Development of Inhibitors of Human PCSK9 as Potential Regulators of LDL-Receptor and Cholesterol

By

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Abstract

Proprotein Convertase Subtilisin/Kexin 9 (PCSK9) is the ninth member of the Ca^{+2}-dependent mammalian proprotein convertase super family of serine endoproteases that is structurally related to the bacterial subtilisin and yeast kexin enzymes. It plays a critical role in the regulation of lipid metabolism and cholesterol homeostasis by binding to and degrading low-density lipoprotein-receptor (LDL-R) which is responsible for the clearance of circulatory LDL-cholesterol from the blood. Owing to this functional property, there is plenty of research interest in the development of functional inhibitors of PCSK9 which may find important biochemical applications as therapeutic agents for lowering plasma LDL-cholesterol. The catalytic domain of PCSK9 binds to the EGF-A domain of LDL-R on the cell surface to form a stable complex and re-routes the receptor from its normal endosomal recycling pathway to the lysosomal compartments leading to its degradation. Owing to these findings, we propose that selected peptides from PCSK9 catalytic domain, particularly its disulphide (S-S) bridged loop1 $^{323-358}$ and loop2 $^{365-385}$, are likely to exhibit strong affinity towards the EGF-A domain of LDL-R. Several regular peptides along with corresponding all- dextro and retro-inverse peptides as well as the gain-of-function mutant variants were designed and tested for their regulatory effects towards LDL-R expression and PCSK9-binding in human hepatic HepG2 and mouse hepatic Hepa1c1c7 cells. Our data indicated that disulfide bridged loop1-hPCSK9$^{323-358}$ and its H^{357} mutant as well as two short loop2-hPCSK9$^{372-380}$ and its Y^{374} mutant peptides modestly promote the LDL-R protein levels. Our study concludes that specific peptides from the PCSK9 catalytic domain can regulate LDL-R and may be useful for development of novel class of therapeutic agents for cholesterol regulation.

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List of Abbreviations

PCSK9 ....Proprotein Convertase Subtilisin/Kexin 9

NARC1............................Neural Apoptosis Regulated Convertase1

LDL-R ....Low Density Lipoprotein Receptor

LDL-C ....Low Density Lipoprotein Cholesterol

Apo-B ....Apolipoprotein B

aa ....Amino acid

h ....Human

Sh.....................................Short

Inter................................Intermediate

D ....Dextro

Rev ....Reverse

ER ....Endoplasmic Reticulum

TGN ....Trans Golgi Network

SREBP ....Sterol Regulatory Element Binding Protein

EGF-A ....Epidermal Growth Factor like repeat A

CHRD ....Cysteine/Histidine Rich Domain

DRD................................Disulphide Rich Domain

ADH ....Autosomal Dominant Hypercholesterolemia

CHD...............................Coronary Heart Disease

CVD...............................Cardiovascular Disease

AnxA2 ....Annexin A2

FH ....Familial Hypercholesterolemia

HMG-CoA......................Hydroxymethylglutaryl Co-Enzyme A Reductase

mAb ....Monoclonal antibody

MS ....Mass Spectrometry

SELDI-tof ....,....Surface Enhanced Laser Desorption Ionization time of flight

MALDI-tof ....Matrix Assisted Laser Desorption Ionization time of flight

RP-HPLC ....Reverse Phase-High Performance Liquid Chromatography

TFA ....Trifluoroacetic acid

ACN ....Acetonitrile

DMF ....N N'-dimethyl formamide

NMP ....N-methyl pyrolidone

Fmoc ....Fluorenyl methoxy carbonyl

HATU.............................Hexafluorophospho(O-7-azabenzotriazol-1-yl)-1,1,3,3-tetramethyluronium

OD.................................Optical Density

BSA...............................Bovine Serum Albumin

NCLPDS........................Newborn Calf Lipoprotein-Deficient Serum

FBS................................Fetal Bovine Serum

TFR...............................Transferrin Receptor

DMEM...........................Dulbecco's modified Eagle's medium

1x α-MEM......................1x Alpha-Minimum Essential Medium

PBS................................Phosphate Buffered Saline

TBS................................Tris Buffered Saline

DTT...............................Dithiothreitol

PMSF............................Phenylmethanesulfonylfluoride

SDS -PAGE...................Sodium Dodecyl Sulfate - Polyacrylamide Gel Electrophoresis

EDTA............................Ethylenediaminetetraacetic acid

1. Introduction

Cardiovascular or coronary heart disease (CHD) is one of the greatest plagues that continue to be the leading cause of death all over the world, especially in the western world (1). One of the best known recognized risk factors for this disease is high level of circulatory low density lipoprotein cholesterol (LDL-C) in the blood. Therefore in recent years the regulation of LDL-cholesterol has drawn significant research interest for developing therapeutic intervention of coronary heart disease for reducing cholesterol (2). Currently several Hydroxymethylglutaryl Co-Enzyme A Reductase (HMG-CoA) inhibitory compounds called statins have been discovered which are highly effective in lowering LDL-C in the serum. These agents are now routinely used to lower LDL-cholesterol in order to treat cardiovascular diseases in humans. These drugs are highly efficient and work for most patients (~90% of cases), but they cause serious side effects (muscle pain, weakness, myopathy, neuropathy, cognitive loss, pancreatic and hepatic dysfunction, etc) for the rest ~10% of population (3). The side effects are more common particulary for diabetic patients.

Since the discovery of Proprotein Convertase Subtilisin/Kexin type 9 (PCSK9) as a strong negative regulator of hepatic Low Density Lipoprotein Receptor (LDL-R) level, it has shown to play a critical role in the regulation of lipid metabolism and cholesterol homeostasis. Thus it became a major target for developing alternate therapeutic agents for lowering LDL-C (4, 5, 6, 7). Antibody mediated inhibition of PCSK9 that prevents its binding interaction with LDL-R has become a novel therapeutic approach for reducing LDL-cholesterol level. In fact, PCSK9 inhibition has emerged as one of the most active lines of investigation in cholesterol research, with promising results in a number of Phase II trials in heterozygous Familial

Hypercholesterolemia (FH) and in patients intolerant to statins. Homozygous FH – a rare heritable disorder that leads to severely elevated LDL-cholesterol and Cardiovascular Disease (CVD) that typically manifests in childhood – is the latest testing ground for this new class of agent. In this respect, blocking antibodies have been raised against PCSK9 functional activity and are now being tested as indicated in several clinical trials. Two of these antibodies called **Alirocumab** (previously known as REGN727/SAR236553, developed by Regenoron Inc.) and **AMG 145** (developed by Amgen Company) showed highly promising results in Phase II clinical trials. When administered through subcutaneous injections, these drugs with or without statin drugs exhibited > 70% reduction in LDL-C levels in humans. (http://informahealthcare.com/doi/abs/10.1517/13543784.2013.822485). Thus agents are now entering Phase III trial (8, 9). In this regard so far no small molecules PCSK9 inhibitors have been developed or reported in the literature expect only a few that are not yet thoroughly substantiated (10, 11). Small molecule inhibitors are always desirable since they possess drug like characteristics, are less expensive and physiologically stable (12). Thus development of small compound PCSK9-inhibitors is more appealing and attractive.

The objective of this study is to develop peptide and peptide based small compounds as potential PCSK9 inhibitors derived from its own catalytic domain that is known to interact with the LDL-R leading to its degradation in the lysosomes.

1.1. PCSK9

1.1.1 PCSK9 History and Discovery

PCSK9 or "Proprotein Convertase Subtilisin/Kexin 9" – the ninth member of mammalian counterpart of bacterial subtilisin and yeast kexin enzymes – originally named Neural Apoptosis Regulated Convertase-1 (NARC-1) was first discovered in 2003 (13). It is now well established following extensive research studies that low density lipoprotein cholesterol (LDL-C) is cleared from plasma via cellular uptake and internalization processes that are mediated by low density lipoprotein receptor (LDL-R), which has been shown to be degraded by PCSK9 (13, 14, 15, 16). Thus PCSK9 was identified as the third gene along with Low Density Lipoprotein receptor (LDL-R) and apolipoprotein B (Apo-B) that are implicated in Autosomal Dominant Hypercholesterolemia (ADH) and was therefore considered as a potential therapeutic target for lowering LDL-C in the plasma (17, 18, 19). PCSK9 is a modular protein that consists of four major domains: the signal peptide (amino acids 1-30) followed by the prodomain (amino acids 31-152), catalytic domain (amino acids 153-454) and finally C-terminal domain (amino acids 455-692) (20, 21, 22). PCSK9 is synthesized as pre-proprotein which loses its signal peptide segment due to signal peptidase action in the Endoplasmic Reticulum (ER) producing 72-kDa pro-PCSK9 protein (human form). This then undergoes auto catalytic cleavage of its prodomain segment in the endoplasmic reticulum (ER) resulting in the release of 14-kDa prodomain and 62-kDa mature PCSK9 proteins in soluble protein that is secreted into the bloodstream (21, 23, 24). Like the other PCSK family enzymes, the prodomain segment of PCSK9 after its cleavage remains tightly associated with the mature protein and thereby blocks its enzymatic activity. However, unlike other family members, the prodomain of PCSK9 does not contain a target loop for secondary cleavage to activate the

enzyme. Thus, in mature secreted PCSK9, the prodomain remains completely bound non-covalently to the catalytic domain thereby covering its catalytic triad residues (**Asp**^{186}, **His**^{226} and **Ser**^{386}) (20, 21). Though catalytically inactive, the prodomain bound PCSK9 remains functionally active in its ability to bind to LDL-R and together as a complex traffic to lysosomal instead of normal endosomal compartment ultimately leading to LDL-R degradation, as depicted in (**Figure 1.1B**) (14, 15). As indicated earlier, PCSK9 exhibits its major role in degrading LDL-R, which is responsible for clearance of plasma LDL-cholesterol (LDL-C) (13, 16). As a result of this important biological property, PCSK9 has drawn significant attention in cardiovascular research where high level of plasma LDL-C is considered as a major risk factor. Thus PCSK9 is a major target for therapeutic intervention of hypercholesterolemia and associated cardiovascular disease risk.

1.1.2 PCSK9 Physiology

1.1.2.1 Biosynthesis

PCSK9 is expressed abundantly in the liver (Biliary system), followed by kidney (Renal system), intestine (Digestive system including pancreas) and trace amount in the brain (Nervous system) (13). Human (h) PCSK9 is a glycoprotein consisting of of 692 amino acids (aa) that interacts post-translationally with LDL-R leading to latter's transport from the normal endosomal pathway to the lysosomal compartment leading to its degradation. As mentioned earlier PCSK9 contains a signal peptide which is cleaved off in the ER, followed by a prodomain that provides a folding chaperone and an inhibitor of cognate proteolytic activity, a preserved catalytic domain with the catalytic triad of residues, and lastly a C-terminal domain

known as Cysteine/Histidine rich domain (CHRD) or Disulphide rich domain (DRD) which has been found to be essential for LDL-R degradation process as illustrated in (**Figure 1.1A**) (13, 25).

Human PCSK9 is synthesized as a 72-kDa zymogen in the ER and then modified on the way to the cell surface. Following the autocatalytic cleavage at the site (VFAQ_{152}↓SIP), the N-terminus prodomain segment still remains strongly attached with the 62-kDa mature protein and acts as a chaperone to guide PCSK9 through secretory pathway (13). To date all efforts to separate this complex using non-denaturing conditions have failed and its enzymatic (proteolytic) activity has only been exhibited recently *in vitro* using a synthetic fluorogenic peptide that encompasses the prodomain cleavage site (26, 27). The dissociation of the prodomain segment through non denaturing conditions has been found to be a most difficult task despite many attempts.

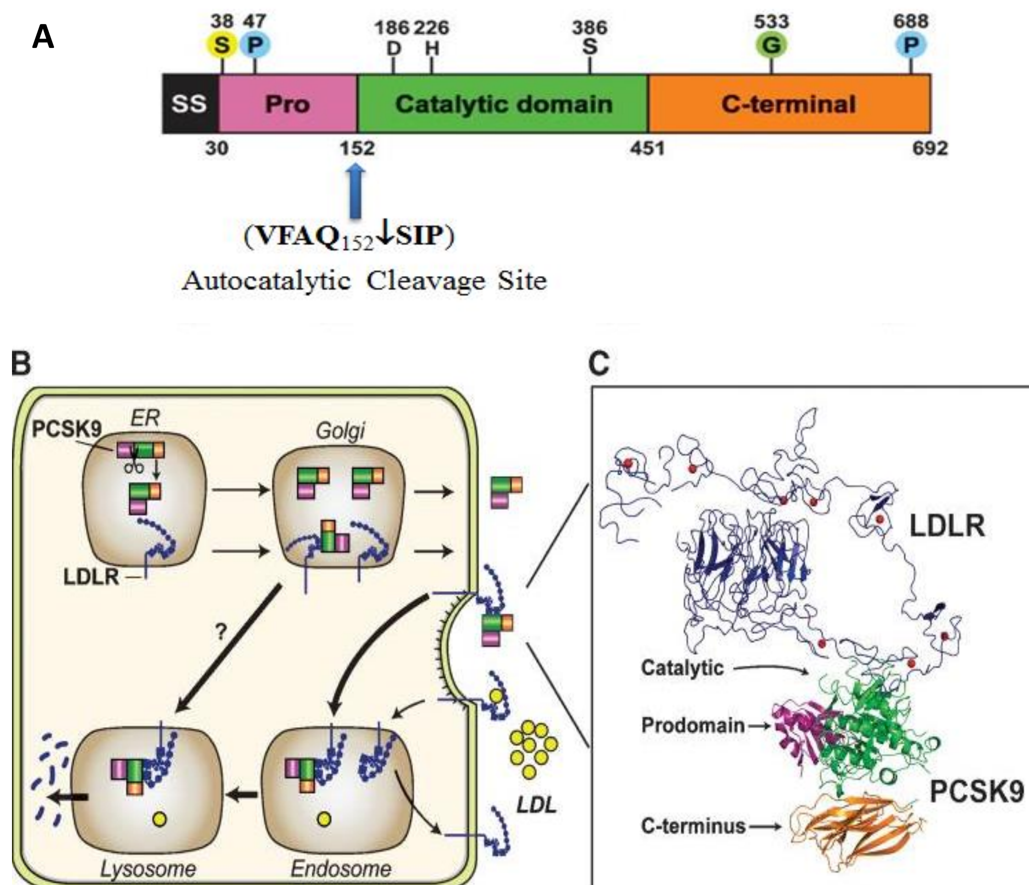
So far no known physiological protein substrates of PCSK9 have been identified. Recently studies revealed that the high acidic character of the prodomain segment might have a role in the regulation the PCSK9 functional activity. In addition, it was also noted that in acidic pH (< 5.4) condition, PCSK9 tends to separate from its prodomain complex and as a result leads to a loss of its functional activity (28). The C-terminal domain of PCSK9 consists of ~ 230 amino acids residues with nine disulfide bridges (3 in each repeat segment). It has been involved in PSCK9-induced LDL-R co-localization at the extracellular surface and in the LDL-R degradation by a mechanism not fully understood yet (29, 30). It has been further revealed that the C-terminus domain is not required for the actual binding of PCSK9 with LDL-R, but it is found to be essential for re-routing LDL-R to the lysosomal degradation pathway. It may also

be involved in the binding with other proteins besides LDL-R (31, 32). Studies also confirmed that PSCK9 lacking this domain is not capable of degrading LDL-R (33).

1.1.2.2 Post-translational modifications

Human PCSK9 undergoes a series of post-translational modifications that may alter its functional activity in either PCSK9 synthesis or its binding to and degrading LDL-R. These include glycosylation, phosphorylation, and tyrosine sulfation (13, 34, 35) as shown in (**Figure 1.1A**). Nevertheless, none of these modifications are required for the PCSK9 secretion. It is now most likely that while transiting through Trans Golgi Network the Asparagine residue at position 533 (**Asn**^{533}) is glycosylated while the Tyrosine at position 38 (**Tyr**^{38}) is sulfated in the prodomain segment (24, 35). So far no known physiological implication of the sulfation has been noticed. In fact it does not seem to have any impact on the PCSK9 functional activity in the degradation of LDL-R nor in the modification of cholesterol homeostasis. Furthermore it has been reported that the only site of glycosylation in PCSK9 found at position 533 (**Asn**^{533}) is not required for LDL-R degradation and PSCK9 secretion from the ER, and its role still remains unclear (20, 36). Currently two phosphorylations in PCSK9 have been located, one in the prodomain region (**Ser**^{47}) and the other in the C-terminal domain region (**Ser**^{688}) (34). Interestingly, those two phosphorylated regions are located in the sites that have not been detected in the crystal structure of hPCSK9 possibly because of lacking of electron density in those sites (20, 34, 37). It is speculated that the phosphorylations in PCSK9 may alter the degradation of LDL-R, but so far no study has been reported in the literature on this significant aspect.

Figure 1.1: **A schematic diagram showing structure of human PCSK9 and its various characteristic domains with their roles in functional activity.** ***A****:* Different regions identified by color coding. The major domains of PCSK9 include; Signal peptide, Pro, Catalytic, and C-terminal domains. The indicated residues in the catalytic domain are the conserved amino acids required for its catalytic activity: Aspartic acid (D), Histidine (H) and Serine (S) and the binding site of the single N-liked sugar (N^{533}). PCSK9 post-translational modifications include glycosylation (G), phosphorylation (P) at S^{47} and S^{688}, and sulfation (S) at Y^{38} and at another unkown tyrosine in the catalytic domain. ***B****:* Cellular itinerary of PCSK9. Following the auto catalytic cleavage in the ER, the prosegment (Purple) remains attached with the catalytic domain (green) and then released to the cell surface. At the cell surface, PCSK9 binds to LDL-R and internalized by adaptor protein ARH leading to its transfer to the lysosomal degradation pathway. A second path may be that PCSK9 binds to LDL-R in post-ER leading to its re-routing for degradation in the lysosomes. ***C****:* Model structure of the complex showing the binding of full length LDL-R with PCSK9. The EGF-A domain (blue) of LDL-R at acidic pH binds to PCSK9. SS: signal peptide sequence; Pro: Prodomain sequence.



This figure was adapted and slightly modified from *Horton et al. 2009* (38)

1.1.3 Enzymatic Activity

The crystal structure of human (h) PCSK9 revealed a strong association of the prodomain segment with the catalytic domain of mature PCSK9. It also shows the blockade of catalytic triad of residues (**Asp**^{186}, **His**^{226} and **Ser**^{386}) (20, 21, 39) by the cleaved prodomain segment. Thus PCSK9 remains enzymatically inactive but functionally active. This means that it has the capability to bind to LDL-R and re-routing it to the lysosomal compartments for degradation, a process shown in detail in (14, 15). To date the only substrate noted for PCSK9 has been its own prodomain segment which has been found to be crucial for its secretion and proper folding during its biosynthesis in the ER and TGN that leads to its LDL-R degradation activity (40). In addition, all efforts in the separation of prodomain of PCSK9 from its complex with mature PCSK9 have failed. Nonetheless, the catalytic activity of PCSK9 has indeed been demonstrated *in vitro* for the first time in both total cell lysates and cultured medium of primary mouse hepatocytes and immortalized human hepatocytes (IHH) by using a fluorogenic substrate designed from its autocatalytic cleavage site (**VFAQ**$_{152}$**↓SIP**) and the same assay was not shown to be suitable for plasma samples (23, 27).

Despite the demonstration of proteolytic activities of various proteases of PCSK-family towards a variety of proprotein substrates such as hormones, neuropeptides, enzymes, growth factors and their receptors as well as host of other proteins (13, 41), the protease activity of PCSK9 in relation to its protein substrates remained elusive. To fully understand PCSK9 functional activity in regulating LDL-R level, the determination of other substrates and illuminating any activity will remain as the objective of intense research in coming months in the study of PCSK9.

1.1.4 Regulation of PCSK9 Transcription

PCSK9 is primarily up-regulated and increased through the activity of the transcription factor Sterol Regulatory Element Binding Proteins SREBP-2 (25). The SREBPs are members of the basic helix-loop-helix leucine zipper (bHLH-Zip) family of transcription factors that regulate genes encoding proteins required for cholesterol, fatty acid, and phospholipid synthesis (42, 43, 44). SREBPs family consists of three isoforms: SREBP-1c is mainly involved in the synthesis of fatty acids and insulin-mediated glucose metabolism, SREBP-2 is directly associated with the synthesis of cholesterol and SREBP-1a seems to activate genes in both biosynthetic pathways (42, 44, 45, 46).

Recently it has been demonstrated that the expression level of both PCSK9 and LDL-R is up-regulated via the activation of SREBP-2 when the content of cholesterol in cells is depleted, such as during the course of statin therapy (23). In addition it was shown that SREBP-1c and SREBP-2 have been involved in the regulation of PCSK9, but mainly SREBP-2 as reported in human hepatocytes (HepG2 cells) study (47). PCSK9 expression is down-regulated by suppression of SREBP-2 and cholesterol feeding according to mice model study reported in (48, 49).

Interestingly, Berberine – a natural plant extract - was shown to decrease PCSK9 expression in a time- and dose-dependent fashion (50). It was further indicated that the combination of berberine and mevastatin lead to an elevation in LDL-R protein level and suppression in PCSK9 level which is caused only by mevastatin (51). The natural hypocholesterolemic agent berberine acts as repressor for the transcription of PCSK9 protein; however, to date the molecular pathway involved remains unclear (51).

1.1.5 Binding Partner Proteins

Currently it was identified that PCSK9 has only two well-known binding partner proteins. The first partner protein is of course LDL-R, which is responsible for clearance of plasma circulatory LDL-cholesterol (LDL-C). PCSK9 binds to LDL-R via latter's epidermal growth factor like-repeat A (EGF-A) domain and its own catalytic domain promoting its degradation and internalization in lysosomal compartment instead of normal endosomal route (6, 52). The second partner protein for PCSK9 is Annexin-A2 which binds to the C-terminal domain (CHRD) of PCSK9 leading to a loss of the degradation of LDL-R activity (33).

