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Novel Regulators of Low-Density Lipoprotein Receptor and Circulating LDL-C for the Prevention and Treatment of Coronary Artery Disease

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1. Introduction

Low-density lipoprotein cholesterol (LDL-C) is a major risk factor for atherosclerosis and coronary artery disease (CAD). The role of LDL-C in CAD has been established through experimental studies, epidemiological and genetic studies and the elucidation of the low-density lipoprotein receptor (LDLR) pathway (Brown and Goldstein, 1986). LDLR deficient mice develop frank hypercholesterolemia and atherosclerosis on western diet (Ishibashi et al., 1994). In humans, people with low LDL-C have very low risk of developing CAD and plasma LDL-C is positively associated with CAD (Stamler et al., 2000). Mutations in LDLR in humans form the molecular basis for familial hypercholesterolemia and patients with this disease develop premature CAD (Hobbs et al., 1990). Statins are a class of small molecule compounds that inhibit HMG-CoA reductase, a rate-limiting enzyme in cholesterol biosynthesis, thereby reducing LDL-C in humans. Statins effectively reduce LDL-C and the associated cardiovascular disease risk by about 30%, but residual risks for developing cardiovascular disease remain. In recent years, clinical studies have suggested that further lower circulating LDL-C is closely associated with additional reduction of cardiovascular risk. The majority of the high risk patients often fail to reach their LDL-C goal, and thus, novel targets and medications are highly desirable for the prevention and treatment of cardiovascular disorders. In the last few years, novel regulators of LDLR and/or circulating LDL-C have emerged that suggest potentially more therapeutic opportunities. These include proprotein convertase subtilisin/kexin type 9 (PCSK9) and the inducible degrader of LDLR (Idol) that regulate LDLR and LDL-C levels. Novel therapeutic modalities thus may provide additional clinical options to further reduce LDL-C and cardiovascular disease risk.

2. Cholesterol homeostasis and the management of cholesterol

While cholesterol is an essential component of cellular membranes, excessive cholesterol is detrimental to cells. Accordingly, the cellular cholesterol level is tightly controlled

through intricate regulatory mechanisms. The steady state cholesterol level is dictated by cholesterol biosynthesis, cellular cholesterol uptake, and cholesterol efflux. The cholesterol biosynthetic pathway involves more than 30 enzymes to make cholesterol from acetyl-CoA, while cholesterol uptake is primarily mediated by the receptor-mediated endocytosis. Cholesterol carried in LDL particles is readily taken up by its cognitive plasma membrane receptor, LDLR. Cholesterol is delivered eventually to the lysosomal compartment, and LDLRs either recycle back to plasma membrane or are degraded in lysosomes. Cholesterol efflux is mediated by ATP-binding cassette transporters (ABC proteins). ABCA1 primarily mediates apolipoprotein AI dependent cholesterol efflux while ABCG1 is largely responsible for apolipoprotein E/high-density lipoprotein (HDL) particle mediated cholesterol efflux. Cholesterol biosynthesis and LDLR levels are feedback-regulated by sterol-responsive element binding protein 2 (SREBP2) (Goldstein et al., 2006), while ABCA1/ABCG1 are both regulated through liver X receptors (LXRs) (Cao et al., 2004).

Physiologically, the circulating LDL-C concentration is determined by the rate of LDL-C production and its clearance. Very low-density lipoprotein (VLDL) particles are assembled and secreted from liver and are converted to LDL particles in circulation through triglyceride lipolysis by lipoprotein lipase (LPL). Proteins involved in this process are intimately related to the rate of VLDL secretion and contribute to circulating LDL-C levels. For instance, apolipoprotein B (apoB) deficiency leads to impaired VLDL assembly and secretion, and accordingly very low levels of plasma cholesterol. LDL particle clearance is primarily mediated through hepatic LDLR, and thus proteins or other agents that affect liver LDLR levels greatly impact LDL-C. In hypercholesterolemic patients, dysfunctional LDLR results in increased circulating LDL-C and premature coronary artery disease.

LDL-C is a major cardiovascular risk factor that has been established through epidemiological, genetic, and pharmacological studies. Statins are small molecule inhibitors of the rate-limiting enzyme HMG-CoA reductase in the cholesterol biosynthetic pathway. Statins reduce intracellular cholesterol and increase hepatic LDLR levels through the feedback mechanism of SREBP2 activation. Statins decrease circulating LDL-C by 20-50% in humans and effectively reduce cardiovascular risk by approximately 30%. Additionally, the cholesterol absorption inhibitor ezetimibe inhibits an intestinal epithelial membrane protein Niemann-Pick C1-Like 1 (NPC1L1) to reduce cholesterol absorption and accordingly lowers LDL-C by about 20% in humans. The cardiovascular risk reduction from ezetimibe, however, is yet to be proven in clinical studies.

In recent years, it has been found that additional reduction of LDL-C has been associated with further reduced cardiovascular risk, suggesting a strategy of "the lower, the better" for cardiovascular disease prevention and treatment (O'Keefe et al., 2004). However, LDL-C lowering efficacy is limited by statins, since doubling the dose of statins typically results in only an additional 6% reduction in LDL-C, a so-called "6% rule". As a result, many patients cannot achieve their cholesterol goal. Additionally, in high risk patients, an LDL-C of 50-70 mg/dl is recommended, which poses more challenges for patients and physicians to achieve this goal. Novel therapeutic modalities are thus highly desirable. The recent discoveries of novel regulators of LDL-C therefore provide new opportunities for such development and will be reviewed in this chapter.

