PROPROTEIN CONVERTASES AND SERINE PROTEASE INHIBITORS: DEVELOPING NOVEL INDIRECT-ACTING ANTIVIRAL STRATEGIES AGAINST HEPATITIS C VIRUS

by

Andrea D. Olmstead
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Abstract

Hepatitis C virus (HCV) utilizes host lipids for every stage of its lifecycle. HCV hijacks host lipid droplets (LDs) to coordinate assembly through the host lipoprotein assembly pathway; this facilitates uptake into hepatocytes through the low density lipoprotein receptor (LDLR). Induction of host lipid metabolism by HCV supports chronic infection and leads to steatosis, exacerbating liver dysfunction in infected patients. One pathway activated by HCV is the sterol regulatory element binding protein (SREBP) pathway which controls lipid metabolism gene expression. To activate genes in the nucleus, SREBPs must first be cleaved by host subtilisin kexin isozyme-1/site-1 protease (SKI-1/S1P). Proprotein convertase subtilisin/kexin type 9 (PCSK9) is one SREBP-regulated protein that post-translationally decreases LDLR expression in the liver. The overall aim of this thesis was to determine the potential application of these two important regulators of host lipid homeostasis, PCSK9 and SKI-1/S1P, as targets for inhibiting HCV infection.

The first hypothesis tested was that inhibiting SKI-1/S1P would block HCV hijacking of the SREBP pathway and limit sequestration of host lipids by HCV, blocking virus propagation. To inhibit SKI-1/S1P function, an engineered serine protease inhibitor (serpin) and a small molecule inhibitor were employed. Both inhibitors were shown to block SKI-1/S1P cleavage of SREBPs, reduce LD accumulation in hepatoma cells and inhibit HCV infection. The next hypothesis explored was that amplifying PCSK9 expression or function in hepatoma cells would increase their resistance to HCV infection through downregulation of LDLR. It was confirmed, using overexpression of wild-type PCSK9 or treating cells with gain-of-function PCSK9, that PCSK9 can be used to prevent HCV entry into hepatoma cells. Finally, studies are presented detailing the discovery and characterization of a non-inhibitory serpin variant with potent antiviral activity against HCV infection. It is hypothesized that inhibition may be related to antiviral functions exhibited by other human serpins or serpin-derived peptides possessing diverse regulatory properties.

Host lipid metabolism is a critical component of the lifecycle of HCV and many other viruses. These studies confirm that lipid metabolism pathways can be rationally targeted to inhibit viral infection and may lead to the development of novel, indirect-acting therapies against HCV and related viruses.
Preface

A version of the research presented in Chapter 2 has been conditionally accepted pending minor revisions to the journal PLoS Pathogens (Olmstead, A.D., Knecht, W., Lazarov, I., Dixit, S. B., Jean, F. (2011) Human subtilisin kexin isozyme-1/site-1 protease (SKI-1/S1P) is a key host factor for robust HCV infection).

In the last year of my PhD, I supervised a co-op student, Ina Lazarov who provided technical assistance for Figure 2.3 C and D and performed the western blots for Figure 2.4 E. The serpin model presented in Figure 1.6, Figure 2.1 and Figure 4.1 was generated with the program pyMOL by Dr. Surjit Dixit (Zymeworks Inc., Vancouver, BC, Canada). Dr. Wolfgang Knecht (Lead Generation - Target Production, AstraZeneca R&D, Mölndal, Sweden) contributed to this work by generating and purifying the recombinant protein SKI-1/S1P used in Figure 2.2 and Figure 4.2. A fellow PhD graduate student, Steven McArthur, performed curve fitting analysis presented in Figure 2.7 A and Figure 3.6 A and stick models generated in pyMOL which are presented in Figure 4.8.

All reagents provided by external research groups are indicated in the materials and methods.

I designed all the experiments described in this thesis together with my supervisor Dr. François Jean. I conducted all of the research, analyzed the data, and wrote the first drafts of the manuscripts presented in Chapters 2, which was then revised together with Dr. Jean.

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List of Abbreviations
AAT – α-1-antitrypsin
Ad – Adenovirus
ADRP – Adipose differentiation related protein
ALLN – α-N-acetyl-leucine-leucine-norleucinal-aldehyde or Calpain inhibitor I
Apo – Apolipoprotein
ATF-6 – Activating transcription factor 6
ATIII – Antithrombin III
bHLH-zip – Basic-helix-loop-helix leucine zipper
BSA – Bovine serum albumin
CD81 – Cluster of differentiation 81
cDNA – Complementary deoxyribonucleic acid
CDRD – Center for Drug Research and Development
CPP – Cell penetrating peptide
Ct – C-terminal
DGAT – Diglyceride acyltransferase
DMEM – Dulbecco’s modified eagle medium
DMSO – Dimethyl sulfoxide
EDTA – Ethylenediaminetetraacetic acid
EGFP – Humanized green fluorescent protein
EGTA – Ethylene glycol tetraacetic acid
E*I* – Enzyme inhibitor complex
ER – Endoplasmic reticulum
FACS – Fluorescence-activated cell sorting
FBS – Fetal bovine serum
FDA – Food and Drug Administration
FFU – Focus forming units
FH – Familial hypercholesterolemia
GP – Glycoprotein
HAART – Highly active antiretroviral therapy
HCMV – Human cytomegalovirus
HCS – High content screening
HCV – Hepatitis C virus
HIV – Human immunodeficiency virus
HMG-CoA – 3-Hydroxy-3-methyl-glutaryl-coenzyme A reductase
HS-GAG – Heparan sulfate glycosaminoglycan
Hsp47 – Heat shock protein 47
HSV – Herpes simplex virus
Huh – Human hepatoma
IDL – Intermediate density lipoprotein
IFN – Interferon
INSIG – Insulin induced gene
IRES – Internal ribosome entry site
JAK/STAT – Janus kinase/signal transducer and activator of transcription
JFH – Japanese fulminant hepatitis
kDa – Kilo Dalton
LCMV – Lymphocytic choriomeningitis virus
LD – Lipid droplet
LDL – Low density lipoprotein
LDLR – Low density lipoprotein receptor
LPDS – Lipoprotein depleted serum
LRP – LDLR-like protein
LVP – Lipoviroparticle
Mhtps – Membrane-bound transcription factor peptidase, site 1
Moi – Multiplicity of infection
mRNA – Messenger ribonucleic acid
MTP – Microsomal triglyceride transfer protein
NANB – Non-A, non-B hepatitis
NFκB – Nuclear factor κ B
NS – Non-structural
PAGE – Polyacrylamide gel electrophoresis
PAI-1 – Plasminogen activator inhibitor 1
PBS – Phosphate buffered saline
PC – Proprotein convertase
PCR – Polymerase chain reaction
PCSK9 – Proprotein convertase subtilisin/kexin isozyme type 9
PDX – α-1-antitrypsin Portland
PTGS2 – Prostaglandin synthase 2
RCL – Reactive center loop
RdRp – RNA-dependent RNA polymerase
RFP – Red fluorescent protein
RIPA – Radioimmunoprecipitation assay
RNA – Ribonucleic acid
S2P – Site-2 protease
SCAP – SREBP cleavage-activating protein
SDS – Sodium dodecyl sulfate
SEC – Serpin enzyme complex
SI – Stoichiometry of inhibition
SKI-1/S1P – Subtilisin kexin isozyme-1/site-1 protease
SP – Signal peptidase or signal peptide
Spn4A – Serpin 4A
SPP – Signal peptide peptidase
SRE – Sterol response element
SREBP – Sterol regulatory element binding protein
SRB1 – Scavenger receptor B 1
SVR – Sustained virological response
TG – Triglyceride
UTR – Untranslated region
VIRIP – Virus inhibitory peptide
VLDL – Very low density lipoprotein
VLDLR – Very low density lipoprotein receptor
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Dedication

To my mom, it is the least I can do.
Chapter 1: Introduction

1.1 Hepatitis C virus

Hepatitis C virus (HCV) is a human pathogen of immense importance in North America and throughout the globe. It is estimated that 2% of the global population is chronically infected with this blood-borne, hepatotropic virus (1). HCV is a very unique pathogen in that it hijacks and manipulates host cholesterol and fatty acid metabolism to assemble, hide, circulate and spread within an infected host. The clever adaption of HCV to the human liver is closely linked to its successful persistence and also leads to pathologies that make this virus one of the world’s leading health concerns. New scientific advances are expanding the repertoire of tools for conducting HCV research and leading to the identification of new potential therapeutic targets for treating HCV infection. Understanding further how HCV is able to successfully manipulate host lipid metabolism may unearth potential Achilles heels in the virus lifecycle and allow the design of effective weapons to combat this cunning virus.

1.1.1 Emergence and characterization

In 1989 the genome of the virus causing non-A, non-B (NANB) hepatitis was identified in a cDNA library derived from human plasma using a random priming method (2). Prior to the identification of the causative agent of this disease the virus could not be screened for and thus made its way into the transfusion blood supply. This lead to uninterrupted spread of the virus and was responsible for worldwide spread of HCV genotype 1b (3). Intravenous drug use, linked with the spread of HCV genotype 3a (3), is currently the main mode of HCV transmission in developed countries including Canada (4). However in undeveloped countries, unsafe needle practices remain a leading cause of HCV transmission (5). Egypt is the country reporting the highest prevalence of HCV infections (15 – 20 %) which is attributed to past widespread schistosomiasis treatment campaigns utilizing unsterilized glass syringes (6).

Throughout the world extensive heterogeneity exists within the HCV genome. HCV genomes are divided into 6 major genotypes (differing by ~ 30%) consisting of over 80 subtypes total (differing by ~ 20%) (7). Within an individual infected with one predominant HCV subtype, high variability in HCV composition exists; this diverse viral population is referred to as quasispecies (8). High sequence variation within HCV genomes is directly linked to error-prone HCV replication via the virus encoded RNA-dependent RNA polymerase (RdRp). RdRps lack 5’ to 3’ exonuclease activity, an essential component of polymerase proofreading function,
thus mutations are continually incorporated into nascent viral genomes. HCV replicates at a high level (9) and persists for several decades in an infected individual allowing an astounding accumulation of HCV genetic variants. The existence of quasispecies within an infected person has many important consequences including escape of host neutralizing immunity, unamenability to vaccine development and rapid resistance to direct-acting antiviral therapeutics (10-12).

1.1.2 HCV pathogenesis

The urgent need for antiviral agents against HCV derives from the potential outcomes associated with infection. Chronic hepatitis is defined as immune-mediated destruction of hepatocytes leading to progressive scarring that eventually alters the overall architecture and function of the liver, known as cirrhosis (13). Up to 80% of individuals who become infected with HCV do not resolve infection and thus chronic infection of the liver develops (14). In the majority of cases infection goes unnoticed for up to two decades before pathological consequences manifest (15). Active viral persistence within the liver leads to accumulation of fibrosis or scar tissue. Cirrhosis of the liver can develop in up to 42% of individuals; this value depends on the type of study conducted but increases with duration of chronic infection (15). Those who progress to cirrhosis have a higher risk of developing hepatocellular carcinoma and without a liver transplant, morbidity can result (16,17).

An additional pathological feature detected in over 50% of HCV infected individuals is the accumulation of fat within the liver, a condition known as steatosis (18-21). Research suggests that viral deregulation of host lipid metabolism leads to a notable increase in hepatosteatosis (22-27). This is assumed in part because the degree and frequency of steatosis development is higher in those infected with HCV than in those infected with hepatitis B virus (HBV). There is also a genetic influence of HCV on steatosis which occurs most severely in those infected with HCV genotype 3 (28-30). The significance of this condition extends from steatosis association with the degree of liver pathology (31,32) i.e. fibrosis, and correlation with HCV response to antiviral therapy (33,34).

A coinciding condition observed in HCV infected individuals, which likely results from viral induced changes in host lipid metabolism, is hypobetalipoproteinemia. A low abundance of lipoproteins in the blood may result from HCV-controlled reductions in host lipoprotein secretion (35). As discussed below, HCV interrupts assembly of host very low density lipoproteins (VLDL) as a means of integrating itself within this host lipid packaging and
exporting pathway (25). Reduced secretion of these neutral lipid vehicles from the liver into plasma is likely a major contributing factor to advanced intracellular lipid accumulation and steatosis (30).

1.1.3 Current antiviral strategies

The current, most widely used therapeutic regimen against HCV consists of a combination of pegylated-interferon-α (peg-IFN-α) with ribavirin (36). IFN treatment serves to boost the host innate immune response to viral infection by signaling naïve cells to establish an antiviral state. IFN acts through the JAK/STAT signaling pathway (a series of signaling kinases and transcriptional activators) to activate production of interferon stimulated genes (37). The mechanism of ribavirin action against HCV is unclear but many mechanisms have been proposed including shifting the TH1/TH2 adaptive immunity balance, causing premature RNA chain termination through misincorporation by the RdRP and by increasing HCV RNA mutations past an ‘error catastrophe’ threshold (36).

IFN-α was shown to be effective in treating HCV infection prior to discovery of the infectious agent causing NANB-hepatitis (38). Since then, improvements in anti-HCV treatments have been implemented including the pegylation of IFN to increase its half-life and the addition of ribavirin, which enhances the efficacy of IFN but cannot cure HCV on its own (39,40). The goal of antiviral therapy is to achieve a sustained virological response (SVR) where no plasma viral RNA is detected after 6 months of treatment termination. With HCV genotype 1, the most prominent genotype in North America, SVR rates are achieved in only ~40% of individuals (41). Less prevalent genotypes of HCV (i.e. 2 and 3) have a higher response to the anti-HCV therapeutic regimen. The outcome of treatment is influenced by a variety of other factors including initial plasma viral load, age, race, gender, obesity and degree of fibrosis (36). In addition to poor treatment response rates, peg-IFN-α and ribavirin cause an array of intolerable side-effects and the regimen is contraindicated in many patients with additional medical conditions.

Fortunately, we have entered a new age of anti-HCV therapeutics. Two new direct-acting antivirals targeting the HCV encoded serine protease, non-structural (NS) 3/4A, have been approved for use in conjunction with peg-IFN-α and ribavirin (42). These new agents increase the SVR rate in both treatment naïve and treatment non-responder patients (patients who did not achieve SVR with peg-IFN-α and ribavirin). Telaprevir and Boceprovir, from Vertex Pharmaceuticals and Merck respectively, were designed to specifically target HCV genotype 1
and are only approved for use against this HCV variant. Many additional HCV antivirals are currently in clinical trials and target a variety of HCV components (Table 1.1). Of particular interest is the indirect-acting (i.e host-directed) antiviral agent Debio 025 from Novartis, which may have an advantage over other pharmaceuticals in its potential to sidestep viral resistance. This drug is directed at a host protein, cyclophilin A, which is required by HCV for replication and has passed Phase 2 clinical trials (12,43,44).

1.2 The liver and host lipid metabolism

A very fundamental and critical aspect to consider when studying a pathogen of interest is the tissue(s) or organ(s) targeted by the infectious agent. Tissue tropism influences mode of transmission, determines the type and potential severity of symptoms resulting from infection, and also limits the types of therapies that can be utilized for fighting the pathogen. The tissue targeted by HCV is of course the liver, an organ playing a central role in regulating energy metabolism throughout the body. HCV infection seems to be highly limited to this organ, hepatocytes specifically, although negative strand viral RNA has been detected in extrahepatic sites (45).

Following inoculation of HCV into the blood stream it reaches the liver through sinusoidal vessels. Sinusoidal endothelial cells may capture HCV particles and promote infection of neighboring hepatocytes (46). Productive infection of the liver is initiated by binding and uptake of HCV via a complex set of cell surface receptors, some playing important roles in lipoprotein metabolism (see section 1.3.2). Once inside hepatocytes, HCV takes full advantage of metabolic pathways important for liver regulation of host lipid homeostasis; deregulation of these pathways supports virus propagation and leads to pathologies associated with liver dysfunction including cirrhosis and steatosis (reviewed in (47)).

1.2.1 Lipoproteins

Cholesterol that passes through the liver can be obtained through diet or it can be synthesized de novo (48). Nearly everything that passes through the intestines is processed first by the liver thus plasma metabolites are controlled by this organ (49). The liver is also the major site of cholesterol synthesis in the body (50); 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase catalyzes the rate limiting step towards cholesterol biosynthesis and its expression and stability are controlled by intracellular cholesterol levels through several mechanisms ((49,51,52)).
When lipid levels are plentiful the liver will esterify dietary or newly synthesized fatty acids and cholesterol. These are stored within lipid droplets (LD) as triglycerides and cholesterol esters. These LDs are dynamic lipid storage organelles thought to form from the ER and are made up of a core of neutral lipids surrounded by a phospholipid monolayer and specific perilipin proteins (53). Any excess cholesterol or fatty acids not required within the liver are packaged into lipid carrying vehicles called VLDL for secretion into the plasma and delivery to extrahepatic tissues (Figure 1.1) (49,54).

VLDLs are assembled onto an apolipoprotein B (ApoB) backbone; lipids are loaded onto ApoB while it is being synthesized and translocated into the ER lumen. Transfer of lipids onto ApoB in the ER requires microsomal triglyceride transfer protein (MTP) activity (55). Final lipidation and maturation of VLDL occurs within the Golgi prior to secretion of these particles (56) and may involve fusion of LDs with assembling VLDL (53). The final VLDL particles consist of a core of neutral lipids, stabilized by the ApoB backbone that is surrounded by a phospholipid monolayer. Following secretion of VLDL the constituent lipids are hydrolyzed by lipases for absorption by tissues; this increases the lipoprotein density generating intermediate density IDL (57) that can be endocytosed back into the liver. Further IDL hydrolysis generates LDL, the main carrier of cholesterol in human circulation. Unlike ApoB, other apoproteins can freely exchange between lipoproteins; ApoE for example can associate with assembled VLDL particles (56,58).

1.2.2 Low density lipoprotein receptor

Assembly of lipoproteins by the liver is a major means of cholesterol delivery to extrahepatic tissues. Utilization of the packaged cholesterol requires both recognition and uptake of circulating lipoproteins into a cell. The LDL receptor (LDLR) is a cell surface receptor playing a critical role in LDL uptake (51). LDLR binds ApoB and ApoE with high affinity via the extracellular domain. Binding induces clathrin dependent endocytosis of the bound lipoprotein which is followed by fusion with lysosomes. The low pH triggers the release of the LDL, which is hydrolyzed, from LDLR, which is recycled back to the cell surface (59,60). The physiological relevance of the LDL/LDLR interaction is evident from people with autosomal dominant mutations in LDLR or ApoB which interferes with LDLR-dependent LDL uptake. These mutation results in a genetic disorder called familial hypercholesterolemia (FH) which is associated with greatly elevated plasma LDL cholesterol levels and early onset of heart attacks ((61) and reviewed in (51)).
1.2.3 Sterol regulatory element binding protein pathway

Due to the importance of LDLR regulation of host cholesterol homeostasis it is unsurprising that the expression of this protein is tightly regulated by both transcriptional and post-translational mechanisms (62-65). The major pathway controlling transcription of LDLR, HMG-CoA reductase and other lipid metabolism genes is called the sterol regulatory element binding protein (SREBP) pathway (Figure 1.2). SREBPs are members of the basic-helix-loop-helix leucine zipper (bHLH-zip) family of transcription factors. They are initially localized to the ER and pass through the ER membrane via two transmembrane domains which are connected by a luminal loop and flanked by two cytosolic domains (Reviewed in (66,67)). The N-terminal bHLH-zip domain acts as the transcription factor and binds sterol response elements (SRE) in the promoters of lipid metabolism genes such as LDLR (68). The C-terminal domain interacts with regulatory proteins in the ER such as SREBP cleavage-activating protein (SCAP) that respond to changes in intracellular sterol levels (69). When sterol levels in the cell are high, SREBPs are retained, inactive, in the ER (70). This retention is due to SREBP interaction with the sterol responsive protein SCAP which in turn interacts with insulin induced genes (INSIG)-1 and -2 (71). When sterol levels drop and the need for cholesterol uptake and synthesis is induced SCAP is released from INSIGs and escorts SREBPs through the secretory pathway to the Golgi (69). In the Golgi apparatus, SREBP encounters two proteases that target and cleave SREBP molecules. A proprotein convertase called subtilisin kexin isozyme-1/site-1 protease (SKI-1/S1P) mediates the first cleavage in the luminal loop of SREBPs (72). The separation of SREBP into two halves permits intramembrane proteolysis of the N-terminal half of SREBP by a cysteine metalloprotease called site-2 protease (S2P) (73). This cleavage liberates the N-terminal, soluble, SREBP active domain whereupon it is translocated into the nucleus and can bind to and activate SREs (68,74). Three SREBP isoforms have been characterized; SREBP-1a and 1c are splice variants from the same gene (75). SREBP-1c primarily regulates genes involved in fatty acid synthesis (76). SREBP-2 is expressed separately and mainly controls genes involved in cholesterol metabolism (77). Research continues to accumulate revealing additional layers of complexity in the regulation of SREBP expression and function and in the genes controlled by SREBP activation (67).

1.3 Hepatitis C virus molecular biology

HCV is a hepacivirus member of the Flaviviridae family which are enveloped viruses encoded by a single-stranded positive sense RNA genome (78). The HCV genome is
approximately 9.6 kb (~3000 amino acids) with flanking 5’ and 3’ untranslated regions (UTR) (Figure 1.3). The UTRs are highly conserved, exhibit extensive secondary structure and are required for HCV replication (79-82). Translation is cap independent and is initiated within the internal ribosome entry site (IRES) located within the 5’ UTR (83). Translation occurs at the rough endoplasmic reticulum (ER) membrane and yields production of one long polyprotein containing 10 multifunctional viral proteins that must be liberated by host and virus mediated cleavages (79,84).

1.3.1 HCV proteins

The viral core and envelope proteins (E1 and E2) are located at the N-terminus of the polyprotein (85) followed by the putative ion channel protein, p7 (Figure 1.3). Each of these proteins is released from the polypeptide by host signal peptidase cleavage. The core protein is initially localized to the ER but is released following a second cleavage mediated by host signal peptide peptidase allowing it to rapidly associate with LDs (86). The core protein acts as the major capsid subunit of the virion and also possesses RNA binding properties. The envelope proteins, E1 and E2, are Type 1 transmembrane glycoproteins that are mainly retained within the ER prior to virus egress. E1 and E2 form non-covalent heterodimers that act as the main structural component of the virus envelope and mediate entry of virus particles (87). A putative viroporin is formed by p7 which forms oligomers possessing cation channel activity (88,89). The HCV cysteine protease, NS2 releases itself from the polyprotein (90) and plays an emerging role in HCV assembly together with p7 (91,92). NS3 is a well-studied serine protease that releases itself and the remainder of the viral proteins from the polyprotein (93). NS3 also possesses helicase and NTPase activity (94). NS4A, located next to NS3 in the polyprotein is a transmembrane protein that acts principally as a cofactor for the NS3 protease, tethering it to intracellular membranes and increasing proteolytic activity (95,96). NS4B is most well-known for its ability to induce membranous web formation within the cell, an important feature of HCV replication (97). Research on NS5A is currently revealing it as a critical member of virus assembly (98) in addition to playing indispensable roles in virus replication (99). The phosphorylation status of NS5A may act as a switch between viral replication and assembly (100,101). NS5B is the HCV RdRp responsible for replicating nascent viral genomic RNA (102).
1.3.2 The HCV lifecycle

The HCV RNA genome is capable, on its own, of initiating the fully infectious lifecycle of HCV. This is demonstrated by the spread of HCV infection in chimpanzees inoculated with HCV RNA into their liver (103). Also, susceptible hepatoma cell lines transfected with full length HCV RNA can produce infectious HCV particles (104). During infection, when HCV RNA is released into the cell cytoplasm it is recognized by host ribosomal machinery and translated into a single polyprotein (83) (Figure 1.4). The viral proteins are released at the ER as described above and initiate various events required for propagation. The non-structural proteins NS3, NS4A, NS4B, NS5A and NS5B are required for replication of the virus genome (105) which occurs at reorganized intracellular membranes on cholesterol enriched, lipid raft-like structures (79,106,107). The membranous web harbors viral replicase complexes consisting of viral and host proteins and nascent HCV RNA (106,108,109). This web is predicted to shield replicating RNA and prevent activation of the intracellular innate immune response.

The core protein when it is released from the ER, moves to host LDs (86), a process which requires host diglyceride acyltransferase (DGAT)-1 (110). Host LDs surrounded by the core protein have been proposed as the main site of HCV assembly (111-113). This is based on the fact that blocking HCV association with LDs blocks release of infectious virus particles (114,115). Association of HCV core with LDs is associated with increased LD size and reorganization of cytosolic LDs to the host perinuclear region (23,24,116). NS5A may act as a switch delivering replicating RNA to the core protein for encapsidation (111). Encapsidation of HCV RNA and assembly of viral particles is likely dependent on the concerted action of core with NS2, NS5A and p7 (91,98,111,117). In particular, p7 may mediate a connection between the envelope proteins and the replicase components (118) and NS2 may mediate multiple contacts bringing all components required for HCV assembly together (119).

Following initial nucleocapsid formation, HCV recruits components of the host VLDL synthesis and secretion pathway to produce fully infectious HCV particles (25,120,121). Although the precise steps remain unclear, virion formation likely involves initial budding of core particles into the ER where the viral envelope is acquired. Subsequently the virus accumulates neutral lipids and apoprotein components. The activity of MTP has been shown to be necessary for infectious virus production as have ApoB and ApoE (120-123).

Virus particles are secreted from the cell as a very heterogeneous population with varying degrees of density and composition (124-126). Low density HCV particles, enriched in neutral
lipids and associated with host lipoproteins, have been named lipoviroparticles (LVP). It has been proposed that the lowest density fraction of HCV has the highest specific infectivity (126,127). This has been attributed to the association of virions with LDL components which promotes interactions of nascent viral particles with cell surface lipoprotein receptors (123,128-131).

Infection of naïve hepatocytes is initiated by LVP binding to heparan sulfate glycosaminoglycans (HS-GAG) and LDLR (128,132-134). HCV binding to the cell surface can be blocked by pretreatment with LDL or heparin, supporting the importance of HS-GAGs and LDLR for early HCV interactions. Following initial binding, HCV interacts with the lipoprotein receptor SRB1 and the tetraspanin CD81 via direct interactions with E2 (131,135,136). SRB1 may act by multiple facets to promote HCV uptake including interactions with HCV associated lipoprotein components (130,137). These receptors likely act post-attachment to support HCV entry. This is supported by the fact that HCV entry can be blocked by antibodies against SRB1 and CD81 added post-adsorption of HCV particles to the cell surface (132,138). In addition, two host tight junction proteins have been implicated in post-attachment steps of HCV entry. Claudin-1 has not been shown to directly interact with HCV envelope proteins but instead may function through direct interactions with CD81 (139-141). Occludin may be responsible for the strict host specificity of HCV infection as expression of this protein in mouse cells renders them permissive to HCV entry (142). Each of these host proteins are thought to act in a well-coordinated manner, likely as a complex, to mediate clathrin-dependent endocytosis of HCV (140,141,143,144). Following endocytosis, the viral envelope is proposed to fuse with host endosomal membranes by a process that is not well defined (127). Low pH and putative envelope fusion peptides are likely to be important for release of the HCV RNA genome and continued propagation of HCV within the newly infected cell (127,145,146).

1.3.3 HCV and host lipid metabolism

Hijacking of host lipids and their biosynthetic pathways is a common strategy utilized by many pathogenic microbes to support infection. HCV may be the best example of a virus that depends on host lipid metabolism for successful propagation. Each stage of the HCV lifecycle is intricately dependent on different aspects of host fatty acid, cholesterol and related metabolite metabolism (Reviewed in (47,147-150)). Replication of viral RNA occurs on detergent resistant membranes reminiscent of lipid rafts (106,107). Geranylgeranylation of the host protein FBL2 is absolutely required for successful viral replication (151,152). As mentioned above, host LDs are
important for virus assembly. LDs provide a platform for virus assembly and permit integration of the virus lifecycle with host VLDL assembly (Figure 1.4) (25,111,120,121). Lipids from LDs, triglycerides and cholesterol esters, are incorporated into nascent HCV particles (153).

Unintuitively, it appears that HCV slows or impedes the host VLDL assembly pathway blocking the activity of its components (25) and reducing lipoprotein secretion (30,35). It has been proposed that this delay is required for HCV to piggy-back onto this pathway. Hijacking of the VLDL pathway is not only important for assembly but has downstream consequences. Virus particles are secreted into plasma as a very heterogeneous population with varying densities and are associated with various lipoprotein components (124-126,154). Association with lipoproteins may shield HCV from the immune system and permit enhanced entry into hepatocytes; the lowest density fraction of HCV has been determined to be the most infectious in both cell culture and in chimpanzees (123,126,155,156).

1.3.4 HCV hijacking of the SREBP pathway

The intimate dependence of HCV on host lipid metabolism has resulted in evolution of mechanisms allowing HCV to stimulate the production and accumulation of lipids within liver cells (26). It is unsurprising then that HCV has developed mechanisms to control one of the most important cholesterol regulatory pathways in the human body; the SREBP pathway. Numerous studies indicate that HCV controls SREBP-1 and -2 activation and expression via several mechanisms such as by inducing SREBP phosphorylation (27,157) and transcriptional activation (158,159). This activation leads to increased expression of proteins involved in synthesizing and taking up lipids into hepatocytes and is likely a contributing factor to HCV-induced steatosis (160).

1.4 Proprotein convertases regulating lipid homeostasis

The members of the proprotein convertase (PC) family of secretory pathway serine proteases are required for maturation and activation of a variety of host and pathogen precursor propeptides (Figure 1.5; reviewed in (161)). Precursors include but are not limited to bacterial protoxins, viral glycoproteins, prohormones and growth factors. Tissue specific expression and compartment specific cleavages mediated by the PC members, allows spatial and temporal activation of proteins with important regulatory and signaling functions. In humans, nine PC members have been identified and characterized. The PC members possess homology to bacterial subtilisin and yeast kexin. Each of the PC members must undergo autocatalytic
cleavage events that remove the N-terminal prosegment which assists protein folding and regulates the protease active site. Seven members, furin, PC3, PC1/3, PC4, PC5/6, PACE 4 and PC7 cleave substrate peptides after basic residues. The two most recently recognized members of this family SKI-1/S1P and PCSK9 cleave after non-basic residues and make important contributions to host lipid homeostasis (Figure 1.5).

1.4.1 Subtilisin kexin isozyme-1/site-1 protease

SKI-1/S1P plays a critical role in host lipid metabolism as it mediates an essential cleavage event required for SREBP activation as described in Section 1.2.3 (72,162). SKI-1/S1P is a ubiquitously expressed transmembrane secretory pathway enzyme that cleaves after the amino acid consensus motif RX(V,L)(K,F,L) (163). In the ER, SKI-1/S1P undergoes autocatalytic cleavages to release the N-terminal propeptide domain yielding a mature ~106 kDa membrane anchored protein (164) or an additional cleavage causes shedding of SKI-1/S1P ectodomain (165). Following activation, SKI-1/S1P moves to the Golgi apparatus where it can mediate proteolytic cleavages of its targeted substrates. The most well studied substrates are the SREBP family members but other substrates have been identified including ATF-6 which plays a role in the ER-stress response (166). SKI-1/S1P has also been implicated in the cleavage of hemorrhagic fever virus glycoproteins (167-169). Liver specific SKI-1/S1P knockout in mice results in 50% reductions in circulating cholesterol and fatty acid levels confirming its importance in regulation of host plasma cholesterol homeostasis. These studies also suggest that targeting SKI-1/S1P activity specifically in the liver is a viable therapeutic opportunity for the treatment of hypercholesterolemia (170,171).

1.4.2 Proprotein convertase subtilisin/kexin type 9

Recently, the proprotein convertase subtilisin/kexin type 9 (PCSK9) was identified as the third major locus contributing to autosomal dominant hypercholesterolemia after LDLR and ApoB (172). The mechanism by which hypercholesterolemia results was elucidated to PCSK9 being a major post-translational regulator of LDLR levels in the liver (63,64,173). PCSK9 is most abundant in hepatocytes and in small intestine enterocytes (174) where it binds LDLR at the cell surface (175) separately from the LDL binding domain (176). Following receptor mediated endocytosis, the tight binding of PCSK9 to LDLR prevents LDLR recycling back to the plasma membrane (177); instead, LDLR is degraded in lysosomes (176). This occurs because PCSK9 prevents an acid-dependent conformational change in LDLR that normally signals
recycling to the cell surface. Mutations in PCSK9 leading to disease thus have varying effects on liver LDLR cell surface expression disrupting normal uptake of plasma cholesterol into the liver. Gain-of-function mutations in PCSK9 have been associated with hypercholesterolemia (172,178,179) due to lower levels of LDLR and reduced clearance of plasma LDL. Loss-of-function PCSK9 mutations are conversely associated with abnormally low circulating cholesterol levels due to increased LDLR abundance on the surface of liver cells (180,181).

PCSK9 is also synthesized as a luminal proprotein in the ER where it undergoes autocatalytic cleavage to remove its N-terminal prosegment (Figure 1.5) prior to secretion and cell surface association with LDLR. In contrast to the other PC members, the prodomain of PCSK9 remains associated to the active site of the protein throughout secretory pathway transit and secretion, steadily inhibiting catalytic activity. Catalytic activity of PCSK9 plays no role in regulating LDLR levels, in fact the only known function of PCSK9 catalysis is for removal of the prodomain, and no other substrates have been identified. PCSK9 expression is also regulated by sterol levels, specifically by SREBPs (182,183). Paradoxically, both PCSK9 and LDLR are upregulated in response to low sterol levels in the liver. Coinduction of these two proteins may control reuptake of liver secreted lipoproteins by LDLR and instead allow their delivery extraneous tissues.

Further research has shown that PCSK9 can interact with other members of the LDLR family of receptors such as VLDLR (184). In addition, one putative natural regulator of PCSK9 has been identified; Annexin A2 was shown to directly interact with and regulate PCSK9 mediated turnover of LDLR (185).

1.4.3 Host proprotein convertases as antiviral targets

Many researchers have conducted proof-of-concept studies highlighting the potential application of inhibiting PCs to block virus infection. Many viruses and bacteria utilize host proteinases to activate critical pathogenic components. Influenza virus, HIV-1, HCMV and Lassa virus for example express precursor glycoproteins that must undergo host-dependent cleavage events within the secretory pathway to produce functional, fusion competent, glycoproteins essential for virus infectivity (167,186-189). For this reason much research has been directed towards developing methods for therapeutically inhibiting these cleavage events.