1.1.5.1 Low Density Lipoprotein Receptor (LDL-R)

Low density lipoprotein receptor (LDL-R) has emerged as a major regulator of cellular LDL-cholesterol uptake. LDL-R protein is synthesized in the endoplasmic reticulum (ER) as a 120-kDa precursor form. Subsequently, the LDL-R precursor is transported to the Golgi apparatus where it undergoes extensive O-linked glycosylation leading to the mature 160 kDa form found at the cell surface (53). LDL-R binds to circulating LDL-cholesterol at the cell surface and the complex is internalized in the lysosomes, where the pH is low that allows LDL-cholesterol to be released for its degradation and redirection of LDL-R to the cell surface, where it continues to remove LDL-cholesterol from circulation (54).

The membrane bound receptor LDL-R protein has eight main structural domains: the signal peptide (amino acids 1-22), the 7 ligand binding repeat class A domains (amino acids 23-312), two EGF domains called EGF-A (amino acids 313-352), EGF-B (amino acids 353-393),

the 6 ligand binding repeats of class B domain (amino acids 397-657), EGF-C domain (amino acids 663-712), β-propeller domain (amino acids 713-721), O-linked sugar rich peptide domain (amino acids 722-768), Trans-membrane domain (amino acids 789-810) and lastly C-terminal cytoplasmic domain (amino acids 811-860) as depicted in (**Figure 1.2**) (55, 56). Accumulated studies show that PCSK9 protein acts as a negative regulator of LDL-R and consequently of circulatory LDL-cholesterol through degradation activity (57).

Moreover, changes in pH levels can lead to some conformational alterations in LDL-R. When the pH is neutral, similar to that found in the cell surface, LDL-R undergoes a conformational change assuming a linear extended structure where it can bind to LDL-C; whereas when the pH is low (~ 5.4), similar to pH found in the lysosomes, LDL-R acquires a hairpin structure, which allows the release of LDL-R into the lysosomal degradation and be re-cycled back to the cell surface (29).

The crystal structure of PCSK9 revealed that its catalytic domain (possibly the residues 367-381) contains the main binding site for the epidermal growth factor-like repeat A (EGF-A) domain of LDL-R and together as a complex traffic to lysosomal compartments leading to degradation as depicted in (**Figure 1.1C**) (21, 31, 58). The binding surface of PCSK9 with LDL-R is believed to involve various hydrophobic, key polar interactions as well as, multiple hydrogen bonds, and a calcium coordination site (31).

1.1.5.2 Annexin A2

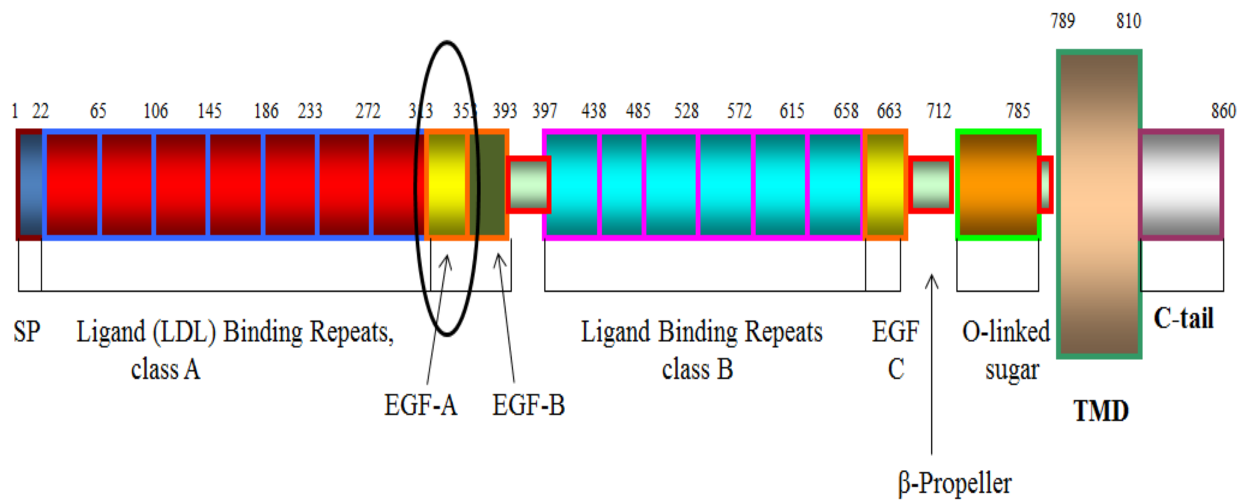
Annexin A2 (AnxA2) has been identified as a cytosolic and a linkage of membrane-associated protein through phospholipid binding. It has been found to be translocated to the cell surface and is known to interact with diverse extracellular proteins (59, 60). Annexin A2 is

highly expressed in lungs, aorta, heart, adrenal and small intestine (33). The binding of PCSK9 with Annexin A2 has been demonstrated in recent studies and this interaction is believed to mediate through the C-terminal domain (CHRD) tail of PCSK9, a segment which has not been considered important until recently (33). As shown in the crystal structure, the CHRD, more specifically the disulphide rich domain (DRD) within it, is not directly involved in the PCSK9=LDL-R complex formation (31). Nevertheless, the CHRD domain is considered as providing essential structural support for the binding of the catalytic segment of PCSK9 with the EGF-A domain of LDL-R (21, 31). The discovery of AnxA2 and its capability to act as an endogenous regulator of LDL-R degradation is the first knowledge of another binding partner protein for PCSK9, and it is the only known so called natural inhibitor of PCSK9 functional activity (33). The AnxA2 and CHRD domain of PCSK9 binding interaction has been shown to decrease PCSK9 induced degradation of LDL-R via an unidentified pathway (33). Currently it has been delineated the first model peptide of Annexin A2 (amino acids 25-97) that can significantly inhibit the PCSK9 functional activity in LDL-R degradation through its binding interaction with the CHRD domain of PCSK9 (61). Although AnxA2 is barely expressed in liver, it has been shown to play as a regulator of LDL-R degradation endogenously in extra-hepatic tissues (61).

1.1.5.3 Other partner proteins

Recently it has been reported that PCSK9 is independently and positively associated with other partner protein called γ-glutamyl transferase in diabetic patients (62). So far no other partner proteins for PCSK9 has been reported in the literature and further studies are needed on this significant aspect.

Figure 1.2: **A schematic diagram showing the protein structure of human LDL-Receptor (LDL-R).** It shows all the major domains of human LDL-R protein that include, Trans-membrane bound domain shown in a large box at the C-terminal tail as well as the EGF-A domain that is involved in the binding interaction with PCSK9 protein shown in an elliptical circle.



This figure was taken from *Basak et al. 2012* (63)

1.1.6 Mechanism of LDL-R Degradation

The mechanism of recognition and degradation LDL-R by PCSK9 protein is beginning to be understood (32). It has now been indicated that the catalytic segment of PCSK9 binds to the EGF-A domain of LDL-R promoting its degradation through two possible pathways: exogenously at the cell surface and endogenously in the Trans Golgi Network (TGN) as depicted in (**Figure 1.1B**) (7, 15, 64). In recent years it was demonstrated through co-immunoprecipitation, co-crystallization and co-expression studies with PCSK9, and LDL-R that PCSK9-mediated degradation of LDL-R occurs through binding of EGF-A domain of LDL-R and the catalytic segment of PCSK9 (16). It was also revealed that PCSK9 specifically binds to the EGF-A domain of LDL-R in a calcium-dependent manner (16).

Even though the binding interaction is between the EGF-A domain of LDL-R and the catalytic domain of PCSK9, other PCSK9 domains including the prodomain and C-terminal segment may also be crucial for LDL-R degradation in an indirect manner. Thus it has been shown that PCSK9 lacking the N-terminal 52 amino acids enhances the binding between both proteins (31). In addition PCSK9 lacking the C-terminal domain does not seem to be involved in LDL-R degradation (16). So far the detail mechanism about how PCSK9 binds to and degrades LDL-R still remains to be fully elucidated.

It was noted that the mature secreted PCSK9 destroyed LDL-R via its binding with the EGF-A domain of LDL-R through an endocytotic internalization pathway. This leads to the transport and co-localization of the complex into the lysosomal compartments and subsequent degradation of LDL-R protein as depicted in (**Figure 1.3**) (16, 65). Furthermore, it was revealed that the binding affinity between both proteins is enhanced in the lysosomal compartments by about ~ 150 fold in an acidic environment with a pH of approximately 5.2

similar to that found in the endo/lysosomal compartment (16, 26) and that this binding is also calcium dependent (16). Despite the fact that autocatalytic action of PCSK9 is necessary for its secretion and maturation (40), the catalytic activity is not required for secreted PCSK9 to bind to degrade LDL-R (40, 66).

1.1.6.1 Domain(s) of LDL-R Crucial for its binding with PCSK9

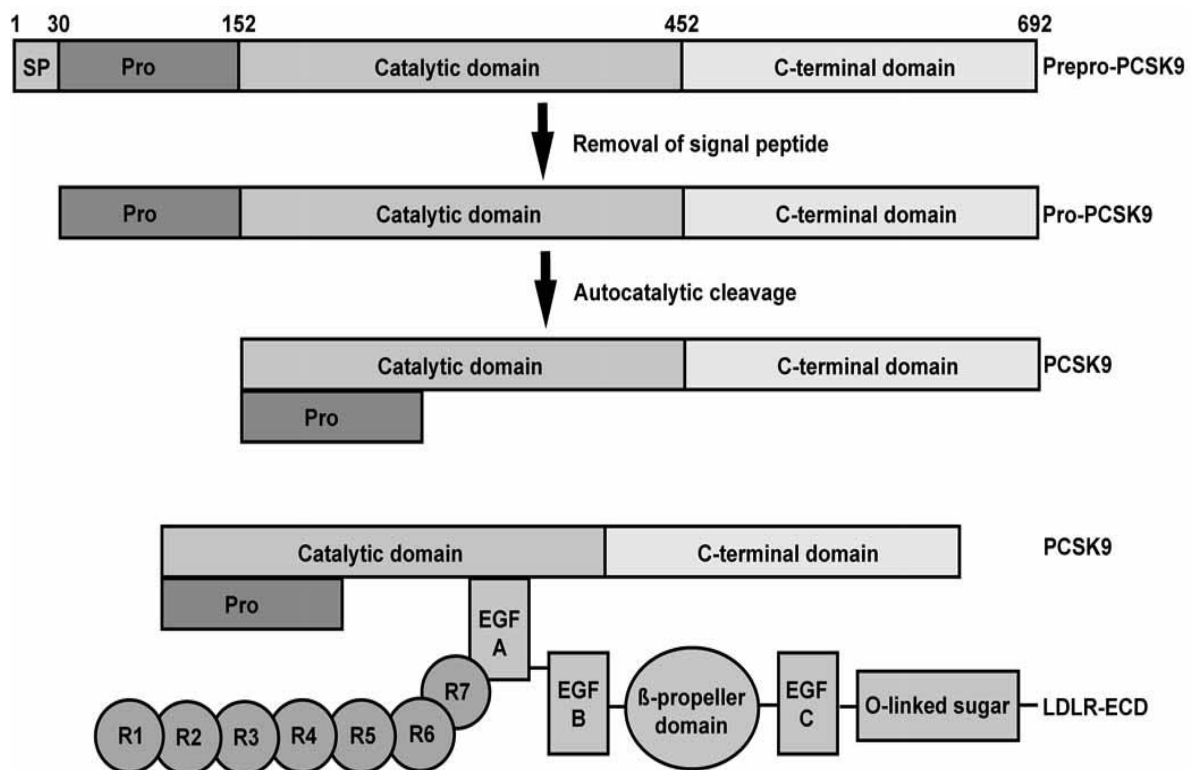
The binding interaction between PCSK9 and LDL-R is specific and requires multiple domains of both proteins although some domains are not physically implicated at all in this binding. There are domains of LDL-R protein that do not make contacts with PCSK9 but may be required for PCSK9-mediated degradation. These include its β-propeller domain and at least three of its seven ligand binding repeat domains as depicted in (**Figure 1.3**) (29). It has been shown that PCSK9 is able to bind with LDL-R even when LDL-R has less than three of its seven ligand binding domains, or is lacking a β-propeller domain. However this binding does not promote LDL-R degradation (29, 33, 67).

The crystal structure of PCSK9 indicated that the pro- and C-terminal domains of PCSK9 are not directly involved in the binding with the EGF-A of LDL-R. In fact PCSK9 catalytic domain was found to make physical contacts with the N-terminal segment of the EGF-A domain, but not the C-terminus as revealed by crystal structure data (31). It was also demonstrated that the C-terminus portion of PCSK9 is necessary for destroying LDL-R (29). Interestingly, it has been suggested that PCSK9 may contain a lysosomal sequence that induces the re-routing of LDL-R to the lysosome, or it may preclude the conformation of LDL-R which cycles it back to the cell surface (7, 15, 32, 65).

It is now believed that PCSK9 binds to LDL-R and this in turn will prevent LDL-R from taking on a "closed" conformation, thereby re-directing the complex to the lysosomal degradation (29, 68). In cell based protein-protein binding experiments it was reported that PCSK9 when added exogenously to the medium of culture cells such as human hepatoma cells (HepG2 and HuH7) or human embryonic kidney cells (HEK-293) dramatically decreases LDL-R level (6, 35, 69).

Through direct binding interaction with the LDL-R, PCSK9 prevents the receptor from recycling into the cell surface and mediates the re-distribution of LDL-R to late endosomes/lysosomes for degradation. Nevertheless, the precise mechanisms by which PCSK9 re-routes LDL-R to the lysosome and mediates its degradation have not yet been fully defined. This information about the exact mechanism of inhibition will be essential in developing any future therapeutic agents for autosomal dominant hypercholesterolemia, ADH (22, 70).

Figure 1.3: **A schematic diagram showing the binding interaction of human PCSK9 protein with the extracellular domain of human LDL-Receptor.** PCSK9 contains a canonical signal peptide (SP), an inhibitory prodomain (pro) that is cleaved off in the ER, a subtilisin-like catalytic domain, and a C-terminal domain. PCSK9 is synthesized as a 72 kDa zymogen (pro-PCSK9) that undergoes autocatalytic action in the ER to release the prodomain (~14-kDa), which remains attached to the ~62-kDa mature protein. The secreted PCSK9 forms a complex with the EGF-A domain of the LDL-R extracellular domain (ECD), leading to endocytosis of the PCSK9=LDL-R complex and subsequent degradation of the LDL-R. The catalytic domain of the mature secreted PCSK9 protein which contains the main binding structure for the epidermal growth factor-like repeat A (EGF-A) domain of LDL-R extracellular domain (ECD), and together as a complex traffic from endosomes to lysosomes leading to degradation. The LDL-R-ECD consists of seven **Cysteine-Rich Repeats (R1-R7)**, three EGF-precuror homology domains, in which EGF-C is separated from EGF-A and B by a β-propeller, and an O-linked sugar (71, 72).



This figure was taken from *Li et al. 2009* (73)

1.1.7 PCSK9 Knockout and Overexpressed Mouse Studies

1.1.7.1 Complete knockout

Complete knockout mouse model studies of PCSK9 have clearly demonstrated the importance of PCSK9 in the regulation of lipid metabolism and the degradation of LDL-R. The first complete PCSK9 mouse knockout model has been examined with the anticipation of enhancement in the LDL-R level and in turn diminishing the plasma LDL cholesterol (74). As a matter of fact, it was shown that there was a 50% reduction in plasma cholesterol level without any significant changes in the phenotype between the wild type mice and the knockout mice (74). This total knockout mouse model study confirmed the functional activity of PCSK9 in the degradation of LDL-R.

It was also indicated the absence of PCSK9 results in up-regulation of LDL-R level and thereby down-regulation of the plasma LDL-cholesterol. Therefore down-regulation or elimination of PCSK9 caused an impact similar to that observed with statin compounds which are drugs well-known for treating hypercholesterolemia. Statin compounds are cholesterol lowering drugs that inhibit the rate limiting enzyme called hydroxymethylglutaryl co-enzyme A (HMG-CoA) reductase responsible for the biosynthesis of cholesterol in the liver (75, 76). Statins were found to up-regulate the transcription of not only LDL-R protein but PCSK9 as well, which decreases LDL-R (77). The ability to inhibit the functional activity of PCSK9 in concurrence with statins may have the beneficial effects on cholesterol levels by enhancing the transcription of only LDL-R and hence diminish circulatory LDL cholesterol without affecting PCSK9 level. This may provide an alternate option for possible treatment of high cholesterol and associated cardiovascular disease.

1.1.7.2 PCSK9 conditional knockout and transgenic mouse models

Mice lacking PCSK9 in total and specific tissues were carried out and suggested that the circulating PCSK9 is exclusively synthesized and expressed in the liver, and this contributes to 2/3rd increase in the circulatory LDL-cholesterol due to the enhancement of LDL-R degradation (78). Transgenic mice over-expressing PCSK9 in the liver has been shown to exhibit 57% increase in plasma cholesterol as a result of mean decrease in the LDL-R level (78). Total PCSK9 knockout mice were shown to exhibit 42% reduction of circulating plasma cholesterol while liver specific PCSK9 deficiency exhibited 27% lower LDL-cholesterol (78). By developing inhibitors targeting PCSK9 in the liver, one can lead to an increase in LDL-R protein expression and hence decrease in plasma cholesterol, without effecting normal physiology of the animal.

Even though the main source of circulating PCSK9 that target LDL-R is the hepatocytes, a significant amount of both proteins PCSK9 and LDL-R may present endogenously in the kidney. In spite of successful overexpression of PCSK9 in the kidney, PCSK9 still reduced the LDL-R level in the liver preferentially (79). To date no data or study has been reported in the literature about conditional knockout of PCSK9 in the kidney or other tissues. Obviously additional studies are needed to understand the function of PCSK9 expression in the kidney, gut and small intestine.

1.1.8 PCSK9 Mutations and Their Implications

Since the discovery of PCSK9 in 2003, numerous natural mutations have been reported in human PCSK9 all across its domains including the signal peptide as depicted in (**Table 1.1**). These mutations either increase its ability to degrade LDL-R, which are termed as "gain of function mutations", or those that decrease degradation of LDL-R are termed as "loss of function mutations" while there are others that do not affect LDL-R degradation (6, 52). The gain of function mutations lead to the enhancement of the activity of PCSK9 protein in degrading LDL-R and hence elevated LDL-cholesterol level in the blood circulation - a condition termed as hypercholesterolemia or autosomal dominant hypercholesterolemia ADH. This can lead to a greater risk factor for stroke and cardio-vascular disease (80). PCSK9 gain-of-function mutations have an effect on PCSK9's functional activity by increasing or providing it a new activity (25). Individuals who carry these mutations are at a higher risk of hypercholesterolemia linked to cardiovascular disease. The gain of function mutations were characterized by increased LDL-cholesterol level and associated with a higher risk of familial hypercholesterolemia (FH) (81).

The first gain of function mutation was identified in a French family as **$S^{127}R$**. The second mutation is **$F^{216}L$** (**Table 1.1**) (82). Recently it has been reported that PCSK9 **$S^{127}R$** mutation leads to an elevation of LDL-R degradation via its enhanced binding affinity to LDL-R on the cell surface (83) while the PCSK9 **$F^{216}L$** mutation results in binding to LDL-R at the same affinity compared to the wild type PCSK9 (21). Furthermore, two additional gain-of-function mutations of PCSK9 namely **$D^{374}Y$** and **$N^{157}K$** were found in Norwegians with clinical symptoms of familial hypercholesterolemia (84). It has now been fully established that PCSK9 **$D^{374}Y$** is the most potent gain of function mutation of PCSK9 (**Table 1.1**) (85). Its property

results from its high binding capacity to LDL-R (25 times greater binding affinity than the wild type) at physiological pH 7.4 and low pH 5.0 as depicted in (**Figure 1.4**) (14, 21). The differential functional activity of PCSK9 **N^{157}K** gain of function mutant still requires additional investigation for further clarification about its implication in LDL-R degradation (24).

The loss of function mutations in PCSK9 has the opposite effect; they decrease the PCSK9's functional activity in degrading LDL-R, leading to up-regulation of LDL-R protein by recycling back to the cell surface. This will result in a greater clearance of LDL-cholesterol from blood circulation instead of being directed towards the lysosomal degradation compartment (86). Consequently, these mutations are believed to be beneficial and protective for the individuals from the risk of cardiovascular disease, this condition is known as **hypocholesterolemia** (15, 87). PCSK9 loss of function mutations were first observed in African Americans subjects with clinical diagnose of hypocholesterolemia as a result of low plasma levels of LDL cholesterol (88, 89). In addition, loss of function mutation of PCSK9 **R^{46}L** was considered as the most potent mutation in the prodomain of PCSK9 found in Caucasians subjects who exhibited a 42% reduced level of LDL-C (**Table 1.1**) (90).

More recently another novel mutation has been discovered this time in a French Canadian family PCSK9 **Q^{152}H** loss of function mutation at the prodomain cleavage site of PCSK9. This resulted in a significant decrease in both concentrations of circulating PCSK9 (79%) and LDL-C (48%) (91). Another study also revealed that PCSK9 **Q^{152}H** variant is not capable of cleaving its prodomain at the cleavage site (VFA$\underline{H}_{152}$↓SIP) (91). It is interesting to point out that patients who exhibit loss of function mutations in PCSK9 are shown to be hypersensitive to treatments with cholesterol lowering HMG-Co A reductase inhibitors, such as statin drugs (84).

