

The role of scavenger receptor B-I in hepatitis C  
virus attachment and entry.

by

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### **Abstract.**

With 170 million infected individuals worldwide, Hepatitis C Virus (HCV) poses a global health problem. The primary site of virus replication is the liver, leading to progressive disease often culminating in life threatening conditions such as hepatocellular carcinoma. HCV has a propensity to persist, with 70-80% of infected individuals failing to clear the virus.

Intriguingly, HCV associates with host lipoproteins to form lipo-viro-particles, these structures are predicted to exhibit the characteristics of both lipoproteins and virus particles. Recent evidence suggests that HCV entry is dependent on at least three cellular entry factors: a tetraspanin, CD81; a lipoprotein receptor, Scavenger Receptor B-I (SR-BI) and the tight junction protein Claudin-1. How these molecules coordinate HCV entry and the role(s) of particle associated lipoproteins in this process is unknown. This study investigates the role of SR-BI in HCV particle attachment and entry.

SR-BI is the major physiological receptor for high density lipoprotein (HDL), it is predominantly expressed in the liver and steroidogenic tissue, where it mediates the selective uptake of cholesterol to the plasma membrane from HDL. HCV particles are believed to interact with SR-BI via the viral envelope protein E2, interestingly the SR-BI ligands HDL and oxidised low density lipoprotein, enhance and inhibit HCV infection, respectively.

In this study we have investigated the interaction of HCV soluble glycoprotein with CHO cells exogenously expressing human SR-BI and, the splice variant, SR-BII. We have shown that over expression of SR-BI/II in human hepatoma Huh-7.5 cells enhances HCV infection, indicating that SR-BI/II surface expression levels limit infection. Furthermore, anti-SR-BI serum inhibits HCV, suggesting that it plays an important role in virus attachment and entry.

We demonstrate that a cell culture adapted HCV mutant, with a single amino acid change in E2, has a reduced dependency on SR-BI. This altered receptor dependency is accompanied by an increased sensitivity to neutralisation by soluble CD81 and enhanced binding of recombinant E2 to cell surface expressed CD81. The cell culture adapted variant also exhibits an altered relationship with lipoproteins and a heightened sensitivity to neutralising antibodies. Our data suggest that a balanced interplay between HCV particles, lipoprotein components and viral receptors allows the evasion of host immune responses.

**Dedication.**

I would like to dedicate this thesis to my parents, John and Deborah Grove, and to two excellent science teachers, Nigel Keates and Luke Rake.



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I have been very fortunate to work within the field of HCV research in this period of new and exciting discoveries. I would like to acknowledge the contributions made by individuals from across the world over the last 30 years, in particular; Harvey Alter, Michael Houghton, Charles Rice, Stanley Lemon, Robert Purcell, Ralf Bartenschlager, Jane M<sup>c</sup>Keating and Takaji Wakita.

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## 1 Introduction

### 1.1 *The disease.*

During the 1970s specific diagnostic tests for hepatitis A, B and D virus(es) established that a significant fraction of blood transfusion acquired hepatitis was caused by other uncharacterised pathogens. Evidence suggested that a single agent, termed non-A non-B hepatitis virus (NANBHV), accounted for up to 90% of undiagnosed cases. NANBHV remained largely uncharacterised until 1989, when it was demonstrated that cDNA, synthesised from the plasma of an infected chimpanzee, could encode for proteins that were recognised by antibodies from NANBH patients. The cDNA clones were found to originate from an RNA genome of ~10kb containing a single open reading frame, this new etiological agent was named hepatitis C virus (HCV) (66, 354).

Expression of HCV encoded peptides in yeast allowed the development of immunoassays capable of screening suspected subjects and it soon became apparent that large numbers of individuals were infected. The major route of infection is via contact with contaminated blood or blood products. Consequentially HCV positive antibodies were found in up to 80% of patients receiving treatment for haemophilia, ~65% of those with transfusion acquired hepatitis and ~60% of intravenous drug users (201, 354). Fortunately, the advent of a reliable assay for the detection of HCV allowed the screening of blood products, therefore reducing transmission.

The occurrence of HCV varies throughout the world, in high prevalence regions such as Northern Africa the majority of new infections occur due to unsafe therapeutic treatments with contaminated needles. In countries with a low incidence of HCV, such as Northern Europe, transmission is predominantly between intravenous drug users. Other routes of infection include sexual and perinatal transmission, however these are relatively inefficient and therefore make only a minor contribution to the prevalence of HCV (7, 201).

Characterising the clinical progression of HCV infection has been difficult as ~70% of infected individuals remain asymptomatic for many years. However it has become clear that only 20-30% of subjects clear the virus, with the majority of individuals developing a persistent infection (5, 49, 251, 329). In these chronically infected patients clinical outcome can vary, ranging from mild liver disease through to liver cirrhosis (20-30%) and hepatocellular carcinoma (1-7%) (5, 61).

There is a poor understanding of what defines the progression and severity of HCV associated liver disease. Pathogenesis can be monitored by the detection of markers of hepatic damage such as alanine transaminase (ALT) and the inspection of liver tissue (92). However, viral load does not necessarily correlate with pathogenesis and in many cases chronic infection with HCV is subclinical. There is also limited evidence for cytopathology using in vitro models of HCV replication (49, 61, 113, 200). These and other findings have led to the broad conclusion that HCV associated disease is

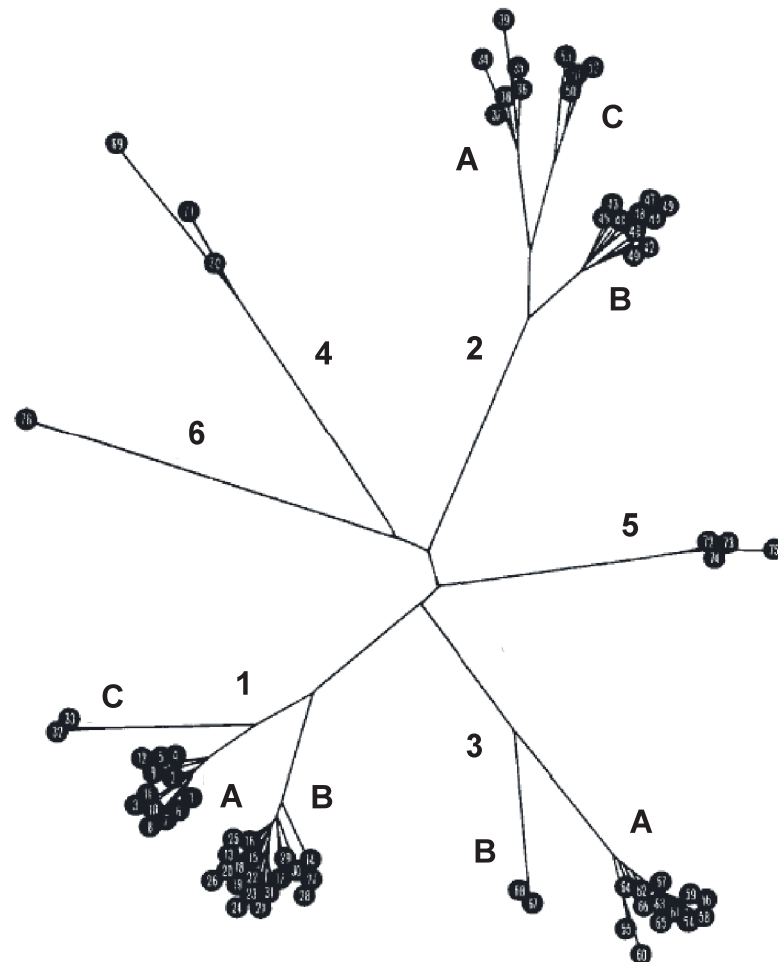


predominantly immuno-pathogenic in nature (61). However the field has yet to reach consensus, with recent findings using cell culture proficient HCV suggesting that the virus may induce cell death (198, 353). Host factors that promote severe disease progression include age at infection, alcohol consumption and co-infection with human immunodeficiency virus (HIV) or hepatitis B virus (HBV) (5).

RNA viruses display broad genetic variability when compared to DNA viruses, this is most likely due to the lack of a proof reading ability in RNA polymerases (106); HCV is no different. There are 6 major HCV genotypes displaying around 30% total divergence (289, 290). A phylogenetic tree displaying the six major genotypes of HCV can be seen in Figure 1-1. Although the genotypes differ in their geographical distribution and prevalence, their basic virology is the same (289) . However there are clinically significant differences in their pathogenesis and response to treatment. A common feature of chronic HCV infection is steatosis, an abnormal accumulation of lipids within the liver, this is particularly prevalent in genotype 3 infected individuals and in these cases has been linked to liver fibrosis (266, 267).

For those patients diagnosed before severe liver injury has occurred a relatively effective treatment regime is available. Administration of Interferon-alpha (IFN $\alpha$ ), a drug capable of inducing an anti-viral state within cells, in combination with the nucleoside anti-metabolite Ribavirin (276, 277), results in a sustained virological response in 60-80% of patients with genotype 2 or 3

infections (12). However in individuals infected with the highly prevalent genotype 1, approximately 40% will respond to treatment (12, 96).



**Figure 1-1 Phylogentic analysis of HCV**

“Phylogenetic analysis of NS-5 sequences from 76 isolates of HCV, showing six major HCV types and subsidiary groupings within some HCV types.” (290)

Aside from considerations of the infecting viral genotype, patient characteristics are also important to consider when administering treatment. A significant proportion of patients are in 'hard to treat' groups, whereby a patient's lifestyle or health makes therapeutic intervention difficult. Such individuals include intravenous drug users, who may continually expose themselves to HCV and patients with alcohol associated liver damage (278). As a result, even in societies with active treatment programs, infection and disease remain difficult to control, especially as there is no vaccine currently available against HCV (12, 278).

For patients who present with cirrhosis, treatment options are more limited as they are typically less responsive to anti-viral therapy, although treatment does slow disease progression (275). The prognosis for individuals presenting with decompensated cirrhosis or hepatocellular carcinoma (HCC) is poor; with 5 year survival rates of 50% in untreated patients (275). Liver transplantation is generally the only suitable course of action in these cases, however the allograft is rapidly re-infected and the clinical features of hepatitis reoccur within 100 days (29), as such 5 year survival rates only increase to ~70% patients post transplant (275).

About one fifth of patients will experience a self-limiting acute infection, culminating in the clearance of the virus. Elucidating the determinants of spontaneous resolution has been difficult, as the vast majority of individuals who clear infection are asymptomatic (87). However, studies using both

animal models and patients identified at the time of exposure to HCV, have shed some light on successful immune responses.

A multi-specific and sustained T-cell response associates with viral clearance; data suggests a role for both CD4 + and CD8 + T cells (70, 112, 118, 167, 174, 283, 311). Subjects who have cleared HCV are not necessarily resistant to re-infection, with approximately 50% of individuals acquiring protective immunity (118, 140, 165, 191, 207). Again, resistance to re-infection is correlated with a prolonged and robust T-cell response to HCV infection (118, 191, 283).

Although the humoral response to HCV is relatively delayed (64), antibodies that neutralise HCV infection in vitro can be detected in both acutely and chronically infected individuals (182, 210, 327). However, there is limited data supporting a role for humoral immunity in viral clearance (87, 140, 182, 245, 295) and as such there is a lack of consensus over the role of B cells and neutralising antibodies in controlling infection. HCV infected patients suffering from hypogammablobulinemia are reported to display a rapid and severe disease progression with poor response(s) to anti-viral treatment (33), suggesting that neutralising antibodies (nAb) may help control HCV. However, chronically infected patients exhibit high titre anti-HCV antibodies (87, 140, 182, 210), suggesting that in the majority of HCV infections nAbs fail to curtail viral replication.

Current attempts to design a HCV vaccine are focusing on eliciting appropriate T-cell activity (43, 61, 154, 298). However two recent publications, using mice with humanised livers, demonstrate passive immunity to HCV following injection with polyclonal patient immunoglobulin G (IgG) or monoclonal anti-E2 antibodies (172, 322), revitalising hopes for a B-cell dependent vaccine.

Unfortunately, for the majority of individuals the virus persists in spite of an immune response. In many cases the viral load declines as if following an acute progression, only to recover over time, establishing a chronic infection capable of exhausting T cell responses (154). As discussed earlier, HCV induced liver disease is believed to be substantially immunopathogenic in nature. Therefore, although a strong T cell response is favourable during early infection, once chronicity is established, it may drive rapid progression to liver fibrosis and cirrhosis (61, 154).

In 2000 the World Health Organisation estimated there to be 170 million infected individuals worldwide; ~3% of the global population. The resulting cases of liver disease will number in the tens of millions; HCV is already the leading indicator for liver transplantation in the United States (source: Centre for Disease Control and Prevention website). Some communities have extremely high seroprevalence, for instance ~40% of people in the Nile Delta region of Egypt became infected after a poorly conducted schistosomiasis vaccination campaign (254). In such areas, the impact of HCV will be apparent throughout the whole of society.

Recent advances in the field have facilitated detailed in vitro studies of HCV, yet without a vaccine or completely effective treatment for the disease, the medical, social and economic impetus to understand the virus remains.

## **1.2 *An elusive pathogen.***

The emergence of a new human pathogen presents the greatest of challenges to modern virology and the discovery of human immunodeficiency virus (HIV) in the early 1980s set a precedent for the field. The discipline of virology was not quite 100 years old when HIV was first isolated (15). However, proficient replication of HIV in vitro allowed the scientific community to characterise the virus exceptionally quickly. From the identification of HIV, to approval of the first specific therapy (AZT) by the Food and Drug Administration took less than 5 years (213, 226) (and FDA website). Developments came so quickly that CD4 was identified as the major entry receptor before the politics of naming the new pathogen had been resolved (178, 199, 272).

As illustrated by HIV, rapid progress in the study of a virus requires an appropriate model system capable of supporting virus replication; from its inception virology has been dominated by species for which such platforms exist. Early research focused on viruses that infect hosts such as bacteria, plants and birds. During the mid 20<sup>th</sup> century, the advent of mammalian cell culture allowed fast and consistent in vitro studies of viral replication. However, not all viruses replicate in vitro, which presents a problem when studying pathogens with host ranges limited to humans and higher primates (78).

In vivo, it is thought that HCV replicates in hepatocytes within the liver. However, the hepatocyte is a highly specialised cell type and the liver a

complicated organ, therefore it is difficult to recreate the hepatic environment in vitro. As a result, HCV within the serum from infected subjects has been difficult to propagate in primary human hepatocytes or hepatoma derived cell lines (16). As a result, advances in HCV research have been hard fought; the suggestion of CD81 as HCV receptor came 10 years after the identification of the virus (248). Prior to addressing the virology of HCV, it is important to explain the circumstances in which the work was carried out. The following section will attempt to outline the technical challenges and breakthroughs encountered in the past two decades of HCV research.

There is little consensus over the frequency of HCV positive hepatocytes in an infected liver (3, 163, 166), however, the total virus production rates are in the order of  $1 \times 10^{12}$  RNA copies per day (173), resulting in high levels of viremia. Consequently, the virus was first isolated from the serum of an experimentally infected chimpanzee (66). This is characteristic of the first decade of HCV research; without any alternative system, many of the early advances came from studies carried out in this ethically and economically controversial animal model. Identification of the virus led to the development of reliable diagnostic tests allowing the identification of infected patients. Observations of virus taken from human plasma shed light on the classification, evolution and immunology of HCV, but provided minimal information on basic virology (354).

Early attempts to propagate HCV in primary or immortalised hepatocytes proved difficult. HCV replication was severely attenuated and detection could only be achieved using reverse transcriptase polymerase chain reaction (RT-



PCR). This technique is highly sensitive making it difficult to discriminate between passive cellular uptake of HCV particles and 'true' virus entry followed by genome replication. Studies were able to eliminate false positives by carrying out RT-PCR for both plus strand HCV genomes and negative strand replicative intermediates (59, 133, 304). Although these reports demonstrated in vitro HCV replication the techniques did not allow detailed characterisation of the virus (18).

Investigating the expression and function of viral proteins in isolation was a more straightforward task. The existence of gene delivery systems such as recombinant plasmids, vaccinia virus and adeno virus, allowed the expression of HCV gene(s) in bacterial and mammalian cells (17, 248, 340). Observation(s) of the proteins' enzymatic and regulatory functions contributed to a basic understanding of the viral component parts. Indeed, such techniques remain a vital tool in the ongoing investigation of HCV. However, the absence of in vitro model supporting replication prevented mechanistic studies to address how these viral proteins acted in concert. As a result elucidating processes such as genome replication or particle assembly remained out of reach.

Another limitation of early HCV research was the lack of an infectious clone. Studies using chimpanzees or primary human hepatocytes relied on HCV positive patient sera as a source of infectivity, however as patient derived particles are genetically diverse there was little understanding of what constituted an infectious HCV particle. In 1997, Kolykhalov et. al. derived a

consensus viral sequence from the serum of a patient carrying highly infectious virus. This was used to create a HCV cDNA template for the transcription of full length RNA genomes, which, when introduced into Chimpanzees, initiated authentic HCV replication (158). This infectious clone, termed H77 (Hutchinson, 1977), provided a bone fide consensus HCV sequence allowing the field to work towards understanding the determinants of infectivity (158, 341).

1999 brought a major technological breakthrough in the form of HCV sub-genomic replicons (SGR). Comprised of a truncated HCV genome and a selectable marker gene, this recombinant construct was capable of autonomous replication when introduced into cultured hepatoma cell lines (35, 183). The SGR system allowed the identification of adaptive mutations that promote HCV replication in vitro (35, 37). When introduced in to full length HCV, these adaptations permitted the replication of the complete viral genome (36, 52). However, these mutations abolished HCV infectivity for chimpanzees (52); representing the challenges faced when studying a virus in vitro. The replicon system made it possible to inspect the viral and host processes necessary for genome replication and maintenance. It also heralded a new era in the search for specific inhibitors of HCV infection, as it was suitable for high throughout screening of candidate compounds (76). However, even replicons carrying the entire HCV coding region were unable to assemble and release infectious particles (35, 37, 183). The process of viral entry and the events surrounding particle assembly could not be studied at this time.

Within six years of the description of HCV SGR two major advances shed light on these elusive stages of the HCV life cycle. Pseudo-particles are recombinant virions encoding a reporter gene such as green fluorescent protein (GFP). They are constructed around a replication deficient retroviral capsid and can be engineered to express the envelope proteins of various viruses. In 2003 HCV pseudo-particles (HCVpp) were reported to be infectious and allowed the functional characterisation of HCV entry, facilitating studies on viral receptors, neutralising antibodies and mechanisms of viral fusion with host membranes (24, 82, 130). As work continued with this new technique a unique HCV clone, termed Japanese Fulminant Hepatitis-1 (JFH-1), was isolated. The complete genome of JFH-1 was seen to replicate in vitro when introduced in to cultured hepatoma cells (142). Unlike all previous HCV genomes tested, infected cells released viable particles that could be used to inoculate naïve hepatoma cells, thus completing a full viral life cycle (179, 328, 351).

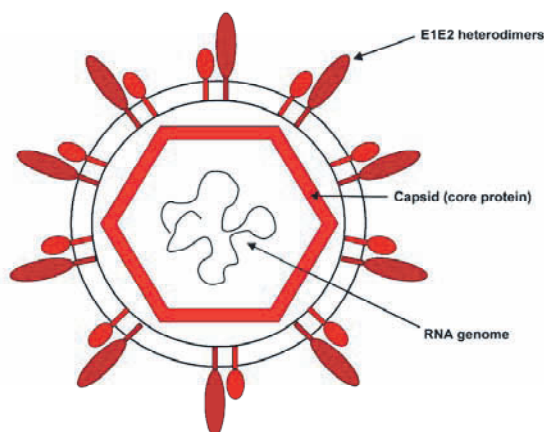
HCV capable of in vitro replication are commonly termed HCVcc, as they are cell culture proficient; their use has brought about a new and exciting stage in the field. Using the JFH-1 non-structural genes as a backbone, numerous studies have successfully made infectious chimeric genomes that produce virus particles consisting of the structural proteins of other HCV strains (143, 179, 344). Importantly, HCVcc is infectious in vivo, both in the chimpanzee and in immuno-compromised mice carrying humanised livers (141, 180).

Current work with HCVcc is focused on defining the determinants of infectivity and in particular viral entry, assembly and release. However, efforts are also being made to reconcile 15 years of observations on viral subunits with this complete model system and to understand how well it represents the situation in vivo. The observation that HCVcc replicates in vivo provides gravity to findings using this system, however the artificial nature of synthesising virus in cultured hepatoma cells will, at best, be a poor mimic of virus produced from an infected liver. Consequently, attempts to recreate the hepatic environment within a culture flask are ongoing (51).

In conclusion, the resistance of HCV to replicate in vitro has hindered its characterisation. However the technological and intellectual tools developed to overcome this problem have made an important contribution to the field of virology as a whole.

### 1.3 Basic virology.

Like other members of the flaviviridae HCV encodes three structural proteins consistent with a 50-60nm particle, comprised of a capsid, an internal complex of core protein and an RNA genome, surrounded by a phospholipid bilayer envelope (181, 281). Functional heterodimers of the E1-E2 glycoproteins sit in the envelope and mediate virion-target cell interactions (Figure 1-2). Interestingly, it is becoming clear that HCV does not fit this classical model of flavivirus structure and that it complexes with host components, this is discussed in detail later. The HCV RNA genome is a single stranded positive sense molecule of 9.6kb in length, comprised of a long coding sequence flanked by 5' and 3' un-translated regions (UTRs) (181). Particle structure and genome organisation suggest that HCV is related to the Flavi and Pestiviruses, as such HCV is classed as the sole member of the Hepacivirus genus within the Flaviviridae (234, 332).