3. PCSK9

3.1 Cell biology, biochemistry, and physiology of PCSK9

Proprotein subtilisin kexin type 9 (PCSK9) was originally named NARC-1 for neural apoptosis-regulated convertase 1 and was found expressed in hepatocytes, kidney mesenchymal cells, intestinal and colon epithelial cells and brain telencephalen neurons (Seidah et al., 2003). PCSK9 belongs to and is the ninth member of the proprotein convertase family that also includes S1P/SKI-1, an essential serine protease that cleaves SREBPs within the luminal region of the endoplasmic reticulum (ER). PCSK9 encodes a protein of 692 amino acids whose structure includes a signal peptide at its amino terminus, followed by a prodomain, catalytic domain, and carboxyl terminal cysteine and histidine rich domain of unknown function (Horton et al., 2009) (Figure 1). PCSK9 is synthesized as a proprotein/zymogen, and the prodomain of PCSK9 is self-cleaved between glutamine 152 and serine 153 producing a prodomain fragment of 14 kD. After self-cleavage, the prodomain remains associated with the catalytic domain to form a non-covalent protein complex. The protein complex is about 74 kD, and in a denaturing polyacrylamide gel electrophoresis (PAGE) system, two fragments of 60 kD (mature protein) and 14 kD (prodomain) are observed. The association of the prodomain with the rest of the catalytic domain is fairly tight, and as a result, catalytic activity is completely inhibited by the presence of the prodomain (Horton et al., 2009). The details of the molecular basis of the protein complex have been described in recent reports that elaborated the crystal structure of the protein complex (Kwon et al., 2008). PCSK9 is glycosylated at Asn 533 and Tyr-sulfated at Tyr 38, but mutants without glycosylation and sulfation retain their secretion and function in degrading LDLR. PCSK9 is also phosphorylated at Ser 47 and 688. Loss of phosphorylation at Ser 47 leads to increased degradation of PCSK9, suggesting its potential role in regulating PCSK9 protein levels and function (Dewpura et al., 2008).

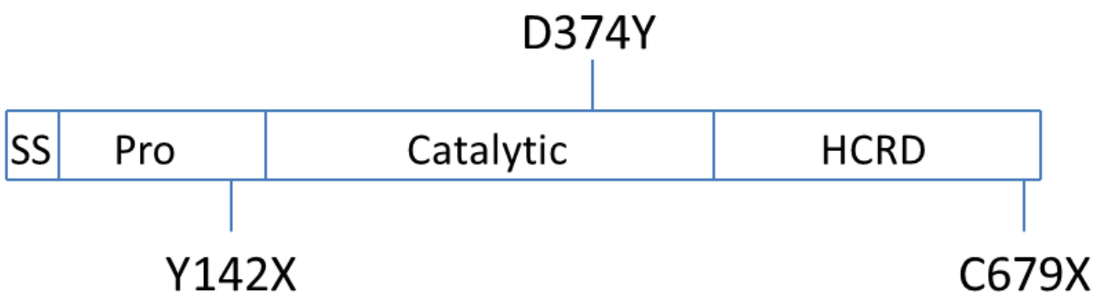

Fig. 1. Domain structure of PCSK9. SS, signal sequence; Pro, prodomain; catalytic, catalytic domain; CHRD, Cys/His rich domain. The gain-of-function mutation D374Y and the two loss-of-function mutations were highlighted.

The amino terminal signal sequence serves to lead the protein into the endoplasmic reticulum and the secretory pathway. The prodomain serves in general as an inhibitor of the mature, catalytic domain before it is cleaved. In PCSK9, however, the prodomain continues to serve as an inhibitory factor even after its auto-processing from the catalytic domain in a

non-covalent fashion. This implies that the catalytic activity is either regulated and activated at some specific physiological condition or is not essential for the function. Indeed, in an important paper, Horton and colleagues reported making a secreted form of catalytically inactive mutant PCSK9 through transexpression of both the prodomain and the catalytic domain and showed that the catalytically inactive mutants were able to degrade cellular LDLR proteins (McNutt et al., 2007). This elegant work along with the reports from other laboratories indicates that the protease activity of PCSK9 is not essential for its physiological function. In addition, it was observed recently that the acidic stretch within the prodomain functions to inhibit PCSK9 degradation of LDLR. Deletion of these acidic residues further increases PCSK9 protein binding to LDLR (Benjannet et al., 2010).

The carboxyl terminus of PCSK9 contains a Cys and His rich domain, the function of which is not well understood. This domain is speculated to be important for protein interaction, and it was recently reported that the carboxyl terminus of PCSK9 binds annexin A2, but not closely related annexin A1. Annexin A2 co-localizes with PCSK9 at the cellular surface and serves to inhibit PCSK9 function in degrading LDLR (Mayer et al., 2008). This speculated role of annexin A2 is consistent with the observation that some monoclonal antibodies with epitopes against the carboxyl terminus of PCSK9 inhibit PCSK9 function (Ni et al., 2010), possibly through mimicking annexin A2 function.

PCSK9 binds to the EGFA domain of the extracellular portion of LDLR, and the binding affinity increases by more than 50-fold when the pH is reduced to 5.5 (Zhang et al., 2007). Upon binding to LDLR, the PCSK9/LDLR complex undergoes receptor mediated endocytosis and traffics to the endosomal compartment, where an acidic environment facilitates tighter binding of the two proteins. This increased affinity between the two proteins presumably results in the escorting of LDLR to lysosomal degradation instead of recycling back to plasma membrane (Lagace et al., 2006; Qian et al., 2007). LDLR and PCSK9 co-localize to endosomal/lysosomal compartments, and the binding of two proteins have been studied in detail with the resolution of co-crystal structure of PCSK9 and the EGFA domain (Kwon et al., 2008). EGFA binds to the catalytic domain of PCSK9 (Figure 2).