Table 1.1: **Some selected known PCSK9 mutations with their phenotypic functional consequences.**

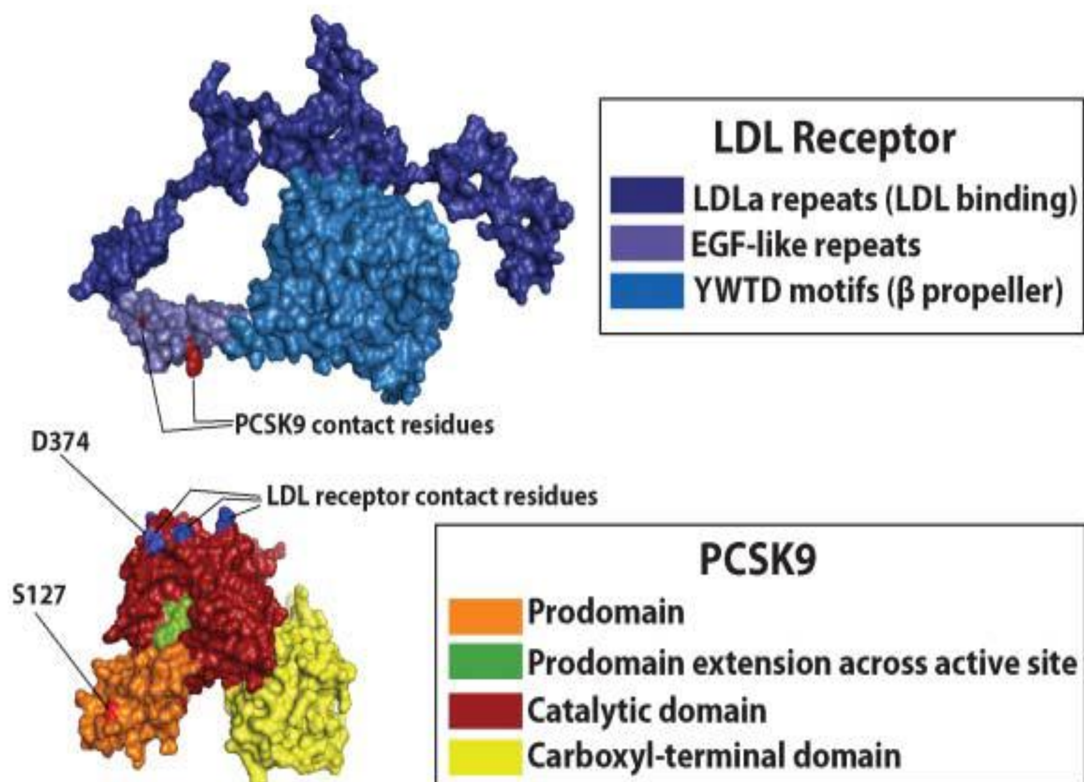
Functional domain	Mutation	Function	References
Signal peptide	**$V^{4}I$**	Gain	Miyake et al. 2008; Shioji et al. 2004
Prodomain	**$E^{32}K$**	Gain	Miyake et al. 2008
Prodomain	**$R^{46}L$**	Loss	Abifadel et al. 2003; Cohen et al. 2006
Prodomain	**$A^{53}V$**	Loss	Kotowski et al. 2006
Prodomain	**$E^{57}K$**	Loss	Kotowski et al. 2006
Prodomain	**$A^{68}T$**	Loss	Miyake et al. 2008
Prodomain	**$R^{104}C$**	Gain	Miyake et al. 2008
Prodomain	**$G^{106}R$**	Loss	Berge et al. 2006
Prodomain	**$V^{114}A$**	Loss	Fasano et al. 2007; Cariou et al 2009
Prodomain	**$S^{127}R$**	Gain	Abifadel et al. 2003; Cameron et al. 2008; Homer et al. 2008
Catalytic	**$F^{216}L$**	Gain	Abifadel et al. 2003
Catalytic	**$N^{157}K$**	Loss	Berge et al. 2006; Cameron et al. 2006
Catalytic	**$A^{168}E$**	No effect	Homer et al. 2007
Catalytic	**$R^{237}W$**	Loss	Benjannet et al. 2004; Berge et al. 2006
Catalytic	**$L^{253}F$**	Loss	Kotowski et al. 2006; Nassouri et al. 2007
Catalytic	**$R^{357}H$**	Gain	Allard et al. 2005
Catalytic	**$D^{357}Y$**	Gain	Bourbon et al. 2008
C-terminal	**$R^{469}W$**	Gain	Allard et al. 2005; Kotowski et al. 2006
C-terminal	**$R^{496}Q$**	Gain	Cameron et al. 2006
C-terminal	**$H^{553}R$**	Gain	Kotowski et al. 2006; Nassouri et al. 2007
C-terminal	**$V^{624}M$**	Gain	Miyake et al. 2008
C-terminal	**$S^{668}R$**	Loss	Miyake et al. 2008

This table was adopted following slight modifications from *Ferri et al 2012* (90)

Furthermore it was shown in wild-type and PCSK9 transgenic mice studies that two gain-of-function mutations of PCSK9 **$S^{127}R$** and **$D^{374}Y$** significantly decreased exogenous levels of the LDL-R protein by 23% and LDL internalization by 38% as compared with wild type PCSK9 (shown in **Figure 1.4**). However, the four loss-of-function mutations **$R^{46}L$**, **$G^{106}R$**, **$N^{157}K$**, and **$R^{237}W$** resulted in ~ 16% increase of cell surface levels of LDL-R and ~ 35% increase of LDL internalization (15). It was also noted that the binding segments of PCSK9 and LDL-R are located within a small region in both proteins. However mutations in non-binding domains of PCSK9 have also shown significant effects on the LDL-R levels. These include the loss-of-function mutation **$R^{46}L$** in the prodomain and gain-of-function mutation **$H^{553}R$** in the C-terminus region (15, 35, 65).

A few mutations were found to be silent without having any significant effects on the functional activity of PCSK9 (**Table 1.1**) (92). To date the location of all types of mutations in PCSK9 and their functional consequences on the degradation of LDL-R still need further investigation and studies (15). It is now clear that the elucidation of PCSK9's functional activity in degrading LDL-R and the discovery of PCSK9 inhibitors will be of major interests in the development of alternate therapeutic agents for the treatment of hypercholesterolemia.

Figure 1.4: **3Dimensional (D) structure of the hPCSK9 and LDL-Receptor at endosomal pH.** The (EGF-A) domain of LDL-R binds to PCSK9 in acidic pH of around 5.2 (68). Gain-of-function mutations at residues $\mathbf{D^{374}Y}$ located at the PCSK9 catalytic domain and $\mathbf{S^{127}R}$ in the prodmain potently reduce LDL-R protein in a significant manner. The $\mathbf{D^{374}Y}$ mutation is close to the LDL-R binding site, while $\mathbf{S^{127}R}$ is quite far from the binding interaction (80).



This figure was taken from *Peterson et al. 2008* (94)

1.2 Current and Future Directions

Certainly, the past decade has elucidated the critical role of PCSK9 in the regulation of lipid metabolism, cholesterol homeostasis as well as in other fields. Since PCSK9 is now considered a major target for providing an alternate innovative approach for therapeutic intervention of hypercholesterolemia, a number of pharmaceutical companies are engaged in developing potent inhibitors of circulatory PCSK9 that could disrupt its capability to degrade LDL-R. Despite the fact that cholesterol lowering HMG-Co A reductase inhibitors (statins) represent the most commonly prescribed drugs used for treating hypercholesterolemia, many individuals do not achieve optimum lipid or cholesterol levels, particularly those with high LDL-cholesterol levels and those intolerant to statin medications alone due to various reasons (95). This in turn led to the development of alternate strategy that may reduce LDL-cholesterol plasma level effectively for those where statin therapy is either less effective or causing serious side effects (96).

The clear link between PCSK9 and its binding to LDL-R in the regulation of cholesterol homeostasis have drawn serious attention from researchers in order to develop inhibitors with therapeutic use for treating hypercholesterolemia (97). Consequently, developing any compound or molecule that is able to disrupt this binding may be potentially beneficial for lowering plasma cholesterol (98). Currently there are a few distinct strategies that have emerged for developing inhibitors of PCSK9. The most promising approach at present that inhibit the formation of PCSK9=LDL-R complex at the cell surface is the use of blocking **monoclonal antibodies (mAb)** or **fibronectin fragments (adnectins)** (97, 99). Among these the blocking monoclonal antibodies (mAb) against PCSK9 have already shown

very promising results even in Phase III clinical trials and are very close to becoming drugs (97, 100). Despite this success and owing to some intrinsic negative features of mAb as potential medicine, there has been a growing interest in the design of small molecule compounds that can alter the function of PCSK9 in degrading LDL-R. Very recently, a recombinant chimera protein called Fcpro based on the constant Fc-region of the human immunoglobulin G (hIgG1) and the recombinant PCSK9 prodomain has been developed which inhibited PCSK9 quite efficiently (101). This data articulated that this chimera protein Fcpro effectively down-regulated PCSK9 activity to mediate LDL-R destruction (101).

In general, the new alternative strategies to inhibit the PCSK9 functional activity in the degradation of LDL-R may provide a glimmer of hope for possible treatment of hypercholesterolemia as an alternative to statin. In addition, small peptide inhibitors are more attractive because of their proteolytic and thermal stability, great compatibility, high aqueous solubility as well as drug like properties. In addition these molecules can also be chemically modified to make them more bioavailable and membrane transportable if necessary (76).

2. Objectives, Hypothesis and Rationale of The Research Project

2.1. Hypothesis

Since PCSK9 discovery only a limited number of strategies have been reported in the literature to develop compounds that can inhibit its biosynthesis or its fuctional activity in degrading LDL-R. These are based on siRNA, antibodies and small peptides. Among these, the development of PCSK9 inhibitory peptides approach is considered as most desirable and innovative. Therefore we focused our attention to this approach and our hypothesis is that peptide based inhibitors derived from the catalytic domain of hPCSK9 may likely to up-regulate LDL-R and thereby cholesterol.

2.2 Objectives

The objective of this research study for my MSc thesis is:

Aim 1: To develop peptide based inhibitors of human PCSK9 based on its own catalytic domain

Aim 2: To examine the regulatory effects of above peptides towards PCSK9 and LDL-R *in virto* and *ex vivo* conditions

2.3. Rational

Peptide/Peptide analogs derived from the catalytic domain of human PCSK9 can disrupt the association of PCSK9 with LDL-R leading to up regulation of LDL-R activity and in turn increased clearance of cholesterol from circulation.

3. Materials and Methods

3.1 Materials

3.1.1 Chemicals and Reagents

3.1.1.1 Peptide Synthesis Work

All coupling and other agents for solid phase peptide synthesis such as HATU, DIEA, piperidine as well as organic solvents such as DMF (NN'-dimethyl formamide), NMP (N-methyl pyrolidone) were purchased from Applied Biosystems (Framigham, Mass, USA), Aldrich-Sigma Chemical company (St. Louis, Missouri, USA) or Chem Impex International (USA). Trifluoroacetic and Acetonitrile needed for purification process (RP-HPLC) were bought from Aldrich-Sigma Chemical Company (St. Louis, Missouri, USA). All protected amino acid derivatives Fmoc-PAL-PEG-PS resin and the solvents were purchased from Applied Biosystems (Foster city, CA, USA), Calbiochem Novabiochem AG (San Diego, CA, USA), Chem-Impex International (Wood Dale, IL, USA) and Aldrich Chemical (Milwaukee, WI, USA).

3.1.1.2 Tissue Culture

Cell culture medium DMEM and 1x α-MEM, fetal bovine serum (FBS), and newborn calf serum were obtained from Gibco – Life Technologies. We also purchased EDTA-free Complete™ Protease Inhibitor Tablets from Roche; all reagents for western blotting were

obtained from Invitrogen – Life Technologies. All other chemicals and reagents were obtained from Aldrich-Sigma Chemical Company.

3.1.2 Synthesis of Peptides

All PCSK9 catalytic domain derived regular peptides with their amino acid sequences and precise locations as well as their dextro and/or retro-inverse analogs from the crucial disulfide bridge loop segments of human PCSK9 (Called Loop1 and Loop2) are shown in (**Table 4.1**). All peptides were synthesized chemically by an automated solid-phase peptide synthesizer instrument (Pioneer, PE-Perspective Biosystem Inc, Framingham, MA, USA and Intavis, Multiprep, Germany) using Fmoc (Fluorenyl methoxy carbonyl) mediated chemistry and HATU (O-7-azabenzotriazol-1-yl-1,1,3,3-tetramethyluronium hexafluorophosphate) / DIEA (N, N'-Diisopropyl ethyl amine) as coupling reagents. Synthesis proceeded from carboxy to amino terminal end direction. Following completion of synthesis, the peptides were cleaved off from the resin and fully deprotected using the cleavage cocktail called Reagent B (88 % TFA + 5 % Tri-isopropyl silane + 2 % H_2O + 5 % Phenol) for 4 hours at room temperature (102).

3.1.3 Peptides Purification

Peptides were then purified using reverse phase HPLC chromatography using a C_{18} semi preparative and analytical columns. The gradient method and the solvents used were according to the protocols as described in (102, 103). The buffer system used for the elution

consisted of double distilled water containing 0.1% (v/v) TFA as the aqueous phase (Solvent A) and 0.1% (v/v) TFA containing Acetonitrile (ACN) as the organic phase (Solvent B). The elution of peptide was carried out by using a 1% increase in Solvent B/min gradient from initial 0 to 65% Solvent B. All major peaks were collected, and characterized by mass spectrometry for their identities.

3.1.4 Characterization of peptides

To verify the purity of each product collected and also to follow any possible chemical modifications of the peptide such as oxidation, MALDI-TOF (matrix assisted laser desorption ionization time of flight) or SELDI-TOF (surface enhanced laser desorption ionization time of flight) mass spectrum was recorded for each collected material by using the Voyageur DE pro (PE Biosystems Inc, Framingham, Ma, USA) instrument using the GRAM software with about 4 μl of each sample solution containing at least 4μg peptide and 2 μl of the CHCA (α-cyano hydroxy cinnamic acid) solution as matrix.

3.1.5 Antibodies

Monoclonal anti-LDL-R antibody rabbit anti-serum 3143 against the C-terminal 14 amino acids of LDL-R was the kind gift of Joachim Herz (University of Texas Southwestern Medical Center, Dallas, TX, USA); a mouse anti-human transferrin receptor (TFR) antibody was purchased from Life Technologies; monoclonal anti-FLAG M2 antibody were from Sigma-Aldrich. Secondary IRDye-labeled goat anti-mouse and anti-rabbit IgG antibodies were from LI-COR Biosciences. Monoclonal antibody 15A6 recognizing an epitope in the C-

terminal domain of human PCSK9 was purified by Protein G affinity chromatography from culture of mouse hybridomas that were the kind gift of Jay Horton (University of Texas Southwestern Medical Center, Dallas, TX, USA).

3.2 Methods

3.2.1 Protein Assay

Protein assay of various cell culture samples was performed by using the Pierce^{TM} Thermo Scientific BCA protein assay dye kit (Thermo Scientific). BCA consists of two reagents: Reagent A (sodium carbonate, sodium bicarbonate, bicinchonic acid and sodium tartrate in 0.1M sodium hydroxide), and Reagent B which contains 4% cupric sulfate. Both reagents were mixed together as B: A in a 1:50 ratio to prepare the BCA working reagent, which is appears as a clear, transparent green solution. The assay was performed to determine the total amount of protein present in each sample. The samples were diluted with distilled H_2O to a final volume of 50 μl, in which the sample volume depends on its anticipated concentration. Standard curves were generated using pure bovine serum albumin (BSA) protein with concentrations ranging from 4-32 μg/ml. Later on 1 ml of the BCA working reagent was added to all samples that were then incubated in water bath at 37^0C for 30 minutes. Absorbance was read at 562 nm on a BIOWAVE II (Biochrom Ltd.,) instrument and the measured values were ploted against the protein amount to finally get the standard curve.

3.2.2 Cell Culture Medium

Medium A contained DMEM, 4.5 g/Litre glucose, supplemented with 100 units/ml penicillin and 100 µg/ml streptomycin sulfate. **Medium B** contained medium A supplemented with 10% (v/v) FBS. **Medium C** contained medium A with 5% (v/v) newborn calf lipoprotein-deficient serum (NCLPDS), 50 µM sodium mevalonate, and 10 µM pravastatin. Sterol-supplemented **Medium D** contained medium A with 5% (v/v) NCLPDS, 1 µg/ml 25-hydroxycholesterol, and 10 µg/ml cholesterol. **Medium E** contained 1x α-MEM (without nucleosides + 2mM Glutamine), supplemented with 100 unit/ml penicillin and 100 µg/ml streptomycin sulfate. **Medium F** contained medium E supplemented with 5% (v/v) NCLPDS. **Medium G** contained medium E with 5% (v/v) NCLPDS, 50 µM sodium mevalonate, and 10 µM parvastatin. Sterol-supplemented **Medium H** contained medium F with 5% (v/v) NCLPDS, 1 µg/ml 25-hydroxycholesterol, and 10 µg/ml cholesterol.

3.2.3 Tissue Culture

All cell culture reagents were obtained from (Invitrogen Corporation, ON Canada). HepG2 cells were grown in Dulbecco's modified Eagle's medium (DMEM) containing 10% FBS (fetal bovine serum), 1:100 dilution of antibiotic penicillin-streptomycin (P/S) under 5% CO_2 condition. Moreover, Hepa1c1c7 cells were grown in 1x alpha Minimum Essential Medium (MEM) containing 5 % NCLPDS (newborn calf lipoprotein-deficient serum) and 50µM Sodium Mevalonate and 10µM Pravastatin and 1:100 dilution of antibiotic penicillin-streptomycin (P/S) plus 3mM $CaCl_2$ under 5 % CO_2 condition. Later on the cells were grown to ~ 80% confluency when the medium was removed. Following treating the cells with MEM +

5% NCLPDS with Mevalonate and Pravastatin for another 18 - 24 hour before the addition of peptides in DMSO solution.

3.2.4 SDS-PAGE

Tris glycine gel with 8% resolving and 4% stacking phase was used for SDS PAGE. Denaturation of the protein samples were carried out by heating to 96^0C for 15 min with sample loading buffer 5x SDS (5% SDS, 25% glycerol, 0.25M Tris and 50 mM EDTA at pH 6.8) plus the addition of β-mercaptoethanol. Following separation with SDS-PAGE the proteins were transferred to a nitrocellulose membrane for 1.5 hour at 100 V (Millipore Corporation, Bedford USA) for immunological detection. The membranes were then blocked for 30 minute in 5% skim milk and incubated overnight with the primary antibody, anti-LDL-R at a dilution of (1:1000), anti-PCSK9 at a dilution of (5µg/ml I5A6) and anti-transferrin receptor at a dilution of 1:1000. The membranes were then incubated with the secondary antibody for 45 minute, with anti-rabbit 1:1000 for detection of LDL-R (Li-Cor Biosciences, USA), or anti-mouse at a dilution of 1:1000 for PCSK9 and Transferrin (Li-Cor Biosciences, USA). Images were then captured using Li-Cor scanner (Odyssey Infrared Imaging System).

3.2.5 Western Blotting

Cells were washed with cold distilled PBS and lysed using Tris lysis buffer (50mM Tris-Cl, PH 7.4, 150mM Nacl, 1% NP-40, 0.5% sodium deoxycholate, 5mM EDTA and 5mM EGTA) with 1M reducing agent Dithiothreitol (DTT) and 25x Protease Inhibitor cocktail (Roche Applied Science, Laval, QC, Canada) and 0.5 M PMSF

(phenylmethanesulfonylfluoride). Proteins were then separated on an (8%) reducing SDS gel and transferred to a nitrocellulose membrane. Membranes were then blocked for 30 minute in PBS containing 5% milk. LDL-R, PCSK9 and transferin receptor levels were determined using anti-LDL-R (1:1000), anti-PCSK9 (5µg/ml I5A6) and anti-transferrin receptor (1:1000) respectively.

3.2.6 Ligand Binding Assay

LDL-R content in the presence of PCSK9 peptides was determined through slot blotting analysis. A nitrocellulose membrane was treated with 1µg - 500 ng of purified ECD LDL-R-692 (i.e. extracellular domain), a kind gift from Dr. Ross Milne at the University of Ottawa Heart Institute. Membranes were blocked in buffer containing 2.5% w/w non-skim milk powder and preincubated with the peptide at the various concentrations (20 µM, 50 µM and 100 µM) for 30 min – 1 hour. Membranes were then further incubated with different concentrations of fluorophore-labelled PCSK9 (800-PCSK9- IRDye) (0.5 µg, 0.2 µg and 0.1 µg) plus peptides for an additional 1 – 2 hours. The membrane was then examined directly using Li-Cor scanner (Odyssey Infrared Imaging System).

3.2.7 Dose-response study of peptides

Approximately $100x10^5$ cells/well was plated in 6 well plate and the cells were grown to approximately 80% confluency (24 - 48 hours). The media containing FBS was removed and fresh DMEM with 5% NCLPDS and pravastatin were added for 24 hour as described above. The peptides were then added at concentrations of 40, 60 and 80 µM in 2 ml of medium.

Following 4 hour incubation the medium was removed and the cells were lysed with Tris lysis buffer. BCA protein analysis was done on all samples and sample containing ~30 - 50 μg of total protein was loaded in each lane and then separated on a gel. It was then transferred for western blotting. Blotting was done for LDL-R, PCSK9 and transferrin receptor in the lysates.

3.2.8 Effects of peptides on LDL-R level in the presence of added recombinant PCSK9

Approximately $100x10^5$ cells/ well were plated in 6 well plate and the cells were grown to approximately 80% confluency for 18 – 24 hour. The medium containing FBS was removed and fresh serum free media was added for 24 hour. Recombinant wild type PCSK9 was added at a concentration of 1μg/ml to all samples with the exception of the control. The peptides were then added at concentrations of 40, 60 and 80 μM in 2 ml of culture medium. Following 4 hour incubation the media were removed and the cells were lysed using Tris lysis buffer. BCA protein analysis was done on all samples and sample containing ~30 - 50 μg of total protein was loaded in each lane and then separated on a gel. It was then transferred for western blotting. Blotting was done for LDL-R, PCSK9 and transferrin receptor in the whole cell extract.

3.2.9 Statistical analysis

All presented values are mean ± standard deviation based on at least 3 repeat experiments and the results were evaluated by GraphPad Prism 5 software. No detail statistical analysis was performed.