The role of furin in cleaving glycoprotein precursors of various pathogens has made it a potential broad-based antiviral target. Natural serine protease inhibitors (serpins; see section 1.5 and Figure 1.6) such as α-1-antitrypsin (AAT) have been engineered to target furin (i.e. α-1-
antitrypsin Portland or PDX) and can block glycoprotein maturation and reduce infectivity of HIV-1 and HCMV (190-192). Interestingly, recombinant PDX can be applied extracellularly and cause depletion of furin from treated cells. This reversible process is sufficient to block HCMV glycoprotein B (gB) processing and HCMV infectious virion production. The *Drosophila melanogaster* serpin Spn4A, which is the most potent inhibitor of furin identified to date (described in Section 1.5.4), has also proven effective for blocking HIV-1 and highly pathogenic Influenza virus propagation in cell culture models of infection ((193) and unpublished data).

Lassa virus, a hemorrhagic fever virus of the *Arenaviridae* family, requires functional SKI-1/S1P activity for glycoprotein precursor (pre-GPC) cleavage into GP1 and GP2. An engineered serpin variant of human AAT was designed to inhibit SKI-1/S1P and was shown to block Lassa virus GPC processing, and to inhibit Lassa virus replication and viral spread (194). An engineered variant of Spn4A targeting SKI-1/S1P can also block Lassa virus GPC processing (M. Chan *et al.*, unpublished data). Recently, a mouse model was developed whereby a missense mutation was introduced into the SKI-1/S1P gene, *Mbtps1* (195). Research demonstrated that these mice were resistant to chronic LCMV infection, a virus that belongs to the same family as Lassa virus (196). Peptide-based and small molecule inhibitors against the PCs have also been developed and confirm that inhibiting viral glycoprotein activation is a promising therapeutic avenue to explore (197-200).

### 1.5 Serine protease inhibitors (serpins)

Serine protease inhibitors (serpins) are a large family of proteins found throughout nature that are classified according to a conserved tertiary structure ([Figure 1.6](#)) (201). The functions of serpins are very diverse, and many serpins are in fact non-inhibitory (202,203). Inhibitory serpins play important roles in regulating coagulation, fibrinolysis, inflammation and complement activation through inhibition of serine proteases involved in these important host pathways. The prototype human serpin is α-1-antitrypsin (AAT) which is an acute phase response protein upregulated and secreted by the liver during inflammation. AAT inhibits neutrophil elastase which contributes to connective tissue turnover in the lung. Inherited mutations in AAT are associated with the development of pulmonary emphysema (204) and can also lead to liver cirrhosis and hepatocellular carcinoma (205). Another serpin of clinical interest is the human neuroserpin which functions within the brain and has been shown to limit damage to nerve cells following a stroke by controlling neurovascular permeability (206). Non-inhibitory serpins such
as the heat shock protein, Hsp47, and hormone binding globulins function through regulatory interactions with other target host proteins (202,207).

### 1.5.1 Serpin structure

The sequence homology between serpin family members can be quite limited however the overall secondary and tertiary structures are very similar consisting of a highly conserved core (201,208). Serpins average approximately 50 kDa but can be as large as 100 kDa depending on the length of N- and C-terminal extensions and the degree of glycosylation. **Figure 1.6** shows the structure of a typical serpin in the native state (Spn4A, see Section 1.5.4) which is largely a globular protein with an extended, exposed loop projected away from the main body. This strand, called the reactive center loop (RCL) is located near the C-terminus of the serpin and dictates proteinase specificity (209,210). The RCL acts as an enzyme substrate as discussed below and is cleaved following proteolytic attack. The largest structural feature of the serpin body is β-sheet A – formed of 5 strands in the native state but, upon cleavage of the RCL, the N-terminal portion of the loop becomes inserted into β-sheet A converting it into a 6-stared fully anti-parallel β-sheet. Two other β-sheets, B and C, exist within the serpin but are smaller that β-sheet A. The rest of the molecule consists of 8 α-helices which are also highly conserved. Relative to β-sheet A, all of these helices are behind the structure with the exception of helix F which lies across the front. Variations in this core structure exist within other serpins, including additional domains mediating interactions with regulatory cofactors such as heparin and ER-retention sequences at the C-terminus of secretory pathway neuroserpins (211).

### 1.5.2 Mechanism of action

The serpin’s native folding state is a characteristic metastable structure possessing all of the necessary features linked to its elegant inhibitory mechanism; each component of the serpin acts in concert to target, trap and disable a specific protease (reviewed in (212)). First, the serpin must be recognized by a target protease. This is achieved through presentation of the nonstructured reactive center loop (RCL) which is displayed above the main body of the serpin (**Figure 1.8**) (213,214). The amino acid sequence of this serpin is the primary determinant of inhibitory specificity and dictates which enzyme is targeted. Typically the RCL will contain a “bait sequence” that is recognized by the target enzyme as an inconspicuous substrate. The designation P1 indicates the amino acid directly preceding the cleavage site whereas P1’ indicates the amino acid to the C-terminal side of the sissile bond (215). Thus a reversible
interaction between the serpin reactive bond and the enzyme active site (EI) initiates the proteinase reaction (Figure 1.7). Following initiation of RCL cleavage an intermediate covalent bond forms between the enzyme’s active site serine and the P1 residue of the RCL (EI’, Figure 1.7) (216). In a normal enzyme substrate scenario, deacylation of the enzyme occurs following cleavage of the enzyme substrate (E + I*). The serpin however is poised to trap the enzyme in this intermediate acyl-enzyme complex stage before the serpin RCL can be released (217,218). The result is formation of a covalent enzyme-inhibitor complex (EI*) that is essentially irreversible and is both heat- and SDS-stable.

The key to this mechanism is the translocation of the N-terminal portion of the RCL from its pre-cleavage location above the serpin to the opposite pole of the serpin (Figure 1.8) (217,219). The conformational shift is energetically driven by the release of the serpin from its native metastable fold allowing it to reach a thermodynamically stable conformation (220-222). Initiation of this major conformational change is dependent on the hinging of RCL residues near the main body of the serpin which bring the RCL down and direct integration of the RCL into the hydrophobic core of the serpin (223,224). In fact, the cleaved RCL is integrated into the main structural β-sheet of the serpin (β-sheet A) and forms a perfect anti-parallel β-strand within this nascent 6-stranded structure (217). The faster the rate of serpin RCL insertion within β-sheet A in relation to the enzyme’s inherent rate of deacylation dictates the propensity of serpin passage through the inhibitory as opposed to the substrate pathway as depicted in Figure 1.7 (224). In other words, if the cleaved RCL cannot translocate fast enough into β-sheet A or if translocation is blocked in some other manner then the serpin will simple be cleaved and released by the enzyme as a substrate (E + I*).

1.5.3 Carboxy-terminal serpin activities

Cleavage of a serpin by a protease logically generates two fragments, a C-terminal and an N-terminal fragment. While the portion of the RCL that becomes integrated within β-sheet A is part of the N-terminus, the C-terminus forms two β-strands within β-sheet B that is buried inside of the serpin (217). The C-terminus of AAT and related serpins has been the focus of many studies due to its ability to mediate uptake and clearance of serpin-enzyme complexes (SECs) (225-227), its ability to modulate several important cellular and biological functions (228-232), and because of its hydrophobic cell penetrating peptide (CPP) properties (233,234).

The C-terminus of many serpins, including AAT and antithrombin (ATIII), has been proposed to carry an epitope required for clearance of SECs from circulation (227,235) via a
putative SEC-receptor (235). This receptor has been well characterized biochemically but has never been purified or cloned (236). A series of studies demonstrated that the C-terminus of AAT carries a hydrophobic, 5 amino acid, epitope (FVFLM) that is sufficient and necessary to mediate binding and uptake of ATT, ATIII and to a lesser extent C1 inhibitor (all in a cleaved or complexed state) into hepatoma cells (225-227). An intact purified C-terminal peptide or a portion thereof containing the FVFLM or a closely related sequence can compete for binding, uptake and lysosomal degradation of SECs (225). Importantly, this pentapeptide is sufficient to upregulate the expression of AAT, an activity previously attributed to AAT cleavage and clearance (225,235). Additionally, this pentapeptide domain is highly conserved within the C-terminus of many human serpins (226). It has been put forth that this epitope only becomes exposed once the serpin becomes cleaved, as native serpins cannot compete for SEC or C-terminal peptide binding to the SEC receptor (225) and in vivo native serpins are cleared more slowly than their SEC counterparts (237-239). On the other hand, arguments have been made that the integration and burying of the C-terminal peptide within the intact cleaved serpin precludes it from binding to this unidentified receptor (217,240).

Research to date has shown that clearance of all SECs does not occur through one conserved mechanism or pathway; many different receptors, binding domains and accessory proteins have been implicated for different serpin-protease pairs (241). Several other receptors have also been implicated in clearance of SECs and interestingly, most of them belong to the LDLR family of receptors and include the LDLR-like protein (LRP), the very low density lipoprotein receptor (VLDLR) and gp330 (235,241-246). Importantly, these receptors have been cloned and characterized in vivo for their roles in SEC clearance. The interaction of SECs with these receptors is also linked to serpin activation of biological signaling pathways by serpins (247).

The C-terminus of many serpins has been shown to activate signaling pathways within the cell through binding to SEC receptors such as induction of cellular proliferation (229), chemoattraction of neutrophils (247) and upregulation of AAT expression (225,232). The C-terminus of AAT has also been shown to act directly by interacting with intracellular components. These activities, which are discussed in detail in Section 4.4.2, include interactions with host transcription factors (248), localization to the cellular nucleolus (233) and binding to HIV-1 glycoprotein (gp41) which disrupts viral envelope fusion (249). Direct modulation of intracellular events by the C-terminal peptide of serpins presumably requires dissociation of this
peptide from the N-terminus of the serpin. How this may occur is largely unexplored experimentally, but isolated C-terminal peptides (i.e. AAT and C1 inhibitor) have been detected in human body fluids (249-251). Once dissociated this peptide may act as a CPP, due to its highly hydrophobic nature (233) or alternatively it may act through interaction with cell surface receptors as discussed above.

1.5.4 The Drosophila melanogaster secretory pathway serpin, Spn4A

A novel, unique serpin discovered in Drosophila melanogaster was originally cloned by Han et al. (252). This novel serpin, originally named Spn4, was hypothesized to be an inhibitory serpin due to the presence of conserved residues within the hinge region important for RCL translocation and insertion into β-sheet A (See pyMOL model of Spn4A, Figure 1.6 and section 1.5.2). It was subsequently determined that the A variant of Spn4 (Spn4A) expresses a signal peptide at the N-terminus and predicted to direct translocation into the cellular secretory pathway (253). Richer et al. discovered a unique feature of Spn4A being that it has an 18 residue RCL instead of the usual 17 residues (212,254). It was determined that the length of this RCL was critical for inhibition of the Drosophila PC, dPC2. It was also discovered that Spn4A encodes within its RCL a consensus cleavage sequence for the human secretory pathway PC furin (254). Biochemical and cellular studies revealed that Spn4A is the most efficient serpin against the PCs identified to date. It was shown that Spn4A partitions exclusively through the inhibitory pathway when incubated with furin and inhibited the enzyme with a stoichiometry of inhibition (SI) of ~ 1 i.e. only 1 mol of serpin was required to inhibit 1 mol of enzyme. Mass spectrometry analysis confirmed the P1 cleavage site to be 18 residues from the conserved glutamate hinge residue. Expression of Spn4A in eukaryotic cells revealed its functionality in the context of these cells. An additional feature noted for Spn4A was the presence of a putative ER-retention signal at the C-terminus, HDEL. The unique features of Spn4A have rendered it a useful molecular tool for studying host secretory pathway proprotein convertases in the context of both cellular functions and viral infections.

1.6 Research hypothesis and rationale

Targeting virus encoded factors does not always avoid the induction of serious side effects in patients undergoing antiviral treatment. This has been clearly seen in HIV-1 infected patients undergoing HAART regimens as well as those utilizing the Influenza virus ion channel inhibitor, Oseltamivir (255,256). In addition to side effects associated with direct-acting
antivirals there is a continuous and seemingly unavoidable development of viral resistance against these agents especially viruses encoded by an RNA genome. Due to poor fidelity and lack of proofreading function of RdRps, RNA viruses continually alter their genetic code; those mutations conferring selective advantages are efficiently selected and passed on to millions of viral progeny. HCV is one of the best examples of a virus that can mutate and alter its composition and respond to changing environments. As discussed, HCV is an RNA virus and exists in patients as a quasispecies; over decades of viral replication novel, highly adapted HCV strains are generated and permit continual replication and spread within the host liver. The first two direct-acting antiviral agents against HCV were FDA approved in 2011. It remains to be seen whether or not these will stand up to the plethora of HCV variants circulating within the human population.

Antiviral agents targeting host factors are making their way into research and development and potentially offer a great advantage over direct-acting antivirals due to the reduced likelihood of resistant viral strains emerging. Host cell factors linked with lipid metabolism are increasingly being implicated in the lifecycle of HCV. The pathways outlined above represent only a subset of regulatory pathways controlling host cholesterol and fatty acid homeostasis which have all been linked to HCV pathogenesis and propagation. Disregulation of these pathways by HCV may contribute to the metabolic syndrome observed in many virus infected individuals including but not limited to steatosis and hypobetalipoproteinemia. It is predicted that disregulation of host metabolic pathways is required to support the HCV lifecycle. Here we hypothesize that targeting pathways disrupted by HCV, in a manner that opposes virally induced changes, can be a useful strategy for both inhibiting various stages of the HCV lifecycle and for reversing virus induced metabolic imbalances.

### 1.6.1 Aim 1

One guiding principle exploited in choosing indirect antiviral host targets is that many viruses alter the intracellular environment to suit their own propagation. We propose that targeting virus manipulated host cell pathways may permit reversal of virus induced pathologies and limit virus spread across hepatocytes. In Chapter 2 we propose to target the host protein SKI-1/S1P to block HCV hijacking of host lipid metabolism. SKI-1/S1P acts as a checkpoint in the hosts’ lipid metabolic system through control of SREBP activation. HCV-mediated activation of the SREBP pathway contributes to accumulation of fat within the liver both supporting the virus lifecycle and exacerbating liver damage by inducing steatosis. We hypothesized that inhibiting
SKI-1/S1P activation of SREBP, using an engineered Spn4A variant or a small molecule inhibitor, would block activation of host lipid metabolism, prevent HCV from hijacking this pathway and ultimately inhibit HCV infection.

1.6.2 Aim 2

A major hurdle in the development of novel antivirals is controlling the specificity and targeting of therapeutic agents. Antivirals that are targeted to specific cell types and those that can act at the cell surface may limit toxicity and circumvent problems with intracellular delivery. PCSK9 is a host protein that acts extracellularly and is primarily expressed and acts upon liver cells. PCSK9 controls cell surface expression of LDLR, a putative receptor for HCV entry. As presented in Chapter 3 we hypothesized that overexpressing PCSK9 or gain-of-function PCSK9 mutants could be employed as a means to prevent HCV entry and propagation in hepatoma cells. Developing new strategies for inhibiting HCV entry has therapeutic potential in HCV infected patients undergoing liver transplantation, as a means of preventing HCV from establishing infection of newly allografted livers.

1.6.3 Aim 3

Non-inhibitory mutant serpins were designed to be used as negative controls in studies where SKI-1/S1P activity was inhibited by recombinant Spn4A variants. As discussed, the hinge mechanism of the serpin RCL is critical to capture and disable a targeted protease. Non-inhibitory serpins were constructed which possess mutations within a conserved hinge residue. During our studies we discovered that a putative negative control Spn4A variant significantly inhibited HCV infection in hepatoma cells. In Chapter 4 research is presented that aims to investigate the basic serpin-like properties and regulatory characteristics of this serpin and ultimately provide a framework for investigating the specific antiviral activity of this non-inhibitory serpin variant. Spn4A hinge-mutant variants are efficiently cleaved thus they act as enzyme substrates rather than inhibitors of serine proteases. The C-terminus of the hinge-mutant may be released from the serpin following enzymatic cleavage as it will no longer be covalently linked to the N-terminus. The Spn4A C-terminus possesses notable homology to the C-terminus of AAT, thus we hypothesize that the antiviral activity of non-inhibitory Spn4A variants may be related to that of AAT. VIRIP is a natural, AAT derived C-terminal peptide that circulates in human plasma during inflammatory events (249). The discovery that this peptide inhibits HIV-1 entry through direct interaction with HIV-1 gp41 has led to the development of an optimized
synthetic peptide that has undergone a phase 1 clinical trial against HIV-1 (257). Thus, exploring the unexpected antiviral properties of non-inhibitory Spn4A variants may lead to the discovery of novel anti-HCV therapeutic targets or agents.
Table 1.1 – New Hepatitis C virus inhibitors in development

<table>
<thead>
<tr>
<th>Drug name</th>
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<td>Phase 2</td>
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</tr>
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<tr>
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*Table adapted from Vermehren et al. 2011 (12)*
Figure 1.1. HCV hijacks host VLDL assembly.

The liver is an organ playing a central role in regulation of total body cholesterol and fatty acid homeostasis. The liver internalizes free fatty acids and lipoprotein associated lipids (triglycerides (TG)) and also synthesizes lipids de novo which are all shunted through lipid droplets (LDs) before being packaged into assembling very low density lipoproteins (VLDL). The VLDL assembly process begins with Apolipoprotein B (ApoB) translation and translocation into the ER where it is coordinately lipidated by microsomal triglyceride transfer protein (MTP) to generate pre-VLDL. Further in the secretory pathway there is a fusion between LDs and pre-VLDL to generate highly lipidated VLDLs that are released into secretion. ApoE may promote VLDL production or may associate with VLDL in the plasma. VLDL-associated lipids are hydrolyzed via the action of lipoprotein lipases. The resulting intermediate and low density lipoproteins (IDL and LDL) can be internalized back into the liver via low density lipoprotein receptor (LDLR). HCV controls this pathway by increasing LD formation, regulating MTP activity, interacting with ApoB, ApoE and neutral lipids and interacting with LDLR at the cell surface for infection of naïve cells (56,147).
Figure 1.2. The sterol regulatory element binding protein (SREBP) pathway.
1. SREBPs are initially localized to the ER and pass through the ER membrane via two transmembrane domains which are connected by a luminal loop and flanked by two cytosolic domains. The N-terminal bHLH-zip domain (N) acts as the transcription factor and binds sterol response elements (SREs) in the promoters of lipid metabolism genes. The C-terminal domain interacts with SCAP, a sterol sensing protein, in the ER that responds to changes in intracellular sterol levels. When sterol levels in the cell are high, SREBPs are retained, inactive, in the ER. 2. When sterol levels drop SREBPs are escorted by SCAP through the secretory pathway to the Golgi. In the Golgi apparatus, SREBP encounters two proteases that target and cleave SREBP molecules. 3. A proprotein convertase called SKI-1/S1P mediates the first cleavage in the luminal loop of SREBPs. 4. The separation of SREBP into two halves permits intramembrane proteolysis of the N-terminal half of SREBP by a cysteine metalloprotease called site-2 protease (S2P). 5. This cleavage liberates the N-terminal SREBP domain which is translocated into the nucleus and activates transcription of genes with SREs in their promoter regions i.e. LDLR, PCSK9 and SREBPs (67).
The HCV genome is a positive sense RNA molecule which is directly translated by the host ribosomal machinery which interacts with the HCV RNA internal ribosome entry site (IRES) located at the 5’ end. The HCV RNA lacks a poly-A tail and instead carries a poly-U tract at the 3’ end. Translation yields a single polyprotein carrying at least 10 individual viral proteins. These are released by host signal peptidase (SP), HCV non-structural (NS) 2 and HCV NS3 proteases. Host signal peptide peptidase (SPP) mediates a secondary cleavage of the HCV core protein which releases it from the ER membrane (79).

Figure 1.3. HCV RNA, polyprotein and viral protein functions.
Figure 1.4. The HCV lifecycle.
1. HCV entry is mediated by a series of host cell receptors interacting with lipoprotein components and viral envelope proteins to trigger clathrin-dependent endocytosis. 2. Low pH triggers viral and endosomal membrane fusion releasing the HCV RNA into the cytoplasm. 3. The positive sense HCV RNA is translated by host ribosomal machinery recognizing the internal ribosome entry site located in the 5’ untranslated region to yield a single polyprotein which is cleaved by host and virus proteases. 4. This releases all of the individual viral proteins which associate with intracellular membranes. 5. The non-structural proteins coordinate on the NS4B-induced membranous web, derived from ER membranes, to replicate nascent genomic RNA in lipid-raft enriched replicase complexes. 6. The core protein which associates with lipid droplets (LDs) following release from the ER recruits newly replicated RNA and other viral protein to initiate assembly of the virus nucleocapsid. 7. By an unknown process the nucleocapsid acquires the viral envelope and envelope proteins. 8. LDs, lipoprotein assembly factors and the secretory pathway are coordinated to mediate non-lytic release of very low density, lipoprotein-associated, “lipoviroparticles” (LVP) (79,147).
Figure 1.5. SKI-1/S1P and PCSK9 are members of the proprotein convertase family.

The proprotein convertase family of serine protease inhibitors possess homology to bacterial subtilisin encoding a conserved catalytic triad (aspartate, histidine and serine). The figure depicts *Bacillus amyloliquefaciens* subtilisin BPN’, human subtilisin kexin isozyme-1/site-1 protease (SKI-1/S1P) and human proprotein convertase subtilisin/kexin type 9 (PCSK9). Subtilisin and related proprotein convertases encode N-terminal signal peptides (SP – dark grey) followed by a prodomain (Pro – light grey), which acts as a chaperone for folding, followed by the proprotein catalytic domain (green). Proprotein convertases must undergo an autocatalytic cleavage to remove the inhibitory prodomain. SKI-1/S1P encodes a transmembrane domain (TM – black) while PCSK9 does not (161).
Figure 1.6. Serpin structural features.

An *in silico* homology model of Spn4A.RRLL(r) is presented displaying the basic structural features conserved across the serpin superfamily. The serpin reactive center loop (RCL) is displayed in wheat colour above the main body of the serpin. The RCL inhibitory mechanism requires hinging of the cleaved N-terminal side of the RCL at the highly conserved “hinge region” into the center of the main structural element of the serpin, β-sheet A (yellow). B-sheet B (blue) is where the C-terminal portion of the serpin (liberated following cleavage) is integrally located. B-sheet C (cyan) is connected to the C-terminal end of the RCL. A-helices are displayed in red and loops are displayed in green.
Figure 1.7. Serpin branched pathway reaction mechanism.

Enzymes (E) recognize serpins (I) through their reactive center loop bait sequence and form an initial stable and reversible complex (EI). Upon initiation of the cleavage reaction a transitory acyl-enzyme covalent intermediate, typical of a normal substrate proteolytic reaction, is formed (EI’). This is followed by complete cleavage (E + I*) of the serpin or alternatively by formation of a stable complex between the enzyme and the serpin (EI*). The ratio of pathway flux between E + I* and EI* is highly dependent on the relative rates of enzyme deacylation and reactive center loop insertion into β-sheet A (258).
Figure 1.8. Formation of the EI* complex.

Ribbon depictions of native α-1-antitrypsin (259) with trypsin aligned above it in the docking orientation (left), and of the complex showing the 71 Å shift of the P1 methionine of α-1-antitrypsin, with full insertion of the cleaved reactive center loop into β-sheet A (right). Regions of disordered structure in the complexed trypsin are shown as interrupted coils projected from the native structure of trypsin. Red, α-1-antitrypsin β-sheet A; yellow, reactive center loop; green ball-and-stick, P1 Met; cyan, trypsin (with helices in magenta for orientation); red ball-and-stick, active serine 195. Reprinted by permission from Macmillan Publishers Ltd: Nature, Huntington, J.A., Read, R. G., Carrell, R. W. Structure of a serpin-protease complex shows inhibition by deformation, copyright 2000 (217).
Chapter 2: Identification and characterization of the human subtilisin kexin isozyme-1/site-1 protease as a key host factor for hepatitis C virus infection

2.1 Introduction

Host lipids are an indispensible component of the HCV lifecycle at several crucial phases of propagation. One element in particular that HCV requires is host lipid droplets (LDs) which are utilized during the assembly stage of HCV manufacture of nascent particles (110,111,113,114). During infection, HCV-encoded proteins promote reorganization and accumulation of LDs at the perinuclear region of the cell (116). The HCV core protein is targeted to these LDs (86,260) and orchestrates the assembly and release of infectious viral particles during the late stages of infection. Disrupting the interaction of the HCV core protein with LDs inhibits this essential lifecycle stage (110,111,113).

Overstimulation of host lipid metabolism and concurrent engorging of host LDs during HCV infection is achieved by a variety of mechanisms (reviewed in (18)). For example, HCV employs various strategies to activate the sterol regulatory element binding protein (SREBP) pathway, which is important for regulation of host lipid homeostasis (26,27,158,159). SREBPs are endoplasmic reticulum (ER) localized transcription factors that sense intracellular sterol levels through interactions with sterol-sensing proteins (reviewed in (67)). When sterol levels are high, SREBPs are retained as inactive precursors in the ER (69,71). Under low sterol conditions, SREBPs are escorted to the Golgi, where resident proteases cleave and release the N-terminal portion of SREBPs (72,73). The released SREBP peptide translocates to the nucleus and activates transcription of proteins required for fatty acid and cholesterol biosynthesis and uptake (68,261). Activation of this pathway by HCV supports the virus lifecycle and may lead to the development of steatosis in chronically infected individuals (160,262).

The subtilisin-like proprotein convertase (PC) superfamily of calcium dependent serine proteases is responsible for regulated cleavage and subsequent maturation of various proprotein precursors (231,263). The PC family member subtilisin kexin isozyme-1 (SKI-1), or Site-1 protease (S1P), is a critical activator of SREBP signaling. In the Golgi, SKI-1/S1P performs the first of two essential endoproteolytic cleavages required for release and subsequent nuclear translocation of the N-terminal SREBP domain (72). Disrupting SKI-1/S1P function ultimately reduces overall lipid biosynthesis (170) and blocks transcriptional activation of numerous genes.

1 A version of Chapter 2 has been conditionally accepted pending minor revisions to Plos Pathogens: Olmstead, A.D., Knecht, W., Lazarov, I., Dixit, S. B., Jean, F. (2011) Human subtilisin kexin isozyme-1/site-1 protease (SKI-
including that of the low-density lipoprotein receptor (LDLR) (171), which is one of several receptors implicated in HCV entry (128,133). Given that SKI-1/S1P is an essential regulator of host lipid metabolism, we hypothesized in this study that inhibiting this enzyme would impede HCV infection by interrupting pathways that are intricately tied to the virus lifecycle.

Two strategies were employed to dissect the role of this enzyme in the HCV lifecycle. First, a novel bioengineered serine protease inhibitor (serpin) was designed and used to investigate the role that SKI-1/S1P plays in HCV infection. Serpins belong to a large superfamily of proteins found throughout nature that exhibit a conserved structure permitting regulation of endogenous protease activity and downstream physiological processes (reviewed in (212,264)). Inhibitory serpins display a C-terminal reactive center loop (RCL) that is specifically recognized by a target protease based on the encoded amino acid “bait” sequence. Following proteolytic cleavage of the scissile bond between amino acid positions P1 and P1’ the serpin acts as a suicide substrate inhibitor and binds covalently to the target enzyme. The protease becomes permanently trapped in a complex with the serpin that is both heat- and SDS-stable (192,254). Previously, we characterized the *Drosophila melanogaster* secretory pathway serpin variant, Spn4A as a potent inhibitor of kexin-like enzymes of the subtilase superfamily, PC2 and furin (254). Kinetic analysis revealed that Spn4A is the most potent secretory pathway subtilase inhibitor reported to date (254). Here, we hypothesized that bioengineering Spn4A to inhibit SKI-1/S1P, by modifying the “bait” region of the serpin RCL, would allow effective disruption of host lipid hijacking and propagation of HCV within liver cells. We also employed a novel small molecule inhibitor of SKI-1/S1P called PF-429242 to block proteinase cleavage during infection. This inhibitor has been shown to block SREBP cleavage and nuclear translocation and reduces plasma cholesterol and fatty acid levels in treated mice (171).

Our studies demonstrated that inhibiting SKI-1/S1P enzymatic activity blocks HCV infection in a dose dependent manner in human hepatoma cells. We confirmed the functionality of our recombinant Spn4A variant, Spn4A.RRLL(s), directed at SKI-1/S1P which effectively blocks SREBP-controlled protein expression. SKI-1/S1P inhibition dramatically reduced intracellular host LD abundance, neutral lipids and the LD marker, ADRP/perilipin-2. Single cell analysis of Spn4A.RRLL(s) treated, HCV infected cells revealed mislocalization of the HCV core protein to cell nuclei suggesting SKI-1/S1P inhibitors are effective therapeutic agents blocking HCV assembly. This is further supported by the finding that PF-429242 blocks HCV even after infection is established. With an increasing number of human enveloped viruses being
shown to employ host LDs for infection (265,266), our results suggest that SKI-1/S1P-directed inhibitors may allow the development of novel broad-spectrum antiviral agents.
2.2 Materials and methods

Cell culture and other reagents – Human hepatoma Huh-7.5.1 cells were kindly provided by Dr. Francis Chisari (Scripps Research Institute, La Jolla, CA, USA) (104). Cultured cells were maintained in Dulbecco’s Modified Eagle Medium (DMEM) supplemented with 1% penicillin, streptomycin, glutamine, non-essential amino acids, HEPES, and 10% fetal bovine serum (FBS) (Gibco/Invitrogen, Burlington, ON, Canada) or 10% lipoprotein-depleted serum (LPDS) (Biomedical Technologies Inc., Stoughton, MA, USA). Bovine serum albumin (BSA), saponin, cholesterol, α-mevalonic acid, lactone, and oleic acid were from Sigma-Aldrich Corp. (St. Louis, MO, USA). Formaldehyde, 37% w/v was from Fischer Scientific (Pittsburg, PA, USA).

Antibodies and dyes – Antibodies used to detect HCV infection included a mouse anti-core antibody (1:500 for western blot and 1:50 for immunofluorescence, Abcam, Cambridge, MA, USA) and a mouse anti-NS5A antibody (1:50, gift of Dr. Chen Liu, University of Florida, Gainesville, FL, USA) (267). Antibodies used to detect cellular proteins included rabbit anti-human proprotein convertase subtilisin/kexin type 9 (PCSK9) (1:500, Abcam), rabbit anti-LDLR (1:250, Fitzgerald Industries International, Inc., Concord, MA, USA), mouse anti-SREBP-2 (1:200, Santa Cruz Biotechnology, Santa Cruz, CA, USA), rabbit anti-adipose differentiation-related protein (ADRP) (1:50, Abcam), mouse anti-ADRP (1:20, Progen, Heidelberg, Germany), rabbit anti-β-tubulin (1:3000, Abcam), mouse anti-β-tubulin (1:500, Lab Vision Corporation, Montreal, QC, Canada), and mouse anti-GM130 (1:200, BD Biosciences, Mississauga, ON, Canada). Tagged proteins were detected using mouse anti-FLAG M2 (1:500 for western blot and 1:50 for immunofluorescence, Sigma-Aldrich Corp.), rabbit anti-FLAG (1:500 for western blot and 1:50 for immunofluorescence, Thermo Scientific, Nepean, ON, Canada), and mouse anti-his antibodies (1:500, Applied Biological Materials, Richmond, BC, Canada). Secondary antibodies used for immunofluorescence were Alexa Fluor-488-conjugated or Alexa Fluor-568-conjugated donkey anti-mouse or donkey anti-rabbit antibodies (1:100, Molecular Probes/Invitrogen). Secondary antibodies used for western blot were IRDye 680-conjugated (red bands) or 800-conjugated (green bands) donkey anti-mouse or goat anti-rabbit antibodies (1:10000, LI-COR Biosciences, Lincoln, NE, USA). Hoechst 33258 (10 µg/ml) and BODIPY (4,4-difluoro-1,3,5,7,8-pentamethyl-4-bora-3a,4a-diaza-s-indacene) 493/503 (1 µg/ml, Molecular Probes/Invitrogen) were used for detection of nuclei and LDs, respectively.

HCV RNA and infectious stock production – A plasmid containing the cDNA of an HCV consensus clone isolated from a Japanese patient with fulminant hepatitis (JFH-1) (GenBank
accession number AB047639) (268) cloned behind a T7 promoter (pJFH-1; a generous gift from Dr. Takaji Wakita, National Institute of Infectious Diseases, Tokyo, Japan) was used to generate genomic HCV RNA and infectious HCV stocks as previously described in (269).

**Transfection of HCV RNA** – Purified HCV RNA was used to transfect Huh-7.5.1 cells as a means of studying HCV infection independently of receptor mediated entry. Five micrograms of purified RNA was incubated with 10 μl of lipofectamine 2000 (Invitrogen) in minimal essential media (MEM) for 30 minutes. The RNA-lipid complexes were then added to cells in MEM for 16 hours, then cells were washed with phosphate-buffered saline (PBS) and complete media was added for the remainder of the experiment.

**HCV titer determination** – The amount of infectious HCV particles generated for viral stocks or in the described experiments was determined using a modified, previously described protocol (104). Briefly, 1 x 10^4 Huh-7.5.1 cells were plated in each well of a 96-well plate and infected with 10-fold serial dilutions of HCV-infected cell media. Three days post-infection cells were fixed and probed as described in the ArrayScan Quantification methods section. An ArrayScan VTI High Content Screening (HCS) Reader (Thermo Scientific) was used to acquire images of the entire infected wells. Titers were determined by manually counting foci (fluorescence forming units (FFU)) in the lowest dilutions with positive signal.