While its potential physiological role was initially speculated in neuronal cell differentiation and development, it was found later through primarily human genetic studies that PCSK9 plays a major role in regulating hepatic LDLR protein levels and thus circulating LDL-C in humans (Abifadel et al., 2009; Abifadel et al., 2003). In addition to the potential role of degrading LDLR intracellularly, secreted PCSK9 protein is functional in degrading cellular and hepatic LDLR and thus elevates LDL-C *in vivo*. Recombinant PCSK9 protein, when added to cell cultures, dose dependently reduces total cellular LDLR protein and cell surface LDLR levels. This results in reduced LDL particle uptake into the cells as evaluated by a fluorescent labeled LDL particle uptake assay. The half-maximum effective concentration (EC_{50}) is achieved at 0.8 μg/ml. When injected into wild type mice intravenously, PCSK9 decreased primarily hepatic LDLR protein levels and increased circulating LDL-C, as evaluated by fast protein liquid chromatography (FPLC) (Qian et al., 2007). When used at the pharmacological levels, PCSK9 appears to degrade LDLR in other tissues as well (Schmidt et al., 2008). When grossly over expressed in mouse liver through adenovirus, PCSK9 dramatically reduces hepatic LDLR protein levels. Circulating LDL-C is thus elevated to a level similar to that seen in LDLR deficient mice (Maxwell and Breslow, 2004). Conversely, PCSK9 deficient mice have a significant increase in hepatic LDLR protein levels.

Importantly, statin treatment of PCSK9 deficient mice further augmented the liver LDLR protein levels, demonstrating the additive effects of PCSK9 deficiency and statin treatment on LDLR protein expression (Rashid et al., 2005).

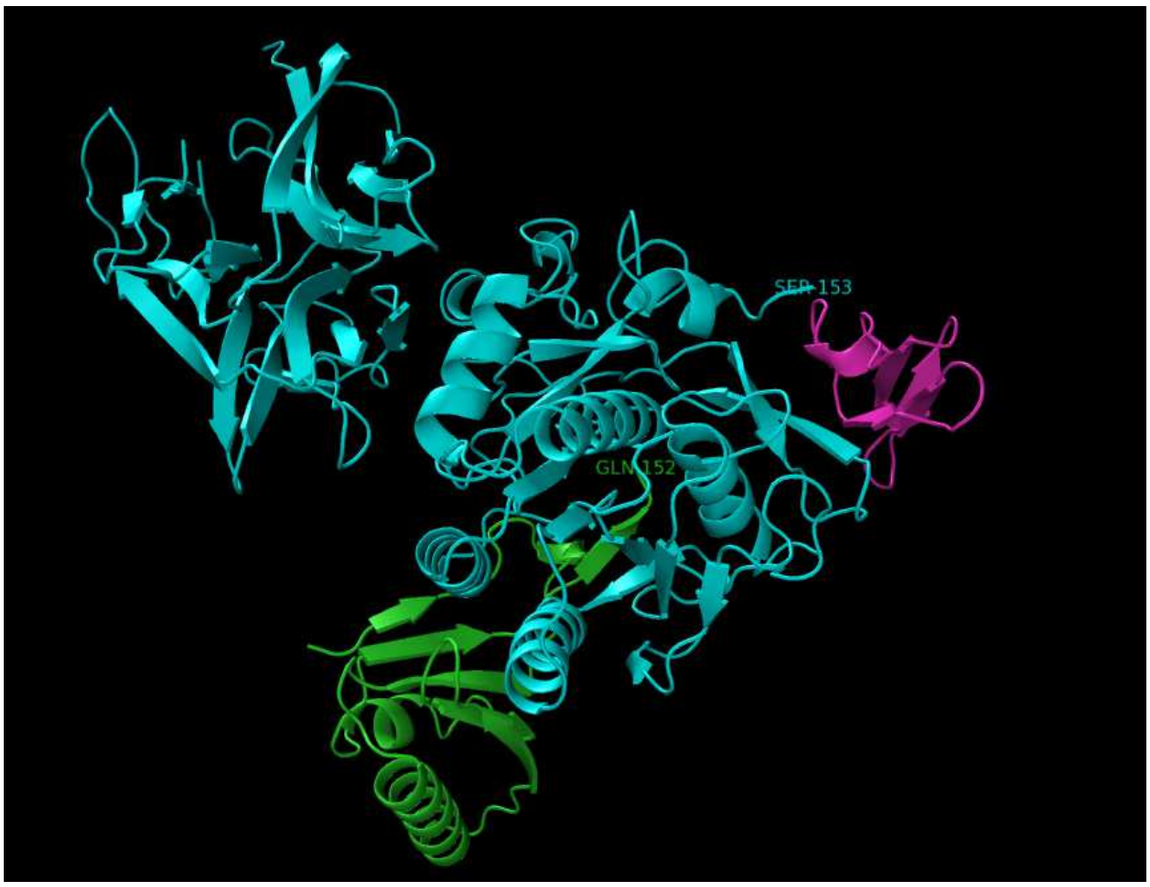

Fig. 2. Co-crystal structure of PCSK9 and EGFA domain of LDLR. Green, prodomain. Blue catalytic and CHRD. Purple, EGFA. Gln152 and Ser153 were labeled (Courtesy of Dr. Yong Wang and Dr. Yue-Wei Qian).