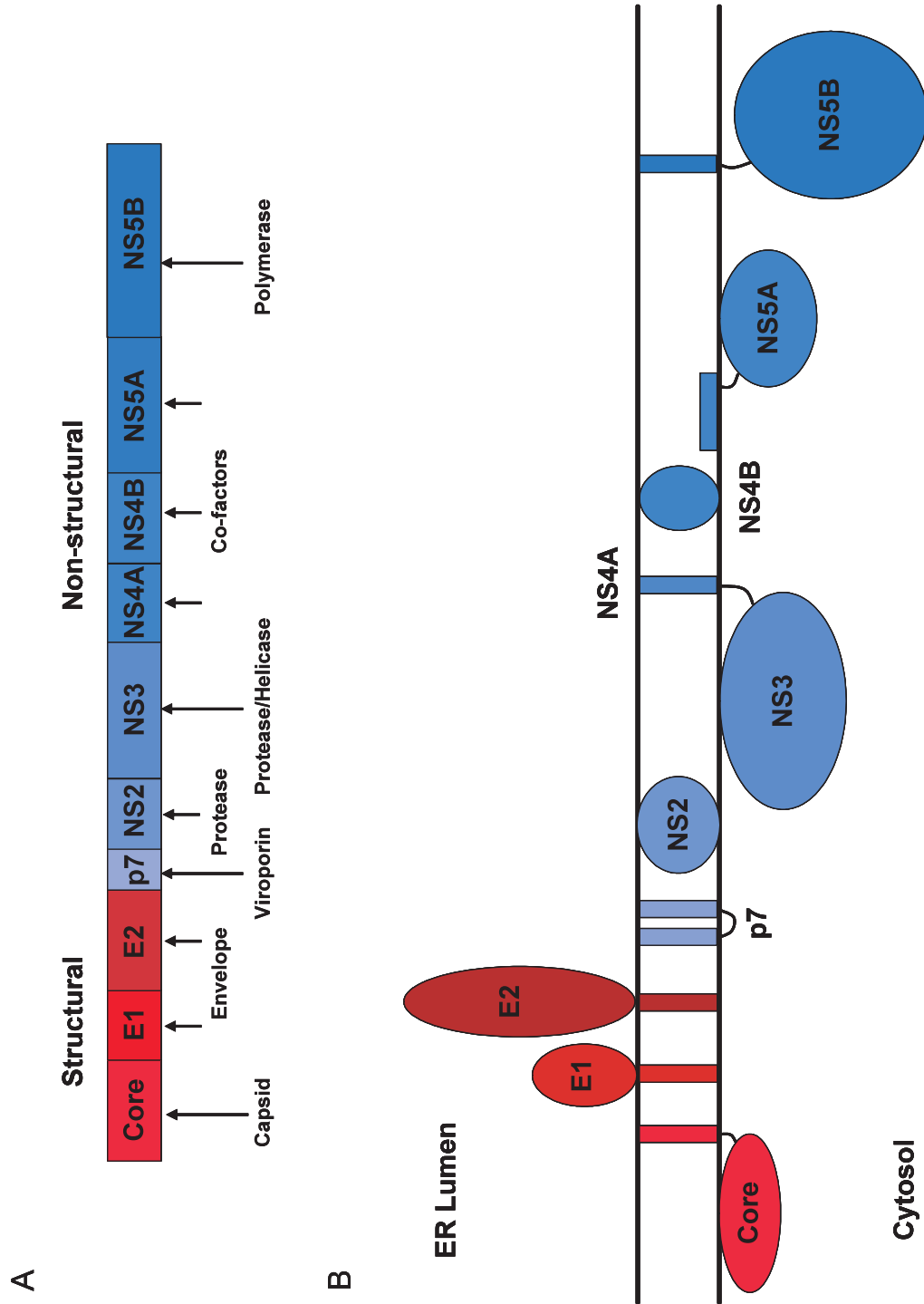


**Figure 1-2 A cartoon of a Hepatitis C virion.**

Not drawn to scale.

HCV targets and enters hepatocytes via at least 3 receptors; CD81, scavenger receptor B-I (SR-BI) and claudin-1 (CLDN1) (91, 120, 179), this process will be covered in section 1.4. After entry to the cytoplasm the HCV genome acts as an mRNA for the production of a single 3000 amino acid polyprotein. Unlike some Flaviviruses such as dengue, HCV lacks a 5' cap and as such initiates translation via an internal ribosomal entry site (IRES). The IRES is found in the 5' UTR and contains a series of RNA stem loop structures which initiate translation by bringing the 40s ribosomal subunit into contact with the viral start codon (18, 251, 318)

The polyprotein comprises 10 proteins, the structural proteins, Core, E1 and E2 are found at the amino terminus and the non-structural proteins p7-NS5B comprise the remainder. Upon its translation a series of trans-membrane domains anchor the polyprotein to the endoplasmic reticulum (Figure 1-2) (181, 241). There is an alternate reading frame within the HCV genome whereby a ribosomal frameshift within the core region yields a series of protein products of unknown function (46, 255).



**Figure 1-2 HCV genome and polyprotein.**

**A.** HCV genome organisation and protein functions **B.** HCV polyprotein cleavage products depicted in a membrane within the endoplasmic reticulum.

***HCV structural proteins: Core.***

HCV core is highly conserved across all HCV genotypes and is found at the N terminus of the HCV polyprotein (203), its maturation is dependent on signal peptide peptidase cleavage at 2 sites at the C-terminal core-E1 interface (306). By analogy to related viruses core is believed to form the nucleocapsid of HCV, indeed, core is seen to homodimerise and possesses sites capable of binding HCV RNA (203). However there is little information about the assembly and structure of HCV capsids, although in vitro expression studies suggest that core is capable of self-assembly into icosahedral particles of 30-40nm (153). In contrast, the ability of core to interact with numerous cellular factors, such as tumour necrosis factor receptor-1(203), DEAD box helicase DDX3 (192) and dicer (65), is much better understood. These observations suggest that core has numerous roles within the replication cycle aside from as a virion component. One of the most striking characteristics of core is its ability to localise to the lipid droplets (41, 42, 215), intracellular stores of neutral lipids and cholesterol, and it has been suggested that this may contribute to the occurrence of steatosis in HCV infected individuals (203). This characteristic is important for HCV particle assembly and is discussed later.

***The envelope proteins; E1 and E2.***

Viral envelope proteins mediate particle interaction with cellular receptors and in doing so may define the tissue tropism and host range of a virus. Following genome translation, HCV E1 and E2 are cleaved by host endoplasmic reticulum signal peptidases, thus liberating them from the polyprotein (168,



241). E1 and E2 are anchored to the ER membrane by C-terminal transmembrane domains, with the ectodomain of either protein extending in to the ER lumen (168). Mature HCV envelope proteins exist as non-covalently linked heterodimers, and it is believed that the correct conformational folding of either protein is dependent on the expression of the other (75, 212). Additionally, a host ER chaperone, Calnexin, has been shown to interact with E1 and E2, suggesting that HCV exploits cellular factors during protein maturation (48). Apart from anchoring the envelope proteins, the transmembrane domains of E1 and E2 are critical for ER retention and heterodimer formation (168).

Unlike the envelope proteins of related flaviviruses, E1 and E2 are highly glycosylated, with each protein possessing 6 and 11 potential sites respectively (168). It is believed that these glycans play a role in protein folding, E1E2 interactions with receptors and sensitivity to neutralising antibodies (116, 126, 168, 187). Furthermore, the infectivity of a great number of viruses is dependent on further proteolytic cleavage of their envelope proteins in to active conformations (102). Current evidence suggests that HCV E1 and E2 do not undergo such processing (168). The crystal structure of the HCV envelope proteins has yet to be solved, thus hindering further dissection of their interactions with one another, viral receptors and neutralising antibodies.

p7 is also processed by signal peptidases, it is a highly hydrophobic molecule that belongs to a broad family of virally encoded ion channels termed

viroporins (117). It forms heptameric ion channels in the ER membrane and is thought to be important for infectious virion release (119, 296, 297).

***HCV non-structural proteins and RNA replication.***

The primary function of the non-structural proteins is genome replication, they undergo autolytic processing by viral proteases. Cleavage of the polyprotein between NS2 and 3 is achieved by the NS2/3 auto-protease. This frees NS3, which in complex with NS4A is the major viral protease, allowing the full processing of the remaining non-structural proteins (17, 241). The precise role of each non-structural protein is not yet understood, however some are better understood than others. For instance, NS5B exhibits all the characteristics of a classical RNA dependent polymerase, whereas the role of NS5A is less well defined, though it has been implicated in RNA replication, particle assembly and virus sensitivity to interferon treatment (301).

Genome synthesis is performed by the NS5B RNA polymerase (28, 340), it uses the 3' hydroxyl group of the positive sense RNA genome to prime the production of a negative sense replicative intermediate. This in turn acts as a template for the production of nascent HCV genomes (18, 181). However, in vitro studies have demonstrated that, when expressed alone, NS5B does not display the required level of template discrimination and fidelity (6, 301). In fact, HCV genome replication is carried out by NS5B in concert with viral and host components; in so called replication complexes (RC).

The characterisation of HCV genome replication has primarily been carried out in cells expressing sub-genomic replicons, in this system it has been estimated that each cell harbours 50-100 RC producing a total of ~1000 HCV genome copies per day (252). HCV RC are thought to contain NS3-5B and are localised to proteinase and nuclease resistant membranous vesicles associated with the ER and golgi network (220, 252, 301). These viral components are retained in this environment either by transmembrane domains (NS4A,4B,5A and 5B) or through tethering to one another (NS3 to NS4A) (220, 301).

Studies to elucidate the role that each non-structural protein plays in the RC are on going. NS3, along with a proteolytic domain, contains a helicase capable of separating double stranded RNA structures, although it is unknown how this contributes to replication (18, 181, 241). NS4B is thought to facilitate the formation of a membranous web associated with the ER, believed to be a major site of genome replication and particle assembly (90, 301, 334). The phosphoprotein NS5A exhibits an RNA binding capacity and numerous cell culture adaptations within the protein are seen to enhance RNA replication (35, 301). These data suggest an important role for NS5A in the RC, indeed its phosphorylation status has been linked to replication efficiency and may be an important regulator in the viral life cycle (35, 301, 310). Host factors that are thought to contribute to the HCV RC include the DEAD-box helicase DDX3 (10), cyclophilin B (125) and miRNA-122 (138). The crystal structures of many of the NS proteins have been solved, allowing the design of specific inhibitors of viral replication (132).

HCV assembly and release is intrinsically linked to the production of very low density lipoproteins (VLDL) by hepatocytes (63, 109, 131). Lipoproteins are spherical particles that deliver dietary lipids to tissues throughout the body (321). The mechanisms by which this occurs are pertinent to the work documented in this thesis and will be addressed in greater detail in sections 1.5 and 1.7.

As discussed earlier, HCV is genetically diverse, however variability is not confined to the global level; an infected individual will harbour a population of viruses known as a quasispecies swarm (44, 197). A HCV patient will produce around  $10^{12}$  RNA copies per day (229), such a large population will contain genetic variants covering each base position of the entire genome (289). Although the swarm will display a sequence consensus at any given time, it is highly able to respond to new evolutionary pressures as they arise (44). This phenomena will become increasingly important as new anti-HCV treatments become available. As seen in HIV, quasispecies swarms are quick to develop drug resistance if treatment is not managed appropriately (69, 176). Rapid quasispecies evolution also contributes to the ability of HCV to continually escape host immune responses (85, 327).

Analysis of genetic variation within genotypic or quasispecies populations has identified highly conserved and diverse regions of the genome. There is pressure to conserve sequences that encode indispensable functions such as the IRES or the NS5B polymerase (289). Whereas other regions, particularly

in the E1 and E2 glycoprotein genes, display high variation (292, 332). Two particular stretches of E2, known as the hypervariable regions I and II (HVR-I and HVR-II), are thought to represent neutralisation epitopes under persistent immunological pressure (reviewed in (292)).

Our understanding of HCV has expanded greatly since the turn of the century, largely due to the technological breakthroughs of HCV replicons and HCVcc in vitro assays. However, many processes remain poorly understood, none more so than the events leading to HCV internalisation.

#### **1.4 Attachment and entry.**

To gain access to a host cell a virus must first attach to its surface and cross the cell membrane. The strategies viruses employ to achieve this are wide and varied, but in all cases the process can be divided into three generic stages. 1) Attachment; this can occur via low specificity interaction(s) with ubiquitous cell surface molecules and by high affinity engagement specific receptors. 2) Penetration; to replicate the virus must gain access to the cell interior. This process always requires transport of viral genetic material across a cellular membrane, this can occur at the cell surface or within a sub-cellular compartment. 3) Uncoating; in this the final stage of entry, the viral genome must become exposed to the appropriate cellular environment to allow replication. Delivery of the genome to a non permissive cellular compartment can result in non-productive entry (150, 193, 194).

In executing the above processes viruses have to overcome an evolutionary dilemma. A virus particle has to be hardy enough to survive the passage to a target cell, with all the environmental and immunological hazards that entails. However, as it enters a cell it must relinquish its genome as soon as the opportunity arises. Therefore to successfully reach and infect a naïve host, a virion must exist in an equilibrium between stability and fragility. An ultra-stable particle may have little trouble surviving the journey only to find its genome irretrievably trapped in the capsid. Conversely an ultra-labile particle would barely make it out of a host before succumbing to one pressure or another (194).

The solution reached by a great many species is to exist in a series of meta-stable conformations. The virus reaches a target cell primed to attach to its surface. This primary interaction will trigger conformational change(s), preparing the particle for the next stage of entry, whether it be co-receptor engagement or penetration to the cell interior (114, 194, 227, 253). Further stimuli will prompt step-wise alterations in the particle structure until the ultimate event of uncoating. In this way a virus can respond to appropriate host surroundings as it moves through an entry pathway. For a general review of virus entry strategies see Marsh 2006 (194).

The cellular receptors used by various viruses include signalling molecules (102). By interacting with these, some species exert another level of control over their entry, inducing signalling events that remodel the target cell to suit their purposes. A prime example of such an entry process is the group B Cocksackieviruses (CVB). CVBs share a receptor with Adenoviruses; the Cocksackievirus and Adenovirus receptor (CAR). CAR is a component of the tight junction and as such is inaccessible to CVB as it approaches the apical surface of epithelial cells. To overcome this, CVB interacts with the apically expressed decay-accelerating factor (DAF) and in doing so induces a signalling cascade. The resulting cyto-skeletal remodelling translocates DAF to the tight junction, delivering CVB to its co-receptor. Subsequent CVB-CAR interaction induces a conformational change allowing particle penetration to the cell interior (71). The details of HCV attachment and entry are poorly understood. However from what little we know, the specific events involved

may be as complicated and fascinating as the pathway employed by viruses such as CVB.

#### **1.4.1 Co-receptors**

The attachment and entry of HCV is dependent on at least three host cell molecules, or receptors, the following section will detail their chronological discovery and what we know of their relationship with HCV.

##### **CD81**

As a member of the tetraspanin family of integral membrane proteins, CD81 has 2 extracellular (EC) loops anchored by 4 transmembrane domains, it is widely expressed throughout the human body (177). Tetraspanins play a role in membrane organisation, forming cholesterol dependent tetraspanin webs. These act as a scaffold for the formation of functionally active membrane domains (40). CD81 facilitates signalling events involved in immune cell differentiation, adhesion and activation, it also plays a role in the acrosome reaction necessary for sperm-egg fusion (177, 268). CD81 has been implicated in the infection of hepatocytes by Plasmodium, the causative agent of malaria (288).

The search for putative HCV receptors began with the development of a soluble form of the major HCV envelope protein (sE2). Pileri et al noted that although sE2 bound to cells of human origin it would not bind to murine cells (248). They utilised sE2 to screen a human cDNA library expressed in mouse fibroblasts; the cDNA clone encoding CD81 successfully conferred sE2



binding to mouse cells. sE2 interacted with the large extracellular loop (LEL) of CD81 and could be perturbed by anti-HCV sera (248, 336). Systems supporting HCV entry were not available at this time and studies continued to characterise recombinant forms of E2 and CD81. Comparison of human CD81 to those of other mammals unable to bind sE2, supported earlier data that the LEL of CD81 is important (99). Subsequent studies using alanine scanning mutagenesis within these regions of CD81, identified positions L162, I182, N184 and F186 to be involved in the interaction with E2 (83, 84, 99, 127).

The advent of the HCVpp system to measure viral entry (24, 82, 130) allowed unequivocal demonstration of the importance of CD81 in HCV infection. Soluble recombinant forms of CD81 LEL, anti-CD81 mAbs and siRNA knockdown of CD81 efficiently inhibited HCVpp infection, confirming its importance for viral entry (130, 171, 348). Furthermore, expression of CD81 in the CD81 negative HepG2 hepatoma cell line rendered the cells permissive to HCVpp infection (171, 202, 348). These observations were soon corroborated using HCVcc system (179, 328). HCVpp bearing diverse glycoproteins showed varying abilities to infect HepG2 cells expressing CD81, suggesting genotype specific differences in E2-CD81 interaction or the involvement of other receptor components, however CD81 was an absolute requirement for infection in each case suggesting a critical role in HCV entry (171, 202).

An interesting study by Flint et. al. demonstrated that although CD81 from African green monkey and hamster showed little or no interaction with sE2, both were capable of supporting HCVpp and HCVcc infection when expressed

in HepG2 cells (101). These data indicate that using recombinant soluble proteins to measure E2-receptor interactions can be a poor model of infection. They also established that soluble CD81 LEL neutralisation of HCVpp occurs after virion binding to target cells (101), suggesting that CD81 is not the primary HCV attachment receptor, a view supported by other findings (31, 91, 346).

The use of anti-E2 mAbs and comparison of diverse envelope proteins allowed the identification of putative CD81 recognition domains within the major HCV glycoprotein. Mutagenic studies within these regions has both confirmed and eliminated candidate residues. The currently accepted CD81 binding regions are 436-443, 527-535 and 612-619, residue 420 is also involved (81, 99, 237, 265). However, without a crystal structure of HCV E2 it remains unclear how these distant regions engage CD81 and whether other receptors are required to promote CD81 interaction. Antibodies that recognise sites within CD81 binding regions are neutralising and work to develop a vaccine that targets these epitopes is ongoing (145, 147, 236, 237, 307).

### ***Scavenger receptor B-I (SR-BI)***

SR-BI is a multi-ligand lipoprotein receptor, it is expressed throughout the body but is predominantly found in the liver and steroidogenic tissue (161). It resides at the plasma membrane, with two transmembrane domains separated by a large extracellular region responsible for ligand binding (161). Its functions are varied and will be covered in more detail in a later section. However its major role within the liver is the uptake of cholesterol from high

density lipoprotein (HDL), allowing selective sorting and if necessary excretion into the bile (161, 286).

Identification of SR-BI as a HCV co-receptor also relied on recombinant viral glycoproteins. Scarselli et. al. noted that sE2 bound to CD81 negative HepG2 cells, indicating another means of attachment (274). sE2 pull down of biotinylated HepG2 cell surface molecules, isolated an 82kDa glycoprotein found to be SR-BI. To confirm this Chinese hamster ovary (CHO) cells were transfected to express human SR-BI and were found to bind HCV sE2 (274).

E2-SR-BI interactions are currently believed to occur via HVR1, as deletion of this region and anti-HVR1 mAbs ablate sE2 binding to CHO SR-BI cells (26, 274). HCVpp virus lacking the HVR ( $\Delta$ HVR) are poorly infectious (25), however it has been shown that  $\Delta$ HVR HCV RNA is still infectious when inoculated in Chimpanzees, although this was accompanied with the rapid occurrence of adaptive mutations within the envelope proteins (104). Maillard et. al. reported that patient serum derived HCV may interact with SR-BI via particle associated lipoproteins and this may provide an explanation for why  $\Delta$ HVR HCV is still infectious (190).

The major SR-BI ligand HDL enhances HCVpp and HCVcc infection (80, 325), although not all HCV genotypes respond to treatment (326). This phenotype is dependent on HVR1 interaction(s) with SR-BI and on the ability of SR-BI to transfer cholesterol from lipoproteins (25, 80, 324, 325). The mechanism by which this occurs is still unclear, however it is thought to

involve the HDL component apoprotein C-I (ApoC-I) (79). It has been suggested that HDL enhancement offers partial protection from anti-glycoprotein neutralising antibodies by increasing the speed of particle uptake (80, 325). Conversely, another SR-BI ligand, oxidised low density lipoprotein (oxLDL) inhibits HCV infection, however this does not occur via perturbation of E2-SR-BI interactions (326). Anti-SR-BI antibodies have been reported to reduce HCV infection as does SR-BI siRNA knock down, however the sensitivity to treatment is genotype specific (60, 120, 139, 171, 346). Over expression of SR-BI in Huh-7.5 cells enhances HCVcc infection (120), suggesting that its expression levels limit viral entry, this work is covered in the following chapters.

sE2 binds to SR-BI from the tree shrew tupaia but does not interact with murine SR-BI, furthermore primary tupaia hepatocytes are permissive to HCV infection (19). This suggests that SR-BI may determine the host range exhibited by HCV. The lack of an SR-BI negative cell line that supports HCV RNA replication has hindered attempts to characterise its role in infection. Consequently there is no definitive proof that SR-BI is an absolute requirement for HCV attachment and entry. However, the field is still developing and current findings suggest that SR-BI plays an important role in the HCV life cycle.

### ***Claudin-1***

The function of all organs within the human body are dependent on the selective and directional sorting of solutes and metabolites, for instance the

blood brain barrier is permeable only to relatively small molecules such as sugars. This is achieved by tissue polarisation; the cells found within tissue layers have distinct 'top' and 'bottom' surfaces, termed apical and basolateral respectively. Each surface will have a specific protein expression profile suited to the tasks it performs. The maintenance of tissue integrity and polarity is largely mediated by tight junctions. These are lateral structures that form at cell junctions, binding adjacent cell membranes together in a way that prevents the free movement of solutes across the tissue layer. Tight junctions are comprised of transmembrane proteins such as occludins, claudins, junctional adhesion molecules and e-cadherin, these interact with the cell interior via cytoplasmic partners, for instance ZO-1 and cingulin (27, 160). Other types of cell junctions include, gap and adherence junctions which mediate selective solute exchange and the attachment of adjacent cells, respectively (74).

