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**Regulatory Effects of Peptides from the pro and Catalytic Domains of Proprotein Convertase
Subtilisin/*Kexin*⁹ (PCSK9) on Low Density Lipoprotein Receptor (LDLR)**

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RECEPTOR (LDLR)**

By

Heather Palmer

Thesis submitted to the Department of Biochemistry, Microbiology
and Immunology in partial fulfillment of the requirements for the
degree of Master of Science.

Department of Biochemistry, Microbiology and Immunology
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ABSTRACT

Proprotein Convertase Subtilisin/Kexin 9 (PCSK9) is the latest member of the PCSK enzyme family that is structurally related to the bacterial subtilisin and the yeast kexin. It plays a major role in the regulation of cholesterol by degrading low-density lipoprotein receptor (LDLR) responsible for the endocytosis of LDL-cholesterol. Thus, there is a great deal of research interest in the development of PCSK9 functional inhibitors which may have potential applications as therapeutic agents for lowering plasma LDL-cholesterol and the associated risk of cardiovascular disease. PCSK9 degrades LDLR by first binding to form a complex and then rerouting to the lysosomal compartment leading to its degradation. In addition it was shown that following the autocatalytic cleavage, the prodomain of PCSK9 remains strongly attached to the mature PCSK9 via its catalytic domain. Owing to these findings, we proposed that selected peptides from hPCSK9 pro and catalytic domains are likely to affect the LDLR binding. Using human hepatic HepG2 and Huh7 cells we showed that the acidic N-terminal and the mid-basic segments of the prodomain enhanced the LDLR protein level significantly, without altering the PCSK9 protein level. The physiologically relevant phospho-Ser⁴⁷ peptide decreased the LDLR protein level suggesting that Ser⁴⁷-phosphorylation leads to a gain of functional activity of PCSK9. Addition of recombinant PCSK9 to the culture medium decreased the LDLR protein level that was restored by the addition of PCSK9³¹⁻⁴⁰, ³¹⁻⁶⁰ or ⁹¹⁻¹²⁰ peptides. Two catalytic domain peptides PCSK9¹⁸¹⁻²⁰⁰ and PCSK9³⁶⁸⁻³⁹⁰ decreased LDLR content confirming their interactions with LDLR. Our study concludes that specific peptides from the pro- and catalytic domains of PCSK9 can regulate LDLR and may be useful for development of novel therapeutics for cholesterol regulation.

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List of Abbreviations

aa- Amino Acid

ADH-Autosomal Dominant Hypercholesterolemia

CD- Circular Dichroism

CHRD-Cys/His-Rich Domain

DMF- NN'-dimethyl formamide

DIEA- N, N'-Diisopropyl ethyl amine

DMEM- Dulbecco's modified Eagle's medium

DTT- Dithiothreitol

EGF-Epidermal Growth Factor

ER- Endoplasmic Reticulum

FBS- Fetal bovine serum

Fmoc- Fluorenyl methoxy carbonyl

HATU- 2-(1H-7-Azabenzotriazol-1-yl)-1,1,3,3-tetramethyl uronium hexafluorophosphate

Kex2-Kexin 2

LDL-C-Low Density Lipoprotein Cholesterol

LDLR- Low Density Lipoprotein Receptor

γ LPH- Gamma lipotropin

β MSH- β melanocyte - stimulating hormone

NMP- N-methyl pyrrolidone

PBS-Phosphate Buffered Saline

PCSK- Proprotein Convertase Subtilisin/Kexin

PCs- Proprotein/Prohormone Convertases

POMC- Pro-opiomelanocortin protein

RIPA- Radio Immuno Precipitation Assay

SREBP- Sterol regulatory element binding protein

TBS- Tris Buffered Saline

TGN- Trans Golgi Network

1. Introduction

1.1 Proprotein convertase subtilisin/kexin (PCSK) family enzymes

1.1.1 Historical Background

Proprotein convertase subtilisin/kexins (PCSKs) constitute a family of mammalian endo-proteolytic enzymes that are structurally related to yeast kexin and the bacterial subtilisin (1). The discovery that most hormones, growth factors and biologically or functionally active polypeptides/proteins are derived from their larger inactive precursor proteins following post-translational cleavages prompted the launch of research studies worldwide for the search of such enzymes. The first evidence in favor of this novel proprotein theory came in 1967 from the work of Steiner et. al and Chretien et. al done almost concurrently, who demonstrated that the hormone insulin as well as the neuropeptide γ lipotropin (γ LPH), and β melanocyte stimulating hormone (β MSH), originated respectively from proinsulin and pro-opiomelanocortin (POMC) (2, 3). The identity of the enzymes responsible for the processing of these precursor proteins was not known for many years until the advent of cloning. The first member of the PCSK family was found during the discovery and cloning of the yeast protein kex2, more commonly referred to as kexin (4). The identification and subsequent characterization of the yeast Kex2 gene, (5-7) led to the discovery of the mammalian homologues the PCSKs.

The cleavage of inactive precursor proteins at specific sites is a regulatory mechanism responsible for the generation of a wide variety of biologically active proteins at the right time and at the right place. This processing event assists the cell in regulating the amount of active proteins generated at one given time. Thus, the proteolytic activation of proneuropeptides, prohormones, precursor growth factors, cell adhesion molecules, surface

proteins, enzymes, viral glycoproteins, bacterial toxin proteins, etc is critical for the production of the corresponding bioactive proteins. Accumulated research over the past several decades revealed that the nine mammalian PCSK enzymes perform the activation. It was further demonstrated that in most cases, this cleavage takes place at the carboxy terminal of a basic amino acid mostly Arg defined by the consensus motif R-X-K/R-R↓ or R-X-X-R↓ (8), where X = any amino acid except Cys, R = Arginine and K = Lysine. Out of the nine PCSKs identified, seven belong to the kexin-type. All PCSKs contain a catalytic domain characterized by the presence of a catalytic triad consisting of Serine, Aspartic acid, Histidine and the oxyanion hole Asparagine, a characteristic feature of all subtilases (9, 10). The first two members of this family are PCSK1/PC1/PC3 and PCSK2. They were discovered in 1990/1991 (5, 11). Later on 7 additional PCSKs were unraveled, these are **PCSK3/furin, PCSK4/PC4, PCSK5/PC5/PC6, PCSK6/PACE4; PCSK7/PC7/PC8/LPC (Lymphatic Proprotein Convertase), PCSK8/SKI-1 (Subtilisin Kexin Isozyme 1)/S1P (Site 1 Protease) and PCSK9/NARC-1 (Neural Apoptosis Regenerative Convertase 1)**. While PCSK1-7 belong to the kexin type cleaving peptide bonds C-terminus to Arg residue, PCSK8 and PCS9 belong to Pyrolysins and Proteinase-K types respectively.

The biosynthesis and expression of the yeast gene kexin, was the first major advancement in the field demonstrating that kexin belonged to a family of calcium dependent serine proteases that exhibit cleavage specificity after single or paired basic amino acids (4, 7, 12). However the discovery of the mammalian counterpart of this enzyme came much later in 1990/91 following extensive research.

1.1.2 PCSKs and human diseases

The expression of most PCSKs, particularly furin/PCSK3 and PC5/PCSK5 was found to correlate with the rapid growth of tumors, tumor invasiveness as well as the metastatic potential of tumors. Many physiological proproteins such as precursor growth factors like proPDGF-A/B and proVEGF-C associated with tumor growth contain a PCSK recognition site in their sequence at which they are cleaved by one or more members of the PCSK family leading to the formation of mature proteins (active growth factor molecules) responsible for cell proliferation, migration and adhesion (13). PCSKs can also cause degradation of the extracellular matrix and enhanced motility (13). Thus evidence suggests that PCSKs particularly PCSK3 and PCSK5 play a major role in cancer and tumor development (14).

The understanding of precursor proteins, their proteolytic activation and mode of action will be important in the understanding of physiological disorders such as Alzheimer's disease (15), obesity (16) and bacterial/viral infections including HIV (17).

1.1.3 PCSK structure and biological function

Each member of the PCSK family exhibits unique characteristic structural features, however in general each contains a signal peptide responsible for directing the protein to the endoplasmic reticulum (ER), the inhibitory prodomain which acts as a chaperone ensuring proper folding of the protein, followed by a catalytic domain containing the characteristic catalytic triad of residues, Aspartic acid, Serine and Histidine, as well as Asparagine for the oxyanion hole. With the exception of PCSK9, c-terminal to the catalytic domain, all eukaryotic PCSKs contain the P (Protease) domain. This domain is important for proper folding, as well as stability and determining the optimum pH for its activity (5). Followed

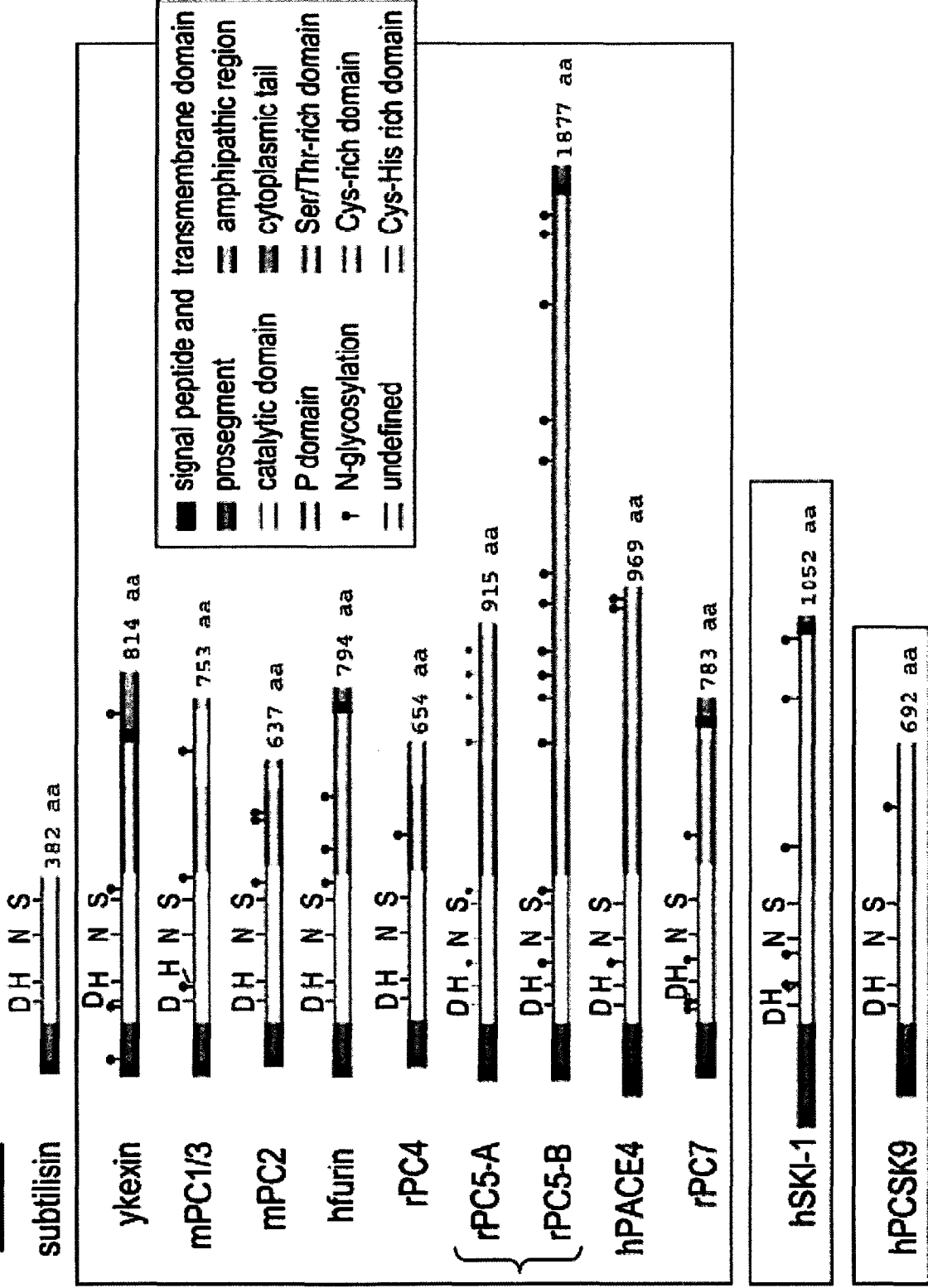
lastly by the C-terminal domain where most of the variability within the PCSK proteins exist (1). Although overall similar in nature and structure, there are many variations in the primary sequence and conformation of the PCSK family, and also to their closest non-mammalian counterparts: bacterial subtilisin and yeast kexin (**Figure 1**).

Thus, starting in the secretory pathway the PCSKs are synthesized as pre-proteins containing both the signal peptide and the prodomain (19). The signal peptide leads the PCSK through the secretory pathway into the ER lumen, wherein it is cleaved and the mature enzyme undergoes post-translational modifications such as, sulfation, glycosylation, phosphorylation, ubiquitination, etc (18, 20). Normally, two cleavages: primary and secondary occur in the N-terminal prodomain. The primary cleavage releases the inhibitory prodomain from the catalytic domain but it remains attached to the mature protease inhibiting its protease activity until the second cleavage takes place within the prodomain leading to its dissociation and the formation of active enzyme (13, 21).

The primary amino acid sequence of the PCSK family of serine proteases has yet to explain the reason behind the high specificity of the PCSKs in cleaving only selected physiological substrates (22). Specific proteolysis of inactive precursors by 7 of the 9 members of the kexin family occurs at the C-terminus of an Arginine residue characterized by the consensus motif **H/K/R-X-X/K/R-R↓Y** (8), where X = any amino acid other than Cys and Y = P' residue. The P1' position (Y) plays a key role in the recognition of the cleavage site for many of the PCSKs, and many amino acids can be present at this site. However, Thr, Val, Ile, Leu, Pro, Cys, Met and Trp are rarely present (18). It is also noted that basic residues at P1 and P2, P4, P6 and P8 are crucial for both recognition and cleavage (23).

Figure 1. Schematic diagram showing the structure of various PCSK enzymes and their characteristic domains. Different regions identified by color including; the signal peptide, pro domain, catalytic, P, transmembrane and C-terminal domains. The indicated residues in the catalytic domain indicate conserved amino acids for catalytic activity. (The figure was obtained from *Prat et al. 2007* following modification and minor changes.

Enzyme



The secondary structure of substrates covering the cleavage site is also important in PCSK recognition and ultimate cleavage. It is generally believed that β -turns located near the cleavage site may help in recognition, binding and cleavage as it exposes the surface. The presence of β -turn breakers usually prevents such cleavage (24, 25). The precise secondary structures surrounding the site of cleavage, provides the PCSK family a high degree of specificity (18).

PCSK8 also known as SKI or S1P and **PCSK9** are quite different and unique from the other PCSK family members known as the kexin type. These two PCSKs possess an unusual recognition motif compared to the others. As a result they cleave after non-basic usually hydrophobic amino acids (13). Thus PCSK8/SKI-1/S1P cleaves after the hydrophobic amino acids characterized by the sequence **R/K/H-X-L/I/V-L/I/G**↓ while for PCSK9, it is only known to cleave its own prodomain at **VFAQ**↓**SIP**. The cleavage specificity, the recognition motifs, as well as, their current accepted nomenclatures are shown in **Table 1**.

Table 1. Different recognition, cleavage motifs and current united accronyms for all known PCSKs.

PCSK	Alternate name	Cleavage Specificity	
PCSK1	PC1/PC3	(H/R/K) -(X) _n - R ↓ X	
PCSK2	PC2		
PCSK3	Furin/PACE		
PCSK4	PC4		(K) - X - X- R ↓ X
PCSK5A	PC5		(H/R/K) -(X) _n -K/R ↓ X
PCSK5B	PC6		
PCSK6	PACE4		
PCSK7	PC7/PC8/LPC		
PCSK8	S1P/SKI	R/K-X- (L,I,V)-Z ↓ X	
PCSK9	NARC-1	V-F-A-Q ↓ S-I-P	

Although the PCSKs of kexin type are similar in nature with overlapping specificities, they also have their differences. For instance PCSK1 and PCSK2 are structurally similar; they have over 50% sequence similarity in their catalytic domain. However, their activation mechanism is different, the activation of PCSK2 does not merely depend on the cleavage of the prodomain but it also requires a partner protein 7B2, which must bind to PCSK2 for its activation (26, 27). Later on the SAAS protein was discovered as the partner protein of PCSK1 (28). The discovery of 7B2 and SAAS proteins raised the question as to whether there are other binding proteins responsible for activating the PCSKs, especially in the case of PCSK9 whose protease activity has not yet been fully demonstrated.

1.1.4 Tissue and subcellular distributions of PCSKs

The PCSKs are not only a diverse group of serine proteases, with different characteristics; they are also expressed as a group or individually throughout mammals (Table 2). The PCSKs are expressed throughout the central nervous system, brain, and other organs depending on the nature of the enzyme. PCSKs can be divided into two major types, those expressed in the constitutive pathway and those in the regulatory pathway.

Table 2 The PCSK enzymes and their tissue distributions.

PCSK	Alternate name	Tissues
PCSK1	PC1/PC3	Nervous system, brain
PCSK2	PC2	Nervous system, brain
PCSK3	Furin/PACE	Ubiquitous
PCSK4	PC4	Gonadal, testes
PCSK5A	PC5	Nervous system, adrenal gland, widespread
PCSK5B	PC6	Widespread
PCSK6	PACE4	Ubiquitous
PCSK7	PC7/PC8/LPC	Ubiquitous
PCSK8	Site 1 protease S1P/SKI	Ubiquitous
PCSK9	NARC-1	Liver (major), Intestine, Kidney, brain

In the regulatory pathway, proteins are cycled from the ER to the trans golgi network (TGN) where they become activated into mature enzymes that are stored and packaged in secretory granules until they are ready to exert their proteolytic actions on physiological proteins. There are four PCSKs that belong to this category. These are PCSK1, 2, 4 and 5A (29). In the nervous system and brain, PCSK2 is the most abundant (11, 30) followed by PCSK1 (31). PCSK1 and 2 are expressed almost exclusively in the brain, and have been shown to co-localize with their physiological precursor proteins in specific cell types (20). In the adrenal gland PCSK5A is expressed most abundantly, PCSK3 is expressed throughout the adrenal cortex, whereas PCSK1 and PCSK2 are primarily expressed in the medulla (1). Unlike other PCSKs that are more ubiquitous in nature, PCSK4 is expressed almost exclusively in reproductive tissues or organs such as testicular germ cells, ovary and placenta, although, both PCSK7 and PCSK3 (1) are present to some degree in the reproductive organs. PCSK4 is the only member of this family whose expression is most selective and is important in male fertilization and reproduction events. PCSK5 exists in two alternatively spliced isoforms known as PCSK 5A, and 5B and both are expressed widely throughout the body (32).

The remaining PCSKs: 3, 5B, 6, 7, 8 and 9 act via the constitutive pathway within the TGN and are also expressed widely in mammalian tissues (33). In the constitutive pathway, proteins are transported from the TGN to plasma membranes by small transport vesicles. Within the brain PCSK3, also known as furin (34, 35), and PCSK5, are expressed weakly (32). Within the digestive system PCSK5 and PCSK3 are the major PCSKs (1). However, PCSK9 is also found in small quantities in the intestine (36). PCSK3 is the most ubiquitous among all the PCSKs and is expressed in all major organs. A complete knockout

of PCSK3 in animals is not viable, since its function is crucial for fetal growth and development (37, 38). PCSK6 is expressed as multiple splice variants and expressed in high levels in the brain and the anterior pituitary (39). PCSK8 more commonly known as S1P or SKI is expressed ubiquitously throughout the body. It is a key player in many physiological disorders particularly cholesterol homeostasis and lipid synthesis (40, 41). PCSK9 is expressed primarily in the liver with minor quantities in the small intestine, gut, plasma and kidney (42, 43).

Thus the nine PCSKs are expressed in a variety of tissues throughout the body and they play important physiological roles either as enzymes or as non-enzyme proteins in activating precursor proteins or as a binding partner. The elucidation of the function of the PCSK enzymes is considered important for any future development of therapeutic agents in the treatment or prevention of various illnesses.

1.2. PCSK9

1.2.1 Background

PCSK9 is the ninth member of the mammalian proprotein convertase family of serine endoproteases (36). Discovered in 2003 as NARC-1 (36, 43), PCSK9 was found to be closely related to Proteinase K, (43), a subtype of bacterial subtilisin. The physiological importance of PCSK9 was revealed when it was discovered as the third gene, along with the Low Density Lipoprotein Receptor (LDLR) and Apolipoprotein B (Apo-B) to be responsible for Autosomal Dominant Hypercholesterolemia (ADH) (43, 44, 45). PCSK9 is synthesized as a pre-proprotein of 72 kDa which loses its N-terminal 30 mer signal peptide. Further removal of the pro-domain takes place during its exit from the ER. This cleavage leads to

the loss of the prodomain via autocatalytic cleavage at VFAQ¹⁵²↓SIP producing a 63 kDa mature protein (44, 45). This site differs from that of the other kexin-type PCSKs that normally cleave after basic amino acids such as H/K/R-X-X/K/R-R↓, or hydrophobic amino acids like PCSK8, with the motif R/K-X-L/I/F-L. In contrast to all other PCSKs, the prodomain of PCSK9 remains strongly attached to the mature protein blocking its proteolytic activity and potential binding to other substrates. The prodomain remains non-covalently attached and covers part of catalytic triad residues, Ser, Asp, and His (46, 47). PCSK9 continues to remain functional and is still capable of binding to LDLR and routing the complex to the lysosome instead of its normal endosomes, for degradation through a still poorly understood mechanism (48, 49). The major function of PCSK9 is to degrade LDLR (36), the primary clearance route of LDL-cholesterol (LDL-C) (50). Since its discovery in 2003 as one of the proteins responsible for cholesterol homeostasis, its function in LDLR degradation, and its possible inhibition PCSK9 has caught attention of researchers worldwide and became an exciting and important area of present day research involving hypercholesterolemia and associated cardiovascular disease risk.

1.2.2 PCSK9 Structure and Function

(i) *Biosynthesis*: Human (h) PCSK9 is a 692 amino acid long glycoprotein that acts in the secretory pathway to post-translationally reroute LDLR from the endosome to the lysosome for degradation. Like other PCSKs, it contains a signal peptide, cleaved in the ER, a prodomain that acts like a chaperone to guide PCSK9 through the secretory pathway, a catalytic domain with the catalytic triad of residues similar to that of subtilisin and a C-terminal domain that is essential for LDLR degradation (36). However its C-terminal

segment contains a cysteine/histidine rich domain (CHRD) that is specific to the proteinase K subfamily (36). The function of the C-terminal domain of PCSK9 still remains to be elucidated (51). Recently it has been shown that PCSK9 lacking the C-terminal domain is not able to degrade LDLR, suggesting that this region is important in either attracting accessory proteins or it may contain a lysosomal signal to re-route LDLR (52). PCSK9 lacks the P domain unlike other eukaryotic members of the PCSK family. This region is believed to regulate protease activity and is required for proper folding of the protein (43). Although PCSK9 shares many similarities with the other PCSK family members, its structure is unique and the function of its specific domains and how they affect LDLR degradation still remains to be elucidated.

The prodomain of PCSK9 is cleaved in the ER, releasing the mature enzyme; however, unlike the other members of the PCSK family, the prodomain of PCSK9 remains non-covalently attached to the catalytic domain covering the catalytic triad of residues (46, 47). So far all attempts to dissociate the two using non-denaturing conditions have failed and its proteolytic activity has only been demonstrated *in vitro* (53, 54) recently with the report by Kourimate et al (54). The site of cleavage of PCSK9 namely VFAQ↓SIP also differs from other PCSKs in that it is not after a basic residue and the cleavage does not require calcium (21, 55). So far no physiological substrates of PCSK9 have been identified.

(ii) Post-translational modifications of PCSK9

PCSK9 also undergoes post-translational modifications that may alter its function in the degradation of LDLR. In the golgi apparatus, an Asparagine residue at position 533 is glycosylated, and a Tyrosine at position 38 is sulfated (21, 56). To date, the function of the sulfation remains unknown; however it does not appear to have any role in degrading LDLR

or in altering cholesterol homeostasis. Asn533 is the only site of N-glycosylation in PCSK9 and is not required for its degradation of LDLR and its retention or secretion from the ER; however, its function remains unclear (46, 57). Recently two phosphorylations were discovered in PCSK9, one in the prodomain, and the other in the C-terminal domain (58). Interestingly both phosphorylations have been found in regions that have not yet been crystallized, due to a lack of electron density in the region (47, 58, 59). It has been speculated that phosphorylations in PCSK9 may alter LDLR degradation but so far no data or study has been presented to support this claim. This forms an important objective of this study. The structural difference between PCSK9 and the other PCSKs link it closely to the bacteria Proteinase K, which may explain in part its specificity in binding and re-routing LDLR for degradation.