Besides the well-documented role of PCSK9 in regulating circulating LDL-C primarily through LDLR, a proposed role for PCSK9 in VLDL secretion has been suggested. It appears that increased PCSK9 levels through adenoviral expression in mice accelerates the VLDL secretion rate while PCSK9 deficiency in mice reduces postprandial hypertriglyceridemia. These observations, however, were not substantiated by reports from other labs and remain controversial. Beyond LDLR as the substrate for PCSK9, some *in vitro* data indicate that very low-density lipoprotein receptor (VLDLR) and apolipoprotein E receptor 2 (apoER2), two proteins that share about 60% homology with LDLR, can be substrates for PCSK9 as well (Poirier et al., 2008). VLDLR is primarily expressed in heart, muscle, and adipose tissue and possibly plays a role in mobilizing triglyceride (TG) from VLDL to these tissues as the energy source. The role of PCSK9 in regulating VLDLR implies a role in regulating energy homeostasis in muscle, heart, and fat. Indeed, a recent study reported increased fat mass in

PCSK9 deficient mice and increased cell surface VLDLR receptor expression in adipose tissue. Conversely, hepatic PCSK9 expression dramatically reduced VLDLR in adipose tissue (Roubtsova et al., 2011). ApoER2 is primarily expressed in brain and is speculated to play a major role in neuronal differentiation and brain development. Regulation of apoER2 by PCSK9 suggests its potential role in brain development and function. In zebra fish, PCSK9 deficiency leads to impaired brain development. In mice, PCSK9 reduces LDLR during brain development and ischemic stroke, whereas in adult mice, LDLR protein levels in the brain are similar in wild type and PCSK9 deficient mice (Liu et al., 2010; Rousselet et al., 2011).

3.2 Human genetics, physiology, and regulation of PCSK9

PCSK9 was cloned as the ninth member of the proprotein convertase family. Its potential role in cholesterol homeostasis was discovered through human genetic studies. In 2003, through extensive genetic mapping studies, mutations in PCSK9 were discovered and reported as the third locus for autosomal dominant familial hypercholesterolemia (ADH) in addition to the well-established loci of apoB and LDLR in this disease (Abifadel et al., 2003). Following the original study, many mutations or polymorphisms within PCSK9 have been reported. These have been categorized into gain-of-function (GOF) mutations, loss-of-function (LOF) mutations, or polymorphisms that result in either elevated or reduced circulating LDL-C levels in humans (Abifadel et al., 2009).

The molecular basis for most GOF mutants is not clear at the moment. However, a D374Y mutation, which was originally described in an Anglo-Saxon family, led to a more severe clinical hypercholesterolemic phenotype than other heterozygous GOF mutations of PCSK9 or LDLR mutations, and is associated with a very early onset of coronary artery disease. Asp374 of PCSK9 is intimately involved in EGFA domain binding as was revealed by the co-crystal structure of PCSK9 and the EGFA domain. The D374Y mutation results in a conformational change that enables PCSK9 to bind LDLR more tightly (Kwon et al., 2008). In cultured cells, the D374Y mutant demonstrated a more than a 10-fold increase in potency in degrading cellular LDLR proteins, highlighting the molecular basis for this GOF mutant (McNutt et al., 2007). Even the plasma level of PCSK9 is decreased in patients carrying this mutation, which probably reflects its tight binding to LDLR and faster clearance through receptor-mediated endocytosis. It is conceivable that such an increase in affinity towards LDLR would dramatically increase LDLR degradation. Indeed, in heterozygous carriers of this mutation, statin use actually worsens the hypercholesterolemia. This occurs presumably as a result of statins inducing more D374Y mutant PCSK9, which significantly reduces hepatic LDLR proteins. In a transgenic mouse model, PCSK9 D374Y mutant significantly elevated plasma LDL-C and led to a more accelerated development of atherosclerosis under hypercholesterolemic conditions (Herbert et al., 2010).

Contrary to the GOF mutations, LOF mutants were found at a relatively high frequency in humans. The combined incidence of LOF mutations of a Y142X mutant, which encodes a truncated protein and a C679X mutant, that leads to lack of protein secretion, was 2.6% in blacks in the ARIC (Atherosclerosis Risk In Communities) study. The two heterozygous LOF mutants in blacks resulted in a 28% lower plasma LDL-C and an 88% reduction in CAD risk. The R46L mutation, which results in reduced secretory efficiency and lower plasma

protein levels, was present in 3.2% whites in the ARIC study and was associated with a 47% reduction in CAD risk (Cohen et al., 2006). These data suggest that the life-long reduction in LDL-C appears to have greater benefit in CAD risk reduction than drug therapy, thus highlighting the potential benefit of reducing LDL-C at an early stage of the disease progression for CAD prevention.

Circulating PCSK9 is largely produced hepatically based on the liver-specific PCSK9 knockout mouse studies, although it is also expressed in intestines and kidney. Circulating PCSK9 exists either in the form of the 74 kD complex or can be further cleaved by furin at Arg 218 to result in a truncated, inactive form of PCSK9 (Benjannet et al., 2006; Konrad et al., 2011). The ELISA method our labs have been using detects both the wild type and the furin cleaved forms. The circulating PCSK9 levels in humans vary significantly and the average level is estimated around 100-500 ng/ml. Not surprisingly, circulating PCSK9 levels are significantly associated with total cholesterol and LDL-C, but not high-density lipoprotein cholesterol (Konrad et al., 2011). Although PCSK9 plays a critical role in regulating hepatic LDLR protein levels and plasma LDL-C, PCSK9 only explains about 7% of the variability in circulating LDL-C levels (Lakoski et al., 2009). In large population studies, PCSK9 is also associated with plasma TG, implying its possible role in VLDL metabolism as suggested by some of the studies in animal models. PCSK9 is positively associated with body mass index in these studies, which is not entirely consistent with the observation in PCSK9 deficient mice that increased visceral adipose mass was observed. Plasma PCSK9 is associated with plasma glucose levels, although the role of PCSK9 in glucose homeostasis has been controversial. Contradicting results have been reported from two different labs, suggesting that in PCSK9 deficient mice, there was either compromised or unchanged insulin sensitivity, as evaluated by an oral glucose tolerant test (Cui et al., 2010; Lakoski et al., 2009; Langhi et al., 2009; Mbikay et al., 2010).