**Recombinant adenoviruses** – Empty adenovirus (Ad-Empty), which was kindly provided by Dr. Jan Breslow (Rockefeller University, New York, NY, USA) (64), was amplified by Viraquest Inc (North Liberty, IA, USA). Spn4A, isolated from *Drosophila melanogaster* [59] (GenBank Accession number NM_165496), demonstrates potent inhibitory activity against the human PC, furin [45] and was used as a scaffold for engineering a SKI-1/S1P directed-inhibitor. First, the N-terminus of Spn4A was modified to encode the AAT signal peptide (residues 1–24, pre-AAT) followed by a tandem his-tag (HHHHHHH) and FLAG-tag (DYKDDDDK) sequence (HF) (254). The Spn4A-variants directed at furin contain the P4 – P1 cleavage sequence (215) RRKR in the reactive center loop (RCL). The ER “retained” (r) variant of the furin-directed serpin designated Spn4A.RRKR(r) (Figure 3.2 A) presents the HDEL ER retention motif at the C-terminus. A “secreted” (s) variant of the serpin Spn4A.RRKR(s) was generated in house by mutating the his codon in the HDEL sequence to a stop codon. SKI-1/S1P was reported to cleave the Lassa virus glycoprotein sequence RRLL [60]. Thus, to specifically target this enzyme, we altered the furin-directed RCL sequence to RRLL through site-directed mutagenesis using QuikChange (Stratagene, La Jolla, CA, USA) to generate Spn4A.RRLL(r) and Spn4A.RRLL(s)
(Figure 3.2 A and B). To produce Ad-Spn4A.RRKR(s), Ad-Spn4A.RRLL(r), and Ad-Spn4A.RRLL(s), all Spn4A constructs were cloned into adenovirus vectors, then were amplified by and purchased from ViraQuest Inc. Adenovirus titers were determined or confirmed in human embryonic kidney (HEK293T) cells using Adeno-X Rapid Titer kit (Clontech, Mountain View, CA, USA).

**In silico homology model of Spn4A.RRLL variant** – The *Drosophila melanogaster* Spn4A sequence (Genebank accession number gi|24586105|ref|NP_524955.2) exhibits 34% sequence homology with the human neuroserpin for which a crystal structure (270) is available in the Protein Data Bank (271) (PDB ID: 3F5N). Of the five chains in this pentameric structure of Neuroserpin, chain B is most well resolved with the fewest missing residues and was used as the template for the homology model presented in Figure 3.2 B. The model was built and refined using the SwissPDB Viewer (272). The C-α residues in this model structure align to 1.9 Å RMSD with reference to the human neuroserpin structure.

**Recombinant enzymes** – The first 997 amino acids of human SKI-1/S1P lacking the C-terminal transmembrane domain but having a C-terminal 8 x his-tag (PGDDDDKHHHHHHHHHSGS) were expressed in Sf9 insect cells as described previously (273). Two liters of cell culture supernatant were used for purification. Two hundred milliliters of 200 mM Tris/HCl pH 8.0, 500 mM NaCl was added and then the pH was adjusted to pH 8.0 by further addition of 2 M NaOH. The resulting precipitate was removed by centrifugation at 10000 x g for 30 minutes and subsequent filtration through a glass filter. The cleared supernatant was then applied to a small (0.9 ml column volume) IMAC column (Ni-Sepharose, GE Healthcare, Freiburg, Germany) by continuous flow (1 ml/minutes). The column had been equilibrated in 50 mM Tris/HCl pH 8.0, 500 mM NaCl (buffer A) and bound recombinant SKI-1/S1P was eluted with a continuous gradient over 30 column volumes to buffer A plus 300 mM imidazole. Collected fractions were assayed for enzymatic activity of SKI-1/S1P using Ac-RRLL-pNA (custom synthesized by Peptides International (Louisville, Kentucky, USA)) as the substrate, as previously described (273). The most active fractions were pooled. Concentration and buffer exchange to buffer A was then done using spin concentrators (Millipore, Billerica, MA, USA) with a molecular weight cutoff of 30 kDa. The final preparation was, after addition of 30% v/v glycerol, stored at -80°C and had a specific activity of 0.018 U/mg (measured as above). Recombinant his-tagged furin (0.432 mg/ml) was purchased from R & D Systems (Minneapolis, MN, USA).
Western blot analysis – Cultured cells were washed with ice-cold PBS and re-suspended in cold radioimmunoprecipitation assay (RIPA) buffer (50 mM Tris-HCl pH 8, 150 mM NaCl, 1% octylphenyl-polyethylene glycol (IGEPAL), 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS)) containing 1 X Complete, EDTA-free, protease inhibitor cocktail (Roche, Laval, QC, Canada). Whole cell extracts were vortexed, and then clarified by centrifugation at 12000 x g for 15 minutes. Soluble extracts mixed with 2 X sample buffer (62.5 mM Tris-HCl, pH 6.8, 25% glycerol, 2% SDS, 0.01% bromophenol blue, and 5% β-mercaptoethanol) were electrophoresed on 8-15% SDS polyacrylamide gels and transferred to nitrocellulose membranes. Membranes were blocked in odyssey-blocking buffer (LI-COR Biosciences) for one hour, and proteins of interest were detected by probing with the appropriate primary and secondary antibodies diluted in odyssey blocking buffer containing 0.1% Tween 20. Protein bands were relatively quantified using the two-colour (700 and 800 nm wavelength) Odyssey Infrared Imaging System (LI-COR Biosciences). β-tubulin was always used as a loading control and for normalizing protein expression. Media samples analyzed for secreted Spn4A variants were taken directly from cultured cells, mixed with 2 X sample loading buffer, and subjected to the described western blot analysis.

Stable enzyme-inhibitor (EI*) complex detection between serpin variants and SKI-1/S1P – Huh-7.5.1 cells were plated at 5 x 10^5 cells per well in a 6-well plate and infected after 24 hours with Ad-Spn4A.RRLL(s), Ad-Spn4A.RRLL(r), Ad-Spn4A.RRKR(s), or Ad-Empty at moi 50. After 72 hours, media and cell extracts (harvested in hypotonic buffer containing 20 mM Tris, pH 7.4, 10 mM MgCl_2, and 10 mM CaCl_2) were mixed 1:1 with enzyme reaction buffer (SK1-1/S1P buffer contains 25 mM Tris-HCl, 25 mM MES, pH 7.4, 2.5 mM CaCl_2 (273); and furin buffer contains 100 mM HEPES, pH 7.5, 1 mM CaCl_2, 0.5% Triton X-100 (254)), 1 X complete EDTA-free protease inhibitor cocktail, and 11.6 ng/µl SKI-1/S1P or 2.4 ng/µl furin. The enzyme mixture was incubated at 30°C for 30 minutes and the reaction was stopped with 12.5 mM EDTA (final concentration) (254). The samples were then processed, as described above, for western blotting.

ArrayScan quantification – In black flat-bottom 96-well plates (BD Biosciences) cells were plated and infected as described in the methods below. Following infection, cells were fixed in 4% formaldehyde v/v diluted in PBS and blocked in PBS containing 3% BSA, 0.3% Triton X-100, and 10% FBS. Cells were first probed with HCV anti-core antibody (1:500) in PBS containing 3% BSA and 0.3% Triton X-100 (Binding Buffer), then incubated with Alexa Fluor-
568-conjugated donkey anti-mouse secondary antibody (1:1000) and 10 µg/ml Hoechst dye. Cells were analyzed by a quantitative, high-throughput, fluorescence microscope system called the Cellomics ArrayScan VTI High Content Screening (HCS) Reader (Thermo Scientific) using the BioApplications software, Target Activation protocol (274). The protocol was used to count the total number of cells (Hoechst-stained nuclei) and the percentage of those cells that were infected with HCV (positive signal at 568 nm wavelength).

**Nuclear fractionation** – On Day 1, 2.5 x 10^6 Huh-7.5.1 cells were plated into 100 mm petri plates. On Day 2, cells were infected with recombinant adenoviruses expressing Spn4A.RRLL(r) or Spn4A.RRLL(s) or with Ad- Empty (moi 50). On Day 3, separate cells were treated with the inhibitor PF-429242 or the control DMSO. On Day 5, all samples (except the untreated control) were treated for 1 hour with 25 µM α-N-acetyl-Leu-Leu-Nle-CHO ((2S)-2-[[((2S)-2-acetamido-4-methylpentanoyl]amino]-4-methyl-N-[(2S)-1-oxohexan-2-yl]pentanamide). Nuclear fractionation of cell lysates was performed at 4°C as described previously with modifications (171,275). Cells were harvested in 400 µl buffer C (10 mM HEPES/KOH, pH 7.6, 10 mM KCl, 1.5 mM MgCl₂, 1 mM EDTA, 1 mM EGTA, 250 mM sucrose) containing 1 X complete protease inhibitor cocktail (Roche, Laval, QC, Canada). To shear DNA, the cells were passed 20 times through a 23-gauge needle. The lysate was centrifuged at 1100 x g for 7 minutes and the resulting supernatant was centrifuged again at 25000 x g for 60 minutes to obtain the membrane pellet. The pellet containing membrane-bound SREBP-1 was resuspended in 75 µl of SDS-lysis buffer (10 mM Tris HCl, 100 mM NaCl, 1% SDS, 1 mM EDTA, 1 mM EGTA, pH 6.8). The pellet from the 1100 x g spin was resuspended in 100 µl buffer D (20 mM HEPES/KOH, 420 mM NaCl, 1.5 mM MgCl₂, 2.5% glycerol, 1 mM EDTA, 1 mM EGTA, pH 7.6) containing 1 X complete protease inhibitor cocktail (Roche). Nuclear pellets were rocked for 1 hour, after which the samples were centrifuged at 25000 x g for 60 minutes to obtain the clarified supernatant containing the nuclear fraction.

**Confocal microscopy and immunofluorescence** – After Huh-7.5.1 cells were seeded onto coverslips for 24 hours, they were either infected with adenovirus (moi 50) for 72 hours or infected with HCV (moi 0.1) for 48 hours. Cells were fixed in 4% v/v formaldehyde in PBS, then permeabilized and blocked in PBS containing 0.05% saponin (wash buffer) and 1% BSA (binding buffer). Blocking of cells stained with BODIPY 493/503 was done in the presence of 0.2 M glycine to reduce background fluorescence. Cells were probed with primary antibodies in binding buffer, then incubated with a secondary antibody, Hoechst dye (10 µg/ml), and BODIPY
493/503 (1 µg/ml; when indicated) diluted in PBS. Cells were mounted onto slides with an anti-fade solution and sealed with clear nail-polish. The slides were then imaged using a Leica TCS SP5 confocal microscope (Leica Microsystems, Wetzlar, Germany) or an Olympus Fluoview FV1000 laser scanning confocal microscope (Olympus Corporation, Tokyo, Japan). Leica MM AF Software (Leica Microsystems) was used to count the number of LDs (green channel) in cells expressing Spn4A.RRLL(r) (n=21) or Spn4A.RRLL(s) (n=15) (cells positive in the red channel). LDs in cells treated with Ad-Empty (n=23) were also enumerated. All quantified images were acquired using the same laser intensity and gain settings, and LDs were enumerated by applying the same threshold setting to each image.

**Adenovirus infections** – Huh-7.5.1 cells were infected with recombinant adenovirus at various moi in complete media with or without exogenous sterols (50 µM sodium mevalonate, 20 µM sodium oleate, 5 µg/ml cholesterol). After 48 hours, the cells were infected with HCV moi 0.1 or transfected with purified HCV genomic RNA for 3 days and then cells were analyzed by Cellomics ArrayScan, or total RNA was isolated from cell extracts using the RNeasy plus kit (Qiagen, Mississauga, ON, Canada) including on-column DNase digestion. Media from treated and infected cells were harvested for HCV titer determination as described above. To examine the Spn4A.RRLL(s) mediated block in PCSK9, LDLR, and SREBP-2 expression, Huh-7.5.1 cells were grown in media supplemented with LPDS for 24 hours, infected with adenovirus variants, and harvested 72 hours later.

**Quantitative real-time (Q) PCR** – Purified total RNA was reverse transcribed to cDNA using TaqMan reverse transcription reagents (random hexamers; Applied Biosystems, Foster City, CA, USA). Real-time quantitative PCR was carried out using Brilliant II Fast QPCR reagents (Stratagene, La Jolla, CA, USA) according to the manufacturer’s instructions on an Mx3005P QPCR system (Stratagene). Online ProbeFinder software (Roche Applied Science) was used to find primers that would allow amplification of the HCV RNA 5’ end in combination with the Human Universal Probe Library from Roche (Roche Applied Science). For amplification of the HCV RNA 5’ region, 400 nM of both forward primer (5’-CATGCGCGTTAGTGATGAGTGTCG-3’) and reverse primer (5’-GGTTCCGCAGACCACCATAT-3’) were used in combination with 200 nM of probe #75 from the Human Probe Library (Roche). HCV RNA levels were relatively quantified across samples and normalized to β-actin RNA levels using 500 nM primers (forward: 5’- GCC CTG AGG CAC TCT TCC and reverse: 5’ GGA TGT CCA CGT CAC ACT
TC-3") and 250 nM probe (5’AC TCC ATG CCC AGG AAG GAA GGC-3’ with a 5’ Cy5 fluorophore and 3’ black hole quencher).

**Cell viability** – Cell viability was determined using CellTiter 96® AQueous One Solution Cell Proliferation Assay (Promega, Madison, WI, USA). This assay employs a tetrazolium compound [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt; MTS], which is bioreduced by cells into a coloured formazan product which can be detected in tissue culture media at 490 nm wavelength.

**Treatment with PF-429242** – PF-429242, a reversible, selective and competitive small molecule inhibitor of SKI-1/S1P (164,171,198), was synthesized by Dr. Peter Chua at the Center for Drug Research and Development (CDRD) at The University of British Columbia (Vancouver, BC, Canada) using a previously described protocol (276). To investigate the antiviral activity of the small molecule, Huh-7.5.1 cells were treated with PF-429242 for 24 hours. After 24 hours of treatment the media was removed and then cells were infected with HCV (moi 0.1) for 48 or 72 hours. Alternatively, cells were first infected with HCV for 24 hours. Then, the media was removed and replaced with media containing various concentrations of PF-429242 for a further 48 hours. Intracellular HCV infection levels were determined using Cellomics HCS ArrayScan. Infectious extracellular titers were determined in media of 72 hour HCV infected cells.

**Intracellular lipid quantification** – To measure the level of intracellular lipids following 72 hour recombinant adenovirus expression or 48 hour PF-429242 treatment, cellular extracts were harvested in 1% triton-X 100 in PBS (for phospholipid, cholesterol and protein assay) or 5 % triton-X 100 in H$_2$O (for triglyceride assay). To extract triglycerides, samples were slowly heated to 90°C and brought to room temperature, twice. Total cholesterol, cholesterol esters (Amplex Red Cholesterol Assay kit, Invitrogen), phospholipids and triglycerides (EnzChrom, BioAssay Systems, Hayward, CA, USA) were quantified using commercially available kits. Lipid levels were normalized to cellular protein content (DC Protein Assay, Bio-Rad, Hercules, CA, USA).

**Curve fitting, half maximal effective concentration (EC$_{50}$) determination and statistics** – The sigmoidal fit function in Igor Pro software (WaveMetrics, Portland, OR, USA) was used for fitting HCV and PF-429242 inhibition curves and for determining EC$_{50}$ values. The reported EC$_{50}$ values are the average of the values calculated from three independent experiments plus or minus the standard deviation. The student’s $t$-test (unpaired) was used to calculate significance
which is represented in the figures by the following notation: * denotes $p < 0.05$, ** denotes $p < 0.01$, and *** denotes $p < 0.005$. 
2.3 Results

2.3.1 Engineering of secretory pathway Spn4A scaffolds to target the human proprotein convertase SKI-1/S1P

Our laboratory previously demonstrated that the Drosophila melanogaster serpin variant, Spn4A, is the most potent inhibitor of the PC furin characterized to date (254). Based on our previous success with engineering serpin scaffolds to target furin (192,277) and the virally encoded HCV NS3 protease (278) we hypothesized that we could modify Spn4A to inhibit SKI-1/S1P. First, we modified the “bait” region sequence of the serpin RCL (Figure 2.1 A and B). Spn4A is a unique secretory pathway “retained (r)” serpin that presents at its C-terminus an HDEL sequence (Figure 2.1 A), a functional variant of the KDEL retention motif, that directs protein retention in the ER (279). We cloned a pre-his/FLAG-tagged Spn4A construct ((254) and see Materials and methods) into an adenoviral shuttle vector to generate Spn4A.RRKR(r) (Figure 2.1 A). To generate a “secreted (s)” variant of Spn4A, Spn4A.RRKR(s), a stop codon was inserted before the C-terminal HDEL sequence. Next, we employed site-directed mutagenesis to alter two residues of the Spn4A “bait” sequence ArgP4-ArgP3-LysP2-ArgP1↓ to generate ArgP4-ArgP3-LeuP2-LeuP1↓ which mimics a SKI-1/S1P consensus sequence (167) (Figure 2.1 A and B: Spn4A.RRLL(r) and Spn4A.RRLL(s)). Since SKI-1/S1P cleavage of its substrates (i.e. SREBPs (69) and viral glycoproteins (167,194)) occurs in the Golgi apparatus (72), we hypothesized that only Spn4A.RRLL(s), which traffics through the late secretory pathway prior to secretion would inhibit SKI-1/S1P.

2.3.2 Cellular expression of recombinant adenoviruses encoding Spn4A-directed SKI-1/S1P variants

To test the properties of our Spn4A variants in cellulo, recombinant adenoviruses expressing the Spn4A constructs were produced, purified and titered using HEK293T cells (permissive for adenovirus vector propagation). Determination of recombinant virus titers allows for dose dependent analysis studies using various multiplicities of infection (moi) and ensures equal amounts of the recombinant adenoviruses are used in each experiment. As adenoviruses display strong tropism for the liver (144), the major site of HCV infection (280), these recombinant adenoviruses are especially useful molecular tools for HCV research.

The level of expression of Spn4A variants was first optimized by infecting human hepatoma Huh-7.5.1 cells (highly permissive for HCV JFH-1 infection) with several moi of
adenovirus expressing Spn4A.RRLL(r) (predicted to be retained, and thus easily detected, within the cells). Cellomics HCS analysis revealed that over 90% of Huh-7.5.1 cells expressed Spn4A.RRLL(r) at a moi of 50 (Figure A1.1). Cellomics HCS was also employed to enumerate the total number of cells (nuclei) following 48 hours of Ad-Empty (control), Spn4A.RRLL(r) and Spn4A.RRLL(s) expression (Figure A1.2) plus 72 hours of HCV infection. No significant reductions of total cell numbers were seen following expression of the Spn4A variants compared to the control indicating that Spn4A.RRLL(r) and (s) do not induce cell death evident when cells detach from the plate.

The secretion profile of recombinant Spn4A variants in Huh-7.5.1 cells was examined by western blotting of cell lysates and extracellular media. Clear, prominent, 45 kDa bands representing the Spn4A variants were detected using anti-FLAG antibody. As hypothesized, Spn4A.RRLL(r) is found only in lysed cell extracts and is not found secreted into extracellular media (Figure 2.2 A, Lane 2). Conversely, Spn4A.RRLL(s) is detected in both whole cell extracts and in cell media (Figure 2.2, Lane 3). These results confirm both robust expression and proper secretion profile of the SKI-1/S1P-directed Spn4A variants.

2.3.3 Specific and selective inhibition of SKI-1/S1P by Spn4A.RRLL(s)

A stable, acyl-enzyme complex is formed between an enzyme and a functional inhibitory serpin following RCL cleavage which allows for detection of the high molecular weight and heat- and SDS-stable complex by standard SDS-PAGE and western blot (192,254). To determine if Spn4A.RRLL(s) is a functional and selective inhibitor of SKI-1/S1P, Spn4A variants (furin- and SKI-1/S1P-directed inhibitors) were expressed in Huh-7.5.1 cells for 72 hours. Cell media and extracts expressing the recombinant serpins were then harvested and incubated with purified, enzymatically active, his-tagged, human SKI-1/S1P (273) or furin (254) (Figure 2.2 B) as described previously (192,277). Reaction mixtures were analyzed by western blot and probed for enzyme-inhibitor (EI*) complex formation with anti-his antibody (samples were also probed with anti-FLAG antibody to distinguish Spn4A variant bands from the recombinant enzymes; Figure A1.3). The results presented in Figure 2.2 B demonstrate EI* complex formation between recombinant SKI-1/S1P and Spn4A.RRLL(s) present in cell media and in cell extracts (Lane 5, upper and lower panels). Spn4A.RRLL(s) did not form a complex with furin (Figure 2.2 B, Lane 10) whereas the furin-directed serpin, Spn4A.RRKR(s), formed an EI* complex with furin but not with SKI-1/S1P in cell media and extracts (Figure 2.2 B, Lane 9 and Lane 7 respectively). Lysed cellular extracts expressing Spn4A.RRLL(r) also demonstrate EI* complex
formation with SKI-1/S1P (Figure 2.2 B, Lane 12, bottom panel). These results confirm our hypothesis that recombinant, adenovirus-expressed Spn4A.RRLL(s) selectively inhibits SKI-1/S1P by trapping the enzyme in a heat- and SDS-stable complex.

2.3.4 Expression of secretory pathway Spn4A.RRLL(s) in Huh-7.5.1 cells inhibits SREBP regulated protein expression and intracellular cholesterol-ester accumulation

To determine whether expression of Spn4A.RRLL(s) in Huh-7.5.1 cells would inhibit endogenous SKI-1/S1P-mediated cleavage of SREBPs, we examined nuclear N-terminal SREBP-1 levels in cells infected with adenovirus-expressed Spn4A variants. As a positive control we also treated cells with the selective, reversible and competitive small molecule inhibitor of SKI-1/S1P, called PF-429242 (171). This compound was recently synthesized and characterized both in vitro and in vivo for its anti-lipidemic properties including efficient inhibition of nuclear SREBP accumulation. As previously described (171), the proteasome inhibitor, α-N-acetyl-Leu-Leu-Nle-CHO or ALLN, was employed to facilitate the detection and accumulation of the N-terminal activated fragment of SREBP-1 in the nucleus. Nuclear extracts were prepared and analyzed by western blot (Figure 2.3 A). As expected, nuclear extracts (positively identified based on detection of the nucleolar marker fibrillarin) from cells treated with 10 μM of PF-429242 (PF-429242 + ALLN) revealed a complete block of SREBP-1 accumulation in the nucleus. Nuclear extracts from cells infected with Ad-Spn4A.RRLL(s) (RRLL(s) + ALLN) also exhibited a dramatic decrease in nuclear SREBP-1 accumulation when compared to Ad-Empty (control + ALLN) and Ad-Spn4A.RRLL(r) (RRLL(r) + ALLN) infected cells. These results confirm that expression of recombinant Spn4A.RRLL(s) in the secretory pathway of Huh-7.5.1 cells inhibits endogenous SREBP-1 processing by SKI-1/S1P.

Next, to determine whether the Spn4A.RRLL(s)-mediated reduction in nuclear SREBPs was associated with a concomitant decrease in the protein levels of SREBP-target genes, we examined the fate of three SREBP-dependent gene products: SREBP-2, LDLR and PCSK9. A time course analysis of cells expressing Spn4A.RRLL(s) in complete media showed the most significant block in SREBP-regulated LDLR expression after 72 hours (Figure A1.4). As HCV is known to induce SREBP activation, we analyzed the expression of various SREBP-regulated proteins under SREBP-activated conditions (74). Huh-7.5.1 cells were depleted of exogenous sterols for 24 hours to induce SREBP translocation to Golgi prior to infection with Ad-Empty, Ad-Spn4A.RRLL(r) or Ad-Spn4A.RRLL(s). The levels of LDLR, PCSK9, and SREBP-2 (all regulated by nuclear SREBPs (51,183,281)) were then measured using western blot analysis of
lysed cell extracts (Figure 2.3 B). After 72 hours of Spn4A.RRLL(s) expression, mature LDLR (160 kDa) levels were reduced by 74% compared to Ad-Empty-treated cells. Similarly, an 85% block in mature PCSK9 (60 kDa) expression and a 79% reduction in full-length SREBP-2 expression were observed. No significant reductions in LDLR or SREBP-2 levels following Spn4A.RRLL(r) treatment were observed. Interestingly, a significant reduction in PCSK9 expression was detected in Spn4A.RRLL(r) expressing cells (Figure 2.3 B). The expression of β-tubulin and the Golgi marker GM130 were not affected by Spn4A.RRLL(r) or Spn4A.RRLL(s) expression (Figure 2.3 B). These results confirm that Spn4A.RRLL(s) specifically inhibits SREBP dependent activation of LDLR, PCSK9, and SREBP-2 expression.

A critical function of the SREBP pathway and the genes that it regulates is to control lipid homeostasis (282). We investigated the impact of inhibiting SKI-1/S1P using PF-429242 or Spn4A.RRLL(s) on total, steady state intracellular lipid levels; specifically on cholesterol, cholesterol-esters, triglycerides and phospholipids (Figure 2.3 C and D). Among the cell lipids examined, PF-429242 and Spn4A.RRLL(s) had the most dramatic impact on cholesterol-ester levels, a major constituent of cellular lipid storage droplets (53); these were reduced by 74% in Spn4A.RRLL(s) treated cells compared to Ad-Empty treated cells (Figure 2.3 D). PF-429242 reduced cholesterol-ester levels ~ 63% compared to cells treated with DMSO (Figure 2.3 C). A 14% reduction in triglycerides was also induced by Spn4A.RRLL(s), although this reduction did not reach significance, whereas PF-429242 caused a significant 51% reduction in total intracellular triglycerides. A significant 5% reduction in free cholesterol levels was also observed in Spn4A.RRLL(s) treated cells and 25 % reduction in PF-429242 treated cells compared to respective controls. No significant reduction in phospholipid levels were detected by any of the treatments (Figure 2.3 C and D). These results suggest that inhibition of host lipid metabolism by blocking SKI-1/S1P activation of SREBP signaling causes increased cellular utilization of lipid stores.

2.3.5 Expression of secretory pathway Spn4A.RRLL(s) reduces lipid droplet abundance in Huh-7.5.1 cells

LDs are dynamic intracellular lipid storage compartments made up of triglyceride and cholesterol esters, surrounded by a phospholipid membrane and associated with specific marker proteins including adipose differentiation-related protein (ADRP) also known as perlipin 2 (53). Because SREBP activation controls the expression of genes directly involved in intracellular fatty acid and cholesterol biosynthesis (reviewed in (282)) and as cholesterol-ester levels were
reduced by Spn4A.RRLL(s), we investigated the effect of serpin-mediated SKI-1/S1P inhibition on cellular LD abundance. Fluorescent, confocal microscopy was employed to examine the abundance of LDs (stained using BODIPY 493/503) in Ad-Empty, Spn4A.RRLL(r), and Spn4A.RRLL(s) (detected with anti-FLAG antibody) treated Huh-7.5.1 cells. After 72 hours, the level of BODIPY-stained LDs in Spn4A.RRLL(s) expressing cells was reduced compared to empty vector treated cells (Figure 2.4 A and B). By contrast, Spn4A.RRLL(r) expressing cells had no reduction in LD size or abundance compared to Ad-Empty-treated cells. Quantification of confocal images demonstrated that on average LD abundance is reduced by 80% in Spn4A.RRLL(s) expressing cells compared to controls (Figure 2.4 B). The effect of Spn4A variants on LD abundance was further supported through confocal examination of the LD marker ADRP/perilipin 2 (Figure 2.4 C), where similar effects were seen as described for BODIPY 493/503. Additionally, Huh-7.5.1 cells were treated for 72 hours with recombinant adenoviruses, whereupon cell extracts were subjected to western blot analysis and probed for total ADRP/perilipin 2 expression. Spn4A.RRLL(s) was observed to reduce ADRP/perilipin 2 expression by 50% compared with control cells (Figure 2.4 D), whereas there was no reduction in ADRP/perilipin 2 expression following Spn4A.RRLL(r) treatment. We confirmed that reduced cellular LD levels were due to inhibition of SKI-1/S1P using 10 µM PF-429242 whereupon ADRP levels were reduced by 62% compared to DMSO treated cells (Figure 2.4 E). These results confirm that PF-429242 and Spn4A.RRLL(s) mediated inhibition of SKI-1/S1P enzymatic activity effectively reduces intracellular LD abundance in Huh-7.5.1 cells.

2.3.6 Expression of secretory pathway Spn4A.RRLL(s) in Huh-7.5.1 cells results in a dose-dependent inhibition of HCV infection

SREBP signaling is actively increased by HCV-encoded proteins during infection (26,27,158,159), thus we explored the effect of blocking this pathway on the HCV lifecycle in human hepatoma cells. Huh-7.5.1 cells were treated with increasing moi (1-50) of Ad-Spn4A.RRLL(r), Ad-Spn4A.RRLL(s), or Ad-Empty (control) for 48 hours in complete media with or without exogenously added sterols followed by 72 hours of infection with HCV. The number of HCV-infected cells, as evidenced by positive core protein expression, was measured using Cellomics HCS (Figure 2.5 A). It was determined that Spn4A.RRLL(s) expression inhibited HCV infection in a dose-dependent manner compared to control treated cells. HCV infection was not significantly reduced in cells infected with Ad-Spn4A.RRLL(s) at a moi of 1. A moi of 12.5, however, caused a 40% reduction, a moi of 25 caused a 60% reduction, and a moi
of 50 caused a 75% reduction in the number of HCV-infected cells compared to controls. Supplementing sterol and lipid metabolites significantly restored infectivity when cells were treated with moi 50 of Ad-Spn4A.RRLL(s) where a 2-fold increase in infection compared to non-supplemented cells was observed (Figure 2.5 A). Spn4A.RRLL(r) had no significant impact on HCV infection when compared to the control. These results indicate that SKI-1/S1P-dependent activation of SREBP signaling is required for efficient HCV infection in Huh-7.5.1 cells. These results also show that this requirement is, in part, directly dependent on the availability of lipids within the cell.

2.3.7 Expression of Spn4A.RRLL(s) inhibits both viral entry and post-entry steps of the HCV life cycle

The effect of inhibiting SKI-1/S1P on the different steps of the HCV life cycle was further investigated by examining various markers of HCV infection. Intracellular HCV core protein was quantified using western blotting, intracellular HCV RNA was quantified using QPCR and extracellular infectious HCV was titered from cells treated with Spn4A variants for 48 hours prior to 72 hours of HCV infection (Figure 2.5 B and C). First, western blot analysis revealed a 64% reduction in the expression of intracellular HCV core protein in Spn4A.RRLL(s) expressing cells compared to the control (Figure 2.5 B). Similarly, Spn4A.RRLL(s) treatment was found to reduce extracellular infectious HCV titers by 76% (Figure 2.5 B). A 65% reduction in intracellular HCV RNA levels was also observed using QPCR analysis (Figure 2.5 C). To determine if decreased HCV RNA is due to reduced viral replication or alternatively due to reduced HCV entry, we also examined cells transfected directly with total genomic HCV RNA for 3 days following 48 hours of adenovirus-mediated serpin expression. Under these experimental conditions, when the receptor dependent entry step of HCV infection is bypassed, no significant changes in HCV RNA levels are detected (Figure 2.5 C). This suggests that the detected reduction in HCV RNA levels following HCV infection is due to reduced viral entry. Spn4A.RRLL(r) expression did not significantly impact any aspect of the HCV infection examined. Overall, these results demonstrate that selective inhibition of SKI-1/S1P using Spn4A.RRLL(s) blocks HCV infection in human hepatoma cells.
2.3.8 Expression of secretory pathway Spn4A.RRLL(s) causes accumulation of the HCV core protein within the nucleus of infected cells

Due to the dose-dependent decrease in HCV core expression following Spn4A.RRLL(s) treatment, it was expected that HCV infection in Spn4A.RRLL(s) expressing cells would be a rare event. To increase the likelihood of HCV infecting a Spn4A.RRLL(s)-expressing cell, Huh-7.5.1 cells were infected with Ad-Spn4A.RRLL(r) and (s) for only 24 hours and then infected with HCV for 48 hours. Examining these cells by confocal microscopy confirmed that HCV infection in Spn4A.RRLL(s) expressing cells was very uncommon. However, cells positive for FLAG and HCV core were identified and in every case a dramatic relocalization of the core protein to the cell nucleus was observed (Figure 2.6 A and B). This is in striking contrast to cells within the same field of view which do not express Spn4A.RRLL(s) and have the expected perinuclear staining of the HCV core protein (Figure 2.6 B). Cells expressing Spn4A.RRLL(s) and infected with HCV were also probed for HCV NS5A protein expression where no nuclear localization of NS5A was detected (Figure 2.6 C). In cells expressing Spn4A.RRLL(r), no dramatic differences in core expression were seen compared to cells not expressing serpin (Figure 2.6 D). These observations demonstrate that when cellular LD abundance is reduced by Spn4A.RRLL(s) expression, localization of the HCV core protein to LDs, an essential aspect of HCV assembly (111,113,114), is prevented and the HCV core protein is instead mislocalized to cell nuclei.

2.3.9 PF-429242 inhibits HCV infection and extracellular infectious virus secretion in a dose-dependent manner.

PF-429242 was used to investigate if a small molecule inhibitor of SKI-1/S1P would mimic the antiviral effects observed with the protein-based inhibitor, Spn4A.RRLL(s) thereby confirming the importance of this enzyme in the HCV lifecycle. Using PF-429242 can confirm that inhibition of HCV infection by Spn4A.RRLL(s) is not a result of non-specific inhibition due to overexpression of this protein in the secretory pathway. First, an MTS-based cell viability assay was used to demonstrate that PF-429242 minimally reduces cell viability at higher concentrations used, i.e. 10 % at 10 µM (Figure A1.5), likely due to reduced availability of lipids required for cell proliferation. Cells were treated with increasing concentrations (0.05 µM to 50 µM) of PF-429242 for 24 hours before the cell media was replaced and cells were infected for 48 hours with HCV. The number of HCV-infected cells, indicated by positive core protein expression, was measured using Cellomics HCS. As shown in (Figure 2.7 A), pretreatment of
host cells with PF-429242 resulted in a dose-dependent decrease in the number of HCV infected cells (EC\textsubscript{50} 6.4 ± 1.3 µM). A near complete block in HCV infection was observed following treatment with 40 µM of PF-429242.

In Huh-7.5.1 cells, from the time of inoculation, approximately 72 hours are required for production of infectious HCV particles. To examine the impact of PF-429242 treatment on HCV infectious particle release, cells were infected for 72 hours with HCV following 24 hours of PF-429242 pre-treatment. The antiviral activity of PF-429242 was still apparent after 72 hours of being removed as the number of HCV infected cells was 10-fold lower in cells treated with 40 µM PF-429242 compared to cells treated with DMSO (EC\textsubscript{50} value of 6.5 ± 4.8 µM) (Figure 2.7). Under these conditions a 16-fold reduction in extracellular viral titers was also observed (Figure 2.7) with an EC\textsubscript{50} concentration of 1.03 ± 0.3 µM. These results indicate that PF-429242 is a potent inhibitor of HCV infection and downstream production of infectious virus particles.