4. Results

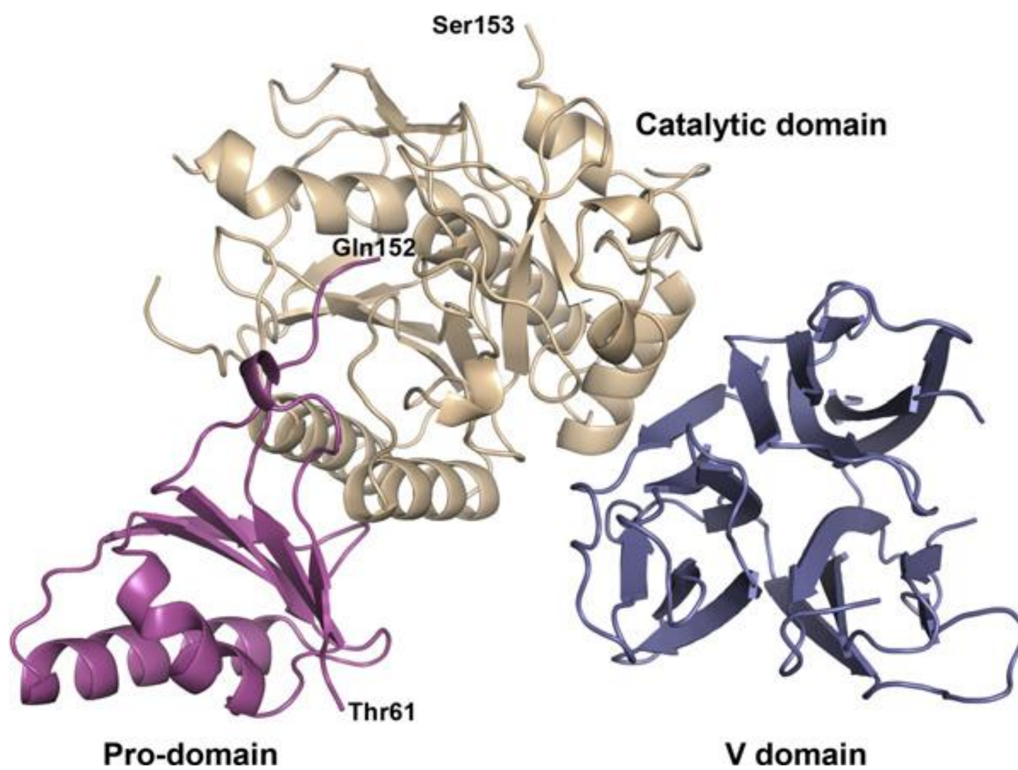
4.1 Peptides Design From hPCSK9 Catalytic Domain

The design of various peptides used in this project was based on the published crystal structures of h (human) PCSK9 protein alone and its complex with LDL-R/its peptides as well as various biochemical studies which were all extremely useful for this purpose. In recent years the crystal structures of h (human) PCSK9 protein along with its complex with LDL-R and its EGF-A domain peptide have been elucidated (20). These clearly revealed many important structural features about PCSK9. Thus it was observed that the interaction between the pro- and catalytic domains of the protein is quite strong and potent with the C-terminal segment of the prodomain forming deep and strong non-covalent interactions with the catalytic domain. This made the active site of hPCSK9 completely inaccessible to its potential exogenous substrates (20, 21, 104). Furthermore, the crystal structure also demonstrated that hPCSK9 protein is composed of subtilisin-like prosegment and catalytic domain, and a novel C-terminal domain (now called V domain) that actually consists mainly of CHRD or Cysteine Histidine Rich Domain as depicted in (**Figure 4.1**).

Human PCSK9 prodomain consists of two α-helices and four-stranded anti-parallel β sheet structure, which forms the interface between the prodomain and catalytic domain through several hydrophobic and key polar interactions as well as hydrogen bonds (21, 105). In particular, the four extreme C-terminal amino acids of the prodomain (residues 149-152) bind to the catalytic site as a β strand, in a corresponding pattern as an inhibitory peptide (20, 21). The crystal structure of hPCSK9 revealed the presence of unique secondary structure in the

prodomain and its strong interaction with the catalytic domain of PCSK9 as depicted in (**Figure 4.1**) (20). In fact after the autocatalytic cleavage of pro-PCSK9 at the site ($VFAQ_{152}\downarrow SIP$), the terminal **Gln**^{152} of the prodomain forms a strong hydrogen bond with the catalytic **His**^{226} and occupies the oxyanion hole located between the backbone nitrogen on the catalytic **Ser**^{386} and the side chain amide of **Asn**^{317}. In addition, stabilization of the bonds that keep the pro- and catalytic segments bound together was provided by a 14- amino acid extension at the N-terminus of prodomain which further blocks the access to the catalytic triad and thereby prevents any subsequent autocatalytic processings from occurring (20, 21).

Figure 4.1: **3D Model structure of hPCSK9 protein.** Ribbon diagram of the structure clearly showed that the prodomain blocks the active site residues (magenta) of the catalytic domain (grey), and the novel C terminal domain (V domain) in blue. **Thr**-61 marks the first seen residue, and **Gln-**152 points the C terminus of the prodomain. **Ser-**153 marks the N terminus of the catalytic domain.



This figure was adapted from *Piper et al. 2007* (20)

The core part of the catalytic segment consists of a seven-stranded parallel β sheet structures, flanked on both sides by α helices. Of note, the N-terminus α helix of this domain undergoes a considerable conformational change following the autocatalytic cleavage of pro-hPCSK9, moving more than 25Å from **Gln**-152 as shown in (**Figure 4.1**). This conformational shift is thought to be a consequence of autocatalysis process and necessary for secretion (20, 104). At least three disulfide bonds have also been characterized in the crystal structure of hPCSK9 within its catalytic segment. Moreover, it has been found that the catalytic triad of PCSK9 is highly conserved even though minor differences were observed in their exact locations, resulting from differences in the surrounding amino acid residues. Interestingly, it has been suggested that **Asn**-137 forms the oxyanion hole critical for catalysis action (20). However, it has been noted that the substrate-binding groove of PCSK9, which governs the substrate specificity, is mostly neutral in lieu of being negatively charged (21).

The C-terminal Cysteine/Histidine Rich Domain (CHRD) is mainly composed of three sub domains of anti-parallel β strands folded in an abridged jellyroll motif. Each sub domain is stabilized by three internal disulfide (-S-S-) bridges respectively between the first number six Cysteine residues, followed by the one between the second and number fifth Cysteine, and lastly the one between the third and number fourth Cysteine which led to the cross-linking β-sheets (β1 – β6, β2 – β6, and β3 – β5, respectively). This domain is also rich in Histidine residues, with the majority of them cluster on a surface between sub domains 2 and 3 (20, 104).

The co-crystallization of hPCSK9 and LDL-R proteins demonstrated that the catalytic domain of PCSK9 directly interacts and binds with the EGFA domain of LDL-R, promoting the degradation of the latter in the lysosomal pathway (16). For reasons already indicated earlier about the strong affinity of prodomain towards the catalytic domain and the presence of

a wide variety of natural mutations particularly in the catalytic domain of hPCSK9 and post-translational modifications, we became attentive and interested in this domain. The rationale is that selected peptides from the catalytic domain of hPCSK9 may likely block at least partly its interaction with the EGF-A domain of LDL-R. The above potential domains may be located in those catalytic segments that have been shown by crystallography to make direct physical contacts with the EGF-A domain of LDL-R. Consequently these peptides may act as competing peptides and therefore interfere in the binding of hPCSK9 with LDL-R protein. If that happens then one can develop a peptide based approach for promoting LDL-R which ultimately lead to regulation (up or down regulation) of circulatory LDL-Cholesterol. Such peptides may constitute as the first generation of lead competing peptides for possible therapeutic applications in cholesterol suppression.

As indicated, the crystal structure has revealed that hPCSK9 binds to LDL-R via its EGF-A domain. However, the specific segment/s of PCSK9 that are involved in this binding is not fully identified. However a recent study and more crystal structure data have implicated its catalytic domain as the candidate segment (20, 66, 106). Thus the exact characterization of this/these domain/s is crucial for development of any effective competing peptides that can target hPCSK9:LDL-R complex formation. Earlier our group demonstrated that two selected peptides from hPCSK9 catalytic domain (hPCSK9$^{181-200}$ and $^{368-390}$) up-regulate LDL-R level without significantly affecting hPCSK9 level in human hepatic cells (107). These effects along with crystal structure and modeling studies confirm the involvement of catalytic domain of hPCSK9 in binding with LDL-R.

In our efforts to identify short catalytic peptide segments of hPCSK9 protein that may induce modification of PCSK9's ability to bind with the EGF-A domain of LDL-R, we

designed and synthesized several regular peptides as well as their dextro and retro-inverse analogs from the crucial disulfide bridge loop regions of hPCSK9 (named loop1$^{323-358}$ and loop2$^{372-380}$). The list of the loop peptides and their analogs are shown in (**Table 4.1**). In this design paradigm and strategy we chose 35 amino acids (aa) long size (for loop1$^{323-358}$ which is the minimum length that can accommodate the S-S bridge) or less as reasonable size (for loop2$^{372-380}$ peptides). In one case, we have included one more short size peptide (9 aa) that contain the crucial S-S Bridge and the so called key amino acid residues based on crystal structure and various biochemical studies (38, 67, 94, 95, 108). The catalytic domain of PCSK9 also includes a variety of natural mutations and post-translational modifications that are likely to regulate the functional activity of PCSK9. Interestingly, the two most potent gain-of-function mutantions of PCSK9 **D^{374}Y** and **R^{357}H** are included within these loop1$^{323-358}$ and loop2$^{372-380}$ peptide sequences. Owing to these observations and recent findings, the catalytic domain drew our attention most and became the focus of this current research study. Consequently, the above-mentioned loop peptides were selected to examine their possible regulatory effects towards LDL-R protein level. We hypothesized that our designed catalytic domain peptides of PCSK9 through its binding interaction with EGF-A domain of LDL-R may function as potential inhibitors by inhibiting the activity of hPCSK9 towards the degradation of LDL-R.

In one of our peptide designs, we have included all dextro-rotatory (D) amino acids instead of regular Leuvo rotatory (L) amino acids which are considered as proteinaceous amino acids. We rationalize that incorporating D instead of L amino acids will significantly enhance the stability of the peptide and possibly its bio- or functional activity. Overall incorporating D- instead of L- will not alter the chemical structure and 3D geometry or conformation of the peptide at all and this will explain why such a substitution is not expected to negatively alter

the bioactivity of the peptides. Such a strategy has been used in the past with great success to develop potent peptide inhibitors and blockers (Reviewed in Ref. 109).

In another design strategy we have inserted reverse or retro inverse peptide bond also called isopeptide bond, -NH-CO- instead of regular –CO-NH- peptide bond. Herein we basically reverse the peptide sequence leading to each peptide bond being substituteds by a retro inverse peptide bond. Such an approach has been used in the past to enhance the bio- and functional activity of short peptides (110,111). It is also interesting to point out that D-amino acid incorporation as well as Retro-inverse peptide bond insertion both enhances the cell penetration property of the peptide. This is also another reason for our design.

Table 4.1: **List of all the loop peptides synthesized for the present study and the calculated molecular weight (MW) of each peptide.** Two Cysteines in each peptide squence are bonded via disulfied bridge (S-S).

Catalytic Domain Based Loop Peptides		
Loop Peptides	**Amino acid sequence**	**Molecular weight (Da)**
Loop1: Cyclic-hPCSK9$^{323-358}$	CLYSPASAPE VITVGATNAQ DQPVTLGTLG TNFG**R**C	3653.10
Dextro-Loop1: Cyclic-hPCSK9$^{323-358}$	CLYSPASAPEVITVGATNAQDQPVTLGTLGTNFGRC	
Reverse-Loop1: Cyclic-hPCSK9$^{358-323}$	C**R**GFNTGLTGLTVPQDQANTAGVTIVEPASAPSYLC	
Dextro-Loop1: Cyclic-hPCSK9$^{323-358}$ H^{357}mut	CLYSPASAPEVITVGATNAQDQPVTLGTLGTNFGHC	3634.05
Loop1: Cyclic-hPCSK9$^{323-358}$ H^{357}mut	CLYSPASAPE VITVGATNAQ DQPVTLGTLG TNFG**H**C	
Loop2: Cyclic-hPCSK9$^{372-380}$	S S**D**CSTCFV	948.03
Dextro-Loop2: Cyclic-hPCSK9$^{372-380}$	S S**D**CSTCFV	
Reverse-Loop2: Cyclic-hPCSK9$^{380-372}$	VFCTSC**D**SS	
Loop2: Cyclic-hPCSK9$^{372-380}$ Y^{374}mut	S S**YC**STCFV	996.12
Dextro-Loop2: Cyclic-hPCSK9$^{372-380}$ Y^{374}mut	S S**Y**CSTCFV	
Reverse-Loop2: Cyclic-hPCSK9$^{380-372}$ Y^{374}mut	VFCTSC**Y**SS	
Dextro-Loop2: Cyclic-hPCSK9$^{365-384}$	APGEDIIGAS S**D**CSTCFVSQ SG	1963.07

4.1.1 Characterization of Catalytic Domain Loop Peptides

The syntheses of all the above peptides were accomplished by Fmoc based solid phase automated peptide synthesizer (See Materials and Methods for details) (102, 103). All crude peptides thus prepared were purified by reverse phase HPLC chromatography. Peaks were collected, lyophilizedand analyzed by mass spectrometry for their molecular weights. The measured values for each collected peaks were compared with that of the calculated value in order to detect the identities of the peaks. We used SELDI-tof (surface enhanced laser desorption ionization time of flight) mass spectrum for all the peaks generated from this proteome analysis. In order to run HPLC chromatography for each peptide, the peptide powder was weighed and dissolved in 0.1% TFA/H_2O buffer (final concentration ~ 1mg/ml) and about 4 µl of each sample was spotted on the gold plate and then 2 µl of the CHCA (α-cyano hydroxy cinnamic acid) matrix solution was added. The results were in excellent agreement with those of the corresponding molecular mass for each catalytic domain loop1 $^{323-358}$ and loop2 $^{372-380}$ peptides. The results are shown in (**Figure 4.2**) and (**Figure 4.3**).

Figure 4.2: **SELDI-tof mass spectra of various hPCSK9 catalytic domain loop2 short sequence peptides.** Each panel shows the mass spectrum peak (major one) and the corresponding m/z (mass/charge) value which agrees well with the corresponding calculated value (see **Table 4.1**). The observed molecular weight value as determined by SELDI tof MS have been indicated in each peak.

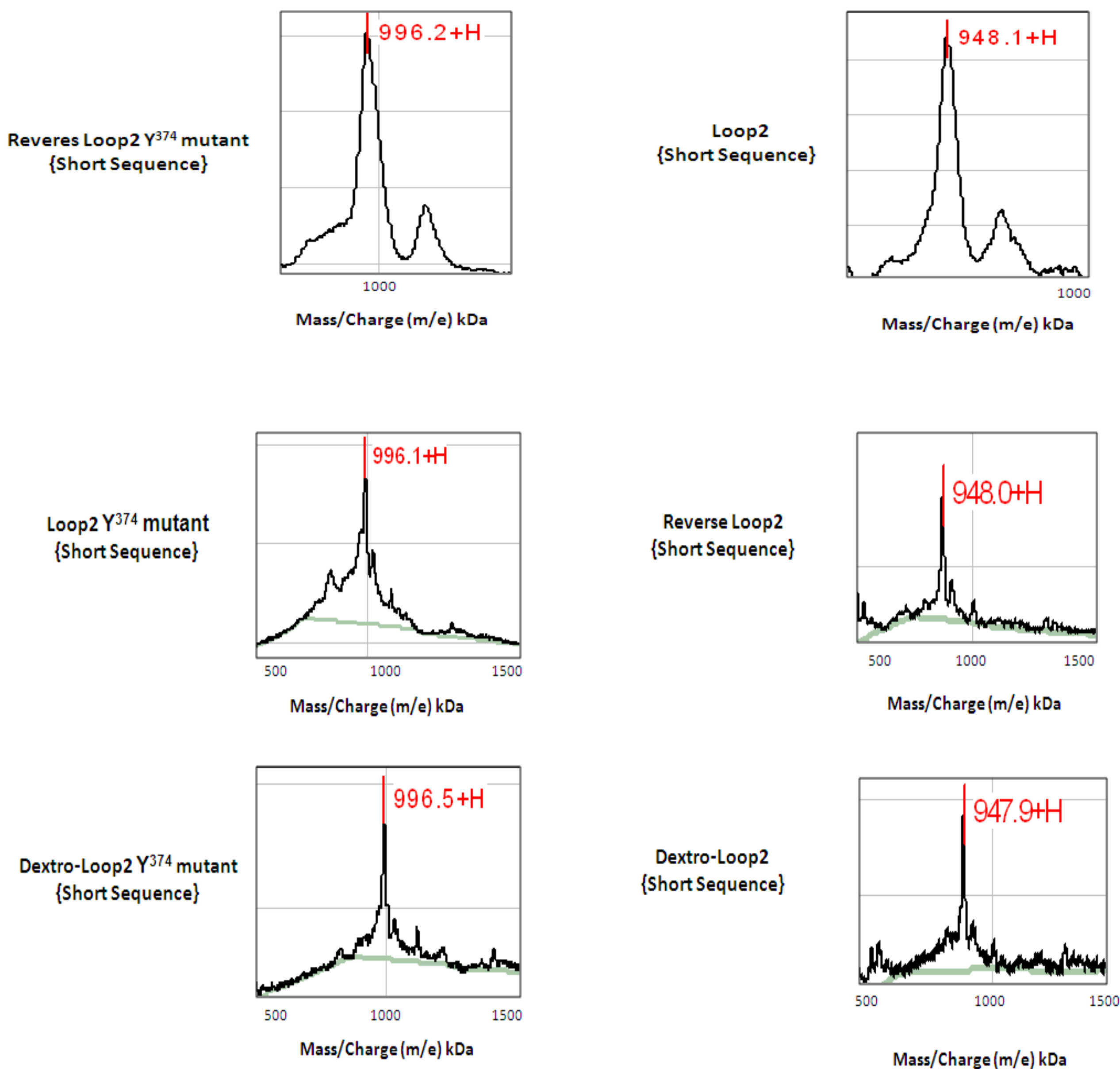
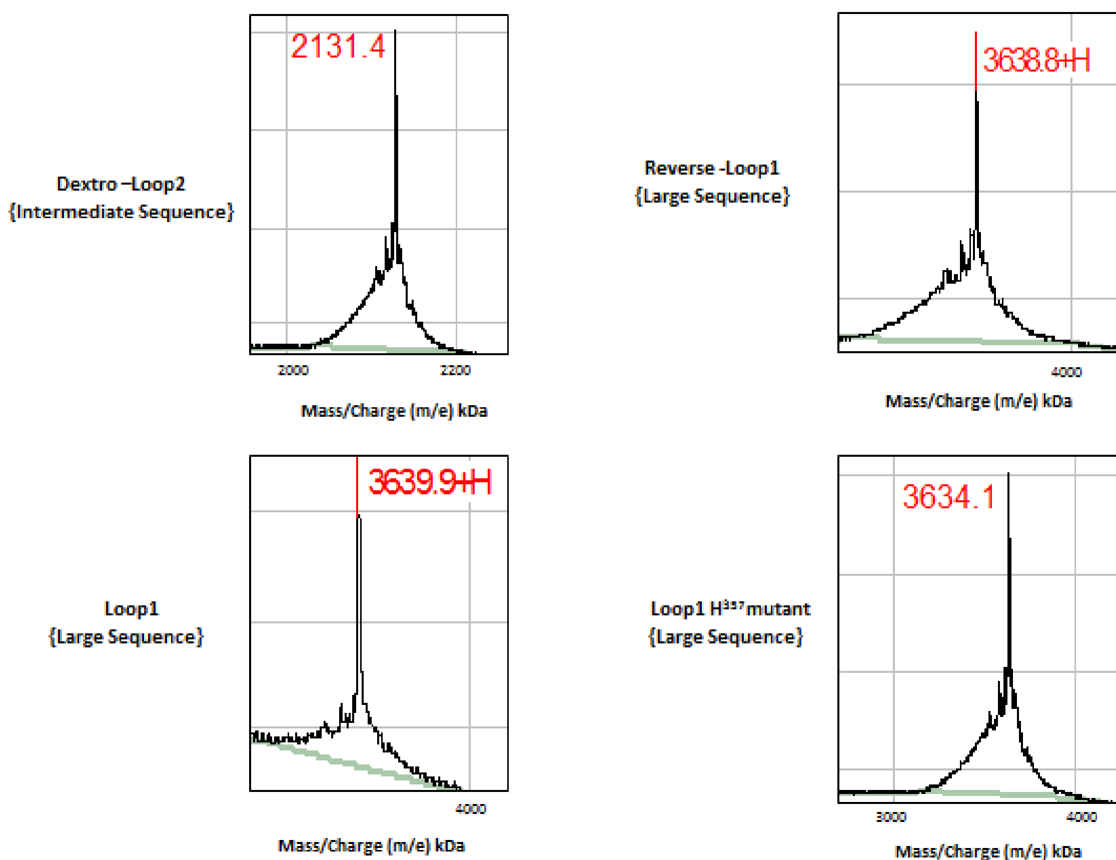


Figure 4.3: **SELDI-tof mass spectra of the PCSK9 catalytic domain loop2 (intermediate) and loop1 (large) sequence peptides.** The panels show the peaks of hPCSK9 loop 2 {intermediate sequence peptide} and loop1 {large sequence peptide}. The peaks are labeled with the measured molecular masses (m/z values) which agree quite well with the corresponding calculated values (see **Table 4.1**).



4.2 PCSK9 catalytic domain loop peptides can regulate LDL-R level

4.2.1 Effect of catalytic domain peptides on LDL-R level in hepatic cell lines

In recent years our preliminary work and others have reported that short peptides derived from hPCSK9 can modify the functional activity of PCSK9 and consequently the total LDL-R level based on animal and cellular studies (112). Some of these peptides caused increased degradation of the membrane receptor hepatic LDL-R protein level during the period of continuous infusion in control mice, while the others did the opposite (79).