1.2.3 Protease Activity

All other PCSKs exhibit protease activity, in cleaving substrates which result in the maturation of inactive precursor proteins such as pro-growth factors, neuropeptides, pro-hormones, surface proteins, cytokines, transcription factors as well as viral glycoproteins and bacterial toxins (36, 60). To date the protease activity of PCSK9 in relation to its physiological protein substrates has not been demonstrated. Elucidating the protease activity of PCSK9 has been increasingly difficult since the prodomain of PCSK9 remains strongly attached after secretion.

Once the crystal structure of PCSK9 was elucidated, it was observed that the prodomain remains attached to PCSK9 and covers the catalytic triad of residues blocking potential substrates (47). PCSK9 still remains functional in binding to LDLR and routing the

complex to the lysosome for degradation through a still unknown mechanism, regardless of catalytic activity (48, 49). So far the only known substrate of PCSK9 is itself, since the auto catalytic cleavage of PCSK9 is required for its secretion and proper folding (61). For these reasons, the catalytic activity of PCSK9 was not shown until recently, when PCSK9 present in the human hepatic cell line, HepG2, and in recombinant form, was able to cleave a small intra-molecularly quenched fluorogenic peptide containing the autocatalytic cleavage site of PCSK9 (54). In order to fully understand PCSK9 and to inhibit its activity in degrading LDLR, the elucidation of any activity and the determination of other substrates will be important.

1.2.4 PCSK9 binding partners

Currently, PCSK9 has two known binding partners; LDLR that is responsible for clearing LDL-C from the blood. PCSK9 binds to LDLR through the epidermal growth factor domains (EGF) domain and promotes its degradation (62, 63). The other partner is Annexin-A2 which binds to the C-terminal domain of PCSK9 and decreases the degradation of LDLR as confirmed by co-expression studies in cell lines (52). PCSK9 and LDLR are regulated transcriptionally through the same pathway. When cholesterol levels are low the sterol regulatory element binding protein-2 (SREBP-2) becomes activated, increasing the transcription of both PCSK9 and LDLR (64). LDLR has five characteristic structural domains: the ligand binding domains 1-7, the EGF-A, B and C domains, a β propeller domain, an O-linked sugar domain, as well as a C-terminal cytoplasmic tail (65, 66). LDLR undergoes a conformational change when there is a change in pH, which is believed to allow the release of LDL-C into the lysosome and recycle LDLR to the cell surface. At a neutral

pH, similar to the cell surface, LDLR adopts a linear structure where it can bind LDL-C. When the pH is lowered to ~5.5, similar to that found in the lysosome, LDLR undergoes a conformational change adopting a hairpin structure, which is believed to allow the release of LDL-C into the lysosome, and be recycled back to the cell surface (51). The binding between PCSK9 and LDLR occurs between the N-terminal portion of the EGFA domain and residues 367-381 of PCSK9 (67, 68). The binding groove of PCSK9 in the crystal structure has been shown to be neutral, unlike other PCSKs that are primarily negatively charged (46,68). The binding is very specific; PCSK9 does not bind or degrade other close family members of LDLR.

The interaction of PCSK9 with Annexin A2 was recently established by Mayer et al (52) who found that the binding of PCSK9 with Annexin A2 occurs at the C-terminal end of PCSK9, namely the CHRD domain, a region whose function has not yet been fully elucidated. The CHRD domain, like the prodomain, is not directly involved in PCSK9: LDLR binding as indicated in the crystal structure (68). However, the CHRD domain is believed to provide structural support for PCSK9 as it binds with the EGFA domain of LDLR through its catalytic domain (46, 68). The binding between Annexin A2 and PCSK9 decreases PCSK9 mediated degradation of LDLR, through an unknown mechanism (52). The discovery of Annexin A2 and its ability to act as an endogenous inhibitor is the first report of another binding partner (52).

1.2.5 Binding of PCSK9 with LDLR

The mechanism by which PCSK9 recognizes and degrades LDLR is beginning to be slowly understood, due in part to the co-crystallization of PCSK9 and LDLR (69) and the

discovery of an intracellular route (70, 71). PCSK9 binds LDLR by two possible routes, one extracellular, where PCSK9 exits the cell and binds LDLR on the cell surface and the second an intracellular route, where PCSK9 enters the endosome directly from the golgi and binds LDLR re-routing it to the lysosome (48, 49, 71). Through co-immunoprecipitation, co-crystallization and co-expression studies with PCSK9, and LDLR, the localization of the PCSK9 mediated degradation of LDLR was determined to be between the EGFA domain of LDLR and the catalytic region of PCSK9 (50). Although the N-terminal part of PCSK9 from amino acids 1-454 could not degrade LDLR, it was shown that this region binds to the EGFA domain of LDLR, whereas the C-terminal domain of PCSK9 does not interact with LDLR (51). Although the binding between LDLR and PCSK9 occurs through the catalytic domain of PCSK9 other domains of PCSK9 including the pro and C-terminal may be important for LDLR degradation. It has been shown that the lack of the N-terminal 52 amino acids of PCSK9 increases the binding between the two proteins (68) and PCSK9 lacking the C-terminal domain will not degrade LDLR (36). To date the detailed mechanism of how PCSK9 binds to LDLR and routes it for degradation remains to be elucidated.

The primary route for LDLR degradation by PCSK-9 occurs on the cell surface before internalization (72, 50). The binding between PCSK9 and LDLR leads to their co-localization to the lysosome, where the binding strength is enhanced by about 150 fold in an acidic environment with a pH of ~ 5.2 (53, 50) and that this binding is also calcium dependent (50). Catalytic activity is not required for PCSK9's binding with and its degradation of LDLR (61, 72), even though the autocatalytic cleavage of PCSK9 is required for PCSK9 secretion and proper folding (61).

PCSK9 binding to LDLR is specific and requires multiple domains on both LDLR and PCSK9, even though the domains do not participate in the binding. PCSK9 continues to bind to LDLR even if LDLR has less than three of its seven-ligand binding domains, or is lacking a β propeller domain; however it will not degrade LDLR (51). The binding of PCSK9 to LDLR has been concentrated to a small segment in both PCSK9 and LDLR; nevertheless, mutations in non-binding regions of PCSK9 have profound effects on the level of LDLR. For instance the R⁴⁶L loss of function mutation in the prodomain and the H⁵⁵³R gain of function mutation in the CHRD domain (42, 73, 74). Neither the prodomain nor the CHRD domain are involved in the binding between LDLR and PCSK9 as shown in the crystal structure of PCSK9 and the EGFA domain (68), indicating that there may be more players in this interaction than was previously thought.

The mechanism of LDLR degradation by PCSK9 is complex since PCSK9 can bind LDLR without leading to its degradation. PCSK9 may contain a lysosomal sequence that causes the rerouting of LDLR to the lysosome, or it may be disrupting the conformation of LDLR which routes it back to the cell surface (50, 51, 51, 75). It is believed that if PCSK9 binds to LDLR this will no longer allow LDLR to form the hairpin structure, thereby rerouting the complex to the lysosome for degradation (51, 76). The effect of PCSK9 on LDLR is through a post-translational mechanism, PCSK9 over expression will reduce the protein levels of LDLR without affecting its mRNA levels (77), and this over-expression of PCSK9 will primarily affect the glycosylated mature form of LDLR (63). The mechanism through which PCSK9 re-routes LDLR to the lysosome for degradation is still poorly understood, and the discovery of the mechanism and inhibition of any step will be crucial in developing any future therapeutics for ADH (78, 79).

1.2.6 PCSK9 knockout mouse model study

Complete knock out

Much of the importance of PCSK9 function and its impact on normal physiology has been revealed by the study of PCSK9 knock out mouse models. Horton et al. (81) reported the first total PCSK9 mouse knockout model in 2005 with the expectation of increasing the level of LDLR and thereby reducing plasma LDL cholesterol. Not only was there a 50% reduction in plasma cholesterol, there were no other phenotypic changes between the wild type mice and the knockout mice (80). This complete knockout mouse model confirmed PCSK9's function for degradation of LDLR and that in its absence there is a greater amount of LDLR and a faster clearance of plasma cholesterol. Thus eliminating or down regulating PCSK9 produced an effect similar to that observed with statin compounds, approved drugs for treating hypercholesterolemia. Statins alone are not as effective as they could be since they induce the transcription of not only LDLR but PCSK9 as well, which reduces LDLR (81). Inhibitors of PCSK9's functional activity in conjunction with statins will have the most beneficial effects on plasma cholesterol levels by increasing the transcription of LDLR without increasing its degradation by PCSK9. This may be the best option for treating high cholesterol and associated cardiovascular disease.

Conditional knock out in tissue-specific manner

The majority of circulating PCSK9 is synthesized in the liver (36, 43), and this contributes to 2/3rd of the circulating cholesterol that is not cleared as a result of increased degradation of LDLR (82). A knockout of PCSK9 in the liver generated a 27% decrease in

total plasma cholesterol, compared to a 42% decrease in the total knockout and had 2-3 times more LDLR than wild type mice (82). By creating inhibitors that target PCSK9 in the liver, there could be an increase in LDLR protein expression, leading to a drop in plasma cholesterol, without affecting the normal physiology of the animal.

Although circulating PCSK9 in the plasma targets mainly LDLR present in the liver, there is still a significant level of both PCSK9 and LDLR present endogenously in the kidney. To date nothing has been published in the literature about conditional knock outs of PCSK9 in the kidney, although there have been successful over-expressions of PCSK9 in the kidney which showed that PCSK9 will still reduce the amount of LDLR protein in the liver preferentially (83). Currently researchers are in the process of creating knockdowns of PCSK9 in the kidneys, gut and intestine to further understand the function of PCSK9 in these organs.

1.2.7 Mutations in PCSK9 and therapeutic benefits or risks

A large variety of PCSK9 mutations in humans have been reported (**Table 3**). Other than PCSK1, no other PCSKs had been shown to have natural mutations. Since its discovery in 2003, PCSK9 has been shown to exhibit numerous natural mutations that either increase LDLR degradation, which are termed as “gain of function mutations”, or those that decrease its ability to degrade LDLR are termed as “loss of function mutations” (62, 63). The gain of function mutations will increase the degradation of LDLR leading to decreased clearance of plasma LDL-C from the blood. This condition is termed hypercholesterolemia. This in turn can promote a higher risk of cardiovascular disease (84). The gain of function mutations, act either by increasing PCSK9s function on LDLR or by providing a new activity (77).

Table 3: 36 Known PCSK9 mutations, amino acids involved, domain and the defect.

Amino acid variation	Protein domain	Functional defect	References
p.L15_L16ins2L	Signal peptide	High total and LDL cholesterol	Abifadel et al 2008
p.V ⁴ I	Prodomain		Miyake et al. 2008
p.E ³² K	Prodomain		Miyake et al. 2008
p.R ⁴⁶ L	Prodomain	Increase in LDLR	Abifadel et al. 2003 Cohen et al. 2006
p.A ⁵³ V	Prodomain	Increase in LDLR	Kotowski et al. 2006
p.E ⁵⁴ A	Prodomain		Miyake et al. 2008
p.E ⁵⁷ K	Prodomain	Increase in LDLR	Kotowski et al. 2006
p.A ⁶⁸ T	Prodomain		Miyake et al. 2008
p.T ⁷⁷ I	Prodomain		Fasano et al. 2007
p.R ⁹³ C	Prodomain		Miyake et al. 2008
p.R ⁹⁷ del	Prodomain	Decrease in PCSK9	Zhao et al. 2006
p.R ¹⁰⁴ C	Prodomain		Miyake et al. 2008
p.V ¹¹⁴ A	Prodomain		Fasano et al. 2007
p.N ¹⁵⁷ K	Catalytic	Increased LDLR	Berge et al. 2006 Cameron et al. 2006
p.A ¹⁶⁸ E	Catalytic		Homer et al. 2007
p.Q ²¹⁹ E	Catalytic		Miyake et al. 2008
p.G ²³⁶ S	Catalytic	Decrease in PCSK9	Cameron et al. 2008
p.R ²³⁷ W	Catalytic	Increase in LDLR	Benjannet et al. 2004 Berge et al. 2006
p.A ²³⁹ D	Catalytic		Miyake et al. 2008
p.A ²⁴⁵ T	Catalytic	No LDL internalization	Cameron et al. 2008
p.L ²⁵³ F	Catalytic	Decrease in PCSK9	Kotowski et al. 2006 Nassouri et al. 2007
p.R ²⁷² Q	Catalytic	No LDL internalization	Cameron et al. 2008
p.N ³⁵⁴ I	Catalytic	Decreased PCSK9, no autocatalytic cleavage	Cameron et al. 2008
p.H ³⁹¹ N	Catalytic	Decrease in PCSK9, no autocatalytic cleavage	Kotowski et al. 2006
p.H ⁴¹⁷ Q	Catalytic		Kotowski et al. 2006
p.I ⁴²⁴ V	Catalytic		Miyake et al. 2008
p.N ⁴²⁵ S	Catalytic	Increase in PCSK9	Pisciotta et al. 2006
p.G ⁴⁵² D	CHRD		Miyake et al. 2008
p.R ⁴⁶⁹ W	CHRD		Allard et al. 2005 Kotowski
p.I ⁴⁷⁴ V	C-terminal		Kotowski et al. 2006 Shioji et al. 2004
p.E ⁴⁸² G	C-terminal		Kotowski et al. 2006
p.R ⁴⁹⁶ Q	C-terminal	LDLR unchanged	Cameron et al. 2006
p.A514T	C-terminal		Miyake et al. 2008
p.A ⁵²² T	C-terminal		Fasano et al. 2007
p.H ⁵⁵³ R	C-terminal	Decrease in PCSK9: LDLR interaction	Kotowski et al. 2006 Nassouri et al. 2007
p.Q ⁵⁵⁴ E	C-terminal	Decrease in PCSK9: LDLR interaction	Fasano et al. 2007
p.P ⁶¹⁶ L	C-terminal		Rs28362277 Kotowski et al. 2006

The loss of function mutations, have the opposite role resulting in a high level of LDLR, they reduce the degrading effect of PCSK9 on LDLR, allowing LDLR to be recycled back to the cell surface instead of being routed to the lysosome for degradation (85). As a result these mutations are considered as beneficial and protect the individuals from cardiovascular disease (57, 86). This condition is referred to as hypocholesterolemia. In general both types of mutations have been described in various domains of PCSK9's protein sequence. Thus these were found in the signal peptide domain, prodomain, the catalytic domain as well as in the C-terminal domain and to date the reason for this widespread location as well as their mechanism of action on LDLR degradation is still largely unknown (86).

The “gain of function mutations” are associated with a greater risk of familial hypercholesterolemia, a disorder characterized by an elevated level of LDL-C (87). Of particular note, the most extreme gain of function mutation in PCSK9 D³⁷⁴Y has been found in 3% of the population with ADH (88). Loss of function mutations were first characterized in African Americans with low levels of circulating LDL-C, where these mutations resulted in a 30% decrease in LDL-C, a truly protective effect against hypercholesterolemia (89, 90). One of the loss of function mutations R⁴⁶L, a mutation in the prodomain of PCSK9, resulted in a 42% decrease in LDL-C (91). Patients who exhibit loss of function mutations in PCSK9 are also more sensitive to treatments with HMG-CoA reductase inhibitors, such as statins (92). There are a few mutations that were found to be silent, having no effect on the functional activity of PCSK9. The elucidation of PCSK9's role in degrading LDLR and the discovery of PCSK9 inhibitors will play a key role in designing therapeutic agents for the treatment of hypercholesterolemia and high blood pressure.

1.2.8 Currently available treatments for hypercholesterolemia

PCSK9 is now recognized as one of the major players in the future of pharmaceuticals for the treatment of hypercholesterolemia since it enhances the degradation of LDLR by rerouting it to the lysosomes (21, 63). Currently, cholesterol lowering HMG-CoA reductase inhibitors known as statins are the most common drugs used for the treatment of hypercholesterolemia (93). Statins lower the level of LDL-C by increasing the transcription of LDLR via the blockade of HMG-CoA reductase. However, many individuals do not exhibit a strong enough response to statin treatment alone due to various reasons (93). PCSK9 and LDLR are coordinately regulated by SREBP2, an important transcription factor linked to cholesterol synthesis and metabolism (64). When lowering the level of circulating cholesterol, statins activate the SREBP-2 pathway that increases not only LDLR expression but also the expression of PCSK9 (94).

Attention may be devoted to the observed fact that stimulus that leads to higher levels of LDLR including, statins, will also co-coordinately increase the level of PCSK9 circulating in the body (95). Studies have shown that PCSK9 reduces the efficiency of statins by blocking their effects (96). Thus by finding specific inhibitors that can block PCSK9's binding to LDLR, there will be a lower LDL plasma cholesterol level and a cholesterol state better than that observed with statins alone (97).

1.2.9 Future direction of treatments for hypercholesterolemia

Numerous clinical, biochemical, knock out, knock down as well as over expression studies have recognized PCSK9's involvement in cholesterol homeostasis. It is now reasonable to understand why the development of PCSK9 functional inhibitors and

regulators has become so attractive and the primary objective to many researchers in the field (98, 99). Several naturally occurring activators, inhibitors and binding partner proteins have been described in the literature for a number of PCSKs, but so far the full length Annexin A2 is the only known physiological regulator of PCSK9 *in vivo* (99).

The clear link between PCSK9 in cholesterol homeostasis and its binding to LDLR has made it an interesting target for the development of therapeutic agents for inhibition. Any compound or molecule capable of blocking this binding may be potentially useful for lowering plasma cholesterol (100). Currently there are three primary approaches in developing inhibitors of PCSK9. These strategies include (i) the delivery of single stranded anti-sense DNA which will degrade PCSK9 *in vivo* and was shown to reduce LDL-C in mice (101), (ii) the delivery of siRNAs that will inhibit the mRNA of PCSK9 *in vivo* and lower LDL-C in both rats and mice (102), and lastly (iii) the development of small molecule peptide inhibitors, as already observed with the development of the EGF-A peptide that decreases the degradation of LDLR *in vitro* (50, 103). Inhibitors of PCSK9's function could potentially increase the clearance of LDL-C if properly delivered to the organelles.

Small peptide inhibitors are normally favored as drug candidates since they are generally more stable than the full-length protein counterpart and can be chemically modified to make more bioavailable and membrane transportable (95). For these reasons, there is a growing interest in the design and synthesis of small peptide inhibitors that can alter the function of PCSK9 in degrading LDLR.

2.0 Hypothesis, Objectives and Rationale of the research project

Since the discovery of PCSK9, its inhibition has been studied as a mode for controlling LDLR, however to date there is still no effective PCSK9 inhibitor on the market. Only a limited number of potential inhibitors of PCSK9 have been studied in the literature. These are based on siRNA, antibodies and a small peptide. These methods suffer from a lack of selectivity, stability and potency. Therefore, there is a need for stronger and more selective PCSK9 inhibitors. The development of PCSK9 inhibitors based on its own inhibitory prodomain has not been pursued. We hypothesize that peptide inhibitors will decrease the PCSK9 mediated degradation of LDLR.

2.1 Objectives

The objectives of this study that formed the basis of the MSc research were:

Aim 1: To develop small molecule peptide inhibitors of PCSK9 based on its own inhibitory prodomain sequence,

Aim 2: To identify specific amino acids in the prodomain of PCSK9 required for LDLR regulation.

We anticipate that specific peptides from prodomain of PCSK9 are involved in regulating its function in degrading LDLR and in turn LDL-C levels. In addition, the prodomain of PCSK9 and its peptides may produce its effects on LDLR by binding to a partner protein and organizing the interaction.

3.0 Materials and Methods

3.1 Materials

Chemicals

All coupling and other agents for peptide synthesis such as HATU (O-7-azabenzotriazol-1-yl-1,1,3,3-tetramethyluronium hexafluorophosphate), DIEA (N, N'-Diisopropyl ethyl amine), Piperidine as well as organic solvents such as DMF (NN'-dimethyl formamide), NMP (N-methyl pyrrolidone) were purchased from Applied Biosystems (Framingham, Mass, USA), Aldrich-Sigma Chemical company (St. Louis, Missouri, USA) or Chem Impex Internaional (USA). Trofluoroacetic (TFA) and acetonitrile needed for RP-HPLC were bought from Aldrich-Sigma Chemical Company (St. Louis, Missouri, USA). All amino acids, coupling reagents namely HATU, DIEA, 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). The buffers (PBS and RIPA), and all reagents for gel electrophoresis and western blot analysis were purchased from Invitrogen. Chemiluminescence reagents (PerkinElmer LAS Inc USA) were used for detection of immuno reactive bands. Images were then captured using Kodak X-OMAT Blue autoradiography film (PerkinElmer LAS Inc., USA).

Recombinant PCSK9

Purified hexa-histidine-tagged recombinant hPCSK9 with S²¹⁸R mutation was obtained as a gift from Dr. Robert Day, Sherbrooke University, Que, Canada. A non-viral large scale

production of recPCSK9 was done using *Drosophila* having small plasmid DNA vectors for rapid transient expression in macrophage-like S2 cells developed in Dr. Day's lab (104). This His-tag recPCSK9 contained R²¹⁸S mutation in order to protect its cleavage by endogenous furin at **RFHR**²¹⁸↓QA (the two crucial Arg residues at P1 and P4 positions for furin recognition shown in bold character) site within the catalytic domain of PCSK9 which results in an increase in half life of PCSK9. His tag recPCSK9-R²¹⁸S variant was purified by using a combination of Nickel-chelating, FPLC and hydrophobic column chromatography (Robert Day, unpublished). The purity of the final eluted material was checked by both coomassie staining as well as by western blot analysis using PCSK9 antibody.

Synthesis of peptides

All PCSK9 prodomain derived peptides with their amino acid sequences and precise locations are shown in **Table 4**. All peptides were synthesized 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 on an Fmoc-PAL-PEG PS [Polyamino linker polyethylene glycol poly styrene with the actual chemical nomenclature being 5-(4-aminomethyl-3, 5-dimethoxy-phenoxy) valeric acid] linker. Following completion of synthesis, the peptides were cleaved off from the resin and at the same time fully deprotected by treatment with the deprotection cocktail called Reagent B

consisting of 90% TFA, 2.5% phenol, 5% water and 2.5% tri-isopropylsilane) for 3 hours at ambient temperature (105).

Peptides were then purified using reverse phase HPLC chromatography using a C₁₈ semi preparative and analytical columns. The gradient method and solvents used are as described in (104, 105). 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 peptides was followed by using a 2% increase in Solvent B/min gradient from initial 0-65% Solvent B. All major peaks were collected and analyzed by mass spectrometry for their identities.

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 ionisation time of flight) mass spectrum was recorded for each collected material by using the Voyager DE pro (PE Biosystems Inc, Framingham, Ma, USA) instrument using the GRAM software with 4 µl of each sample and 2 µl of the CHCA (α-cyano hydroxy cinnamic acid) matrix.

3.2 Methods

3.2.2 Bradford Protein assay

Bradford protein assay of various cell culture samples was performed by using the BioRad protein assay dye kit (BioRad). The assay was done to determine the amount of total amount of protein present in each sample. Sample fractions were diluted 1000 fold and the intensity of absorption at wavelength 595 nm was measured using a UV spectrophotometer. Triplicates of each sample and the control were used and an average value was used for each

experiment. A standard curve was generated by using various known amounts ranging from 0 µg-10 µg of a standard protein sample such as BSA (bovine serum albumin) then plotting the amount protein against the absorbance intensity.

3.2.3 Cell Culture.

HepG2 and Huh7 cells were grown in Dulbecco's modified Eagle's medium (DMEM) (Sigma Aldrich, Saint Louis, Missouri, USA) containing 10% FBS (fetal bovine serum) (Gansera International Inc, ON, Canada) and 0.6µl/ml of antibiotic gentamicine sulphate (GTM) (Invitrogen Corporation, ON Canada) under 5% CO₂ condition. Phenyl red indicator was added to the cell culture medium in order to detect any possible bacterial growth or cell death resulting from high growth of the cells or toxicity, which would be indicated by color change as a result of change in pH of the medium. The cells were grown to ~80% confluency when the medium was removed. Serum free culture medium was added and the cells were further grown for another 24 hrs before the addition of peptides in aqueous solution.

3.2.4 SDS-PAGE and immunoblotting

Tris glycine gel with 8% resolving and 4% stacking phase was used for SDS PAGE. Denaturation of the protein samples were achieved by heating to 95⁰C for 10 min with sample loading buffer contain 1% SDS, 10% glycerol, 10 mM Tris-Cl and 5% of reducing agent dithiothreitol (DTT) from Sigma Aldrich at pH 6.8. Following separation with SDS-PAGE the proteins were transferred to a nitrocellulose membrane for 3 hrs at 100 V (Millipore Corporation, Bedford USA) for immunological detection. The membranes were

then blocked for 1 h using 5% skim milk and incubated overnight with the primary antibody, anti-LDLR at a dilution of 1:250 (Fitzgerald, USA), anti-PCSK9 at a dilution of 1:500 (Cayman, USA) and anti-transferrin receptor at a dilution of 1:2000 (Invitrogen, USA) the membranes were then incubated with the secondary antibody for 1 h, with anti-rabbit 1:1000 for detection of PCSK9 and LDLR (Santa Cruz, USA), or anti-mouse at a dilution of 1:1000 for Transferrin (Santa Cruz, USA). Chemiluminescence reagents (PerkinElmer LAS Inc USA) were used for detection. Images were then captured using Kodak autoradiography film (PerkinElmer LAS Inc., USA).