2.3.10 Inhibition of SKI-1/S1P by PF-429242 interferes with host cell production of infectious HCV particles when added post-HCV entry

Our analysis of Spn4A.RRLL(s) and its inhibition of HCV infection as presented in Figure 2.5 C, suggests that inhibiting SKI-1/S1P activity impedes HCV entry. We hypothesized, based on our observation of reduced LDs, the detection of HCV core in the nucleus and on previous publications demonstrating a requirement for LDs in HCV assembly (110,111,113,114), that SKI-1/S1P inhibition will also block late stages of the HCV lifecycle. Cells were first infected with HCV for 24 hours, allowing uninterrupted HCV entry and establishment of infection. If only HCV entry is blocked when SREBP cleavage is inhibited then PF-429242 should have no further impact on the progression of HCV infection. HCV containing media was then removed and the cells were treated with 0.05 µM to 50 µM of PF-429242. In this scenario, HCV infection was reduced by up to 78% in cells treated with 40 µM PF-429242 compared to DMSO treated cells (Figure 2.7 B). Infectious virus production (extracellular viral titer) was reduced by 90% following treatment with 40 µM PF-429242 (EC\textsubscript{50} of 6.0 ± 3.5 µM) (Figure 2.7 B). Comparing the two sets of data presented in (Figure 2.7 B), which demonstrate a strong antiviral effect of PF-429242 when added either pre- or post-HCV inoculation, we can confirm that inhibiting SKI-1/S1P inhibits post-entry stages of the HCV lifecycle.
2.4 Discussion

A relationship between HCV infection and lipid metabolism has been apparent since early histology studies revealed that infection was associated with hepatic accumulation of LDs (20), a pathology known as steatosis. Research over the past 20 years has continued to demonstrate a key role for fatty acids, cholesterol, and related metabolites and proteins in the HCV lifecycle (18,47,149,215). LDs are important for assembly and release of infectious HCV particles (111,113,114), lipoproteins are required for release (120,121,123) and they facilitate attachment and entry of new virions (129,133), cholesterol uptake receptors play roles in HCV internalization (128,131), and HCV replicase complexes are localized to ER-associated lipid-rafts (106,107). It is this intricate association of the HCV lifecycle with host lipid metabolism that prompted our targeting of SKI-1/S1P, a key lipid metabolism regulator, to block HCV infection in human hepatoma cells.

SKI-1/S1P enzymatic cleavage of host SREBP proteins is essential for activation of host lipid biosynthesis and uptake (282) (summarized in Figure 2.8, left panel). Here we provide experimental evidence that blocking this central step in lipid metabolism activation inhibits the HCV lifecycle. For the first time we show that inhibiting SKI-1/S1P endoproteolytic activity before or after establishment of HCV infection results in a dose dependent reduction in HCV propagation. Inhibiting SKI-1/S1P also reduces intracellular LD abundance which mislocalizes the HCV core protein to cell nuclei and impedes HCV assembly and secretion (summarized in Figure 2.8, right panel).

To dissect the contribution of SKI-1/S1P enzymatic activity to the HCV lifecycle, we first employed a protein-based inhibitor strategy and engineered the natural serpin, Spn4A, which is the most potent inhibitor of furin known (254), to target SKI-1/S1P. We demonstrate that by deleting the ER-retention signal and altering the RCL primary sequence to mimic a SKI-1/S1P polypeptide substrate, Spn4A can form a selective, SDS-stable and heat-stable complex with recombinant SKI-1/S1P. Spn4A.RRLL(s) blocks the expression of SREBP-2, PCSK9, and LDLR in sterol-depleted cells, all of which are transcriptionally regulated by nuclear localization of the transcription activating N-terminal SREBP peptide (51,183,281), confirming effective inhibition of SKI-1/S1P enzymatic activity.

Interestingly, each of these proteins has been implicated in the HCV lifecycle. SREBP-2, along with the other SREBP isoforms SREBP-1a and -1c, are activated by HCV-encoded proteins or during HCV infection (26,27,157-160,262,283,284). PCSK9 has been implicated in
HCV infection through its regulation of two HCV receptors: CD81 and LDLR ((285) and see Chapter 3). As LDLR levels are disrupted by SKI-1/S1P inhibition, a decrease in HCV infection in Spn4A.RRLL(s) treated cells may be partially explained by decreased HCV entry into these cells. This is supported by our finding that when HCV RNA is transfected into cells expressing Spn4A.RRLL(s), less than a 20% reduction in HCV RNA is seen compared to Ad-Empty-treated cells whereas a 66% reduction in HCV RNA was seen in HCV-infected, SKI-1/S1P-inhibited cells (Figure 2.5 C).

In addition to blocking SREBP-mediated gene expression, we hypothesized that blocking SKI-1/S1P would also deplete intracellular lipid stores. As LDs are a key component of the HCV lifecycle (111), we investigated the effect of blocking SKI-1/S1P proteolysis on intracellular LD abundance. Analysis of intracellular lipid levels revealed a 74% reduction in cholesterol-esters, a component of LDs following Spn4A.RRLL(s) expression. Also, when using confocal microscopy it was clear that in Spn4A.RRLL(s) expressing cells LDs were largely absent. Spn4A.RRLL(s)-expressing cells also had reduced ADRP abundance compared to control treated cells. ADRP/perilipin 2, a member of the perilipin family of LD-associated proteins, plays an important role in LD structure and formation (286). The level of ADRP/perilipin-2 expression within a cell is tightly correlated with triglyceride mass (287). Our observed reduction in ADRP/perilipin 2 expression in itself does not demonstrate a block in LD formation, however when ADRP/perilipin 2 is not bound to LDs, it is rapidly degraded (288). A reduction in LD abundance will result in a concomitant decrease in ADRP/perilipin 2 levels as we have observed. Importantly, ADRP/perilipin 2 is found to be upregulated in fatty liver of humans and mice with liver steatosis (289,290). Our observed reduction in LD and ADRP/perilipin 2 abundance suggests that inhibiting SKI-1/S1P offers an attractive target for reducing HCV-induced liver steatosis.

As hypothesized, blocking SKI-1/S1P-mediated SREBP cleavage using Spn4A.RRLL(s) allowed for a dose-dependent decrease in HCV infection. Supplementing Spn4A.RRLL(s)-expressing cells with mevalonate, oleate, and cholesterol, as described for culturing of a SKI-1/S1P deficient cell line (291), allowed for a partial rescue of HCV infection at moi 50, suggesting that the effect is partially, but not completely due to decreased availability of lipids in these cells. As discussed, inhibition of HCV may also be partially due to reduced HCV entry due to LDLR down-regulation. Western blotting confirmed that HCV core expression is reduced by Spn4A.RRLL(s) and that HCV core protein processing was unaltered as the size of the protein
was unaltered. Spn4A.RRLL(s) was also determined to significantly reduce HCV RNA levels following HCV infection. Reduced HCV RNA and HCV core protein levels in SKI-1/S1P inhibited cells lead to a significant reduction in extracellular infectious HCV levels.

We corroborated the importance of SKI-1/S1P to HCV infection using the pharmacologic inhibitor of SKI-1/S1P, PF-429242, which has recently been characterized for its effectiveness in blocking cleavage of SREBPs, blocking expression of SREBP activated genes and also for inhibiting Arenavirus glycoprotein processing (171,198). We confirmed that PF-429242 blocks endogenous SREBP cleavage and showed that neutral lipids and ADRP/perilipin 2 levels are reduced following PF-429242 treatment. Subsequently, it was confirmed that inhibition of SKI-1/S1P using PF-429242 blocks HCV infection and extracellular infectious virus production in a dose-dependent manner. The magnitude of inhibition was greater than that observed with Spn4A.RRLL(s) which may be due to efficient and homogeneous uptake of the compound. Inhibition of HCV infection was particularly striking in that only 24 hour pre-treatment of the compound was required to induce an intracellular state which was greatly resistant to HCV infection and this effect was still apparent 72 hours after the compound had been removed.

As the inhibitory effect of PF-429242 on HCV infection and neutral lipid accumulation was extremely efficient, we next investigated whether HCV infection could be blocked post-establishment of viral entry. HCV infection was allowed to progress for 24 hours in Huh-7.5.1 cells prior to removal of the HCV-containing media and treatment with PF-429242. After 48 hours it was clear that late stages of the HCV lifecycle are inhibited by blocking SKI-1/S1P activity. As extracellular infectious virus production was reduced when the inhibitor was added post-HCV entry and as we determined previously that viral replication is not directly affected, we can conclude that inhibiting SKI-1/S1P prevents HCV assembly and secretion in infected cells. This finding is unsurprising and supports our observation of reduced LD abundance and numerous reports which clearly implicate this organelle in HCV assembly (111,113,114).

Confocal microscopy analysis of Spn4A.RRLL(s) expressing cells infected with HCV provided insight into a mechanism likely contributing to reduced HCV assembly in SKI-1/S1P inhibited cells. The observation that HCV core protein is only found in the nucleus and not in the perinuclear region of Spn4A.RRLL(s)-expressing cells suggests that LD depletion reduces HCV core protein stability directly preventing HCV assembly and secretion. This hypothesis is based on previous reports showing that unstable forms of HCV core, i.e., C-terminally truncated (292-294) or those with mutations in LD targeting domains (112,114), are targeted to cell nuclei
and/or are rapidly turned over. Proteasomal inhibition in these cells recovers core levels and increases detection in cell nuclei (292,295). Thus, proteasome-mediated degradation of the core protein appears to take place in the nucleus in a manner dependent on the proteasome activator PA28-γ (293). Interestingly, association of the HCV core protein with PA28-γ in the nucleus has also been implicated in HCV core-mediated activation of SREBP promoters and the development of steatosis and hepatocellular carcinoma in a mouse model (283). Knocking out PA28 prevents turnover of the nuclear core protein, but it also prevents the development of steatosis in this core transgenic mouse. These results elucidate a potential scenario where, when LDs are absent, the core protein is targeted to the nucleus to activate lipid synthesis for efficient propagation. However, if SREBP-controlled lipid biosynthesis is blocked as in our system, the core protein will continue to accumulate in the nucleus. An absence of LDs and mistargeting of the core protein ultimately leads to reduced HCV assembly and secretion as seen in our analysis of HCV titers in Spn4A.RRL(s)-expressing and PF-429242 treated cells (see model presented in Figure 2.8).

One caveat in targeting SKI-1/S1P is that it is an essential component of lipid metabolism, and lipid metabolism is an essential aspect of cell growth and survival. For dividing cells, a supply of new lipids is clearly important for formation of new membranes and cellular organelles. However, in HCV-infected individuals, there is an over-induction of these processes in the liver (20,262,296). Inhibiting the SREBP pathway in HCV-infected cells, which have exacerbated lipid production and that are steatotic, may relieve symptoms caused by chronic HCV infection in addition to inhibiting multiple stages of the virus lifecycle. SKI-1/S1P is a highly researched target for the treatment of hypercholesterolemia and many therapeutic approaches for inhibiting this enzyme, such as the development of small molecule inhibitors, are being investigated (171,276,297). We have confirmed that a small molecule targeting SKI-1/S1P effectively inhibits HCV infection and can reduce viral spread even after infection is established. Thus, pharmacologic inhibition of SKI-1/S1P offers a very promising avenue for the development of effective antiviral therapeutics.

Investigating the role of SKI-1/S1P is extremely important for many other serious global pathogens. Steatosis can also develop in hepatitis B virus infection, which has been linked to SREBP activation (298,299). Rotaviruses and dengue virus also use LDs for propagation (265,266) and may be disrupted by SKI-1/S1P inhibition. Additionally, Spn4A.RRL(s) or related inhibitors may be applied to study other aspects of lipid metabolism such as lipid rafts in
virus lifecycles. The use of statins to regulate host cholesterol metabolism in individuals with high cholesterol levels (300) suggests that the targeting of lipid metabolism pathways disregulated during microbial infection that correlate with disease severity is a promising potential antimicrobial therapy. Master regulators of these lipid metabolism pathways such as SKI-1/S1P are thus excellent targets for investigating the potential effect of lipid metabolism-directed therapeutics against a wide array of important human pathogens.
Figure 2.1. Engineering the Spn4A scaffold to target the human subtilase SKI-1/S1P.

(A) Recombinant Spn4A variants and amino acid sequences of the engineered reactive center loop (RCL) “bait” region. Spn4A.RRKR(r) encodes for the wild type serpin Spn4A, isolated from Drosophila melanogaster, with potent inhibitory activity against the human proprotein convertase furin. Spn4A.RRKR(r) contains the α-1-antitrypsin (AAT) signal peptide (SP) at the N-terminus followed by a tandem his-tag (HHHHHH) and FLAG-tag (DYKDDDDK) sequence (HF). The P4 – P1 furin cleavage sequence in the RCL is RRLL. Spn4A.RRKR(r) also contains the HDEL ER retention motif (r) at the C-terminus. The secreted (s) serpin, Spn4A.RRKR(s), contains a stop codon before the HDEL signal. The RCL of Spn4A-RRKR(r) and (s) was modified to mimic the predicted SKI-1/S1P target cleavage site present in the Lassa virus glycoprotein pre-GP-C, which is RRLL. Thus, Spn4A.RRLL(r), which is also retained in the ER, encodes the P4 – P1 RRLL cleavage recognition sequence in the RCL. Spn4A.RRLL(s) contains a stop codon before HDEL allowing the serpin to be secreted. (B) In silico homology model of Spn4A.RRLL(r) variant. The molecular model was generated using pyMOL. The side chains of the RRLL residues within the flexible “bait region” of the RCL are shown as sticks in wheat colour. Sheet A is shown in yellow, sheet B is shown in blue, and sheet C is shown in cyan. Α-helices are shown in red and loops are shown in green.
Figure 2.2. Cellular expression and serpin-like properties of recombinant, adenovirus-expressed Spn4A variants.

(A) The cellular expression of serpin variants Spn4A.RRLL(r) and Spn4A.RRLL(s) were examined using infectious adenoviral-mediated expression (moi 50) in Huh-7.5.1 cells. An empty adenovirus vector (Ad-Empty) was used as a control. After 48 hours, cell media (upper panel) and lysates (lower panels) were subjected to western blot analysis. Spn4A variants were detected with mouse anti-FLAG antibody and, to ensure equal loading of samples, extracts were also probed with rabbit anti-β-tubulin antibody. (B) Huh-7.5.1 cells were infected with recombinant adenovirus expressing the his- and FLAG-tagged Spn4A variants indicated or the Ad-Empty control for 72 hours. Media alone (upper panels) or cell extracts (lower panels) lysed in RIPA buffer were combined with recombinant his-tagged SKI-1/S1P or his-tagged furin (273) for 30 minutes at 30°C. Samples were prepared for western blot analysis and probed with mouse anti-his antibody to detect SDS- and heat-stable enzyme-inhibitor (EI*) complex formation as described in the materials and methods.
Figure 2.3. PF-429242 and Spn4A.RRLL(s) inhibit SREBP activation and reduce neutral lipid abundance in Huh-7.5.1 cells.

(A) Huh-7.5.1 cells were treated with DMSO or 10 µM PF-429242 for 24 hours or infected with Ad-Empty (control), Ad-Spn4A.RRLL(r) or Ad-Spn4A.RRLL(s) for 48 hours. One hour prior to harvesting, cells were treated with 25 µM of the calpain inhibitor, ALLN. Cell extracts were harvested and subjected to nuclear fractionation as described in the materials and methods. Nuclear extracts were probed for N-terminal SREBP-1 expression and for enrichment of the nucleolar marker, fibrillarin. The * denotes a non-specific band detected by polyclonal anti-SREBP-1 antibody. (B) Huh-7.5.1 cells were grown in LPDS-supplemented media for 24 hours and were then infected with Ad-Spn4A.RRLL(r), Ad-Spn4A.RRLL(s), or Ad-Empty (control) for 72 hours and cell extracts were harvested and subjected to western blot analysis. Anti-PCSK9, LDLR, SREBP-2, and GM130 antibodies were used to detect protein expression levels, and β-tubulin was probed for normalizing band intensities. Values are plotted relative to protein expression in control cells (left panel). The right panel shows representative western blots for the effect of serpin on each protein examined. (C) Cells were treated with DMSO (control) or 10 µM PF-429242 for 24 hours. The compound was removed and the cells incubated for an additional 24 hours. Cells were then harvested and the relative levels of intracellular lipids were determined in treated cells relative to control cells. Lipid levels were normalized to total intracellular protein content. (D) Huh-7.5.1 cells were infected with Ad-Spn4A.RRLL(r), Ad-Spn4A.RRLL(s) or Ad-Empty (control) for 72 hours. Cells were then harvested and the relative levels of intracellular lipids were determined in treated cells relative to control cells. Lipid levels were normalized to total intracellular protein content. Values are plotted relative to protein or lipid level in control cells. In (A), a representative western blot of 3 independent experiments is shown. For (B), (C) and (D) results (mean ± SEM) from 3 independent experiments are shown. * denotes p < 0.05, ** denotes p < 0.01, and *** denotes p< 0.005
Figure 2.4. LD abundance is dramatically reduced in Huh-7.5.1 cells expressing Spn4A.RRLL(s).

(A-C) Huh-7.5.1 cells were infected with Ad-Empty (control), Ad-Spn4A.RRLL(r), or Ad-Spn4A.RRLL(s) for 72 hours. Fixed cells were stained for cell nuclei using Hoechst dye (blue) and probed for expression of Spn4A variants using mouse anti-FLAG antibody (red). (A) LDs were detected using BODIPY 493/503 (green) and images were acquired using a Leica TCSSP5 confocal microscope. (B) MetaMorph imaging software was used to quantify the number of BODIPY-stained LDs in control cells (n=23), and individual cells expressing Spn4A.RRLL(r) (n=21) or Spn4A.RRLL(s) (n=15). Acquisition and analysis was performed using the same intensity and threshold settings across all images. (C) The LD marker ADRP was detected in Ad-Spn4A.RRLL(r), Ad-Spn4A.RRLL(s) and Ad-Empty (control) treated cells using rabbit anti-ADRP antibody (green), and images were obtained using an Olympus Fluoview FV1000 laser scanning confocal microscope. (D) Huh-7.5.1 cells infected with Ad-Empty (control), Ad-Spn4A.RRLL(r) or Ad-Spn4A.RRLL(s) for 72 hours were harvested and subjected to SDS-PAGE and western blot analysis. Mouse anti-ADRP antibody was used to detect protein expression levels in serpin-treated cells compared to control-treated cells. Relative protein expression was quantified by normalizing to β-tubulin expression. The inset shows a representative western blot. (E) Huh-7.5.1 cells were treated with DMSO (control) or with 10 μM PF-429242 for 24 hours, the compound was removed and the cell lysates harvested after an additional 48 hours. Relative ADRP expression (normalized to β-tubulin) in inhibitor treated cells compared to control cells, was quantified by subjecting total cell lysates to western blot analysis. Values are plotted relative to protein expression in control cells, which are set to 1. Results (mean ± SEM) from 3 independent experiments are shown. * denotes p < 0.05.
Figure 2.5. Inhibition of SKI-1/S1P using Spn4A.RRLL(s) results in a dose-dependent inhibition of HCV infection and compromises HCV entry in Huh-7.5.1 cells.

(A) Huh-7.5.1 cells were infected with various moi (1 - 50) of Ad-Empty (control), Ad-Spn4A.RRLL(r), or Ad-Spn4A.RRLL(s) for 48 hours in regular media or media supplemented with sterols. Treated cells were infected with HCV (moi 0.1) and fixed 72 hours post-HCV infection. Cells, probed with HCV anti-core antibody (red) and stained with Hoechst dye (nuclei; blue), were quantified using Cellomics HCS to determine the percentage of HCV-infected cells. Sample images of cells infected with adenovirus moi 50 acquired with Cellomics HCS using the 10 X objective are shown below the graph. (B) Huh-7.5.1 cells were infected with Ad-Empty (control), Ad-Spn4A.RRLL(r) or Ad-Spn4A.RRLL(s) (moi 50) for 48 hours in complete media and then infected with HCV (moi 0.1) for 72 hours. Relative HCV core expression (normalized to β-tubulin) in serpin-treated cells compared to control-treated cells, was quantified by examining total cell lysates through western blot analysis. Infectious HCV titer in the extracellular media was also determined and relatively quantified. (C) Control and serpin treated cells were infected with HCV or transfected with genomic HCV RNA and total cellular RNA was harvested 72 hours later. HCV RNA levels, normalized to β-actin transcript levels, were relatively quantified in cell extracts using real-time PCR. All values are expressed as relative HCV infection in treated cells compared to control treated cells. Results (mean ± SEM) from 5 (A) or 3 (B and C) independent experiments are shown. Statistical significance was calculated for Ad-Spn4A.RRLL(r) or Ad-Spn4A.RRLL(s) infection compared to Ad-Empty infection of the same moi. * denotes p < 0.05, ** denotes p < 0.01, and *** denotes p< 0.005.
Figure 2.6. Expression of Spn4A.RRLL(s) induces accumulation of the HCV core protein in the nucleus of HCV infected human hepatoma cells.

(A) Huh-7.5.1 cells were infected with Ad-Empty (control) for 72 hours. (B-D) Huh-7.5.1 cells were infected with Ad-Spn4A.RRLL(r) or Ad-Spn4A.RRLL(s) for 24 hours and then infected with HCV for 48 hours. Fixed cells were probed for expression of (A, B, and D) HCV core (mouse anti-core antibody; green) or (C) HCV NS5A (mouse anti-NS5A; green). In all samples Spn4A.RRLL(r) or Spn4A.RRLL(s) expressing cells were detected using rabbit anti-FLAG antibody (red) and were stained for cell nuclei using Hoechst dye (blue). Images were obtained using a Leica TCSSP5 confocal microscope.
A

DMSO + HCV

PF-429242 + HCV

Nuclei

Core

B

Relative HCV extracellular titer

Control 0.1 5 10 40

Relative intracellular HCV core

Control 0.1 5 10 40

PF-429242 → HCV → Harvest

HCV → PF-429242 → Harvest

EC_{50} = 6.4 ± 1.3 \mu M
Figure 2.7. PF-429242 inhibits HCV propagation in a dose dependent manner, pre- and post-establishment of HCV infection in Huh-7.5.1 cells.

(A) Huh-7.5.1 cells were treated with various concentrations (0.01 to 50 µM) of PF-429242 for 24 hours. The inhibitor was removed and the cells were then infected with HCV (moi 0.1) in complete media for 48 hours. Cells, probed with HCV anti-core antibody (red) and stained with Hoechst dye (nuclei; blue), were quantified using Cellomics HCS to determine the percentage of HCV-infected cells. The average EC50 value from 3 independent experiments is displayed above the graph. Sample images of cells treated with DMSO or 50 µM PF-429242 acquired with Cellomics HCS using the 10 X objective are shown to the left of the graph.

(B) Cells were treated with various concentrations (0.1 to 40 µM) of PF-429242 either 24 hours before (left half of graph) or 24 hours after infection (right half of graph) with HCV (moi 0.1). Seventy-two hours post-HCV infection cells were fixed and probed with HCV anti-core antibody (red) and stained with Hoechst dye (nuclei; blue) to determine the percentage of HCV-infected cells (core) using Cellomics HCS. Also, media from treated and HCV-infected cells was harvested and the amount of extracellular, infectious HCV was titered (HCV titer; FFU/ml). Results (mean ± SEM) from 3 independent experiments are shown. Statistical significance was calculated for PF-429242 treated cells compared to DMSO treated cells. * denotes p < 0.05, ** denotes p < 0.01, and *** denotes p < 0.005.
Figure 2.8. Protein-based and small molecule inhibitors of SKI-1/S1P disrupt host lipid metabolism and compromise the HCV lifecycle.

(1) The inactive SKI-1/S1P zymogen is biosynthesized in the ER and traffics to the Golgi apparatus following intramolecular autocatalytic maturation of the proenzyme (69,163,165). (2) During HCV infection, the SREBP pathway is activated by a variety of molecular mechanisms (26,27,158,159). (3) For SREBP to activate genes involved in lipid biosynthesis, its N-terminal domain must be released through sequential cleavage by SKI-1/S1P and then by S2P (72,73). (4) The released N-terminal domain translocates to the nucleus and activates various aspects of lipid metabolism (282). (5) Activation of lipid biosynthesis increases LD formation where the HCV core protein localizes to orchestrate HCV assembly and subsequent secretion (111,113,116). (6) LDLR, a proposed receptor for HCV entry, is also activated by SREBP signaling (65,128,133). (7) Spn4A.RRLL(s) is a secretory pathway-expressed serpin. (8) Spn4A.RRLL(s) interacts and forms a covalent complex with active SKI-1/S1P (Figure 2.2) in the Golgi preventing SKI-1/S1P cleavage of SREBP protein (Figure 2.3 A). (9) A small molecule PF-429242 also inhibits SKI-1/S1P (10). SKI-1/S1P inhibition blocks expression of LDLR (Figure 2.3 B) and reduces HCV entry (Figure 2.5 C). (11) The expression of other SREBP-regulated genes such as PCSK9 and SREBP-2 are also blocked (Figure 2.3). (12) Downstream lipid synthesis is interrupted resulting in overall reduced intracellular cholesterol-ester and triglyceride abundance (Figure 2.3 C and D). This is then detected as a decrease in LD abundance (Figure 2.4) which impedes viral assembly and secretion (Figure 2.5 and Figure 2.7) (13) and mislocalizes the HCV core protein to cell nuclei (Figure 2.6).
Chapter 3: PCSK9 mutations and abundance impact hepatitis C virus entry into human hepatoma cells

3.1 Introduction

Hepatitis C virus (HCV) infection is restricted to the liver, an organ playing a central role in regulating whole-body cholesterol homeostasis through controlled expression of factors required for cholesterol synthesis, uptake and release (Reviewed in (301)). HCV employs an array of strategies to hijack these integral lipid metabolism processes which allows for efficient viral propagation and persistence (26,110,111,152). Assembly of HCV particles requires and is orchestrated around host lipid droplets (LD) (111-114,116,295) which normally provide lipids to assembling very low density lipoproteins (VLDL) (53). HCV subverts the VLDL synthesis and secretion pathway for its own release from an infected cell (120-122). The secreted viral particles are found in association with host lipoprotein components and are termed lipoviroparticles (LVP) (124,126,302,303). This stealthy escape process from the cell allows LVP interaction with and potentially uptake by the low density lipoprotein receptor (LDLR) into hepatocytes (See Figure 3.1)(123,128,133,134,304). It remains unclear how LDLR cooperates with other well studied HCV receptors CD81 (131,135,305,306), SR-B1 (131,305), Claudin-1 (139) and occludin (142,307) to mediate HCV entry.

Recently, proprotein convertase subtilisin/kexin type 9 (PCSK9) was shown to be a major post-transcriptional regulator of LDLR in the liver (63,64,173). PCSK9 is a member of the proprotein convertase (PC) family of serine proteases involved in the maturation and activation of a variety of host and pathogen encoded proteins (263). PCSK9 is expressed primarily in the liver and intestine (308) and is synthesized as a luminal proprotein in the ER. There, it undergoes autocatalytic cleavage to remove its amino-terminal prodomain (309) which remains associated to the active protein and inhibits catalytic activity (174,310). PCSK9 is then secreted and binds LDLR at the cell surface (175) (see Figure 3.1). Following receptor mediated endocytosis PCSK9 prevents LDLR recycling back to the plasma membrane (177); instead, LDLR is degraded in lysosomes (176). Thus, increasing PCSK9 expression or its LDLR binding-affinity results in lower than normal cell surface LDLR expression. In humans, gain-of-function mutations in PCSK9 have been associated with hypercholesterolemia (172,178,179) due to

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decreased clearance of plasma LDL by the liver, while loss of function PCSK9 mutations are associated with abnormally low circulating cholesterol levels (180,311).

The unique features of PCSK9 prompted our investigation into the potential application of modulating HCV infection through strategic manipulation of PCSK9 activity. Here we confirm that PCSK9 overexpression can be effectively used to inhibit HCV infection and downstream infectious virus production. We also demonstrate that PCSK9 gain- and loss-of-function mutants allow various degrees of HCV infection inhibition in a human hepatoma cell line. We provide evidence that PCSK9 acts primarily at the cell surface despite potential effects on intracellular phases of HCV propagation which also supports the importance of LDLR in mediating HCV entry. These studies suggest that PCSK9 may be employed in the development of novel therapeutic avenues against HCV infection through inhibition of viral entry and ensuing infection.
3.2 Materials and methods

Cell culture and other reagents – Human hepatoma Huh-7 and Huh-7.5.1 cells were kindly provided by Dr. Francis Chisari (Scripps Research institute, La Jolla, CA, USA). Cultured cells were grown in Dulbecco’s Modified Eagle Medium (DMEM) supplemented with 1% of each penicillin, streptomycin, glutamine, non-essential amino acids, HEPES and 10% fetal bovine serum (FBS) (Gibco/Invitrogen, Burlington, ON, Canada) or 10% lipoprotein depleted serum (LPDS) (Biomedical Technologies Inc., Stoughton, MA, USA). Recombinant, his-tagged, wild-type PCSK9 and gain-of-function mutant PCSK9-D374Y are from Circulex at MBL International (Woburn, MA, USA). Bovine Serum Albumin (BSA) and Saponin are from Sigma-Aldrich Corp. (St. Louis, MO, USA) and 37% formaldehyde is from Fischer Scientific (Pittsburg, PA, USA).

Antibodies and dyes – Antibodies used to detect HCV infection include a mouse anti-core antibody (1:500 for western blot and 1:50 for immunofluorescence, Abcam, Cambridge, MA, USA) and a mouse anti-NS5A antibody (1:50, gift of Dr. Chen Liu, University of Florida, Gainesville, FL, USA). Antibodies used to detect cellular proteins include rabbit anti-human PCSK9 (1:500 for western blotting, Abcam) and rabbit anti-mouse PCSK9 (1:50 for immunofluorescence, Cayman chemical, Ann Arbor, MI, USA), rabbit anti-LDLR (1:250, Fitzgerald Industries International, Inc., Concord, MA, USA), rabbit anti-β-tubulin (1:1000, Abcam), mouse anti-β-tubulin (1:500, Lab vision corp. Montreal, QC, Canada). Secondary antibodies used for immunofluorescence include Alexa Fluor-488-conjugated or Alexa Fluor-568-conjugated donkey anti-mouse or donkey anti-rabbit antibodies (1:100, Molecular Probes/Invitrogen). Secondary antibodies used for western blot were IRDye 680-conjugated (red bands) or 800-conjugated (green bands) donkey anti-mouse or donkey anti-rabbit antibodies (1:10000, LI-COR Biosciences, Lincoln, NE, USA). Hoechst 33258 (10 µg/ml, Molecular Probes) was used for detection of nuclei.

HCV RNA and infectious stock production – A plasmid encoding the cDNA of an HCV consensus clone isolated from a Japanese patient with fulminant hepatitis (JFH-1) (GenBank accession number AB047639) (268) cloned behind a T7 promoter (pJFH-1; kindly provided by Dr. Takaji Wakita, National Institute of Infectious Diseases, Tokyo, Japan) was used to generate genomic HCV RNA and infectious HCV stocks as described previously (269).

Transfection of HCV RNA – Purified HCV RNA was used to transfect Huh-7.5.1 cells to study HCV infection independently of receptor mediated entry. Five micrograms of purified RNA was
incubated with 10 μl of lipofectamine 2000 (Invitrogen) in minimal essential media (MEM) for 30 minutes. The cells were transfected in MEM overnight and then replaced with complete media for the remainder of the experiment.

**HCV titer determination** – Infectious HCV titers of viral stocks or of HCV in media harvested in experiments were determined using a modified, previously described protocol (104). Briefly, 1 x 10^4 Huh-7.5.1 cells were plated in each well of a 96-well plate and infected with 10-fold serial dilutions of HCV-infected media. Three days post-infection cells were fixed and probed as described in the ArrayScan methods section. A Cellomics ArrayScan VTI High Content Screening (HCS) Reader (Thermo Scientific) was used to acquire images of the entire infected wells. Titers were determined by counting foci (fluorescence forming units (FFU)) in the lowest dilutions with positive signal.

**Recombinant adenoviruses** – Empty adenovirus (Ad-Empty) and recombinant adenovirus expressing mouse wild-type PCSK9 (GenBank accession no. AY273821) (Ad-PCSK9) or catalytic mutant PCSK9 (Ad-PCSK9-S402A), was kindly provided by Dr. Jan Breslow (The Rockefeller University, New York, NY, USA) (64). Adenovirus expressing EGFP (Ad-CMV-EFGP) was a gift from Dr. Lin Liu (Oklahoma State University, Stillwater, OK, USA). All viral constructs (except Ad-CMV-EGFP) were amplified by and purchased from ViraQuest Inc (North Liberty, IA) to generate high titer adenovirus stocks. Adenovirus stocks were titered in HEK293T cells using Adeno-X Rapid Titer kit (Clontech, Mountain View, CA, USA).

**Western blot analysis** – Cells were rinsed with ice-cold, phosphate-buffered saline (PBS) before resuspending in cold radioimmunoprecipitation (RIPA) buffer (50 mM Tris-HCl pH 8, 150 mM NaCl, 1% octylphenyl-polyethylene glycol (IGEPAL), 0.5% sodium deoxycholate, 0.1% SDS) containing 1 X Complete, EDTA-free, protease inhibitor cocktail (Roche, Laval, QC, Canada). Whole cell extracts were vortexed and then clarified by centrifugation at 12000 x g for 15 minutes. Cell extracts were mixed with 2 X sample buffer (62.5 mM Tris-HCl, pH 6.8, 25% glycerol, 2% SDS, 0.01% bromophenol blue, and 5% β-mercaptoethanol), electrophoresed on SDS-polyacrylamide gels and transferred to nitrocellulose membranes. Membranes were blocked in odyssey-blocking buffer (LI-COR Biosciences) for one hour, and probed with appropriate primary and secondary antibodies in odyssey blocking buffer containing 0.1% Tween-20. Protein bands were relatively quantified using the Odyssey two-colour, infrared imaging system (LI-COR Biosciences). B-tubulin was used as a loading control and for normalizing protein
expression. Media samples analyzed for secreted PCSK9 were taken directly from cultured cells, mixed with 2 X sample loading buffer, and analyzed by western blot.

**ArrayScan quantification** – In black flat-bottom 96-well plates (BD Biosciences) cells were plated and infected as described. Cells were fixed in 4% formaldehyde diluted in PBS then blocked in PBS containing 3% BSA, 0.3% Triton X-100, and 10% FBS. Cells were probed with anti-core antibody (1:500) in PBS containing 3% BSA and 0.3% Triton X-100 (Binding Buffer) then incubated with Alexa Fluor-568-conjugated donkey anti-mouse secondary antibody (1:1000) and 10 µg/ml Hoechst dye. Cells were analyzed by a quantitative, high-throughput, fluorescence microscope system called the Cellomics ArrayScan VTI High Content Screening (HCS) Reader (Thermo Scientific). The BioApplications software, Target Activation protocol was used to count the total number of cells (Hoechst-stained nuclei) and the percentage of those cells that were infected with HCV (positive signal at 568 nm wavelength).