Encouraged by these findings we began our investigation by first synthesizing the catalytic domain peptides of PCSK9 and then studying their possible regulatory effects on hPCSK9 and LDL-R levels in specific hepatic cell lines. Our hypothesis is driven by the fact that the catalytic segment peptides of PCSK9 may behave as exogenous inhibitors by blocking hPCSK9's interaction with the EGF-A domain of LDL-R protein. This will subsequently elevate the clearance of circlatory LDL-cholesterol.

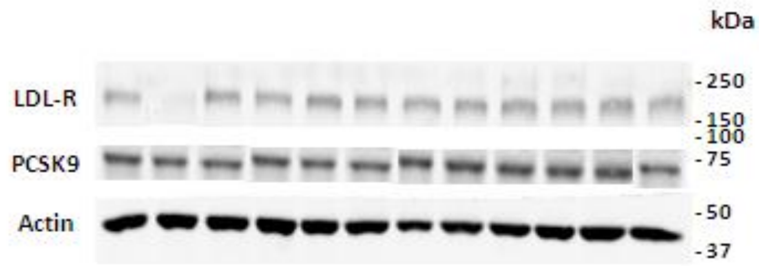
4.2.1.1 Effect on LDL-R level in human hepatic cell line HepG2

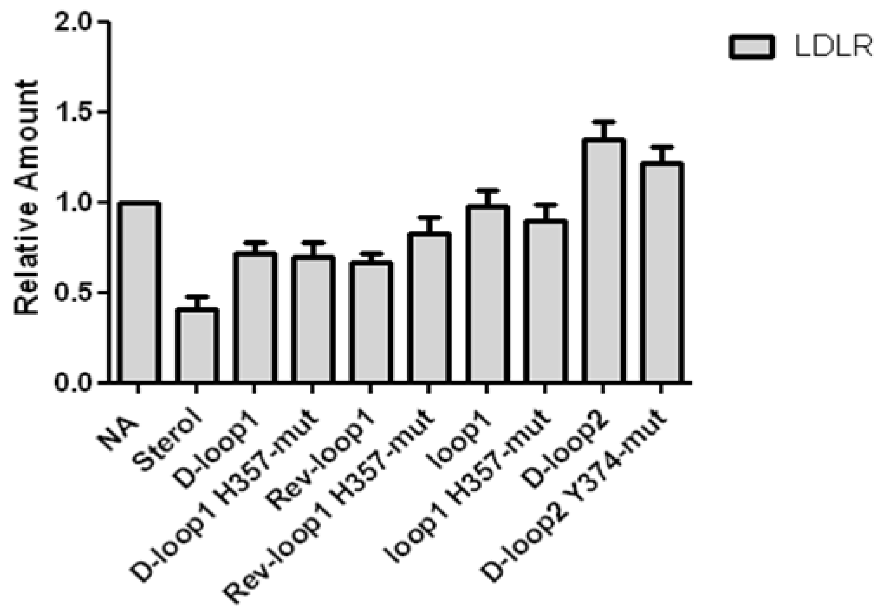
In order to test our hypothesis, we assessed the effects of hPCSK9 catalytic domain peptides on the levels of LDL-R using human HepG2 hepatic cell line that endogenously expresses both PCSK9 and LDL-R proteins. The cells were cultured in the medium B (see Material Methods for composition) and in the sterol-depleted medium containing the cholesterol lowering statin drug, pravastatin for 18 - 24 hour before PCSK9 catalytic domain peptides were added to the culture medium. Cells were treated with each peptide at 5μM final concentration which was about 30 μg/ml of the medium for a period of 18 hour incubation as

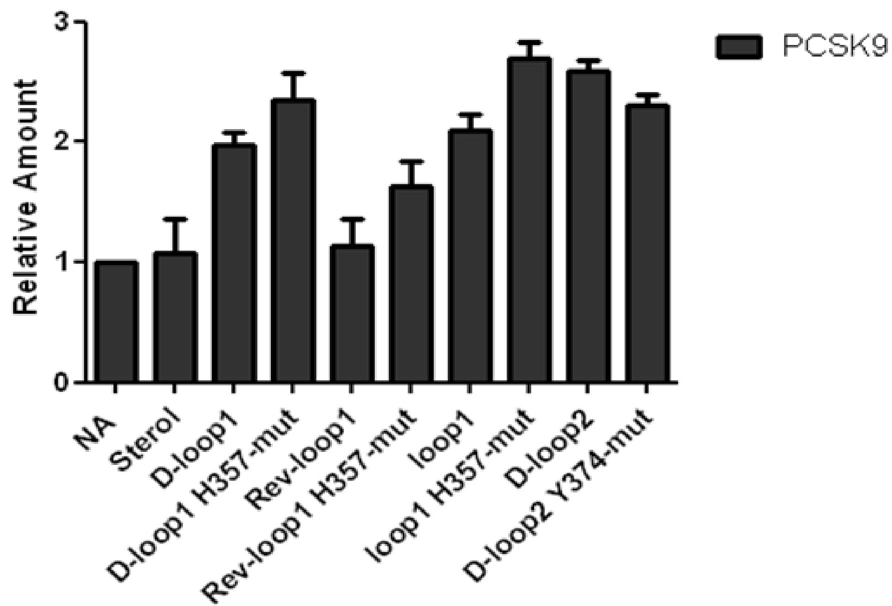
described in the Materials and Methods section. Following the removal of the medium and 1X wash with buffer, the whole cells were lysed and extracted with lysis buffer (see Materials and Methods for instructions). The collected cell lysates were examined for exogenous levels of PCSK9 and LDL-R by immunoblotting analysis.

Immunoblotting analysis of the whole cell proteins extract indicated that in response to the various PCSK9 catalytic peptides tested, our designed dextro short Loop2 $^{372-380}$ and its Y^{374} mutant peptides showed slight increase in LDL-R level (~1.4 - 1.5 fold). This is observed in three repeat experiments despite an unexpected increase of PCSK9 levels (2 - 2.5 fold) as compared to control untreated cells. The increase of both LDL-R and PCSK9 at the same time is very surprising and could not be explained at the first sight. It is also surprising to note that two other peptides derived from loop1 $^{323-358}$ sequence namely D-loop1 $^{323-358}$ and its H^{357}mutant also enhanced PCSK9 expression by nearly 2.4 - 2.5 fold and as expected decreased LDL-R level (20 - 30%) compared to control. These results will be discussed in more detail in the Discussion section. The data obtained for all the peptides tested are depicted in (**Figure 4.4**). All results are represented following standardization against the control protein actin and calculated by Graph Pad Prism 5 software. Taken together, the PCSK9 catalytic peptides from loop1 $^{323-358}$ and loop 2 $^{372-380}$ regions can affect the level of LDL-R protein in the cell lines with some modest effect on the PCSK9 protein levels.

Figure 4.4: **Effect of various peptides of human PCSK9 on LDL-R and PCSK9 levels when added to growing HepG2 cells at 5 µM (final) concentrations.** HepG2 cells were grown to ~80% confluence in medium B and in sterol-depleting medium D, and then were incubated for 18 - 24 hours to allow the expression of both proteins LDL-R and PCSK9 during the cell growth. HepG2 cells were then treated with hPCSK9 loop2 $^{372-380}$ - short sequence peptides and loop1$^{323-358}$ –large sequence peptides and incubated for 18 hours. The whole cell lysates were collected and subjected to 8% SDS-PAGE followed by immunoblotting analysis of LDL-R, PCSK9 and Actin as an internal control (~30 µg/ lane). Secondary infrared dye (IRDye-800)-labeled antibodies were used for detection using the LI-COR Odyssey infrared imaging system. Results are plotted relative to the control Actin. *All experiments were performed in triplicate independently.* **WT:** Wild type**; mut:** mutant; **NA:** Not Applicable.


kDa
LDL-R
PCSK9
Actin
250
150
100
75
50
37

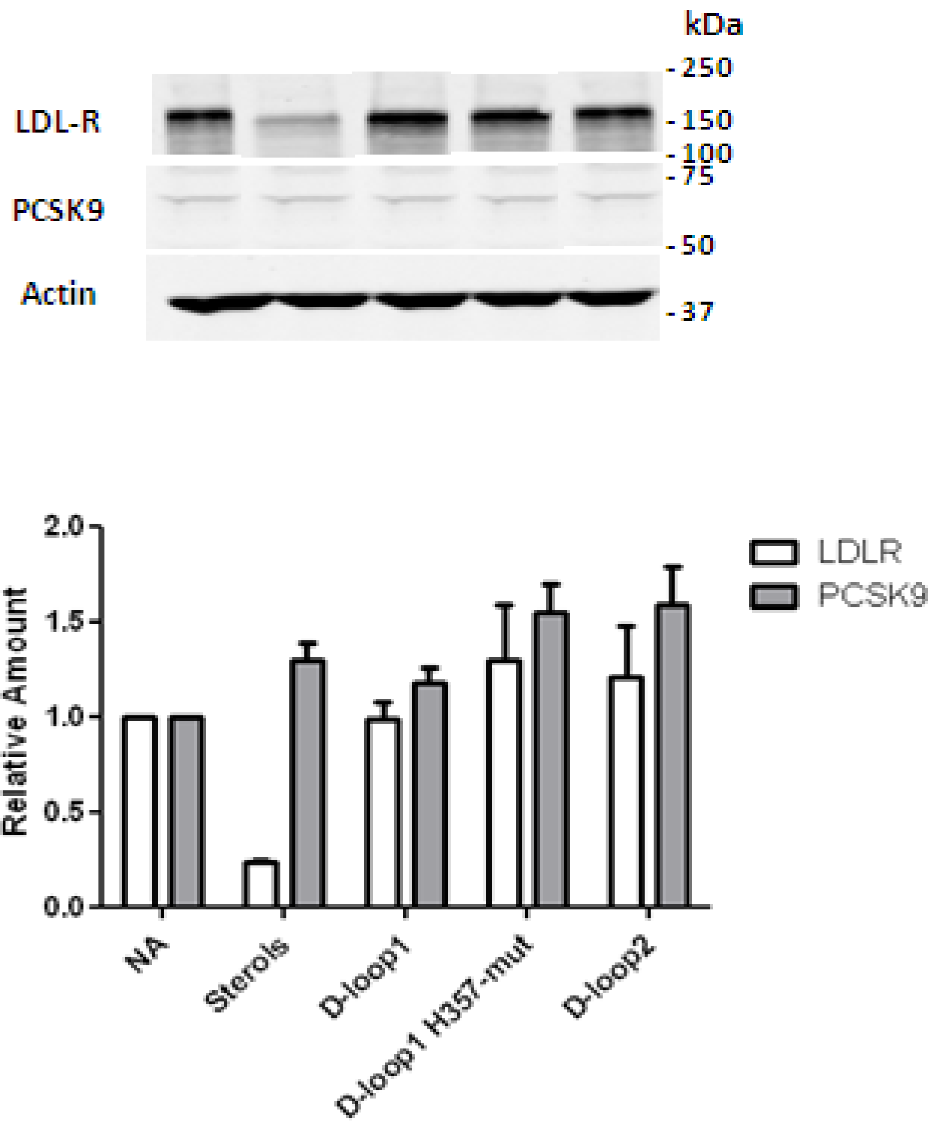

LDLR
Relative Amount
2.0
1.5
1.0
0.5
0.0
NA
Sterol
D-loop1
D-loop1 H357-mut
Rev-loop1
Rev-loop1 H357-mut
loop1
loop1 H357-mut
D-loop2
D-loop2 Y374-mut

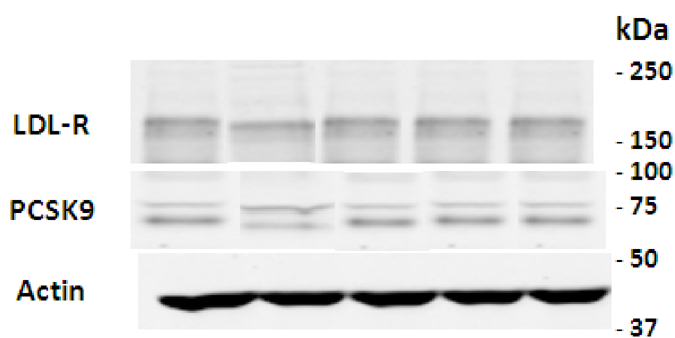

PCSK9
Relative Amount
3
2
1
0
NA
Sterol
D-loop1
D-loop1 H357-mut
Rev-loop1
Rev-loop1 H357-mut
loop1
loop1 H357-mut
D-loop2
D-loop2 Y374-mut

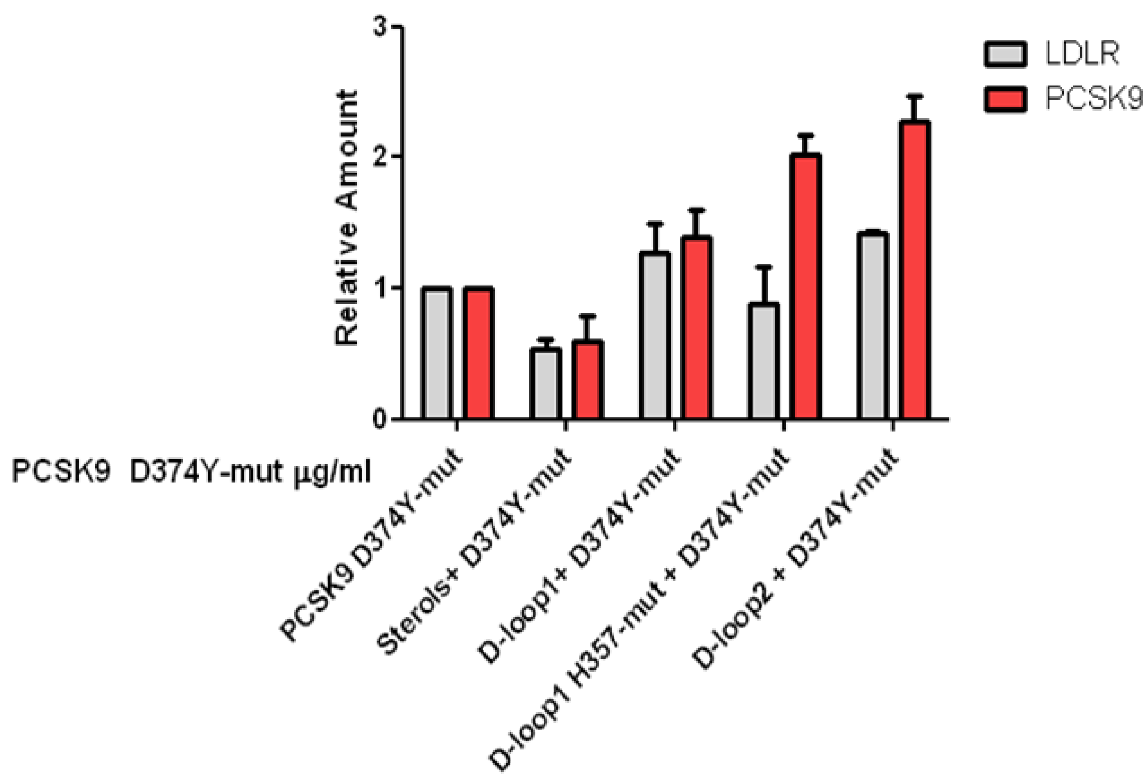
In order to further confirm our results we have decided to carry out the above cell culture experiments in the presence of added purified recombinant hPCSK9 and its gain of function mutant PCSK9-D^{374}Y containing a C-terminal FLAG tag (kindly provided by my co-supervisor Dr. Tom Lagace, U Ottawa Heart Institute). The recombinant PCSK9 was added at physiological concentration (1µg/ml) to the medium of grown cells and incubated for 18 hours in the presence and absence of various PCSK9 catalytic peptides at 5µM concentration which was about 30 µg/ml and incubated for a period of 18 hour as indicated in (**Figure 4.5A**). The mutant PCSK9-**D^{374}Y** has been characterized to be about 10-fold more potent than the wild type PCSK9 in degrading LDL-R in cells under identical conditions (7).

Notably, PCSK9-D^{374}Y at 1 µg/ml markedly reduced exogenous LDL-R and PCSK9 levels in the presence of sterols as shown in (**Figure 4.5 B**).

Figure 4.5: **Effect of various peptides of hPCSK9 on LDL-R and PCSK9 levels in the absence and presence of the recombinant mutant PCSK9-D^{374}Y protein when added to growing HepG2 cells at 5 µM (final) concentration levels.** (**A**) HepG2 cells were grown to ~80% confluence in medium B and in sterol-depleting medium D, and then were incubated for 18 - 24 hours to allow the expression of both proteins LDL-R and PCSK9 during the cell growth. HepG2 cells were then treated with hPCSK9 loop2$^{372\text{-}380}$ - short sequence peptides and loop1$^{323\text{-}358}$ –large sequence peptides and incubated for 18 hours. The whole cell lysates were collected and subjected to 8% SDS-PAGE followed by immunoblotting analysis of LDL-R, PCSK9 and Actin as an internal control (~30 µg/ lane). Secondary infrared dye (IRDye800)-labeled antibodies were used for detection on the LI-COR Odyssey infrared imaging system. (**B**) HepG2 cells were cultured to ~80% confluence in medium B and in sterol-depleting medium D, and then were incubated for 18 - 24 hours to allow expression of both proteins LDL-R and PCSK9 during cell growth. The cells were then treated with hPCSK9 loop2 $^{372\text{-}380}$ - short sequence peptides and loop1$^{323\text{-}358}$ –large sequence peptides plus the addition of the recombinant mutant PCSK9-D^{374}Y protein at physiological concentration (1µg/ml) and incubated for 18 hours. The whole cell lysates were collected and subjected to immunoblotting analysis of LDL-R, PCSK9 and Actin as an internal control (~30 µg/ lane). Secondary infrared dye (IRDye800)-labeled antibodies were used for detection on the LI-COR Odyssey infrared imaging system. Results are plotted relative to the control Actin. *All experiments were performed three times independently with similar results.* **WT:** Wild type; **DY:** purified recombinant hPCSK9 D^{374}Y mutant; **Loop2 sh:** Loop 2 short; **mut:** mutant; **NA:** Not Applicable.


A
kDa
LDL-R
PCSK9
Actin
250
150
100
75
50
37
Relative Amount
2.0
1.5
1.0
0.5
0.0
LDLR
PCSK9
NA
Sterols
D-loop1
D-loop1 H357-mut
D-loop2


B
kDa
LDL-R
PCSK9
Actin
- 250
- 150
- 100
- 75
- 50
- 37


Relative Amount
3
2
1
0
LDLR
PCSK9
PCSK9 D374Y-mut µg/ml
PCSK9 D374Y-mut
Sterols+ D374Y-mut
D-loop1+ D374Y-mut
D-loop1 H357-mut + D374Y-mut
D-loop2 + D374Y-mut

4.2.1.2 Studies on LDL-R regulation in mouse hepatic cell lines Hepa1c1c7

To further study the effects of catalytic loop peptides observed in HepG2 cells, we wanted to examine the effects in another hepatic cell line this time the one derived from mouse. Hepatoma cells are similar to HepG2 cells and thus mouse hepatic Hepa1c1c7 cells also express endogenous PCSK9 and LDL-R. This fact encouraged us to examine the effect of the catalytic segment peptides of PCSK9 in this mouse cell line and hope to compare the results with those observed in HepG2 human hepatic cell line.

To assess the effects of exogenously added catalytic domain peptides of PCSK9 on LDL-R levels in Hepa1c1c7 hepatic cells, we cultured the cells in the medium G and in the sterol-depleted medium containing statin (pravastatin) for 18 - 24 hour before PCSK9 catalytic domain peptides treatment to up-regulate LDL-R expression. Following treating the cells with each peptide at 5µM (final) concentration which represents~ 30 µg/ml for a period of 4 hour incubation, the whole cell extracts were collected and examined for exogenous levels of PCSK9 and LDL-R by immunoblotting analysis.

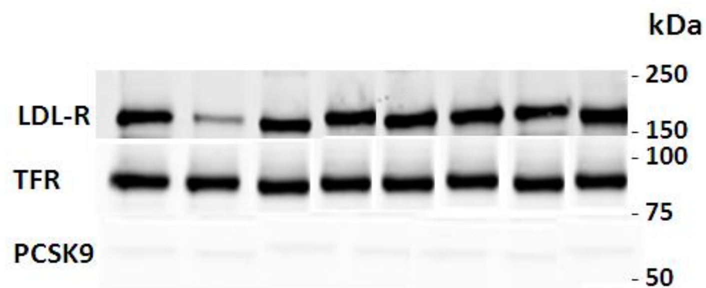
Immunoblotting analysis of the whole cell lysates showed that in response to the addition of hPCSK9 catalytic domain peptides, LDL-R levels did slightly change with modest effect with our designed short loop2$^{372-380}$ and its Y^{374}-mutant peptides as seen in (**Figure 4.6 A**). In parallel we also noted some effects on PCSK9 level with other peptides as well.

