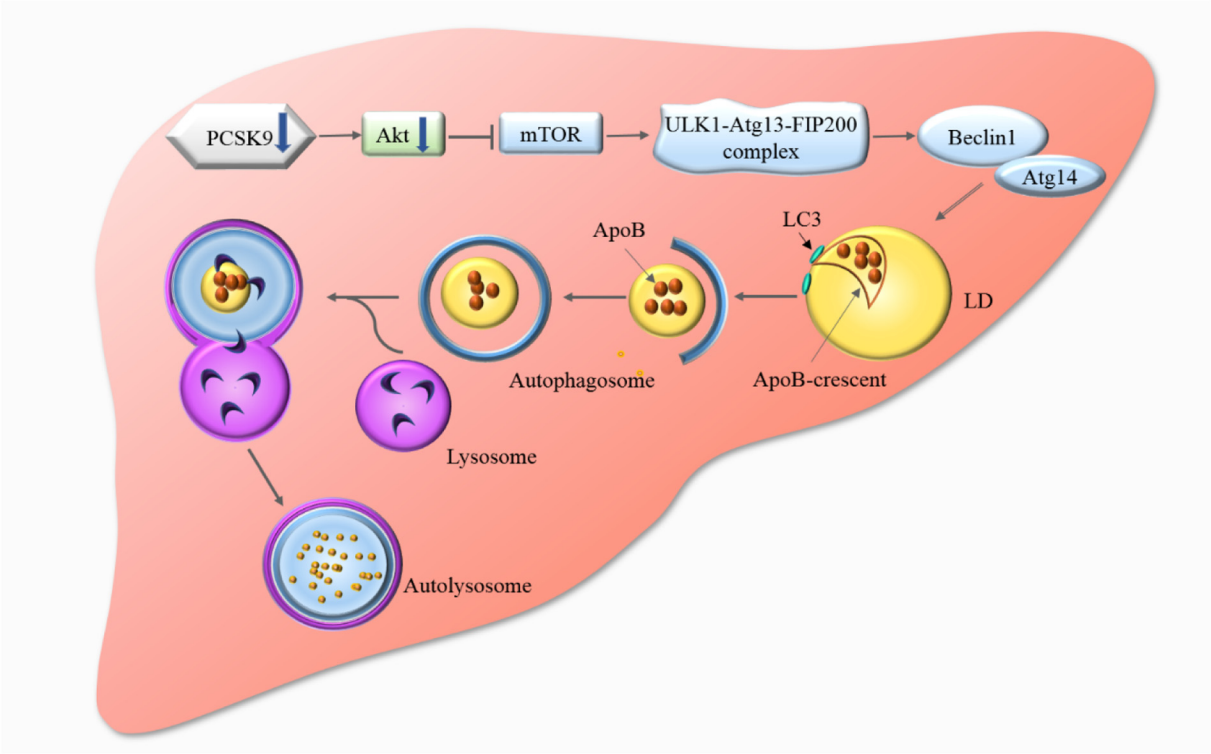

Fig. 2. The speculated mechanism of PCSK9 regulating lipid metabolism via the lipophagy pathway. PCSK9 plays an important role in increasing the expression of Akt and ULK1 proteins. Akt inhibits lipophagy by activating mTOR and inhibiting the ULK1 kinase complex. Under conditions of PCSK9 deletion, Akt protein activity is reduced, mTOR is inhibited and the ULK1 kinase complex is activated. Activated ULK1 kinase complex activates Beclin-1 complex to activate lipophagy, thereby degrading intracellular lipids and apoB to regulate lipid metabolism.

diseases, especially for the development of new therapeutic approaches for atherosclerosis.

Declaration of interest

There are no conflicts of interest.

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