**Confocal microscopy and immunofluorescence** – Fifteen thousand Huh-7.5.1 cells were seeded onto round, sterile coverslips in a 24-well plate for 24 hours. Cells were then infected with adenovirus (moi 100) for 24-hours followed by HCV infection (moi 0.5) for 48 hours. Cells were fixed for 20 minutes in 4% formaldehyde in PBS and then washed with PBS. Cells were permeabilized and blocked for 1 hour in PBS containing 0.05% saponin (wash buffer) and 1% BSA (binding buffer). Cells were washed with PBS and then probed with primary antibodies for 1 hour at room temperature in binding buffer. Cells were washed 3 times then incubated for 1 hour with secondary antibody and Hoechst dye (10 µg/ml) diluted in PBS. Cells were mounted onto slides with an anti-fade solution and sealed with clear nail-polish. Slides were imaged using a Leica TCSSP5 confocal microscope (Leica Microsystems).

**Adenovirus infections** – All adenovirus infections were done at moi 50 unless otherwise indicated. To determine the effect of PCSK9 overexpression on LDLR expression and HCV infection an adapted previously described protocol was used (64). Huh-7.5.1 cells were infected with Ad-PCSK9, Ad-PCSK9-S402A or Ad-Empty for 24 hours then complete media was changed to media containing LPDS and the cells were reinfected with adenovirus. After a further 60 hours samples were harvested for western blot analysis. Alternatively, treated cells were infected with HCV at a moi of 0.1 or transfected with 5 µg HCV genomic RNA (diluted in Opti-MEM with Lipofectamine 2000 as described above, media changed after 16 hours) and harvested 3 days later for western blot, PCR or titer determination.
**Soluble extracellular PCSK9 treatments** – In a 96-well plate 7 X 10^3 Huh-7.5.1 cells were plated in complete media, cells were then switched to LPDS supplemented media and after 24 hours were treated with various concentrations of PCSK9, PCSK9-D374Y or BSA; after 8 hours, the cells were infected with HCV at moi 0.5. Recombinant PCSK9 was replaced 24 hours after the first PCSK9 treatment, the cells were then fixed 48 hours following HCV infection and the percentage of HCV infected cells were determined using Cellomics ArrayScan VTi HCS reader.

**Quantitative real-time PCR** – Purified total RNA was reverse transcribed to cDNA using Taqman reverse transcription reagents (random hexamers; Applied Biosystems, Foster City, CA, USA). Real-time quantitative PCR was carried out using Brilliant II Fast QPCR reagents (Stratagene, La Jolla, CA, USA) according to the manufacturer’s instructions on a Stratagene Mx3005P QPCR system. For amplification of the HCV RNA 5’ region, 400 nM of both forward (5’-CATGGCGTTAGTATGAGTGC-3’) and reverse (5’-GGTTCGCAGACCACACTAT-3’) primers were used in combination with 200 nM of probe #75 from the human probe library (Roche). HCV RNA was relatively quantified using β-actin as an endogeneous control amplified using 500 nM primers (forward: 5’- GCCCTGAGGC TCTTCC and reverse: 5’ GGATGTCCACGTCA CACTTC-3’). The probe sequence for β-actin is 5’ACTCCATGC CCAGGAAGGAAGGC-3’ and has a 5’ Cy5 fluorophore and 3’ black hole quencher.

**Curve fitting, half maximal effective concentration (EC50) determination and statistics** – The sigmoidal fit function in Igor Pro software was used for fitting HCV and recombinant PCSK9 or PCSK9-D374Y inhibition curves and for determining EC50 values. The reported EC50 values are the average of the values calculated from three independent experiments plus or minus the standard deviation. The student’s t-test (unpaired) was used to calculate significance which is represented in the figures by the following notation: * denotes p < 0.05, ** denotes p < 0.01, and *** denotes p< 0.005.
3.3 Results

3.3.1 Huh-7 and Huh-7.5.1 cells express different levels of PCSK9 and LDLR

To investigate the potential role of PCSK9 in HCV infection we used the genotype 2a JFH-1 HCV strain and Huh-7 and Huh-7.5.1 human hepatoma cell lines which are infected by HCV with different efficiencies (104). The difference in susceptibility of the two cell lines was confirmed by infecting Huh-7 and Huh-7.5.1 cells in parallel with HCV for 72 hours. Cell extracts were subjected to western blot analysis and probed for relative HCV core and HCV NS5A expression. HCV core expression was 16-fold greater and NS5A expression was 15-fold greater in Huh-7.5.1 cells compared to Huh-7 cells (Figure 3.2 A). The amount of infectious virus produced in cell media was titered and found to be 117-fold greater in Huh-7.5.1 cells than in Huh-7 cells (Figure 3.2 A). We then probed naïve Huh-7 and Huh-7.5.1 cell extracts for endogenous PCSK9 expression levels using western blotting. Interestingly, Huh-7 cells expressed, on average, 2.3-fold more PCSK9 than Huh-7.5.1 cells (Figure 3.2 B). This correlated with significantly lower expression (3.2-fold) of mature LDLR (160 kDa cell surface form) in Huh-7 cells compared to Huh-7.5.1 cells. Claudin-1 expression was also examined and was found not to differ significantly across the two cell lines. The reduced PCSK9 and increased LDLR abundance in Huh-7.5.1 cells could potentially contribute to greater HCV infection efficiency due to more efficient attachment and entry.

3.3.2 Wild-type PCSK9 and the catalytic mutant PCSK9-S402A are secreted and reduce mature LDLR expression in Huh-7.5.1 cells

To investigate the potential use of PCSK9 for modulating the susceptibility of liver-based cell lines to HCV infection we examined PCSK9 overexpression in Huh-7.5.1 cells. Infectious recombinant adenoviruses, which display strong tropism for the liver (144), the natural site of HCV infection (280), were employed to overexpress wild-type PCSK9 or a catalytic mutant form of PCSK9 (PCSK9-S402A). Previously, the catalytic mutant PCSK9-S402A was shown to be unprocessed in rat hepatoma cells and exhibited a reduced capacity for LDLR turnover (173) compared to wild-type PCSK9. Huh-7.5.1 cells which have low levels of PCSK9 expression compared to Huh-7 cells were infected with Ad-PCSK9, Ad-PCSK9-S402A or the control empty vector (Ad-Empty) and the cell extracts and media were analyzed by western blotting for PCSK9 expression. As expected, wild-type PCSK9 overexpression caused an intracellular increase in both the 75 kDa, proprotein form of PCSK9 and the mature, processed, 60 kDa PCSK9 which
demonstrates autocatalytic cleavage of the prodomain from the catalytic domain (Figure 3.3 A, Lane 5 and 6, bottom panel). Only the mature form of PCSK9 was secreted, as evidenced by detection of the 60 kDa band in the media (Figure 3.3 A, Lane 5 and 6, top panel). Western blotting of cell extracts expressing PCSK9-S402A revealed an expected increase in only the unprocessed 75 kDa form of PCSK9 (Figure 3.3 A, Lane 3 and 4, bottom panel); unexpectedly this form was also found secreted into the media (Figure 3.3 A, Lane 3 and 4, top panel). The presence of PCSK9-S402A in cell media did not appear to result from lysis of treated cells as the cells were visibly healthy when observed by light microscopy. Also, β-tubulin was not detected in the same media subjected to western blot analysis (Figure 3.3 A). Secretion of PCSK9-S402A also occurred in Huh-7 and HepG2 hepatoma cells (Figure A2.1).

Next, the effect of wild-type and mutant PCSK9 overexpression on LDLR levels in Huh-7.5.1 cells was examined using western blotting. Initial time course studies where Huh-7.5.1 cells were treated with Ad-PCSK9 for varying amounts of time revealed that these cells were resistant to LDLR turnover compared to HepG2 cells; in a published study using these same constructs only 36 hours of PCSK9 overexpression was required to dramatically reduce whole cell LDLR levels (64). Here, to achieve a 66% reduction in mature LDLR expression, 86 hours of PCSK9 treatment were required (Figure 3.3 B). This may reflect the fact that Huh-7 and Huh-7.5.1 cells express high levels of Annexin II, a PCSK9 antagonist, compared to HepG2 cells (185 and Figure A2.2). PCSK9-S402A caused a 32% reduction in LDLR expression compared to cells infected with Ad-Empty. Although unexpected, a slight reduction in LDLR by this mutant was previously reported in rat hepatoma cells (173). These results confirm that overexpressing PCSK9 increases LDLR turnover in Huh-7.5.1 cells and that the degree of turnover can be altered using different PCSK9 variants.

3.3.3 Overexpression of PCSK9 and PCSK9-S402A inhibits HCV infection in Huh-7.5.1 cells

As LDLR is important for HCV entry (128,129,132-134,312) we next investigated the effect of overexpressing PCSK9 on HCV infection in hepatoma cells. Huh-7.5.1 cells were infected with Ad-PCSK9, Ad-PCSK9-S402A or Ad-Empty and then infected with HCV. Western blotting analysis of cellular extracts showed an 80% decrease in HCV core expression in wild-type PCSK9 overexpressing cells compared to control treated cells (Figure 3.4 A). Following PCSK9-S402A overexpression a 31% reduction in HCV core expression was observed (Figure 3.4 A). The level of infectious virus secreted from HCV infected cells was also
experimentally determined, and shown to be reduced by 74% and 40% in PCSK9 and PCSK9-S402A overexpressing cells respectively compared to Ad-Empty infected cells (Figure 3.4 B). The total amount of intracellular HCV RNA was quantified and determined to be 64% lower in wild-type PCSK9 expressing cells compared to Ad-Empty infected cells (Figure 3.4 C). Altogether these results confirm that increasing PCSK9 expression renders Huh-7.5.1 cells resistant to HCV infection.

3.3.4 Individual cells overexpressing PCSK9 are resistant to HCV infection

To gain further insight into the mechanism of PCSK9 mediated inhibition of HCV infection we examined HCV infected cells following PCSK9 and PCSK9-S402A overexpression using immunofluorescence and confocal microscopy. Using a PCSK9-directed antibody we detected two subsets of cells when Ad-PCSK9 or Ad-PCSK9-S402A cells were examined: those with high PCSK9 expression and those with low, apparently endogenous, PCSK9 expression (See Figure 3.5 A and B, left panels). Cells with more intense staining are predicted to be those cells infected with Ad-PCSK9. Infection of Huh-7.5.1 cells with Ad-PCSK9 and then HCV revealed that cells overexpressing PCSK9 (high level of PCSK9 staining) consistently demonstrated little to no HCV core or NS5A expression (Figure 3.5 A). HCV appeared to only infect cells with low or endogenous levels of PCSK9. A similar phenotype was observed in cells infected with Ad-PCSK9-S402A, where cells exhibiting more intense PCSK9 staining were less frequently infected with HCV (Figure 3.5 B). As a control we also imaged cells that were first infected with Ad-CMV-EGFP (adenovirus expressing humanized green fluorescent protein) and then infected with HCV. HCV core and NS5A protein expression was consistently detected in EGFP expressing cells (Figure 3.5 C). These results support our hypothesis that cells overexpressing PCSK9 are resistant to HCV infection.

3.3.5 Increasing PCSK9 activity inhibits HCV at the entry step of infection

Little is known about the function of PCSK9 outside of its capacity for LDLR and related receptor (i.e. VLDLR) down regulation (184). As such, it was unknown whether PCSK9 may also modulate intracellular aspects of cholesterol metabolism that may impact an intracellular stage of the HCV lifecycle. To investigate, PCSK9 and PCSK9-S402A were overexpressed in Huh-7.5.1 cells. Treated cells were then transfected with purified, genomic, HCV RNA which can be replicated and translated by the host machinery. After 72 hours, the level of HCV core produced in Ad-PCSK9 and Ad-PCSK9-S402A infected cells was not reduced compared to Ad-
Empty infected cells (Figure 3.6A), indicating that PCSK9 overexpression does not affect translation of the HCV genome. Real-time PCR analysis demonstrated that HCV RNA levels were also unaffected by PCSK9 and PCSK9-S402A overexpression when HCV receptor mediated endocytosis is bypassed (Figure 3.6B). These findings suggest that the reduction in HCV core and HCV RNA that result following PCSK9 overexpression result from disrupted HCV entry into Huh-7.5.1 cells.

To provide further evidence that PCSK9 inhibits HCV entry we took advantage of a unique aspect of PCSK9 function. Many studies have confirmed that PCSK9 can efficiently downregulate LDLR expression in cell culture when recombinant, soluble PCSK9 is added extracellularly (177,313). The ability of extracellular PCSK9 to inhibit HCV infection through extracellular targeting of cell surface LDLR in Huh-7.5.1 cells was examined. A time course analysis of soluble PCSK9 added to Huh-7.5.1 cells showed the most efficient knockdown of mature LDLR expression after 8-hours over a 24-hour period (data not shown). Huh-7.5.1 cells were thus treated for 8 hours with seven different concentrations of purified PCSK9 (from 0.15 µg/ml to 25 µg/ml) prior to HCV infection (Figure 3.7A and B). Pretreatment of cells with 1.5 µg/ml was required to inhibit HCV infection by 26 % whereas 25 µg/ml PCSK9 nearly completely inhibited HCV infection (98.6 % or 42-fold). The EC$_{50}$ value for PCSK9 inhibition of HCV infection under these conditions is 4.6 ± 0.06 µM. These results confirm that interaction of PCSK9 with receptors at the cell surface is sufficient to block HCV infection and further supports a role for PCSK9 in regulating HCV entry.

3.3.6 The gain of function mutant, PCSK9-D374Y, blocks HCV infection more efficiently than wild-type PCSK9

The gain-of-function mutant PCSK9-D374Y is a well-studied PCSK9 mutant found within the human population. This mutant form of PCSK9 has been shown to bind LDLR with increased affinity (314,315) and increases LDLR turnover at least 10-fold compared to wild type PCSK9 (313). This increased activity is associated with hypercholesterolemia in patients expressing the PCSK9-D374Y mutant (178,179,316). Purified recombinant PCSK9-D374Y was used in this study to pretreat cells before infecting with HCV as described for wild-type PCSK9. Only 0.15 µg/ml PCSK9-D374Y was required to inhibit HCV infection by 29 % (compared to BSA treated cells). This level of inhibition required 10-fold more (1.5 µg/ml) of wild-type PCSK9. With 10 µg/ml of PCSK9-D374Y or wild-type PCSK9 a similar level of HCV infection inhibition was observed (75 % and 70 % respectively). Interestingly at the highest PCSK9-
D374Y concentration used only 90% inhibition of HCV infection was observed compared to 97.6% inhibition by wild-type PCSK9. The EC$_{50}$ value for PCSK9-D374Y inhibition of HCV infection under these conditions is 0.66 ± 0.05 µM. These studies confirm that PCSK9-D374Y can regulate HCV entry more efficiently than wild-type PCSK9 at concentrations lower than 10 µg/ml. Our research also suggests that natural mutations in PCSK9 found within the human population may regulate susceptibility to HCV infection.
3.4 Discussion

The discovery of PCSK9 and its unique capacity for post-translational LDLR regulation has led to many advances in potentially antagonizing PCSK9 function as a means of treating hypercholesterolemia (reviewed in (317)). The biological significance of PCSK9 activity is apparent from the effect of mutations in PCSK9 in the human population and how they impact plasma LDL and cholesterol levels. The gain of function mutant PCSK9-D374Y, for example, is associated with autosomal dominant hypocholesterolemia resulting from reduced liver LDLR levels and defective uptake of LDL from patient circulation (178,179,313-316). The prominent effect of PCSK9 on LDLR regulation and overall cholesterol homeostasis combined with the dependence of HCV infection on LDLR expression led us to investigate the role of PCSK9 in HCV infection (Summarized in Figure 3.1). Here we report several findings which demonstrate the importance of PCSK9 in regulating HCV entry and subsequent infection of hepatoma cells.

First, we examined the levels of PCSK9 and LDLR in two related cell lines that are susceptible to JFH-1 HCV infection with differing efficiencies. We discovered that higher PCSK9 levels are expressed in Huh-7 cells than in Huh-7.5.1 cells which correlate with lower LDLR levels in the Huh-7 cell line. This may explain in part, the increased susceptibility of Huh-7.5.1 cells to HCV infection. Based on this information we chose to increase the levels of PCSK9 in the Huh-7.5.1 cells and expectedly saw a reduction in LDLR protein expression. Substantially reducing PCSK9 expression however, required a longer pre-incubation time in Huh-7.5.1 cells compared to previous studies performed in HepG2 cells (64). One potential reason for this may be the expression of Annexin A2, an antagonist of PCSK9 function (185), in Huh-7.5.1 cells which is not expressed in HepG2 cells. Expression of this endogenous inhibitor may impede PCSK9 mediated downregulation of LDLR which would not occur in HepG2 cell-based experiments.

A reduction in LDLR expression was observed when using both wild-type and a catalytic mutant form of PCSK9. It has been previously suggested that PCSK9 autocatalytic cleavage is necessary for proper folding and exit of PCSK9 from the ER (174). We thus predicted that PCSK9-S402A would remain trapped in the ER and be unable downregulate LDLR. Unexpectedly, we did detect uncleaved PCSK9 in the media which may be due over saturation of the secretory pathway or the ability of this particular mutant to fold in such a way that allows it to be secreted. Although exit of PCSK9 from the ER is necessary for PCSK9 downregulation of LDLR (64), catalytic activity is not (318,319). Thus, if an interaction between LDLR and PCSK9
is permitted it is reasonable that this mutant is able to downregulate LDLR expression as seen here and previously in rat hepatoma cells (173).

As hypothesized, disrupting LDLR levels by overexpressing PCSK9 allowed a dramatic reduction in HCV infection in Huh-7.5.1 cells as evidenced by decreased core production, viral RNA and reduced extracellular infectious virus. We also visualized cells overexpressing PCSK9 or PCSK9-S402A and infected with HCV using immunofluorescence and confocal imaging. Staining cells for PCSK9 and HCV core or HCV NS5A expression revealed HCV consistently infected cells with low levels of PCSK9 while cells overexpressing PCSK9 were resistant to HCV infection. This supports research done by Poirier et al. which showed that wild-type PCSK9 acts locally on LDLR cell surface expression immediately upon secretion and not on neighboring cells (320).

We extended these experiments and investigated the stage of the HCV lifecycle that is inhibited by increased PCSK9 expression. Although the predominant function of PCSK9 appears to be modulation of LDLR levels at the cell surface, PCSK9 can also modulate intracellular levels of LDLR (320). Research has shown that intracellular LDLR levels control host lipoprotein secretion (280,321,322) which could also potentially impact intracellular phases of the HCV lifecycle (25,120,121,323). Huh-7.5.1 cells overexpressing PCSK9 were thus transfected with genomic HCV RNA, bypassing the requirement for receptors to initiate the HCV lifecycle. In this situation we did not observe any downregulation of HCV core or viral RNA levels three days post-transfection. Following HCV RNA transfection, approximately 7 days are required for infectious virus production thus viral titers were not examined. Further support for PCSK9 regulating HCV entry was provided by the use of a recombinant form of PCSK9 added extracellularly which regulates cell surface LDLR levels. Recombinant, purified, his-tagged PCSK9 was able to block HCV infection in a dose dependent manner. This supports our hypothesis that regulation of cell surface LDLR levels is sufficient for PCSK9 mediated inhibition of HCV infection.

A prominent number of individuals in the human population encode PCSK9 mutant alleles within their genomes which leads to hyper or hypocholesterolemia (172,178-180,311,316). Our finding that PCSK9-S402A inhibits HCV infection with a lower efficiency than wild-type PCKS9 suggests that mutations within the human population may be linked with different susceptibilities to HCV infection. Studies have shown that the well-known mutant PCSK9-D374Y reduces LDLR levels 10-fold more efficiently than wild-type PCSK9 and that
this may be due to its increased affinity for LDLR (313,324). In support of this, it was found that PCSK9-D374Y inhibited HCV infection much more efficiently (estimated EC\textsubscript{50} of 0.92 µg/ml) compared to PCSK9 wild-type (estimated EC\textsubscript{50} of 5.55 µg/ml) and was particularly evident at lower and more physiologically relevant concentrations which have been shown to range between 0.033 µg/ml to 3 µg/ml (270). These results have important implications as a variety of mutations found within the human population have profound impacts on the levels of liver LDLR and may impact susceptibility to HCV infection.

During our investigation into the role of PCSK9 during HCV infection, Labonté et al (285) also demonstrated that stable overexpression of PCSK9 or a chimeric membrane anchored overactive PCSK9 mutant (PCSK9-ACE2) could be used to render Huh-7 cells resistant to HCV infection. Using flow cytometry analysis a decrease in cell surface CD81 expression was found in addition to the expected LDLR reduction. Nearly a complete block of HCV infection in cells expressing PCSK9-ACE2 was observed but only a 5-fold reduction in pseudoviral particle entry was achieved in PCSK9-ACE2 expressing cells. Pseudoviral particles do not utilize LDLR for entry as they are not associated with lipoproteins and thus the effect on pseudoviral particles was likely due solely to lowered CD81 expression whereas the enhanced reduction of HCV infection likely resulted from reduced LDLR expression and potentially altered cholesterol homeostasis. Due to the enhanced nature and time of PCSK9 expression in these stable cell lines abnormal cholesterol uptake by these cells due to reduced LDLR, VLDLR and ApoE2 expression (184,325) may alter the normal plasma membrane expression of CD81, as this is a lipid raft targeted protein (272,305,326).

A direct interaction between PCSK9 and CD81 has not yet been demonstrated to indicate whether such a regulation would also occur in humans. Mazumdar et al. (327) did not detect a significant reduction in cell surface CD81 levels in Huh-7 cells transfected with PCSK9-D374Y-expressing plasmids. However, they did observe a reduction in entry of pseudoviral particles expressing the HCV E1 envelope glycoprotein. In our studies we cannot rule out the effect of CD81 in PCSK9-mediated block in HCV infection but it is clear that LDLR is playing a role. This notion is supported by studies done by Owen et al (128) where knockdown of CD81 and LDLR had a synergistic effect in blocking HCV entry. One last important point is that it has been shown that CD81 also affects intracellular aspects of HCV infection (144,271) and we have determined that there is no effect of short term PCSK9 expression on post-entry stages of HCV infection. Overall, our studies confirm the role of PCSK9 and LDLR on HCV entry.
In addition to directly interrupting HCV entry, enhancing or inducing PCSK9 function may also have therapeutic benefits for those infected with HCV. During chronic HCV infection the levels of circulating plasma lipoproteins are reduced, a pathology known as hypobetalipoproteinemia. In relation to this, retention of lipids in the liver is grossly increased, leading to steatosis. Hypobetalipoproteinemia and steatosis have both been linked to increased severity of chronic HCV-induced liver disease and steatosis correlates with a poor response to antiviral therapy (30,31,33,35). Both of these pathologies are linked to LDLR, which is responsible for clearing circulating lipoproteins (51) and prevents cholesterol-rich lipoproteins from being secreted by reducing ApoB stability (321). Therefore, reducing PCSK9 expression and function could potentially restore lipid homeostasis by reducing LDLR mediated clearance of lipoproteins and supporting secretion of stored lipids from hepatocytes.

In summary, PCSK9 mediates a precisely controlled regulation of LDLR; PCSK9 acts effectively when applied extracellurally and single mutations dramatically alter its capacity for LDLR regulation and turnover. As a high degree of variability in PCSK9 function exists within the human population it is feasible that PCSK9 influences natural susceptibility and progression of HCV infection. It follows then that PCSK9 is also a promising therapeutic target for treating or preventing the progression of HCV infection and may increase the effectiveness of, if targeted in combination with, the current antivirals pegylated-interferon-α and ribavirin or other direct-acting antiviral agents.
Figure 3.1. PCSK9 is a post-translational regulator of low density lipoprotein receptor (LDLR).

(Left panel) LDLR is a cell surface transmembrane protein which plays a role in internalization of host lipoproteins from plasma. Highly infectious HCV particles produced in cell culture, chimpanzees and in humans are complexed with and resemble host lipoproteins and are termed lipoviroparticles (LVPs). These particles bind LDLR at the cell surface and as such this receptor is proposed to play a role in HCV entry. Following binding of lipoproteins, LDLR and attached ligands are internalized in a clathrin dependent manner. Lipoproteins are released and metabolized intracellularly while LDLR is recycled back to the cell surface. (Right panel) PCSK9, a member of the proprotein convertase family, is synthesized in the ER where it undergoes autocatalytic cleavage which permits proper folding and passage through the secretory pathway. The prodomain of PCSK9 remains associated with the catalytic domain throughout secretory pathway transit and upon extracellular secretion of PCSK9. At the cell surface PCSK9 binds LDLR, separately from the lipoprotein binding domain. When the complex is internalized, LDLR recycling is prevented by the interaction with PCSK9; instead LDLR is degraded. The effect of increased PCSK9 expression or affinity for LDLR is an overall reduced LDLR abundance at the cell surface.
Figure 3.2. Huh-7.5.1 cells express higher LDLR and lower PCSK9 levels than Huh-7 cells, correlating with higher susceptibility to HCV infection.

Huh-7 and Huh-7.5.1 cells were grown in parallel in complete media and infected (or not infected) with HCV at a moi of 0.1. Three days post infection total cell extracts were harvested in RIPA buffer and subjected to SDS-PAGE and western blot analysis. (A) Infected cell extracts were analyzed for relative HCV core and relative HCV NS5A expression in Huh-7 and Huh-7.5.1 cells using monoclonal anti-core and anti-NS5A antibodies respectively. The amount of infectious virus (fluorescence forming units (FFU)/ml) produced in cell media was also determined in both cell lines as described in the materials and methods. (B) Non-infected cell extracts were analyzed for relative expression of PCSK9, LDLR and Claudin-1 in Huh-7 and Huh-7.5.1 cells using polyclonal anti-PCSK9, anti-LDLR and monoclonal anti-claudin-1 antibodies. In all cases, relative protein expression was determined by quantification of protein band intensities normalized to β-tubulin band intensities (polyclonal or monoclonal anti-β tubulin) using LI-COR odyssey infrared imaging system. Values are plotted relative to protein expression in Huh-7 cells, which is set to 1. Results (mean ± SEM) from 3 or more independent experiments are shown. * denotes p < 0.05 and ** denotes p< 0.005.
Figure 3.3. PCSK9 and PCSK9-S402A are secreted and downregulate LDLR expression in Huh-7.5.1 cells.

Huh-7.5.1 cells were infected with Ad-Empty (Empty), Ad-PCSK9-S402A (S402A) or Ad-PCSK9-WT (WT) as described in the materials and methods. (A) Conditioned cell media (top panel) and total cell extracts (bottom panel) were harvested for western blot analysis to confirm overexpression and secretion of PCSK9 (using polyclonal anti-PCSK9 antibody from Abcam) in adenovirus infected cells. (B) Total cell extracts were also probed for mature LDLR (160 KDa) expression upon PCSK9 and PCSK9-S402A overexpression using polyclonal anti-LDLR antibody. Relative LDLR expression was determined by quantification of LDLR band intensities normalized to β-tubulin band intensities (monoclonal anti-β tubulin) using LI-COR odyssey infrared imaging system. Values are plotted relative to Ad-Empty treated cells, which are set to 1. Results (mean ± SEM) from 3 independent experiments are shown. *** denotes p< 0.005.
Figure 3.4. PCSK9 overexpression inhibits HCV infection in Huh-7.5.1 cells.

Huh-7.5.1 cells were infected with Ad-Empty, Ad-PCSK9-S402A (Ad-S402A) or Ad-PCSK9-WT (Ad-WT) as described in the materials and methods. Adenovirus was removed and cells were infected with HCV at a moi of 0.1. (A) Cell extracts were harvested 3 days post-HCV infection and subjected to western blot analysis to probe for HCV core expression using monoclonal anti-core antibody (Abcam). Relative core expression was determined by quantification of core band intensities normalized to β-tubulin band intensities (polyclonal anti-β tubulin) using LI-COR odyssey infrared imaging system. (B) Cell media was also harvested 3 days post-HCV infection to determine relative infectious HCV (FFU/ml) produced by Ad-Empty, Ad-PCSK9-S402A or Ad-PCSK9-WT treated cells as described in the materials and methods. (C) Cell lysates were also harvested for total RNA extraction. Purified total cellular RNA was analyzed by real-time PCR to determine relative HCV RNA expression normalized to β-actin transcript levels in adenovirus treated cells. (A – C) All values are plotted relative Ad-Empty treated cells, which is set to 1. Results (mean ± SEM) from 3 independent experiments are shown. * denotes p < 0.05 and *** denotes p< 0.005.
Figure 3.5. Individual cells overexpressing PCSK9 are resistant to HCV infection.

Huh-7.5.1 were seeded onto glass coverslips and after 24 hours were infected with (A) Ad-PCSK9-WT, (B) Ad-PCSK9-S402A or (C) Ad-CMV-EGFP for 24 hours and then infected with HCV for an additional 48 hours. Cells were fixed, permeabilized and probed for expression of (A and B) NS5A (green) or core (green) and PCSK9 (red) using monoclonal anti-NS5A, monoclonal anti-core and polyclonal anti-mouse PCSK9 antibodies, respectively. (C) Cells expressing GFP were also probed for NS5A (red) expression. All cell nuclei (blue) were stained using Hoechst dye. Fluorescently labeled cells were visualized using a Leica TCSSP5 confocal microscope.
Figure 3.6. PCSK9 does not inhibit HCV replication or core expression in HCV RNA transfected cells.

Huh-7.5.1 cells were infected with Ad-PCSK9-WT (Ad-WT), Ad-PCSK9-S402A (Ad-S402A) or Ad-Empty as described in the materials and methods. Adenovirus was removed and cells were transfected with 5 µg of full length HCV genomic RNA. Cell lysates were harvested 3 days post-transfection and subjected to (A) western blot analysis to probe for HCV core expression using monoclonal anti-core antibody. Relative core expression was determined by quantification of core band intensities normalized to β-tubulin band intensities (polyclonal anti-β tubulin) using LI-COR odyssey infrared imaging system. (B) Total RNA was also purified and used for real-time PCR analysis of relative HCV RNA levels normalized to β-actin levels in adenovirus treated cells. (A and B) Values are plotted relative to Ad-Empty treated cells, which are set to 1. Results (mean ± SEM) from 3 and 2 independent experiments are shown for panel A and B respectively.
Figure 3.7. Recombinant PCSK9 and the gain of function mutant PCSK9-D374Y inhibit HCV infection in a dose dependent manner.

(A) Huh-7.5.1 cells grown in LPDS supplemented media were treated with varying concentrations of recombinant BSA, PCSK9 and PCSK9-D374Y for 8 hours. Treated cells were infected with HCV moi 0.5 and cells were fixed 48 hours post-infection. Cells, probed with HCV core specific antibodies and stained with Hoechst dye to visualize cell nuclei, were counted using Cellomics HCS to determine the percentage of total HCV infected cells. Values are expressed as relative HCV infection in treated cells compared to untreated cells, which are set to 1. Results (mean ± SEM) from 3 independent experiments are shown. (B) Representative images acquired 48 hours post HCV infection. HCV infected Huh-7.5.1 cells (HCV core in red and Hoechst dye in blue) which were untreated (top left), treated with 25 µg/ml BSA (bottom left) or treated with the indicated concentration of recombinant wild-type PCSK9 or PCSK9-D374Y for 8 hours prior to HCV infection are shown. Images were acquired by the Cellomics HCS reader using the 10X objective.
Chapter 4: Discovery of non-inhibitory serine protease inhibitors as novel, potent anti-hepatitis C virus agents

4.1 Introduction

Serine protease inhibitors (serpins) are a large family of proteins found throughout nature that are classified according to a conserved tertiary structure (201). The native serpin folds into a characteristic metastable structure which is purposefully poised to target, trap and disable a specific protease (Reviewed in (212)). First, the serpin lures in a target protease through presentation of the reactive center loop (RCL) displayed above the main body of the serpin (213,214). The serpin RCL presents a “bait sequence” which is recognized by an enzyme as a substrate (Figure 4.1) (209,210). Following initiation of enzymatic cleavage an intermediate covalent bond forms between the enzyme’s active site serine and the P1 residue of the RCL (215,216). In a normal enzyme substrate scenario, deacylation of the enzyme occurs following cleavage of the enzyme substrate. An inhibitory serpin however traps the enzyme in this intermediate acyl-enzyme complex stage before the serpin RCL can be released (217,218). The result is formation of a covalent enzyme-inhibitor complex that is essentially irreversible and is both heat- and SDS-stable.

The key to this mechanism is the translocation of the N-terminal portion of the RCL from its pre-cleavage location above the serpin to the opposite pole of the serpin (See Figure 1.8) (217,219). The conformational shift is energetically driven by the release of the serpin from its native metastable fold allowing it to reach a thermodynamically stable conformation (220-222). Initiation of this major conformational change is dependent on the hinging of RCL residues near the main body of the serpin which bring the RCL down and direct integration of the RCL into the hydrophobic core of the serpin (217,223). In fact, the cleaved RCL is integrated into the main structural β-sheet of the serpin (β-sheet A) and forms a perfect anti-parallel β-strand within this nascent 6-stranded structure (217). Decreasing the rate of serpin RCL insertion within β-sheet A can bide the target enzyme enough time to release the serpin before it becomes permanently trapped (224).

Using Plasminogen Activator Inhibitor 1 (PAI-1) as a model, Lawrence et al. (224) demonstrated that the hydrophobicity and lack of charge in the critical P14 hinge residue of PAI-1 is required for rapid RCL insertion into the serpin hydrophobic core. Mutation of this P14

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residue to a charged amino acid abolishes the inhibitory capacity of PAI-1 and instead it is efficiently cleaved by known target proteases. It is predicted that disrupting the hydrophobicity and charge of this residue slows the rate of loop insertion into β-sheet A and allows the enzyme enough time to deacylate before it can be permanently trapped by the serpin.