PCSK9 is regulated by many physiological processes and pharmacological agents. The most prominent regulating factor is SREBP2. PCSK9 is a direct target gene of SREBP2, with a sterol-responsive element residing in its proximal region of its promoter. In cultured cells, statins inhibit cholesterol biosynthesis, reduce intracellular cholesterol levels, and activate SREBP2 and its target genes, including PCSK9. The parallel regulation of cholesterol biosynthesis, LDLR, and PCSK9 represents a complicated and intricate regulatory system to fine tune cholesterol homeostasis. In humans, statins significantly increase plasma PCSK9 levels, which attenuates statin efficacy (Careskey et al., 2007; Konrad et al., 2011). In this regard, therapeutic agents that inhibit PCSK9 expression or function will additively reduce LDL-C with statin therapy. The bile acid binding resin cholestyramine, expectedly increased circulating PCSK9 levels through accelerating the conversion of cholesterol to bile acid in liver and depleting hepatic cholesterol (Persson et al., 2010). Conversely, cholesterol or sterols in cultured cells repress SREBP2 activity and accordingly PCSK9 expression (Schmidt et al., 2006).

Circulating PCSK9 has a very distinct diurnal rhythm that peaks around 4:00 am and has a nadir from 4-9 pm with a fluctuation of 15% of its plasma levels. This diurnal rhythm is completely paralleled by the cholesterol biosynthesis surrogate lathosterol, suggesting that circulating PCSK9 is tightly controlled together with hepatic cholesterol biosynthesis (Persson et al., 2010). Although cholesterol biosynthesis fluctuates during the 24 hour shift, plasma cholesterol levels remain constant, and this may be achieved through paralleled

expression of PCSK9, which implies a physiological role of PCSK9 in maintaining a constant plasma cholesterol level during the daily fluctuation of cholesterol biosynthesis (Persson et al., 2010). In animal models and humans, fasting reduces SREBP2 activity, and the plasma PCSK9 level is reduced accordingly. Re-feeding results in recovery of PCSK9 expression and circulating PCSK9 levels (Browning and Horton, 2010; Persson et al., 2010). PCSK9 is also reported to be regulated by insulin, LXRs, and the SREBP1c axis in cultured cells. Bile acids, on the other hand, reduce PCSK9 expression presumably through the farnesoid X receptor (Langhi et al., 2008). Fenofibrate reduces PCSK9 expression in cultured cells and in animal models. In the clinic, however, the majority of available data point to the fact that fibrates significantly elevate PCSK9 levels in both diabetic and non-diabetic subjects, which possibly attenuates fibrate effects in reducing LDL-C levels (Konrad et al., 2011). Berberine, a compound that reduces LDL-C in humans and is derived from a Chinese herb (Coptis sinensis), dramatically reduces PCSK9 mRNA and protein expression in cultured hepatoma cells (Cameron et al., 2008). PCSK9 is regulated by several hormones. In humans, females have higher circulating PCSK9 levels than males, and post-menopausal women have higher PCSK9 levels than premenopausal women (Cui et al., 2010; Lakoski et al., 2009). Estradiol reduces PCSK9 in rats but not in humans, while growth hormone significantly reduces PCSK9 levels in humans (Persson et al., 2010).

3.3 Therapeutic approaches to modulate PCSK9 expression and function

An effective therapy is based on a solid hypothesis and human validation. LDL-C as a major risk factor for CAD is firmly established, and in addition, genetic data from PCSK9 mutations in humans have provided the strongest validation to pursue PCSK9 modulators to either suppress its expression or inhibit its function. Furthermore, data collected in recent years indicate that the lower the LDL-C, the more CAD protection, thus PCSK9 also provides a unique opportunity for LDL-C lowering since statins increase PCSK9 in humans, which attenuates their efficacy. Thus, novel agents targeting PCSK9 that function in an additive fashion to statins are highly desirable and will be a valuable addition for physicians to effectively manage circulating LDL-C.

Anti-sense oligonucleotides (ASO) have been explored as a potential therapeutic agent for years, and recently an ASO against apoB has proven successful in phase III clinical studies that effectively reduced LDL-C. In mice, an ASO effectively reduced liver PCSK9 mRNA and thus its production, leading to significantly elevated hepatic LDLR levels (Graham et al., 2007). Recently, a new generation of ASO called locked nucleic acid (LNA) modified gap-mer antisense oligonucleotides has been developed. These LNA oligonucleotides are short in length, single stranded, and have high affinity towards mRNA and microRNA. LNAs are readily delivered in saline and have been shown to safely and potently inhibit mRNA targets in mice and humans. LNA against PCSK9 effectively reduced PCSK9 expression in cultured cells. In mice, LNA reduced PCSK9 expression by 60%, and the effect lasted more than two weeks. Hepatic LDLR levels were elevated by more than 2-fold (Gupta et al., 2010). These studies suggest that ASO or LNA have potential to reduce circulating LDL-C in humans through inhibiting PCSK9 expression. An LNA against PCSK9 is currently in phase I clinical studies.

A similar approach involves inhibiting PCSK9 expression through RNA interference. A small interfering RNA (siRNA) was delivered to animals through lipidoid nanoparticles.

Liver specific knockdown of PCSK9 in mice and rats reduced PCSK9 mRNA by 50-70% and reduced circulating cholesterol concentrations by 60%. In non-human primates, a single dose of siRNA targeting PCSK9 reduced plasma PCSK9 by more than 70% and caused an accompanying reduction of LDL-C by more than 50%. The reduced LDL-C only returned to baseline 21 days following the siRNA delivery (Frank-Kamenetsky et al., 2008). These data suggest that siRNA targeting of PCSK9 to reduce LDL-C is technically feasible and support the possibility of LDL-C lowering through suppressing PCSK9 expression. A clinical trial application for ALN-PCS, an RNAi therapeutic targeting PCSK9 for the treatment of severe hypercholesterolemia was filed recently.