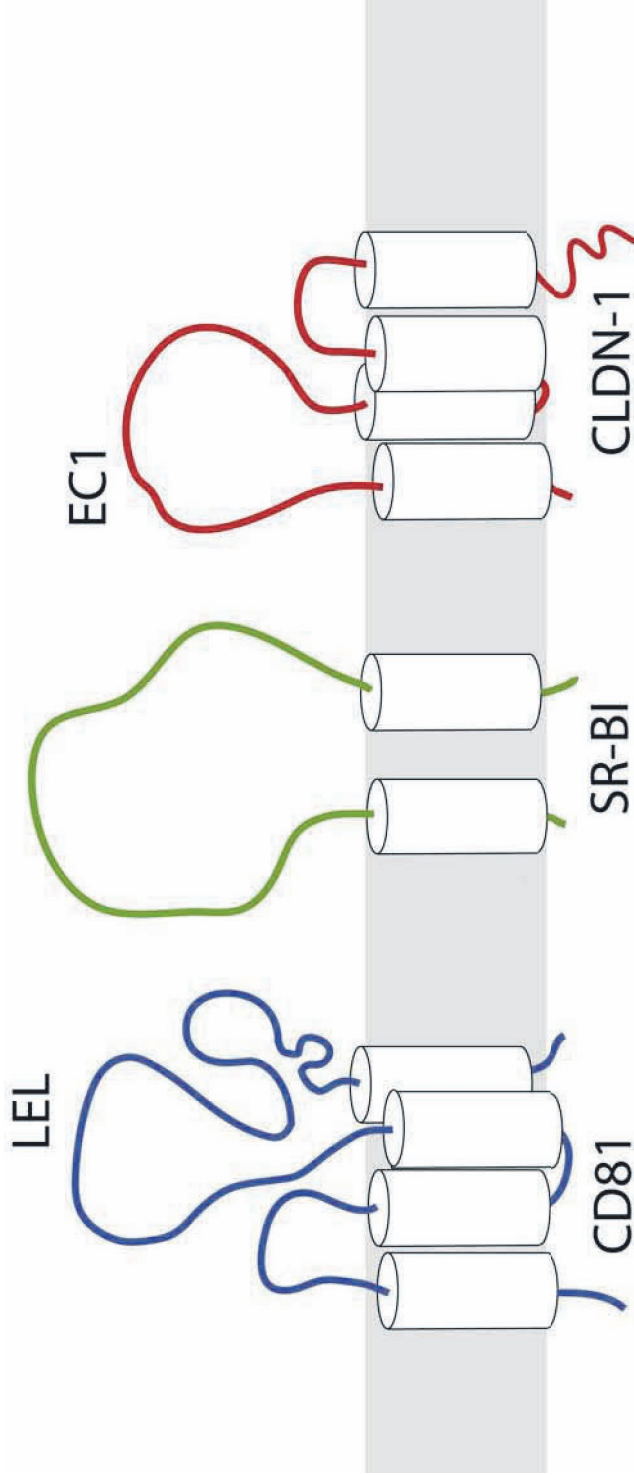
There are 24 members of the claudin family and they share a basic structure with tetraspanins; 4 transmembrane domains anchoring 2 extracellular (EC) loops. The larger of the loops, EC1, is responsible for sealing the tight junctions and the formation of selective ion channels, whereas the other mediates lateral and oppositional organisation of the protein (160). Intracellular domains interact with adaptor molecules that tether the proteins to the cytoskeleton (160). The liver is composed of highly polarised units called lobules and claudin-1 (CLDN1) is expressed in the tight junctions between hepatocytes that maintain these structures (157, 160).

The discovery of CLDN1 as a HCV receptor, unlike CD81 and SR-BI, utilised the HCVpp system. Evans et. al. expressed a cDNA library from a permissive hepatoma cell line in CD81<sup>+</sup>/SR-BI<sup>+</sup> human embryonic kidney cells and screened the transduced cells for their ability to support HCVpp infection (91). Consecutive rounds of screening identified CLDN1 as a HCV co-receptor. This was confirmed by HCVcc infection of 293T cells expressing CLDN1 (91). Whereas siRNA silencing of CLDN1 in hepatoma cells rendered the cells non-permissive for viral infection (91, 205, 350) .

Generation of chimeric CLDN1 molecules expressing domains of CLDN7 demonstrated that the CLDN1 EC1 domain is necessary for receptor activity. Only five residues differ between the CLDN1 and CLDN7 EC1 domains, mutation at these sites revealed that residues I32 and E48 are critical for CLDN1 receptor activity (91). Due to the unavailability of an appropriate anti-CLDN1 antibody, a flag tag was inserted into the CLDN1 EC1, permitting the inhibition of HCVpp infection with an anti-flag antibody. Anti-flag or anti-CD81 mAbs were used to inhibit HCV entry at various time points, demonstrating that HCV-CD81 interaction precedes CLDN1 engagement. The anti-flag mAb also inhibited E1E2 dependent fusion suggesting that CLDN1 facilitates the molecular events preceding particle penetration (91).

In the year following the identification of CLDN1 as a HCV receptor, studies have indicated that 2 other claudin family members, CLDN6 and CLDN9, confer permissivity to 293T cells (205, 350). It is not yet clear how CLDN1/6/9

act as receptors for viral entry and studies to characterise their interaction with HCV are ongoing (91, 342). Figure 1-2 depicts the HCV receptor complex.



**Figure 1-2 Cellular receptors for HCV.**

The receptor activity of CD81 and CLDN1 is dependent on critical residues within the LEL and EC1 domains, respectively. The regions of SR-BI responsible for receptor activity have yet to be reported.



### **1.4.2 Attachment factors**

The true definition of a virus receptor is somewhat debatable. There are some types of molecules used by viruses to aid tethering to the cell surface that are not thought to initiate specific entry. These so called attachment factors are important for infection as they bring a virus into proximity with the cell surface and its entry receptors. However virus interaction with them may be of low specificity and have little role in viral entry (102, 194). The following section discusses molecules employed by HCV that, as current understanding allows, fall into this category.

#### ***Heparan sulphate***

Glycosaminoglycans (GAGs) are polysaccharide moieties that are post-translationally added to certain types of protein known as proteoglycans (30). Heparan sulphate (HS) is a long chain, highly sulphated GAG, that is commonly found on proteoglycans in the extracellular matrix and at the plasma membrane and offers a ubiquitous target for viral attachment (30, 102).

HCV E2 association with HS (20, 22), is believed to occur via electrostatic interactions involving basic residues possibly including the HVR (20, 22), although some evidence disputes this (57). Soluble highly sulphated HS inhibits the binding of E2 to cells and reduces HCVpp entry, as does removal of cellular HS by heparinase (20, 22). However, to date there is little evidence

that these treatments inhibit authentic HCV particle infection, leaving the importance of HS in some question (221).

### ***L/DC-SIGN***

Liver or dendritic cell specific intercellular adhesion molecule 3-grabbing nonintegrins (L/DC-SIGN) are C-type lectins capable of recognising glycoproteins via mannose residues (186). They are believed to play a role in liver sinusoidal/dendritic cell interactions with immune cells by virtue of their ability to bind Inter-Cellular Adhesion Molecules (ICAMs) (149). DC-SIGN has also been implicated in the attachment and entry of HIV, Dengue and severe acute respiratory syndrome (SARS) corona virus (62, 111, 149, 175, 309).

L/DC-SIGN facilitate binding of sE2, HCV virus-like particles (HCV VLPs), HCVpp and patient serum derived virions, and this can be inhibited using exogenous mannan ligand or by L/DC-SIGN antibodies (23, 107, 185, 187, 250). Dendritic cells (DCs) are central to antigen processing and presentation, it has been shown that HCV VLPs bind to DC-SIGN on the surface of DCs and in doing so avoid targeting to the lysosome, suggesting an ability to evade the antigen processing pathway (188). Liver sinusoidal cells can bind HCV sE2 via L/DC-SIGN, although they are not permissive to HCVpp infection (162). However, pseudo-particles bound to L/DC-SIGN can be cross-presented to hepatoma cells (93, 185). The prevailing hypothesis is that these molecules do not take part in HCV entry but allow concentration of virions on dendritic cells or liver endothelium allowing presentation to permissive hepatocytes (162).

### **1.4.3 Endocytosis and fusion**

After successful binding to a target cell a virion must cross a cellular membrane to gain entry to the interior. HCV has an enveloped particle and most likely achieves this by fusion of its own lipid membrane with that of the host cell, allowing free passage of the capsid into the cytoplasm (150). Viral fusion events occur either at the plasma membrane (e.g. HIV) or in an intracellular compartment after endocytosis (e.g. Influenza) and normally involve a conformational change in an envelope protein exposing a fusion peptide (150, 194). These hydrophobic domains insert into the target membrane as homotrimers, allowing the formation of a 'hair-pin' structure that bring the virus and host lipid bilayers into contact, as reviewed by Kielian and Rey (150).

The penetration of a number of viruses has been well characterised and as such theorising over the fusion strategy employed by HCV was possible long before an in vitro model of entry was available. For instance proteomic analysis of HCV E1 suggests it is a class II fusion protein similar to that of another Flavivirus, tick-borne encephalitis virus (108). Using well established tools for investigating virus fusion, the HCVcc/pp systems are now allowing the elucidation of HCV fusion events.

Class II fusion proteins typically initiate virion penetration after endocytosis in acidified endosomal compartments; low pH being necessary for the exposure of the fusion peptide (150, 195). Indeed compounds that disrupt the regulation

of pH within the endosome inhibit HCVpp infection, consistent with this model (34, 68, 130, 206). The initial endocytosis of an enveloped virus can occur in a number of ways, for instance via clathrin or caveolae dependent pathways (195). HCV endocytosis is via the clathrin pathway as demonstrated using dominant negative and siRNA suppressors of the clathrin heavy chain protein (34, 68, 206). The virus is thought to initiate fusion in the early endosome, although unlike other similar viruses there appears to be a delay between endocytosis and full penetration, suggesting that low pH alone is not sufficient to trigger fusion and that other molecular events, such as receptor engagement, are necessary (206). In support of this low pH pre-treatment does not affect the infectivity of HCVcc particles (317).

We know very little of the precise molecular events involved in HCV mediated fusion other than pH and temperature dependence and possible requirement for prior interaction with CD81 and CLDN1 (91, 155, 169). Identification of membranotropic regions within E1 and E2 have provided numerous putative fusion domains (238, 242-244). Although mutagenesis studies have shown some regions to be necessary for HCVpp fusion and/or infection, a consensus has not yet been reached (155, 170, 242). Interestingly, Dreux et. al. established that enhancement of HCV infection by HDL involves the incorporation of a lipoprotein component, ApoC-I, into HCV particles. This in turn increases HCVpp-liposome membrane mixing (79) suggesting that HCV may sequester host factors to facilitate fusion.

#### ***1.4.4 Co-receptor interplay and localisation.***

Although there is some evidence to support extra hepatic reservoirs of HCV (72), the primary site of HCV replication is the liver, however it is unknown what determines this tropism. The major HCV co-receptors discussed above are expressed to varying degrees throughout the entire body, furthermore some hepatic cell lines expressing all three remain non-permissive to HCVpp (91). In this respect they can be regarded as necessary but not sufficient for HCV infection. The initial interpretation of this observation is that there is another co-receptor yet to be identified; indeed this possibility is under continuous scrutiny. However other explanations must also be considered and one such candidate for determining liver tropism is tissue specific co-receptor interplay and localisation. The information and tools necessary to address co-receptor interactions and organisation have only recently become available and as such it remains a burgeoning field of interest. This section will detail our as yet limited understanding of how the HCV co-receptors behave within a permissive cell.

Current evidence suggests that CD81 is not the primary HCV receptor (31, 91, 101, 346). There is little information as to when SR-BI is involved, although it too may play a role after virus attachment to factors such as heparan sulphate (346). It has been demonstrated that anti-CD81 and SR-BI antibodies act synergistically to inhibit HCV infection, suggesting an interplay between these molecules in defining viral entry (139, 346).

The entry of a great many viruses is dependent on cholesterol levels within host cell and particle membranes (150, 194). Indeed depletion of cholesterol by treatment with methyl- $\beta$  cyclo-dextran inhibits HCV infection although reports differ as to whether this occurs via relocalisation of CD81 or CLDN1 (47, 139). SR-BI is unique in its ability to deliver cholesterol from lipoproteins directly to the plasma membrane (161). Recent publications implicate SR-BI in the infection of hepatocytes by *Plasmodium* (262, 339), the CD81 dependent causative agent of Malaria. It was shown that the delivery of cholesterol to the plasma membrane by SR-BI promotes CD81 localisation to the cholesterol dependent tetraspanin domains, facilitating *Plasmodium* sporozoite invasion (339). It is possible that SR-BI may contribute to HCV entry in a similar manner by introducing cholesterol at defined locations within the membrane.

Current data suggests that CLDN1 acts at a point downstream of virus-CD81 engagement (91), however the transition of HCV between its receptors is not yet understood. Populations of CLDN1 and CD81 have been shown to co-localise in hepatoma cells and fluorescent resonance energy transfer (FRET) analysis indicates they are within ~5nm of one another (124), suggesting the formation of CLDN1-CD81 complexes. Although there is no evidence that HCV infection alters CD81-CLDN co-localisation, addition of recombinant E1E2 envelope proteins reduces the distance between CLDN1 and CD81 as measured by FRET (124).

The summer of 2008 brought two publications that provide evidence of signalling events involved in HCV entry. Farquhar et. al. have shown that

inhibition of protein kinase A (PKA) activity in target cells reduces HCVcc and HCVpp infection, correlating with a re-localisation of CLDN1 into intracellular vesicles (94). Brazzoli et. al. used siRNA silencing of Rho family GTPases to abrogate HCV infection, they attributed this phenotype to the ability of HCV E2 to induce Rho dependent re-localisation of CD81, suggesting that HCV may be able to orchestrate molecular events necessary for viral entry (47).

Hepatocytes, as discussed earlier, are a polarised cell type and reside in a highly ordered three dimensional structure, it is unknown what implications this has for HCV infection. Whether the cultured hepatoma cells used for in vitro HCV infection are capable of polarisation remains a controversial topic (204, 342). In this respect they may represent a poor mimic of the hepatic environment and work has begun to address HCV receptor expression in polarised systems.

The most comprehensive study thus far involved the infection of Caco-2 (colorectal adenocarcinoma) cells by HCVcc/pp (204). Although these cells express the 3 major HCV co-receptors they are minimally permissive to infection. However, this is balanced by the fact that Caco-2 cells are a widely recognised model of a polarised cell and provide a stepping stone to understanding HCV interactions with hepatocytes. The expression levels and localisation of the HCV receptors altered greatly depending on the degree of Caco-2 polarisation. HCVpp entry occurred preferentially via the apical surface and disruption of tight junctions enhanced infection, suggesting laterally located co-receptors are inaccessible to the virus in Caco-2 cells

(204). Other investigations have correlated CLDN1 junctional localisation to HCV permissivity, however these studies were carried using minimally or non-polarised cell types (342). The search for an appropriate polarised hepatic cell line is ongoing.

A recent report has raised the prospect of tissue specific factors that may interfere with the interaction of HCV and its co-receptors (261). The tetraspanin web in which CD81 resides contains numerous partner molecules that contribute to the characteristics of the membrane domain. EWI-2 is a member of the immunoglobulin super-family and contains a unique glutamine-tryptophan-isoleucine (EWI) motif, it is a major partner of CD81 and is believed to mediate interactions between the web and underlying cytoskeleton (271). Interestingly a tissue specific EWI-2 cleavage product, EWI-2wint (EWI-2 without its N-terminus), is able to block HCV E2 interaction with CD81 and introduction of EWI-2wint into permissive hepatoma cells reduces HCVcc/pp infection (261). The proteolytic cleavage necessary for EWI-2wint expression does not occur in permissive cells, suggesting that an inhibitory protein may determine HCV liver tropism.

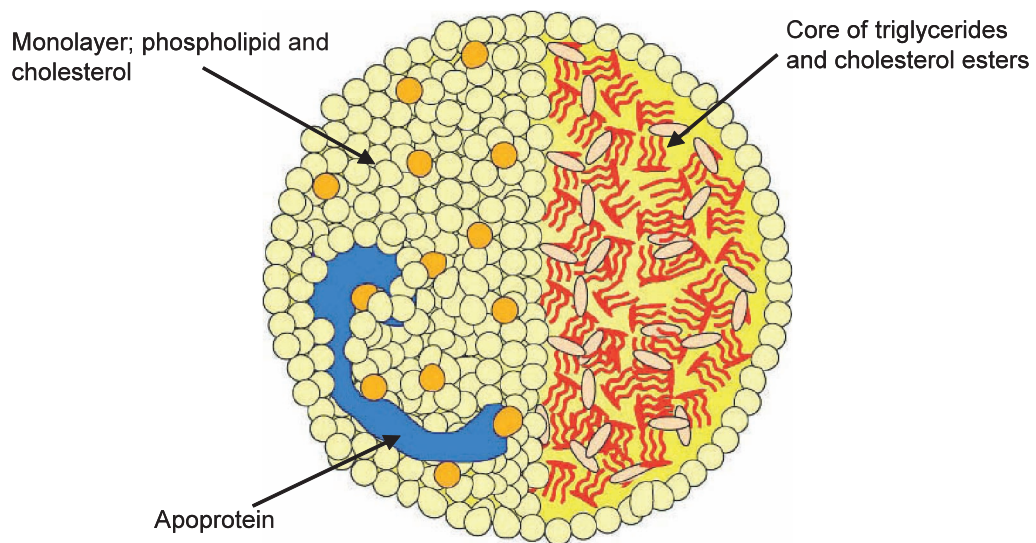
The process of characterising the series of virus-receptor and receptor-receptor interactions necessary for HCV infection has only just begun; the rather disparate nature of our current knowledge reflects this. However, as the field develops we will be able to consolidate our understanding of the complex relationships within a permissive cell allowing a global perspective on HCV attachment and entry.



### **1.5 Lipoproteins**

Since 1992 there has been a steady accumulation of evidence implicating lipoprotein metabolism in the HCV life cycle, specifically during viral entry, via receptors such as SR-BI and the subsequent secretion of particles from infected cells (25, 109, 312). Therefore prior to discussing these matters, the following section will give an overview on the structure, physiological importance and metabolism of lipoproteins. The majority of the material covered is summarised from Lipoproteins in Health and Disease by Betteridge et. al. (32) and Biochemistry of Lipids, Lipoproteins and Membranes by Vance et. al. (321).

Lipids play an essential role in the maintenance of the human body, however they are by their very nature insoluble in water, making their transport problematic. Specific tissues overcome this by complexing them with proteins into organised soluble structures known as lipoproteins, which circulate in the plasma. Although diverse in nature, all lipoproteins have the same basic structure; a core of neutral, therefore hydrophobic, triglycerides (TG) and cholesterol esters, surrounded by a monolayer of polar phospholipids. The protein component of these structures, termed apoproteins, sit in the monolayer penetrating the central core to varying degrees. Whilst in the circulation, these apoproteins determine the interaction of lipoproteins with the mediators of their metabolism (Figure 1-3).



**Figure 1-3 The basic structure of a lipoprotein**

Name	Abbreviation	Density (g/ml)	Diameter (nm)	Lipid:Protein	Major Lipid Species
Chylomicron	CM	<0.94	1000-500	100:1	Triglycerides
Very Low Density Lipoprotein	VLDL	0.94-1.006	100-50	10:1	Triglycerides
Low Density Lipoprotein	LDL	1.006-1.063	~25	4:1	Cholesterol
High Density Lipoprotein	HDL	1.063-1.210	~10	1:1	Cholesterol

**Table 1-1 The major species of lipoprotein.**

Lipid:protein ratios are calculated by weight.

Apoprotein	Type	Mw (kDa)	Lipoprotein class
ApoB-48	Non-exchangable	242	CM
ApoB-100	Non-exchangable	512	VLDL, LDL
ApoA-I	Exchangable	28	CM, HDL
ApoC-I	Exchangable	9	CM, VLDL, HDL
ApoE	Exchangable	34	CM, VLDL, HDL

**Table 1-2 Major forms of apoprotein.**

Lipoproteins exist in a dynamic spectrum of size and composition, consequently their classification can be approached in a number of ways, for example by apoprotein content. However, as lipids have a lower density than proteins, lipoproteins can also be separated by their lipid to protein ratio via buoyant density centrifugation, allowing simple classification of distinct subsets. They range from large, triglyceride rich Chylomicrons through to small, cholesterol and protein rich High Density Lipoproteins (summarised in Table 1-1).

The apoproteins are a diverse class of molecule that contribute to lipoprotein structure and mediate their specific interaction(s) with a wide array of enzymes and receptors. They fall into two subsets; exchangeable and non-exchangeable. The former are soluble proteins that readily transfer between lipoproteins in circulation, the latter are large insoluble proteins that remain within a lipoprotein for the duration of its catabolism. The non-exchangeable ApoB proteins are the major structural elements of CM, VLDL and LDL. The exchangeable forms, such as ApoC, have transient relationships with lipoproteins, directing their traffic and hydrolysis in response to the requirements of specific tissues and dietary conditions (Table 1-2).

Lipids need to be delivered to tissues in a highly specific manner as inappropriate or excessive lipid delivery is pathogenic, as seen in coronary heart disease. An array of lipase enzymes and cellular receptors allow different tissue types to extract the appropriate lipids in the correct amounts from the same circulating pool. To meet these varied demands, there is an

intricate network of interactions between the lipoproteins, apoproteins, enzymes and receptors, which naturally lends it self to adaptability. It would be inappropriate to cover this in great detail here, therefore the following paragraphs summarise the journey of dietary lipids entering and exiting the lipoprotein pathway.

Upon entering the intestine, dietary lipids are emulsified, taken up by enterocytes and used to assemble CM, each containing a single ApoB-48 molecule. CM enter circulation via the lymphatic system and quickly acquire exchangeable ApoC-II molecules. ApoC-II facilitates the action of lipoprotein lipase (LPL), an enzyme that releases fatty acids from TG. As the TG is depleted from CM ApoC-II is replaced by ApoE, which facilitates the uptake of CM by the liver, delivering the remaining TGs and cholesterol to the hepatic lipoprotein assembly pathway.

As the site of VLDL assembly the liver is central to controlling plasma lipoprotein concentrations. Microsomal triglyceride transfer protein (MTP), loads ApoB-100 molecules with lipid to form nascent VLDL particles. As these move through the secretory pathway they become increasingly lipidated a process facilitated by the addition of ApoE (122). ApoB-100 is constitutively produced by hepatocytes however poorly lipidated forms are degraded before secretion. Once in the circulation the action of lipases such as LPL depletes VLDL of tri-glycerides, leading to a gradual enrichment of cholesterol and loss of the exchangeable apoproteins. This culminates in LDL, a smaller, more dense particle. LDL is the major carrier of cholesterol throughout the body,

and delivers its cargo via the well characterised clathrin dependent LDL receptor (LDLR) pathway.