Previously, our laboratory constructed a recombinant serpin called Spn4A.RRLL(s) to inhibit the host proprotein convertase (PC) SKI-1/S1P, an important regulator of host lipid metabolism (See Chapter 2). Spn4A is a secretory pathway serpin originating in Drosophila melanogaster which expresses an HDEL C-terminal sequence directing retention within the ER (254). The P4-P1 bait sequence in the RCL of Spn4A is RRKR and enables this serpin to inhibit the human PCs furin and PC2. To alter Spn4A specificity towards SKI-1/S1P the P4-P1 sequence RRKR was mutated to RRLL. SKI-1/S1P cleaves its substrates that regulate lipid metabolism in the Golgi apparatus (69) thus we generated a secretory pathway, secreted (s) variant that would pass through this cellular compartment. Blocking host cholesterol and fatty acid metabolism using Spn4A.RRLL(s) significantly reduced HCV infection in a dose dependent manner. Spn4A.RRLL(s) was also shown to deplete host lipid droplets (LD) and cholesterol esters within human hepatoma cells. These host factors are critical to HCV infection and their absence prevents HCV assembly and downstream virus release. Using these constructs we demonstrated that SKI-1/S1P function is required for HCV infection in human hepatoma cells and that it may be a novel cellular target for treating HCV infection.

Based on research by Lawrence et al, hinge-mutant counterparts of recombinant Spn4A variants, including those targeting SKI-1/S1P, were generated for use as controls in studies of HCV infection. Here, we report that these recombinant, hinge-mutant Spn4A variants possess potent yet unexpected anti-HCV activity. We confirmed first that these non-inhibitory Spn4A variants do not inhibit, but instead act as substrates of SKI-1/S1P. We showed that no complex forms between the hinge-mutant serpins and SKI-1/S1P. SREBP-regulated proteins and LDs were shown to not be disrupted by the non-inhibitory variant of Spn4A.RRLL(s). Antiviral activity is also independent of the RCL bait sequence as an non-inhibitory mutant directed at furin also inhibits HCV infection. Our findings suggest that an unidentified component of the cleaved Spn4A mutants possesses intrinsic antiviral activity. An extensive review of the literature and potential mechanism by which cleaved serpins may inhibit viral infection are discussed at the end of this chapter. We propose that the activity may be mediated by the released C-terminal peptide of Spn4A. Spn4A possesses homology to the human serpin α-1-
antitrypsin (AAT) which possesses many regulatory properties and has been shown to inhibit human immunodeficiency virus (HIV)-1 entry and other aspects of the HIV-1 life cycle (228,229,231,249). Research into the mechanism of Spn4A hinge-mutant inhibition of HCV infection may lead to discovery of new host targets against HCV and may also allow rational design of novel, potent HCV antivirals.
4.2 Materials and methods

Cell culture and other reagents – Human hepatoma Huh-7.5.1 cells were kindly provided by Dr. Francis Chisari (Scripps Research Institute, La Jolla, CA, USA) (104). Cultured cells were maintained in Dulbecco’s Modified Eagle Medium (DMEM) supplemented with 1% penicillin, streptomycin, glutamine, non-essential amino acids, HEPES, and 10% fetal bovine serum (FBS) (Gibco/Invitrogen, Burlington, ON, Canada). Bovine serum albumin (BSA), saponin, Formaldehyde, 37% w/v was from Fischer Scientific (Pittsburg, PA, USA). SKI-1/S1P deficient CHO, SRD12B, cells were maintained in a 1:1 mixture of DMEM and Ham’s F12 medium supplemented with 1 % penicillin and streptomycin, 5 % FBS, 50 μM sodium mevalonate, 20 μM sodium oleate and 5 μg/ml cholesterol (ref: Rawson, Goldstein 1998 J Biol Chem 273(43): 28261) and subjected to weekly amphotericin B selection as previously described.

Antibodies and dyes – Antibodies used to detect HCV infection included a mouse anti-core antibody (1:50 for immunofluorescence, Abcam, Cambridge, MA, USA) (267). Antibodies used to detect cellular proteins included rabbit anti-human proprotein convertase subtilisin/kexin type 9 (PCSK9) (1:500, Abcam), rabbit anti-LDLR (1:250, Fitzgerald Industries International, Inc., Concord, MA, USA), mouse anti-SREBP-2 (1:200, Santa Cruz Biotechnology, Santa Cruz, CA, USA), rabbit anti-adipose differentiation-related protein (ADRP) (1:50, Abcam), mouse anti-ADRP (1:20, Progen, Heidelberg, Germany), rabbit anti-β-tubulin (1:3000, Abcam), mouse anti-β-tubulin (1:500, Lab Vision Corporation, Montreal, QC, Canada), and mouse anti-GM130 (1:200, BD Biosciences, Mississauga, ON, Canada). Tagged proteins were detected using mouse anti-FLAG M2 (1:50, Sigma-Aldrich Corp.), rabbit anti-FLAG (1:50, Thermo Scientific, Nepean, ON, Canada), and mouse anti-his antibodies (1:500, Applied Biological Materials, Richmond, BC, Canada). Secondary antibodies used for immunofluorescence were Alexa Fluor-488-conjugated or Alexa Fluor-568-conjugated donkey anti-mouse or donkey anti-rabbit antibodies (1:100, Molecular Probes/Invitrogen). Secondary antibodies used for western blot were IRDye 680-conjugated (red bands) or 800-conjugated (green bands) donkey anti-mouse or goat anti-rabbit antibodies (1:10000, LI-COR Biosciences, Lincoln, NE, USA). Hoechst 33258 (10 μg/ml) and BODIPY (4,4-difluoro-1,3,5,7,8-pentamethyl-4-bora-3a,4a-diaza-s-indacene) 493/503 (1 μg/ml, Molecular Probes/Invitrogen) were used for detection of nuclei and LDs, respectively.

HCV RNA and infectious stock production – A plasmid containing the cDNA of an HCV consensus clone isolated from a Japanese patient with fulminant hepatitis (JFH-1) (GenBank
accession number AB047639) (268) cloned behind a T7 promoter (pJFH-1; a generous gift from Dr. Takaji Wakita, National Institute of Infectious Diseases, Tokyo, Japan) was used to generate genomic HCV RNA and infectious HCV stocks as previously described in (269).

**Transfection of HCV RNA** – Purified HCV RNA was used to transfect Huh-7.5.1 cells as a means of studying HCV infection independently of receptor mediated entry. Five micrograms of purified RNA was incubated with 10 µl of lipofectamine 2000 (Invitrogen) in minimal essential media (MEM) for 30 minutes. The RNA-lipid complexes were then added to cells in MEM for 16 hours, then cells were washed with phosphate-buffered saline (PBS) and complete media was added for the remainder of the experiment.

**HCV titer determination** – The amount of infectious HCV particles generated for viral stocks or in the described experiments was determined using a modified, previously described protocol (104). Briefly, 1 x 10^4 Huh-7.5.1 cells were plated in each well of a 96-well plate and infected with 10-fold serial dilutions of HCV-infected cell media. Three days post-infection cells were fixed and probed as described in the ArrayScan Quantification methods section. An ArrayScan VTI High Content Screening (HCS) Reader (Thermo Scientific) was used to acquire images of the entire infected wells. Titers were determined by manually counting foci (fluorescence forming units (FFU)) in the lowest dilutions with positive signal.

**Recombinant adenoviruses** – Empty adenovirus (Ad-Empty), which was kindly provided by Dr. Jan Breslow (Rockefeller University, New York, NY, USA) (64), was amplified by Viraquest Inc (North Liberty, IA, USA). Spn4A, isolated from *Drosophila melanogaster* [59] (GenBank Accession number NM_165496), was modified to express the AAT signal peptide (residues 1–24, pre-AAT) followed by a tandem his-tag (HHHHHH) and FLAG-tag (DYKDDDDK) sequence (HF) at the N-terminus (254). The Spn4A-variants directed at furin contain the P4 – P1 cleavage sequence (215) RRKR in the reactive center loop (RCL). The ER “retained” (r) variant of the furin-directed serpin designated Spn4A.RRKR(r) (**Figure 4.1 A**) presents the HDEL ER retention motif at the C-terminus. A “secreted” (s) variant of the serpin Spn4A.RRKR(s) was generated in house by mutating the his codon in the HDEL sequence to a stop codon. The furin-directed RCL sequence was modified to RRLL through site-directed mutagenesis using QuikChange (Stratagene, La Jolla, CA, USA) to generate Spn4A.RRLL(r) and Spn4A.RRLL(s) (**Figure 4.1 A and B**). Lastly, Spn4A.RRKR.T328D(r) and (s) and Spn4A.RRLL.T328D(r) and (s) were also generated using site-directed mutagenesis. All recombinant Spn4A variants were cloned into pShuttle and recombinant adenoviruses were
generated in the laboratory (using AdEasy XL Adenoviral Vector System from Stratagene) or by ViraQuest Inc and are called Ad-Spn4A.RRKR(r etc. Adenovirus titers were determined in human embryonic kidney (HEK293T) cells using Adeno-X Rapid Titer kit (Clontech, Mountain View, CA, USA).

**In silico homology model of Spn4A.RRLL** – The *Drosophila melanogaster* Spn4A sequence (Genebank accession number gi|24586105|ref|NP_524955.2) exhibits 34% sequence homology with the human neuroserpin for which a crystal structure (270) is available in the Protein Data Bank (271) (PDB ID: 3F5N). Of the five chains in this pentameric structure of Neuroserpin, chain B is most well resolved with the fewest missing residues and was used as the template for the homology model presented in Figure 4.1 B. The model was built and refined using the SwissPDB Viewer (272). The C-α residues in this model structure align to 1.9 Å RMSD with reference to the human neuroserpin structure.

**Recombinant SKI-1/S1P** – The first 997 amino acids of human SKI-1/S1P lacking the C-terminal transmembrane domain but having a C-terminal 8 x his-tag (PGDĐĐĐKHHHHHHHHHSGS) were expressed in Sf9 insect cells as described in (273). Two liters of cell culture supernatant were used for purification. Two hundred milliliters of 200 mM Tris/HCl pH 8.0, 500 mM NaCl was added and then the pH was adjusted to pH 8.0 by further addition of 2 M NaOH. The resulting precipitate was removed by centrifugation at 10000 x g for 30 minutes and subsequent filtration through a glass filter. The cleared supernatant was then applied to a small (0.9 ml column volume) IMAC column (Ni-Sepharose, GE Healthcare, Freiburg, Germany) by continuous flow (1 ml/minutes). The column had been equilibrated in 50 mM Tris/HCl pH 8.0, 500 mM NaCl (buffer A) and bound recombinant SKI-1/S1P was eluted with a continuous gradient over 30 column volumes to buffer A plus 300 mM imidazole. Collected fractions were assayed for enzymatic activity of SKI-1/S1P using Ac-RRLL-pNA (custom synthesized by Peptides International (Louisville, Kentucky, USA) as the substrate and as described in (273). Most active fractions were pooled. Concentration and buffer exchange to buffer A was then done using spin concentrators (Millipore, Billerica, MA, USA) with a molecular weight cutoff of 30 kDa. The final preparation was, after addition of 30% v/v glycerol, stored at -80°C and had a specific activity of 0.018 U/mg (measured as above).

**Western blot analysis** – Cultured cells were washed with ice-cold PBS and re-suspended in cold radioimmunoprecipitation assay (RIPA) buffer (50 mM Tris-HCl pH 8, 150 mM NaCl, 1% octylphenyl-polyethylene glycol (IGEPAL), 0.5% sodium deoxycholate, 0.1% sodium dodecyl

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sulfate (SDS)) containing 1 X Complete, EDTA-free, protease inhibitor cocktail (Roche, Laval, QC, Canada). Whole cell extracts were vortexed, and then clarified by centrifugation at 12000 x g for 15 minutes. Soluble extracts mixed with 2 X sample buffer (62.5 mM Tris-HCl, pH 6.8, 25% glycerol, 2% SDS, 0.01% bromophenol blue, and 5% β-mercaptoethanol) were electrophoresed on 8-15% SDS polyacrylamide gels and transferred to nitrocellulose membranes. Membranes were blocked in odyssey-blocking buffer (LI-COR Biosciences) for one hour, and proteins of interest were detected by probing with the appropriate primary and secondary antibodies diluted in odyssey blocking buffer containing 0.1% Tween 20. Protein bands were relatively quantified using Odyssey Infrared Imaging System (LI-COR Biosciences). B-tubulin was always used as a loading control and for normalizing protein expression. Media samples analyzed for secreted Spn4A variants were taken directly from cultured cells, mixed with 2 X sample loading buffer, and subjected to the described western blot analysis.

**Stable enzyme-inhibitor (EI*) complex detection between serpin variants and SKI-1/S1P** – SRD12B cells were plated at 5 x 10^5 cells per well in a 6-well plate and infected after 24 hours with Ad-Spn4A variants (Ad-Empty, Spn4A.RRLL(r) and (s) – moi50; Spn4A.RRLL.T328D(s) moi 100; Spn4A.RRLL.T328D(r) – moi 35). After 72 hours, media and cell extracts (harvested in hypotonic buffer containing 20 mM Tris, pH 7.4, 10 mM MgCl_2, and 10 mM CaCl_2) were mixed 1:1 with enzyme reaction buffer (SK1-1/S1P buffer contains 25 mM Tris-HCl, 25 mM MES, pH 7.4, 2.5 mM CaCl_2 (273); and furin buffer contains 100 mM HEPES, pH 7.5, 1 mM CaCl_2, 0.5% Triton X-100 (254)), 1 X complete EDTA-free protease inhibitor cocktail, and 11.6 ng/µl SKI-1/S1P or 2.4 ng/µl furin. The enzyme mixture was incubated at 30°C for 30 minutes and the reaction was stopped with 12.5 mM EDTA (final concentration) (254). The samples were then processed, as described above, for western blotting.

**ArrayScan quantification** – In black flat-bottom 96-well plates (BD Biosciences) cells were plated and infected as described in the methods below. Following infection, cells were fixed in 4% formaldehyde v/v diluted in PBS and blocked in PBS containing 3% BSA, 0.3% Triton X-100, and 10% FBS. Cells were first probed with HCV anti-core antibody (1:500) in PBS containing 3% BSA and 0.3% Triton X-100 (Binding Buffer), then incubated with Alexa Fluor-568-conjugated donkey anti-mouse secondary antibody (1:1000) and 10 µg/ml Hoechst dye. Cells were analyzed by a quantitative, high-throughput, fluorescence microscope system called the Cellomics ArrayScan VTI High Content Screening (HCS) Reader (Thermo Scientific) using the BioApplications software, Target Activation protocol (274). The protocol was used to count
the total number of cells (Hoechst-stained nuclei) and the percentage of those cells that were infected with HCV (positive signal at 568 nm wavelength).

**Confocal microscopy and immunofluorescence** – After Huh-7.5.1 cells were seeded onto coverslips for 24 hours, they were either infected with adenovirus (moi 50) for 72 hours or for 24 hours (moi 200) followed by HCV infection (moi 0.1) for 48 hours. Cells were fixed in 4% v/v formaldehyde in PBS, then permeabilized and blocked in PBS containing 0.05% saponin (wash buffer) and 1% BSA (binding buffer). Blocking of cells stained with BODIPY 493/503 was done in the presence of 0.2 M glycine to reduce background fluorescence. Cells were probed with primary antibodies in binding buffer, then incubated with a secondary antibody, Hoechst dye (10 µg/ml), and BODIPY 493/503 (1 µg/ml; when indicated) diluted in PBS. Cells were mounted onto slides with an anti-fade solution and sealed with clear nail-polish. The slides were then imaged using a Leica TCS SP5 confocal microscope (Leica Microsystems, Wetzlar, Germany) or an Olympus Fluoview FV1000 laser scanning confocal microscope (Olympus Corporation, Tokyo, Japan).

**Adenovirus infections** – Huh-7.5.1 cells were infected with recombinant adenovirus at various moi in complete media. After 48 hours, the cells were infected with HCV moi 0.1 or transfected with purified HCV genomic RNA for 3 days and then cells were analyzed by Cellomics ArrayScan, or total RNA was isolated from cell extracts using the RNaseasy plus kit (Qiagen, Mississauga, ON, Canada) including on-column DNase digestion. Media from treated and infected cells were harvested for HCV titer determination as described above. To examine the Spn4A.RRLL(s) mediated block in PCSK9, LDLR, and SREBP-2 expression, Huh-7.5.1 cells were grown in media supplemented with lipoprotein depleted serum (LPDS) for 24 hours, infected with adenovirus variants, and harvested 72 hours later.

**Quantitative real-time (Q) PCR** – Purified total RNA was reverse transcribed to cDNA using TaqMan reverse transcription reagents (random hexamers; Applied Biosystems, Foster City, CA, USA). Real-time quantitative PCR was carried out using Brilliant II Fast QPCR reagents (Stratagene, La Jolla, CA, USA) according to the manufacturer’s instructions on an Mx3005P QPCR system (Stratagene). Online ProbeFinder software (Roche Applied Science) was used to find primers that would allow amplification of the HCV RNA 5’ end in combination with the Human Universal Probe Library from Roche (Roche Applied Science). For amplification of the HCV RNA 5’ region, 400 nM of both forward primer (5’-CATGGCGTTAGTATGAGTGCG-3’) and reverse primer (5’-GGTTCCGCAGACCACTAT-3’) were used in combination with 200
nM of probe #75 from the Human Probe Library (Roche). HCV RNA levels were relatively quantified across samples and normalized to β-actin RNA levels using 500 nM primers (forward: 5’- GCC CTG AGG CAC TCT TCC and reverse: 5’ GGA TGT CCA CGT CAC ACT TC-3’) and 250 nM probe (5’AC TCC ATG CCC AGG AAG GAA GGC-3’ with a 5’ Cy5 fluorophore and 3’ black hole quencher).

Cell viability – Cell viability was determined using CellTiter 96® AQueous One Solution Cell Proliferation Assay (Promega, Madison, WI, USA). This assay employs a tetrazolium compound [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt; MTS], which is bioreduced by cells into a coloured formazan product which can be detected in tissue culture media at 490 nm wavelength.

Statistics – The student’s $t$-test (unpaired) was used to calculate significance which is represented in the figures by the following notation: * denotes $p < 0.05$, ** denotes $p < 0.01$, and *** denotes $p < 0.005$. 
4.3 Results

4.3.1 Engineering non-inhibitory, hinge-mutant counterparts of inhibitory Spn4A variants targeting human proprotein convertases (PCs) SKI-1/S1P and furin

Lawrence et al. (224) previously demonstrated that the hydrophobicity and lack of charge in the critical P14 (threonine) hinge residue of the RCL of PAI-1 is required for rapid insertion of the cleaved loop into the serpin’s hydrophobic core. Mutation of this P14 residue to a charged amino acid abolishes the inhibitory capacity of PAI-1 and instead it is efficiently cleaved by known target proteases. We employed a similar strategy to generate non-inhibitory, hinge-mutant counterparts to recombinant Spn4A variants generated in our laboratory (328).

Spn4A is a natural, secretory pathway serpin in Drosophila melanogaster that expresses a C-terminal HDEL sequence directing retention of the protein in the ER. The RCL P4-P1 bait sequence is RRKR and it was discovered that this sequence permits Spn4A to target and inhibit the human PC furin with unparalleled efficiency. Recombinant variants of Spn4A were constructed with a his/FLAG tag at the N-terminus proceeded by the α-1-antitrypsin (AAT) signal peptide (for efficient secretory pathway expression). The recombinant, ER retained (r) Spn4A variant is called Spn4A.RRKR(r) (Figure 4.1 A). Many enzymes function late in the secretory pathway and thus retention within the ER renders the serpin non-functional within the context of an intact cell. Inserting a stop codon prior to the ER-retention sequence generates a secretory pathway serpin Spn4A.RRKR(s) (Figure 4.1 A) that is efficiently secreted (refer to Figure 2.2, Lane 6).

Engineering of the P4-P1 bait sequence in the RCL (See Figure 4.1 B) of Spn4A.RRKR(r) and (s) allowed us to alter the target specificity of these serpins to inhibit the PC SKI-1/S1P (Figure 4.1 A). SKI-1/S1P is absolutely required for activation of host cholesterol and fatty acid metabolism through the sterol regulatory element binding protein (SREBP) pathway (74). This pathway and its control of lipid metabolism are critical for the propagation of HCV. Since SKI-1/S1P cleaves SREBPs within the Golgi, SREBP activation is only blocked by the secreted variant, Spn4A.RRLL(s).

Spn4A.RRKR(r) and Spn4A.RRLL(r), although retained within the ER following expression, retain their inhibitory capacity if active enzyme is encountered. Thus, genuine non-inhibitory serpin variants to be used as negative controls in cell biology and virology-based studies were also constructed. A threonine residue at position 328 (P15) in Spn4A is located within the hinge region of the serpin RCL (Figure 4.1 B). Based on studies by Lawrence et al.
we expected this residue to be critical for rapid conformational inactivation of target enzymes by recombinant serpins. This threonine residue was mutated to aspartic acid in all four of the above described recombinant Spn4A variants to generate four novel hinge-mutants: Spn4A.RRKR.T328D(r) and (s) and Spn4A.RRLL.T328D(r) and (s) (Figure 4.1 A)(328). It is expected that the bait sequence in the serpin RCL will still be recognized and cleaved by the target enzyme. However, insertion of a charged amino acid in this hinge region should hinder rapid insertion of the cleaved RCL into β-sheet A of the serpin; thus, the enzyme should simply release the cleaved serpin and retain its enzymatic function.

4.3.2 Cellular expression and differential secretion of Spn4A.RRLL.T328D(r) and Spn4A.RRLL.T328D(s) in human hepatoma Huh-7.5.1 cells

To test the impact of the T328D mutation in Spn4A, we first characterized the recombinant serpins targeting SKI-1/S1P due to our interest in the role this enzyme plays in HCV infection. To confirm that the hinge-mutant variants Spn4A.RRLL.T328D(r) and Spn4A.RRLL.T328D(s) are stable, can be detected following intracellular delivery and exhibit the expected secretion profile, they were expressed using an infectious adenovirus delivery system. Recombinant adenovirus vectors expressing the inhibitory Spn4A variants, Spn4A.RRLL(r) and Spn4A.RRLL(s), and the hinge-mutant variants, Spn4A.RRLL.T328D(r) and Spn4A.RRLL.T328D(s), were used to infect the human hepatoma, Huh-7.5.1 cell line. Spn4A variant expression was analyzed using western blot analysis of cell extracts and cell media probed with an anti-his tag antibody. As previously shown (Figure 2.2 A) Spn4A.RRLL(r) is retained within cell extracts and Spn4A.RRLL(s) is secreted into extracellular media. Spn4A.RRLL.T328D(r) was also shown to be retained as it is only found within cell extracts while Spn4A.RRLL.T328D(s) is found to be secreted (Figure 4.2 A).

A significant differentiating feature between the inhibitory and hinge-mutant variants is the size of the predominant bands detected by western blot analysis (Figure 4.2 A). The intact, full length Spn4A variants are predicted to be ~ 45 kDa. Cleavage of the serpin reactive center bond in the RCL should release a his-tagged N-terminal fragment that is ~ 41 kDa. In cell extracts, the majority of Spn4A.RRLL.T328D(r) appears as the lower molecular weight (~41 kDa) form (Figure 4.2 A, lane 4). This is in contrast to Spn4A.RRLL(r) which is predominantly 45 kDa (Figure 4.2 A, lane 2). Similarly, in cell media, Spn4A.RRLL(s) is 45 kDa while Spn4A.RRLL.T328D(s) appears to be completely processed prior to secretion (Figure 4.2 A, upper panel, compare lanes 3 and 5). Another noticeable difference between the SKI-1/S1P
inhibitory and non-inhibitory Spn4A variants is their abundance. Protein bands representing Spn4A.RRLL.T328D(r) and Spn4A.RRLL.T328D(s) are less abundant than Spn4A.RRLL(r) and Spn4A.RRLL(s) respectively which indicates altered stability of the hinge-mutants. The effect of the proteasome inhibitor MG132 on Spn4A.RRLL.T328D(s) expression was tested but did not result in restoration of hinge-mutant serpin levels (data not shown). Overall, these results confirm that Spn4A.RRLL.T328D(r) and Spn4A.RRLL.T328D(s) are abundantly expressed and differentially secreted in Huh-7.5.1 cells. Also, the detection of Spn4A.RRLL.T328D(r) and Spn4A.RRLL.T328D(s) as lower molecular weight forms supports their function as substrates rather than inhibitors of SKI-1/S1P.

4.3.3 Spn4A.RRLL.T328D(r) and Spn4A.RRLL.T328D(s) partition into the substrate rather than inhibitory pathway following cleavage by SKI-1/S1P

To investigate whether cleavage of the hinge-mutants detected by western blot analysis is mediated by SKI-1/S1P, the four Spn4A.RRLL variants were expressed in a SKI-1/S1P deficient, CHO-based cell line called SRD12B. The left panels in Figure 4.2 B show that the serpins are not cleaved in the absence of SKI-1/S1P expression. All variants in media and cell extracts appear as 45 kDa bands, with the exception of a very minor portion (< 1%) of cleaved Spn4A.RRLL.T328D(s) detected in cell media (Figure 4.2 B, top left panel, lane 5). Surprisingly, Spn4A.RRLL.T328D(r) and Spn4A.RRLL.T328D(s) expression appears more stable in the SKI-1/S1P-deficient cell line than in Huh-7.5.1 cells relative to the non-inhibitory mutants. This suggests that Spn4A.RRLL.T328D(s) is being turned over in Huh-7.5.1 cells but may be cleared by a mechanism independent of the proteasome. Incubation of serpin-expressing media and cell extracts with recombinant SKI-1/S1P resulted in cleavage of the 45 kDa Spn4A variants (Figure 4.2 B right panels) into the lower molecular weight form detected in Huh-7.5.1 cells.

Incubation of Spn4A.RRLL(r) from lysed SRD12B cell extracts (Figure 4.2 B, bottom panel lane 7) or of Spn4A.RRLL(s) from cell media (Figure 4.2 B, top panel lane 8) with recombinant SKI-1/S1P resulted in detection of a heat- and SDS-stable, high MW complex by western blot, as we previously demonstrated in Huh-7.5.1 cells. In contrast, no enzyme-inhibitor (EI*) complex was detected when Spn4A.RRLL.T328D(r) or Spn4A.RRLL.T328D(s) were incubated with recombinant SKI-1/S1P. These results confirm that Spn4A.RRLL.T328D variants are cleaved by and do not form a complex with SKI-1/S1P, confirming that they are substrates rather than inhibitors of SKI-1/S1P.
4.3.4 Evidence that Spn4A.RRL.L.T328D(s) does not inhibit SKI-1/S1P or host lipid metabolism

Our previous characterization of Spn4A.RRL(s) revealed that it effectively blocks the expression of proteins regulated by SREBP activation which results in reduced abundance of neutral lipids and lipid storage droplets within hepatoma cells. Parallel studies examining the impact of Spn4A.RRL.T328D(s) on the SREBP pathways were conducted in Huh-7.5.1 cells. First, we examined whether Spn4A.RRL.T328D(s) mediates a reduction in the expression of three proteins shown to be regulated by SREBP activation which require SKI-1/S1P cleavage. No reduction in the abundance of PCSK9, LDLR or SREBP-2 were seen using western blot analysis of Spn4A.RRL.T328D(s) treated compared to empty adenovirus vector (control) treated cells (Figure 4.3 A). In addition, the expression of the Golgi marker, GM130 was unaffected (Figure 4.3 A).

Next, both confocal analysis and western blotting were employed to examine the integrity of LDs within treated cells. The LD marker ADRP/perilipin 2 is well known to associate with LDs and the abundance of this protein reflects the quantity of neutral lipids storage within hepatocytes (287,289,290). Using confocal microscopy of Spn4A.RRL.T328D(s) treated cells no reduction in ADRP/perilipin 2 (Figure 4.3 B) expression was detected. Western blot analysis of total cell extracts following 72 hour Spn4A.RRL.T328D(s) treatment confirmed that total cellular ADRP/perilipin 2 levels were not reduced compared to control (Ad-Empty treated) cells (Figure 4.3 C). We also examined cells directly for the abundance of LDs using a hydrophobic, fluorescent dye called BODIPY 493/503 which immerses rapidly into LDs and can be detected using fluorescent microscopy. Confocal analysis of BODIPY 493/503 stained cells confirmed that Spn4A.RRL.T328D(s) does not decrease intracellular LD abundance (Figure 4.3 D).

Cellomics HCS was used for preliminary investigations into the antiviral activity of the SKI-1/S1P inhibitor Spn4A.RRL(s). This technique was employed for enumeration of HCV infected cells following 48 hours of recombinant adenovirus (moi 1, 12,5, 25 and 50) followed by 72 hours of HCV infection. At this stage we predicted that Spn4A.RRL.T328D(s) would act as the most appropriate control for ruling out non-specific effects of expressing the secreted variant Spn4A.RRL(s) within host cells. Cellomics HCS analysis revealed no significant reductions in the number of HCV infected cells (cells immunoreactive for HCV core protein) following Spn4A.RRL.T328D(s) treatment compared to Ad-Empty treated cells (Figure 4.4 A). Also, confocal analysis of cells expressing Spn4A.RRL.T328D(s) (detected with anti-
FLAG antibody) demonstrated proper localization of the HCV core protein to the perinuclear region of the cell (Figure 4.4 B and C). No differences in HCV core protein localization were apparent between untreated, HCV infected cells (Figure 4.4 B) and HCV infected and Spn4A.RRLL.T328D(s) treated cells (Figure 4.4 C). Overall, these results confirmed that Spn4A.RRLL.T328D(s) is an appropriate negative control for studies investigating the role of SKI-1/S1P in the HCV lifecycle.

4.3.5 The SKI-1/S1P directed hinge-mutant serpin Spn4A.RRLL.T328D(s) exhibits potent antiviral activity against HCV replication and virion production

Successive experiments examining the impact of Spn4A.RRLL(s) on HCV replication were also conducted where Spn4A.RRLL(r), Spn4A.RRLL.T328D(s) and Ad-Empty were used as controls. Real-time PCR analysis of Huh-7.5.1 cells infected with HCV following recombinant adenovirus treatment was conducted. These experiments revealed an unexpected and dramatic 80 % reduction in HCV RNA levels in cells treated with the putative control Spn4A.RRLL.T328D(s) compared to Ad-Empty treated cells (Figure 4.5 A). HCV RNA levels in Spn4A.RRLL.T328D(s) treated cells were in fact 1.6-fold lower than in cells treated with the SKI-1/S1P inhibitor, Spn4A.RRLL(s) which reduced HCV RNA levels by 67 % (Refer to Figure 2.5).

The reduction in HCV RNA levels may result from reduced HCV replication but could also reflect a block in HCV entry. To clarify, Huh-7.5.1 cells treated with Spn4A.RRLL.T328D(s) were transfected with HCV genomic RNA to circumvent the requirement of HCV entry via receptor-mediated endocytosis. Three days post-transfection HCV RNA levels were measured and found to be reduced by nearly 50% (Figure 4.5 B). Thus, HCV replication or HCV RNA stability is negatively impacted by Spn4A.RRLL.T328D(s) expression. As the magnitude of decreased RNA abundance is less than when receptor mediated entry is required (compare Figure 4.5 A and B), it remains that Spn4A.RRLL.T328D(s) may also negatively impact HCV entry. However, the fact that no reductions in HCV core levels were seen using Cellomics HCS suggests that the impact of Spn4A.RRLL.T328D(s) on entry is minimal. Overall, QPCR analysis of HCV RNA levels suggests that Spn4A.RRLL.T328D(s) blocks HCV replication or decreases HCV RNA stability.

The consequence of reduced HCV replication is underscored by dramatic reductions in infectious HCV titers produced by Spn4A.RRLL.T328D(s) treated cells compared to control (Ad-Empty treated) cells. As shown in Figure 4.5 C, extracellular infectious HCV production
was reduced by 95%. This is again in contrast to HCV titers produced in Spn4A.RRLL(s) treated cells which were only reduced by ~70% compared to controls (Refer to Figure 2.5). These results strongly suggest that the hinge mutation in Spn4A.RRLL.T328D(s) confers antiviral properties to this “non-inhibitory” serpin which are absent in the inhibitory serpin Spn4A.RRLL(s).

To demonstrate that Spn4A.RRLL.T328D(s) is not cytotoxic to Huh-7.5.1 cells, both an MTS based cell-viability assay and Cellomics HCS analysis of Huh-7.5.1 cell numbers were conducted. A colourimetric, MTS-based cell viability assay was conducted on Huh-7.5.1 cells after 72 hours of infection with Ad-Spn4A.RRLL.T328D(s) and Ad-Empty at moi 1, 12.5, 25 and 50. Cell viability was not reduced by Spn4A.RRLL.T328D(s) under these conditions (Figure 4.6 A). Additionally, we examined the fate of Huh-7.5.1 cells treated with Ad-Spn4A.RRLL.T328D(s) or Ad-Empty (moi 1, 12.5, 25 and 50) for 48 hours followed by an additional 72 hours of HCV infection. Cellomics HCS was used to count the number of cell nuclei after this time reflecting the number of adherent cells at the end of the experiment; cell death results in rounding and lifting of cells directly off of the tissue culture plate. Figure 4.6 B shows that cell numbers were not reduced by Spn4A.RRLL.T328D(s) under these conditions. In fact, the control Ad-Empty has a greater impact on cell viability as cell numbers are reduced with increasing amounts Ad-Empty (Figure 4.6 B). Ad-Spn4A.RRLL.T328D(s) in contrast does not reduce cell numbers under the examined conditions. These results confirm that Spn4A.RRLL.T328D(s) inhibition of HCV infection is not due to a negative impact on cell viability.

4.3.6 Spn4A.RRKR.T328D(s) also inhibits HCV replication

Although Spn4A.RRLL.T328D(s) does not inhibit SKI-1/S1P regulation of lipid metabolism (Figure 4.3 A-D), this serpin is still recognized by SKI-1/S1P (Figure 4.2 B). The possibility remains that Spn4A.RRLL.T328D(s) is regulating an aspect of SKI-1/S1P function that has not yet been examined and which is leading to reduced HCV infection. To shed light on this possibility we tested the furin directed hinge-mutant serpin Spn4A.RRKR.T328D(s) (Figure 4.1 A) for anti-HCV activity. This adenovirus expressed mutant serpin was previously demonstrated to not form a complex with furin in HEK-293 cells (328). As with Spn4A.RRLL.T328D(s), cells were treated with Spn4A.RRKR.T328D(s) for 48 hours followed by HCV infection for 72 hours. QPCR analysis of HCV RNA levels revealed an 87% reduction in HCV RNA levels by Spn4A.RRKR.T328D(s) (Figure 4.7 A). Also, HCV extracellular titers
were dramatically reduced by 91% following Ad-Spn4A.RRKR.T328D(s) infection compared to Ad-Empty infection. These results reveal that the serpin P4-P1 bait sequence is not important for the antiviral effects of Spn4A.RRLL.T328D(s) and Spn4A.RRKR.T328D(s), but that a common feature between the two non-inhibitory serpins hinders HCV infection in Huh-7.5.1 cells.
4.4 Discussion

4.4.1 Spn4A derived hinge-mutants possess powerful antiviral activity against hepatitis C virus

The serpins are a fascinating group of proteins that play roles in a wide array of cellular functions. Inhibitory serpins are powerful modulators of proteolytic activity and can be engineered to exhibit a variety of properties dictating cellular localization, rate of inhibition, protease targets and additional binding partners. Studies have also revealed secondary non-conventional functions of serpins, mediated through interactions of the serpins and serpin cleavage products with various cellular factors (228,229,231,232,247-249,329-331).