3.2.5 Western blotting

Cells were washed with 1X PBS (phosphate buffer saline) and lysed using RIPA (Radio Immuno Precipitation Assay) buffer (50 mM Tris/HCl, pH 8.0. 1% Nonidet P40, 150 mM NaCl, 0.5% sodium deoxycholate) in the presence of a complete Protease Inhibitor cocktail (Roche Applied Science, Laval, QC, Canada). Proteins were then separated on an (8%) reducing SDS gel and transferred to a nitrocellulose membrane. Membranes were then blocked for 1 hr in TBS (Tris Buffered Saline, 50 mM Tris.HCl, pH 7.4, 150 mM NaCl)-Tween containing 5% milk. LDLR was detected using anti-LDLR (1:250), anti-PCSK9 (1:5000) and anti-transferrin receptor (1:2000).

3.2.6 LDLR protein assay

LDLR content in the absence and presence of various PCSK9 peptides was determined through western blot analysis using both HepG2 and Huh7 cell lysates. The cells were treated with serum free media containing the peptide at the concentration indicated for 4 hr

or the time indicated. Total cell lysate with volume adjusted to that containing 50 µg was loaded on each lane and the samples were run on a reducing 8% SDS-PAGE and transferred to a nitrocellulose membrane.

3.2.6.2 Concentration effect of peptides

Approximately 3×10^5 cells were plated in P6 wells (60 mm diameter) and the cells were grown to approximately 80% confluency (24-48 hr). The media containing FBS was removed and fresh serum free media was added for 24 hr as described above. The peptides were then added at concentrations of 0, 1, 2, 2.5, 5, and 10 µg in 1 ml of medium. Following 4 hr incubation the medium was removed and the cells were lysed with RIPA buffer. Bradford analysis was done on all samples in triplicate and sample containing ~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 LDLR, PCSK9 and transferrin receptor in the lysates, and the media. Cytotoxicity analysis of each sample medium was also performed.

3.2.6.3 Time course effect of peptides

Approximately 3×10^5 cells were plated in P6 wells (60 mm diameter) and the cells were grown to approximately 80% confluency (24-48 hr). The media containing FBS was removed and fresh serum free media was added for 24 hr. The peptides were then added at a concentration 2.5 µg/ml. Following 0, 2, 4, or 24 hr incubation, the media was removed and the cells were lysed with RIPA buffer. Bradford analysis was done on all samples in triplicate and again the sample containing 50 µg of total protein was loaded on each lane and then separated on a gel electrophoresis and finally transferred for western blotting. Blotting

was done for LDLR, PCSK9 and transferrin receptor in the lysate, and the media was analyzed for cytotoxicity.

3.2.6.4 Effects of peptides on LDLR level in the presence of recombinant PCSK9

Approximately 3×10^5 cells were plated in P6 wells (60 mm diameter) and the cells were grown to approximately 80% confluency (24-48 hr). The medium containing FBS was removed and fresh serum free media was added for 24 hr. Purified recombinant His₆-PCSK9 R²¹⁸S was added at a concentration of 0.5 μg/ml to all samples with the exception of the control. The peptides were then added at concentrations of 0, 1, 2, 2.5, 5, and 10 μg in 1 ml of culture medium. Following 4 hr incubation the media were removed and the cells were lysed with RIPA buffer. Bradford analysis was done on all samples in triplicate and ~50 μg protein containing samples were separated on a gel and transferred for western blotting. Blotting was done for LDLR, PCSK9 and transferrin receptor in the lysate, and the media was used for cytotoxicity analysis.

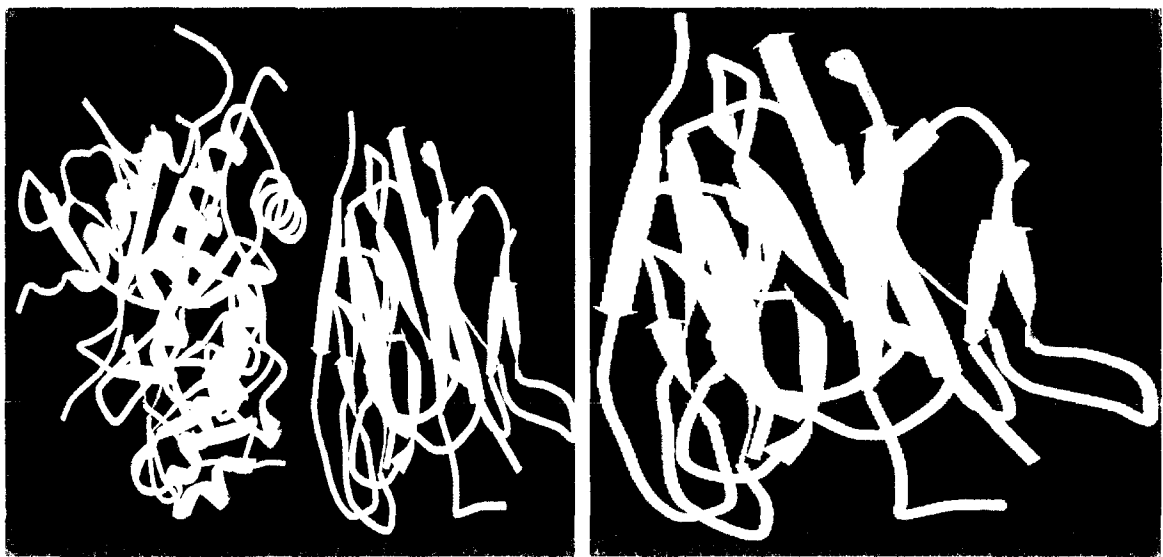
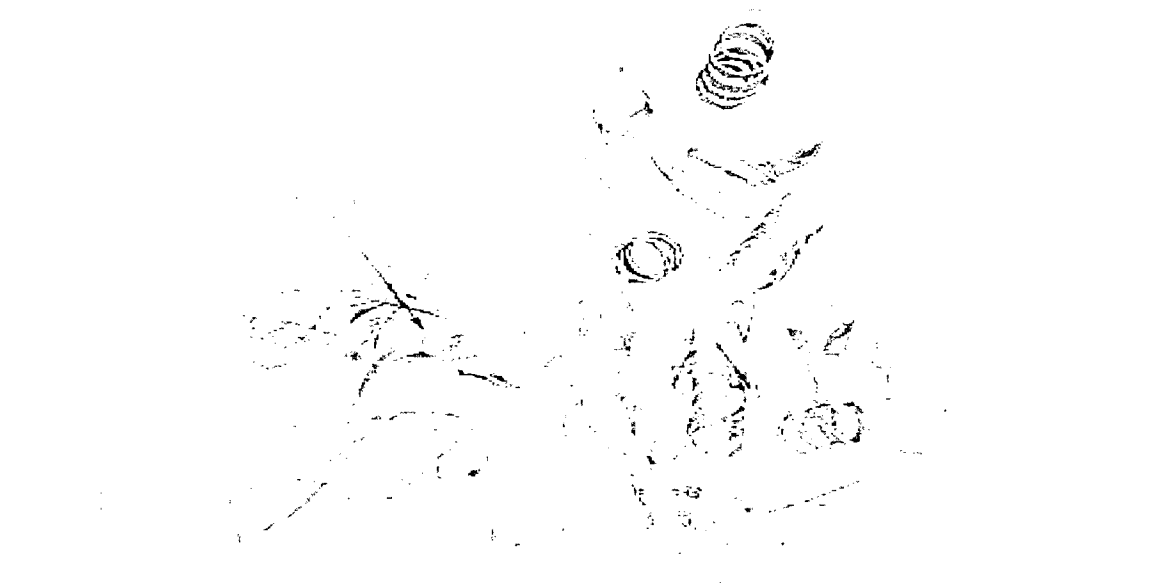
3.2.7 Circular Dichroism

For determination of the secondary structure of the peptides, circular dichroism (CD) studies were performed using the Jasco-810 spectropolarimeter and the spectra were analyzed using the Dichroweb software, available in web for registered users: <http://www.cryst.bbk.ac.uk/cdweb/html/home.html>. The spectra were recorded using a 0.1 mm thick quartz cell, for minimizing the background noise level and also for highest degree of precision and quality with 250 μl of solution. The spectra ranged from 185-240 nm, increasing every 0.1 nm at room temperature.

4.0 Results

Design of peptides. Recently, the crystal structure of PCSK9 was fully elucidated (47). It indicated an extremely stable and potent non-covalent association between the pro and catalytic domains of the protein thereby providing an explanation for the difficulty encountered while demonstrating its protease activity (61). The co-crystallization of PCSK9 and LDLR showed that the catalytic domain of PCSK9 interacts directly with the EGFA domain of LDLR and promotes its degradation (50). We became interested in the prodomain due to the strong affinity of the region towards the catalytic domain, the presence of several natural mutations and post-translational modifications. The rationale is that selected peptides from the PCSK9 prodomain, may exhibit a strong interaction with the region of the catalytic domain that makes physical contact with the EGFA domain of LDLR. These peptides are likely to affect the LDLR protein level either in a positive or negative manner. Such peptides may have useful therapeutic applications in cholesterol suppression. The crystal structure of PCSK9 (47) (**Figure 2**) showed the presence of unique secondary structures in the prodomain and a strong interaction with the catalytic domain of PCSK9. After the cleavage of the prodomain at VFAQ¹⁵²↓SIP, Q¹⁵² forms a hydrogen bond with the catalytic His²²⁶ and occupies the oxyanion hole between the amide nitrogens of catalytic Ser³⁸⁶ and Asn³¹⁷(46). The region occupying residues 61-152 forms five β -strand sheets covered by two α -helices (46), which are believed to be important for its interaction with the catalytic domain and preventing the exposure of the catalytic domain (47).

Figure 2 Structural model of PCSK9 relevant to Ser⁴⁶² (one important site of mutation Ser to Pro) (top). Ser⁴⁶² is positioned after the first beta-strand (pink) of the C-terminal domain (golden). The PCSK9 prodomain blocks the active site residues (magenta) of the catalytic domain (grey), b) the full length PCSK9 structure after cleavage. C) Shows the prodomain after cleavage. Adopted from Cunningham et al 2006, and Zhang et al 2007.



In the crystal structure of PCSK9, the first 24 amino acids covering the prodomain segment {31-54} were not detected and therefore its interaction, if any with the catalytic domain remains unknown. It might be possible that this region represents an additional binding site for LDLR if PCSK9 were to possess multiple binding sites for LDLR (103, 106). Likely the structure of PCSK9, has evolved to act as a complex, in that there will be no secondary cleavage, and the pro-, catalytic and C-terminal domain will act as a unit for their biological function (46, 107)

In our efforts to identify specific segments of the proregion of PCSK9 that may alter the prodomain's affinity towards LDLR, we designed peptides by splitting the entire prodomain of hPCSK9 into three separate segments based on net charge and hydrophobicity. These are (i) the N-terminal acidic, (ii) the mid-basic hydrophobic and (iii) the C-terminal acidic hydrophobic domains. The list of the peptides, two from each domain and their locations are shown in **Table 4**. The prodomain also contains sites for several natural mutations and post-translational modifications that are likely to exhibit regulatory effects on PCSK9's function. Owing to these reasons, the prodomain became the focus of our study. The prodomain peptides are expected to have regulatory effects on LDLR. We rationalize that the prodomain through its interaction with the catalytic domain may play a crucial role in regulating LDLR degradation. Alternatively it may also directly interact with LDLR if PCSK9 does possess multiple binding sites.

Table 4: List of Peptides synthesized for the present prodomain work

PRODOMAIN BASED	
C-terminal hydrophobic/acidic segments	Amino acid sequence
Pep1: hPCSK9 ¹²¹⁻¹⁵²	PGFLVKMSGD LLELALKLPH VDYIEEDSSV
Pep2: hPCSK9 ¹³¹⁻¹⁵²	FA LLELALKLPH VDYIEEDSSV FA
Mid basic/hydrophobic segment	
Pep3: hPCSK9 ⁹¹⁻¹²⁰	QSERTARRLQ AQAARRGYLT KILHVFHGLL
Pep4: hPCSK9 ⁶¹⁻⁹⁰	TTATFHRC AK DPWRLPGTYV VVLKEETHLS
N-terminal acidic/hydrophobic domain	
Pep5: hPCSK9 ³¹⁻⁴⁰	QEDEDGDYE
Pep6: hPCSK9 ³¹⁻⁶⁰	QEDEDGDYE ELVLALRSEE DGLAEAPEHG

4.1 PCSK9 prodomain peptides can regulate LDLR level

4.1.1 Effect on LDLR level in HepG2 cells

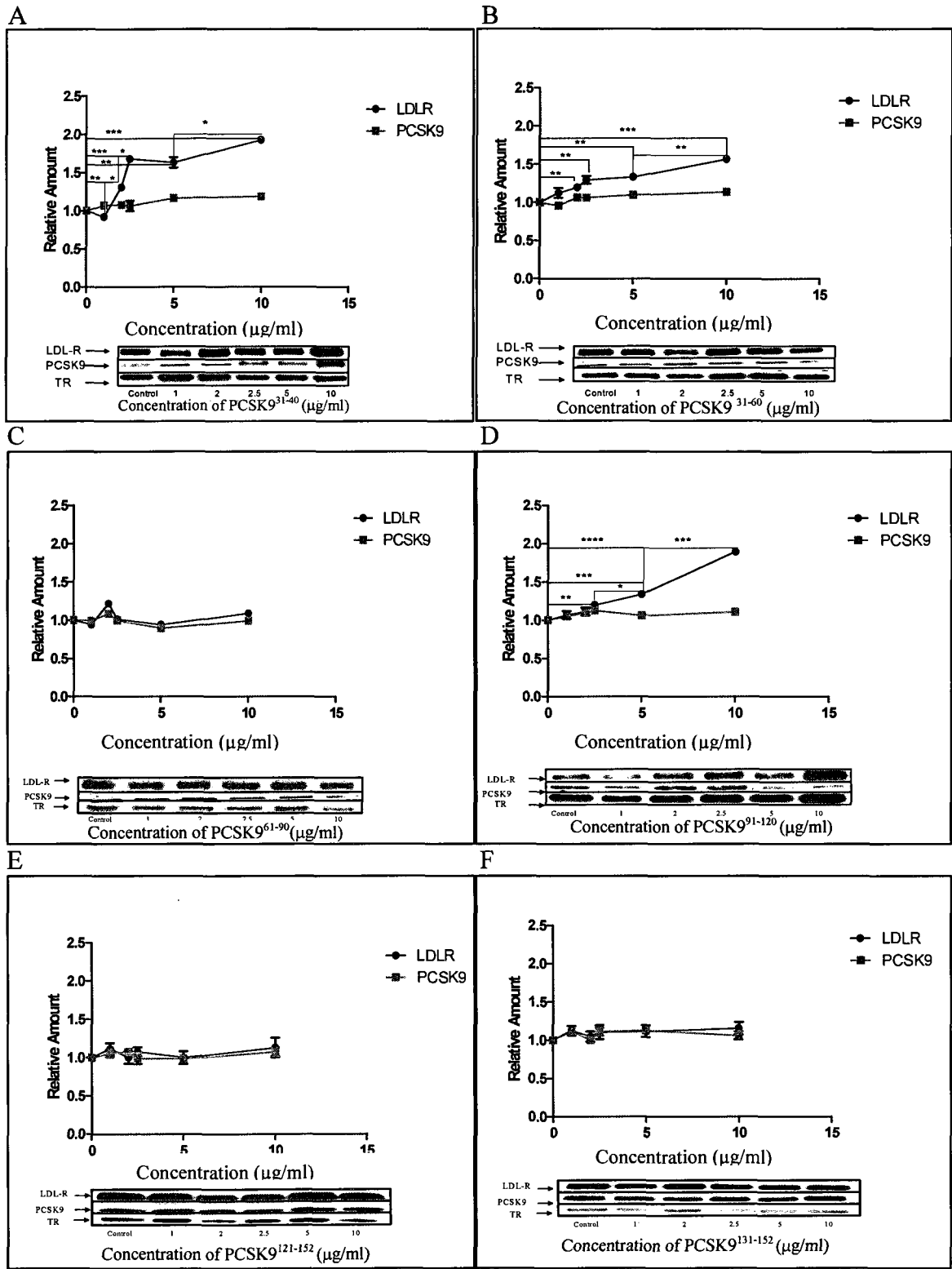
Previous work has reported that small peptide inhibitors of PCSK9 may alter PCSK9's function in degrading LDLR (103). Encouraged by the results we began our investigation by first synthesizing prodomain peptides of PCSK9 and then studying the possible effects on PCSK9 and LDLR protein levels in specific cell lines. Again our hypothesis is driven by the fact that the prodomain of PCSK9 acts as an endogenous inhibitor by remaining non-covalently attached to PCSK9 following its autocatalytic cleavage.

In order to assess the effects of PCSK9 prodomain peptides on the LDLR protein level, we used the human HepG2 hepatic cell line that endogenously expresses both PCSK9 and LDLR. The cells were grown for 24h in serum free medium and treated with each peptide in varying concentrations ranging from 0 - 10 µg/ml for a period of 4h as described in the Materials and Methods section. The cell lysates were examined for endogenous levels

of PCSK9 and LDLR by immunoblot analyses. The data obtained are shown in **Figs 3A-F** for all the peptides tested. The addition of either of the two N-terminal prodomain peptides: hPCSK9³¹⁻⁴⁰ and hPCSK9³¹⁻⁶⁰ produced a statistically significant increase in the LDLR protein level in a concentration dependent manner (**Fig 3A and 3B**) with no significant effect on PCSK9 protein content. At a concentration of 10 µg/ml of peptide, there was 1.6 to 2.0-fold increase in the LDLR protein level respectively. In contrast, the mid-basic hydrophobic hPCK9⁶¹⁻⁹⁰ peptide (**Figure 3C**) produced no significant changes on either the LDLR or the PCSK9 protein levels. However, the addition of another mid-basic hPCSK9⁹¹⁻¹²⁰ peptide to HepG2 cells produced an increase in the LDLR protein level, very similar to that observed with the N-terminal peptides. Like the other peptides, it produced a 40% increase in LDLR protein level at 5 µg/ml concentration and ~2.0 fold increase at a concentration of 10 µg/ml of peptide (**Fig 3D**). With the C-terminal basic peptides hPCSK9¹²¹⁻¹⁵² and hPCSK9¹³¹⁻¹⁵² both ending with the prodomain cleavage residue, no significant changes in the expression of LDLR and PCSK9 were noted (**Fig 3E and F**). All results are represented following standardization against the control protein transferrin receptor (TR) as calculated by densitometry.

Thus, the PCSK9 prodomain peptides can affect the LDLR protein level in the cell without affecting the PCSK9 protein level. In all cases there was no change in the PCSK9 protein level.

Figure 3. PCSK9 prodomain peptides and their effects on LDLR and PCSK9 protein contents. Increasing concentrations of PCSK9 prodomain peptides were used in HepG2 cells to study their effects on LDLR and PCSK9 protein contents. PCSK9 prodomain peptides were added to the serum free media after cells were grown to ~80% confluency after 24 hours. After another 4 hours of incubation, the whole cell lysates and media were collected and analyzed via immunoblotting for PCSK9 and LDLR levels as well as the level of transferrin receptor (TR) as an internal control (~50 µg protein loaded/lane). The plot is a representation of three separate experiments. A significance $p < 0.1$ is denoted with *, $p < 0.05$ ** and $p < 0.005$ ***. Results are plotted relative to the control TR. A) PCSK9³¹⁻⁴⁰, b) PCSK9³¹⁻⁶⁰, c) PCSK9⁶¹⁻⁹⁰, d) PCSK9⁹¹⁻¹²⁰, e) PCSK9¹²¹⁻¹⁵², f) PCSK9¹³¹⁻¹⁵²



4.1.1b Cell Survival

To determine whether the addition of the peptides is effecting the survival of the cells we measured cell survival with a cytotoxicity test. The media of HepG2 cells grown in the presence of various peptides were tested for cell survival using an LDH cytotoxicity assay (Biovision, USA) and compared with the control without the added peptide. No significant cell deaths were observed as determined by cell viability. In fact we found that over 90% of the cells survived compared to the control (**Figure 4A-F**).

4.1.2 Studies on LDLR regulation in Huh7 cells

To further examine the effects seen in HepG2 cells, we tested another cell line. Although liver cells similar to HepG2 cells, Huh7 cells have less endogenous PCSK9 than HepG2. This fact allowed us to see if there was a difference in the effect of the prodomain peptides in the presence of a lower amount of PCSK9, we expected to see similar results to the HepG2 cells and believe the effects observed are widespread.

Similar results were noted with the Huh7 cells as compared to the HepG2 cells based on immunoblotting data. For instance, PCSK9³¹⁻⁴⁰ peptide increased the LDLR protein level; there was approximately a 2-fold increase in LDLR at a concentration 10 µg/ml. Also, the peptide showed a significant increase in LDLR protein levels even at a low concentration of 2 µg/ml. There was no change seen in the PCSK9 protein level (**Figure 5A**). PCSK9³¹⁻⁶⁰ showed results similar to that observed with PCSK9³¹⁻⁴⁰, in that it increased the LDLR protein levels by approximately 60% compared to the control. There was a significant increase in LDLR protein levels with the addition of as little as 2 µg/ml of peptide and no observed change in PCSK9 protein level (**Figure 5B**).