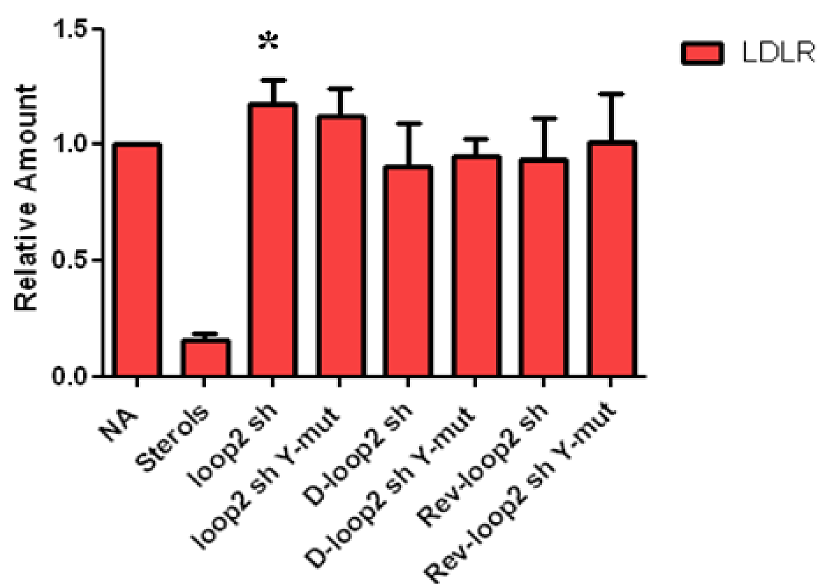
To determine the effects, these catalytic domain peptides were individually added to the media of Hepa1c1c7 cells at a final concentration 5 µg/ml in the presence of purified recombinant wild type hPCSK9 protein. Latter was externally added at concentration 10 µg/ml. This was then incubated for 4 hours. The whole cell lysates were collected and examined for exogenous levels of PCSK9 and LDL-R by immunoblotting analysis. We noted that both LDL-

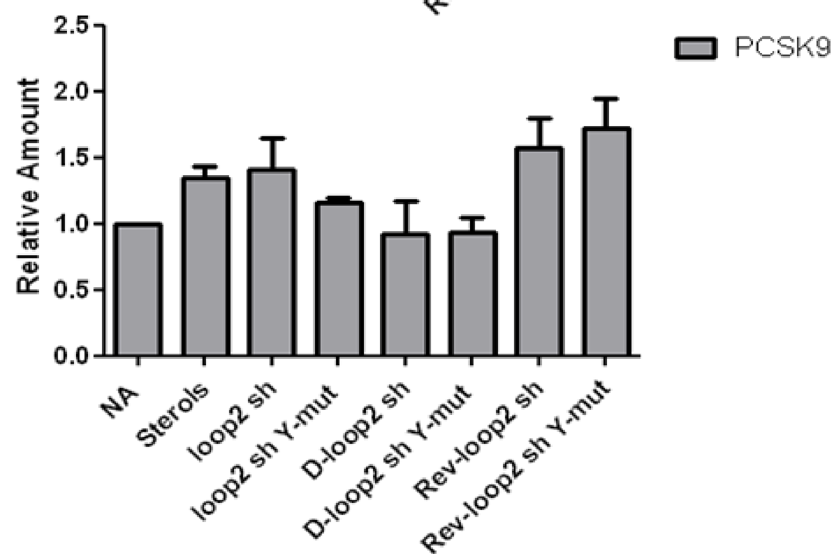
R and PCSK9 levels did not change significantly as depicted in (**Figure 4.6 B**) except for Loop2$^{372-380}$ short peptide and its D^{374}Y mutant peptide where ~ 20% up-regulation of LDL-R was noticed. We also noted a significant up regulatory effects (50 - 60%) on PCSK9 expression following treatment with Reverse loop2 $^{380-372}$ short peptide and its D^{374}Y mutant.

Figure 4.6: **Effect of various peptides (hPCSK9 loop2 $^{372\text{-}380}$ - short sequence derived from human PCSK9) on LDL-R and PCSK9 levels when added to growing Hepa1c1c7 cells at 20 µM (final) concentrations.** (**A**) Hepa1c1c7 cells were cultured to ~80% confluence in medium F and in sterol-depleting medium H, and then were incubated for 18 hours to induce LDL-R expression. Hepa1c1c7 cells were then treated with hPCSK9 loop2$^{372\text{-}380}$ - short sequence peptides and incubated for 4 hours, and then the whole cell lysates were collected and subjected to 8% SDS-PAGE followed by immunoblotting analysis of LDL-R, PCSK9 and TR (Transferrin Receptor) as an internal control (~30 µg/ lane). Secondary infrared dye (IRDye800)-labeled antibodies were used for detection on the LI-COR Odyssey infrared imaging system. (**B**) Hepa1c1c7 cells were cultured to ~80% confluence in medium F. The medium was then switched to sterol-depleted medium H to allow induction of LDL-R expression. Purified recombinant wild-type PCSK9 (10 µg/ml) were added to the medium in the presence of hPCSK9 loop2 $^{372\text{-}380}$ - short sequence peptides and incubated for 4 hours. Whole cell lysates were collected and analyzed via immunoblotting for the presence of PCSK9 and LDL-R levels as well as the transferrin receptor (TFR) as an internal control (~30 µg/ lane). Secondary infrared dye (IRDye800)-labeled antibodies were used for detection on the LI-COR Odyssey infrared imaging system. Results are plotted relative to the control transferrin receptor (TFR). *All experiments were performed three times independently.* **WT:** Wild type; **Loop2 sh:** Loop 2 short; **mut:** mutant; **NA:** Not Applicable.

A







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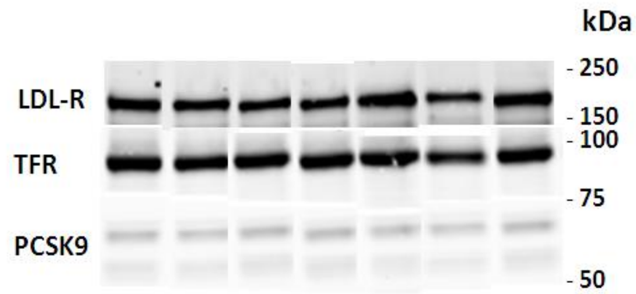
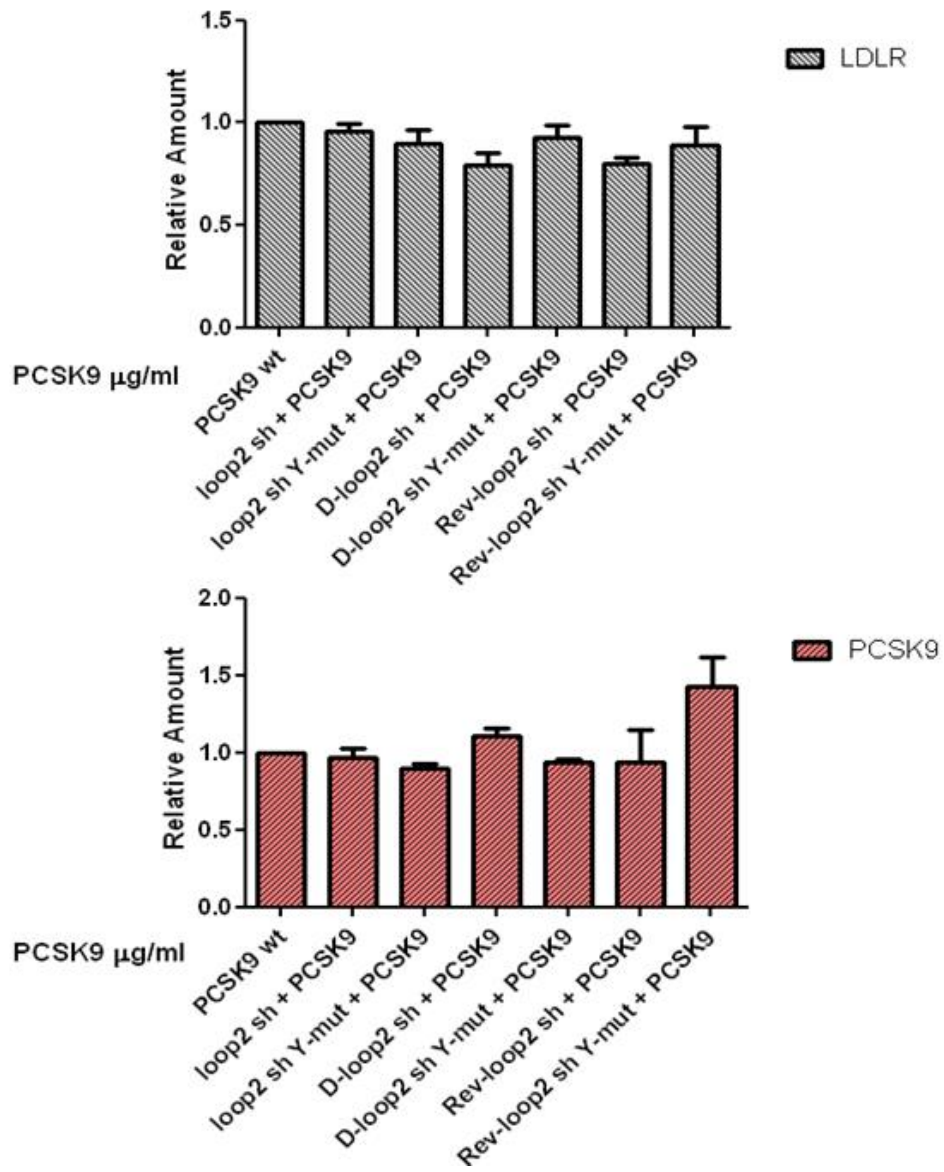
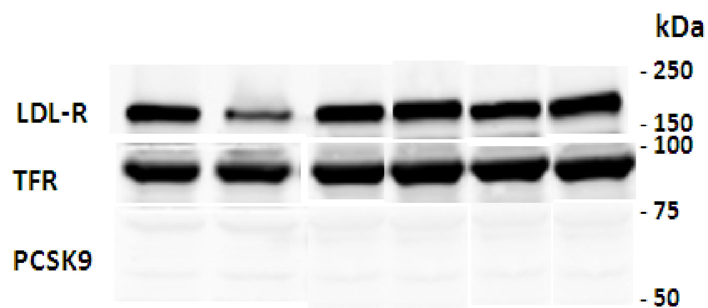


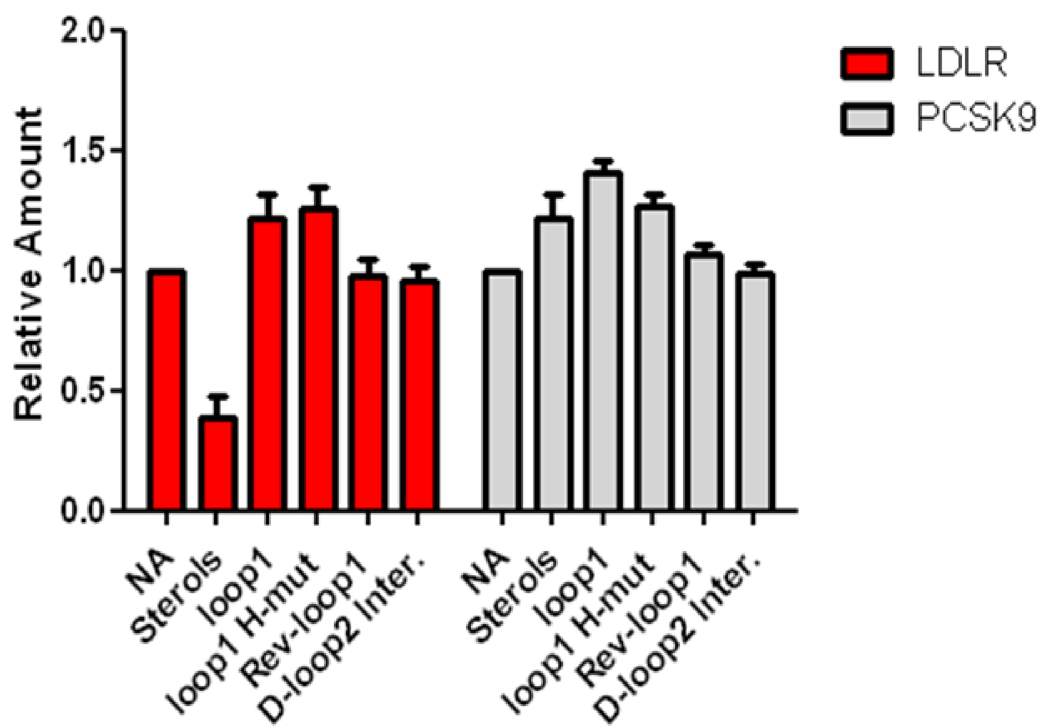


Following the observed results in (**Figure 4.6**), we became interested to determine whether catalytic loop1 $^{323\text{-}358}$ - large sequence peptide and its H^{357}-mutant peptide could counteract the effect on the LDL-R levels. To accomplish this, we added those peptides at a slightly higher concentration level of 20 μM to the medium of Hepa1c1c7 cells and performed the cell culture experiment exactly the way mentioned earlier. The obtained cell lysates were then immunoblotted for LDL-R, PCSK9 and Transferrin receptor. We observed a modest elevation in LDL-R levels (by ~ 20 - 40%) depending on the nature of the peptide used with both peptides as indicated in (**Figure 4.7 A, B**).

Figure 4.7: **Effect of peptides hPCSK9 loop2$^{365\text{-}384}$ - Intermediate sequence and loop1$^{323\text{-}358}$ - large sequence of human PCSK9 on LDL-R and PCSK9 levels when added to growing Hepa1c1c7 cells at 20 µM final concentration.** (**A**) Hepa1c1c7 cells were cultured to ~80% confluence in medium F and in sterol-depleting medium H (see Materials and Methods for buffer compositions), and then were incubated for 18 hours to induce LDL-R expression. Hepa1c1c7 cells were then treated with hPCSK9 loop2$^{365\text{-}384}$ - Intermediate sequence and loop1$^{323\text{-}358}$ - large sequence peptides. After another 4 hours incubation, the whole cell lysates were collected as described earlier and subjected to analyses by 8% SDS-PAGE followed by immunoblotting analysis of LDL-R, PCSK9 and TFR as an internal control (loading amount: ~30 µg/ lane). Secondary infrared dye (IRDye800)-labeled antibodies were used for detection on the LI-COR Odyssey infrared imaging system. (**B**) Hepa1c1c7 cells were cultured to ~80% confluence in medium F. The medium was then switched to sterol-depleted medium H to induce LDL-R expression. Purified recombinant wild-type PCSK9 (10 µg/ml) was added to the medium in the presence of hPCSK9 loop2$^{365\text{-}384}$ - Intermediate sequence and loop1$^{323\text{-}358}$ - sequence peptides and incubated for 4 hours. Whole cell lysates were collected and analyzed via immunoblotting for PCSK9 and LDL-R levels as well as for the transferrin receptor (TFR) as an internal control (~30 µg/ lane). Secondary infrared dye (IRDye800)-labeled antibodies were used for detection on the LI-COR Odyssey infrared imaging system. Results are plotted relative to the control transferrin receptor (TFR). Graphs represent the means ± standard deviation from three independent experiments. * indicates a statistical difference between columns with significance $p < 0.05$ by Student t-test**.** **WT:** Wild type; **D-Loop2 Inter.:** Dextro-Loop 2 Intermediate; **mut:** mutant; **NA:** Not Applicable.

A





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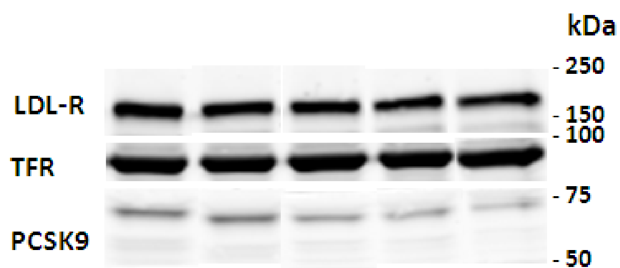
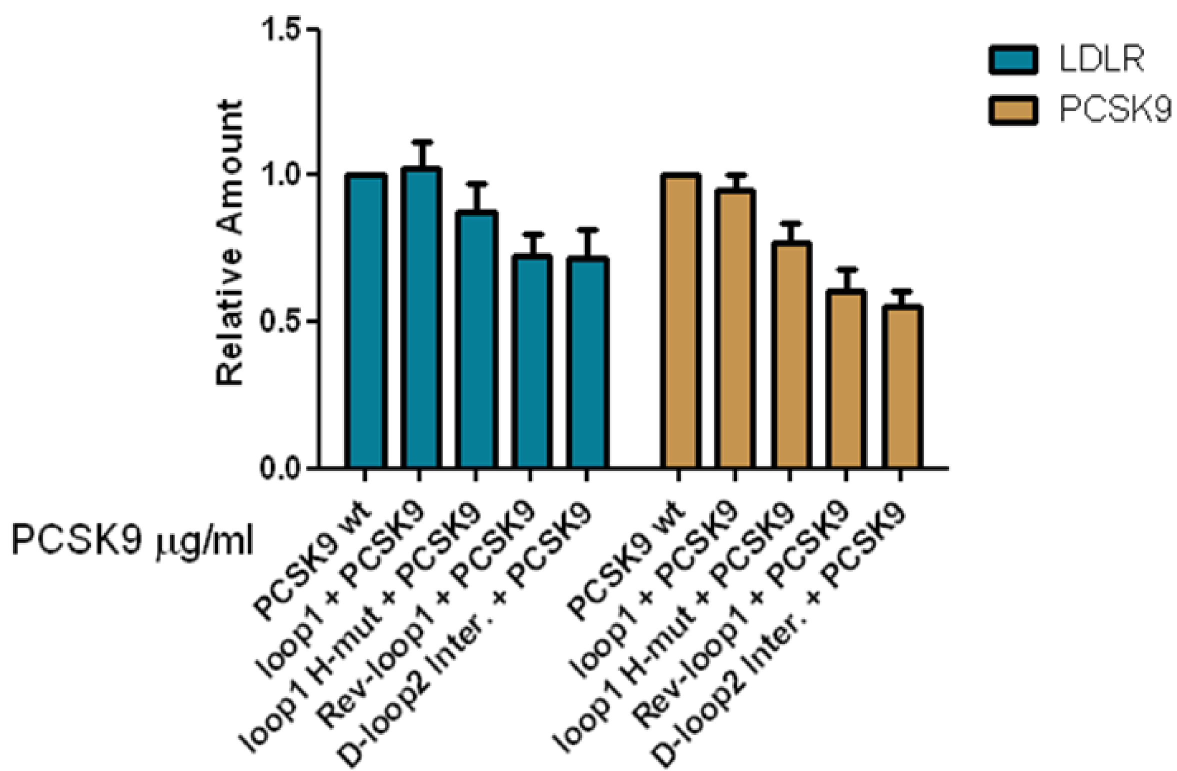




4.3 Dose-respone studies of the catalytic peptides derived from hPCSK9 in the presence of exogenous PCSK9

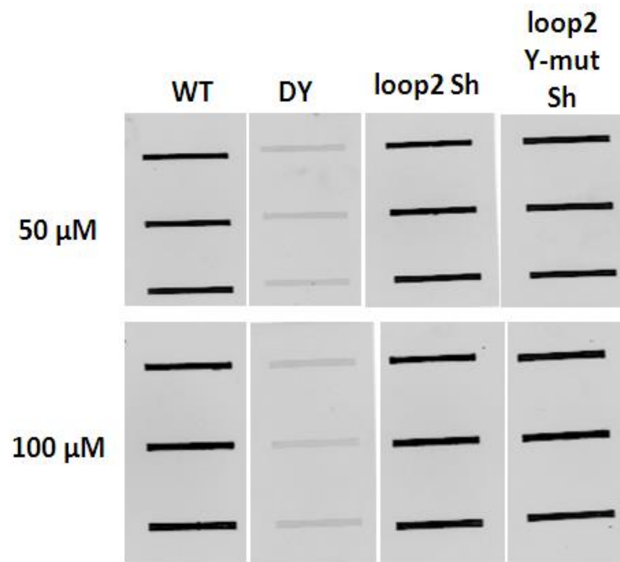
4.3.1 Effect on LDL-R levels *in vitro* conditions

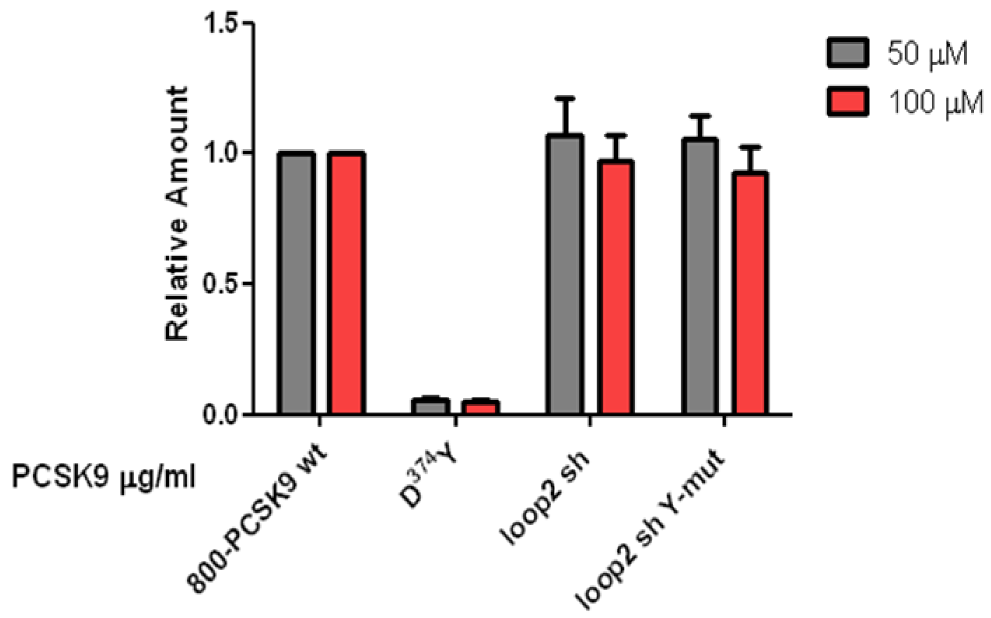
To explore the potential mechanisms of the underlying causes of above observations with the catalytic domain peptides in various hepatic cell lines, we further examined *in vitro* the influence of these peptides in the presence of added recombinant wild type hPCSK9 protein at the physiological concentration after binding to the LDL-R-692 ECD (extracellular domain) using slot blotting assay.

It has been demonstrated that PCSK9 concentrations are average range from 33 ng/ml to 4 µg/ml in plasma healthy individuals (113, 114, 115). Therefore, after slot blot LDL-R-692 ECD at the concentration 500 ng/ml on the membrane, we treated the membrane with (IRDye800)-labeled wild type PCSK9 at physiological concentration (0.1 µg/ml) in the presence of recombinant PCSK9 $D^{374}Y$ mutant and hPCSK9 catalytic $loop2^{372-380}$ - short sequence peptides and then incubated for a period of 1 hour.