Here we report the construction of non-inhibitory, hinge-mutant variants of inhibitory serpins derived from the Drosophila melanogaster Spn4A scaffold. These serpins contain a single mutation of the P15 threonine residue located within the hinge region of the serpin RCL. Replacing the threonine residue with aspartate abolishes previously established inhibitory properties, likely through hindering cleaved RCL translocation and insertion into the main structural β-sheet of the serpin (224) i.e. the serpin partitions into the substrate rather than the inhibitory pathway (Figure 1.7). The serpin of focus in this study, Spn4A.RRLL.T328D(s), is derived from a previously engineered inhibitory serpin Spn4A.RRLL(s) which inhibits SKI-1/S1P proteolytic activity (Figure 2.2 and Figure 2.3). We confirmed that Spn4A.RRLL.T328D(s) is cleaved by but does not form an enzyme-inhibitor complex with SKI-1/S1P. We also confirmed that SKI-1/S1P-activation of SREBP signaling is not blocked by Spn4A.RRLL.T328D(s) as SREBP-regulated protein expression and LD abundance do not decline when this serpin is expressed. These findings led us to predict that Spn4A.RRLL.T328D(s) would be a suitable negative control for studies examining Spn4A.RRLL(s) regulation of the HCV lifecycle. However, we report here that both Spn4A.RRLL.T328D(s) and the furin-directed, hinge-mutant Spn4A.RRKR.T328D(s) possess potent antiviral activity resulting in reduced HCV RNA replication and reduced HCV infectious virion secretion in human hepatoma cells.

The antiviral potency of Spn4A.RRLL.T328D(s) was not revealed in initials studies of HCV infection where the number of HCV core expressing cells was used as an indicator of viral propagation. Also, the localization of the HCV core protein appears undisrupted when examined by confocal and immunofluorescence microscopy. This suggests that Spn4A.RRLL.T328D(s) does not hinder HCV entry and permits uninterrupted release of viral genomic RNA into the
cytoplasm. If entry and genome release occur normally, the cell can initiate translation of the viral RNA into viral proteins i.e. core. Although the actual number of cells infected in the first round of HCV infection is not reduced, the amount of core protein produced in each cell may decline relative to control cells as infection progresses and RNA replication is impeded. As Cellomics HCS analyses provided the number of core expressing cells but not the intensity of core expression, the antiviral effect of Spn4A.RRLL.T328D(s) went undetected.

The negative impact of Spn4A.RRLL.T328D(s) on HCV infection was revealed upon quantitative PCR analysis of HCV RNA abundance. These studies demonstrated that Spn4A.RRLL.T328D(s) reduces HCV RNA levels within the cell more efficiently than Spn4A.RRLL(s) (80% versus 65% inhibition; compare Figure 4.5 and Figure 2.5). The impact of Spn4A.RRLL.T328D(s) on HCV replication was further confirmed by transfection of HCV genomic RNA into Huh-7.5.1 cells following Spn4A.RRLL.T328D(s) treatment. HCV RNA was reduced by 50% even when receptor mediated endocytosis was bypassed which is in contrast to studies with Spn4A.RRLL(s) where no there was no significant reduction in HCV RNA following transfection. The exact implication of this finding is difficult to interpret as the PCR analysis performed does not distinguish transfected RNA from newly replicated RNA. However, the result does point towards Spn4A.RRLL.T328D(s) hindering the stability or amplification of HCV RNA. The nature of and mechanism causing this reduction remains to be determined.

The significance of reduced HCV RNA levels in Spn4A.RRLL.T328D(s) treated cells is underscored by the additional finding that little to no infectious virus is produced. One interpretation of reduced infectious virus production is that the lack of intracellular nascent genomic RNA prevents HCV virion assembly within the cell and thus no infectious particles are secreted. Although this is the most straightforward explanation, other possibilities exist. Spn4A.RRLL.T328D(s) may cause premature but defective virion secretion into the media. This would explain reduced HCV RNA within the cells and lack of infectious HCV particle detection. However this scenario is contradicted by the lack of reductions in HCV core expression in Spn4A.RRLL.T328D(s) treated cells. Experiments quantifying HCV RNA and core production in the extracellular media using PCR and ELISA assays respectively will clarify which scenario is correct. A third possibility is that in addition to an intracellular reduction in HCV replication, Spn4A.RRLL.T328D(s) may also decrease the infectivity or secretion of HCV particles i.e. by altering the integrity of the viral envelope or envelope components. This is supported by the greater magnitude of reduced HCV titers (>90%) compared to reduced HCV RNA (80%).
The SKI-1/S1P-directed serpin Spn4A.RRLL.T328D(s) and the furin-directed serpin Spn4A.RRKR.T328D(s) block HCV infection (HCV RNA and HCV titer) with similar efficiencies. This eliminates the likelihood that a potential undefined impact of Spn4A.RRLL.T328D(s) on SKI-1/S1P is hindering HCV infection. Early on we ruled out Spn4A.RRLL.T328D(s) acting as a competitive inhibitor, limiting the availability of SKI-1/S1P to cleave SREBPs. However, SKI-1/S1P is also known to cleave other host, membrane bound transcription factors. Many of these factors may also play indirect roles in HCV infection through regulation of the ER stress response or lysosome formation for example (166,332). The fact that both non-inhibitory serpin variants block HCV infection alludes to a common feature between these serpins that is regulating HCV infection.

4.4.2 Insights into potential mechanisms of non-inhibitory serpin-mediated inhibition of HCV infection

4.4.2.1 Characteristics of the cleaved serpins

An important feature of the hinge-mutant serpins is that they are efficiently cleaved in vitro (224) and following intracellular expression (Figure 4.2), i.e. they function as substrates rather than as inhibitors. Incubating Spn4A.RRLL.T328D(r) or (s) with recombinant SKI-1/S1P suggests that cleavage is mediated by this enzyme which is predicted to cleave between the P1-P1’ reactive bond in the serpin RCL (Figure 4.2 B). The RCL of many serpins are vulnerable to inactivating cleavages by various host and pathogen proteases at sites distinct from the P1-P1’ cleavage site (250,333). This does not appear to be true for Spn4A.RRLL.T328D(s) in an SKI-1/S1P deficient cell line where little to no cleavage of Spn4A.RRLL.T328D(r) or (s) is detected. We did observe a small amount of Spn4A.RRLL.T328D(s) cleavage product generated in the SRD12B cells (< 1%), but we assume this is due to the high propensity of these cells for reversing the mutation in SKI-1/S1P. Any cells regaining SKI-1/S1P function have a very high selection advantage and thus these cells are subjected to weekly amphotericin B selection to prevent this from occurring. It has not yet been determined whether alternate, Spn4A-derived, cleavage products are generated during HCV infection that are not detected in SRD12B cells or whether Huh-7.5.1 cells express proteases recognizing Spn4A.RRLL.T328D(s) that are not present in SRD12B cells.

A consequence of RCL cleavage, regardless of the protease, is the release of a C-terminal peptide that is predicted to adopt a double stranded β-hairpin structure that is non-covalently
integrated within the serpin β-sheet B (217) (See molecular models in Figure 4.8). Several studies with human serpins indicate that the C-terminal cleavage product exhibits robust and clinically relevant biological activities (228,229,231,248). The biological activities attributed to the serpin C-terminus may provide insight into the potential mechanism of HCV inhibition by our recombinant hinge-mutant Spn4A variants.

4.4.2.2 Properties of the serpin C-terminus

A motif contained within the C-terminus of human serpins is exposed following protease cleavage which is recognized by cell surface receptors (LRP1/CD91, SEC receptor) (introduced in Section 1.5.3) (226,235,246). This interaction mediates clearance of circulating serpin-enzyme complexes (227) and is linked with induction of signaling cascades that promote cellular proliferation (229), cell migration (247), and induce AAT expression (235). One of the most notable properties exhibited by the C-terminus of AAT is that is possesses potent antiviral activity against HIV-1 (228,249).

The isolated C-terminal peptide of AAT has been identified in human spleen and bile samples and that of the C1 protease inhibitor has been identified in human plasma (250). In these studies, N-terminal fragments of the serpins were not identified suggesting that the C-terminal fragments may actually be released from cleaved serpins and mediate biologically relevant activities. It is reasonable to predict then that the C-terminus of Spn4A.RRLL.T328D(s) is released following cleavage in Huh-7.5.1 cells and may regulate processes in the cell leading to reduced HCV propagation.

One important observation relevant to this prediction is seen in Figure 4.2. Here, Huh-7.5.1 cells express Spn4A.RRLL.T328D(r) that is clearly cleaved intracellularly. This dissociates the C-terminus containing the ER-retention HDEL sequence from the remainder of the protein, yet the N-terminal portion of the serpin remains inside the cell. This indicates that the C-terminus is still tightly integrated within the serpin body although it is no longer covalently linked. It remains though that the secreted serpin, which passes through downstream cellular compartments with differing pH, may encounter an environment where dissociation of the C-terminus is possible.

It is also possible that cleavage of hinge-mutant serpins is accompanied by a structural rearrangement which exposes residues or structures that mediate interactions of the intact cleaved serpin with cellular receptors or other cell factors. If this is the case, a purified C-terminal peptide may mimic the activities of a cleaved serpin through presentation of an
important sequence or structure conferring antiviral properties. Whether this sequence is presented in the context of the intact cleaved serpin or as a synthetic C-terminal peptide may not be important.

However, the question then remains: what is the difference between the structures of the inhibitory and non-inhibitory serpins following cleavage? One way cleaved serpins differ from native serpins is that they present a motif recognized by cell surface receptors (i.e. SEC receptor, LRP1/CD91) (226,227,235,245,246). Both serpin-enzyme complexes (EI*) and cleaved, uncomplexed serpins can interact with these cell surface receptors (227,246,247). If this is the case, cleavage of both our inhibitory and hinge-mutant serpins may expose a sequence or motif capable of regulating and inhibiting HCV infection. The inhibitory serpin variants (as part of an EI*) however may be rapidly degraded following cleavage and complex formation; this is supported by the fact that we cannot detect a complex between Spn4A.RRLL(s) and endogenous SKI-1/S1P.

Alternatively, the EI* complex, like the hinge-mutant serpins, may also possess inherent antiviral activity in addition to inhibiting proteases critical for viral propagation (i.e. furin or SKI-1/S1P). In this scenario, inhibition of HCV infection by Spn4A.RRLL(s) may result from inhibition of SKI-1/S1P in addition to modulation of factors impacted by the hinge-mutant serpins. An argument against this is that the hinge-mutant serpins are more potent inhibitors of HCV infection than the inhibitory serpins. Also, the mechanism of action of the serpins differs; experiments monitoring HCV propagation yield different results depending on the marker being examined (i.e. HCV core, RNA, virus titer).

Yet another possibility is that there are different changes in conformation of the C-terminal portion of the inhibitory versus the non-inhibitory serpins following proteinase cleavage. The differences in conformation may permit intra- or extracellular dissociation of the C-terminal peptide in the case of non-inhibitory serpins but limit peptide release from inhibitory serpins. Regardless of which of these hypotheses is correct it remains plausible that the C-terminal portion of recombinant hinge-mutant Spn4A variants harbor anti-HCV activity. Below, research on biological activities of serpins and their C-terminal domains that are relevant to HCV infection are discussed.

4.4.2.3 AAT C-terminal (Ct) peptide regulation of cellular proliferation

The C-terminal, 26 amino acid peptide of AAT possesses mitogenic activity, stimulating cellular proliferation, when exposed to a limited number of cell lines which include the hepatoma
cell line HepG2 (229). The proliferative effect of AAT Ct supports our observation of increased cell numbers in cultures treated with Spn4A.RRLL.T328D(s) or Ad-Empty and infected with HCV over a 5 day period (Figure 4.6). At a moi of 50, Ad-Spn4A.RRLL.T328D(s) treatment prior to HCV infection resulted in 1.8 times more cell growth than in Ad-Empty and HCV infected conditions. This observation suggests that a similar effect within the cell may be activated by components of Spn4A.RRLL.T328D(s) that are activated by AAT and potentially other human serpins. How an induction in cellular proliferation may impact HCV infection is currently unknown, however it has been reported previously that HCV-replicon replication is suppressed in confluent, Huh-7-derived cells (334,335). This block in replication can be partially restored through the exogenous addition of nucleosides to cell media (335). Thus, increased confluence of Huh-7.5.1 cells induced by Spn4A.RRLL.T328D(s) may contribute to reduced HCV replication observed in our studies. This remains to be explored.

4.4.2.4 Human serpins and their C-terminal peptides inhibit HIV-1 infection

Several studies have demonstrated anti-HIV-1 properties mediated by human serpins and their C-terminal peptides (228,249,329-331). Using the C-terminal 26-amino acids of AAT, Congote, L. F. (228) demonstrated the potent anti-HIV-1 activity of this peptide which was associated with increased STAT1 activation (STAT1 phosphorylation) (228). The human serpin PAI-1 has also been shown to induce STAT-1 activation (translocation to the nucleus) in monocytes (247). This induction is dependent on PAI-1 interaction with cell surface LRP1/CD91 which plays a well-defined role in clearing serpin-enzyme complexes (245) and as discussed above recognizes a serpin C-terminal motif that is exposed following serpin cleavage (226,227,245,246). Interestingly, many studies on HCV have revealed that viral proteins (i.e. core) interfere with JAK/STAT signaling to prevent IFN-α mediated induction of the host immune response (336-339). Future studies examining cellular signaling pathways induced by cleaved Spn4A variants and C-terminal peptides should provide insight into these findings.

Antithrombin III (ATIII), an inhibitor of the coagulation cascade, is a serpin with anti-inflammatory properties that has also been identified as an inhibitor of HIV-1 infection (330). In fact, it was determined that activated CD8+ T-cells, from HIV-1 seropositive individuals, process ATIII into a low molecular weight form possessing antiviral activity. Anti-HIV-1 activity of ATIII may result from induction of cellular factors such as prostaglandin synthase 2 activity (PTGS2 aka COX-2) as suggested by Whitney et al. (331). It was determined that PTGS2 gene expression is greatly (>300-fold) upregulated in HIV-1 infected cells treated with
ATIII. Overexpression of PTSG2 was associated with a reduction in NFκB expression which is important for HIV-1 replication. In this study the authors also report that ATIII inhibits HCV, HSV-1 and HSV-2 infection (331). Similarly, it would be interesting to investigate using western blot and mass spectrometry-based analysis whether HCV infected cells process hinge-mutant Spn4A variants differentially from non-infected cells.

Intact, purified AAT can also be used to inhibit HIV-1 in several models of infection, however the amount of AAT required (1 – 5 mg/ml) is much greater than the amount of ATIII that is required (>100 fold) (329). Researchers implicating AAT as a natural inhibitor of HIV-1 infection predicted that full length AAT inhibits HIV-1 infection by disabling undefined proteolytic activity required for HIV-1 entry. The authors also demonstrate reductions in NFκB expression, required for HIV-1 replication, as another mechanism of AAT inhibiting HIV-1 infection (329). The overlap in NFκB modulation by ATIII and AAT supports our prediction that pathways affected by Spn4A hinge-mutants in Huh-7.5.1 cells may overlap with effects seen in studies examining human serpins. Thus these studies should provide suitable starting points for investigation into the anti-HCV propensity of hinge-mutant Spn4A variants.

4.4.2.5 VIRIP – an AAT Ct derived peptide inhibitor of HIV-1 entry

Application of the antiviral activity of AAT has been extended to the development of a short antiviral peptide that has undergone Phase I clinical trials against HIV-1 (257). The key to this development was the discovery of a 20-residue, C-terminally derived subfragment of AAT designated virus inhibitory peptide (VIRIP) isolated from a complex human peptide library that was inhibitory against HIV-1 infection. The N-terminus of the fragment begins upstream of the AAT P1-P1’ cleavage site and ends 22 amino acids prior to the AAT C-terminus. The fragment is not released following an inhibitory cleavage, instead host matrix metalloproteinases have been implicated in mediating these serpin inactivating cleavage events (249,251). The antiviral potency of VIRIP was increased through mutational analysis and screening of hundreds of VIRIP derived peptides. It was also discovered that VIRIP inhibits HIV-1 entry by binding very specifically via hydrophobic contacts to the fusion peptide in the HIV-1 glycoprotein, gp41. A derivative of VIRIP, VIRIP-576, was shown in a short-term Phase I clinical trial to reduce patient plasma HIV-1 load by at least 1 log (257).

The relevance of the antiviral activity of VIRIP to our discovery of the anti-HCV activity of hinge-mutant Spn4A variants is not currently known. The N-terminal lysine residue of VIRIP was shown to be essential to its inhibitory action. We currently predict that Spn4A hinge-mutants
are cleaved at the P1-P1’ binding site. This differs from VIRIP in that the N-terminus precedes the P1-P1’ RCL site in AAT and the C-terminus of VIRIP is 22 amino acids short of the AAT C-terminus (249). As mentioned above, we have not ruled out the possibility that HCV infection may also promote alternate cleavages of Spn4A hinge-mutants releasing variable C-terminal peptides. Also, clearly the structure and composition of HIV-1 and HCV envelope proteins differ (i.e. plasma membrane versus endosomal membrane fusion and class I versus class II fusion proteins (146,340,341)) thus a peptide specifically interacting with HCV glycoproteins will reasonably differ from a peptide interacting with HIV-1 glycoproteins.

If the Spn4A hinge-mutants do interrupt HCV entry or fusion, it cannot be the only mechanism of HCV infection inhibition. We are confident HCV entry does occur in Spn4A.RRLL.T328D(s) treated cells because the HCV core protein is easily detected following infection. HCV RNA replication however appears to be severely inhibited, thus mechanisms beyond inhibition of HCV entry must exist. We suspect that Spn4A.RRLL.T328D(s) inhibits HCV through more than one mechanism, interfering with both replication and virus particle infectivity. This prediction is based on the observation that reductions in viral titers are greater than reductions in HCV RNA levels. If a hydrophobic, Spn4A-derived peptide is released intracellularly it can feasibly interact with components of the HCV envelope (i.e. glycoproteins) within the secretory pathway and negatively impact the infectivity and subsequent entry of nascent particles. In this scenario, a reduction in virus entry would only become apparent upon the second round of infection (i.e. during HCV titration experiments). Inhibition of HCV entry by a hydrophobic portion of Spn4A is a valid and intriguing avenue to explore; this is further supported by research presented below demonstrating serpin regulation of host lipoprotein metabolism.

4.4.2.6 AAT Ct regulation of host lipid metabolism

As repeatedly stressed throughout this thesis, HCV infection is intricately dependent on host lipid metabolism for propagation. It is thus very interesting to find that the C-terminal peptide of AAT has been shown to affect LDL metabolism in HepG2 cells and in monocytes (230-232). AAT Ct was shown to increase LDL uptake and degradation in HepG2 cells and to induce LDL aggregation (230,231). Also, an oxidized, non-inhibitory form of AAT was found to directly interact with LDL particles by binding to ApoB in human plasma. Further studies demonstrated that a fibrillar form of AAT Ct had an increased ability to enhance LDL uptake compared to a non-fibrillar preparation (231). The increased LDL metabolism was shown to be
independent of the serpin-enzyme complex, the SEC receptor or LRP1/CD91. In fact it was determined that LDLR was required for this biological effect. Studies in human monocytes demonstrated an upregulation of LDLR mRNA and protein upon AAT C-terminal peptide treatment (232).

A C-terminal peptide of AAT was previously isolated from the phospholipid fraction of human bile (250). Metabolism of bile in the human body is a major mechanism for controlling cholesterol homeostasis through conversion of excess cholesterol through the bile synthesis and secretion pathway. Research has shown that AAT Ct downregulates bile acid synthesis through interactions with α-fetoprotein, a transcription factor required for the expression of enzymes required for bile acid synthesis (248). Reduced bile acid synthesis results in increased cellular cholesterol and inhibition of the SREBP pathway.

Our research to this point has not revealed regulation of host lipid metabolism by Spn4A.RRLL.T328D(s) thus it is likely that this is not the mechanism of action of HCV infection inhibition. However, in our studies we have not examined the specific pathways, i.e. bile acid synthesis and LDL catabolism shown to be regulated by AAT Ct. It is possible that unexplored changes in lipid metabolism are occurring in Spn4A.RRLL.T328D(s) treated cells that may explain at least in part the antiviral activity of this serpin. For example, if the serpin Ct is interacting with host LDLs, it could disrupt HCV interaction with VLDL assembly pathway which is required for HCV assembly and secretion (147). Data shows that HCV impedes host VLDL production possibly as a means of increasing the potential for the virus to hijack these components (25). A peptide mediated increase in LDL metabolism as discussed above may hinder HCV subversion of this pathway.

4.4.2.7 AAT Ct peptide is a cell penetrating peptide with a nucleolar targeting motif

The range of potential mechanisms of hinge-mutant serpin inhibition of HCV infection is vast and will require an in depth analysis of various properties of both HCV infection during Spn4A hinge-mutant treatment and of Spn4A localization and interactions during HCV infection. Currently, members of our laboratory are investigating the chemical and biological properties of synthetic C-terminal serpin peptides including those derived from Spn4A. Computer modeling of the Spn4A C-terminal peptide indicates that the peptide will adopt a β-hairpin structure similar to that of the AAT C-terminus (Figure 4.8). This β-hairpin of AAT was identified as an integral component of the full length AAT crystal structure (Figure 1.8) (217). The similarities between
the structures of the 2 serpin C-terminuses support our hypothesis that similar activities can be mediated by these two peptides.

The β-hairpin, C-terminus of Spn4A and AAT is predicted to be stabilized by hydrophobic interactions between two highly conserved stretches of amino acids (annotated Box 1 and Box 2 in Figure 4.8). The first hydrophobic stretch (Box 1) contains the motif implicated in SEC receptor interactions. This stretch of amino acids, due to its highly hydrophobic nature may also confer cell penetrating properties, independent of receptor binding, as shown by Rhee, M. and Davis, P. (233). A peptide derived from AAT called C105Y is 17 amino acids long and contains the pentapeptide sequence PFVYLI shown previously to compete for serpin-enzyme complex binding to the SEC receptor (226). In Huh-7 cells, it was shown that this sequence is sufficient to mediate cellular uptake and targeting to the cell nucleolus when labeled with FITC and mediates efficient gene transfer delivery when complexed to polyK condensed DNA particles (233,234). Importantly, a scrambled peptide variant of PFVYLI was also shown to enter the cell nucleolus thus it may not be the sequence but the hydrophobicity or other biochemical properties that are important. It was shown that C105Y is internalized via a clathrin and caveolin-independent mechanism (233). The authors also pointed out that many nucleolar targeted proteins contain the sequence (L/R)VXP (342) which is similar to PFVYLI within C105Y. Thus, Box 1 may be integral to many biological properties associated with the C-terminal portion of AAT such as interaction with host transcription factors and regulation of gene expression (248). Spn4A contains a similar amino acid sequence PFTYVLV in the Box 1 region which may also confer cell penetrating or nuclear targeting properties to the peptide. It remains to be determined whether this sequence in context of the full β-hairpin or the full length C-terminus enables or overrides these properties.

4.4.3 Conclusions

Analysis of the literature reveals that it is very likely that the C-terminus of Spn4A hinge-mutants is conferring anti-HCV activities to the serpin. This is based on the fact that the hinge-mutants are very efficiently cleaved by cellular proteases, likely targeting the P1-P1’ bait bond. As such, an abundance of C-terminal peptide sequences are likely to be present intra- or extracellularly following hinge-mutant serpin expression. We reason that the C-terminus of the hinge-mutants may be released from the intact serpin in a manner facilitated by RCL cleavage and that release of the peptide may also occur following trafficking to the late secretory pathway or following secretion. We propose that two conserved histidine residues within the C-terminus
may act as pH sensors dictating release of the peptide depending on the cellular microenvironment. If the C-terminus is not released, residues may still be exposed following cleavage that are important for inhibiting HCV infection; it may not matter if the sequence is presented in isolation or in context of the intact serpin.

The potential mechanisms exhibited in the literature are vast and much research must be done before a mechanism is revealed. Biotin-labeled serpin derived peptides are now being examined in our laboratory with a focus on detecting cellular pathways affected by the C-terminal peptide using immunoprecipitation combined with proteomics analysis. In fact, preliminary studies are revealing some very pertinent potential binding partners including ApoE. Mass spectrometry or gene expression analysis of cell extracts treated with the hinge-mutant serpins will assist our investigation into which cellular pathways are affected and whether these pathways intersect with those affected by AAT. Western blotting of intracellular peptide expression will be used to determine if Spn4A Ct is a cell penetrating peptide. Confocal imaging is being employed to detect the intracellular localization of these peptides. Most importantly the various biotin-labeled peptides are being examined to determine if a portion or the full length Ct of Spn4A and other related serpins is responsible for the anti-HCV activity of the hinge-mutant serpins. An additional series of experiments that will contribute to our understanding of the hinge-mutant serpin function is the construction of recombinant adenoviruses expressing a C-terminal tagged Spn4A construct. This will assist in determining the cellular localization of the Ct and also reveal whether a purified Ct localizes to the same place in the cell as the Ct generated following hinge-mutant serpin cleavage.

Considering the knowledge that the major HCV lifecycle stage disrupted by Spn4A hinge-mutant variants is replication we may also examine cellular components critical for HCV replication to see if they are affected. For example, we can investigate the integrity of the membranous web during viral infection as this structure is critical to protecting replicating viral RNA. It has also been shown that HCV replication occurs on lipid rafts within the ER so the integrity of these structures should be inspected (106,107). Due to the hydrophobic nature of these peptides and the knowledge that the AAT Ct cofractionates with the phospholipid component of bile and that it interacts with LDL particles it is possible that the peptide can disrupt or interact with cellular structures (i.e. ApoB, ApoE or LDLR) required for HCV infectivity. Also many host proteins have been implicated in replicating HCV RNA that may be
alternately expressed if their transcription is altered by nucleolar targeting of the C-terminal peptide.

The focus of this thesis has been using the host as a target for inhibiting viral infection but it is a great possibility that the serpin is directly targeting a viral component such as the HCV polymerase or the envelope proteins as discussed above. Studies in our laboratory testing these serpins against HIV-1 (193) and Influenza virus (unpublished data) so far indicate that the hinge-mutant serpins only inhibit HCV infection. Thus, that a very specific interaction between Spn4A Ct and the viral envelope is occurring is an intriguing possibility. As both HCV envelope proteins and AAT Ct have been shown to interact with LDL, there exists a very real potential for these two components to encounter each other during the virus lifecycle either within the secretory pathway or extracellularly.

Understanding the mechanism of action of these serpins may provide a starting point for developing new antivirals against HCV or may reveal new targets for treating HCV infection. Understanding the antiviral component of these serpins might lead to rationale design and development of novel potent inhibitors of HCV infection.
A

Spn4A.RRKR(r)  
Spn4A.RRKR(s)  
Spn4A.RRLL(r)  
Spn4A.RRLL(s)  
Spn4A.RRKR.T328D(r)  
Spn4A.RRKR.T328D(s)  
Spn4A.RRLL.T328D(r)  
Spn4A.RRLL.T328D(s)  

B

"Bait" Region

Hinge Region

C₁
Figure 4.1. Engineering recombinant Spn4A scaffolds to generate non-inhibitory hinge-mutant serpins.

(A) Spn4A variants and amino acid sequences of the engineered reactive center loop (RCL) “bait” region. Spn4A.RRKR(r) encodes for the naturally occurring serpin Spn4A, isolated from *Drosophila melanogaster*, with potent inhibitory activity against the human proprotease furin. Spn4A.RRKR(r) contains the α-1-antitrypsin (AAT) signal peptide (SP) at the N-terminus followed by a tandem his-tag (HHHHHH) and FLAG-tag (DYKDDDDK) sequence (HF). The P4 – P1 furin cleavage sequence in the RCL is RRKR. Spn4A.RRKR(r) also contains the HDEL ER retention motif (r) at the C-terminus. The secreted (s) serpin, Spn4A.RRKR(s), contains a stop codon before the HDEL signal. The P4 – P1 bait sequence of the RCL of Spn4A.RRKR(r) and (s) was modified to RRLL to target the PC SKI-1/S1P. Spn4A.RRLL(s) contains a stop codon before HDEL allowing the serpin to be secreted. To generate non-inhibitory, hinge-mutant variants of the four Spn4A inhibitory serpins, site-directed mutagenesis was employed. The critical hinge region residue P15 (threonine) was mutated to a charged aspartate residue resulting in four serpins with a T328D mutation. (B) *In silico* homology model of Spn4A.RRLL(r) variant. The molecular model was generated using pyMOL. The side chains of the RRLL residues within the flexible “bait region” of the RCL are shown as sticks in wheat colour. Sheet A is shown in yellow, sheet B is shown in blue, and sheet C is shown in cyan. α-helices are shown in red and loops are shown in green.
Figure 4.2. Cellular expression and characterization of adenovirus-expressed Spn4A.RRLL inhibitory and non-inhibitory mutants.

The cellular expression of serpin variants Spn4A.RRLL(r), Spn4A.RRLL(s), Spn4A.RRLL.T328D(r) and Spn4A.RRLL.T328D(s), were examined using infectious adenoviral-mediated expression. An empty adenovirus vector (Ad-Empty) was used as a control. (A) In Huh-7.5.1 cells, after 72 hours of adenovirus infection, cell media (upper panel) and lysates (lower panel) were subjected to western blot analysis. Spn4A variants were detected with mouse anti-his antibody. (B) SKI-1/S1P-deficient, SRD12B cells were infected with recombinant adenoviruses expressing his-tagged Spn4A.RRLL variants or the control for 72 hours. Media alone (upper panels) or cell extracts (lower panels) lysed in RIPA buffer was combined with recombinant his-tagged SKI-1/S1P for 30 minutes at 30°C. Samples were prepared for western blot analysis and probed with mouse anti-his antibody to detect SDS- and heat-stable, enzyme-inhibitor (EI*) complex formation as described in the materials and methods.
Figure 4.3. Expression of Spn4A.RRLL.T328D(s) does not inhibit SREBP-2 regulated protein expression or lipid droplet abundance in Huh-7.5.1 cells.

(A) Huh-7.5.1 cells were grown in LPDS-supplemented media for 24 hours and were then infected with Ad-Empty (control) or Spn4A.RRLL.T328D(s) for 72 hours and cell extracts were harvested and subjected to western blot analysis. Antibodies against PCSK9, LDLR, SREBP-2, and GM130 were used to detect protein expression levels, and β-tubulin was probed for normalizing band intensities. Values (mean ± SEM) are plotted relative to protein expression in control treated cells. Results from 3 independent experiments are shown. (B-D) Huh-7.5.1 cells were infected with Ad-Empty (control) or Ad-Spn4A.RRLL.T328D(s) for 72 hours. (B) Fixed cells were stained for cell nuclei using Hoechst dye (blue) and probed for Spn4A.RRLL.T328D(s) expression using mouse anti-FLAG antibody (red). The LD marker ADRP was detected using rabbit anti-ADRP antibody (green), and images were obtained using an Olympus Fluoview FV1000 laser scanning confocal microscope. (C) Huh-7.5.1 cells infected with Ad-Empty (control) or Ad-Spn4A.RRLL.T328D(s) for 72 hours were harvested and subjected to SDS-PAGE and western blot analysis. Mouse anti-ADRP antibody was used to detect protein expression levels in serpin-treated cells compared to control-treated cells. Values (mean ± SEM) are plotted relative to protein expression in control treated cells. Results from 3 independent experiments are shown. (D) Fixed cells were stained for cell nuclei using Hoechst dye (blue) and probed for expression of Spn4A.RRLL.T328D(s) using mouse anti-FLAG antibody (red). LDs were detected using BODIPY 493/503 (green) and images were acquired using a Leica TCSSP5 confocal microscope.
Figure 4.4. Spn4A.RRLL.T328D(s) does not decrease the frequency of HCV core expression or localization following HCV infection in Huh-7.5.1 cells.

(A) Huh-7.5.1 cells were infected with moi 1, 12.5, 25 or 50 of Ad-Empty (control) and Ad-Spn4A.RRLL.T328D(s) for 48 hours followed by HCV infection (moi 0.1) for 72 hours before fixing. Cells were probed with HCV anti-core antibody (red), stained with Hoechst dye (nuclei; blue) and were analyzed using Cellomics HCS to determine the percentage of HCV-infected cells. Values (mean ± SEM) are plotted relative to control treated cells. Results from 5 independent experiments are shown. Sample images of cells infected with adenovirus moi 50 acquired with Cellomics HCS using the 10 X objective are shown on the right. (B) Huh-7.5.1 cells infected with HCV (moi 0.1) for 48 hours were fixed, probed with mouse anti-core antibody (red) and stained with Hoechst dye (nuclei; blue). (C) Huh-7.5.1 cells were infected with Ad-Spn4A.RRLL.T328D(s) for 24 hours and then infected with HCV for 48 hours. Fixed cells were probed for expression of HCV core (green) or Spn4A.RRLL.T328D(s) (rabbit anti-FLAG antibody; red) and were stained for cell nuclei using Hoechst dye (blue). Confocal images were obtained using a Leica TCSSP5 confocal microscope.
Figure 4.5. HCV RNA and HCV extracellular titers are dramatically reduced by Spn4A.RRLL.T328D(s).