Figure 4. Cell survival plot of HepG2 cells in the presence of increasing concentration of PCSK9 prodomain peptides. Increasing concentrations of PCSK9 prodomain peptides were added to the media of HepG2 cells and after 4 hours of incubation, the culture media and cell lysate were collected. 10 μ l of media was used to determine the level of lactate dehydrogenase released from the cells according to the kit instructions from Biovision. A) PCSK9³¹⁻⁴⁰, b) PCSK9³¹⁻⁶⁰, c) PCSK9⁶¹⁻⁹⁰, d) PCSK9⁹¹⁻¹²⁰, e) PCSK9¹²¹⁻¹⁵², f) PCSK9¹³¹⁻¹⁵²

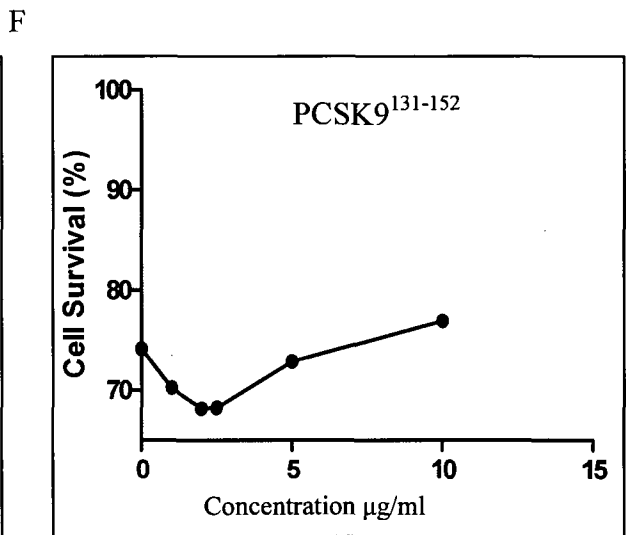
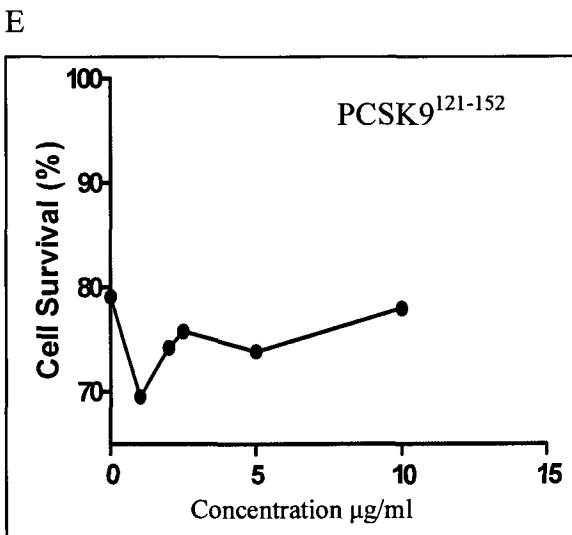
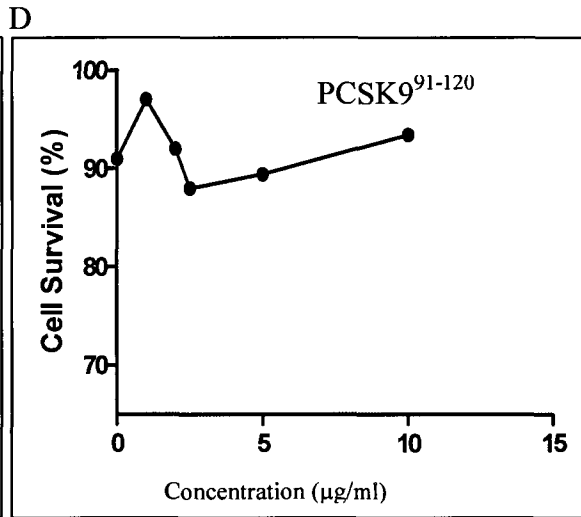
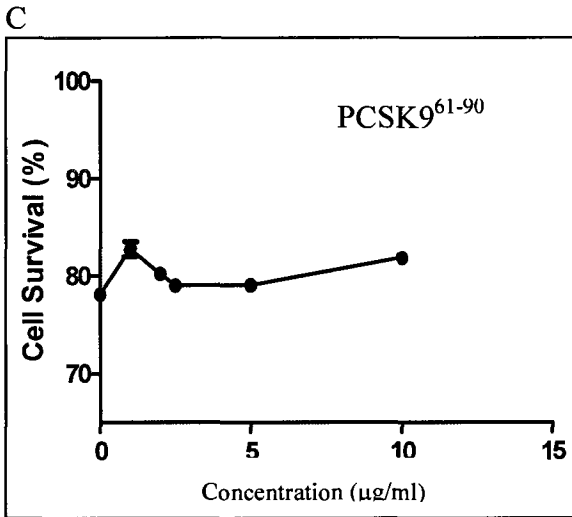
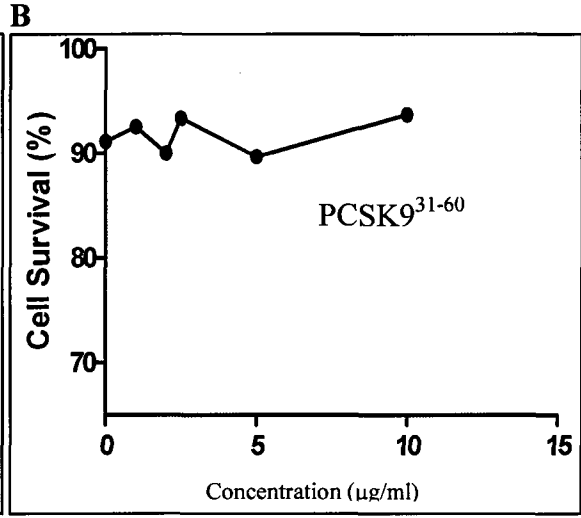
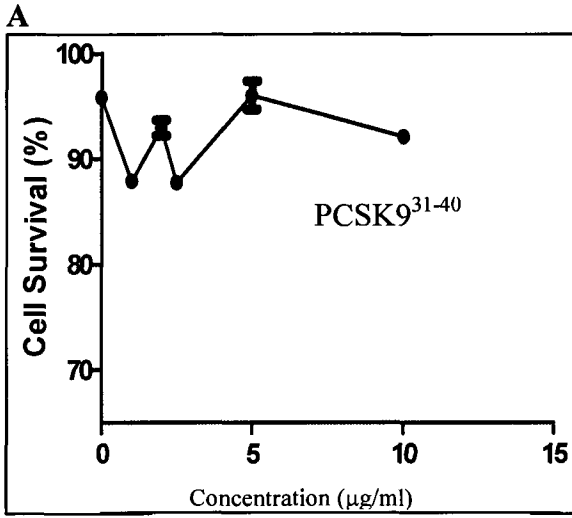
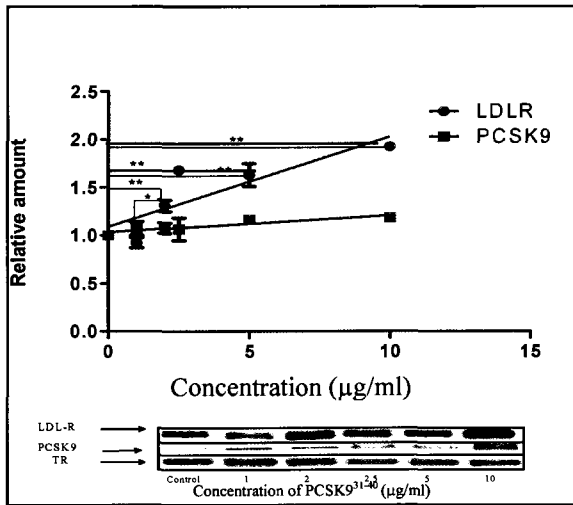
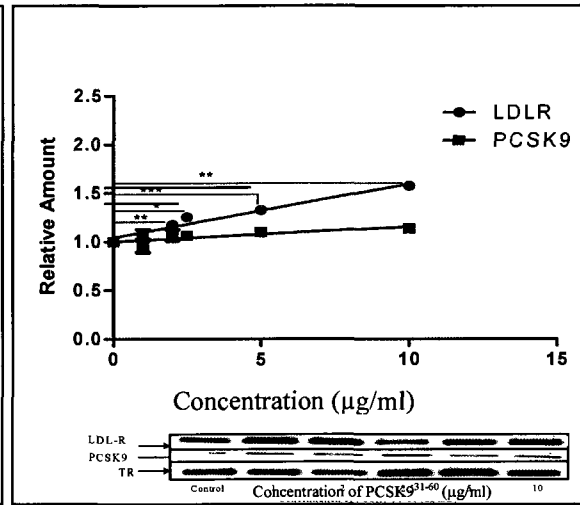


Figure 5. Effect of PCSK9 prodomain peptide on LDLR and PCSK9 levels in Huh7 under increasing concentrations in Huh7 cells. PCSK9 prodomain peptides were added to cells in serum free media after the cells attained ~80% cell confluency in nearly 24 hours. Following another 4 hours of incubation, the whole cell lysates and media were collected and only the lysates were analyzed via immunoblotting for PCSK9 and LDLR levels as well as transferrin receptor (TR) as an internal control (~50 µg protein loaded/lane). Each western blot shown above is a representation of three separate experiments. A significance $p < 0.1$ is denoted with *, $p < 0.05$ ** and $p < 0.005$ ***. Results are plotted relative to the control TR. A) PCSK9³¹⁻⁴⁰, b) PCSK9³¹⁻⁶⁰, c) PCSK9⁶¹⁻⁹⁰, d) PCSK9⁹¹⁻¹²⁰, e) PCSK9¹²¹⁻¹⁵², f) PCSK9¹³¹⁻¹⁵².

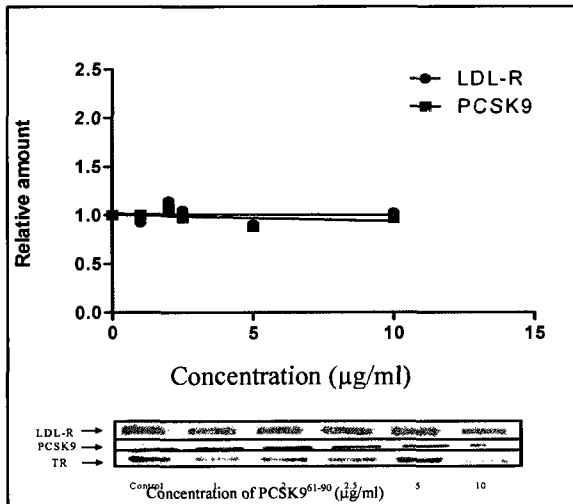
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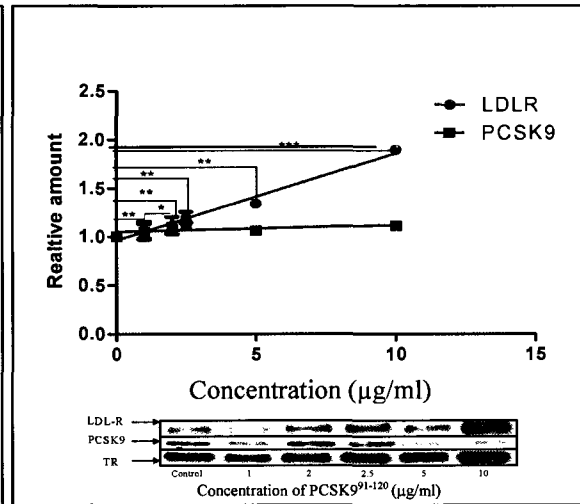
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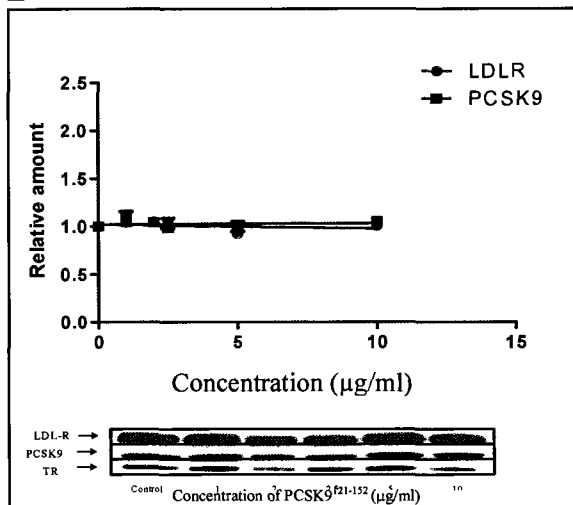
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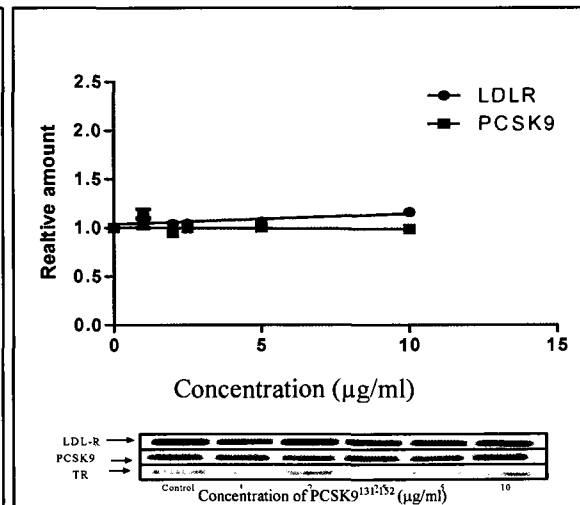
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E



F



The PCSK9⁹¹⁻¹²⁰ peptide synthesized from the mid-basic hydrophobic domain increased the LDLR protein levels by 2 fold. Also there was a significant increase in LDLR protein level even at 1 µg/ml with very little effect on the PCSK9 protein level (**Figure 5D**). For the remaining peptides, PCSK9^{61-90, 121-152} and ¹³¹⁻¹⁵² we observed no changes in either the PCSK9 or LDLR protein levels (**Figure 5CEF**).

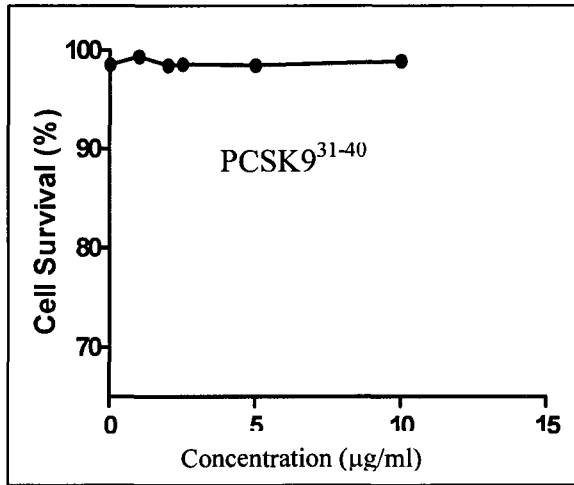
The results observed in Huh7 cells are similar to those observed in HepG2 cells. The PCSK9 prodomain peptides can affect the LDLR protein level in the cell without affecting the PCSK9 protein level.

4.1.2b Cell Survival

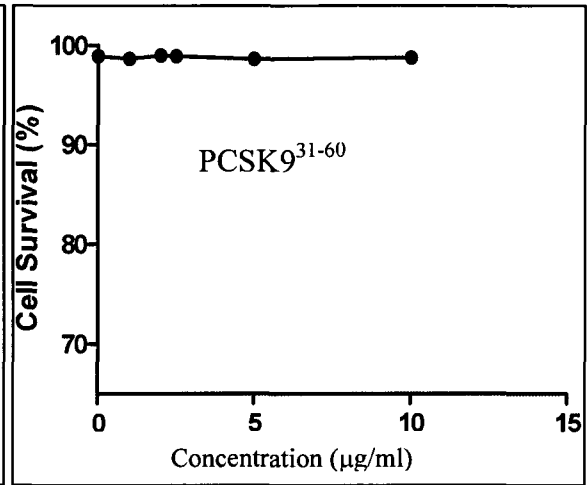
To determine whether the addition of the peptides is effecting the survival of the cells we measured cell survival with a cytotoxicity test. The media of HepG2 cells grown in the presence of various peptides were tested for cell survival using an LDH cytotoxicity assay (Biovision, USA) and compared with the control without the added peptide. No significant cell deaths were observed as determined by cell viability. In fact we found that over 90% of the cells survived compared to the control (**Figure 6**).

Figure 6. Cell survival plot of Huh7 cells in the presence of increasing concentration of PCSK9 prodomain peptides. Increasing concentrations of PCSK9 prodomain peptides were added to the serum free culture media of HepG2 cells as before and both the media and the cell lysate collected after 4 hours of incubation. 10 μ l of media was used to determine the level of lactate dehydrogenase released from the cells according to the kit instructions from Biovision. A) PCSK9³¹⁻⁴⁰, b) PCSK9³¹⁻⁶⁰, c) PCSK9⁹¹⁻¹²⁰

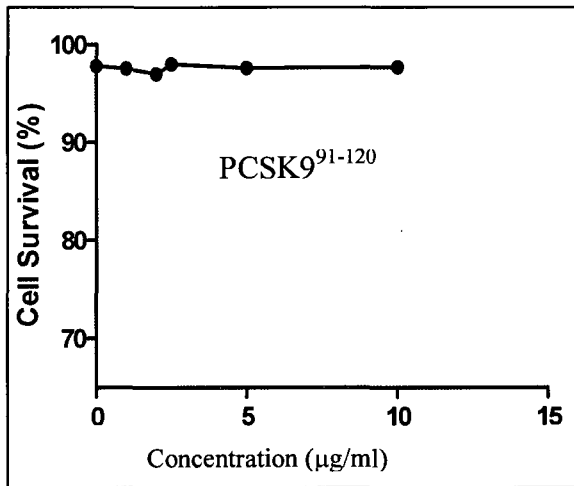
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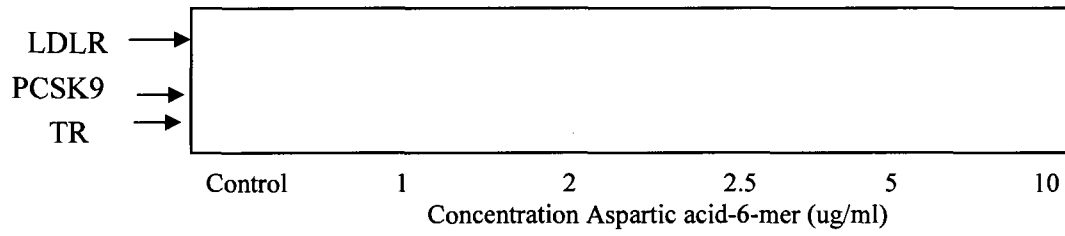
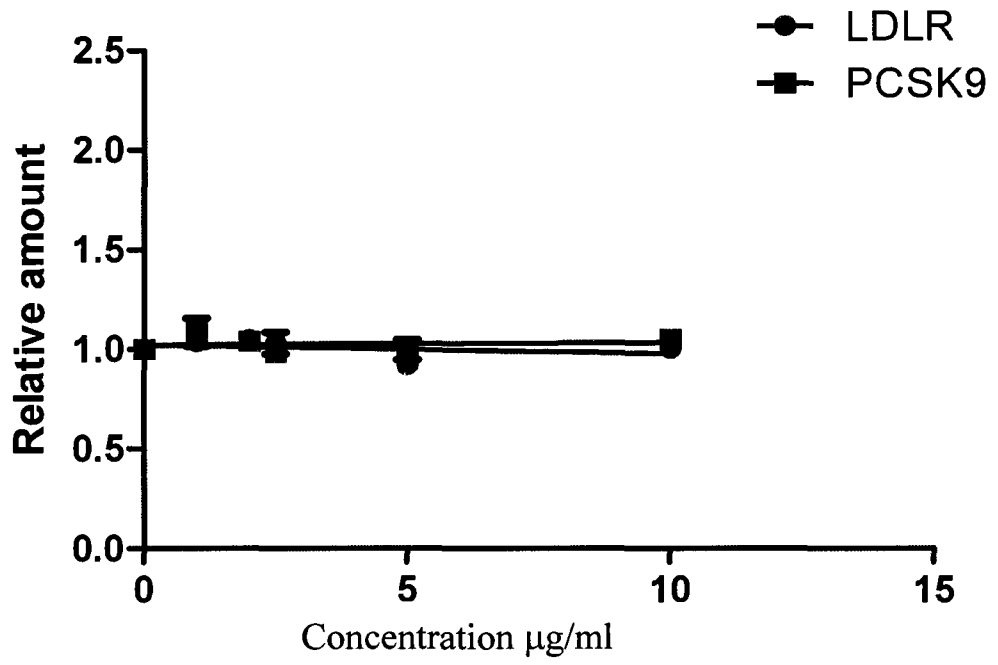
C



4.2 Effect of poly-Aspartic Acid peptide

Two of the prodomain peptides used were highly acidic in nature and these are PCSK9³¹⁻⁴⁰: QEDEDGDYE where 7 out its 10 residues are acidic in nature and PCSK9³¹⁻⁶⁰: QEDEDGDYE ELVLALRSEE DGLAEAPEHG that contains 13 acidic amino acid residues out of a total of 30 residues. Both peptides were highly acidic in nature and both exhibited LDLR enhancing effects in HepG2 and Huh7 cells. Therefore it was proposed that perhaps the high negative charge was responsible for the observed effect on LDLR. To test this notion, we synthesized a 6-mer aspartic acid peptide and added it to the media of HepG2 cells in a concentration dependent manner, 0, 1, 2, 2.5, 5 and 10 $\mu\text{g/ml}$. We found that the hexa-Asp peptide did not alter the level of PCSK9 or LDLR level to any significant extent (**Figure 7**). Although the length of poly-Asp peptide used in the study is not the same as the PCSK9 prodomain peptides, the data seems to suggest that the negative charge alone is not sufficient to explain the LDLR promoting activity of the two N-terminal PCSK9 prodomain peptides. It is likely that the exact amino acid sequence, secondary structure and geometrical conformation may all be important for the display of the observed effect.

Figure 7. Effect of Hexa-Aspartic acid peptide on both LDLR and PCSK9 protein levels in HepG2 cells at varying concentrations. Hexa-Asp peptide was added to serum free culture medium after 24 hours of growth as described previously. After another 4 hours of culture, the whole cell lysates and medium were collected. Only the lysates were analyzed via immunoblotting for PCSK9 and LDLR levels as well as transferrin receptor (TR) as an internal control (50 μ g protein/lane). Each western blot is a representation of three separate experiments. A significance $p < 0.1$ is denoted with *, $p < 0.05$ ** and $p < 0.005$ ***. The dashed line represents significance from the control. Results are plotted relative to the control TR



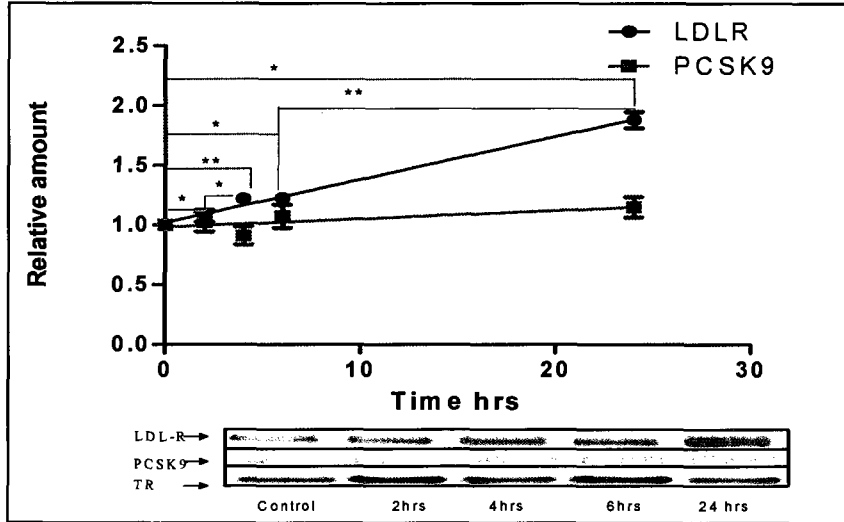
4.3 Time course study: The effect of prodomain peptides over time

Following the positive effects on the LDLR protein level, observed with the three PCSK9 prodomain peptides we became interested in the time course effect of the peptides. Knowing that PCSK9 acts post-translationally to alter the levels of LDLR we intended to see how the LDLR level changed over time in the presence of a fixed amount of PCSK9 prodomain peptides. To monitor the effect on LDLR protein content in a time dependent manner, HepG2 cells were incubated with the peptides at a fixed concentration 2.5 $\mu\text{g/ml}$. The concentration was chosen since all peptides showed effects starting at 2.5 $\mu\text{g/ml}$. The cells were lysed after 2, 4, 6 and 24 hours. The N-terminal hPCSK9³¹⁻⁴⁰ peptide increased the LDLR protein level in a significant manner after 2h and there was >2 fold increase after 24h (**Figure 8A**). The longer N-terminal prodomain peptide hPCSK9³¹⁻⁶⁰ enhanced the LDLR protein level in the cell lysate after 4h and more than a 2-fold increase was noticed after 24h (**Figure 8B**). The mid-basic prodomain peptide hPCSK9⁹¹⁻¹²⁰ increased LDLR protein level in the cell lysate by ~50% in 2h and after 24h, the LDLR protein level was increased by ~ 2.25 fold (**Figure 8C**). Thus, the peptides act on the protein level of LDLR in a time and concentration dependent manner.

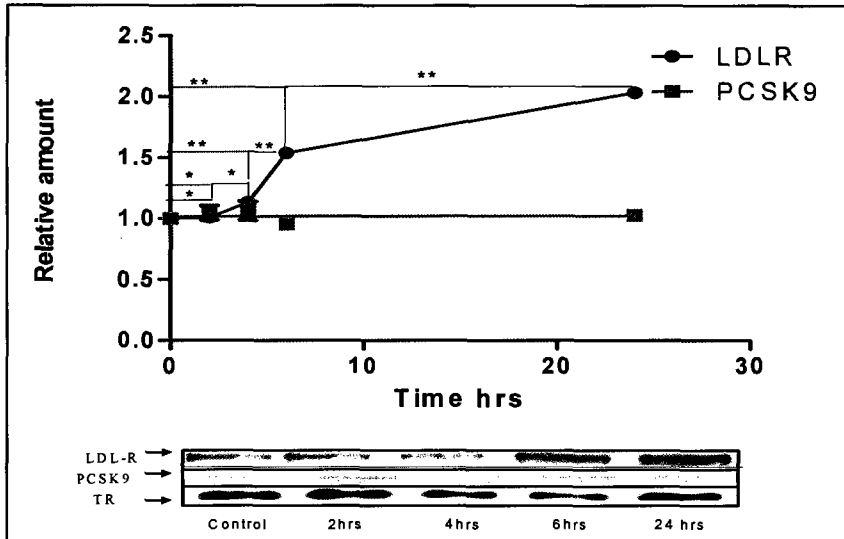
To ensure that the effect we observed was not just due the peptide alone, we used PCSK9¹²¹⁻¹⁵² peptide as a control and collected the lysate after 0, 2, 4, and 24 hours and analyzed by western blot. Our results showed that after 24 hours, there were no significant changes in the LDLR protein level, thus the previous prodomain peptides were having a specific effect, and this effect was not solely due to the presence of a peptide **Figure 9**.

Figure 8. The Effect of PCSK9 prodomain peptides over time on LDLR and PCSK9 protein levels in HepG2 cells by western blot analyses. PCSK9 prodomain peptides were added to cells after 24 hours in serum free media at a concentration of 2.5 µg/ml. After 0, 2, 4, 6 and 24 hours whole cell lysates and medium were collected and lysates were analyzed via immunoblotting for PCSK9 and LDLR levels as well as transferrin receptor (TR) as an internal control (50 µg protein loaded/lane). The plots are a representation of three separate experiments. A significance $p < 0.1$ is denoted with *, $p < 0.05$ ** and $p < 0.005$ ***. Results are plotted relative to the control TR. A) PCSK9³¹⁻⁴⁰, b) PCSK9³¹⁻⁶⁰, c) PCSK⁹¹⁻¹²⁰.