In this experiment, we observed that catalytic loop2 $^{372-380}$ - short sequence peptide and its Y^{374}-mutant peptide slightly increased the LDL-R level at 50 µM concentration compared to the control (800-WT PCSK9) as seen in (**Figure 4.8**). In contrast, we did not notice any significant effect of these peptides even at 100 µM concentration on the LDL-R level. Furthermore, addition of recombinant PCSK9-$D^{374}Y$ mutant alone at 20 µg/ml concentration dramatically reduced LDL-R levels.

Figure 4.8: **Effect of higher concentration levels of hPCSK9 catalytic domain loop peptides (hPCSK9 loop2$^{372\text{-}380}$ - short sequence) on LDL-R levels with lower concentration of added recombinant hPCSK9 protein using slot blotting assay.** The LDL-R-692 ECD (extracellular domain) (500 ng/ml) was spotted onto nitrocellulose membrane using slot blot apparatus. After blocking the membrane for 30 min, it was then pre-incubated with hPCSK9 loop2$^{372\text{-}380}$ - short sequence peptides at 50 µM and 100 µM concentration, and incubated for 30 min. (IRDye800)-labeled Wild-type PCSK9 (0.1 µg/ml) were added to the blocking buffer in the presence of added recombinant hPCSK9 D^{374}Y mutant and hPCSK9 loop2 $^{372\text{-}380}$ - short sequence peptides and incubated for 1 hour. The membrane was then detected on the LI-COR Odyssey infrared imaging system. *The experiment was performed in triplicate and the data averaged.* **WT:** Wild type; **DY:** purified recombinant hPCSK9D^{374}Y mutant; **Loop2 sh:** Loop 2 short; **mut:** mutant.


WT
DY
loop2 Sh
loop2 Y-mut Sh
50 µM
100 µM


1.5
1.0
0.5
0.0
Relative Amount
50 µM
100 µM
PCSK9 µg/ml
800-PCSK9 wt
D374Y
loop2 sh
loop2 sh Y-mut

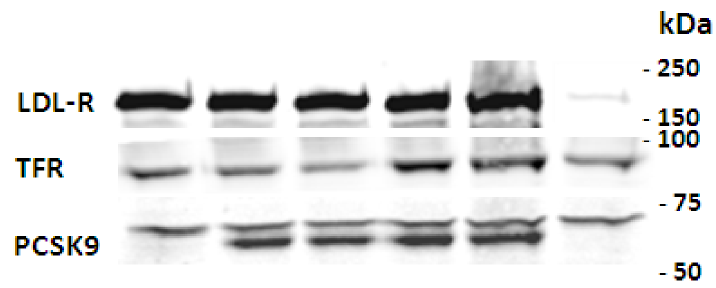
4.3.2 Effect of various hPCSK9 catalytic domain peptides on LDL-R level *in ex vivo* conditions

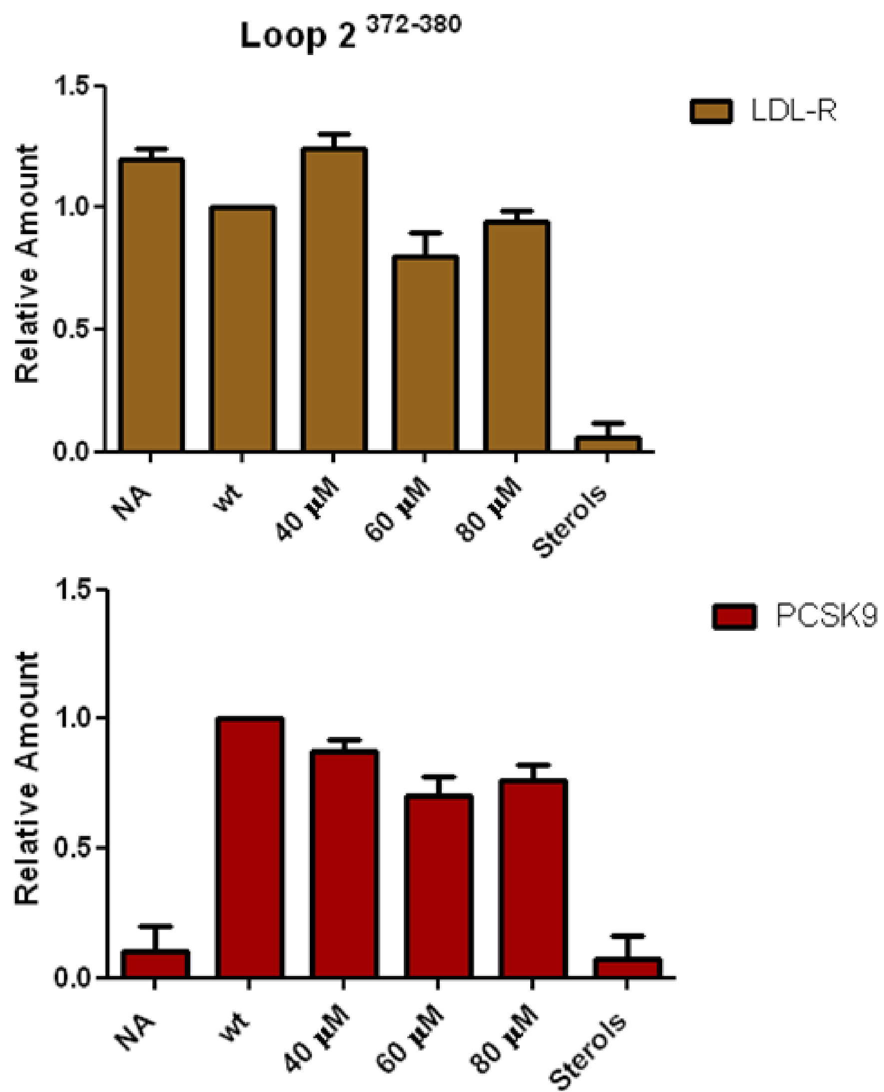
In order to confirm the observations as reported earlier that catalytic loop2 $^{372\text{-}380}$ - short sequence peptide and its Y^{374}-mutant peptide slightly enhance the LDL-R level at 20 µM concentration, we used PCSK9 uptake assay with those peptides in dose-dependent manner. Hepa1c1c7 cells were treated with wild type recombinant PCSK9 at physiological concentration (1 µg/ml) in the presence of hPCSK9 loop2 $^{372\text{-}380}$ - short sequence peptide at different concentrations (40, 60 and 80 µM). The cell lysates were then immunoblotted for the presence of PCSK9, LDL-R and Transferrin receptor as we did before. Our results revealed that both did not display any effect on LDL-R level although we noted a moderate increase in PCSK9 uptake for both the peptides as indicated in (**Figure 4.9 A, B**).

To examine the influence of the catalytic loop1 $^{323\text{-}358}$ - large sequence peptide and its H^{357}-mutant peptide on the LDL-R protein level in Hepa1c1c7 cells, the experiment was done in a manner similar to the way mentioned earlier. Our dose-dependent study involving those selected peptides revealed that there is no dose response effect on the LDL-R levels as indicated in (**Figure 4.10 A, B**).

Figure 4.9: **PCSK9 uptake assay in the presence of various concentrations of catalytic hPCSK9 loop2$^{372\text{-}380}$ and its Y^{374}-mutant peptides in Hepa1c1c7 mouse hepatoma cells.** (**A**) Hepa1c1c7 cells were cultured to ~80% confluence in medium F and in sterol-depleted medium H, and then were incubated for 18 hours to induce LDL-R expression. Hepa1c1c7 cells were then treated with wild type recombinant PCSK9 (1 μg/ml) which was added to the medium in the presence of hPCSK9 loop2$^{372\text{-}380}$ - short sequence peptide at different concentrations (40, 60 and 80 μM). Following an additional 4 hours incubation, the whole cell lysates were collected as described earlier and subjected to 8% SDS-PAGE followed by immunoblotting analysis of LDL-R, PCSK9 and TFR as an internal control (~ 50 μg/ lane). Secondary infrared dye (IRDye800)-labeled antibodies were used for detection on the LI-COR Odyssey infrared imaging system. (**B**) Hepa1c1c7 cells were treated with recombinant wild type hPCSK9 (1 μg/ml) which was added to the medium in the presence of hPCSK9 loop2$^{372\text{-}380}$ –Y^{374} - mutant short sequence peptide at different concentrations (40, 60 and 80 μM) and incubated for 4 hours. Whole cell lysates were collected and analyzed via immunoblotting for PCSK9 and LDL-R levels as well as the transferrin receptor (TFR) as an internal control (~ 50 μg/ lane). Secondary infrared dye (IRDye800)-labeled antibodies were used for detection on the LI-COR Odyssey infrared imaging system. Results are plotted relative to the control transferrin receptor (TFR). The graphical representations were performed using the average and the measured standard deviation values from *three* independent experiments done in parallel.

A





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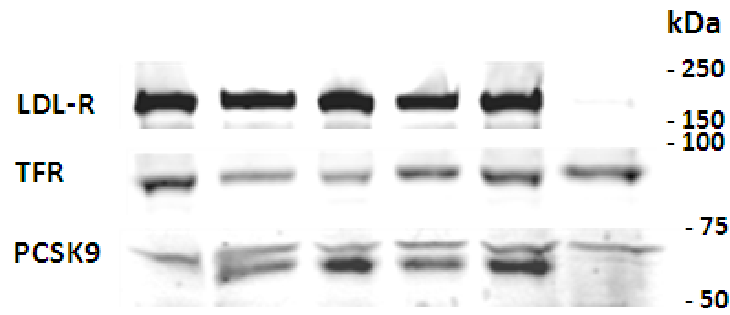
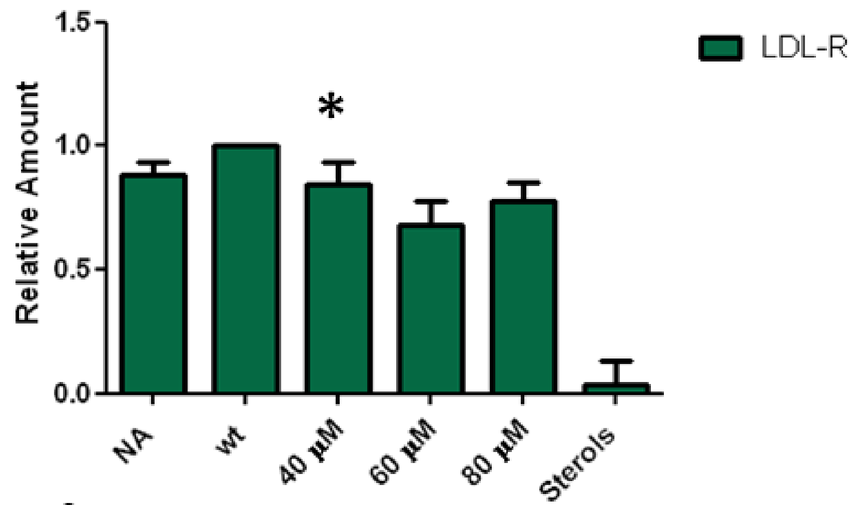




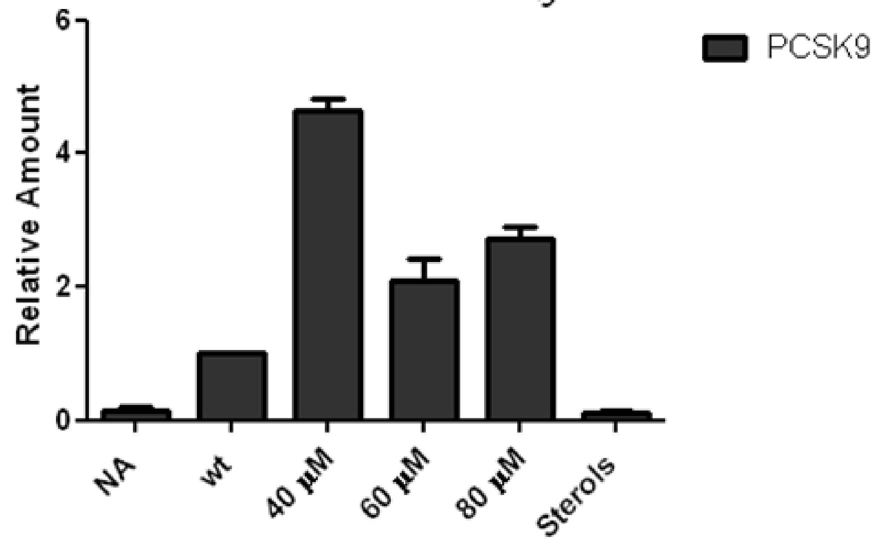
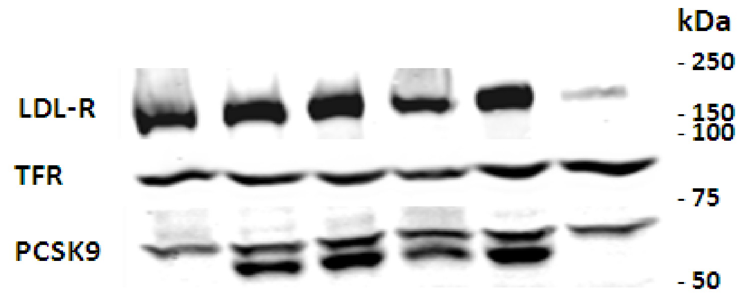
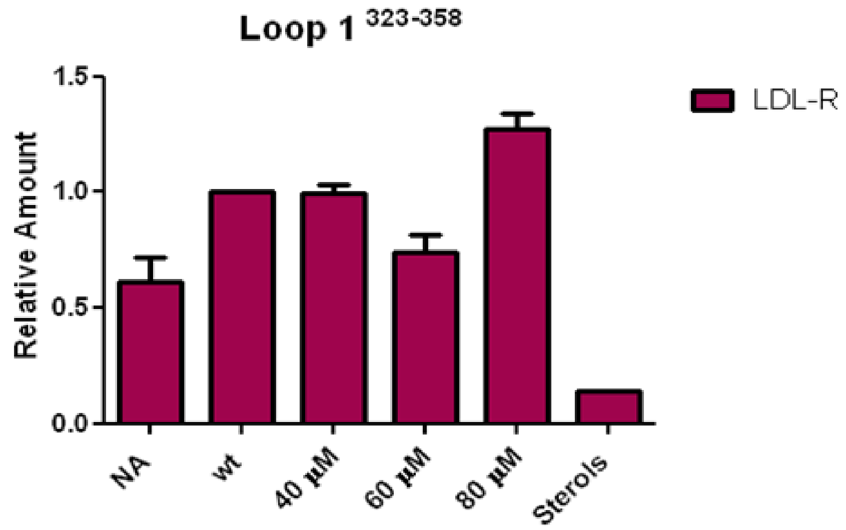
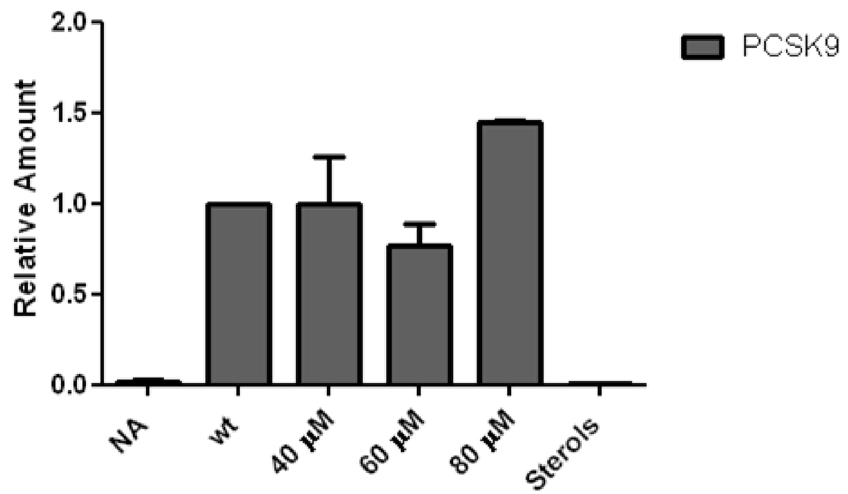


Figure 4.10: **PCSK9 uptake assay in the presence of various concentrations of catalytic hPCSK9 loop1$^{323\text{-}358}$ and its H^{357}-mutant peptides in Hepa1c1c7 mouse hepatoma cells.** (**A**) Hepa1c1c7 cells were cultured to ~80% confluence in medium F and in sterol-depleting medium H, and then were incubated for 18 hours to induce LDL-R expression. Hepa1c1c7 cells were then treated with recombinant wild type PCSK9 (1 µg/ml) which was added to the medium in the presence of hPCSK9 loop1$^{323\text{-}358}$ - large sequence peptide at different concentrations (40, 60 and 80 µM). After another 4 hours of incubation, the whole cell lysates were collected as described before and subjected to 8% SDS-PAGE followed by immunoblotting analysis for LDL-R, PCSK9 and TFR (internal control protein) (~ 50 µg/ lane). Secondary infrared dye (IRDye800)-labeled antibodies were used for detection on the LI-COR Odyssey infrared imaging system. (**B**) Hepa1c1c7 cells were treated with wild type PCSK9 (1 µg/ml) were added to the medium in the presence of hPCSK9 loop1 $^{323\text{-}358}$ –H^{357} -mutant large sequence peptide at different concentrations (40, 60 and 80 µM) and incubated for 4 hours. Whole cell lysates were collected and analyzed via immunoblotting for PCSK9 and LDL-R levels as well as the transferrin receptor (TFR) as an internal control (~ 50 µg/ lane). Secondary infrared dye (IRDye800)-labeled antibodies were used for detection on the LI-COR Odyssey infrared imaging system. Results are plotted relative to the control transferrin receptor (TFR). Graphs were plotted using the mean ± standard deviation values from three independent experiments run in parallel. * indicates a statistical difference between columns with significance $p < 0.05$ by Student t-test.

A







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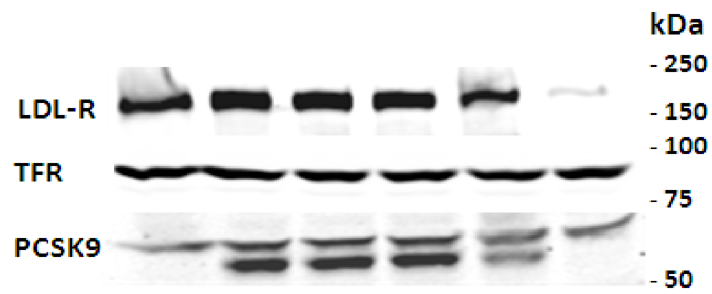
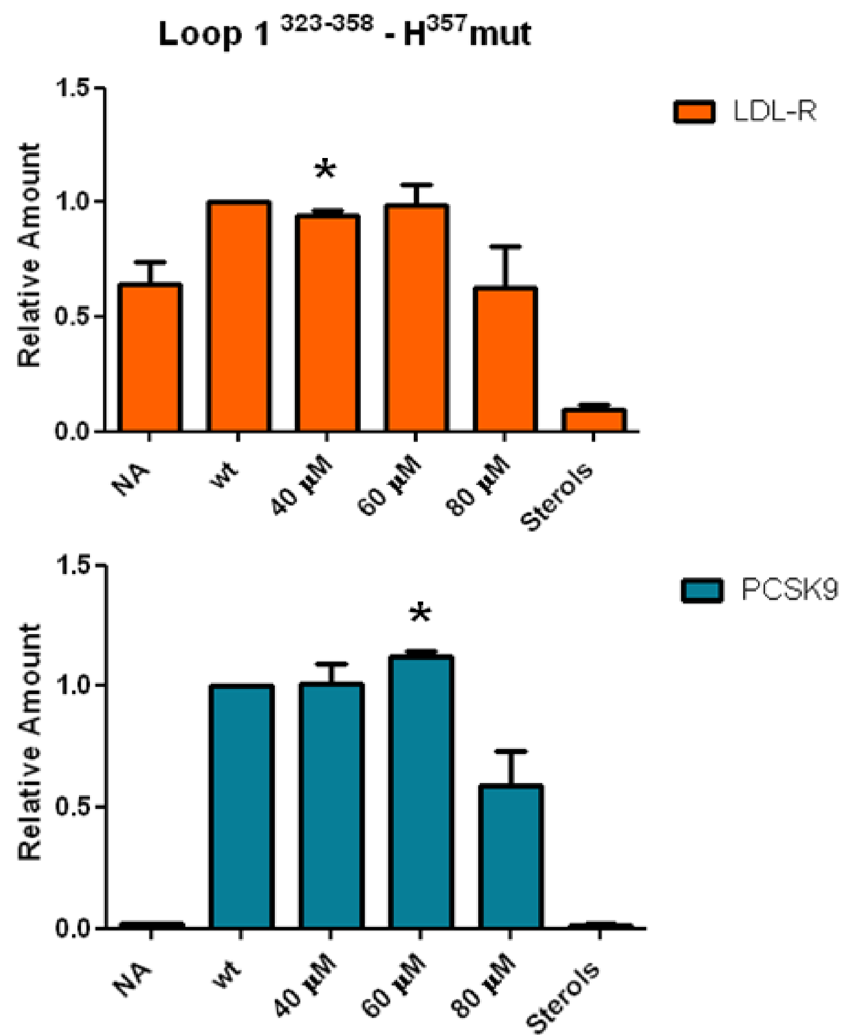