Huh-7.5.1 cells were infected with Ad-Empty (control) or Ad-Spn4A.RRLL.T328D(s) (moi 50) for 48 hours in complete media. Adenovirus infected cells were (A) infected with HCV (moi 0.1) or (B) transfected with HCV RNA for 72 hours. Total cellular RNA was harvested and the levels of HCV RNA, normalized to β-actin transcript levels, were relatively quantified in cell extracts using real-time PCR. (C) Ad-Empty (control) and Ad-Spn4A.RRLL.T328D(s) (moi 50) infected cells were infected with HCV for 72 hours and the infectious HCV titer in the extracellular media was determined and relatively quantified. Results (mean ± SEM) from 3 independent experiments are shown. *** denotes p< 0.005.
**Figure 4.6.** Spn4A.RRL.T328D(s) does not compromise Huh-7.5.1 cell growth. (A) Huh-7.5.1 cells were infected with moi 1, 12.5, 25 and 50 Ad-Empty (control) or Ad-Spn4A.RRL.T328D(s) (moi 50) for 72 hours. The relative cytotoxicity of the adenoviruses was determined using an MTS-based cell viability assay. The absorbance measured at 490 nm is proportional to the amount of living cultured cells. (B) Huh-7.5.1 cells were infected with moi 1, 12.5, 25 and 50 Ad-Empty (control) or Ad-Spn4A.RRL.T328D(s) (moi 50) for 48 hours in complete media. Treated cells were then infected with HCV (moi 0.1) and fixed 72 hours post-infection. Fixed cells were probed with Hoechst dye to stain for cell nuclei, which were then quantified using Cellomics HCS to determine the relative number of cells in each well under the varying conditions. Results (mean ± SEM) from 3 independent experiments are shown.
Figure 4.7. HCV RNA and HCV extracellular titers are dramatically reduced by Spn4A.RRKR.T328D(s).

Huh-7.5.1 cells were infected with Ad-Empty (control) or Ad-Spn4A.RRRK.T328D(s) (moi 50) for 48 hours in complete media. (A) Adenovirus infected cells were (A) infected with HCV (moi 0.1) for 72 hours. Total cellular RNA was harvested and the levels of HCV RNA, normalized to β-actin transcript levels, were relatively quantified in cell extracts using real-time PCR. (B) Ad-Empty (control) and Ad-Spn4A.RRKR.T328D(s) (moi 50) infected cells were infected with HCV for 72 hours and the infectious HCV titer in the extracellular media was determined and relatively quantified. Results (mean ± SEM) from 2 independent experiments are shown.
Figure 4.8. Spn4A and α-1-antitrypsin C-terminal peptides.

The amino acid sequences and stick model comparisons of AAT and Spn4A C-terminal peptides released following serpin cleavage of RCL between P1 and P1’ are shown. The sequences highlighted in yellow (Box 1 on left and Box 2 on right) are predicted to stabilize the modeled two strand β-hairpin structures. Box 1 contains the conserved putative SEC receptor binding sequence. The underlined sequence highlights the section of the C-terminal peptide appearing in the stick model. Residues in green are hydrophobic, blue are basic, red are acidic and black are polar non-charged. Dashed lines depict predicted hydrogen bonding. Stick models courtesy of Steve McArthur were modeled in pyMOL.
Chapter 5: Conclusions and future directions

5.1 Discussion

The aim of this work was to uncover and validate novel host factors, important for the HCV lifecycle, as potential targets for impeding virus infection. Many approaches can be explored for choosing candidate antiviral targets such as monitoring proteins disregulated during infection (genomics and proteomics approaches), determining host proteins that interact with viral components (yeast-two-hybrid or co-immunoprecipitation studies) or by systematically inhibiting gene expression and monitoring the impact on viral fitness (RNA interference or mutagenesis screens). We chose a more selective approach and singled out two host proprotein convertases, SKI-1/S1P and PCSK9, that are linked to pathways utilized by HCV and that possess properties making them favorable for antiviral therapeutic development. The studies presented herein provided evidence that both of these proteins play important roles in the HCV lifecycle. Serine protease inhibitors (serpins) have evolved as a natural host mechanism for controlling endogenous proteolytic function and provided us with a molecular tool for dissecting the endoproteolytic contribution of SKI-1/S1P to HCV hijacking of host lipid metabolism. Investigative studies using different serpin variants revealed that non-inhibitory serpins possess potent and unexpected antiviral function against HCV that remains to be understood. Overall the research presented here has revealed three promising strategies for disrupting HCV infection that may lead to further our understanding of the HCV lifecycle and provide new grounds for building anti-HCV multidrug regimens employing indirect-acting antiviral agents.

5.1.1 SKI-1/S1P and host lipid metabolism

In Chapter 2, SKI-1/S1P is explored as a potential target for inhibiting HCV infection. Two modes of inhibition were employed. First, we used a protein-based inhibitory strategy using an infectious, recombinant adenovirus delivery system. A selective and specific, secretory pathway expressed serpin was designed (Spn4A.RRLL(s), Figure 2.1) and was shown to form a stable EI* complex with SKI-1/S1P (Figure 2.2). Inhibition of SKI-1/S1P resulted in a dramatic reduction of host LD components including ADRP/perilipin 2 and cholesterol-esters (Figure 2.3 and Figure 2.4). LDs are critical to the assembly stage of the HCV lifecycle and are increased in abundance during infection (111), thus it was not surprising that inhibiting SKI-1/S1P blocked HCV infection in a dose-dependent manner (Figure 2.5). We observed reductions in extracellular viral infectivity and also in HCV RNA levels which may be reduced due to
interrupted HCV entry. HCV RNA levels, following transfection of HCV genomic RNA into the cell were not significantly reduced by Spn4A.RRLL(s) (Figure 2.5 C). Using a previously characterized pharmacological inhibitor of SKI-1/S1P we confirmed the importance of this enzyme to HCV infection, observing a dramatic dose-dependent reduction in HCV core and titer levels (Figure 2.7). Inhibiting HCV infection using this small molecule inhibitor demonstrated that HCV infection, post-entry could also be impeded. We also found that the compound acted very quickly and the effect was long lived (at least 72 hours post-removal).

The use of serpin-based inhibitors such as Spn4A.RRLL(s) has many advantages over other molecular inhibitory tools, especially for cell and animal based analyses. One advantage is the amenability of protein-based inhibitors to molecular biology techniques. Protein inhibitors of interest can be cloned and systematically altered to introduce mutations or sequences of interest. In our studies we were able to engineer an inhibitor and directly target it to the secretory pathway for transit through the Golgi apparatus. In theory we could target any compartment of interest based on subcellular localization signals. We successfully designed a suitable control serpin by taking advantage of the putative ER-retention signal, HDEL, and the fact that the enzyme we are targeting is in a post-ER compartment. A second feature of the serpin we took advantage of was the bait sequence to specifically target the SKI-1/S1P active site. In this aspect we were able to rationally design a compartmentally targeted, selective and specific inhibitor against SKI-1/S1P.

The use of a protein-based inhibitor permits the use of standard techniques such as western blotting to confirm the predicted features of the serpin i.e. intracellular expression, secretion and complex formation. Another advantage of using protein-based inhibitors is that they can be monitored at a single cell level through the use of epitope tags, fluorescent tags or protein-specific antibodies. The value of this technique is seen in Figure 2.4 and Figure 2.6 where inhibitor expressing cells and treatment naïve cells can be compared side by side. Concurrently, the impact on a marker of interest, such as lipid droplets or viral proteins, can be observed.

We increased the efficacy of our Spn4A-based inhibitors by expressing them via infectious adenovirus vectors. These vectors proved suitable for studies in cell culture but may provide a further benefit in their applicability to animal-based studies. Many Spn4A variants have been designed in our laboratory that have an N-terminal RFP tag which are confirmed to be functional and which allow monitoring by FACS or live animal imaging. The applications are extraordinary as we can monitor the localization within an animal and determine its stability over
time. Preliminary studies have already been conducted in collaboration with the Centre for Drug Research and Development performing live animal imaging of mice infected with RFP-serpin expressing adenoviruses. Of particular importance for studies on HCV, a prominent accumulation of adenovirus expressed serpin within the liver was observed and expression was detected up to 12 days post-inoculation (data not shown). This feature of adenoviruses has been applied to other advances in HCV research such as the development of a mouse model expressing human proteins in the liver (343). It is of interest to note as well that Phase II gene therapy clinical trials are being conducted whereby the human serpin AAT is being delivered to the human liver via an adenovirus-based system (344).

Of course, there are limitations to using adenovirus-expressed serpins for antiviral studies. We required a high moi to achieve 90% coverage of cell populations expressing the serpins of interest. We suspect this is the reason that the magnitude of HCV infection inhibition is not as high in serpin-based studies as with the small molecule PF-429242. This is a significant limitation in the scenario of viral infection where millions of viral particles can be amplified from a very small subset of treatment naïve, HCV susceptible cells. For this reason it was also important that we examine the effect of inhibiting SKI-1/S1P on HCV infection using a secondary method. The small molecule inhibitor PF-429242 allowed us to confirm the important contribution of SKI-1/S1P to HCV propagation.

5.1.2 PCSK9 and HCV entry

In Chapter 3, the application of employing the unique function of PCSK9 to block HCV entry was explored. PCSK9 is the most recently identified member of the PC family and plays a unique role in the complex regulatory pathway controlling host cholesterol metabolism. The extracellular functionality of this protein and its control of an HCV entry co-factor, LDLR, implied its importance for HCV propagation.

An interesting preliminary observation that was made was the differential expression of PCSK9 in two cell lines that permit HCV infection with differing kinetics (Figure 3.2). Higher LDLR levels and lower PCSK9 expression in the more susceptible cell population encouraged our hypothesis that PCSK9 could be employed to reduce viral fitness. Infectious adenovirus vectors were employed to deliver PCSK9 to Huh-7.5.1 cells. We found a very long pretreatment period was required to reduce LDLR levels by 70% (Figure 3.3) and render cells sufficiently resistant to HCV infection (Figure 3.4). This is in contrast to purified recombinant PCSK9 in which only an 8 hour pretreatment was required to block HCV infection almost completely.
Our finding that overexpressing PCSK9 does not reduce HCV RNA abundance post-HCV RNA transfection (Figure 3.6) confirmed that PCSK9 inhibits HCV infection by inhibiting viral entry. Lastly, we employed two PCSK9 mutants in our studies which showed significant variations in their capacity to inhibit HCV infection. As a plethora of natural mutations in PCSK9 are found throughout the human population, it remains an intriguing possibility that PCSK9 alleles may regulate natural susceptibility to HCV infection.

The potential applications of PCSK9 as an entry inhibitor of HCV infection are significant especially for HCV patients who have reached a point where a liver transplant is required. Recent studies show that the number of people progressing to severe end stage level disease due to HCV infection is rising (345) thus the need for therapies compatible with transplantation is pressing. The need for HCV entry inhibitors is underlined by the fact that newly allografted livers invariably become infected with circulating HCV almost immediately (346). Therapeutic HCV entry inhibitors, such as neutralizing antibodies, are currently in clinical trials with the end goal of treating liver transplant recipients (347,348).

A very interesting finding that supports investigating PCSK9 as an HCV entry inhibitor is that PCSK9 is required for liver regeneration in mice undergoing hepatectomy (308); PCSK9−/− mice were observed to develop necrotic lesions following this procedure. The reason for this is unclear but indicates that overexpressing PCSK9 or mimicking its function may prove beneficial to liver transplant recipients. Also, our studies provide support for using an extracellularly applied PCSK9-mimetic to block HCV infection as opposed to an intracellularly delivered and expressed protein. We found that addition of recombinant PCSK9 to the media of Huh-7.5.1 cells was much more rapid and effective at blocking HCV entry than adenovirus-expressed PCSK9; even more effective was the use of PCSK9-D374Y which blocked HCV infection at lower concentrations than the wild-type variant. Overall these studies support a role for PCSK9 in decreasing susceptibility of hepatocytes to HCV infection.

Therapies modeled after PCSK9 may also be used for patients in a non-transplantation context as part of a multi-drug regimen (i.e. with IFN and ribavirin). It has been calculated that approximately $10^{12}$ HCV particles are produced and cleared from patient plasma each day (9). This implies that new cells are continually being infected in the liver. Thus, in combination with other drugs, an entry inhibitor may slow the spread of infection and enhance the effectiveness of co-administered therapeutics.
PCSK9-based therapies may also ameliorate the metabolic disorder hypobetalipoproteinemia which occurs in many patients infected with HCV. As the name indicates, this disorder is characterized by low circulating plasma lipoprotein abundance, likely a result of their retention in the liver which coincides with the occurrence of steatosis. As presented in Chapter 1, HCV has been shown to inhibit lipoprotein secretion, potentially as a means of commandeering this pathway. LDLR plays a major part in regulating not only lipoprotein uptake but also in controlling the secretion of ApoB-containing lipoproteins. When dietary cholesterol levels are low, LDLR expression is induced to increase lipoprotein uptake. Within the secretory pathway, LDLR also controls the secretion of VLDL from hepatocytes by inducing premature degradation of immature ApoB-containing lipoproteins. Therefore, reducing LDLR levels using PCSK9-based therapies may restore lipoprotein secretion while concurrently reducing HCV and lipoprotein uptake by the liver. This in turn may restore circulating lipoprotein levels and reduce accumulation of esterified-lipids in and steatosis of in the liver.

5.1.3 Spn4A hinge-mutants possess antiviral activity against HCV

In Chapter 3, research demonstrating antiviral properties inherent in the Drosophila melanogaster-encoded, Spn4A hinge-mutants is presented. These serpin variants were designed and constructed by a former laboratory member, Christine Lai, who conducted a pilot study to examine the impact of altering the P15 hinge residue (amino acid 328) in the RCL of Spn4A.RRKR(r) to aspartic acid, glycine or lysine (See diagrams Figure 4.1 and (328)). This residue is highly conserved among inhibitory serpins and is required for displacing the cleaved RCL from the top of the serpin to the opposite pole of the molecule, an integral feature of the serpin inhibitory mechanism. Christine Lai found that both T328D and T328K mutants had lost their capacity for EI* complex formation with overexpressed furin when compared to Spn4A.RRKR(r) (328). From these results she proceeded to construct a small library of hinge-mutant variants to use as negative controls in studies dissecting furin and SKI-1/S1P enzymatic function. These hinge-mutants have proved very useful for studies in our laboratory examining the role of furin in activation of influenza A virus and HIV-1 glycoproteins required for viral infection.

Studies investigating the role of SKI-1/S1P during HCV infection also initially suggested that the hinge-mutant serpin Spn4A.RRLL.T328D(s) was an ideal control for Spn4A.RRLL(s). As shown in Figure 4.2, Spn4A.RRLL.T328D(s) did not form an EI* complex with recombinant SKI-1/S1P in contrast to Spn4A.RRLL(s). This confirms that Spn4A.RRLL.T328D(s) acts as a
substrate rather than an inhibitor of SKI-1/S1P. The studies presented in Figure 4.3 indicate that Spn4A.RRLL.T328D(s) does not hinder SKI-1/S1P activation of the SREBP pathway nor does it reduce LD abundance or ADRP expression. Initially, Spn4A.RRLL.T328D(s) also appeared to have no effect on HCV infection when the number of cells expressing the HCV core protein was monitored (Figure 4.4) following hinge-mutant serpin treatment. It has not been determined why this is the case but this observation suggests that Spn4A.RRLL.T328D(s) does not significantly inhibit HCV entry i.e. HCV RNA is released into the cell uninterrupted and so the core protein is expressed in the early stage of infection.

When HCV RNA levels were examined in Spn4A.RRLL.T328D(s) cells it was unexpectedly revealed that HCV RNA levels were greatly reduced compared to Ad-Empty treated cells (Figure 4.5). It was subsequently found that little to no infectious HCV particles were being secreted into the media of Spn4A.RRLL.T328D(s) treated cells. In contrast to studies examining the impact of overexpressed PCSK9 and Spn4A.RRLL(s), we found that in Spn4A.RRLL.T328D(s) treated cells, HCV RNA levels were greatly reduced following HCV RNA transfection (different from infection) as determined by QPCR analysis. This suggests that the mechanism of inhibition by Spn4A.RRLL.T328D(s) and Spn4A.RRLL(s) is different and that Spn4A.RRLL.T328D(s) inhibits HCV replication. We subsequently confirmed that Spn4A.RRRK.T328D(s), the hinge-mutant variant of the furin-targeting serpin, also blocks HCV infection (Figure 4.7). Thus the antiviral effect is likely unrelated to inhibition of SKI-1/S1P function. Another notable feature of Spn4A.RRLL.T328D(s) is its impact on cell viability. It has been consistently observed that adenoviruses expressing hinge-mutant serpins do not lead to cell death even at very high mois. Empty adenovirus vectors on the hand do lead to observable declines in cell numbers at high mois and following extended treatment periods (Figure A1.2). One potential explanation is that Spn4A.RRLL.T328D(s) stimulates the proliferation of Huh-7.5.1 cells (explored in Section 4.4.2.3). Overall, our studies strongly suggest that hinge-mutant Spn4A variants are effective HCV inhibitors and indicate that these or potentially other homologous serpins may act as scaffolds for designing novel antiviral HCV agents.

Numerous experiments must be conducted to understand the antiviral mechanism of the hinge-mutant serpins which were proposed throughout Section 4.4.3. However, further experiments using the tools available and those currently under development in our laboratory will provide further insight into the specific features of Spn4A.RRLL.T328D(s) that confer anti-HCV activity. Specific experiments and tools that will be investigated (experiments proposed
below in Section 5.2) will help in determining which phase of the lifecycle is affected but many additional tools and experiments are needed to determine why the hinge-mutants block HCV infection. For example, we will determine the anti-HCV potency of the secreted hinge-mutant serpins versus that of the retained variants. This may pinpoint where in the cell the serpins must be localized in order to block the virus lifecycle. Subsequently we will conduct studies whereby cells are infected with Ad-Spn4A.RRLL-T328D(s) or (r), the virus is removed, and the cells permitted to express and secrete the serpin. The serpin-conditioned media will then be used to treat a new subset of cells prior to, during or after HCV infection. This will indicate whether the hinge-mutant serpin acts extracellularly on virus particles or whether it needs to be expressed within the secretory pathway.

New tools are now under construction and validation stages in our laboratory to examine whether isolated C-terminal serpin peptides inhibit HCV infection. A growing library of biotin-labeled peptides comprising different lengths and portions of the C-terminus of the serpin and of other human serpins (i.e. Hsp47 and AAT) is being developed. These are being monitored for cell entry, cell localization and for interactions with host cell proteins. A vector expressing Spn4A.RRLL.T328D(s) with a his/FLAG tag at the C-terminus (all of the constructs so far studied are labeled at the N-terminus) is also being constructed. This will provide another means for monitoring the localization of the C-terminus in the cell and also will provide a tool for purification of this fragment from the media of cells expressing the serpin so that its antiviral potential can be assessed. At this stage it is difficult to interpret the application of our findings to understanding HCV biology or to the development of novel therapeutic avenues. However, the available data on the applications for human AAT to treat HIV-1 infection suggests that this is a very exiting avenue of research to explore; much remains to be learned about this serpin and how it inhibits HCV infection.

Overall the research presented in this thesis has confirmed the importance of the SREBP pathway to HCV infection and has revealed two novel host proteins, tightly integrated into the SREBP signaling pathway, as potential antiviral targets. The relevance of SKI-1/S1P and PCSK9 to HCV infection and the metabolic disorders steatosis and hypobetalipoproteinemia are implied by their direct roles in regulating lipid production and lipoprotein metabolism (Figure 5.1) respectively. Support for their importance is further verified by our experimental analyses. The exiting possibility that non-inhibitory hinge-mutant serpins also block HCV infection through regulation of host cholesterol, bile or lipoprotein metabolism remains to be explored.
5.2 Future directions: further dissecting the roles and applications of proprotein convertases (PCs) and serine protease inhibitors (serpins) for blocking HCV infection

The studies presented in Chapter 2, 3 and 4 provide proof-of-concept studies for employing serpins and targeting PCs to impede various stages of the HCV life cycle. As we propose to target host rather than virus components, these strategies may provide the added benefit of resisting viral resistance in the context of the HCV RdRp which replicates diverse pools of HCV genomes. Our studies as a whole however have left open a wide range of questions that need to be explored to determine further the impacts of these strategies on virus infection i.e. what are the lifecycle stages being affected and what is the in vivo potential of these antiviral approaches.

5.2.1 Antiviral strategies and HCV replication

HCV replication is the source of new and potential antiviral resistant HCV variants. An ideal therapeutic strategy will thus interrupt this phase of the HCV lifecycle. Fortunately, many tools for studying HCV replication exist, and in fact have been available since before the advent of the fully infectious HCV system used here (105). Although it can be argued that using the fully infectious system is more relevant for determining the application of antiviral targets and therapies, other tools must also be employed to dissect the specific lifecycle stages impacted by potential HCV inhibitors.

Many aspects of cholesterol metabolism are critical for HCV replication. It is possible that our examinations of the abundance of transfected HCV RNA within hepatoma cells following treatments with Spn4A and PCSK9 variants overshadows potential changes in nascent replicated RNA levels (see Figure 2.5 C, Figure 3.6 and Figure 4.5 B). To address this issue we will test the effect of all of our antiviral strategies on HCV replicon systems which do not produce infectious particles but allow robust replication of the viral genome under selection. Based on our research up to this point we hypothesize that Spn4A.RRLL(s) and Ad-PCSK9 will not inhibit HCV replicon replication. However, we do anticipate that Spn4A hinge-mutants will negatively impact replicon abundance as our research suggests that they inhibit HCV replication.

5.2.2 Antiviral strategies and cellular pathways in the context of HCV infection

It will also be important to investigate the impact of SKI-1/S1P inhibition and PCSK9 overexpression on different aspects of host lipid metabolism in the context of HCV infection. For example, although we have determined that Spn4A.RRLL(s) and PF-429242 reduce LDs in
uninfected cells, the significance of this physiological effect during HCV infection remains unexplored. It will be interesting to investigate whether slight reductions in cell proliferation observed using MTS-based cell viability assays of PF-429242 treated cells is negated when cells are first allowed to establish HCV infection and accumulate excess lipids.

Similarly, it will be important to conduct studies with PCSK9 and measure various aspects of host lipid metabolism during HCV infection. For example, as discussed, PCSK9 overexpression should reduce LDL uptake and increase LDL secretion due to reduced LDLR levels. We would like to determine how PCSK9 overexpression affects these processes as well as LD abundance (directly linked to VLDL assembly) in HCV infected cells.

In parallel we would also like to test the impact of hinge-mutant serpins on host lipoprotein metabolism. The link between the lipoprotein pathway and human serpins is very intriguing (see section 4.4.2.6). Although we have not observed changes in LDLR or LD levels in Spn4A.RRLL.T328D(s) treated cells, these aspects have not been examined in HCV infected cells treated with this serpin.

Another feature of the hinge-mutant serpins that will be examined during HCV infection is the integrity of the serpin itself. In the case of HIV-1, it has been determined that during inflammation an alternate AAT cleavage occurs which frees the VIRIP peptide (251). This peptide is not generated by enzymes cleaving between P1-P1’ in the serpin RCL. Similarly, Spn4A.RRLL.T328D(s) may be susceptible to a cleavage event that is initiated only during HCV infection, releasing an uncharacterized C-terminal peptide. Monitoring this serpin by western blot and mass spectrometry during HCV infection will reveal whether or not this possibility exists. Examining the hinge-mutants by western blot during infection will also indicate if there is an impact on the stability of the protein, as the expression is very low in HCV naïve cells (Figure 4.2) but the antiviral effect is very prominent.

5.2.3 Antiviral strategies and HCV assembly

The assembly stage of the HCV lifecycle can be further examined using cells that are transfected with HCV genomic RNA. Following HCV RNA transfection we have observed a 6 to 7 day lag in the time it takes for infectious virus to be produced. Thus, cells can be transfected, replication allowed to establish for several days and then cells treated with SKI-1/S1P inhibitors, PCSK9 variants or hinge-mutant serpins. Measuring viral titers ~ day 7 post transfection will allow us to determine with greater certainty whether any of our antiviral strategies affect the assembly stage of the HCV lifecycle. All of our studies examining extracellular HCV titers have
been derived from HCV infected cells, where an impact on entry cannot be fully separated from downstream antiviral effects.

This experimental setup can also allow us to examine if PCSK9 regulation of LDLR has downstream implications for HCV assembly. As explained above, LDLR controls the stability of ApoB and thus the secretion of VLDL from the hepatocyte secretory pathway. Combining PCSK9 overexpression and LDLR downregulation with HCV RNA transfection and examination of viral titers may permit us to understand how important VLDL assembly is to HCV infection. It is difficult to predict the outcome; we know VLDL assembly is required and that reducing LDLR expression in the secretory pathway should increase lipoprotein secretion but we do not know if this will assist or hinder HCV assembly. We hypothesize that PCSK9 overexpression should disrupt HCV’s capacity to hijack this pathway. This would be true if impeding lipoprotein secretion is indeed required for HCV seizure of self-appropriating VLDL assembly.

5.2.4 Antiviral strategies, HCV and mouse models

HCV infection has a very strict host and tissue specificity; only humans, chimpanzees and tree shrews can naturally be infected. Previously, the only method for studying HCV infection in mice required inactivation of the mouse immune system and engrafting the mouse with human liver cells (349). More recently, mice infected with adenovirus vectors expressing human CD81 and human occludin (receptors for HCV entry, Section 1.3.2) were shown to be permissive for HCV infection in the liver (343), although HCV is cleared in under 72 hours by the mouse interferon response. This mouse model was shown to be useful for studying in vivo, cellular factors that are important for HCV entry. Thus, this model may be used to determine the therapeutic potential of PCSK9 for inhibiting HCV entry.

Another useful mouse model that exists is one where the HCV core protein is transgenically expressed in the liver. These mice were shown to develop steatosis and eventually hepatocellular carcinoma (24,350). Using this system we may be able to test the application of Spn4A.RRLL(s) and PF-429242 in relieving HCV core induced steatosis. Alternatively, mice expressing the mutant Mtbps1 (SKI-1/S1P) allele could be infected with adenoviruses expressing CD81 and occludin and then subsequently challenged with HCV infection to determine the in vivo importance of SKI-1/S1P to HCV infection.

In summary, many tools that have not yet been employed will provide valuable information for understanding the contributions of SKI-1/S1P, PCSK9 and Spn4A variants to the HCV lifecycle and elucidate their therapeutic potential.
5.3 Conclusions

There is certainly no shortage of novel HCV antivirals undergoing clinical trials. With the FDA approval of two, direct-acting antiviral agents it will soon become apparent whether these agents maintain a lasting position in the HCV therapeutic repertoire. There is much concern about the development of resistance due to the extreme heterogeneity of circulating HCV genomes. It appears that these direct-acting antiviral agents cannot stand on their own in the fight against HCV and its propensity for resistance and so must be administered in combination with interferon and ribavirin. Many patients, especially those infected with HCV Genotype 1 do not respond to IFN-therapy potentially due to virus encoded factors that oppose immunity or due to the induction of opposing immune factors i.e. IL-8 (41,337,351). In addition, many patients cannot tolerate the side effects induced by both IFN and ribavirin. These points stress the need for novel indirect-acting antiviral agents for implementation of effective anti-HCV multidrug regimens.

The instinct to oppose targeting of host proteins is strong in many researchers and there is no doubt that a unique set of challenges must be faced in order to determine what factors can be targeted in a safe and effective manner. The idea of targeting lipid metabolism in particular raises concerns as there is a clear necessity of lipids for cellular growth, maintenance of membranes and for fuel. However, there is readily available and widespread evidence that lipid metabolism can be targeted to a degree that benefits human health (352). Statins are widely used cholesterol lowering agents that inhibit the host enzyme HMG-CoA reductase, an enzyme that is critical for de novo synthesis of cholesterol (52). In this scenario, cholesterol homeostasis is a priori disrupted due to genetic or diet related factors and administering statins serves to balance cholesterol homeostasis.

An additional point to keep in mind that has been repeatedly stressed throughout this thesis is that HCV greatly alters the host lipid balance to the point of inflicting metabolic disorders including steatosis, hypobetalipoproteinemia as well as insulin resistance (18,19,353). Thus targeting lipid metabolism in this scenario is not equivalent to targeting lipid metabolism in a healthy individual. This situation may be compared to the use of interferon for treating HCV infection. The host immune response is critical for human survival and is activated to control infection; however the immune response must be tightly regulated to prevent intrinsic host damage. HCV and many if not all successful infectious agents have evolved mechanisms to oppose the widespread actions of interferon (37). To treat HCV infection requires external
interferon administration which is associated with side effects intolerable by many but thus far is the only isolated mechanism of successful HCV infection inhibition in humans.

The importance of cholesterol homeostasis to human health makes it a leading area of drug development and research and so any discoveries or advances made in this field may be applied to targeting lipid metabolism disregulation during HCV infection. One great example of this that we have explored is the targeting of SKI-1/S1P which is of great interest for the management of hypercholesterolemic disorders (171). A limited number of studies in mice suggest that inhibiting SKI-1/S1P is a viable option. Liver specific knockouts and small molecule treated mice have reduced plasma cholesterol levels (170,171). Also, SKI-1/S1P mutant mice are viable and resistant to chronic LCMV infection (196). Although PCSK9 overexpression would promote higher blood cholesterol levels in healthy individuals, in HCV patients there is a lower than normal presence of lipoproteins in the blood and so PCSK9 overexpression may balance this deficit. In liver transplant recipients the effects of overexpressing PCSK9 may actually prove beneficial in both recovery of the liver and in preventing reinfection of allografts (308). The use of serpins for treating viral infections is widely explored especially using the human serpin AAT to treat HIV-1 infection (191,228,249,329). Our studies on Spn4A suggest that this serpin, and potentially other serpins, possess a potent antiviral component that may be exploited to treat HCV infection. Gene therapy trials in humans using adenovirus delivery of AAT will reveal the application of targeting exogenous serpin expression to the liver. Studies using VIRIP-based peptides suggest that peptide inhibitors may also prove therapeutically beneficial. Moving our studies to animal models of infection will be critical in further evaluating SKI-1/S1P, PCSK9 and Spn4A in the future of anti-HCV therapeutic medicines. At the very least future studies on these three targets promises to reveal important and novel features regarding the fascinating lifecycle of hepatitis C virus.
Figure 5.1. Host lipid metabolism is a critical component of the HCV life cycle.

1. The SREBP pathway is a major host pathway regulating the expression of host lipid metabolism genes such as LDLR. 2. LDLR plays a role in mediating attachment of HCV lipoviroparticles (LVP) to the surface of hepatocytes. 3. This receptor is controlled at the post-translational level by PCSK9, a proprotein convertase (PC) whose expression is also controlled by SREBP activation. Increasing PCSK9 level or activity reduces cell surface LDLR levels, decreasing hepatocyte susceptibility to HCV infection. 4. Blocking SREBP activation using small molecule or protein-based inhibitors directed at the PC SKI-1/S1P reduces cellular lipid abundance detected as a decrease in host lipid droplets (LDs). 5. LDs are a host organelle critical for HCV assembly and for host VLDL synthesis. Reducing intracellular lipid droplet abundance reduces production of infectious HCV particles by HCV infected cells through mislocalization of the HCV core protein and compromised virus secretion.
Bibliography


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Appendix 1: Chapter 2 supplementary figures

Figure A1.1. Percentage of Huh-7.5.1 cells expressing Spn4A.RRLL(r).

Huh-7.5.1 cells were infected with moi 1, 12.5, 25 and 50 of the intracellularly retained serpin Ad-Spn4A.RRLL(r). Treated cells were fixed 48 hours post-infection and probed for serpin expression using mouse anti-FLAG antibody. Cell nuclei were stained with Hoechst dye to determine the total cell number. The percentage of Spn4A.RRLL(r)-expressing cells was quantified using Cellomics HCS.
Figure A1.2. Effect of Spn4A variant treatment and HCV infection on cell growth.

Huh-7.5.1 cells were infected with various moi (1-50) of Ad-Empty, Ad-Spn4A.RRLL(r) or Ad-Spn4A.RRLL(s) for 48 hours in complete media. Treated cells were infected with HCV (moi 0.1) and fixed 72 hours post-infection. Fixed cells were probed with Hoechst dye to stain for cell nuclei, which were then quantified using Cellomics HCS to determine the relative number of cells in each well under the varying conditions. All values are expressed as relative cell number in serpin-treated cells compared to cells infected with Ad-Empty, which is set to 1. Results (mean ± SEM) from 3 independent experiments are shown.
Huh-7.5.1 cells were infected with recombinant adenovirus expressing the his- and FLAG-tagged Spn4A variants indicated or the Ad-Empty control for 72 hours. Media alone (upper panels) or cell extracts (lower panels) lysed in RIPA buffer were combined with recombinant his-tagged SKI-1/S1P or his-tagged furin (273) for 30 minutes at 30°C. Samples were prepared for western blot analysis and probed with rabbit anti-FLAG antibody to detect SDS- and heat-stable enzyme-inhibitor complex formation and also to distinguish Spn4A bands from recombinant enzyme bands on the Western Blots.
Figure A1.4. Time course analysis of LDLR expression in Spn4A.RRLL(s) treated cells. Huh-7.5.1 cells were grown in complete media for 24 hours and were then infected with Ad-Empty or Ad-Spn4A.RRLL(s). Cell extracts were harvested for western blot analysis 24, 48, and 72 hours post-infection, and lysates were then subjected to western blot analysis. Anti-LDLR antibody was used to detect protein expression levels in Ad-Empty and Ad-Spn4A.RRLL(s) treated cells and β-tubulin was probed for normalizing LDLR expression. Values are plotted relative to LDLR expression in Ad-Empty-infected cells, which was set to 1.
Figure A1.5. Effect of PF-429242 on cell viability.

Huh-7.5.1 cells were treated with DMSO (control) or various concentrations of PF-429242 for 24 hours before the inhibitor was removed and fresh complete media was added to the cells for an additional 48 hours. The relative cytotoxicity of the compound was then determined using an MTS-based cell viability assay. The absorbance measured at 490 nm is proportional to the amount of living cultured cells. Results (mean ± SEM) from 3 independent experiments are shown. Statistical significance was calculated for PF-429242 treated cells compared to DMSO treated cells.
Appendix 2: Chapter 3 supplementary figures

Figure A2.1. PCSK9-S402A is secreted from Huh-7, Huh-7.5.1 and HepG2 cells.
Huh-7, Huh-7.5.1 and HepG2 cells were infected with Ad-PCSK9-S402A for 48 hours following 24 hours of incubation in LPDS containing media. Treated cell media was harvested for western blot analysis to detect secretion of PCSK9-S402A (using polyclonal anti-PCSK9 antibody) in adenovirus infected cells.
Figure A2.2. Huh-7.5.1 cells express annexin A2, a PCSK9 antagonist.

Huh-7.5.1 and HepG2 cells were seeded into 6-well plates and maintained for 72 hours before total cell extracts were subjected to western blot analysis. Annexin A2 was detected with a mouse anti-annexin A2 antibody and, to ensure equal loading of samples, extracts were also probed with rabbit anti-β-tubulin antibody.