Unlike TGs, which are routinely loaded into adipocytes, excess cholesterol cannot be stored. Cholesterol deposits are pathogenic, leading to atherosclerosis and coronary heart disease and thus plasma cholesterol levels must be maintained at a steady state. HDL plays an important role in this process; it can be seen as carrying cholesterol out of the body, a process often referred to as reverse cholesterol transport.

HDL is formed extracellularly around ApoA-I molecules. ApoA-I is secreted from the liver and intestines and it is loaded with excess cholesterol by membrane proteins such as the ATP binding cassette transporter (ABCA-I), forming disc shaped precursor HDL particles. ABCA-I is expressed throughout the body, however it is found at particularly high levels on macrophages, as they are important for clearing excess cholesterol from arterial atherogenic lesions. These particles mature into spherical HDL particles which then deliver cholesterol esters to the liver via SR-BI, permitting excretion in the bile, this process will be covered in detail later.

## **1.6 Scavenger receptor B-I.**

SR-BI is a central player in the regulation of cholesterol levels, the following section details SR-BI in the context of lipoprotein metabolism.

Also known as Cla-1, SR-BI was first described in 1993 by Calvo et. al. due to its homology to CD36, another receptor involved in lipoprotein metabolism (58). 509 amino acids in length, SR-BI has two trans-membrane domains bridging intracellular N and C termini with a large extracellular loop responsible for ligand binding (286). SR-BI is predominantly expressed within the liver and steroidogenic tissue (161), where it mediates the transfer of cholesterol from lipoproteins, it also plays a role in the disposal of cholesterol from foam cells within atherosclerotic lesions (287).

The expression of SR-BI on the surface of hepatocytes is regulated by an interaction with the scaffolding protein PDZK1 (156, 285), via PDZ binding domains within its C-terminus (135). In turn PDZK1 is regulated in response to hormonal stimuli by phosphorylation of key serine residues (225). Notably, PDZK1 does not modulate SR-BI localisation in steroidogenic organs (156), reflecting the differing cholesterol requirements of specific tissues.

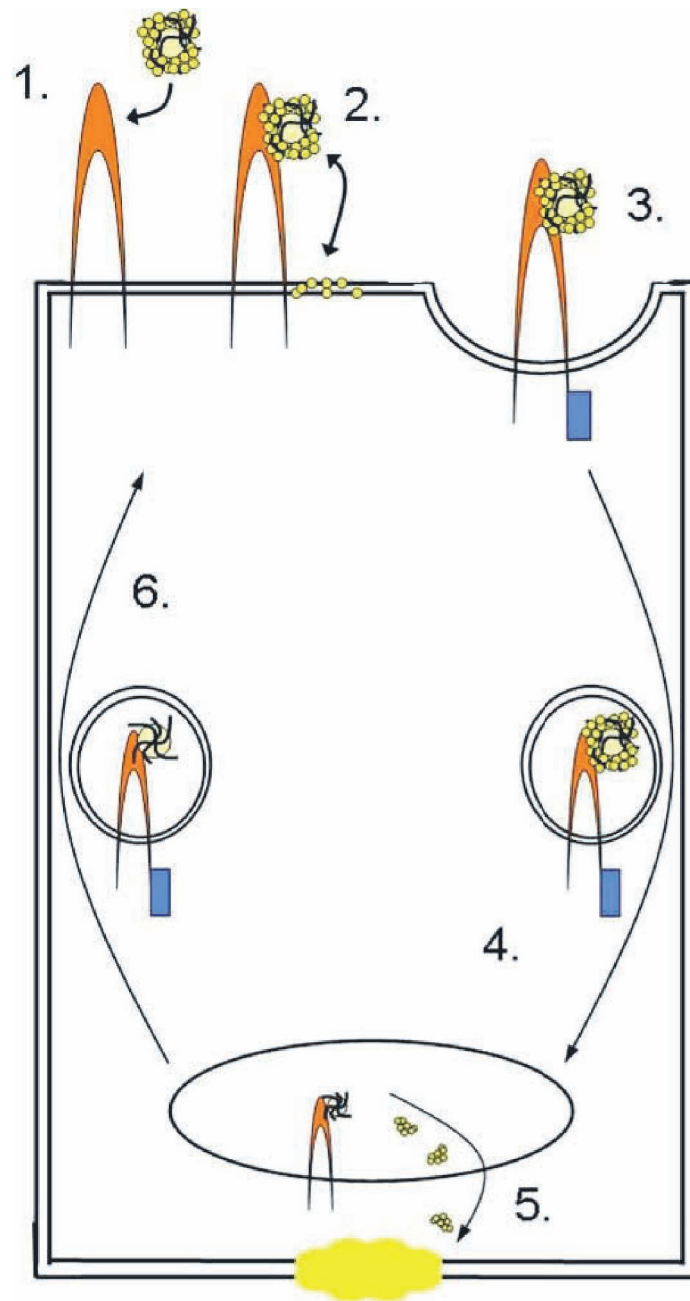
In 1996 Acton et. al. discovered SR-BI to be the previously unidentified physiological receptor for HDL. SR-BI is capable of selective cholesterol uptake from HDL (2), a process whereby cholesterol esters are delivered directly to the plasma membrane independent of the apoprotein component of HDL (11). Swarnakar et. al. reported that SR-BI also mediates selective

uptake of cholesterol from LDL (303). The membranous environment in which SR-BI resides appears to determine its activity in respect of selective uptake; association with lipid rafts favours selective uptake from LDL whereas uptake from HDL occurs preferentially within disordered regions of the membrane (258). It is believed that HDL and LDL possess alternative modes of binding to SR-BI, possibly via distinct domains. However, HDL can compete for the binding of LDL, whereas LDL only partially inhibits HDL binding, suggesting a complex ligand-receptor interplay (161).

Although originally thought to be a strictly non-endocytic receptor, it has become clear that SR-BI is capable of endocytosing both HDL and LDL independent of selective uptake (231, 287, 300). In the case of HDL, whole particle uptake is followed by selective sorting of the constituent apoprotein components within the endosome for resecretion, a process known as retro endocytosis, whereas the cholesterol component exits the body via biliary secretion (239, 286, 287, 338). In polarised hepatocytes SR-BI localises to both the sinusoidal (basal) and canalicular (apical) surfaces; it is thought to transcytose between the two in response to cholesterol requirements (53, 123, 287). Although the precise endocytic pathway employed by SR-BI is unknown, its trafficking is thought to be mediated by factors such as membrane associated protein 17, which targets SR-BI-HDL complexes to the sub apical recycling compartment of the endosomal pathway (286). A recent study suggests that intracellular SR-BI may mediate the trafficking of cholesterol between organelles (4).

The ability of SR-BI to strip HDL particles of CE allows the liver to dispose of excess cholesterol whilst returning de-lipidated apoproteins into circulation. This is the basic principle of reverse cholesterol transport (RCT), whereby cholesterol transporters upon peripheral macrophage efflux excess cholesterol to HDL particles, which in turn deliver it to the liver for SR-BI dependent excretion (319). LDL cannot mediate RCT (260) and thus high LDL levels correlate with atherosclerotic lesions caused by cholesterol accumulation, whereas high levels of HDL are protective against atherosclerosis (2). Although SR-BI can bind VLDL (331) it is not thought to donate cholesterol esters in selective uptake (50), and it is unclear whether VLDL is endocytosed by SR-BI. Lipoprotein metabolism by SR-BI is summarised in Figure 1-4.





**Figure 1-4 SR-BI-lipoprotein interactions within a hepatocyte.**

(1) HDL binds SR-BI. (2) Selective cholesterol uptake and cholesterol efflux occur at the plasma membrane. (3) SR-BI mediated HDL endocytosis. (4) Vesicular trafficking to sub-apical recycling compartment – directed via PDZK1. (5) Selective sorting of cholesterol for excretion via bile canaliculus. (6) Recycling of HDL protein component to the plasma membrane (286).

Alternative splicing of SR-BI mRNA yields the SR-BII isoform; identical in respect to the extracellular ligand binding domain, SR-BII has a unique C-terminal intracellular region that, importantly, lacks the PDZ domain present in SR-BI (330). Whilst the majority of SR-BI is found upon the cell surface, ~85% of SR-BII is expressed intracellularly, a reflection of its altered interaction(s) with adaptor proteins (89). Indeed PDZK1 KO mice show reduced SR-BI expression whilst SR-BII levels remain unaffected (156). Like SR-BI, SR-BII is capable of both selective uptake of cholesterol and receptor mediated endocytosis. However HDL whole particle uptake occurs much more rapidly with SR-BII (89). This was discovered to occur via clathrin coated vesicles, a process mediated by a C-terminal dileucine motif; insertion of this motif into the C-terminus of SR-BI incurs SR-BII like trafficking (88). Recently, Svensson et. al. reported a theoretical third isoform, SR-BIII, although the biological significance of this finding is unknown (302).

As the name suggests, scavenger receptors typically have a diverse range of ligands. Other SR-BI ligands include modified lipoproteins such as oxidised or acetylated LDL, advance glycation end products and serum amyloid A (56, 103, 115, 235). More recently SR-BI has been reported to recognise components of bacterial cell walls (39). Furthermore, expression of SR-BI in non-phagocytic cells facilitates the intracellular accumulation of bacteria. This suggests a role for SR-BI/II in innate immunity possibly via broad pattern recognition abilities (246, 323).

In summary the class B scavenger receptors interact with lipoproteins and are capable of selective cholesterol uptake and receptor mediated endocytosis, making them central to cholesterol metabolism. The way in which these ligands are metabolised and trafficked is determined by the environment of the surrounding membrane and specific interactions between the scavenger receptor C-termini and different adaptor molecules.

### **1.7 HCV and lipoproteins.**

Over the last 15 years there has been a steady accumulation of evidence to implicate lipoproteins in HCV infection. Initial observations came from infected patient sera in the early 1990s, however only with the advent of the HCVcc system has the true nature of HCV-lipoprotein association become apparent. The following section covers the extraordinary ability of HCV to exploit the liver's central role in lipid metabolism.

Differential centrifugation through a continuous gradient of inert medium, such as sucrose, allows the separation of viruses according to their density, which is in turn determined by the relative nucleic acid, protein and lipid composition of individual virions (102). This technique has been used by virologists for over 40 years (230), consequently analysis of HCV by density centrifugation was almost inevitable. As a rule particle density is relatively invariable within viral species, for instance Adenovirus particles have a homogenous density of ~1.34g/ml (55, 102). Therefore it came as some surprise to find that upon fractionation of HCV derived from patient sera, RNA genomes could be found across a broad range of densities; 1.03-1.20 g/ml (214). A large proportion of virions appeared to be of particular low density (~1.08g/ml) suggesting a high lipid content, indeed removal of lipids with detergents increased virion density to 1.25g/ml (214). The density heterogeneity and apparent high lipid composition of serum HCV particles does not conform to the traditional model of an enveloped virion, therefore it was hypothesised that HCV associates with host serum components.

In 1992 Thomssen *et. al.* demonstrated that low density HCV particles could be precipitated with anti-ApoB-100 antibodies, indicating an association with low density lipoproteins (312, 313). However understanding the nature of this association would go little further until 2002 when Andre *et. al.* used electron microscopy to study the sera of HCV infected patients (8). They observed low density spherical structures of ~100nm, atypical of normal human lipoproteins, treatment with detergent revealed central viral capsids that could be recognised using HCV core antibodies, they named these structures HCV lipo-viro-particles (LVPs) (8).

Subsequent studies using infected patient liver and serum samples revealed that LVPs contain HCV RNA and structural proteins, ApoB-100/48, ApoE, ApoC, triglyceride, cholesterol and phospholipids (8, 77, 210, 232, 233, 312). However, analysis of their lipid composition suggests that they are not merely HCV particles carrying surface lipoproteins (8). LVPs have characteristics of both lipoproteins and virus particles and are believed to circulate as functional carriers of dietary lipids (77). Indeed, HCV LVP are reported to interact with the metabolic enzyme lipoprotein lipase, however this is believed to facilitate particle entry along a non-permissive pathway (9).

Prior to an *in vitro* system to measure particle infectivity, observations were made using patient sera and Chimpanzees. These early studies suggested that low density LVP are more infectious than those of a higher density (45, 128). Initial experiments to elucidate the mechanism of LVP formation measured the association of HCV structural proteins with lipoproteins,

although a consensus has not yet been reached. Lambot et. al. used immobilised sE2 to capture VLDL and LDL in vitro (164) , Monazahian et. al. made similar observations using co-precipitation techniques (219), however others have failed to detect a direct interaction (337).

True characterisation of HCV LVPs began with the introduction of cell culture proficient JFH-1 HCVcc. Using HCV genome specific RT-PCR to measure particle distribution throughout the density gradient, it was observed that, like serum virus, HCVcc particles exhibit a broad range of densities. However peak HCVcc genome titre occurred at a slightly higher density than serum virus (~1.15g/ml), suggesting reduced association with lipoproteins (179, 328). The ability to measure infectivity as well as genome titre allows the calculation of specific infectivity, i.e. a measure of the infectious potential of any given particle. By correlation of density with specific infectivity, it was demonstrated that high density particles are less infectious than those of a low density (179), consistent with previous observations in the Chimpanzee (45, 128).

A chimeric HCVcc genome containing the structural genes of strain J6 and the non-structural genes of JFH-1 (J6/JFH) produces particles in vitro that are infectious for Chimpanzees (179, 180). Interestingly, J6/JFH virus recovered from the plasma of infected animals displayed a density distribution profile more similar to that of patient sera derived HCV than HCVcc, accompanied by an increase in specific infectivity. Propagation of Chimpanzee plasma HCV in cultured hepatoma cells restored the density distribution and infectivity to that of cell culture derived HCV (180). Taken together these data suggest that

lipoprotein association promotes HCV infectivity and in vitro HCVcc particles do not fully represent patient serum derived LVPs.

### ***1.7.1 Assembly and release.***

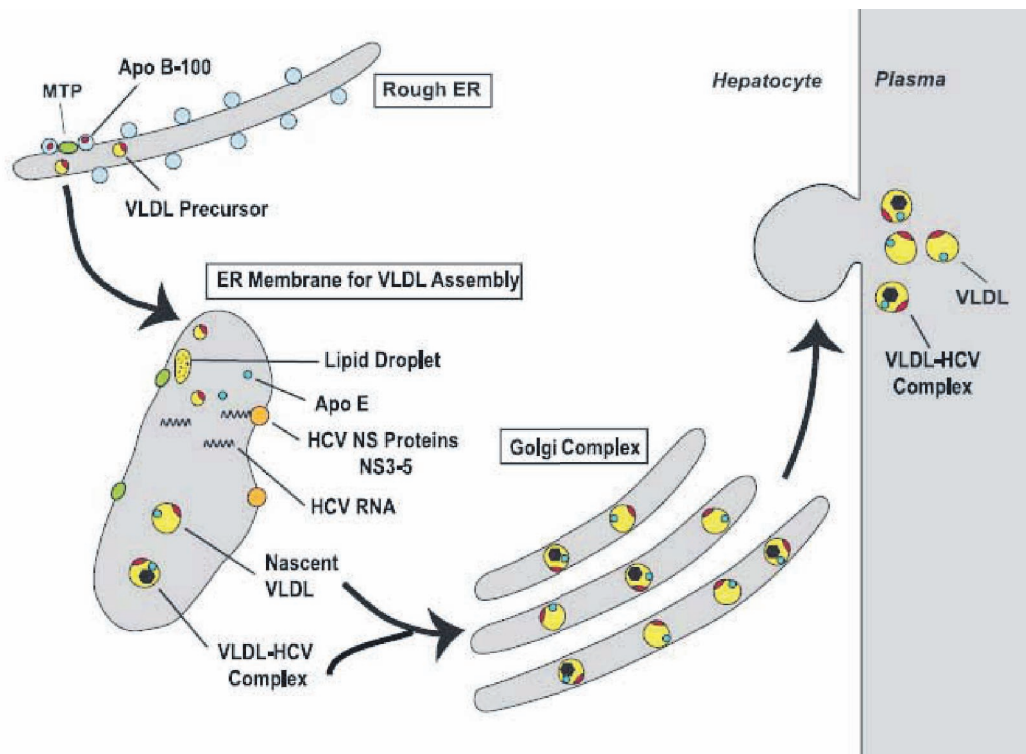
Prior to the development cell culture proficient HCV the assembly and release of infectious particles had been somewhat of a “black box”. We knew of the components entering the process, yet the mechanisms of HCV particle egress remained mysterious. However, publications in the last 12 months have revealed that HCV commandeers the hepatic VLDL assembly machinery to produce infectious LVP.

As covered earlier, the process of VLDL assembly and release is relatively well understood and this information was utilised to investigate HCV production by infected hepatoma cell lines. Intracellular infectious HCV particles are of a higher density than secreted particles, suggesting that low density is gained during viral egress (110). Treatment of infected cells with brefeldin, a broad range inhibitor of the secretory pathway, reduced particle release and led to an accumulation of intracellular virus (109). Inhibition of VLDL assembly using MTP inhibitors or silencing of apoproteins apoB-100 and apoE, prevented both the release of particles and brefeldin induced intracellular accumulation, indicating a block to virion assembly. However these treatments had no effect on viral RNA replication or intracellular genome concentrations (63, 109, 131, 224).

These data point to a requirement for the VLDL assembly machinery for the packaging of viral RNA and structural proteins into infectious HCV LVP, which are trafficked via the secretory pathway to allow particle release. Intracellular particles are vulnerable to pre-secretory degradation (109), suggesting that an infected host cell treats HCV LVPs as VLDLs, targeting lipid poor forms for recycling in the absence of sufficient substrate(s).

It is not yet clear how the virus structural proteins and host apoproteins associate during particle assembly. However current data suggests that lipid droplets (LD), intracellular organelles responsible for lipid storage, are central to this process. HCV genome replication is believed to occur on membranes in and around the endoplasmic reticulum, mature E1E2 heterodimers also reside there (181, 220). Core protein, however, translocates to the LDs and is seen to coat their surface (41, 42, 215), NS5A also localises with the LDs (280), viral protein association with LDs is essential for the production of infectious particles. Core directs the redistribution of LDs along the micro-tubule network bringing them into the vicinity of membranes bearing genome replication complexes (41). It is currently believed that assembly occurs at these juxtapositions (41, 215), indeed numerous studies have demonstrated a accumulation of viral proteins and RNA at these sites (131, 189, 215, 305). Furthermore, LDs provide a source of triglycerides for the loading of lipids on to ApoB (321), suggesting that the VLDL and HCV assembly pathways converge at this location. (Figure 1-5).





**Figure 1-5 Lipo-viro-particle assembly.**

Newly synthesised ApoB is loaded with lipid by MTP in the rough ER, yielding a VLDL precursor. Upon translocation of the precursor to the ER lumen MTP mediated lipidation continues, with the lipid droplets providing the major source of triglycerides and cholesterol. Within an infected liver, these ER membranes are also the site of HCV RNA replication and it is believed particle assembly occurs in parallel with VLDL, allowing virions to become incorporated into lipo-viro-particles. Image taken from Ye et. al. (343)

### ***1.7.2 Low density lipoprotein receptor and virus entry.***

Several reports provide evidence that LDLR may be an additional candidate receptor for HCV attachment and entry, notably, these studies were carried out almost exclusively with patient serum derived virus. Low density serum HCV will bind to cells expressing LDLR, whereas higher density particles do not (218, 336). This interaction is inhibited by the addition of native LDL, alternatively removal of cell associated LDL enhances HCV binding (95, 218, 336), these data suggest that HCV interacts with LDLR in an analogous manner to its natural ligand. A recent study by Molina et. al. demonstrated that infection of primary human hepatocytes by serum HCV is inhibited by anti-LDLR mAbs and recombinant soluble LDLR peptides, furthermore, treatments to modulate LDLR expression similarly affected HCV infection (216). However, the above observations have yet to be recapitulated using the HCVcc/pp systems (24), thus limiting our understanding of HCV-LDLR interactions. Current data suggest that envelope proteins do not interact with LDLR directly (24, 336) and that HCV relies on associated lipoproteins for binding (219, 337), it is likely that HCVcc/pp are not sufficiently lipoprotein-like for this to occur.

As illustrated by investigations on the role of LDLR in HCV entry, in vitro generated HCVcc may not represent native HCV circulating within an infected individual. Cultured human hepatoma cells often have restrictions in their ability to assemble lipid rich VLDL particles (316), this would almost certainly impact on the nature of secreted HCV LVP. Moreover, circulating lipoproteins undergo continual modification in response to metabolic requirements (321).

In vitro culture medium, into which HCVcc LVPs are secreted, would not replicate this environment. Therefore, understanding how to better replicate these systems without the use of animal models is imperative.

### **1.8 *Project Objectives.***

The aim of this study was to investigate the role of SR-BI in HCV attachment and entry, using recombinant soluble HCV glycoproteins and the HCVcc/pp infectious models. In doing this, we also hoped to examine the importance of lipoproteins in HCV infection. The broad objective of the Birmingham HCV Research Group is to characterise the routes of transmission exhibited by HCV, specifically focussing on the interplay between virus particles and cellular receptors.

## 2 **Materials and methods.**

### 2.1 ***Cell lines.***

Table 2-1 details the different cell lines used in this study. Cells were maintained in tissue culture flasks (Becton Dickinson, NJ, USA) in the stated culture media supplemented with 10% foetal calf serum (FCS) (Gibco, CA,USA), 1% non-essential amino acids (Gibco), 1% L-glutamine (Gibco) and 50units/ml penicillin and 50µg/ml streptomycin (P/S) (Gibco).