A



B



C

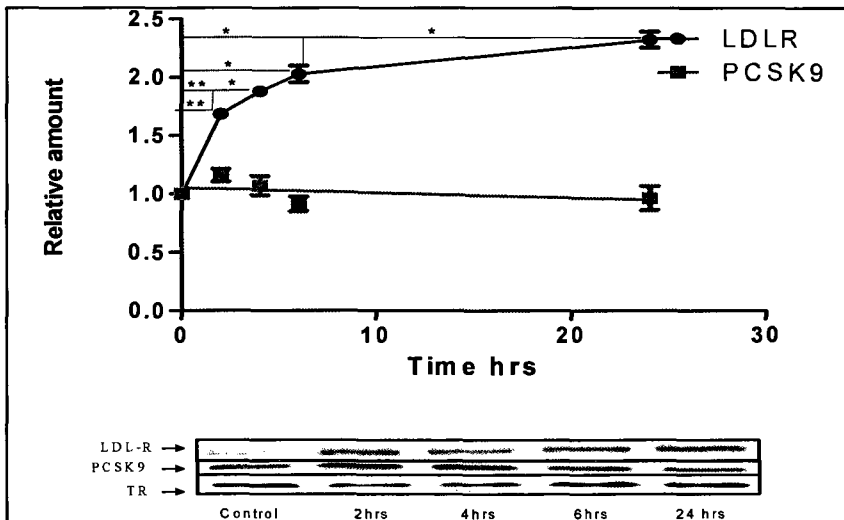
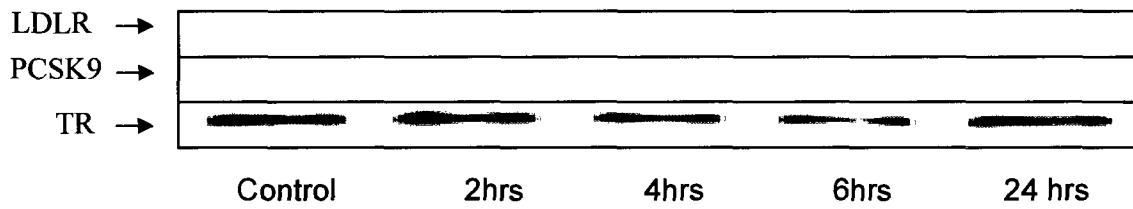
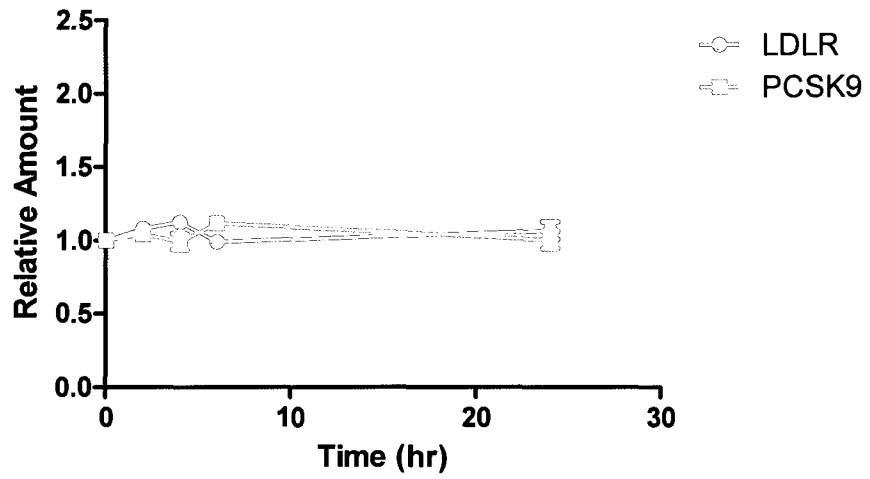


Figure 9. Effect of PCSK9 prodomain peptide PCSK9¹²¹⁻¹⁵² over time on LDLR and PCSK9 protein levels in HepG2 cells by western blot analyses. PCSK9¹²¹⁻¹⁵² was added to cells after 24 hours in serum free media at a fixed concentration of 2.5 µg/ml. After 0, 2, 4, 6 and 24 hours whole cell lysates and medium were collected and lysates were analyzed via immunoblotting for PCSK9 and LDLR levels as well as transferrin receptor (TR) as an internal control (50 µg protein loaded/lane). Western blot is a representation of three separate experiments. A significance $p < 0.1$ is denoted with *, $p < 0.05$ ** and $p < 0.005$ ***. Results are plotted relative to the control TR.



Following the time-course results of PCSK9 prodomain peptides, it appears that the prodomain peptides reduce the effect that PCSK9 exerts on LDLR, in both a time and concentration dependent manner.

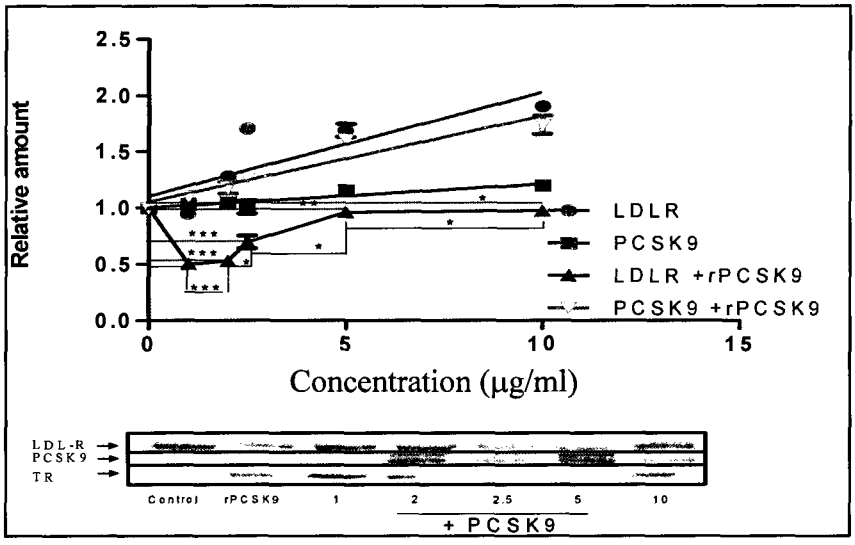
4.4 Restoration of LDLR levels in the presence of recombinant PCSK9

Having observed that certain PCSK9 prodomain peptides could increase LDLR protein levels in both HepG2 and Huh7 cells in a concentration and time-dependent manner, we next decided to examine whether these peptides were able to assist the cells in recuperating LDLR destroyed by an external addition of purified recombinant PCSK9. A concentration of 2.5 μM of rec-PCSK9 and a 4-hour incubation is sufficient to cause a significant drop ($\sim 50\%$) in the LDLR protein level in HepG2 cells. Therefore all peptide experiments were performed in the absence and presence of 2.5 μM of rec-PCSK9 (**Figs 10A, B and C**). Addition of prodomain peptides PCSK9³¹⁻⁴⁰, ³¹⁻⁶⁰ and ⁹¹⁻¹²⁰ to HepG2 cells following treatment with recPCSK9 (2.5 μM) led to a nearly complete recovery of LDLR. For the N-terminal acidic prodomain peptide hPCSK9³¹⁻⁴⁰, the recovery of LDLR was nearly 90% at 10 $\mu\text{g}/\text{ml}$ (**Fig 10A**). For the N-terminal prodomain peptide hPCSK9³¹⁻⁶⁰, the recovery of LDLR was noted after the addition of 1 $\mu\text{g}/\text{ml}$ and $\sim 85\%$ was recovered at a concentration 10 $\mu\text{g}/\text{ml}$ (**Figure 10B**). Finally, for the mid-basic hydrophobic prodomain peptide hPCSK9⁹¹⁻¹²⁰, the LDLR recovery started at 2 $\mu\text{g}/\text{ml}$ concentration with a final recovery of $\sim 85\%$ of the original LDLR level (**Figure 10C**).

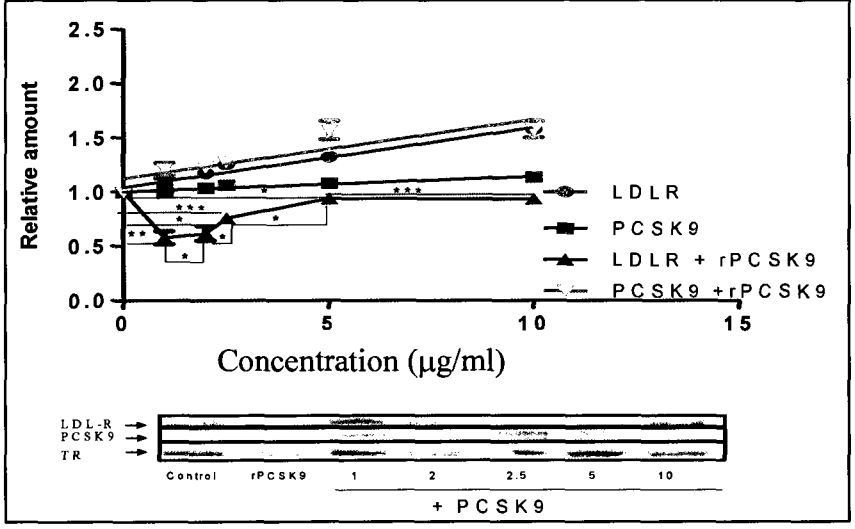
The peptides in the presence of PCSK9 can decrease LDLR degradation, thereby potentially lowering the LDL cholesterol level.

Figure 10. Recovery of LDLR level in the presence of recombinant PCSK9. HepG2 cells were treated with purified recombinant PCSK9 at 2.5 μ M concentration for 4 hours before addition of prodomain peptides. After 4 hours whole cell lysates and medium were collected and the lysates were analyzed via immunoblotting for PCSK9 and LDLR levels as well as transferrin receptor (TR) as an internal control (50 μ g protein loaded/lane). Western blot is a representation of three separate experiments. A significance $p < 0.1$ is denoted with *, $p < 0.05$ ** and $p < 0.005$ ***. The dashed line represents significance from the control. Results are plotted relative to the control TR. A) PCSK9³¹⁻⁴⁰, b) PCSK9³¹⁻⁶⁰, c) PCSK⁹¹⁻¹²⁰.

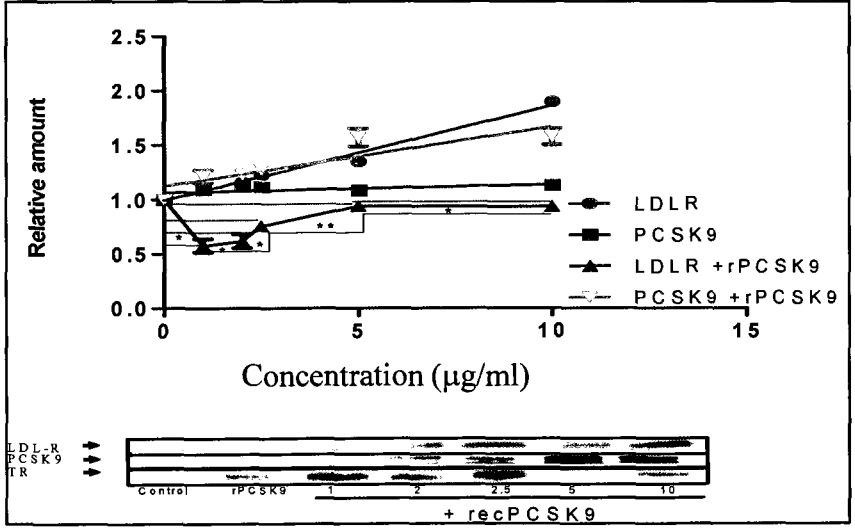
A



B



C



4.5 Key amino acid residues within PCSK9³¹⁻⁶⁰

Following the observation that PCSK9³¹⁻⁴⁰ and PCSK9³¹⁻⁶⁰ (<10 µg/ml) inhibited LDLR degradation by as much as 2-fold, we went on to identify the minimum sequence required for this effect. For this purpose, we synthesized four peptides with 2, 4, 6 or 8 amino acids truncated from the N-terminal side of PCSK9³¹⁻⁶⁰ and one with 10-amino acids truncated from the C-terminus (**Table 5**). This was designed to reveal which amino acids of PCSK9³¹⁻⁶⁰ are critical for the LDLR promoting effect of PCSK9³¹⁻⁶⁰.

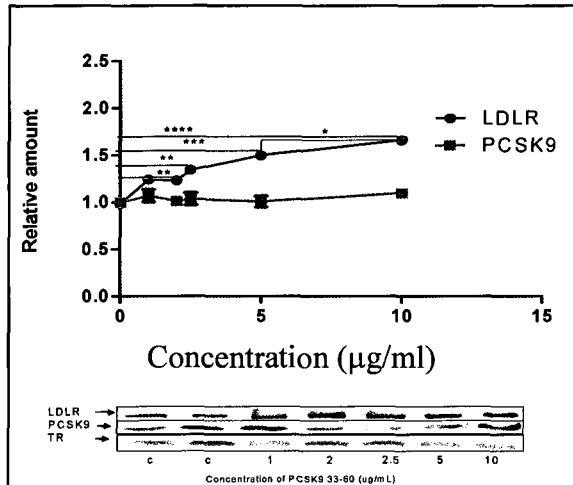
Table 5: List of deletion peptides based on PCSK9³¹⁻⁶⁰ sequence

hPCSK9 ³³⁻⁶⁰	DEDGDYE ELVLALRSEE DGLAEAPEHG
hPCSK9 ³⁵⁻⁶⁰	DGDYE ELVLALRSEE DGLAEAPEHG
hPCSK9 ³⁷⁻⁶⁰	DYE ELVLALRSEE DGLAEAPEHG
hPCSK9 ³⁹⁻⁶⁰	E ELVLALRSEE DGLAEAPEHG
hPCSK9 ³¹⁻⁵⁰	QEDEDGDYE ELVLALRSEE

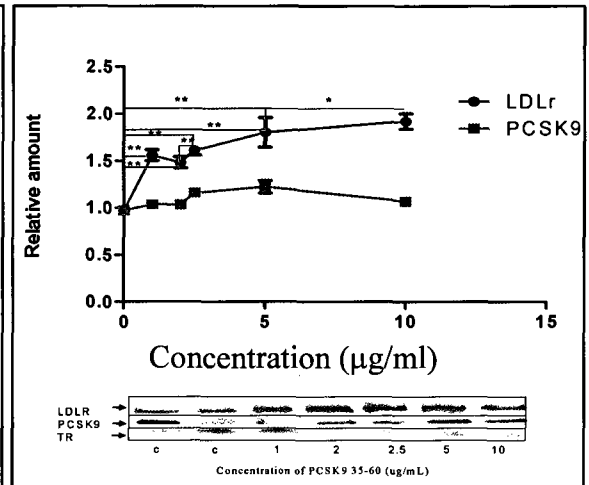
We added each of these peptides to the media of growing HepG2 cells and performed immunoblotting for LDLR and PCSK9 in the cell lysates. In the case of PCSK9³³⁻⁶⁰ peptide we noticed an increase of 80% in the LDLR protein level (**Figure 11A**) similar to that observed with PCSK9³¹⁻⁶⁰. For the peptide with two additional N-terminal amino acids deleted, PCSK9³⁵⁻⁶⁰, we noted a >2-fold increase in the LDLR protein level (**Figure 11B**). Similar results were observed with PCSK9³⁷⁻⁶⁰ which was found to promote a >2-fold increase in the LDLR protein level (**Figure 11C**). However, with further N-terminal deletion there was a reduction in the effect. With PCSK9³⁹⁻⁶⁰ we noted merely a 40% increase in the LDLR protein level (**Figure 11D**). The C-terminal truncated PCSK9³¹⁻⁵⁰ peptide showed only a 50% increase in LDLR (**Figure 11E**). The highest LDLR protein level was observed in the presence of hPCSK9³⁵⁻⁶⁰. These observations revealed that the segment {35-39} of PCSK9³¹⁻⁶⁰ is crucial for the effect on the LDLR protein level

Figure 11. Identification of the amino acid segment of PCSK9³¹⁻⁶⁰ crucial for its LDLR promoting activity. Effects of PCSK9³¹⁻⁶⁰ and its various truncated peptides towards LDLR and PCSK9 protein levels in HepG2 cells at varying concentrations of the peptides. The truncated peptides used were designed by deleting two amino acids at a time from the N-terminal of PCSK9³¹⁻⁶⁰ peptide. One additional truncated peptide was obtained by deleting the last 10 amino acids from the peptide. Each peptide was added exogenously to the serum free media of growing HepG2 cells and the media were collected after 4 hours of culture. The protocol used was the same as described previously for other peptides. The cell lysates were analyzed via immunoblotting for PCSK9 and LDLR protein levels as well for transferrin receptor (TR) as an internal control (~50 µg of protein loaded on each lane). Western blot shown below is a representation of three separate experiments. A significance p<0.1 is denoted with *, p<0.05 **, and p<0.005 ***, p<0.0005 *****. Results are plotted relative to the control TR. A) PCSK9³³⁻⁶⁰, b) PCSK9³⁵⁻⁶⁰ c) PCSK9³⁷⁻⁶⁰ d) PCSK9³⁹⁻⁶⁰ and e) PCSK9³¹⁻⁵⁰

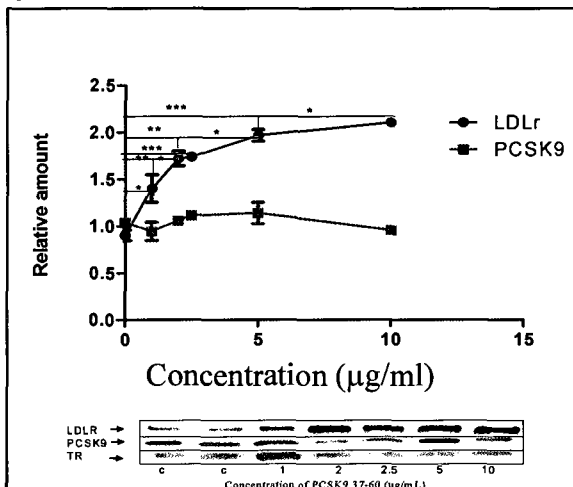
A



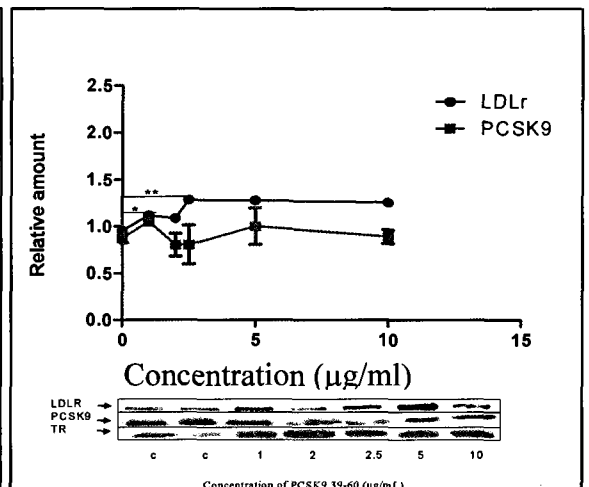
B



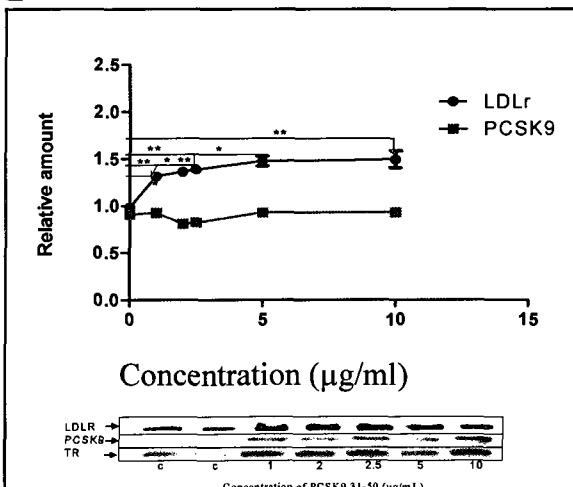
C



D



E



4.6 Effect of Post-translational Modifications of PCSK9 prodomain peptides on LDLR content in HepG2 cells

Three major types of posttranslational modifications of PCSK9 have been reported. These are Ser-phosphorylation, Tyr-sulfation and Asn-glycosylation. Interestingly the first two types are located in the PCSK9 prodomain sequence and within one of the peptides hPCSK9³¹⁻⁶⁰, which exhibited an LDLR promoting effect in HepG2 cells.

4.6.1 Effect of Ser-phosphorylation in the prodomain on LDLR

A recent study (58) has indicated that PCSK9 circulates in human serum as a phosphorylated protein with Ser⁴⁷ of the prodomain and Ser⁶⁸⁸ of C-terminal domain being phosphorylated by a golgi casein kinase type of kinase (59). It was also proposed that the prodomain phosphorylation may protect the propeptide against auto-proteolytic action of the enzyme and therefore may play an important regulatory role in the function of PCSK9. Moreover the LDLR promoting peptide PCSK9³¹⁻⁶⁰ contains one of these Ser phosphorylation sites (Ser⁴⁷ residue). We wanted to examine how this Ser-phosphorylation would affect the ability of PCSK9³¹⁻⁶⁰ to enhance the LDLR protein level in HepG2 cells. In our efforts to study this important aspect of PCSK9 research *in vitro*, we synthesized PCSK9³¹⁻⁶⁰Ser^{47P} peptide that contains the Ser⁴⁷-phosphate. In addition we have also prepared two other peptides that replace Ser⁴⁷ by Ala or Asp residue. The last two peptides used were represented as PCSK9³¹⁻⁶⁰S⁴⁷A, and PCSK9³¹⁻⁶⁰S⁴⁷D as shown in **Table 6**. The rationale for these mutations is that Ala is a neutral residue whereas Asp is an acidic amino acid residue that may mimic the Ser-phosphate moiety in terms of charge distribution. The study on the comparative effects of these peptides on LDLR production is expected to provide information regarding the role of Ser-phosphorylation in the hepatic cell lines. Upon

addition of PCSK9³¹⁻⁶⁰Ser^{47P} we observed a significant decrease in the LDLR protein level of (**Figure 12A**). There was a 50% decrease in the LDLR protein level, at a concentration of 10 µg/ml in comparison to that observed with the corresponding non-phosphorylated peptide under identical conditions, possibly by inducing a significant conformational change in the PCSK9³¹⁻⁶⁰ peptide that affected its LDLR regulatory activity.

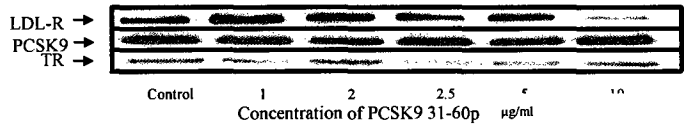
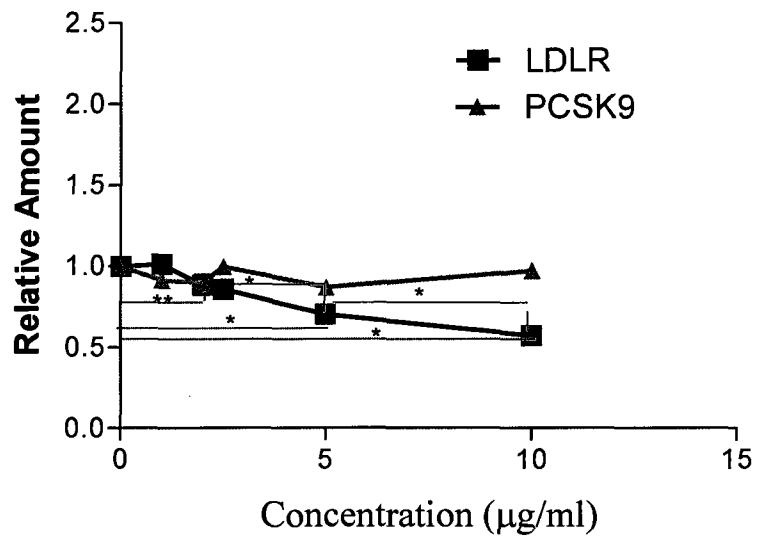
Table 6. Post-translationally modified and various mutated peptides derived from PCSK9³¹⁻⁶⁰

hPCSK9 ^{31-60p}	QEDEDGDYE ELVLALRS(PO4)EE DGLAEAPEHG
hPCSK9 ^{31-60/47A}	QEDEDGDYE ELVLALR A EE DGLAEAPEHG
hPCSK9 ^{31-60/47D}	QEDEDGDYE ELVLALR D EE DGLAEAPEHG
hPCSK9 ^{31-60/38-SO3}	QEDEDGDY(SO3)E ELVLALRSEE DGLAEAPEHG

4.6.2 Effect of Ser⁴⁷ mutations in PCSK9³¹⁻⁶⁰ peptide on LDLR

Following the observation that the phosphorylated PCSK9^{31-60p} peptide negatively affected the LDLR protein level in HepG2 cells, we wanted to determine if Ser⁴⁷ had a critical role in the ability of PCSK9 to degrade LDLR. For this we prepared two peptides with Ser⁴⁷ substituted by an Ala or Asp residue (**Table 6**). Alanine was chosen, as it is expected not to alter the conformation of the PCSK9³¹⁻⁶⁰ peptide and Aspartic acid would mimic the negative charge created by the phosphate group. This will help to determine if the overall charge distribution in the peptide is a factor in regulating the LDLR protein level in HepG2 cells.