An alternative approach is to inhibit PCSK9 function through developing monoclonal antibodies against PCSK9. PCSK9 functions through binding to EGFA domain of LDLR. Blocking the interaction of PCSK9 and LDLR is thus a promising approach. Indeed, a monoclonal antibody that blocks the interaction of PCSK9 and EGFA domain of LDLR blocked PCSK9 function and increased cellular LDLR protein levels in cultured cells. Notably, the effect of preserving LDLR protein level was further enhanced when cultured cells were treated with statins, suggesting the additive effect with statins (Chan et al., 2009). In a mouse model over expressing human PCSK9, this antibody reduced LDL-C significantly (Chan et al., 2009). In non-human primates, a single injection of the antibody at 3 mg/kg reduced LDL-C by 80% within one week of treatment, and the significant reduction of LDL-C was maintained more than 10 days after antibody administration (Chan et al., 2009). In humans, single dose administration through either intravenous or subcutaneous injection of a PCSK9 monoclonal antibody developed by Regeneron dose-dependently reduced LDL-C up to 60% (2011 The Deuel Conference on Lipids). This antibody is currently in phase II clinical trials.

A small molecule approach to inhibit PCSK9 function or its auto-catalytic processing has proven very challenging. However, it was reported that berberine, a natural product, dramatically reduced PCSK9 expression through transcriptional repression (Cameron et al., 2008; Li et al., 2009). While the mechanism is not entirely understood, this observation suggests the possibility that developing small molecule modulators to mediate PCSK9 expression is potentially viable.

PCSK9 appears to be a very safe target. While it was shown in zebra fish that PCSK9 is essential for brain development, PCSK9 deficient mice have appeared normal. In humans, compound heterozygous carriers for LOF mutations for PCSK9 who lack circulating PCSK9 have been reported. These individuals have very low levels of serum PCSK9 levels (below 20 mg/dl) but otherwise appear completely healthy. Thus, PCSK9 is a well validated target in humans for LDL-C reduction and CAD prevention and treatment.

4. The role of Idol in regulating LDLR

The **i**nducible **d**egrader **o**f **L**DLR (Idol) was recently identified as another post-translational regulator of LDLR. Idol was originally cloned from neuronal cells as a myosin regulatory light chain (MRLC) interacting protein (Olsson et al., 1999), and named Mylip (or MIR). Mylip has a FERM (4.1 band, ezrin, radixin and moesin) at its amino terminal end and a RING zinc finger ubiquitin ligase domain at its carboxy terminal end. The FERM domain is thought to mediate protein-protein or protein-membrane interactions. Proteins containing a

FERM domain provide a link between the cell membrane and cytoskeleton. The RING domain is the active site of a large number of E3 ubiquitin ligases which catalyze the transfer of activated ubiquitin to substrate proteins leading to their degradation in the proteasome or lysosome. Mylip is the only FERM-containing protein that also has a RING domain. It has been shown that the RING domain of Mylip can mediate its self-ubiquitination and degradation in the proteasome (Bornhauser et al., 2003). Over expression of Mylip can inhibit nerve growth factor-stimulated neurite outgrowth in PC12 neuronal cells, presumably due to the ubiquitination and degradation of myosin regulatory light chain (Bornhauser and Lindholm, 2005). In the rat, Mylip expression is localized especially to neurons in the hippocampus and cerebellum, both during development and in adult brain (Olsson et al., 2000). Mylip expression can also be detected in many other tissues, suggesting additional functions and targets for this protein.

The Liver X receptors (LXRs) are important transcriptional factors regulating cellular cholesterol content. LXRs. LXR-α and LXR-β are sterol-response nuclear receptors activated by excess cellular cholesterols. Activated LXRs stimulate expression of ATP-binding cassette transporters A1 and G1 (ABCA1 and ABCG1) to promote efflux of cellular cholesterol, resulting in hepatic secretion of cholesterol into the bile and enhancement of reverse cholesterol transport in peripheral tissues. In mouse models of atherosclerosis, LXR agonists suppressed the development of atherosclerosis lesions. However, LXR agonists also stimulate hepatic fatty acid and triglyceride synthesis. This is due to the fact that sterol responsive element-binding protein 1c (SREBP1c) is a down-stream target of LXR. Activation of SREBP1c would lead to increased expression of genes involved in fatty acids and triglyceride synthesis.

In a recent publication, Zecler and co-workers investigated the effect of LXR agonists on cholesterol uptake in cultured liver cells (Zelcer et al., 2009). They found that LXR agonists suppressed LDL-C uptake by these cells. Further investigation revealed that LXR agonist GW3956 did not change LDLR mRNA level. Instead, the compound reduced LDLR protein level and redistributed LDLR from the plasma membrane to intracellular compartments. To explore the mechanism by which LXR agonists reduced LDLR protein, Zecler and colleagues also performed transcription profiling in cells treated with LXR agonists. Mylip is one of the genes whose mRNA is stimulated by LXR agonists. Treatment of primary hepatocytes with the LXR agonist GW3956 resulted in a 4-fold increase in Mylip mRNA level. GW3956 also stimulated Mylip transcription in multiple tissues in mice, including spleen, adrenal gland, intestine, and liver. The fact that Mylip is an ubiquitin E3 ligase capable of self-ubiquitination promoted Zecler and co-workers to investigate if Mylip can also mediate LDLR ubiquitination. Immunoprecipitation coupled with immunoblotting analysis revealed LDLR ubiquitination and subsequent degradation in HEK293 cells co-transfected with LDLR and Mylip. Additionally, Mylip carrying a mutation in the RING domain failed to induce LDLR ubiquitination and degradation when co-transfected into HEK293 cells. MG132, a proteasome inhibitor, did not block Idol-induced LDLR degradation. This observation is consistent with studies of PCSK9-mediated LDLR degradation in which the degradation occurs in the lysosome and not the proteasome. These data suggested that Mylip functions as an E3 ligase to trigger LDLR ubiquitination and degradation. Therefore, Mylip was renamed as inducible degrader of LDLR (Idol).