<b>Cell lines</b>			
<b>Name</b>	<b>Species : Tissue</b>	<b>Growth media</b>	<b>Source</b>
<b>Huh-7.5</b>	Human hepatoma	DMEM	Dr C.M.Rice, Rockefeller University, New York
<b>CHO</b>	Chinese hamster ovary	Ham's F12	American Type Culture Collection
<b>293T</b>	Human embryonic kidney	DMEM	American Type Culture Collection
<b>CHO-SR-BI</b>	Chinese hamster ovary	Ham's F12 + G418	Dr T.Huby, INSERM, Paris
<b>CHO-SR-BI M1</b>	Chinese hamster ovary	Ham's F12 + G418	Dr T.Huby, INSERM, Paris
<b>CHO-SR-BI M2</b>	Chinese hamster ovary	Ham's F12 + G418	Dr T.Huby, INSERM, Paris
<b>CHO-SR-BI M3</b>	Chinese hamster ovary	Ham's F12 + G418	Dr T.Huby, INSERM, Paris
<b>CHO-SR-BI M4</b>	Chinese hamster ovary	Ham's F12 + G418	Dr T.Huby, INSERM, Paris
<b>CHO-SR-BI M5</b>	Chinese hamster ovary	Ham's F12 + G418	Dr T.Huby, INSERM, Paris
<b>CHO-SR-BI M6</b>	Chinese hamster ovary	Ham's F12 + G418	Dr T.Huby, INSERM, Paris
<b>CHO-CD81</b>	Chinese hamster ovary	Ham's F12 + G418	In house

**Table 2-1 List of cell lines.**

### 2.2 ***Antibodies.***

Table 2-2 details the antibodies used in this study.

Primary antibodies					
Antibody name	Antigen	Type	Specificity	Species	Source
Anti-SR-B1	Human SR-B1	Animal sera	Polyclonal	Rabbit	Dr. I Huby, INSERM, Paris
Anti-CLA-1	Human SR-B1	Purified IgG	Polyclonal	Mouse	Environ Dickinson Transduction Laboratories, New Jersey
3D5	Human SR-B1	Purified IgG	Monoclonal	Mouse	Dr. A Nicotia, IREM, Rome
C11	Human SR-B1	Purified IgG	Monoclonal	Humanized	Dr. A Nicotia, IREM, Rome
Anti-SR-B1 (C-terminus)	Human SR-B1	Animal sera	Polyclonal	Rabbit	Dr. I Huby, INSERM, Paris
Anti-SR-B1	Human SR-B1	Animal sera	Polyclonal	Rabbit	Dr. J Owen, Imperial College, London
W38	Human CD81	Hydromax SN	Monoclonal	Mouse	Dr. F. Baecker, DKFZ Cancer Institute, Birmingham
15B21	Human CD81	Hydromax SN	Monoclonal	Mouse	In house
15B21	Human CD81	Hydromax SN	Monoclonal	Mouse	In house
25B39	Human CD81	Hydromax SN	Monoclonal	Mouse	In house
9E10	HCV NS5A	Hydromax SN	Monoclonal	Mouse	Dr. I. Talmon, and Dr. C. M. Rice, Rockefeller University, New York
C1	HCV E2	Purified IgG	Monoclonal	Mouse	Dr. O. Barton, The Scripps Research Institute, La Jolla
5B2	HCV E2	Purified IgG	Monoclonal	Rat	In house - see entry on anti-E2 mAb preparation
2A11	HCV E2	Purified IgG	Monoclonal	Rat	In house - see entry on anti-E2 mAb preparation
1B39	HCV E2	Purified IgG	Monoclonal	Rat	In house - see entry on anti-E2 mAb preparation
11B20	HCV E2	Purified IgG	Monoclonal	Rat	In house - see entry on anti-E2 mAb preparation
5B1a	HCV E2	Purified IgG	Monoclonal	Rat	In house - see entry on anti-E2 mAb preparation
5B1b	HCV E2	Purified IgG	Monoclonal	Rat	In house - see entry on anti-E2 mAb preparation
9B15	HCV E2	Purified IgG	Monoclonal	Rat	In house - see entry on anti-E2 mAb preparation
6B3	HCV E2	Purified IgG	Monoclonal	Rat	In house - see entry on anti-E2 mAb preparation
10B60	HCV gp120	Purified IgG	Monoclonal	Rat	In house - see entry on anti-E2 mAb preparation
P1.6	HCV	Patient sera	Polyclonal	Human	In house - see entry on patient IgG isolation
Secondary antibodies					
Antibody name	Antigen	Type	Specificity	Species	Source
Anti-Mouse Alexa Fluor 488	Mouse IgG	Purified IgG	Polyclonal	Goat	Molecular Probes (Invitrogen), California
Anti-Mouse Alexa Fluor 533	Mouse IgG	Purified IgG	Polyclonal	Goat	Molecular Probes (Invitrogen), California
Anti-Rabbit Alexa Fluor 488	Rabbit IgG	Purified IgG	Polyclonal	Goat	Molecular Probes (Invitrogen), California
Anti-Rat Alexa Fluor 488	Rat IgG	Purified IgG	Polyclonal	Goat	Molecular Probes (Invitrogen), California
Anti-Rat IgG	Rat IgG	Purified IgG	Polyclonal	Donkey	Jackson ImmunoResearch, Pennsylvania

Table 2-2 List of antibodies.

**Preparation of rat anti-E2 mAbs.**

Anti-E2 and control mAbs were cloned from rats immunised with recombinant antigen as previously described (99, 130, 282). Hybridoma cells expressing the panel of antibodies were grown in miniPERM bioreactors (Greiner Bio One, Germany), according to the manufacturers instructions, under the supervision of Dr. Margaret Goodall. Approximately 250ml of cell culture medium containing mAb was harvested from each bioreactor allowing IgG isolation using the following protocol.

1. A 2ml column containing 750µl of Fast-flow protein G conjugated sepharose beads (GE Healthcare, UK) was prepared for each mAb to be isolated.
2. To capture IgG, the column was connected to a peristaltic pump (Pharmacia, Sweden) and 10ml phosphate buffered saline (PBS) (Gibco) passed through to wash, followed by 10ml of harvested culture media.
3. The column was washed once more and the IgG eluted with 10ml 0.1M glycine (Sigma-Aldrich, MO, USA) at pH 2.7, the acidic eluate was immediately neutralised with 650µl 1M TRIS (Sigma-Aldrich) at pH 9.0.
4. Finally, the eluate was dialysed against PBS overnight at 4°C. IgG concentration was determined using a UV spectrophotometer (Amersham, UK).

### Isolation of patient IgG.

The sera of patients infected with HCV was kindly provided by Dr. David Mutimer and Dr. David Adams of the Queen Elizabeth Hospital Liver Unit. Prior to isolation of IgG sera was heat inactivated by treatment at 60°C for 1.5 hrs. IgG was isolated using a 5ml column containing 2.5ml Fast-flow beads following the above protocol. Remaining patient sera was stored in a secure biological hazard container at -80°C.

### 2.3 Plasmids and proteins.

Table 2-3 and Table 2-4 detail the plasmids and recombinant proteins used in this study.

Plasmids	
Name	Source
HCVcc JFH-1	Dr. T.Wakita, National Institute of Infectious Diseases, Tokyo
HCVcc J6/JFH-1	Dr C.M.Rice, Rockefeller University, New York
HCVcc JFH-1 G451R	Dr. F.Chisari and Dr. J.Zhong, The Scripps Research Institute, La Jolla
TRIP SR-BI	In house
TRIP SR-BII	In house - see entry on cloning TRIP SR-BII
TRIP CD9	In house
HIV gagpol 8.2	Aaron Diamond AIDS Research Center
VSV-G	Aaron Diamond AIDS Research Center
PCDNA3.1 GFP murine SR-BI	Dr. D.R. van der Westhuyzen, University of Kentucky
CSGW GFP	Aaron Diamond AIDS Research Center
ADARC HIV gagpol	Aaron Diamond AIDS Research Center
JFH HCV E1E2	Dr. Jie Zhang, Rockefeller University, New York
JFH HCV sE2 - 10/76B	In house - see entry on cloning JFH-1 sE2
JFH G451R HCV sE2 - 10/76B	In house - see entry on cloning JFH-1 sE2

Table 2-3 List of plasmids.

Proteins	
Name	Source
Strain HCV-1 sE2	Chiron, California
Strain JFH-1 sE2	In house - see entry on cloning JFH-1 sE2
Strain JFH-1 G451R sE2	In house - see entry on cloning JFH-1 sE2
Human CD81 LEL	Dr. H.Drummer, Burnet Institute, Melbourne
Human CD81 LEL monomer	Dr. H.Drummer, Burnet Institute, Melbourne
Human CD81 LEL L162P	Dr. H.Drummer, Burnet Institute, Melbourne
Human CD81 LEL L182F	Dr. H.Drummer, Burnet Institute, Melbourne
Human CD81 LEL N184Y	Dr. H.Drummer, Burnet Institute, Melbourne
Human CD81 LEL F186S	Dr. H.Drummer, Burnet Institute, Melbourne

Table 2-4 List of recombinant proteins.



## **2.4 Basic techniques.**

### **Flow cytometry.**

1. Cells of interest were trypsinised (Gibco) for 5 mins, resuspended in appropriate media and counted using a haemocytometer. Cells were then pelleted in a 5804R centrifuge (Eppendorf, Germany) at 1500rpm for 5 mins and diluted to  $2 \times 10^6$  cells/ml in PBS + 0.5% bovine serum albumin (BSA) (Sigma-Aldrich).
2. If cells required fixation prior to staining they were treated for 5 mins with 3% paraformaldehyde (PFA) (TAAB, UK), followed by a PBS wash and resuspension in PBS + 0.5% BSA. If permeabilisation was necessary, fixed cells were resuspended in PBS + 0.5% BSA + 0.5% saponin (Sigma-Aldrich) and all subsequent steps carried out in this buffer.
3. Suspended cells were incubated for 20 mins at RT to block. If using saponin this also served as a permeabilisation step.
4. Antibody staining was performed in a 96 well U bottomed plate (Corning, NY, USA) with  $2 \times 10^5$  cells/well. 100 $\mu$ l of cell suspension was put into each well, the cells were pelleted by centrifugation at 1500rpm for 5 mins and then resuspended in 70-100 $\mu$ l of primary antibody or control diluted in PBS + 0.5% BSA (+ 0.01% saponin).
5. After 45 mins incubation at RT, cells were washed with PBS; 100 $\mu$ l PBS was added to each well and the cells were pelleted by centrifugation. This process was repeated after which the cells were

resuspended in 70-100ul of fluorescently conjugated secondary antibody diluted as above.

6. A further 45 mins incubation completed the antibody staining and the cells were washed in PBS as above. In the case of live staining, the cells were fixed, as in step 2, prior to analysis.
7. Staining intensities were measured using a Facscalibur flow cytometer (Becton Dickinson), the data was captured with Cell Quest (Becton Dickinson) and analysed using FlowJo (Tree Star, OR, USA).

#### **Indirect immunofluorescence.**

1. Cells of interest were seeded at  $3 \times 10^4$  cells/100mm<sup>2</sup> in 48, 24 or 12 well tissue culture plates (Becton Dickinson) 24hrs prior to study.
2. Cells were fixed prior to staining by 5 mins incubation with ice cold methanol (Fisher Scientific, UK), after which the cells were blocked for 20 mins with either PBS + 0.5% BSA or, if permeablising, PBS + 0.5% BSA + 0.01% saponin.
3. Primary antibody staining was achieved by incubation for 45 mins at RT with antibody or control diluted in the appropriate buffer.
4. To wash the cells, diluted antibody was removed by careful aspiration, followed by the addition of excess PBS, the process was then repeated.
5. Secondary antibody staining was achieved by incubation for 45 mins at RT with fluorescently conjugated secondary antibody diluted in the appropriate buffer.

6. Finally, the cells were washed as in step 4 and the staining visualised using a fluorescent microscope (Nikon TE2000, Japan). Images were taken using a digital camera (Hamamatsu, Japan).

### **Enzyme linked immunosorbent assay (ELISA).**

1. Immulon 2HB flat bottom assay plates (Thermo, MA, USA) were first coated with a capture agent of either *Galanthus nivalis* (GNA) lectin (5µg/ml) (Sigma-Aldrich) or recombinant CD81 LEL (5µg/ml). 50-100µl of capture agent diluted in PBS was added to each well followed by an overnight (O/N) incubation at 4°C. The GNA and CD81 capture ELISAs have been previously described (84, 99).
2. The plate was washed 3 times by the addition of excess PBS after which it was blocked for 30 mins at RT with PBS + 5% BSA.
3. The plate was then incubated with 50µl/well soluble HCV E2 glycoprotein diluted in PBS + 5% BSA (0.5-1µg/ml) for 4 hrs at 37°C. Wells incubated with buffer alone served as a negative control.
4. The plate was washed as in step 2 and incubated with 50µl/well anti-E2 or control antibody diluted in PBS + 5% BSA (5µg/ml, or as stated in figure) for 45 mins at RT.
5. Bound antibody was detected using 50µl/well HRP conjugated donkey anti-rat IgG secondary diluted in PBS + 5% BSA (1/1000) for 45 mins at RT.
6. Binding was realised by addition of 50µl/well HRP substrate (BioFix, MD, USA), HCl stop solution (BioFX) being used once the signal had

developed. The absorbance of each well was measured at 450nm using a plate reader (Thermo) and associated Ascent software.

#### **Cell based soluble E2 binding assay.**

1. A flow cytometry based assay was used to assess sE2 binding to cells. Trypsinised cells were resuspended at  $2 \times 10^6$  cells/ml in PBS + 0.5% BSA + 0.01% sodium azide (Sigma) and incubated at RT for 20 mins. Sodium azide inhibits cell metabolism and therefore ensures minimal trafficking of proteins to and from the cell membrane.
2. As detailed in the flow cytometry protocol, sE2 binding was carried out in a 96 well U bottomed plate. Cells were resuspended in 70-100 $\mu$ l of sE2 diluted in the above buffer and incubated for 1 hr at 37°C.
3. Cells were PBS washed as in the flow cytometry protocol, resuspended in rat anti-E2 mAb diluted in above buffer (5 $\mu$ g/ml, or as stated in figure) and incubated for 45 mins at RT.
4. Cells were washed and bound E2-Ab complexes detected using goat anti-rat IgG fluorescently conjugated secondary antibody diluted buffer (1/1000) and incubated for 45 mins at RT.
5. Finally, cells were washed and fixed and analysed as detailed in the flow cytometry protocol. This protocol was first developed by Flint and co-workers (98, 99).

#### **Transfections.**

1. Transfections were carried out using the Fugene (Roche, Switzerland), Lipofectamine (Invitrogen, CA, USA) or Profection

(Promega, WI, USA) kits according to the manufacturer's guidelines.

Briefly, cells were seeded at 800 cells/mm<sup>2</sup> in P/S free media 24hrs prior to transfection.

2. Transfections were carried out at 37°C for 8hrs, using ~1µg of plasmid DNA per 2x10<sup>5</sup> cells, after which the transfection mixture was replaced with DMEM + 3% FCS + P/S.
3. Expression efficiency was typically monitored 48 hrs post transfection.

### **TRIP retrovirus gene delivery system.**

The TRIP system is a retrovirus gene expression vector developed by Zennou *et. al.* (347). It produces virus vector particles, formed around a replication deficient HIV gag-pol core, that bear the envelope glycoprotein of vesicular stomatitis virus (VSVG). These particles can package a gene of interest as an RNA transcript, subsequent transduction of a cell line with the TRIP system results in reverse transcription of the target gene and its insertion into the genomic DNA. In this study transduced cells were not under selection, however they maintained exogenous gene expression for around 1 month, after which they were discarded. The following protocol documents the synthesis and use of TRIP particles, the cloning of SR-BII for use in the TRIP system is detailed later.

1. TRIP particles were produced by Fugene transfection of 293T cells with the following quantities of plasmids encoding the constituent elements; 400ng VSVG envelope, 600ng TRIP gag-pol and 600ng target gene.

Cells were transfected in 6 well tissue culture plates for 8hrs, after which the culture media was changed to DMEM + 3% FCS + P/S.

2. Transfection efficiency was monitored by the inclusion of a TRIP plasmid encoding enhanced green fluorescent protein as a target gene (TRIP EGFP).
3. Culture media containing TRIP particles was harvested at 48 and 72 hrs post transfection and passed through a 0.2 $\mu$ M filter to remove any contaminating 293T cells. Transduction of target cells was performed immediately.
4. Target cells were seeded at  $4 \times 10^5$  cells/well in a 6 well tissue culture plate 24 hrs prior to transduction. To transduce, cells were incubated O/N with harvested TRIP culture media diluted 1:1 in DMEM + 3% FCS + P/S. After which the media was changed to DMEM + 10% FCS + P/S.
5. Transduction efficiency was assessed after 48 hrs by monitoring expression of TRIP EGFP and flow cytometric detection of target gene(s).

### **HCV pseudo particle system.**

The HCVpp system used in this study is similar to that described by Hsu et. al and Pohlmann et. al. (130, 250). Like the TRIP system, HCVpp are based around a replication deficient HIV gag-pol core but carry the E1E2 glycoproteins of HCV. Infection is detected by an EGFP reporter gene packaged into the HCVpp. As the particles do not encode any HCV structural

proteins and are incapable of further rounds of replication they only mimic the entry process of HCV.

1. HCVpp were synthesised in a similar manner to TRIP particles. Briefly, 293T cells were Fugene transfected with plasmids encoding HCV E1E2 (1µg), HIV gag-pol (250ng) and an EGFP reporter (250ng).
2. Culture media containing HCVpp was harvested at 48 and 72 hrs post transfection. Infection of target cells was performed immediately.
3. Target cells were seeded at  $6 \times 10^4$  cells/well in a 12 well tissue culture plate 24 hrs prior to infection. To infect, cells were incubated for 8 hrs with harvested HCVpp culture media diluted 1:3 in DMEM + 3% FCS + P/S. After which the media was refreshed.
4. Infection was assessed after 72 hrs by flow cytometric detection of the EGFP reporter.

## **2.5 Cell culture proficient hepatitis C virus.**

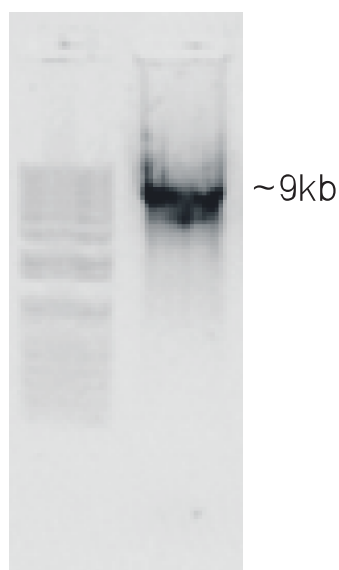
The following section details the synthesis and use of HCVcc particles. Currently, all HCVcc viruses are constructed around the non-structural proteins of HCV strain JFH-1, a unique isolate capable of producing particles in certain hepatoma cell lines (179, 328, 351). The HCVcc viruses used in this study were JFH-1 wild type, cell culture adapted JFH-1 G451R, and a chimeric J6/JFH virus which encodes core, E1, E2, p7 and NS2 of strain J6 HCV (179, 328, 352). In each case virus was produced by transcription of RNA from a plasmid encoding the HCV genome, introduction of RNA genomes into Huh-7.5 cells by electroporation and subsequent harvest of secreted HCVcc particles.

### **RNA synthesis.**

1. RNA transcripts of the HCV genome were produced using the T7 RNA polymerase kit (Ambion, TX, USA) according to the manufacturer's instructions. Briefly, 5µg of plasmid containing a cDNA clone of the HCV genome was linearised by XbaI digest (Promega).
2. 1µg of linearised plasmid was used as a template for RNA transcription, the reaction mixture was incubated at 37°C for 3-4 hrs, after which the RNA was cleaned up using the RNeasy MinElute kit (Qiagen, Netherlands) according to manufacturer's instructions.
3. The quality of the RNA was assessed by gel electrophoresis on a 1% agarose gel (Bioline, UK). Typical yields, as measured by a UV



spectrophotometer (Amersham), were 250-1000ng/μl. A gel image showing HCV RNA transcripts is displayed in Figure 2-1.

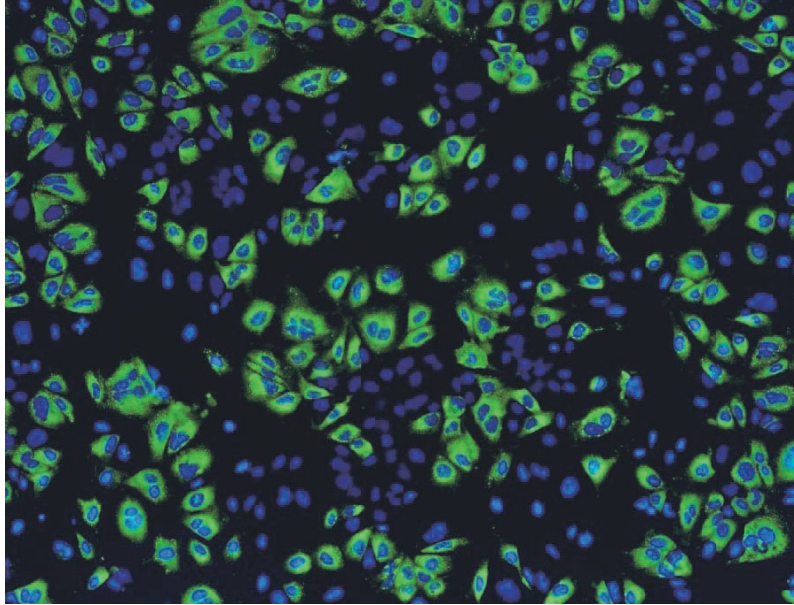


**Figure 2-1 In vitro transcribed HCV RNA.**  
Realised on a 1% agarose gel.

### **Electroporation.**

1. Huh-7.5 cells at 60-80% confluence were trypsinised, resuspended in DMEM and counted.
2. To prepare, the cells were washed with excess ice cold PBS and pelleted by centrifugation at 1250rpm for 5 mins at 1°C, this process was repeated after which the cells were resuspended in ice cold PBS at  $1.5 \times 10^7$  cells/ml and placed on ice.
3. To electroporate, 400μl of cell suspension was mixed with 3μg of genomic RNA and transferred into a 0.2cm EP cuvette (Sigma-Aldrich). EPs were carried out at 780v in an Electro Square Porator (Harvard Apparatus, MA, USA).