Figure 12. Comparison on the effects of phosphorylated PCSK9³¹⁻⁶⁰ and the corresponding non-phosphorylated peptide on LDLR protein level in HepG2 cells. PCSK9^{31-60p} was added to cells as before in serum free media after 24 h growth of HepG2 cells. After 4 hours, the medium was removed and the cells were lysed. The latter were then analyzed via immunoblotting for PCSK9 and LDLR levels as well as for transferrin receptor (TR) as an internal control (50 µg protein loaded/lane). Western blot shown in the figure is a representation of three separate experiments. A significance $p < 0.1$ is denoted with *, $p < 0.05$ **, and $p < 0.005$ ***, $p < 0.0005$ *****. Results are plotted relative to the control TR.



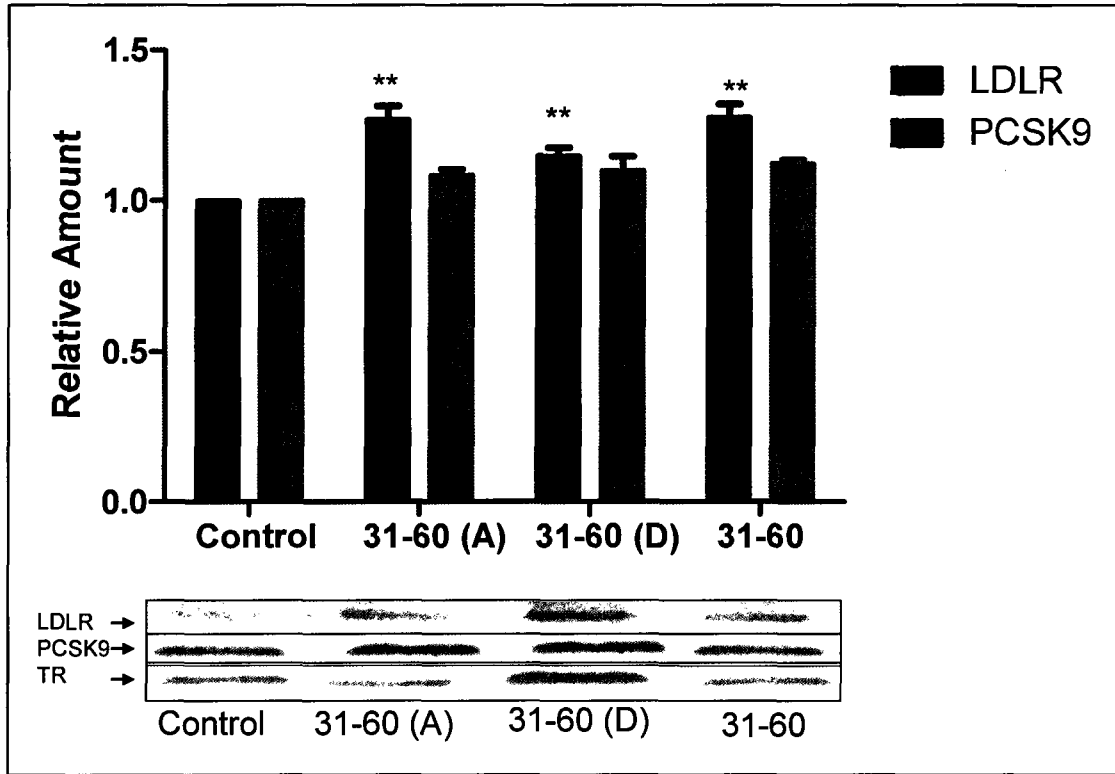
The Alanine mutation did not alter the effect of the peptide PCSK9³¹⁻⁶⁰ on the LDLR protein level in HepG2 cells. When added to the media of HepG2 cells at a concentration of 5µg/ml there was a 30% increase in LDLR level with no significant change compared to the wild type peptide PCSK9³¹⁻⁶⁰ (**Figure 13A**). When Ser⁴⁷ was replaced with Aspartic acid, there was only a modest change in the level of LDLR. Unmodified or wild type PCSK9³¹⁻⁶⁰ peptide enhanced LDLR protein level by approximately 30% at a concentration of 5µg/ml, whereas the Asp-mutant peptide showed a 20% increase in LDLR protein level. This difference is not significant and is not enough to suggest the effect of negative charge if any on LDLR level (**Figure 13A**). Thus we conclude that although charge may play some role in LDLR regulation it is only minor in nature. Possibly the phosphorylation is creating a conformational change in PCSK9³¹⁻⁶⁰ peptide that is in turn affecting its ability to influence or affect LDLR protein level in HepG2 cells.

4.6.3 Effect of Tyr-sulfation in the prodomain on LDLR

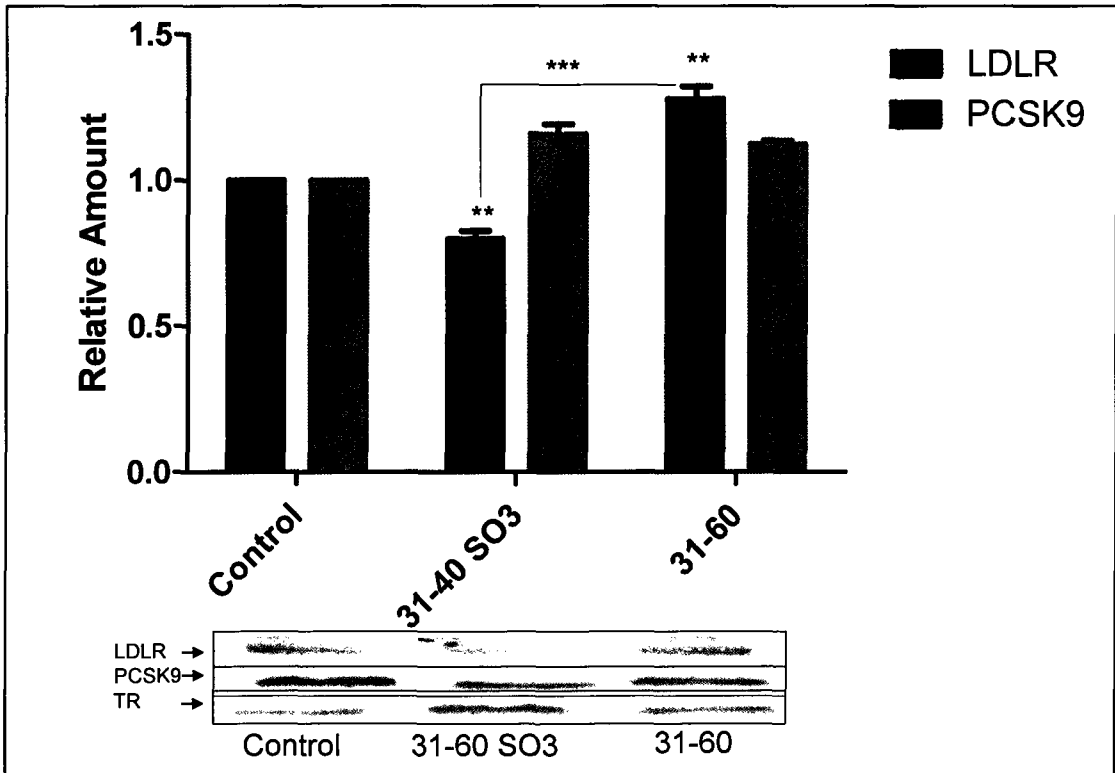
In 2004, a post-translational sulfation was reported at Tyr³⁸ of PCSK9. This modification is within the region of our peptide PCSK9³¹⁻⁶⁰ that exhibited an LDLR promoting effect in HepG2 cells. Thus we became interested to study the effect of this mutation on the LDLR protein level. When added to the media of HepG2 cells at a concentration of 5µg/ml, there was a small but significant ~20% decrease in the LDLR protein level (**Figure 13B**). This effect was similar to that observed with the phosphorylated PCSK9^{31-60p} peptide. Likely again the sulfation may alter the conformation of PCSK9³¹⁻⁶⁰ similar to that observed with PCSK9^{31-60p}.

Figure 13. Two mutations and a post-translational modification from PCSK9³¹⁻⁶⁰ and the effect on LDLR protein expression. PCSK9³¹⁻⁶⁰ peptides were added to cells after 24 hours in serum free media. After 4 hours whole cell lysates and medium were collected and lysates were analyzed via immunoblotting for PCSK9 and LDLR levels as well as transferrin receptor (TR) as an internal control (50 μ g protein loaded/lane). Western blot is a representation of three separate experiments. A significance $p < 0.1$ is denoted with *, $p < 0.05$ **, and $p < 0.005$ ***, $p < 0.0005$ *****. Results are plotted relative to the control TR. A) Shows the effect of two mutations at the Ser47 position of PCSK9³¹⁻⁶⁰, B) Shows the effect of a tyrosine sulfation at position 38 of PCSK9³¹⁻⁶⁰.

A



B



4.7 HepG2 knockdown cells

To determine whether the peptides are acting via PCSK9, we performed further experiments using variant HepG2 cell types where the PCSK9 level has been heavily suppressed. This cell line was kindly provided to us by Dr. Robert Day, Sherbrooke University. Three HepG2 cell lines were obtained (i) PLKO-1 with PCSK9 knockdown, (ii) HLDLR (high LDLR) cells over expressing LDLR and (iii) non-target cells containing the empty vector. Initially we determined the level of PCSK9 in the knockdown cell lysates (**Figure 14**) and found that both PCSK9 knockdown cells, PLKO-1 and over-expressed HLDLR have a 50% decrease in PCSK9 protein level compared to the non-target control cells, and wild type HepG2 cells.

To determine if the PCSK9 prodomain peptides were having an effect on LDLR through PCSK9, we added the peptides to the media of the cells at the fixed concentration of 5µg/ml, to ensure that there was an observable effect. In normal HepG2 cells we noticed a ~60% increase in the LDLR protein level for PCSK9³¹⁻⁴⁰, ~85% for PCSK9³¹⁻⁶⁰ and ~80% for PCSK9⁹¹⁻¹²⁰ treated cells (**Figure 15A**). In the non-target cells we noted a ~ 50% increase in the lysate of LDLR level for PCSK9³¹⁻⁴⁰, 65% for PCSK9³¹⁻⁶⁰ and 60% for PCSK9⁹¹⁻¹²⁰ treated cells (**Figure 15B**), similar to that observed with the control HepG2 cells. Overall we observed that in the PLKO-1 cell line, PCSK9³¹⁻⁴⁰ peptide produced ~60% increase in LDLR level compared to ~75% and ~60% for PCSK9³¹⁻⁶⁰ and PCSK9⁹¹⁻¹²⁰ respectively (**Figure 15C**). In HLDLR cell line, PCSK9³¹⁻⁴⁰ had a 2-fold increase in LDLR level, whereas PCSK9³¹⁻⁶⁰ and PCSK9⁹¹⁻¹²⁰ exhibited ~75% and ~60% increase in LDLR level respectively (**Figure 15D**).

Figure 14. Western blots showing the presence of PCSK9 protein in widely varying amounts in three HepG2 cell types including the wild type. There is approximately a 50% decreased level of PCSK9 in cell lysates obtained from PCSK9-KD HepG2 cells compared to that of control wild type cell. In contrast in HLDLR derived lysate exhibited ~20% decreased level of PCSK9 protein whereas there was no change in the non target cells all relative to the control wild type HepG2 cells

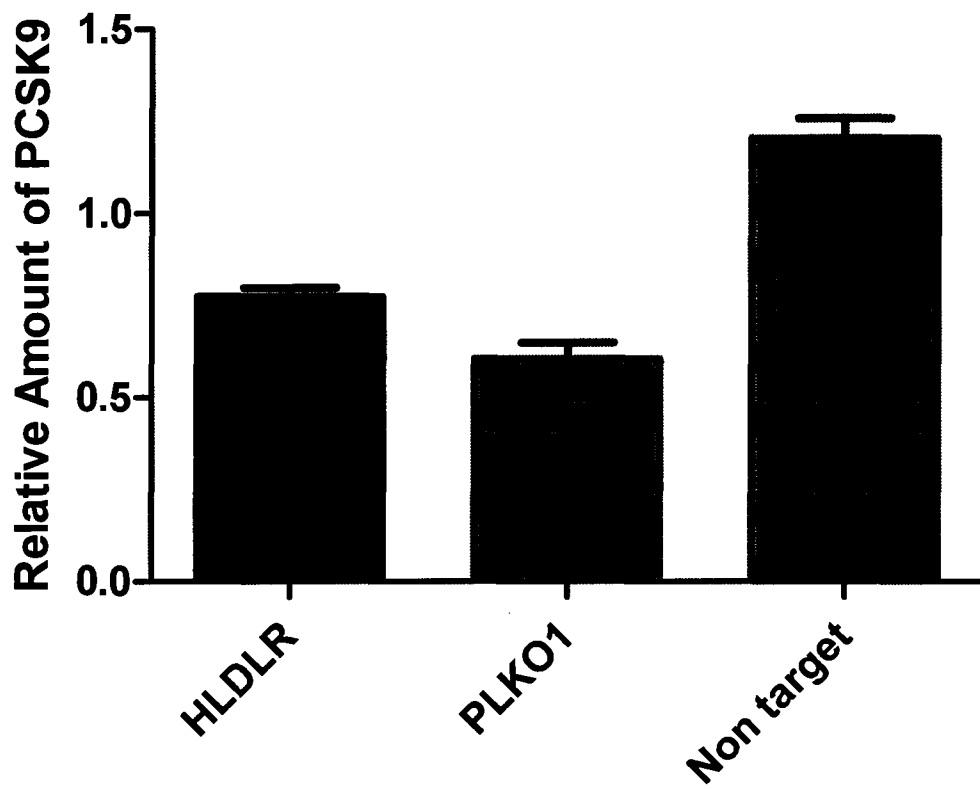
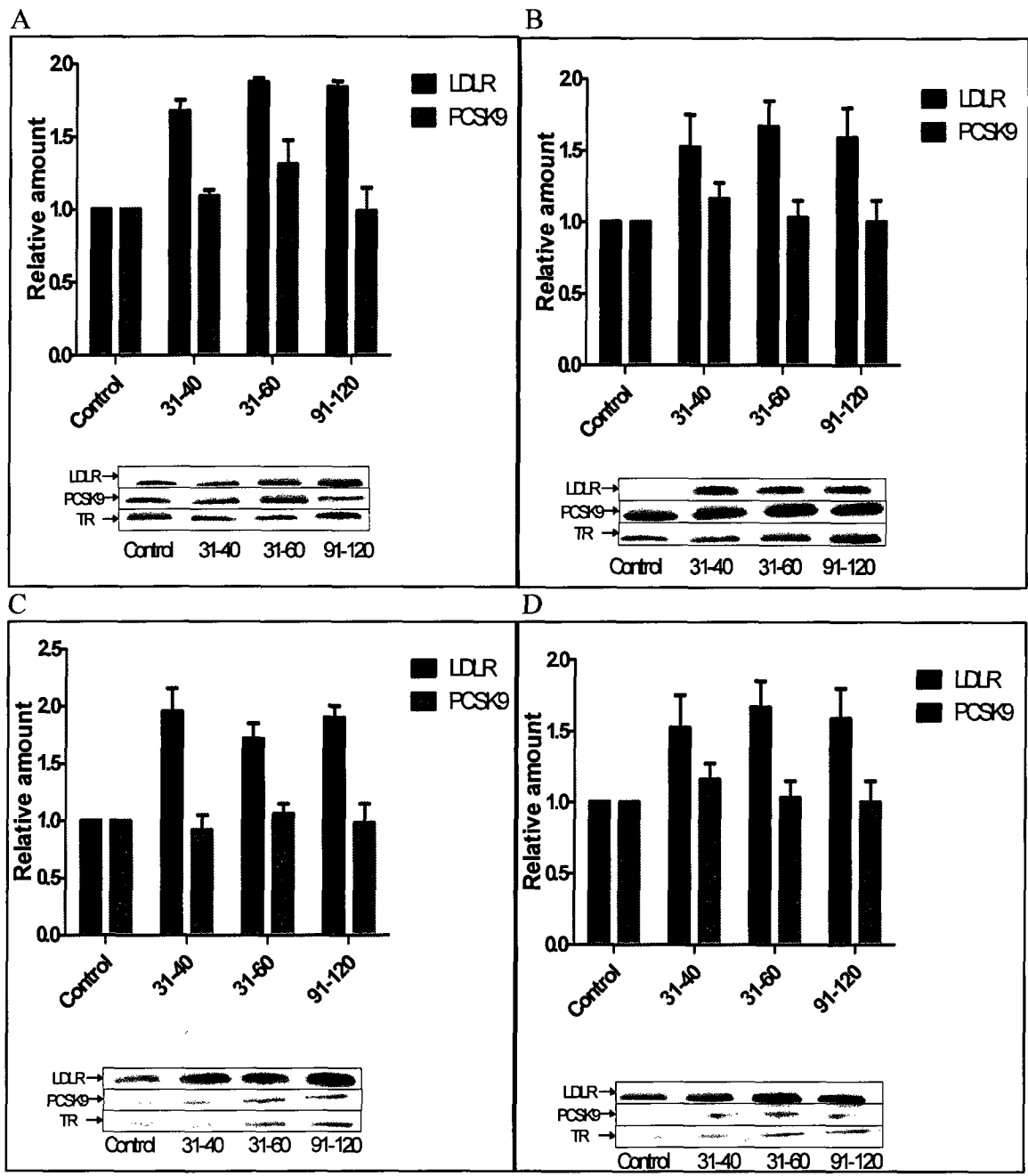


Figure 15 Effect of PCSK9 prodomain peptides on LDLR in various HepG2 cell lines. The figure shows the results with PCSK9³¹⁻⁴⁰, ³¹⁻⁶⁰ and ⁹¹⁻¹²⁰ peptides all at the fixed concentration of 5 µg/ml in various HepG2 cell types. A) Normal or wild type HepG2; B) Non Target cell, C) HDLDR cell type over expressing PCSK9 and d) PLKO1 cells with reduced expression of PCSK9.



The above results suggest that the LDLR-promoting activity of PCSK9 prodomain peptides is similar to that observed with both normal and PCSK9 knock down HepG2 cells. Therefore the observed effects of the peptides are independent of the amount of endogenous PCSK9 present in the HepG2 cell types.

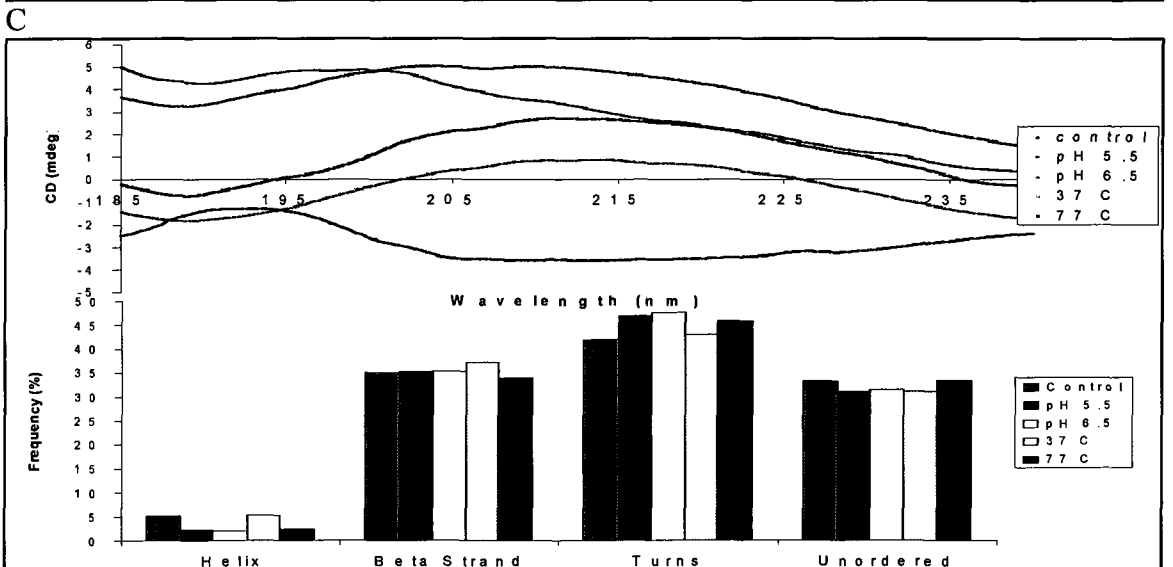
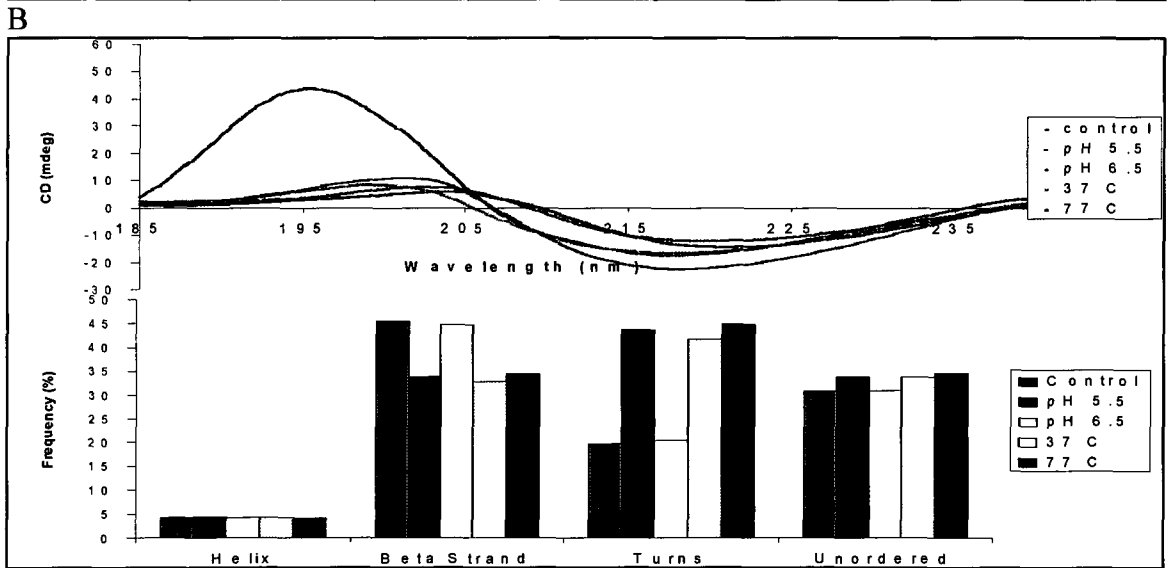
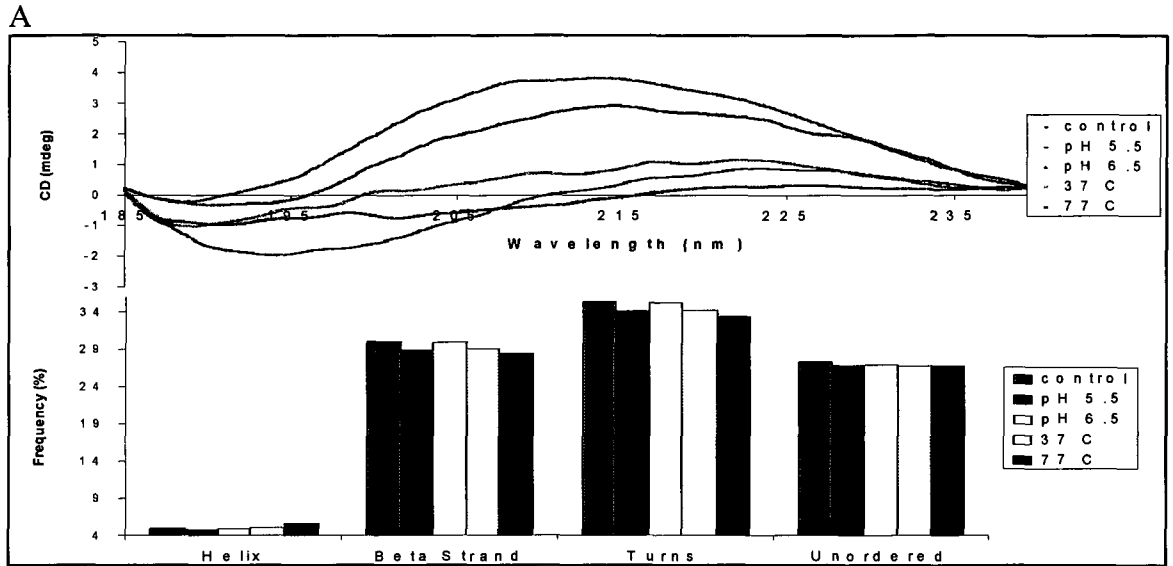
4.8 Secondary structure analysis of PCSK9 peptides

Since the PCSK9 prodomain peptides displayed differential effects on LDLR in hepatoma cells, we became interested in studying their secondary structures *in vitro* by using Circular Dichroism (reviewed by Whitmore 2008) at different pH and temperature conditions. The rationale behind this study is that it might provide a suitable explanation about the conformational role of the Ser-phosphorylation and Tyr-sulfation. The interaction between PCSK9 and LDLR has been shown to be pH dependent, and a reduction in pH value from neutral ~7.4 to an acidic 5.2 increases the binding efficiency of PCSK9 with LDLR by a factor of 150 fold. Also, the functional activity of PCSK9 is destroyed upon heating. These observations may indicate the important role of secondary structure and conformation on its function. This may also hold true for the observed effects of prodomain peptides on LDLR. This prompted us to investigate the secondary structures and geometry of PCSK9 prodomain peptides and how pH and temperature changes affect this. This was accomplished by studying their circular dichroism (CD) spectra. **Figure 16** shows the overlay CD spectra of various PCSK9 peptides that exhibited LDLR promoting effects in the cell experiments. Our data revealed that all peptides tested showed predominantly β -sheet and random structures with low helical content as analyzed by their measured CD spectra using Dichroweb software.