Amino acid sequence alignment revealed a conserved lysine residue in the C-terminal portion of LDLR, immediately following the NPVY endocytotic motif. Mutation of this lysine residue was not able to block Idol-stimulated LDLR ubiquitination and degradation. However, double mutation containing lysine 20 and cysteine 29 (K20R/C29A) rendered LDLR resistant to Idol-modulated ubiquitination and degradation in HEK293 cells (Zelcer et al., 2009). LDLR belongs to the LDLR family of receptors that share sequence and structure homology. The very-low-density lipoprotein receptor (VLDLR) and lipoprotein E receptor 2 (ApoER2) are two members of this receptor family that share a high degree of homology with LDLR. In co-transfection assays, Idol was able to stimulate ubiquitination and degradation of both VLDLR and apoER2. The C-terminal lysine residue (K20) is conserved in both VLDLR and ApoER2, while the cysteine (C29) is not conserved. Single mutation at K20 of VLDLR was able to block Idol-mediated ubiquitination and degradation (Hong et al., 2010). However, other members of this receptor family that also contain this conserved lysine residue, such as LPR1b, are not targets of Idol. It has been reported that PCSK9 can also reduce the protein levels of VLDLR and ApoER2. Thus it appears that the substrate specificities of Idol and PCSK9 overlap.

Using a floxed gene trap cassette system, Idol-deficient embryonic stem (ES) cells were generated (Scotti et al., 2011). LDLR protein level was much higher in Idol$^{-/-}$ ES cells than in wild type ES cells. When the cells were switched to medium containing 10% lipoprotein-deficient serum (LPDS), LDLR protein level increased in both types of ES cells. In Idol$^{-/-}$ ES cells, membrane-bound and intracellular LDLR protein levels both increased, suggesting that Idol can mediate degradation of LDLR before and after it reaches the cell membrane. Remarkably, treatment with the LXR agonist GW3965 reduced LDLR protein levels in wild type, but not in Idol$^{-/-}$ ES cells. This observation confirmed the previous finding that LXR-induced LDLR reduction was mediated through Idol. While PCSK9 binds to the extracellular EGFA domain of LDLR, Idol interacts with the C-terminal region of LDLR. Yet both pathways resulted in LDLR degradation in the lysosome. In Idol-deficient ES cells, recombinant PCSK9 is still able to induce LDLR degradation. This suggests that the Idol and PCSK9 pathways acted independently of each other. It could be speculated that owing to the importance of tightly controlling cellular cholesterol levels and circulating LDL-C levels, mammalian cells evolved two independent post-transcriptional pathways to regulate LDLR protein levels, and hence LDL-cholesterol uptake. These two pathways could complement each other or counterbalance each other depending on the specific situation. As discussed above, statins increase hepatic cholesterol uptake largely due to activation of LDLR transcription through SREBP-2. However, activation of SREBP-2 also increases expression of PCSK9, which in turn functions to reduce LDLR protein level. In a recent report, Dong and colleagues observed that statins suppressed Idol expression in HepG2 cells (Dong B et al., 2011). More interestingly, the ability of statins to increase LDLR protein level in these cells was compromised if the cells were transfected with Idol siRNA. The authors proposed that suppressing Idol expression is one of the mechanisms through which statins increase LDLR expression. This notion, however, was not confirmed by studies in Idol-deficient ES cells. Statins maintain their ability to increase LDLR protein level in Idol$^{-/-}$ ES cells (Scotti et al., 2011). In fact, LDLR levels in Idol$^{-/-}$ ES cells are much higher than wild type cells, even after statin treatment. Thus the exact relationship between these different LDLR regulatory pathways remains to be elucidated.

Studies in rodent models of hypercholesterolemia show that Idol can affect LDLR protein levels and cholesterol metabolism *in vivo*. In mice treated with the LXR agonist GW3965, LDLR protein levels were reduced in multiple tissues, including macrophages, small intestine, and to a lesser degree, liver. Conversely, the LDLR protein level was increased in these tissues in mice deficient for both LXR genes. More direct evidence was provided with studies using adenoviral construct harboring mouse Idol (Ad-Idol). Mice infected with Ad-Idol displayed dramatically reduced hepatic LDLR protein expression and a significantly increased plasma cholesterol levels compared to mice infected with control viral construct. Size fractionation of plasma lipoproteins revealed that the increased plasma cholesterol was largely due to increases in LDL cholesterol (Zelcer et al., 2009).

Idol has also been shown to play a role in impaired cholesterol metabolism in the Niemann-Pick type C and ApoE double knockout mice (NPC$^{-/-}$ApoE$^{-/-}$) (Ishibashi et al., 2010). The plasma cholesterol level is significantly higher in NPC$^{-/-}$ApoE$^{-/-}$ mice than in ApoE$^{-/-}$ mice. A large portion of this elevated plasma cholesterol is in the form of VLDL-C, due to reduced clearance of VLDL-C in the double knock-out mice. The double knock-out mice have significantly less LDLR protein in the liver, despite increased mRNA levels. Further analysis revealed that the expression of both Idol and PCSK9 is increased in livers of NPC$^{-/-}$ApoE$^{-/-}$ mice. In fact, all LXR and SREBP-2 target genes were stimulated in the livers, including LDLR at the transcriptional level. However, increased expression of Idol and PCSK9 resulted in accelerated degradation of hepatic LDLR protein, leading to reduced VLDL-cholesterol clearance in the NPC$^{-/-}$ApoE$^{-/-}$ mice. In this situation, Idol and PCSK9 worked in a complementary fashion to reduce hepatic LDLR levels, which resulted in significant hypercholesterolemia.