4. After EP the cells were allowed to stand for 5 mins at RT prior to being transferred into 10ml of pre-warmed DMEM + 10% FCS + P/S. 8ml of the resuspended cells were placed in a T75 culture flask, and the remainder put into 2 wells of a 24 well tissue culture plate to allow the monitoring of HCV protein expression. The cells were immediately taken into category 3 containment laboratories for culture and harvest of particles.
5. At 48 hrs post EP, the efficiency of viral replication was quantified by detection of the HCV non-structural protein NS5A. Briefly, electroporated cells were stained using the immunofluorescence (IF) protocol with saponin permeabilisation (as detailed above), mouse anti-NS5A mAb 9E10 was used at 1/200 dilution. JFH infected NS5A positive Huh-7.5 cells are shown in Figure 2-2.
6. HCVcc particles were harvested between 4 and 14 days post EP, after which the cells were discarded. To harvest, infected cells were cultured in a minimal volume of DMEM + 3% FCS + P/S and media containing secreted virions collected every 8-14 hrs. Harvested virus was frozen prior to titration using the infection assay.



**Figure 2-2 Electroporated Huh-7.5 cells.**

Huh-7.5 cells 48 hrs post EP with JFH-1 RNA, infection was detected by visualisation of HCV NS5A.

### **Infection assay.**

All HCVcc infectivity data presented in this thesis was obtained using the following protocol.

1. Naïve Huh-7.5 cells were seeded at  $1.5 \times 10^4$  cells/well in 48 well tissue culture plate 24 hrs prior to infection.
2. To infect, the media was removed from the cells and replaced with 100 $\mu$ l of HCVcc virus diluted in DMEM + 3% FCS + P/S.
3. After 8 hrs at 37°C the HCVcc inoculum was removed and the cells refed with 150 $\mu$ l of DMEM + 3% FCS + P/S, infections were allowed to proceed for 48 or 72 hrs, as stated in the figure legends.
4. Infected cells were fixed and NS5A positive cells detected using the IF protocol as stated above.

5. The level of infection was assessed by enumeration of NS5A positive cells using a fluorescence microscope, data is expressed as infectious units (IU) per ml of harvested HCVcc culture media; 1 infected cell = 1 IU. To ensure reliable data read outs, HCVcc virus was always diluted according to harvest titre so that each well contained 150-300IU.

**Neutralisation assay.**

Much of the work covered in this thesis regards the use of antibodies or receptor mimics to inhibit HCVcc infection. These treatments were evaluated using an adapted form of the infection assay.

1. Huh-7.5 cells were seeded for infection, as above.
2. To treat virus particles, titrated HCVcc were diluted in DMEM + 3% FCS + P/S containing the appropriate concentration of inhibitory or control compound. Alternatively, to treat target Huh-7.5 cells, culture media was replaced by 80µl of DMEM + 3% FCS + P/S containing the inhibitory or control compound. In either case pre-treatment was for 1 hr at 37°C.
3. To infect, pre-treated virus was used to inoculate naïve Huh-7.5 cells. If pre-treating target cells, 80µl of appropriately diluted HCVcc was added directly to the culture media containing the treatment.
4. In each case the infection was allowed to proceed for 48 hrs. The original virus inoculum and inhibitory treatment were present for the duration of the assay to ensure evaluation of absolute neutralisation titres.

5. Data is expressed as percentage neutralisation; this was calculated by comparison of the level of infection after inhibitory treatment to that after treatment with a control compound.

### **Statistical analysis of infectivity data.**

To ensure appropriate statistical analysis of infection assays we used a D'Agostino & Pearson omnibus normality test to determine whether typical infectivity data fits a Gaussian distribution. Briefly, the data from n=10 JFH-1 and J6/JFH-1 HCVcc infection assays were analysed using GraphPad Prism (GraphPad Software, CA, USA), both data sets were deemed to be normally distributed as summarised in Table 2-5. We therefore have assumed normal distribution for all infectivity data and statistical analyses were performed using an unpaired t-test.

Virus	Mean IU/ml	SD	D'Agostino & Pearson test		
			P value	K2 statistic	Passed test?
JFH-1	2470	1659	0.5383	1.239	Yes
J6/JFH-1	11960	6210	0.258	2.71	Yes

### **Table 2-5 Normality testing.**

Analysis of JFH-1 and J6/JFH-1 infectivity data with the D'Agostino & Pearson omnibus normality test following the guidelines in the GraphPad statistics handbook.

### **Iodixanol density gradient centrifugation.**

The analysis of HCVcc particle density was achieved by iodixanol density gradient centrifugation. This work was carried out in collaboration with Dr Søren Nielsen of Newcastle University Medical School. The preparation and

use of the iodixanol gradient columns was performed by Dr Nielsen, the remaining work was carried out in Birmingham.

1. 50ml of culture medium containing harvested HCVcc particles was concentrated to 1ml in a Vivaspin 20 column with a 100kDa molecular weight cut off (Sartorius, Germany).
2. Linear iodixanol (Axis-Shield, UK) gradients were prepared using a two-chamber gradient maker (Jencons, UK) with light (6%) and dense (56%) iodixanol solutions (233). Gradients were used immediately after preparation and 0.4 ml of concentrated virus loaded onto each gradient.
3. Samples were centrifuged at 100,000xg for 21h at 4°C in a L80-M ultracentrifuge (Beckman, UK), fractions were harvested and their density determined with a digital refractometer (Atago, Japan).
4. RNA was extracted from each fraction using an RNEasy Mini kit (Qiagen) according to the manufacturer's instructions, allowing the analysis of HCV genome distribution throughout the density gradient.
5. HCVcc infectivity within each fraction was determined with a standard infection assay.

### **Quantitative RT-PCR**

Genome copy number within each iodixanol density fraction was measured by quantitative RT-PCR, using a Cells Direct kit (Invitrogen) according to the manufacturer's instructions. In summary.

1. The Cells Direct reaction mixture was made with HCV specific primers (PrimerDesign, UK) and GAPDH house keeping gene control primers

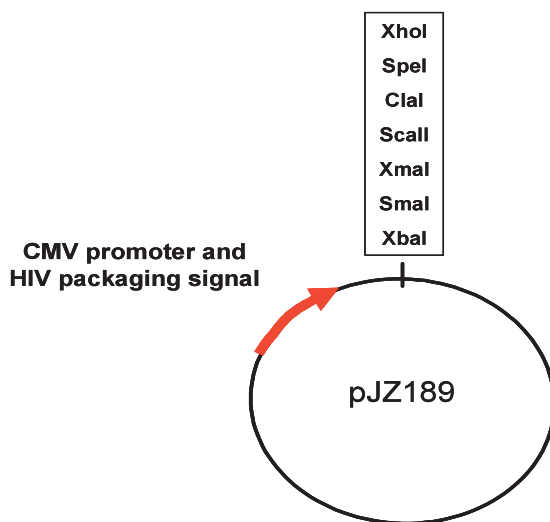
(Invitrogen). The samples to be tested were derived from cell culture media and not cell lysate and do not contain GAPDH mRNA, therefore 0.25µg of HeLa cell RNA (Invitrogen) was added to the mix as a template for the GAPDH control primers.

2. The RT-PCR was carried out in a MicroAmp 96 well optical reaction plate (Applied Biosystems, CA, USA), with samples tested in quadruplicate. A standard curve was made up with in vitro transcribed HCV RNA of known copy number.
3. The reaction was performed in a MX3000P quantitative PCR machine (Stratagene, CA, USA) and the data analysed using the associated MXpro software.

## 2.6 Cloning of *TRIP SR-BII*.

As previously described the TRIP gene vector system can package a target gene for delivery to cultured cells. To create TRIP particles containing a particular gene it is necessary to clone it into a compatible plasmid background. A TRIP plasmid construct must encode an RNA transcript comprised of i) an appropriate promoter, ii) the target gene and iii) a lentiviral packaging signal that drives the transcript's inclusion into budding TRIP particles.

Cloning of TRIP SR-BII was achieved by restriction enzyme excision from a PCDNA3.1 plasmid encoding a fully sequenced human SR-BII cDNA (provided by Dr Jim Owen at University College London). The excised gene was then transfer cloned into an in-house TRIP plasmid, pJZ189, which encodes a packaging signal and a CMV promoter with a downstream polylinker (Figure 2-2).

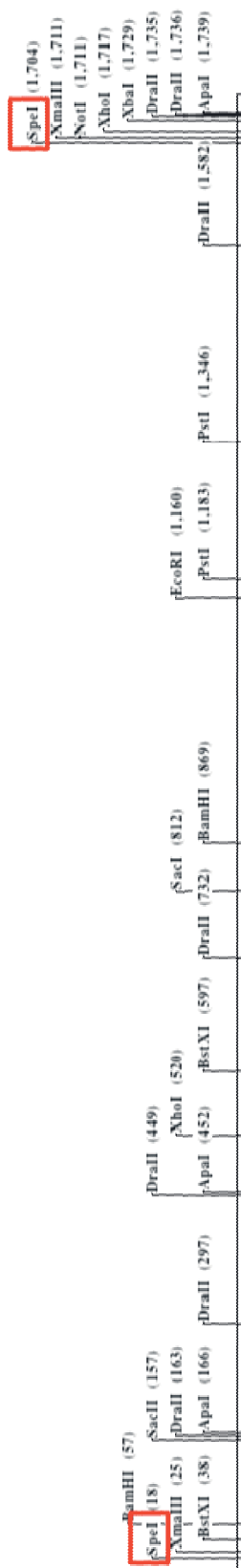


**Figure 2-2 Basic plasmid map of pJZ189.**

Position of promoter, packaging signal and polylinker are shown.



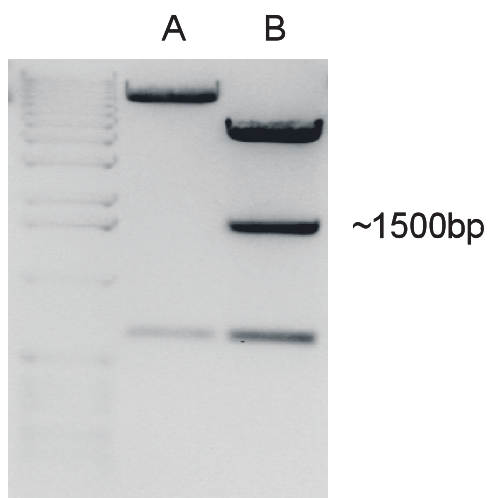
1. The SR-BII coding region was excised from the PCDNA3.1 background using the 3' and 5' flanking *SpeI* sites highlighted in Figure 2-2 .



**Figure 2-2 Restriction Map.**

Restriction map of SR-BII coding sequence, the flanking *SpeI* sites are highlighted in red.

2. The digested DNA was ran on a 2% agarose gel allowing gel purification of the coding sequence using a MinElute gel extraction kit (Qiagen), the excised fragment is shown in Figure 2-2.
3. TRIP pJZ189 was linearised via the XbaI site found within the polylinker (Figure 2-2), creating ends compatible for transfer cloning of the SR-BII coding sequence. The ligation products were then used to transform omniMAX *E.coli* (Invitrogen).
4. As the excised SR-BII sequence had symmetrical 5' and 3' SpeI sites the fragments will have inserted into pJZ189 in both sense and anti-sense orientations. Therefore, clones carrying the correctly orientated open reading frame (ORF) were identified by PCR screening using a forward primer specific to a pJZ189 sequence upstream of the insert and a reverse primer specific to a sequence within the SR-BII insert.
5. Exogenous SR-BII expression efficiency from the selected clones was assessed by transduction of CHO cells with TRIP SR-BII particles, this is documented in section 3.



**Figure 2-2 SR-BII restriction digest.**

PCDNA3.1 SR-BI (A) and PCDNA3.1 SR-BII (B) were digested with SpeI, the excised SR-BII coding sequence is shown at ~1500bp in lane B.

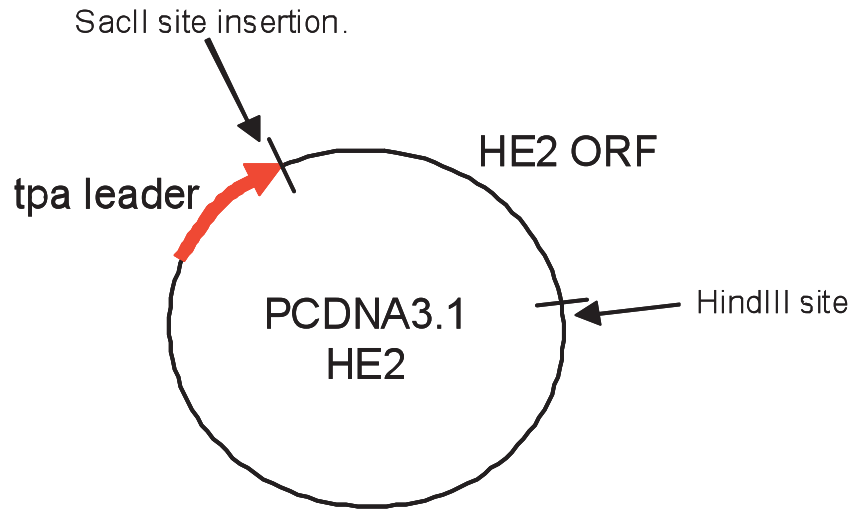
## **2.7 Cloning and synthesis of JFH-1 wt and G451R soluble E2.**

Many early studies into HCV attachment and entry were performed using truncated soluble forms of the E2 glycoprotein (99, 100). These recombinant proteins contain amino acids 384-661 of the HCV polyprotein, a region encompassing all of E2 with the exception of the trans-membrane domain, thus rendering the proteins soluble (99, 100). Our study required the direct examination of JFH-1 wt and G451R glycoprotein–receptor interactions, to achieve this it was necessary to engineer plasmids encoding sE2 for each virus.

Our cloning strategy used an existing PCDNA3.1 plasmid that encodes a strain H sE2 downstream of a tissue plasminogen activator (tpa) leader sequence. When introduced into 293T cells, the tpa leader directs the secretion of synthesised sE2 into the culture media and is therefore an essential element of the construct. By insertion of a silent and unique restriction site at the interface between the tpa leader and sE2 ORF we were able to excise the strain H sE2 sequence and transfer clone JFH-1 sE2 PCR products into the plasmid background. We also introduced a C-terminal epitope tag to the JFH-1 sE2s via an extended reverse primer. This work was carried out partly by Dr Peter Balfe.

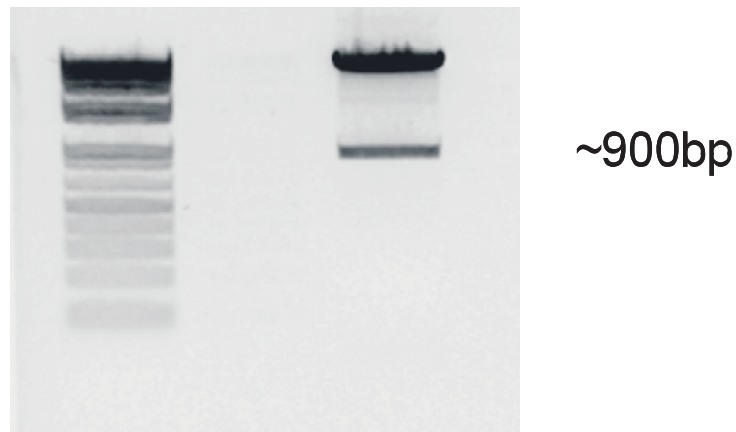
1. A QuikChange II (Stratagene) site directed mutagenesis kit was used to insert a synonymous mutation in the strain H sE2 plasmid at the 3' end of the tpa leader sequence. This alteration created a unique SacII restriction site directly upstream of the H sE2 coding sequence, which

along with a flanking HindIII site was used to excise the strain H sE2 ORF, leaving a linearised PCDNA3.1 + tpa leader background (Figure 2-3). Figure 2-4 displays a gel image of the digested PCDNA3.1 H sE2 plasmid.



**Figure 2-3 Basic plasmid map of PCDNA3.1 H E2.**

The position of the flanking SacII and HindIII sites are shown.



**Figure 2-4 PCDNA3.1 H E2 digest.**

Digested with SacII and HindIII to release the ~900bp H sE2 coding sequence.

The sequence encoding amino acids 384-661 of the HCV polyprotein was PCR amplified from plasmids encoding cDNA copies of the JFH-1 wt and G451R genomes, using the following primers (Invitrogen);

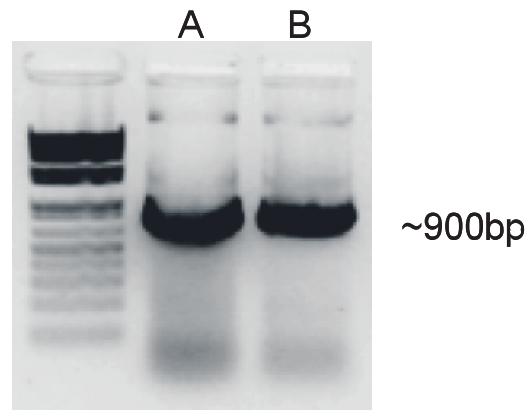
Forward

5'AGCCGCGGAGCCAGATCCGGCA**CCACCACCGTTGGAGGCG**3'

Reverse

5'**GACAGGGACAGGTCCGAGAG**TACTAGTATCCGCGGCAAGGTACAGTG  
AAAGCTTAAGCGCG3' (sequence shown is the reverse complement)

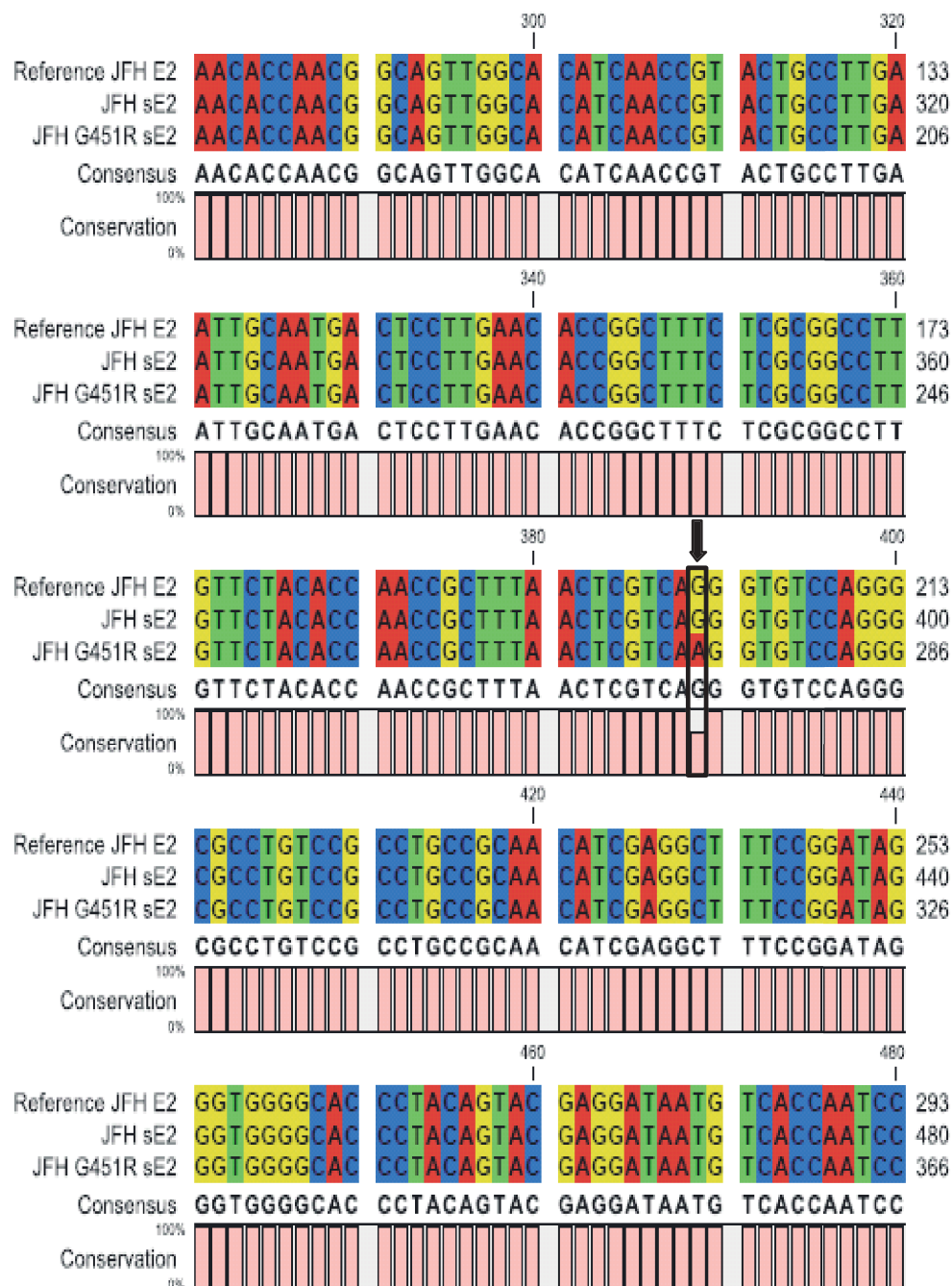
The sections of red text are HCV specific sequences, the bold text indicate the 5' SacII and 3' HindIII sites necessary for transfer cloning, the underlined sequence encodes a HIV gp120 epitope tag (STSIRGKVQ) recognised by mAb 10/76B. Figure 2-5 displays the ~900bp PCR product amplified from JFH-1 wt and G451R plasmids.



**Figure 2-5 sE2 coding region PCR.**

JFH-1 (A) and JFH-1 G451R (B) PCR products encoding amino acids 384-661 of the HCV polyprotein.