Data revealed that PCSK9³¹⁻⁴⁰ (**Figure 16A**) contains primarily β -sheet and β -turn structures. Once the pH was lowered to 5.5 there was a decrease in the structural composition consisting of β -sheet, β -turn and α -helical conformations, at a pH of 6.5, or at a temperature of 37°C. However, upon denaturing the peptide by heating to 77°C, there was a ~12% increase in α -helical structure and a 5% decrease in overall β -pleated sheet and β -turn structures with no significant changes in other structural components. It was also revealed that the secondary structure of PCSK9³¹⁻⁴⁰ is relatively stable within a pH range of 6.5 to 7.4 and at a temperature of 37°C. However, at a temperature of 77°C, and at an acidic pH its structure and conformation is affected, which may provide a reasonable rationale for the observed effect of the PCSK9³¹⁻⁶⁰ peptide on LDLR in HepG2 cells. This prodomain peptide exhibited the highest content of helix and also the beta pleated structures as seen in **Figure 16B**. Upon decreasing the pH to 5.5, there was a reduction in α -helical structure by ~50%, and also a decrease in both β -sheet and β -turns by ~20%. Even at a less acidic pH of 6.5 there was still a 45% decrease in α -helical structure and a 15% decrease in both β -sheet and β -turns compared to the control. PCSK9⁹¹⁻¹²⁰ showed the highest β -turn content and lowest β -sheet contents (**Fig 16C**). Upon a decrease in pH to 5.5 there was a ~57% drop in α -helical structure, and an ~11% increase in β -turn structure. This dramatic decrease in α -helical structure was also observed at a pH of 6.5 and treatment at 77°C with a 61% and 55% loss of α -helical structure respectively. There was little deviation of the structure at 37°C.

The decrease in α -helical structure for PCSK9⁹¹⁻¹²⁰ is similar to that observed in PCSK9³¹⁻⁶⁰ but not PCSK9³¹⁻⁴⁰. In PCSK9³¹⁻⁴⁰, there was no significant deviation in its structure. Thus no consensus structural features were observed with these peptides.

Figure 16. pH and temperature effects on the secondary structure of PCSK9 prodomain peptides. Secondary structure analysis was performed by measuring circular dichroism spectra and then using the dichroweb contin software program. PCSK9³¹⁻⁴⁰, PCSK9³¹⁻⁶⁰ and PCSK9⁹¹⁻¹²⁰ peptides were treated with 25 mM Tris-Mes buffer, pH 7.4, 6.5 or 5.5 and then incubated at a temperature of 37°C or 77°C for one hour before analysis. The frequency of secondary structures is plotted as the average of all matching results. A) PCSK9³¹⁻⁴⁰, b) PCSK9³¹⁻⁶⁰ c) PCSK9⁹¹⁻¹²⁰.

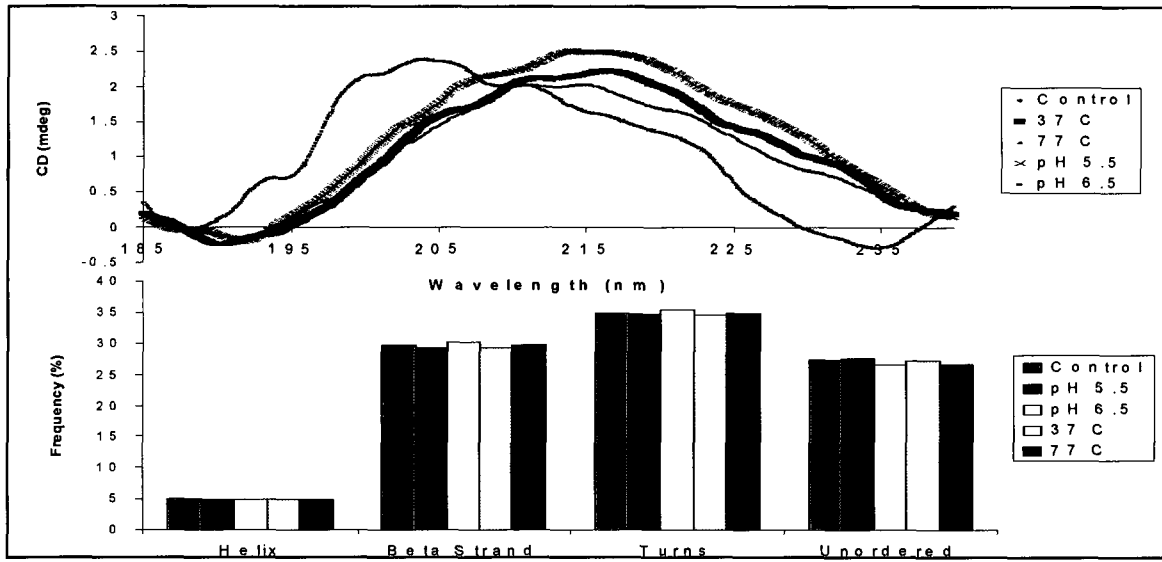


With the control peptides PCSK9¹²¹⁻¹⁵² and PCSK9⁶¹⁻⁹⁰ there was no significant deviation in α -helical structure upon changes in either pH or temperature (**Figure 17ab**). However, upon a drop in pH or a change in temperature there was a 2-fold increase in β -turn structure, which we did not observe in either of the peptides responsible for increasing the LDLR protein level. This change signifies an important role played by the secondary structures between the peptides effecting LDLR degradation and those that do not.

To determine the impact of Ser-phosphorylation on the secondary structure of PCSK9³¹⁻⁶⁰ peptide, we carried out structural analysis as previously mentioned. The findings are shown in **Figure 18b**. Here we found that unlike PCSK9³¹⁻⁶⁰ **Figure 18a** there was no decrease in α -helical structure upon a change in pH or temperature. The structure of the peptide was not significantly altered after treatment at pH 6.5. However, at a pH of 5.5 there was a 2-fold increase in β -turn structure and a 26% decrease in β -sheet structure, this trend was observed both at 77°C and 37°C. This is also accompanied by a decrease in β -sheet structure by ~24% and 29% respectively and a 2-fold increase in β -turn structure for each temperature. The differential structural response to changes in pH and temperature, may explain why PCSK9^{31-60p} affected LDLR level in an opposite manner than unphosphorylated PCSK9³¹⁻⁶⁰.

Figure 17. Effect of pH and temperature on the secondary structure of PCSK9 prodomain peptides that did not affect LDLR level in our cell based assay. Secondary structure analysis was done using circular dichroism and the results analyzed by using dichroweb contin software. PCSK9⁶¹⁻⁹⁰ and ¹²¹⁻¹⁵² were treated with Tris-Mes 25mM buffer at pH 7.4 (control), pH 6.5, and pH 5.5 and incubated at a temperature of 37°C and 77°C for one hour before analysis. The frequency of secondary structures is plotted as the average of all matching results. A) PCSK9⁶¹⁻⁹⁰, b) PCSK9¹²¹⁻¹⁵².

A



B

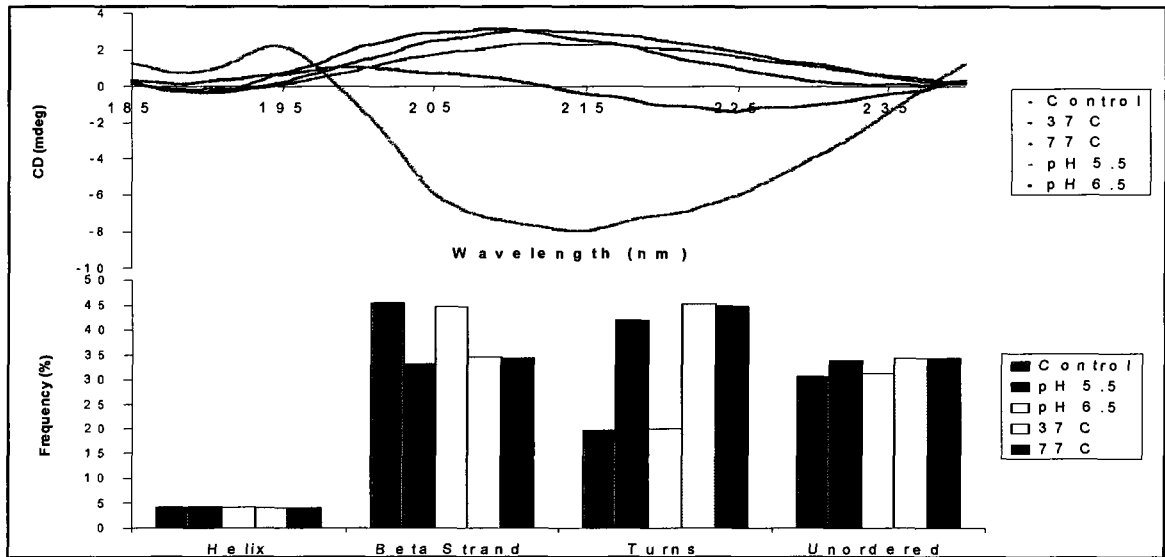
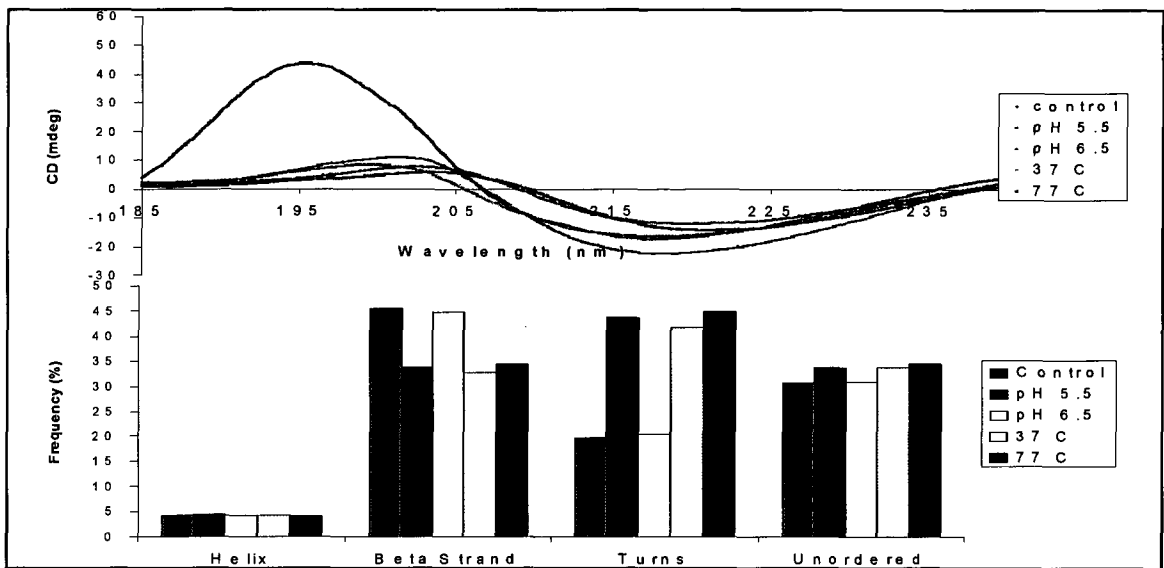
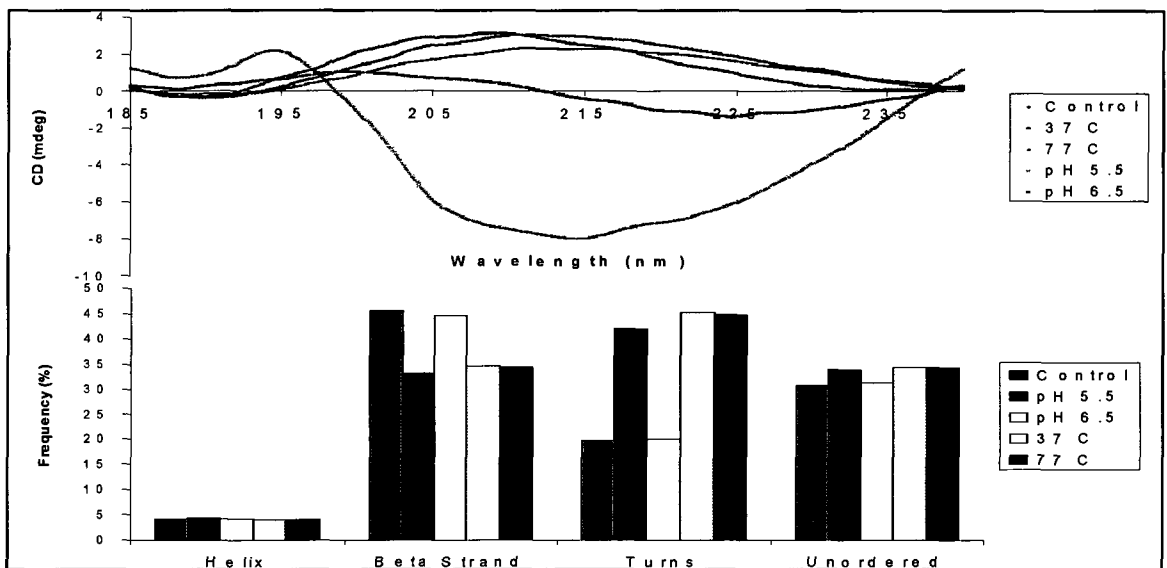


Figure 18. The effects of pH and temperature on the secondary structure of PCSK9³¹⁻⁶⁰ and corresponding Ser⁴⁷-phosphate containing peptide PCSK9^{31-60p}. Secondary structure analysis was achieved by circular dichroism spectra and their analyses by dichroweb contin software. PCSK9^{31-60p} was treated with 25 mM Tris-Mes buffer at pH 7.4, 6.5, 5.5 and incubated at temperature of either 37°C and 77°C for one hour before analysis. The frequency of secondary structures is plotted as the average of all matching results. A) PCSK9³¹⁻⁶⁰ b) PCSK9^{31-60p}.

A



B



4.9 Effect of catalytic domain peptides on LDLR

While our studies on PCSK9 prodomain peptides and their effects on LDLR were in progress, a report was published in the literature where it was revealed for the first time that the catalytic domain of PCSK9 physically interacts with the EGFA domain of LDLR. Two regions in close proximity of catalytic Asp¹⁸⁶ and Ser³⁸⁶ have been identified as the potential sites for the interaction (108). The crystal structure of the complex showed that the amino acids Ser¹⁵³, Asn¹⁵⁷, Arg¹⁹⁴ as well as Asp²³⁸, Tyr³⁰⁶, and Asp³⁷⁴ either interact or provide H-bonding to the EGF-A domain of LDLR (47). It was revealed that the P' helix (N-terminal to the autocatalytic cleavage site) is also important in the interaction. In order to confirm these observations we designed two peptides: PCSK9¹⁸¹⁻²⁰⁰ and PCSK9³⁶⁸⁻³⁹⁰ from the catalytic domain that comprise the above two regions (**Table 7**) and examined their effects on the LDLR protein level in HepG2 cells.

Table 7. List of peptides derived from the catalytic domain of PCSK9. The catalytic **Asp** and **Ser** residues are in bold underlined

Name	Amino acid sequence
hPCSK9 ¹⁸¹⁻²⁰⁰	EVYLL <u>D</u> TSIQ SDHREIEGRV
hPCSK9 ³⁶⁸⁻³⁹⁰	IIGASSDCST CFVSQSGT <u>S</u> Q AAA

Thus to determine the effects, these catalytic domain peptides were individually added to the media of HepG2 cells at a concentration of 5 µg/ml, a concentration found to have an effect with all peptides, and the cell lysates were immunoblotted for the presence of PCSK9, LDLR and Tranferrin receptor. Our data revealed that both catalytic domain peptides decreased the protein level of LDLR (**Figure 19**). There was a 60% and 50% decrease in LDLR for the peptides PCSK9¹⁸¹⁻²⁰⁰ and PCSK9³⁶⁸⁻³⁹⁰ respectively. These

effects are opposite to those observed with the prodomain peptides PCSK9³¹⁻⁴⁰, ³¹⁻⁶⁰ and ⁹⁰⁻¹²⁰.

Following these results, we then became interested in determining whether the prodomain peptides could counteract the effects of the catalytic domain peptides when used in combination. To do this, we added the catalytic domain peptides and prodomain peptides at a concentration of 2.5 μM to maintain a 1:1 ratio of prodomain peptide to catalytic derived peptide, and performed the cell culture experiment. The obtained cell lysates were immunoblotted for LDLR, PCSK9 and Transferrin receptor (**Figure 20**).

Our data indicated that indeed the peptides were able to counteract the effects of the catalytic domain peptides and the level of LDLR protein remained stable at a level observed for the control without the addition of either peptide.

Figure 19. Effect of addition of either catalytic domain peptides PCSK9¹⁸¹⁻²⁰⁰ or PCSK9³⁶⁸⁻³⁹⁰ along with individual prodomain peptides. PCSK9 peptides were added to serum free culture medium for 4 hours in serum free media. After 4 hours whole cell lysates and medium were collected and only the lysates were analyzed by immunoblotting for PCSK9 and LDLR levels as well as transferrin receptor (TR) as an internal control (50 μ g total protein loaded/lane). Western blot is a representation of three separate experiments. A significance $p < 0.1$ is denoted with *, $p < 0.05$ ** and $p < 0.005$ ***, $p < 0.0005$ ****. Results are plotted relative to the control TR.

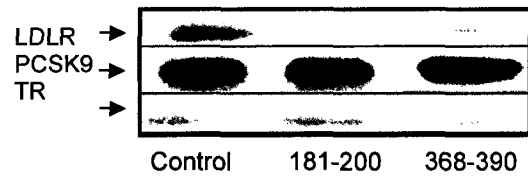
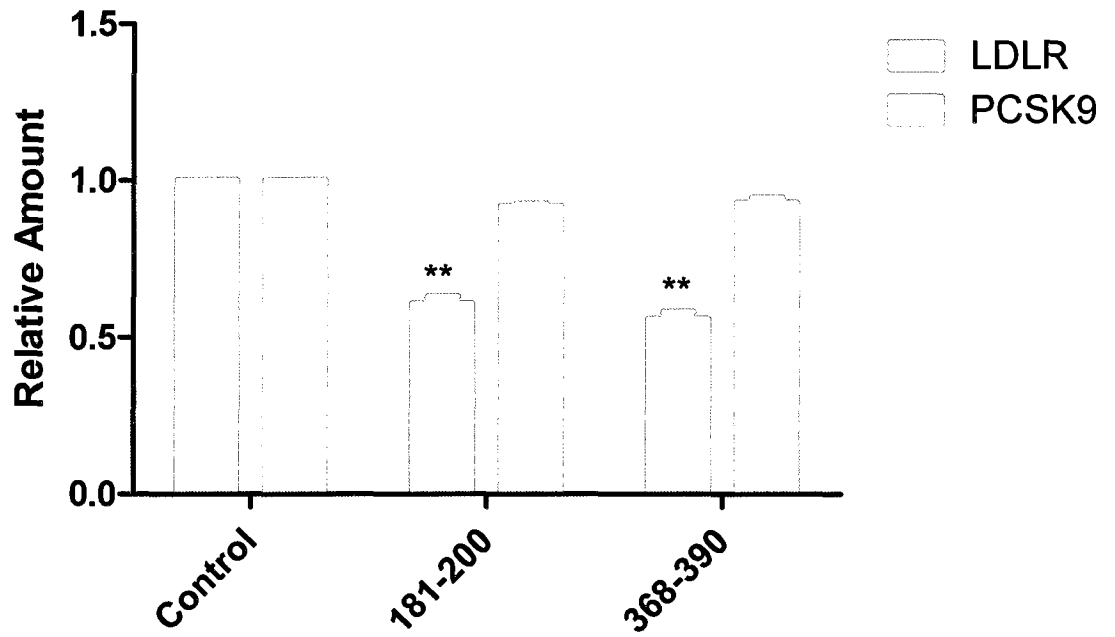
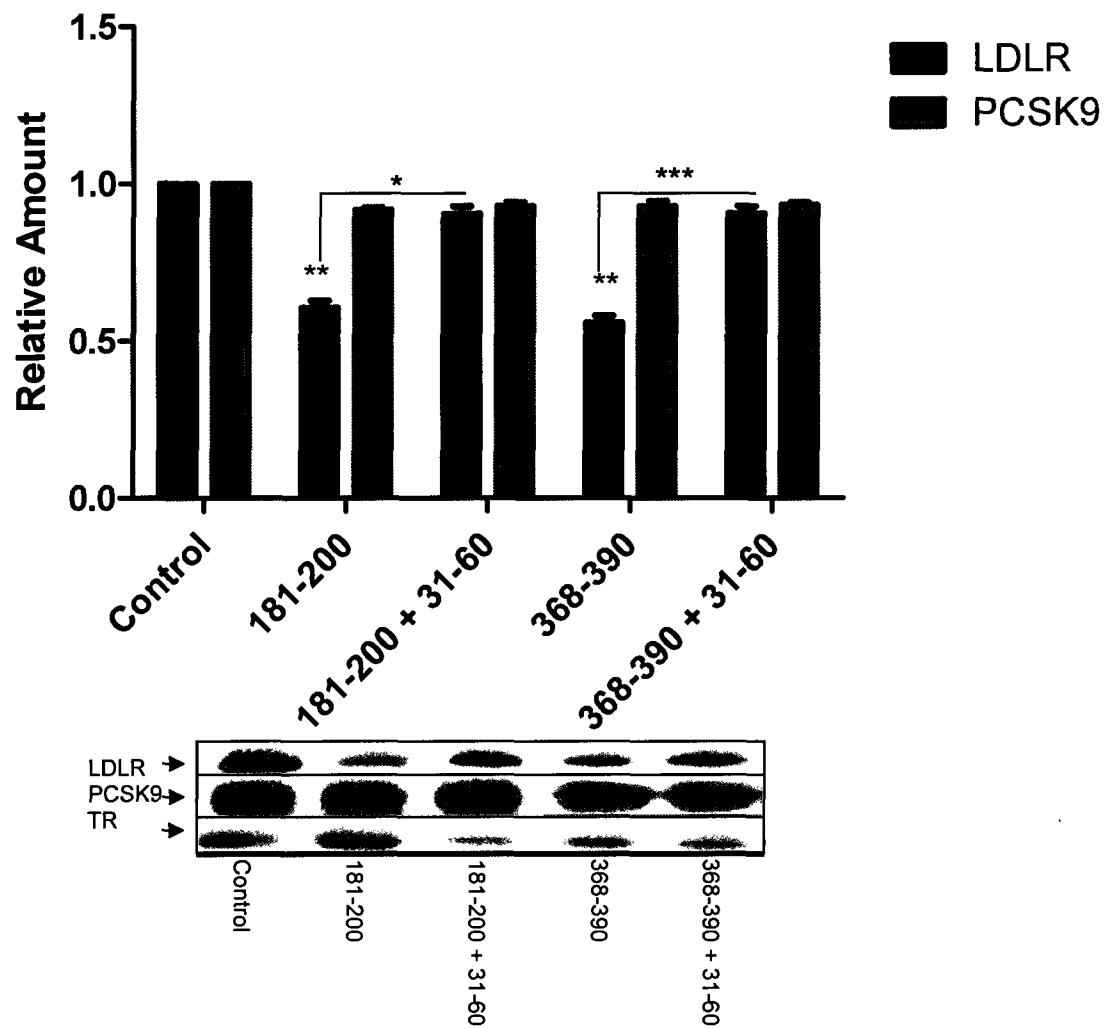


Figure 20. The effect of catalytic domain peptides in HepG2 cells on LDLR in the presence of prodomain peptide PCSK9³¹⁻⁶⁰. PCSK9 peptides were added to cells as described before. The obtained cell lysates were analyzed via immunoblotting for PCSK9 and LDLR levels as well as transferrin receptor (TR) as an internal control (50 μ g total protein loaded/lane). Western blot is a representation of three separate experiments. A significance $p < 0.1$ is denoted with *, $p < 0.05$ **, and $p < 0.005$ ***, $p < 0.0005$ *****. Results are plotted relative to the control TR.