Recent genome-wide association studies (GWAS) provided some clues that Idol might be involved in the regulation of LDL-cholesterol in humans. The Women's Genome Health Study (WGHS) included 17,296 North American women with self-reported European ancestry who were non-diabetic and not on lipid lowering therapy at baseline (Chasman et al., 2009). Plasma lipid profiles and genotyping analysis were performed in these subjects. A total of forty-three loci displayed significant association with at least one lipid marker, including LDL-C, HDL-C, triglycerides, ApoA1, and ApoB100 levels, as well as lipoprotein size. The majority of these loci correspond to genes known to affect plasma lipid levels, such as PCSK9, LDLR, CETP, and HMG-CoA reductase. However, three novel loci were identified that showed significant association with at least one of the lipids measured, and one of the loci (rs2480) associated with LDL-cholesterol resides on chromosome 6q22.3, near the Mylip gene. In a separate report, Waterworth and co-workers performed a meta-analysis of 8 independent GWAS studies with a total of 17,723 participants of Caucasian European descent (Waterworth et al., 2010). This analysis confirmed most of the known genes affecting lipid metabolism as well as new loci identified from other GWAS. Additionally, six new loci were identified that reached genome-wide statistical association with circulating lipids. These are SBPs at Mylip/GMPR and PPP1R3B loci with LDL-C; at SLC39A8, TTC39B and FADS1 loci with HDL-C; and FADS1 loci with triglycerides. SNP rs2142672 lies in a distinct block of high linkage disequilibrium (LD) between 2 genes; Mylip and guanosine monophosphate reductase (GMPR) on chromosome 6q23. The C allele, with a frequency of 74%, is associated with relatively high levels of circulating LDL-C. These studies demonstrated the potential association between Idol gene polymorphisms and circulating

LDL-cholesterol levels, although there have been no reports on gain-of-function or loss-of-function of Idol so far. Therefore, a causal relationship between Idol gene function and LDL-C levels in humans is yet to be established.

To summarize, biochemical studies have demonstrated that Idol functions as an ubiquitin ligase for LDLR leading to its degradation in the lysosome. *In vivo* studies provided evidence that increased Idol expression in the liver could lead to reduced LDL-C clearance and elevated plasma cholesterol levels. GWAS data suggested associations between Idol gene polymorphisms and circulating LDL-cholesterol levels. It is conceivable that compounds capable of inhibiting Idol-mediated LDLR degradation could lead to increased hepatic cholesterol clearance and reduce circulating LDL-C. Idol is an intracellular protein that interacts with the intracellular domain of LDLR. Thus, unlike the situation with PCSK9, small molecular inhibitors that can penetrate cell membrane will have to be developed. Ubiquitin ligases represent one of the largest protein families in mammalian cells. It is estimated that over six hundred ubiquitin ligases exist in humans with each one targeting only a few specific substrates. Dysfunction of the ubiquitin proteasome system (UPS) has been shown to be involved in multiple diseases (Cohen and Tcherpakov, 2010). The development of small molecule inhibitors targeting UPS represents one of the exciting new frontiers in drug discovery (Ceccarelli et al., 2011). A small molecule inhibitor of Idol would be useful as an additional LDL-C lowering agent for those patients who cannot tolerate statins, or those unable to reach an aggressive LDL-C lowering goal with statins.

5. Concluding remarks

In summary, recent evidence has emerged that supports further reducing LDL-C for CAD prevention and treatment. It is expected that the new guidelines for LDL-C will be even lower. Existing therapies to reduce LDL-C, however, do not appear to be sufficient to meet these new guidelines, and therefore, there are significant unmet medical needs in this area. The identification of PCSK9 and Idol as novel regulators of LDLR has provided novel opportunities to develop additional therapeutics to further reduce LDL-C. New experimential drugs particularly targeting PCSK9 are already in clinical development. We anticipate that one or more of these agents will be able to demonstrate significant efficacy in reducing LDL-C and additional benefit in CAD prevention and treatment. Such compounds may likely become the next generation of medicines for managing cardiovascular disease.

6. Abbreviations

- apoER2 ApoE receptor 2
- ApoB Apolipoprotein B
- CAD Coronary artery disease
- EGFA Epidermal growth factor domain A
- Idol Inducible degrader of LDLR
- LDL-C Low-density lipoprotein cholesterol
- LDLR Low-density lipoprotein receptor
- LXR Liver X receptor
- PCSK9 Proprotein convertase subtilisin/kexin type 9
- SREBP2 Sterol-responsive element binding protein 2
- VLDLR Very low-density lipoprotein receptor

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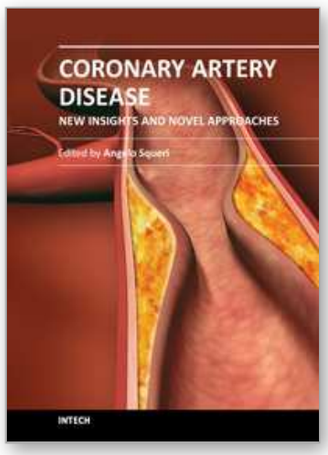

Coronary Artery Disease - New Insights and Novel Approaches
Edited by Dr. Angelo Squeri

ISBN 978-953-51-0344-8
Hard cover, 260 pages
Publisher InTech
Published online 16, March, 2012
Published in print edition March, 2012

Coronary Artery disease is one of the leading causes of death in industrialized countries and is responsible for one out of every six deaths in the United States. Remarkably, coronary artery disease is also largely preventable. The biggest challenge in the next years is to reduce the incidence of coronary artery disease worldwide. A complete knowledge of the mechanisms responsible for the development of ischaemic heart disease is an essential prerequisite to a better management of this pathology improving prevention and therapy. This book has been written with the intention of providing new concepts about coronary artery disease pathogenesis that may link various aspects of the disease, going beyond the traditional risk factors.

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