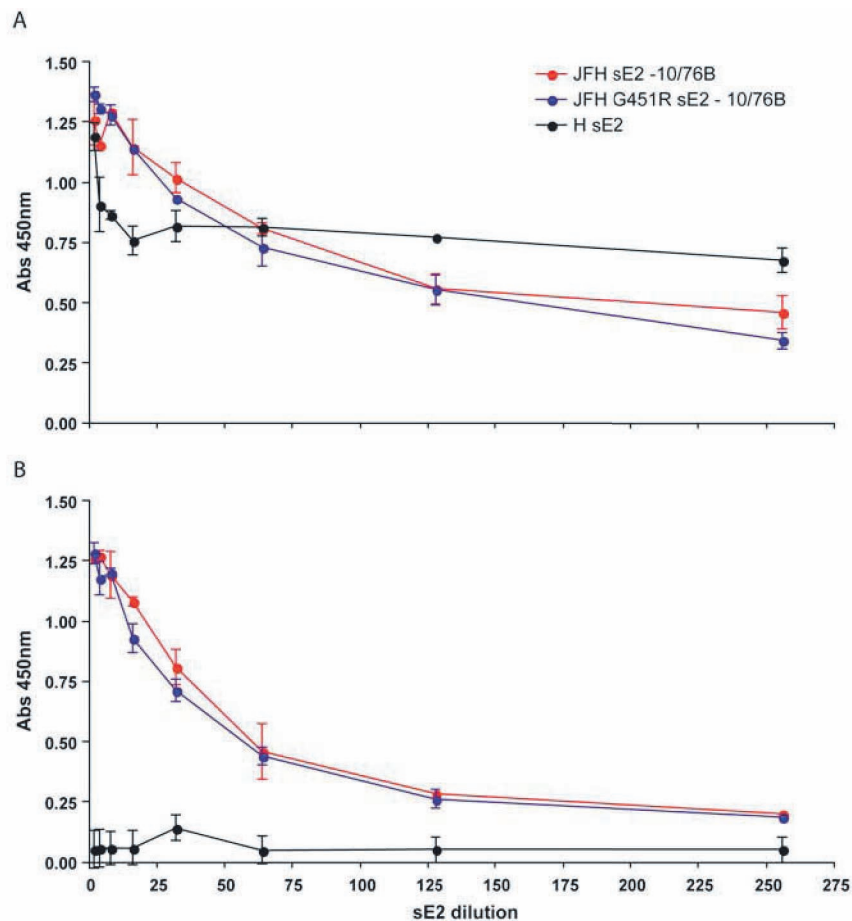
2. The PCR products were gel purified and ligated into the pGEM-T easy plasmid background (Promega) to allowing transformation of *E.coli*.
3. Clones carrying the correct insert were identified by PCR screening using a pGEM-T easy specific forward primer and HCV specific reverse primer. The selected clones were sequenced and subsequently digested with SacII and HindIII for transfer into the PCDNA3.1 + tpa leader background.
4. Candidates recovered from the transfer cloning were PCR screened and sequenced once more prior to evaluating their protein expression. Figure 2-6 displays a sample of the sequencing data obtained; the G451R point mutation is highlighted.



**Figure 2-6 sE2 sequencing.**

JFH-1 and JFH-1 G451R sE2 sequencing data was compared to a reference JFH-1 E2 sequence (accession number AB047639) using the CLC Workbench software (CLCBio, Denmark). The G451R point mutation is highlighted.

5. To investigate whether the selected clones produced sE2, flasks of 293T cells were transfected with 10 $\mu$ g of each plasmid using the Profection kit (as documented earlier). Supernatant containing secreted protein was harvested at 48 hrs post transfection.
6. Supernatants were tested using a GNA capture ELISA (as documented earlier). Figure 2-7 displays the detection of recombinant JFH-1 wt and G451R sE2 by anti-E2 mAb 3/11 and anti-tag mAb 10/76B, untagged strain H sE2 was used as a control.

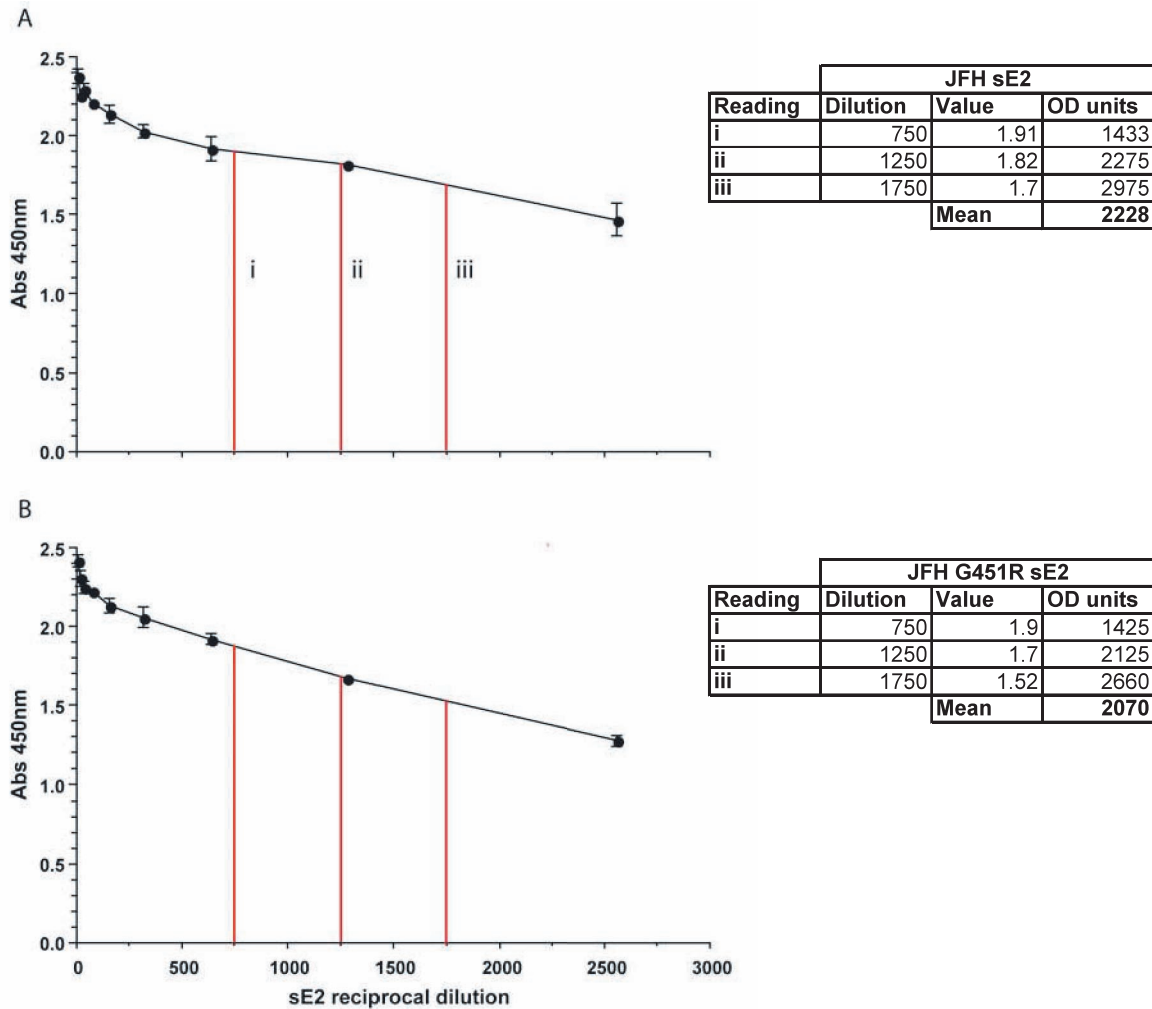


**Figure 2-7 Expression of sE2.**

JFH-1 (red line), JFH-1 G451R (blue) and untagged strain H (black) sE2s were immobilised in an ELISA plate by GNA capture. Bound glycoprotein was detected with 1 $\mu$ g/ml anti E2 mAb 3/11 (A) or anti-tag mAb 10/76B (B). Error bars indicate standard deviation from the mean n=3.



7. Large scale transfections were carried out to produce sufficient sE2 for investigation of glycoprotein-receptor interactions. The involvement of lipoproteins was eliminated by culturing the transfected 293T cells in media containing de-lipidated FBS, supplied by Dr Søren Nielsen of Newcastle University.
8. 50ml of harvested supernatant containing JFH-1 wt and G451R sE2 was concentrated to 2.5ml using Vivaspin 20 columns with a 10kDa molecular weight cut off (Sartorius). The relative concentration of each preparation was assessed by GNA capture ELISA using the anti-tag mAb 10/76B, Figure 2-8 details the calculation of either glycoprotein's concentration, expressed as relative OD units.
9. We assessed sE2-receptor interactions using the cell based E2 binding assay and CD81 capture ELISA (both documented earlier), in each case protein concentrations were matched by appropriate dilution.



**Figure 2-8 Titration of sE2.**

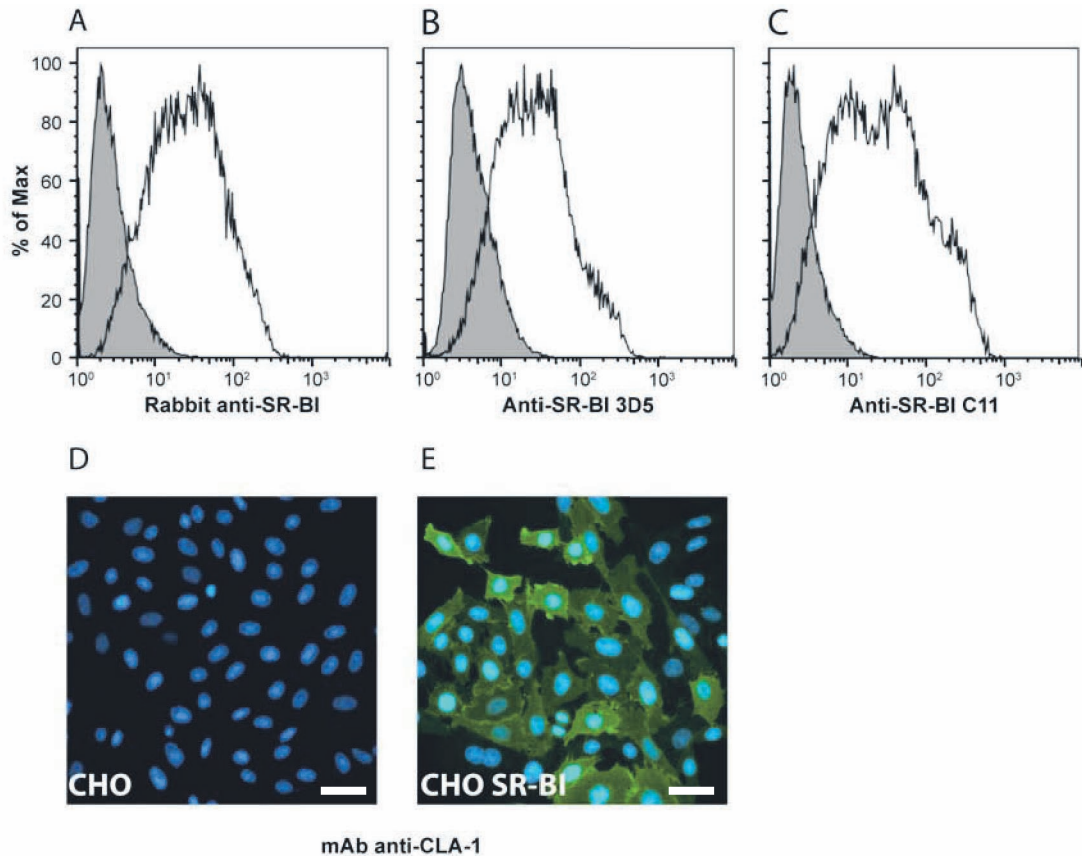
Serial dilutions of JFH-1 (A) and JFH-1 G451R (B) sE2 were captured in a GNA ELISA, bound glycoprotein was detected with 1 $\mu$ g/ml anti-tag mAb 10/76B. The relative concentration of each sE2 was assessed by calculation of OD units; three readings were taken from a linear section of each curve (at 1/750, 1250 and 1750). OD units were calculated by multiplying the absorbance by the reciprocal dilution for each reading. Comparison of the mean OD units for either glycoprotein suggests that JFH-1 sE2 is ~8% more concentrated than JFH-1 G451R sE2, therefore JFH-1 sE2 was diluted appropriately before use in the assays presented in the results section.

### **3 Results: Investigations using CHO cell expressed SR-BI.**

The work documented in this thesis is reliant on a number of techniques and reagents, this chapter covers the validation of these tools along with studies using a panel of SR-BI mutants and the splice variant SR-BII.

#### **3.1 *Expression of SR-BI in CHO cells.***

To investigate the interaction of HCV glycoproteins with SR-BI it was necessary to identify a cell background lacking functioning viral receptors. Hamster derived CHO cells do not bind HCV sE2 (274) and it has been shown that SR-BI expressed in a CHO cell is functional with regards to lipid uptake and trafficking (239, 259, 349). We therefore began work with a CHO cell line stably expressing human SR-BI. We assessed SR-BI expression in CHO cells using a panel of anti-SR-BI antibodies by flow cytometry and immunofluorescent microscopy (Figure 3-1). Figure 3-1 A-C show live cell staining of CHO-SRBI and represent plasma membrane SR-BI expression.

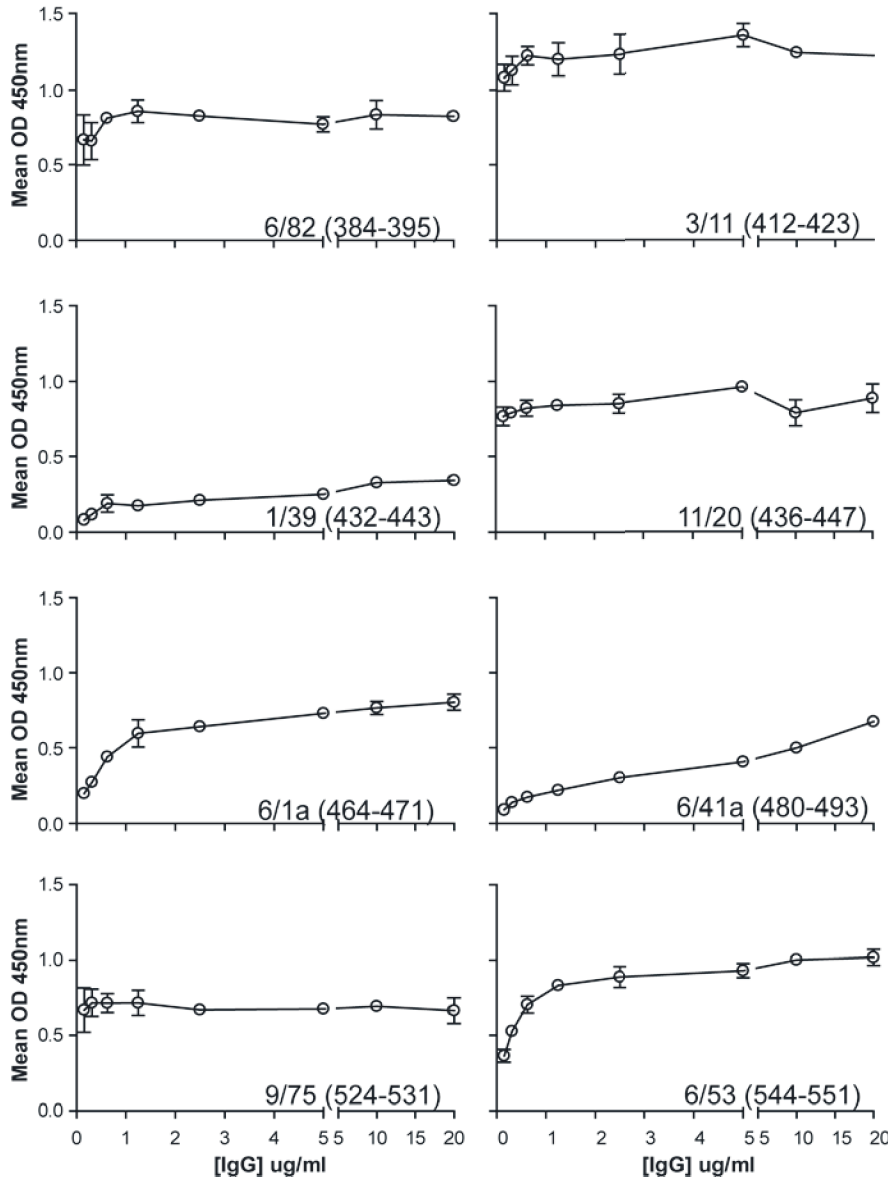


**Figure 3-1 Expression of SR-BI on CHO cells.**

CHO cells (filled histogram) or those stably expressing human SR-BI (unfilled histogram) were analysed by flow cytometry after live cell surface staining using 1/500 rabbit anti-SR-BI sera (**A.**), 1 $\mu$ g/ml mouse anti-SR-BI mAb 3D5 (**B.**) or 1 $\mu$ g/ml human anti-SR-BI mAb C11 (**C.**). Methanol fixed parental CHO (**D.**) or those expressing SR-BI (**E.**) were stained using 1 $\mu$ g/ml mAb anti-CLA1 (SR-BI). The images were taken at X200 magnification, scale bars represent 20 $\mu$ m.

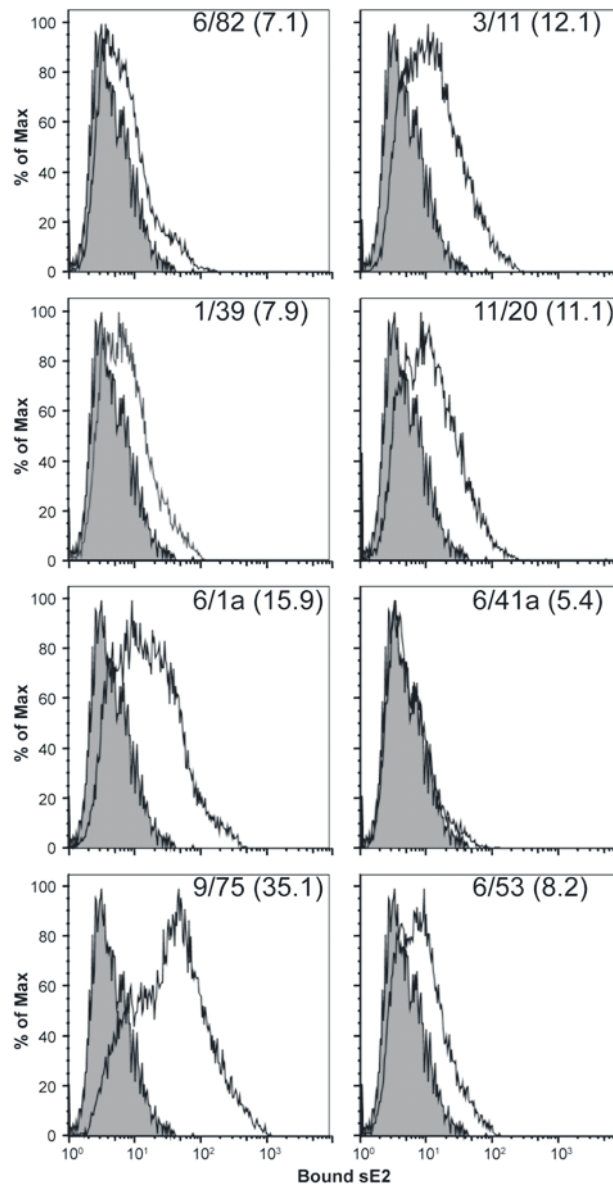
### ***3.2 CHO cells expressing human SR-BI bind soluble HCV E2 glycoprotein.***

Previous studies have established an interaction between soluble HCV E2 and human SR-BI (274), we aimed to recapitulate these findings in our CHO-SR-BI background. Flint et. al. demonstrated limited availability of epitopes on sE2 when bound to CD81 (99), we were interested to identify the epitopes exposed on envelope protein when bound to SR-BI. Eight anti-E2 mAbs specific for non-overlapping epitopes between residues 384-551 of the HCV polyprotein were prepared as documented in section 2. Immunoglobulins isolated from these supernatants were assessed for their reactivity with immobilised strain HCV-1 sE2 by ELISA (Figure 3-2). All of the mAbs bound sE2 and demonstrated a range of relative binding affinities. To investigate the availability of mAb epitopes on E2 bound to SR-BI, CHO-SR-BI cells were incubated with sE2 and the bound protein detected with the mAbs by flow cytometry (Figure 3-3). Recombinant HCV glycoprotein did not bind to parental CHO cells (Figure 3-4) Most of the mAbs were able to detect cell bound E2, however mAb 9/75 recognised sE2 with the highest fluorescence. mAb 9/75 is specific for residues 524-531 of E2 (130), a region reported to be involved in CD81 engagement (81). To further quantify the glycoprotein binding capacity of CHO-SR-BI cells, we performed a titration of sE2 from 1-50µg/ml, bound glycoprotein was detected with a known excess of mAb 9/75. Envelope protein binding to CHO-SR-BI cells reached saturation at 30µg/ml sE2 (Figure 3-4B).



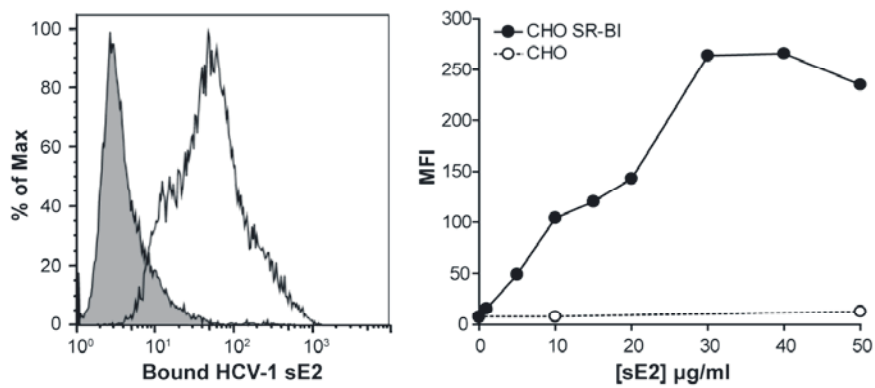
**Figure 3-2 Activity of rat anti-E2 monoclonal antibodies.**

To test the reactivity of a panel of anti-E2 mAb IgG, strain HCV-1 soluble E2 (0.5ug/ml) was captured onto GNA lectin coated ELISA plates. Each mAb was assessed for its ability to bind immobilised sE2, bound IgG was detected using an anti-rat HRP conjugated secondary. The signal recorded for mAb binding in the absence of E2 has been subtracted for each plot. The name of each mAb and its epitope is shown. Although each mAb recognised HCV-1 E2 there was a range of relative binding affinities. An irrelevant rat IgG control gave a signal of 0.05 ( $\pm$ 0.01) at 20 $\mu$ g/ml (data not shown). Error bars indicate standard deviation (n=3).