5.0 Discussion

5.1 Possible rationale and mechanism of action of PCSK9 pro and catalytic peptides

Determining the effects of various regions of the prodomain of PCSK9 on the LDLR protein level was an essential first step in understanding the detailed mechanism of LDLR degradation by PCSK9; as well as, to develop novel functional inhibitors of PCSK9. In the first set of experiments to study the effect of PCSK9 prodomain peptides on the regulation of LDLR, we used human hepatic cell lines HepG2 and Huh7 cells both of which express PCSK9 and LDLR endogenously. In this study we discovered three peptides, covering two regions of the prodomain that increase the protein concentration of LDLR in a dose and time dependent manner (**Figures 3, 5 and 8**). Two of the peptides PCSK9³¹⁻⁴⁰ and ³¹⁻⁶⁰ appear in a region of PCSK9 that were missing in its own crystal structure as well as in the co-crystal structure of PCSK9 and the EGFA complex. We were able to demonstrate that PCSK9³¹⁻⁴⁰ is the most potent peptide in increasing the LDLR protein level, followed by PCSK9⁹¹⁻¹²⁰ and lastly PCSK9³¹⁻⁶⁰ peptides (**Figures 3 and 5**). Furthermore, none of the peptides tested affected the level of the PCSK9 protein in either cell line used suggesting that the observed effect on LDLR is occurring through a pathway that is controlling its functional activity and its level of expression. Our results using various HepG2 cell types suggested that the observed LDLR enhancing effect is independent of the actual amount of PCSK9 (**Figure 14**). These peptides may be directly or indirectly affecting PCSK9's ability to bind to LDLR and transport it to the lysosomal compartment for its ultimate degradation. It is possible that the N-terminal and mid part prodomain peptides might separately interact with the two regions encompassing the catalytic Asp²¹⁸ and Ser³⁸⁶ residues that are implicated in binding with the EGF-A domain of LDLR. Thus although the mechanism of

action of these peptides is not clearly understood at the present time, it requires further studies. However two possible mechanisms may be presented to explain the observed effects. The increase in LDLR level may occur either through enhancing the synthesis of LDLR or by diminishing the degradation of LDLR by PCSK9. The latter possibility seems more likely and logical. Thus we propose that our prodomain peptides showing LDLR promoting effects, first bind to the specific region/s of catalytic domain of PCSK9 particularly the area covering the catalytic Asp¹⁸⁶ and Ser³⁸⁶ regions, and interfere in the binding of PCSK9 with the EGFA domain of LDLR. This leads to less rerouting of LDLR to the lysosomal pathway and therefore less degradation. The effects of these prodomain peptides may be considered as indirect. Further work in support of this PCSK9 mediated mechanism has been in progress in the laboratory. It is important to point out that two of these peptides PCSK9³¹⁻⁴⁰ and ³¹⁻⁶⁰ are highly acidic in nature and are located at the extreme N-terminal region of proPCSK9. Interestingly this region of proPCSK9 was not detectable in the crystal structures of either proPCSK9 (47, 50, 107) or the co-crystal structure of proPCSK9 and synthetic EGFA peptide (50). Our data revealed that PCSK9³¹⁻⁴⁰ containing 70% acidic and 10% hydrophobic residues is the most effective peptide in enhancing the LDLR protein level, followed by PCSK9⁹¹⁻¹²⁰ (with 3% acidic and 26% basic and 40% hydrophobic residues) and PCSK9³¹⁻⁶⁰ (37% basic and 40% hydrophobic residues). A comparison of the effects of {31-40} and {31-60} peptides suggests that the segment {41-60} which is more hydrophobic than acidic is less contributory to the observed effect on LDLR. Additionally it was also noted that the charge alone is not the only determining factor for the observed effect since a 6-mer all Asp peptide was not able to produce similar effect on LDLR in HepG2 cells (**Figure 7**). It is further noted that the middle {61-90} and

C-terminal {121-152} and {131-152} prodomain peptides did not exhibit any significant regulatory effect on LDLR levels (**Figures 3 and 5**). We believe that the secondary structure and geometrical conformation of the prodomain peptides may be playing a more significant role in its possible binding with the catalytic domain of PCSK9 thereby controlling LDLR degradation. This is further confirmed by secondary structure analysis using CD spectroscopy.

In the present study we also determined that not only do these selected propeptides increase the protein level of LDLR in a dose, and time dependent manner but they can also compensate for LDLR degradation brought about by exogenous addition of recPCSK9 (**Figure 10**). In this regard again the PCSK9³¹⁻⁴⁰ was found to be the most effective and rapid. However, among the active prodomain peptides, PCSK9³¹⁻⁶⁰ has drawn our attention, since it contains the sites for four natural mutations, E³²K, R⁴⁶L, A⁵³V, E⁵⁷K (108, 109) as well as two post-translational modifications, PCSK9-Y³⁸SO₄ (21) and PCSK9-Ser⁴⁷PO₄ (58). Following the observed effects of the prodomain peptides on LDLR degradation and the knowledge that PCSK9³¹⁻⁶⁰ has yet to be crystallized, our next goal was to identify the amino acids that are critical for the observed effect of the peptide on the LDLR protein level. Using a series of deletion peptides derived from PCSK9³¹⁻⁶⁰, we were able to show that the first 5 amino acid segment (³¹QEDED³⁵) is the most crucial for its LDLR enhancing effect since its removal led to a significant loss of function (**Figure 11**). Interestingly two known mutations are found within these five amino acids plus one additional unreported PCSK9^{R36G} mutation (*Seidah et. al. unpublished, personal communication*). In addition it also contains the posttranslational modified residue PCSK9^{Y38}SO₃ (21). Another interesting observation of our study is that PCSK9^{S47}PO₄ produced an opposite effect on LDLR

compared to that observed with the corresponding non-phosphorylated peptide (**Figure 12**). This may suggest that the overall structural conformation of the peptide is significantly modified by Ser⁴⁷-phosphorylation.

The prosegment plays a role in the interaction of PCSK9 with LDLR, and post-translational mutations within the region may lead to gain or a loss of function of PCSK9 (110). In fact a mutation of **R⁴⁶L** (this may cause non phosphorylation of adjacent **Ser⁴⁷** residue) and **Y³⁸F** (loss of sulfation), have both been shown to be loss of function mutations, and increase PCSK9s degradation by proteases (110). The biochemical effects of the Tyr³⁸-sulfation and Ser⁴⁷ phosphorylation were unknown until recently. Our study shows that both mutations decrease LDLR protein expression (**Figure 12 and 13**), leading to a gain of function for PCSK9. Tyr³⁸-sulfation is found to be present endogenously in HepG2 cells and has previously been found not to affect zymogen processing, LDLR degradation or PCSK9 secretion/folding (56). It may affect the traffic and routing of PCSK9 to the lysosomal compartment although the functional aspect of this modification is still not fully understood. In regard to the Ser⁴⁷-phosphorylation, it has been suggested that it occurs physiologically and is cell-type dependent. It possibly protects the prodomain from proteolysis and may play an important regulatory role (58). In fact our data using the {31-60} propeptide showed that Ser⁴⁷-phosphorylation decreased LDLR protein expression by 50% (**Figure 12**) in contrast to that observed with the corresponding non-phosphorylated peptide. This could be a result of increased acidity and negative charge and/or conformational effect.

To define whether charge or a change in conformation was responsible for the decrease in LDLR our next step was point mutations of the Serine at position 47. The results with the **Asp⁴⁷** substituted peptide (**Figure 13**) indicate that the observed gain of function

effect is most likely due to conformation and not negative charge alone. When the **Ala⁴⁷** peptide was added to the media of growing cells, there was no significant change in LDLR protein expression compared to PCSK9³¹⁻⁶⁰ (**Figure 13**). Alanine is not expected to alter the conformation of PCSK9³¹⁻⁶⁰ significantly; hence we speculate that any amino acid placed at position 47 that would have an effect on the overall conformation of the peptide. This will potentially affect the ability of PCSK9³¹⁻⁶⁰ to increase LDLR. There appears to be a significant structural change of the peptide induced by either Ser⁴⁷ phosphorylation or Tyr³⁸-sulfation. This will lead to a change in their interactions with LDLR and PCSK9 leading to a change in the LDLR protein level. Among the above natural mutants, all except R⁴⁶L mutant lead to a gain of PCSK9 functional activity. R⁴⁶L mutant leads to a significant loss of PCSK9 function and results in low cholesterol. Overall, our study indicates that these posttranslational modifications of the prodomain of PCSK9 may be a mechanistic cause for the up-regulation of PCSK9 and plasma cholesterol level. This conclusion is supported by the published report that PCSK9 Δ 53 is more potent in degrading LDLR than full-length PCSK9 (67).

Our study with the catalytic domain derived peptides supports the crystal structure of PCSK9 and LDLR where the catalytic domain makes contact with the EGFA domain of LDLR via residues around the catalytic Asp²¹⁸ and Ser³⁸⁶ sites (108). This contact was necessary for the degradation of LDLR by PCSK9. The catalytically derived peptides PCSK9¹⁸¹⁻²⁰⁰ and PCSK9³⁶⁸⁻³⁹⁰ showed a decrease in the LDLR protein level compared to the control (**Figure 19**). As a possible mechanism for the observed LDLR regulatory effects of some PCSK9 prodomain peptides, we speculate that these peptides may bind to a segment of the EGFA domain of LDLR. The peptides may interfere in the PCSK9 LDLR binding

process leading to a decrease of LDLR in the lysosomal pathway for its degradation. It appears that the catalytic domain peptides are acting in a similar fashion to endogenous PCSK9. When the propeptides were added in conjunction with the catalytic peptides, there was a recovery of the LDLR protein level similar to that observed with rec-PCSK9 (**Figure 20**). The catalytic region of PCSK9 is responsible for binding and the increase in degradation of LDLR on the cell surface as seen through the co-crystallization (50) and our present work with peptides.

The completion of this study has shed some light on the importance of the prodomain in PCSK9 as not only a chaperone but also as an inhibitor because of its ability to degrade LDLR. This and other peptides with LDLR enhancing effects in human hepatic cell lines may be further developed as alternate therapeutic agents for controlling plasma cholesterol level particularly for the high risk population. The observed LDLR promoting effects of the three prodomain peptides can be tested in animal models to further examine their abilities to increase LDLR and lower cholesterol levels. The significance of the conformation and secondary structure of the prodomain peptides in terms of their action on PCSK9 is evident from our preliminary 3D model structures of PCSK9³¹⁻⁶⁰, Ser⁴⁷ phosphate, Tyr³⁸ sulfate, as well as the doubly modified Ser⁴⁷-phosphate + Tyr³⁸-sulphate derivatives (**Fig 21**). The models confirm the presence of unique secondary structures and conformational geometry in the molecules that are modified by phosphorylation, or sulfation or both. Clearly our energy minimized models (produced by Hyperchem software program) indicate that the above posttranslational modifications lead to the introduction of turns and twists. Such structures are stabilized by several strong key H-bonds (shown by dotted lines) generated as a result of a modification of Ser and Tyr residues. It can be speculated that

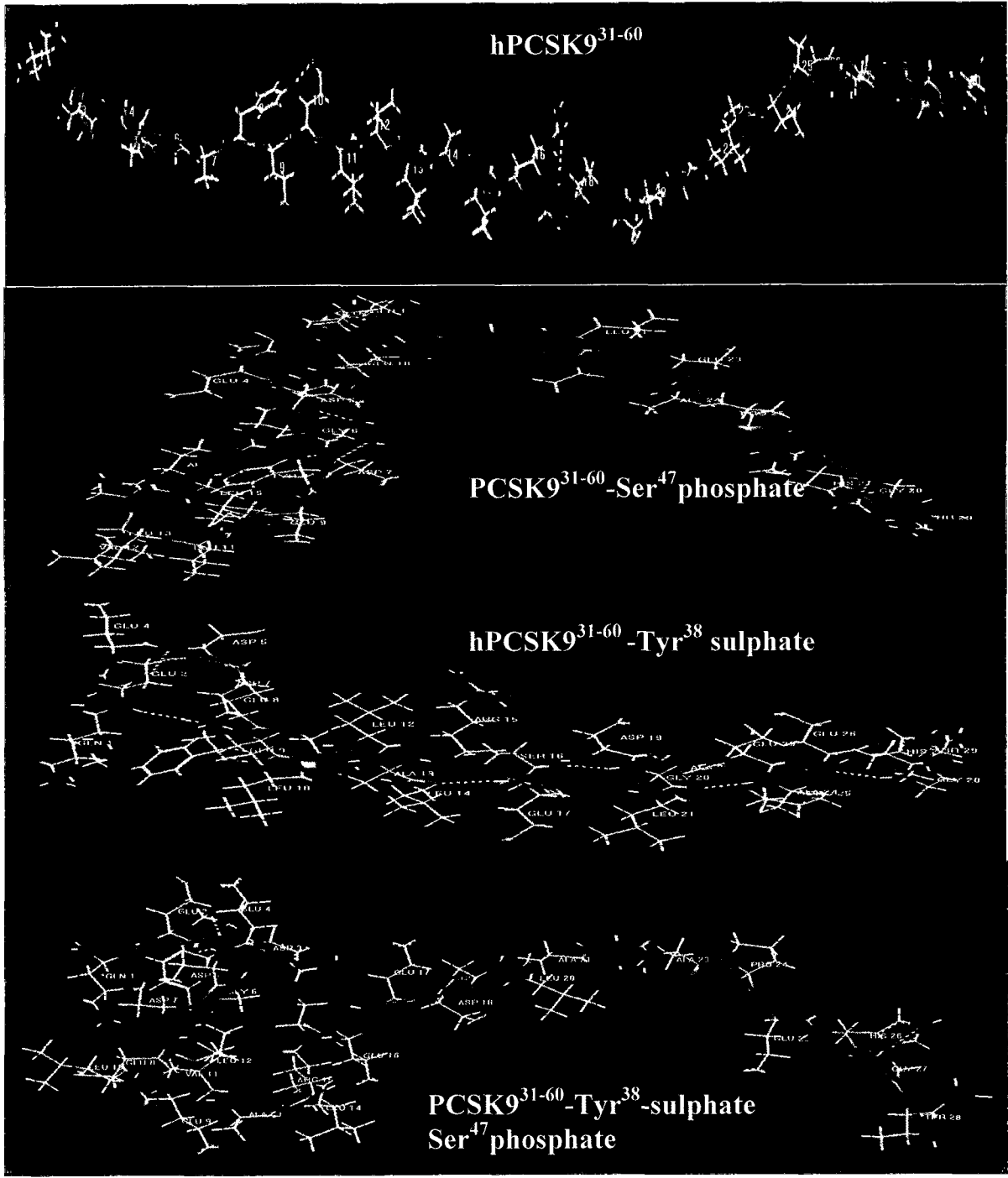
these modifications allow specific regions of the peptide to be more exposed and presented to the PCSK9 molecule thereby affecting their interactions with the catalytic domain of PCSK9.

5.2 Effects of PCSK9 pro and catalytic domain peptides on LDLR level in hepatic cells

Since its discovery in 2003, interest in PCSK9 research exploded tremendously owing to the fact that PCSK9 functional inhibitors are capable of up-regulating plasma LDLR leading to a greater clearance of cholesterol in circulation (84, 111). PCSK9 inhibitors can be useful as potential therapeutic agents for treating hypercholesterolemia with or without the conjunction of other cholesterol lowering drugs like statins (95). The gain of function mutations in human and the over-expression of PCSK9 in animal models both lead to hypercholesterolemia. This was also confirmed by PCSK9 knock out mouse model studies (70, 74) and various cellular experimental studies that lead to hypocholesterolemia. It is generally established that suppression of PCSK9's ability to degrade LDLR with specific inhibitors will be an effective method for treatment of hypercholesterolemia and associated cardiovascular disease. So far PCSK9 gene silencing, monoclonal antibody and small interfering (si) RNA techniques have been demonstrated in the literature as possible strategies for inhibiting PCSK9's function. Currently very limited information is available in the literature about PCSK9 inhibitory compounds. Only a synthetic EGF-A peptide has so far been described (95). In addition another protein called Annexin-A2 that acts as an endogenous inhibitor (52). Currently research on inhibiting PCSK9 has concentrated on the development of monoclonal antibodies and small interfering (si) RNA molecules (97, 102, 103, 112).

Our results show that the prodomain of PCSK9 is of interest as a potential regulator of PCSK9's function. This domain is known to be tightly associated with its own catalytic domain that has been recently implicated in binding with the EGFA domain of LDLR. In fact this association has been found to be so strong that it is not even affected by usual non-denaturing buffer conditions. Most efforts to dissociate the prodomain from mature PCSK9 have ended in failure (107). Studies also indicate that the autocatalytic cleavage of proPCSK9 with the concomitant release of the prodomain is necessary prior to its binding to LDLR leading its degradation (35). Interestingly six "**loss of function mutations**" of PCSK9 were located in the prodomain and one of them **R⁴⁶L** leads to the most severe loss of function mutation (113). These observations justified the present study. So far our studies using the human hepatic cell lines suggest that the peptides derived from the N- and mid terminal prodomain can up-regulate LDLR protein level in contrast to the effects of the two catalytic domain peptides that have been shown to down regulate LDLR level.

Figure 21 3D theoretical model structures of hPCSK9³¹⁻⁶⁰ peptide, its Ser⁴⁷-phosphorylated, Tyr³⁸ sulphate as well as the doubly modified hPCSK9³¹⁻⁶⁰-Ser⁴⁷-Tyr³⁸ sulphate peptide derivatives as indicated. The secondary structures of peptide backbone were shown in thin ribbon sheets. Models were generated Hyperchem software program following energy minimization using Polok-Ribiere algorithm



6.0 Conclusion

This study demonstrates that small peptides derived from the prodomain of PCSK9 as well as its catalytic domain can act *in vitro* as regulators of the LDLR protein level in human hepatoma cell lines. We observed that there were three regions of the prodomain of PCSK9 that can increase the protein levels of LDLR without any significant alteration in PCSK9 levels as observed with statin treatment. As expected, peptides from the catalytic domain will also be of interest in this respect for any future development of cholesterol lowering agents. Our data also revealed the crucial role of Ser⁴⁷-phosphorylation and Tyr³⁸-sulfation in PCSK9 mediated LDLR degradation which will need more in depth study in the future.

In summary, this is the first demonstration of how short and medium peptides derived from the prodomain of PCSK9 can alter its effect in degrading LDLR and work as an endogenous functional inhibitor. More experiments are needed to understand the detailed mechanism of action of these peptides.

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Curriculum Vitae

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Profile

A recent graduate with a Master of Science in Biochemistry at the University of Ottawa, completed in December 2009. Well organized, thorough, efficient, reliable, people oriented and recognized for her leadership roles as a senior graduate student and in customer service work. Fully bilingual in both official languages, a Canadian citizen, and a permanent resident of the National Capital region.

Education

University of Ottawa

December / 2009

Master's Degree - Biochemistry

Ottawa, Ontario, Canada

Current project entitled "Peptides Derived from Proprotein Convertase Subtilisin Kexin 9 (PCSK9) Can Regulate Cholesterol Level by Modulating Its Functional Activity to Degrade LDL-Receptor"

University of Ottawa

April / 2007

Bachelor's Degree - Biochemistry

Ottawa, Ontario, Canada

Honor's in Biochemistry with a specialization in biochemistry

Work Experience

Ottawa Hospital Research Institute, Ottawa

April / 2007 - September / 2009

Graduate Student Completed research on two proteins involved in cholesterol homeostasis, with the goal being to control the level of low density lipoprotein cholesterol in the blood stream.

Ottawa Hospital Research Institute, Ottawa

May / 2007 - September / 2007

Summer Student Studying the relationship between two proteins involved in cholesterol homeostasis. As well, the completion of previous work done with the tau protein in Alzheimer's disease.

Ottawa Hospital Research Institute, Ottawa

September / 2005 - April / 2006

Honor's Student Studied the structural features of the tau protein in Alzheimer's disease using proteomics.

FootLocker, Ottawa

August / 2001 - Present

Sales Associate

Security Clearance

Enhanced Reliability Status

December 2009

Skills

Summary of research experience

Experience in standard molecular biology techniques, including PCR, gel electrophoresis, ELISA and immunoblotting.

Excellent knowledge of immunological assays, including ELISAs, for protein binding, fluorescence microscopy for protein location and mode of action, protein and peptide antibody work through molecular immunology and cellular immunology work.

Extensive knowledge in mammalian cell culture laboratory techniques, and standard experiments, including transfections and peptide addition.

Knowledge of standard bacteriology methods

Experienced in performing mutagenesis to determine the effects of various naturally occurring mutations on the level of proteins expressed in cells, through cell culture methods.

Proficient in Protein extraction and analysis

Experience in protein biochemistry techniques including: purification and identification techniques; Gel electrophoresis, MALDI-TOF and SELDI-TOF mass Spectroscopic methods

Protein secondary structure determination by Circular dichroism analysis.

Experience in designing, conducting experiments as well as, extrapolating and analyzing experimental data.

Responsible for supervision and training of lab personnel including new students

Capable of producing accurate and reliable experimental data

Ability to maintain, handle and calibrate modern laboratory equipment and follow analytical techniques.

Self-motivated, enthusiastic and a strong ability to work within a team and independently.

Knowledgeable of laboratory safety practices

Teamwork

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, personal research, as well as being a supervisor at Footlocker.

Established ability to develop and maintain contacts within the research community to collaborate effectively, persuade, and influence the scientific community. As seen with recent publications, and grant proposals.

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.

Organizational

Excellent organization, time management and communication skills.

Ability to manage and coordinate diverse activities, including personal research, reviewing grants and papers, and instructing students and prioritize under tight deadlines according to policies and procedures set by the graduate school and OHRI.

Strong performance recognized by employers in annual progress reports for reliability, thoroughness,

integrity in work, communication and organization, in both research and customer service work. Strong analytical skills and attention to detail, trained to independently analyze, resolve difficult situations and propose change, evident from university research.

Willing to adapt to a different environment, or to different ideas to respect the integrity of the workplace, and others opinions.

Efficient and thorough in independently analyzing and resolving difficult situations by reviewing, advanced planning and taking initiative to propose change to procedures and policies, shown in graduate studies and customer service training.

Communication

Excellent oral and written communication skills in both official languages, shown in monthly meetings, supervising of associates and paper communication.

Successful interpersonal communication established through customer service training and collaborating within the scientific community by communicating with people of differing backgrounds, respecting diversity, allowing easy communication and increasing productivity by reducing conflicts. Demonstrated talent in communicating effectively under pressure and difficult situations in monthly lab meetings, and twice yearly meetings with grant members unaware of current research results and trends.

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.

Behavioral flexibility, revealed through treatment of staff members, customers and students by building trust and confidence opening the communication and leading to outstanding results for both the team and the individuals.

Technical

Excellent computer knowledge in Microsoft outlook, Microsoft word perfect, Microsoft excel, PowerPoint presentations, and MS Project.

Experienced in using software for data manipulation and analysis

Including GraphPad Prism, Sigma Plot, CIPHERGEN, Soft Sec, AlphaEase, and others.

Customer Relations and Administration

Customer service training (Footlocker, Cosmic Adventures).

Over five years of experience in the customer service industry.

Excellent organization, time management skills and communications.

Strong performance recognized by employers in performance evaluations for reliability, communications and organization.

Structures a balance between my work and athletics interests enabling success in both areas.

Experienced in cash management and inventory control, operation of cash registers, debit card and credit card transactions and preparing large cash deposits.

Excellent computer knowledge in Microsoft outlook, Microsoft word perfect, Microsoft excel, PowerPoint presentations, and MS Project.

Provides friendly, helpful, and courteous service to clients, the public, fellow co-workers, and supervisors.

Works independently with minimal supervision

Awards/Achievements

Admission scholarship for the University of Ottawa - 2002

Ontario scholar - 2002

All Star water polo team - 2003

All Star water polo team - 2004

Ontario summer games gold medalist in rowing - 2002

Interests

A member of the competitive Ottawa University Gee Gee's water polo team from 2004-2006

Assistant coach of high school swim team

Science students associations volunteer from 2004-2006.

Volunteer for the national capital race weekend from 2003-2006.

Leisure activities include, swimming, biking, rowing, water polo, running, and avid reader.