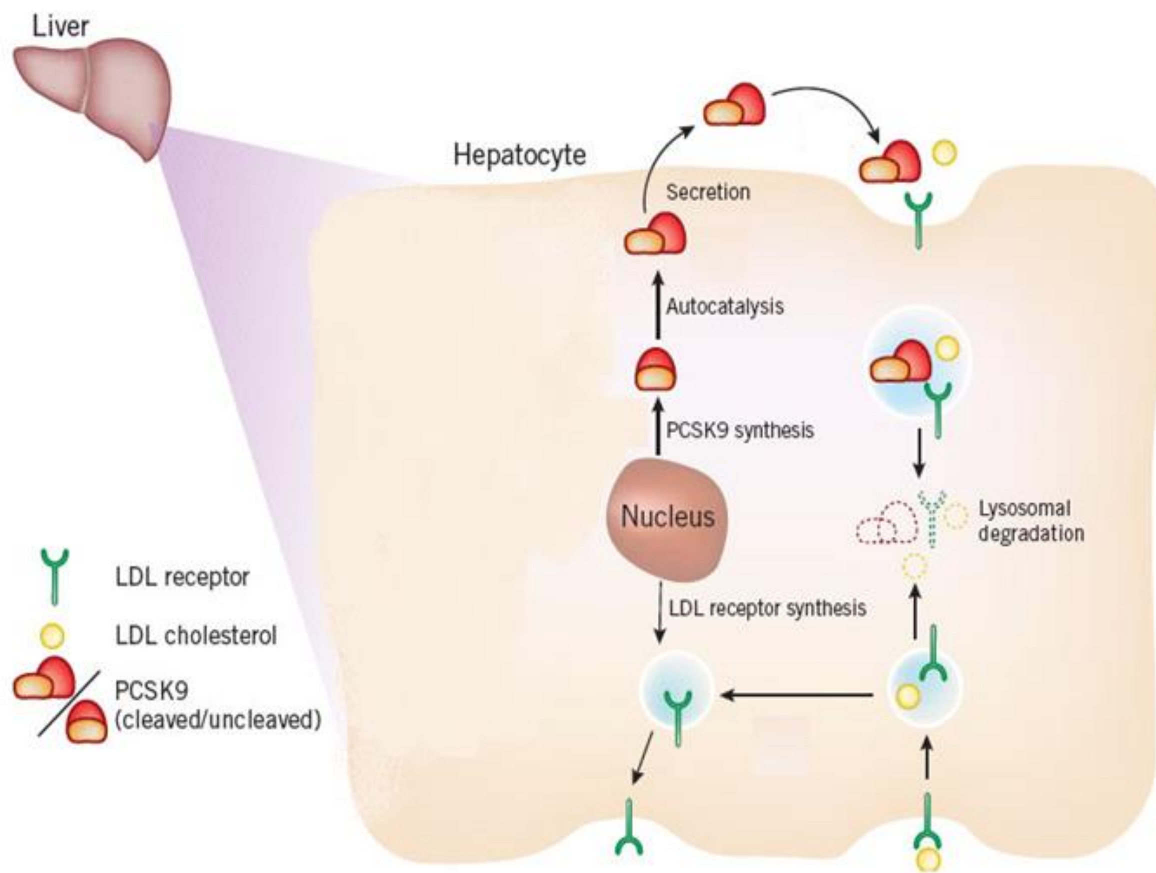


5. Discussion

Determining the regulatory effect of various synthetic peptides used in this study on the LDL-R protein level was served as the first step in understanding the mechanism of PCSK9-mediated LDL-R degradation as well as in developing novel peptide based inhibitors of PCSK9. As indicated earlier in the introduction section, elevated levels of plasma LDL-cholesterol is a major risk factor of cardiovascular or coronary heart disease (CHD) (1). The degradation of LDL-R by PCSK9 is highly detrimental since it affects the uptake of LDL particles into cells which ultimately determines the level of circulatory plasma LDL-cholesterol (116, 117). The detail events and the pathway have already been discussed where it was pointed out that PCSK9 protein is secreted as a complex with its prodomain that promotes LDL-R degradation in hepatocytes following binding. PCSK9 then directly binds with LDL-R on the cell surface, and are internalized in an LDL-R-dependent manner promoting its degradation in the lysosomal compartments (13, 14, 15, 16). The events including the various steps involved have been described schematically in (**Figure 5.1**) (54, 118). As a result of the above observation, PCSK9 became an important target for the development of non-statin based cholesterol lowering agents which remains the principal aim of this research study.

The above rationale was confirmed by knockout mouse studies and the observation of low levels of LDL-cholesterol found in individuals who carry loss-of-function mutations in PCSK9 (89). Consequently, PCSK9 inhibitors can be useful as potential therapeutic agents for treatment of hypercholesterolemia-linked cardiovascular diseases with or without the conjunction of statins and other cholesterol lowering medications (76).

Figure 5.1: **PCSK9-mediated LDL-R Degradation Pathway.** PCSK9 is predominantly expressed in the liver**.** Following its synthesis in the liver, PCSK9 undergoes autocatalytic cleavage, which is required for its secretion from the cell. Though cleaved, the two domains of PCSK9 remain together tightly associated as a complex. When cholesterol-bound LDL receptor is also associated with PCSK9, the LDL receptor is redirected for lysosomal degradation and prevented from recycling to the cell surface. If an internalized LDL receptor is not bound to PCSK9, the receptor is recycled to the cell surface, where it continues to remove LDL cholesterol from circulation.



This figure was adapted from Lou et al. 2009 (119)

To date three major stategies have been pursued to reduce PCSK9 activity. These include antisens nucleotides, specific antibodies and competing peptide approaches (10, 96, 120, 121). Crystal structure revealed that PCSK9 binds to LDL-R via its EGF-A domain. However, the segment(s) of PCSK9 involved in this binding is not fully identified although a recent study and crystal structure have implicated its catalytic domain (20, 67, 106). The exact characterization of this domain is crucial for development of any effective competing peptides that can target PCSK9:LDL-R interaction**.** Recently we reported that selected peptides from PCSK9 prodomain and two peptides from catalytic domain (hPCSK9 $^{181-200}$ and $^{368-390}$) are capable of upregulating LDL-R level without significantly affecting PCSK9 levels in human hepatic cells (107). Owing to thses findings along with crystal structure, cellular and knock out mouse studies confirmed the involvement of catalytic domain of PCSK9 in binding with LDL-R.

The current proposal is designed to identify these domain(s) as novel functional inhibitors of PCSK9 and evaluate their bioactivties. In that presepective our design of loop1 $^{323-358}$ and loop2 $^{372-380}$ peptides and their analogs is understandable and well justified and our results reported in this study though modest may shed some light in our efforts to identify catalytic domain/s of PCSK9 that are involved in binding with LDL-R.

5.1 Possible rationale and site of action of PCSK9 catalytic peptides

In the current study, we have successfully synthesized and fully characterized various catalytic domain derived peptides of hPCSK9 as well as their modified analogs and examined

their effects on LDL-R and PCSK9 levels when incubated in growing hepatic cell lines such bas human HepG2 and mouse Hepa1c1c7.

Our results using HepG2 cells indicated that when used at 5 µM final concentration level, Dextro-loop-$2^{372-380}$ short peptide and its Y^{374} mutant peptides both promoted the expression of LDL-R level by 30 - 50% depending on the peptide used. Dextro-loop-$2^{372-380}$ short peptide appears to be slightly more potent than its Y^{374} mutant peptide under identical condition. The rest of the peptides tested namely Dextro-loop-$1^{323-358}$ and its H^{357} mutant did not exhibit any significant effect on LDL-R level as observed in (**Figure 4.4**).

It is surprising to note that all the above peptides significantly increased the expression of PCSK9 by 2 - 2.6 folds depending on the nature of the peptide used. At the first sight it is difficult to explain how two of the peptides namely Dextro-loop $2^{372-380}$ and its Y^{374} muant enhanced both LDL-R and PCSK9 at the same time. We explain this by assuming that even though here PCSK9 level has increased but it may likely remain in functionally inactive form meaning that they are incapable of degrading LDL-R protein, either due to misfolding or absence of potential binding partners.

For the other peptides enhancement of PCSK9 level with accompanying decrease in LDL-R level are expected as noticed in (**Figure 4.4**). In these studies we examined the effects of our peptides towards the exogenous LDL-R and PCSK9 levels in comparison to Transferrin receptor or Actin as control housekeeping protein.

When recombinant PCSK9 **$D^{374}Y$** mutant protein was added to growing HepG2 cells in absence and presence of various PCSK9 peptides tested at 5 µM final concentration levels, we noted a slightly different result in comparison to that done in parallel in the absence of any added recombinant PCSK9 **$D^{374}Y$** protein. Thus our data seen in (**Figure 4.5**) showed that both

Dextro-loop2 $^{372\text{-}380}$ and Dextro-loop1 $^{323\text{-}358}$ H^{357} mutant peptides enhanced the levels of LDL-R by ~ 30% and ~ 50% respectively in the absence of any added recombinant PCSK9 D/Y protein. In both cases the PCSK9 level is also enhanced which most likely remain in majority in functionally inactive form. It may be pointed out that the functional activity (meaning the degradation of LDL-R by PCSK9) of PCSK9 depends on various factors besides its amount alone. These include its levels of phosphorylation, sulfation, glycosylation, conformational and interaction ability of PCSK9 and finally the extent of autocatalysis at the prodomain site. All these factors can influence the ultimate functional activity of PCSK9. In our experiments these factor/s may play role in explaining why in some cases increased PCSK9 level led to decreased LDL-R level as expected and why in other cases it is not.

Our cell culture data using mouse hepatic cell lines revealed nearly similar results under the condition of no added recombinant PCSK9 D/Y mutant protein. Thus here again Loop-2$^{372\text{-}380}$ short peptide along with its Y^{374} mutant at 5 µM concentration level both exhibited a modest ~ 20% enhancement of LDL-R level. Both these peptides also increased PCSK9 level by nearly 60% as shown in (**Figure 4.6**). Again this is quite unexpected but we rationalize this observation by proposing that this PCSK9 may remain largely in inactive form due to various reasons as explained previously.

It is interesting to note that two other peptides namely Reverse-Loop2 $^{380\text{-}372}$ short and its Y^{374} mutant both promoted PCSK9 level by 50 - 60% yet this is not reflected in LDL-R level which remains relatively unaffected. When the peptides were used at a higher concentration level such as 20 µM (instead of 5 µM) we noted a modest LDL-R promoting (by ~ 20 - 40% depending on the nature of the peptide used) activity with loop1 $^{323\text{-}358}$ and its H^{357} mutant

peptides as seen in (**Figure 4.7**). At a lower concentration level these peptides did not exhibit and LDL-R promoting activity as shown previously in (**Figure 4.5**).

Our *in vitro* displacement experiment using western dot blot methodology where only the two Loop2 $^{372\text{-}380}$ short peptides were used, we did not notice any displacement compared to control as observed in (**Figure 4.8**). This is quite unexpected and it needs to be repeated before any conclusion was made.

Our PCSK9 uptake experiment with the two loop2 $^{372\text{-}380}$ peptides revealed that both did not exhibit any significant effect on LDL-R level although we noted a modest increase in PCSK9 uptake for both the peptides as observed previously in (**Figure 4.9**). Again this experiment needs to be repeated to verify the above results. Also, our dose study (**Figure 4.10**) involving a few selected peptides revealed that there is no dose response effect. This is unexpected and needs to be repeated before any possible explanation is provided.

Importanly, this research project we noted that the mature PCSK9 (62 kDa) is more regulated by some of our synthetic peptide compared to the full length of proPCSK9 (72 kDa). Besides, those peptides used in this study are contain the crucial disulfide S-S bridge and therefore rigid. Interestingly, the two most potent gain-of-function mutantions of PCSK9 **D^{374}Y** and **R^{357}H** are included within these loop1 $^{323\text{-}358}$ and loop2 $^{372\text{-}380}$ peptide sequences suggesting their possible link to the expression of the mature PCSK9 form and not the full length form.

This research study described herein demonstrates that the catalytic domain of PCSK9 is of interest as a potential regulator of LDL-R. This domain is known to be implicated in the binding with EGF-A domain of LDL-R. Recently, very limited data is available in the literature about PCSK9 inhibitory compounds. So far, a synthetic LDL-R's EGF-A peptide has been

described (76). In addition, it has been indicated recently that small size peptide Pep 2-8 derived phage-displayed peptide libraries against PCSK9 can up regulate LDL-R levels (122). Moreover, it has been reported that peptides derived from the Annexin A2 that acts as endogenous inhihibitor can directly inhibited functional activity of PCSK9 (61). Study also indicted that the PCSK9 prodomain-derived peptides can promote LDL-R activity using human hepatocytes HepG2 cells (107). All these observations and findings justified the present resreach study. So far our results using human hepatocytes HepG2 and mouse hepatocytes Hepa1c1c7 suggest that specific peptides derived from the catalytic domain of PCSK9 can up regulate LDL-R protein levels.

6. Conclusion

In conclusion our research demonstrates that short and large medium size peptides derived from the catalytic domain of PCSK9 protein in the loop1 $^{323-358}$ and loop2 $^{372-380}$ can affect both LDL-R and PCSK9 in cell-based assays using human and mouse hepatoma cell lines. In this study we observed that among the various PCSK9 catalytic peptides tested Loop1 $^{323-358}$, its H^{357}mutant as well as our designed short Loop2 $^{372-380}$ and its Y^{374}mutant peptides can modestly promote the LDL-R protein levels. However, in most cases we also noted an increase in PCSK9 amount as determined by western blot analysis using specific PCSK9 antibody. This goes against normal expectation. However in order to explain this observation we propose that most of this enhanced PCSK9 amount remains in functionally inactive form this hypothesis requires further investigation. Our results though modest, are still encouraging because of potential implication in cholesterol clearance. Furthermore, our data indicated the crucial role of the catalytic domain of PCSK9 in regulation LDL-R protein and thereby cholesterol levels which will require more in depth studies in the future. Our catalytic domain peptides with LDL-R promoting activity may be useful as therapeutic agents for lowering cholesterol but it will need more in depth studies in future.

In summary, this is the first demonstration to our knowledge about LDL-R regulatory effects of peptides derived from the catalytic domain of PCSK9. Further investigation and studies are being pursued to better understand the exact influence of these peptides on the cholesterol regulation.

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Rasha Hassen Alghamdi

Profile

A recent graduate with a Master of Science in Biochemistry at the University of Ottawa completed in December 2013. Well organized, thorough, efficient, reliable, people oriented and recognized and always keen to learn something new as a senior graduate student. Fully bilingual in both Arabic and English languages.

Education

University of Ottawa **December 2013**
Master of Science in Biochemistry, Faculty of Medicine
Ottawa, Ontario, Canada

Kaplan-Pacific Language Institute **January/August 2011**
Diploma course in the English Language program
Vancouver, British Columbia, Canada

University of British Columbia **September/December 2010**
Diploma course in the Intensive English Program (IEP)
Vancouver, British Columbia, Canada

Pacific Gateway International College **April/August 2010**
Diploma course in the English Communication program
Vancouver, British Columbia, Canada

King Abdulaziz University **January 2009**
Bachelor of Science in Biochemistry
Jeddah, Kingdom of Saudi Arabia
Honor's in Biochemistry with a specialization in Biochemistry

Internship

Maternity & Children Hospital **February 2009/2010**
Training in Clinical Laboratories Department
Jeddah, Saudi Arabia

Maternity & Children Hospital **August 2008**
Summer Training in Clinical Laboratories Department
Jeddah, Saudi Arabia

King Faisal Hospital Specialist Hospital & Research Centre **June 2008**
Summer Training in the Department of Food Services
Jeddah, Saudi Arabia

Maternity & Children Hospital **August 2007**
Summer Training in Clinical Laboratories Department
Jeddah, Saudi Arabia

Work Experience

University of Ottawa Heart Institute **September 2012/2013**
Graduate Student in Lipoprotein Receptor Biology Laboratory, compeleted research on two major proteins that implicated in cholesterol homestasis, with the goal being to regulate cholesterol levels in the blood circulation.
Faculty of Medicine, University of Ottawa
Ottawa, Ontario, Canada.

Ottawa Hospital Research Institute **September 2011/2012**
Graduate Student in Proprotein Convertase Laboratory, Chronic Disease Program, studying the relationship between two major proteins that implicated in cholesterol homestasis.
Faculty of Medicine, University of Ottawa
Ottawa, Ontario, Canada

Skills

Summary of Research Experience

- Detailed experience in standard molecular biology techniques, including western blot analysis, and gel electrophoresis.
- Extensive knowledge in mammalian cell culture laboratory techniques and standard experiments, including media preparation, transfections and peptide addition.
- Experience in protein biochemistry techniques including Gel electrophoresis, MALDI-TOF and SELDI-TOF mass Spectroscopic methods
- Extensive experiences in peptides synthesis using Automated Solid Phase Peptide Synthesizer and protein purification techniques including Reverse Phase High

Performance Liquid Chromatography (RP-HPLC) as well as enzyme purification using Column Chromatography

- Excellent knowledge of immunological assays, including ELISA's for protein binding, fluorescence microscopy for protein location and mode of action, protein and peptide antibody work through molecular immunology and cellular immunology work
- Experience in enzyme assays (using fluorometer) and protein assays (BCA and Bradford method)
- Experience in designing experiments as well as, extrapolating and analyzing experimental data.
- Capable of producing accurate and reliable experimental data
- Ability to maintain, handle and calibrate modern laboratory equipment and follow analytical techniques.
- Responsible for supervision and training of lab personnel including new students
- Willing to take initiative to complete work in a timely manner, through attention to detail.
- Self-motivated, enthusiastic and a strong ability to work within a team and independently.
- Willing to adapt to a different environment, or to a different ideas to respect the integrity of the workplace, and others opinions
- Knowledgeable of laboratory safety practice

Teamwork

- Established ability to develop and maintain contacts within the research community to collaborate effectively, persuade, and influence the scientific community. As seen with the recent publication.
- Team oriented, well organized, thorough, efficient, and reliable
- Willing to take initiative to complete work in a timely manner, through attention to detail, can work under pressure and on tight deadlines.
- Capable of managing a high volume of activities, analyzing and resolving difficult situations, by working independently or with a team under pressure and on tight deadlines.
- Demonstrated ability to work effectively as part of a complex team setting and independently, through the management of summer and honor's students at the OHRI, and personal research

Technical

- Excellent computer knowledge in Microsoft application (Word, PowerPoint, Excel,

Access)

- Familiar with advanced search techniques
- Excellent typing proficiency, PowerPoint Presentation and MS project
- Experienced in using software for data manipulation and analysis including GraghPad Prism, Sigma Plot, Ciphergen, Soft Sec and others

Communication

- Excellent oral and written communication skills in both languages, shown in monthly meetings, supervising of associate and paper communication.
- Very good team leadership skills and very active in social events
- Excellent time management and interpersonal communication skills
- Confident, creative, and very motivated to work within a team and independently
- Honest, Dependable with Excellent Interpersonal skills
- Work well under pressure and on tight deadlines, flexible, and quick learner
- Results and people oriented, shown with monthly meetings where team members are trained in what is expected, and can participate in any changes of policy.
- Structures a balance between my work and daily interests enabling success in both areas
- Behavioral flexibility, revealed through treatment of staff members and students by building trust and confidence opening the communication and leading to outstanding results of both the team and the individuals.
- Demonstrated talent in communicating effectively under pressure and difficult situations.
- Provides friendly, helpful, and courteous service to supervisors and summer students.
- Works independently with minimal supervision

Certificates

July 24 to Aug 4, 2010: Participation in the preclinical course at King Abdulaziz University Hospital, Jeddah, Saudi Arabia
June 26, 2010: Participation in the Saudi Educational Students Academic Fair in Vancouver, BC, Canada
Feb 28, **2010:** Certificate of Completion of CPR, King Fahad Hospital, Jeddah, Saudi Arabia
Feb 13/16, 2010: Certificate of Attendance “7th Scientific Conference “for Medical Students in the GCC Countries, Jeddah, Saudi Arabia
May 30, 2009: Certificate of Attendance for Attending Lab Day 2 held at Ibn Sena Lecture Hall in King Fahad Research Center, Jeddah, Saudi Arabia

April 19/22, 2009: Certificate of attendance of Second Annual Laboratory Professional Week at King Abdulaziz University hospital in Jeddah

March 30, 2009: Certificate of completion of Infection program of the Department of Infection at Maternity & Children hospital in Jeddah

May 12/13, 2008: Participation in the Second Scientific meeting at King Abdulaziz University

June 10/14, 2006: Certificate of Attendance in the Science of Medical Terminology and abbreviations at Maternity & Children hospital in Jeddah

May 31 to June 30, 2006: Certificate of attendance in self Discovery, leadership skills communication skills and time management at King Abdulaziz University

Scientific Publications

Articles

Sarmistha Basak, Aryan Shiari, **Rasha H. Alghamdi** and Ajoy Basak.
"Post translational modifications of proteins of rare types and their biological implications"
Review article submitted to *Current Medicinal Chemistry, USA,* July 10, 2013.

Abstract Presentations & Conference Participations

- **Rasha H. Alghamdi**, Thomas A. Lagace and Ajoy Basak.
  "Biochemical and interaction studies of fluorescent EGF-A peptide towards recombinant PCSK9: Potential implication in regulation of LDL-R and cholesterol"
  Abstract accepted for poster presentation in the 6th Annual Molecular Function and Imaging (MFI) Symposium, Ottawa University of Heart Institute in Ottawa, Canada, June 27-28, **2013.**

- **Rasha H. Alghamdi**, Thomas A. Lagace and Ajoy Basak.
  "*Design & Development of PCSK9 inhibitors for regulating LDL-R and cholesterol"*
  Abstract accepted for Oral presentation at Biochemistry, Microbiology and Immunology Annual Symposium Day in Ottawa, Canada, March 11, **2013**

- **Rasha H. Alghamdi**, Thomas A. Lagace and Ajoy Basak.
  "Development of PCSK9 inhibitors for regulating LDL- R and cholesterol"
  Abstract accepted for Poster presented in the 5th Annual Molecular Function and Imaging (MFI) Symposium, University of Ottawa Heart Institute in Ottawa, Canada, June 21-22, **2012**

- **Rasha H. Alghamdi**, Thomas A. Lagace and Ajoy Basak.
  "*Development of PCSK9 inhibitors for regulating LDL-R and cholesterol"*
  Abstract accepted for poster presentation at Biochemistry, Microbiology and

Immunology Annual Research Day in Ottawa, Canada, May 17, **2012**

Interests

- Oral communication course in French language from January/April 2012
- Leisure activities include, swimming, playing tennis, esquash, doing aerobics, and avid reader.