**Figure 3-3 Ability of anti-E2 mAb to recognise SR-BI bound E2.**

To determine which mAb is most suitable for detecting sE2 bound to SR-BI, parental CHO cells or those expressing SR-BI were incubated with strain HCV-1 sE2 at 5µg/ml. Bound sE2 was detected using the panel of anti-E2 mAbs at 1µg/ml and an anti-rat fluorescently conjugated secondary. The cells were analysed by flow cytometry. The panels show the detection of sE2 bound to CHO SR-BI cells by each mAb (unfilled histogram) and an irrelevant rat mAb (filled histogram). The name of each mAb is shown along with geometric mean fluorescent intensity (GMFI) in brackets. Optimal detection of sE2 bound to SR-BI was achieved with mAb 9/75. sE2 did not bind to parental CHO cells, giving GMFI=3.6 when detected using 9/75 (data not shown).



**Figure 3-4 Soluble HCV E2 binding to cell expressed SR-BI.**

**A.** CHO (filled histogram) or CHO-SR-BI (unfilled histogram) cells were incubated with 5 µg/ml strain HCV-1 sE2 for 1 hr. Bound sE2 was detected by flow cytometry using rat anti-E2 mAb 9/75 (5 µg/ml) and a fluorescently conjugated secondary. **B.** Titration of sE2 binding; saturation of E2 binding to CHO-SR-BI cells (closed circles) was achieved at 30 µg/ml sE2. No sE2 binding to CHO cells (open circles) could be detected.



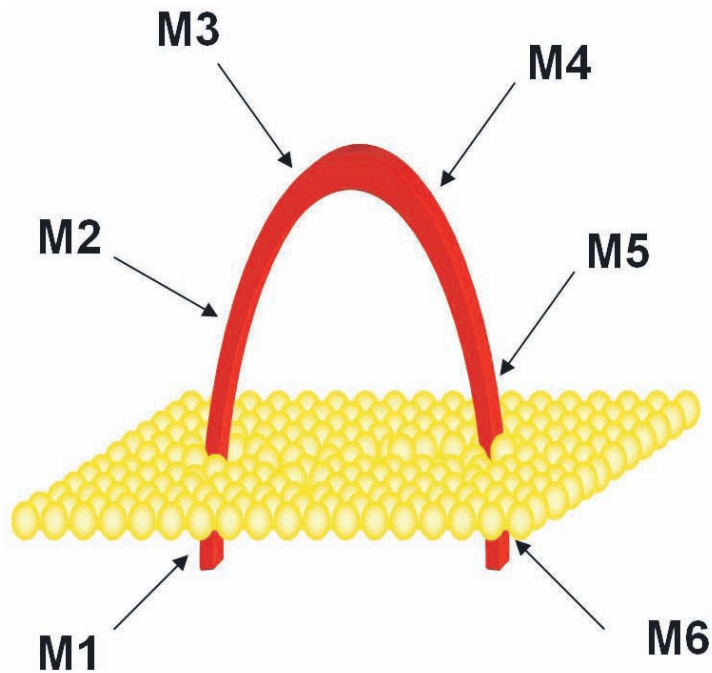
### **3.3 *The interaction of sE2 with a panel of SR-BI mutants.***

Lipoproteins are heavily implicated in HCV-SR-BI interactions; native HCV has been proposed to interact with SR-BI via associated apoproteins (190) and HDL enhances HCV infection in a process requiring selective cholesterol uptake by SR-BI (25, 79, 324). We were therefore interested to investigate whether mutations that perturb SR-BI interactions with lipoproteins affect envelope protein binding. We used a panel of 6 SR-BI mutants expressed in CHO cells, provided by our collaborator Thierry Huby at INSERM in Paris. Of the 6 mutants, 2 are unable to mediate cholesterol transfer (121), 1 bears a common human polymorphism linked to alterations in plasma lipid levels (1) and 3 carry mutations in alpha-helical domains believed to be important for SR-BI conformation (Figure 3-5 and Table 3-1).

We hypothesised that one or more of the mutations would modulate the binding of HCV sE2 and that this may correlate with the mutant's ability to exchange cholesterol with HDL. To test this we performed SR-BI staining and sE2 binding assays in parallel. A saturating concentration of sE2 was used to ensure detection of any changes in glycoprotein binding to the mutants. We observed variation in the cell surface expression of the mutants, in each case sE2 binding was proportional to surface SR-BI levels, this suggests that the mutations do not modulate sE2 interaction (Figure 3-6).

Anti-SR-BI mAbs 3D5 and C11 have been previously shown to inhibit HCVcc infection, sE2 binding to CHO-SR-BI cells and SR-BI mediated cholesterol

transport from HDL (60). The epitopes recognised by these antibodies have yet to be identified, we therefore studied their binding to the panel of SR-BI mutants. We would expect that if a mutation falls within a mAb epitope it would perturb its binding. Table 3-2 displays the geometric mean fluorescent intensity (GMFI) of polyclonal SR-BI, sE2, mAb 3D5 and C11 bound to each of the mutants. Figure 3-7 displays the data expressed as a function of cell surface SR-BI expression. For each reagent tested, GMFI was proportional to mutant SR-BI cell surface expression, indicating that the mutations had not altered interaction with mAb or E2.



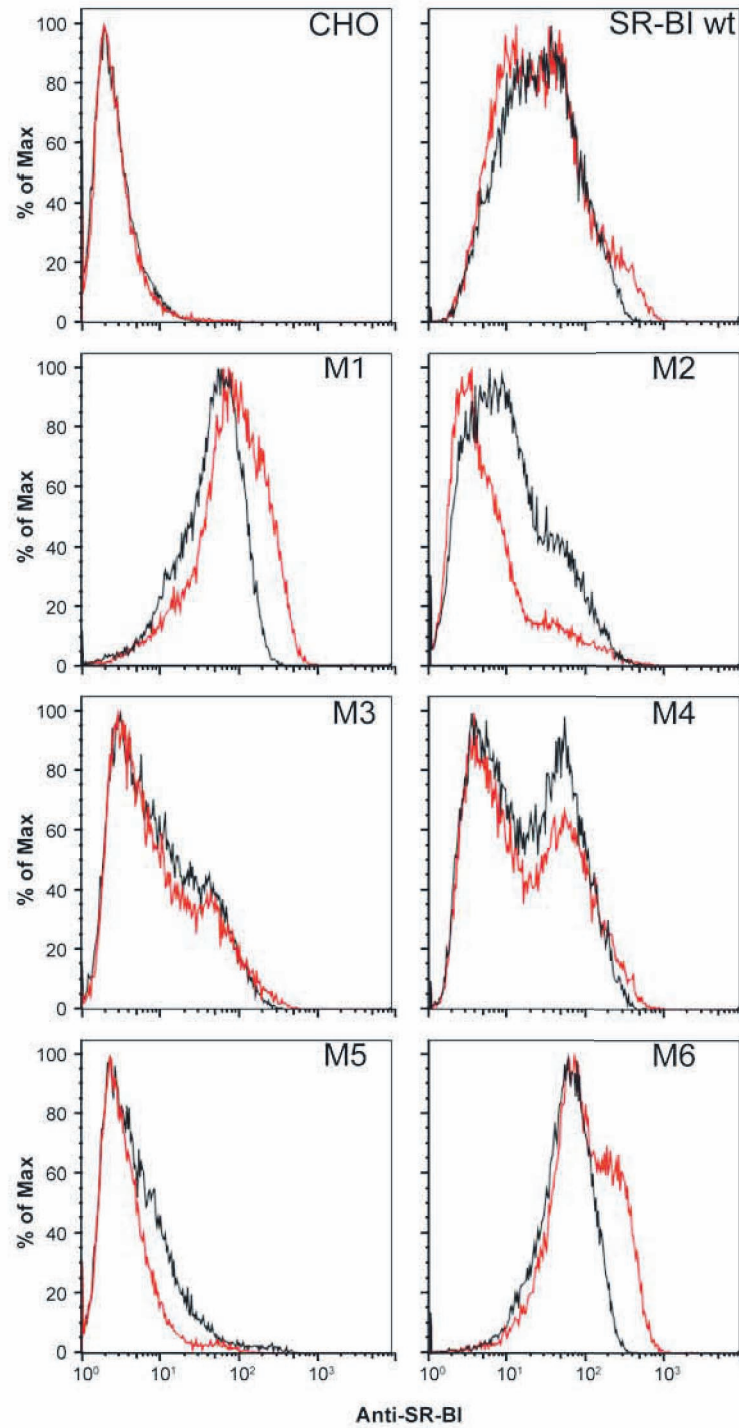
**Figure 3-5 Position of SR-BI mutations.**

A schematic representation of SR-BI displaying the mutations listed in Table 3-1.

<i>Name</i>	<i>Mutation/Position</i>	<i>Target of mutation</i>	<i>Cholesterol transfer</i>
<b>M1</b>	G2S	Human polymorphism	Positive
<b>M2</b>	I158R	Critical for HDL binding	Negative
<b>M3</b>	N189R L190R	Alpha-helix	Positive
<b>M4</b>	Q365R E366R	Alpha-helix	Positive
<b>M5</b>	Q402R E418R	Critical for HDL binding	Negative
<b>M6</b>	I147R Q488R	Alpha-helix	Positive

**Table 3-1 SR-BI mutations.**

The amino acid change and position of each mutation. M1 targets a known human SR-BI polymorphism, M2 and M5 target residues reported to prevent HDL binding, M3, M4 and M6 are in predicted alpha-helical domains. M2 and M5 are unable to mediate cholesterol transfer.



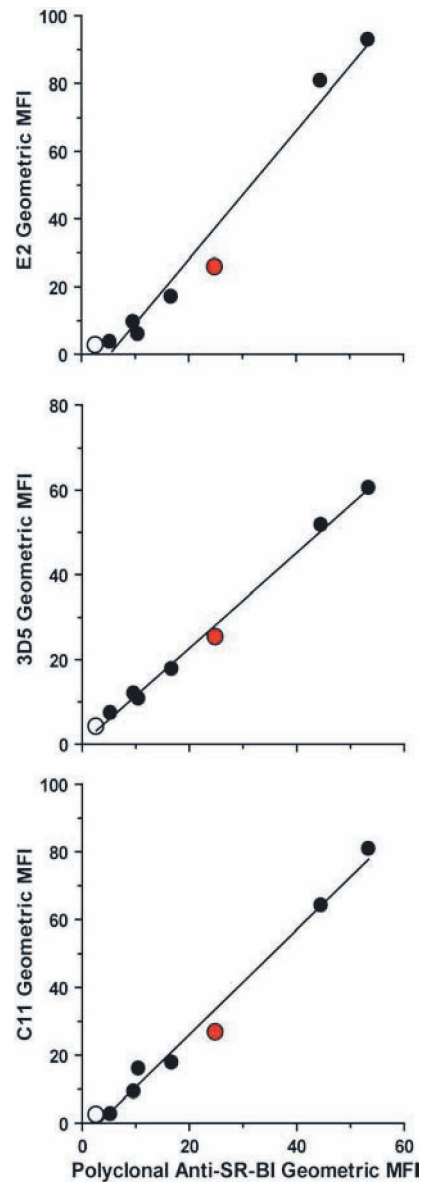
**Figure 3-6 Expression of SR-BI mutants in CHO cells.**

CHO cells stably transfected to express a panel of SR-BI mutants were analysed for expression (black histogram) and ability to bind HCV-1 sE2 (30 µg/ml) (red histogram). Panels indicates the name of each mutant.

<i>Cell</i>	<i>Mutation</i>	<i>Geometric mean fluorescent intensities</i>				
		<i>Rabbit -ve</i>	<i>Anti-SR-BI</i>	<i>HCV-1 sE2</i>	<i>3D5</i>	<i>C11</i>
<b>CHO</b>	<b>N.A.</b>	2.81	2.65	2.73	4.17	2.48
<b>SR-BI wt</b>	<b>wt</b>	3.85	24.87	25.88	25.38	26.77
<b>M1</b>	<b>G2S</b>	4.78	44.52	80.80	51.79	64.17
<b>M2</b>	<b>I158R</b>	2.82	10.50	6.06	10.81	16.06
<b>M3</b>	<b>N189R L190R</b>	2.92	9.62	9.65	12.06	9.26
<b>M4</b>	<b>Q365R E366R</b>	3.85	16.68	17.08	17.85	17.84
<b>M5</b>	<b>Q402R E418R</b>	3.11	5.28	3.75	7.42	2.71
<b>M6</b>	<b>I147R Q148R</b>	3.64	53.37	92.88	60.57	80.91

**Table 3-2 Investigation of cell expressed SR-BI mutants.**

The table displays the binding of rabbit pre-immune serum (1/500), anti-SR-BI serum (1/500), sE2 (30µg/ml), mAb 3D5 (5µg/ml) and mAb C11(5µg/ml) to CHO cells expressing wt or mutant SR-BI. Binding was assessed by flow cytometry and the data shown are GMFIs.

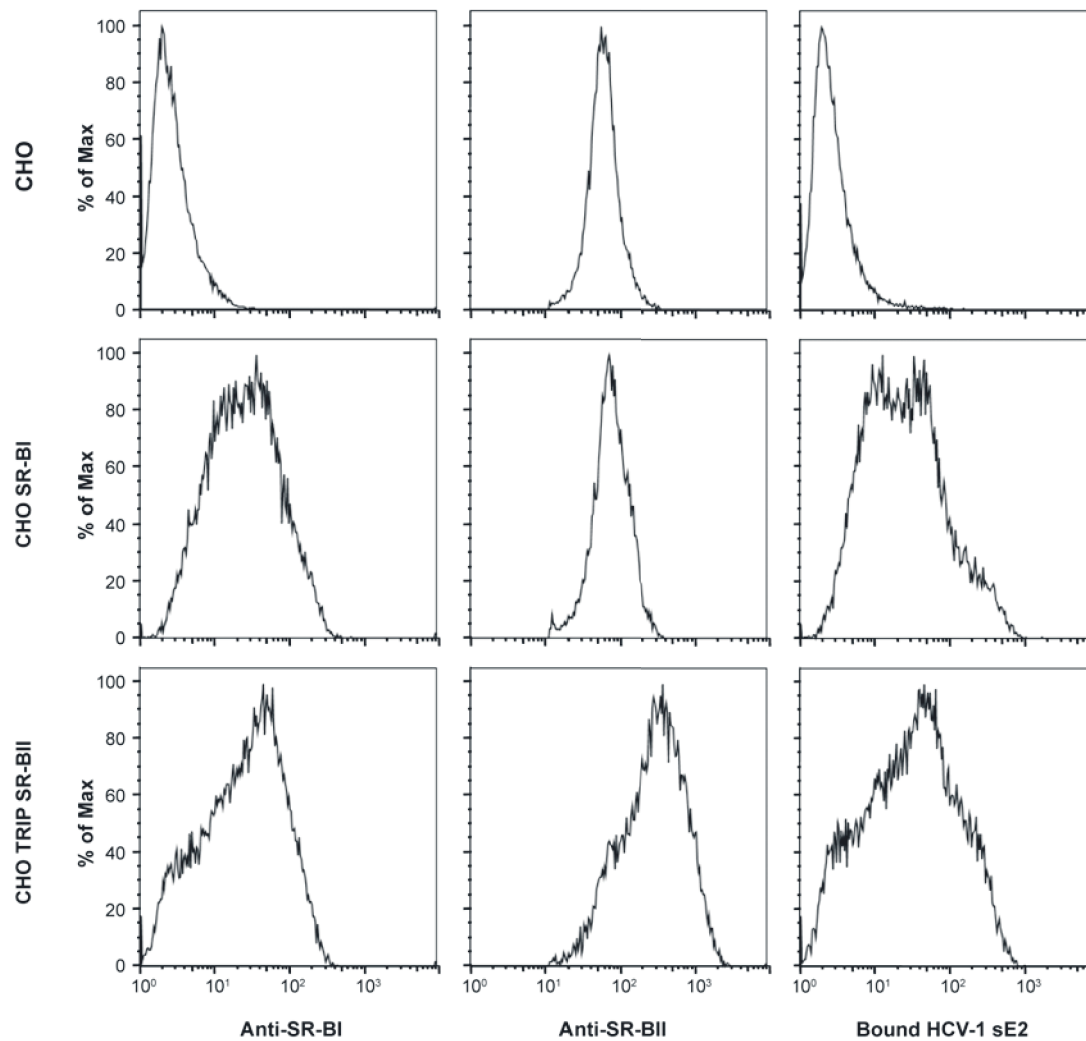


**Figure 3-7 Antigenic characterisation of SR-BI mutants.**

Using the GMFI data shown in Table 3-2, the binding of **A.** sE2, **B.** mAb 3D5 and **C.** mAb C11 to the SR-BI mutants was expressed as a function of surface SR-BI expression: assessed with polyclonal SR-BI sera. Each plot shows parental CHO cells (unfilled circle), those expressing wt SR-BI (red circle) and the SR-BI mutants (filled circles). For each mutant the binding of sE2, 3D5 and C11 was proportional to surface SR-BI expression, suggesting that the mutations do not modulate E2 or antibody recognition. This data set is representative of multiple experiments.

### **3.4 Expression of SR-BII in CHO cells.**

SR-BII is a variant of SR-BI that differs in its C-terminal intracellular region. It is believed to bind all SR-BI ligands, however in rodent livers it has been reported to be expressed predominantly intracellularly, it traffics via clathrin coated vesicles (88, 89, 223, 330). We aimed to investigate whether SR-BII can function as a receptor for HCV entry by generating TRIP lentiviral vectors (347) encoding SR-BII (documented in section 2), allowing exogenous expression in CHO cells. Figure 3-8 displays the binding of polyclonal anti-SR-BI serum and sE2 to parental CHO, CHO-SR-BI and CHO TRIP SR-BII. The anti-SR-BI serum is directed against the extracellular region of the molecule and is cross reactive for SR-BII; cell surface expression of SR-BI and II were comparable. A polyclonal anti-serum specific for the C-terminal region of SR-BII was not reactive with CHO or CHO-SR-BI cells, indicating specific transduction with the TRIP lentivirus vector. Both receptors bound sE2, suggesting that SR-BI and II share HCV envelope protein as a ligand.



**Figure 3-8 Expression of SR-BII in CHO cells.**

CHO cells were transduced with a TRIP lentivirus vector expressing human SR-BII. CHO, CHO-SR-BI and CHO TRIP SR-BII cells were evaluated for their ability to bind rabbit anti-SR-BI serum (1/500), rabbit anti-SR-BII serum (1/100) and HCV-1 sE2 (30 $\mu$ g/ml). Anti-SR-BI bound to CHO SR-BI/II cells, anti-SR-BII sera bound specifically to CHO TRIP SR-BII cells. SR-BII confers sE2 binding to CHO cells.



### **3.5 Discussion.**

We have demonstrated that expression of SR-BI in a CHO cell background permits the examination of HCV glycoprotein-SR-BI interactions in isolation from other receptors such as human CD81. This is consistent with reported findings (274). Using a panel of anti-E2 mAbs specific for non-overlapping, consecutive epitopes we were able to determine epitope availability on glycoprotein bound to SR-BI. mAb 9/75 recognised sE2 bound to SR-BI with the highest signal, however mAbs 3/11, 11/20 and 6/1a also detected glycoprotein (Figure 3-3). Interestingly, the epitopes recognised by three of these antibodies (9/75, 3/11 and 11/20) are critical for E2-CD81 interactions (81, 99, 237), suggesting that interaction of E2 with SR-BI may not preclude CD81 engagement.

SR-BI mediated lipoprotein metabolism has been implicated in HCV attachment and entry (25, 210, 324, 326). We therefore investigated the binding of sE2 to a panel of SR-BI mutants, some of which are unable to mediate cholesterol exchange with HDL. sE2 binding to these mutants was proportional to their cell surface expression, indicating that SR-BI-E2 interactions were unaltered (Figure 3-6 and 3-7) mAbs 3D5 and C11 have previously been shown to inhibit HCVcc infection and SR-BI mediated cholesterol exchange with HDL (60). We attempted to map amino acid residues that are important for mAb binding by monitoring their binding to the panel of SR-BI mutants. Again, binding was proportional to surface expression, suggesting that the mutations did not modulate antibody binding (Figure 3-7 & Table 3-2).

It would be more appropriate to measure mutant SR-BI receptor activity using an in vitro model of viral entry such as the HCVpp system. We therefore attempted to render CHO-SR-BI cells permissive to HCVpp infection by exogenous expression of CD81 and CLDN1. These cells were readily infected by control pseudo-particles expressing VSV glycoproteins, indicating that CHO cells support the expression of the GFP reporter gene, however HCVpp failed to infect the cells (data not shown). This suggests that either the HCV receptors are inappropriately expressed in a CHO cell background or further co-receptor(s) await identification.

In the time since this work was completed reports have indicated that residues 119, 153, 163 and 168 of the SR-BI extracellular loop may be important for E2 interaction (J.Bwanali et. al. 14th International Symposium on HCV and personal communication); the mutants used in this study do not cover these sites. These putative E2 binding residues are not currently believed to be involved in the interaction of SR-BI with any of its natural ligands, however it would be interesting to study whether SR-BI remains biologically functional when engaged by HCV.

A recent publication on Plasmodium infection of hepatocytes suggests that SR-BI mediated delivery of cholesterol directly to the plasma membrane alters CD81 localisation, resulting in increased Sporozoite invasion (339). The two SR-BI mutants unable to mediate selective cholesterol transfer (Table 3-1),

may offer a tool to study the importance of cholesterol delivery for HCV infection and are worthy of re-investigation.

We successfully generated TRIP viral vectors capable of transducing CHO cells to express human SR-BII. Exogenous protein expression was monitored with SR-BI/II specific antibodies and CHO-SR-BII cells bound sE2 (Figure 3-8), indicating that SR-BII may act as a HCV entry receptor. Validation of the TRIP vectors, allowed us to use them in studies to investigate the role of SR-BI/II in HCV entry.

## **4 Results: Scavenger Receptor BI and BII Expression Levels Modulate HCV Infectivity.**

We aimed to use the tools developed during the previous chapter to investigate the importance of SR-BI/II in HCV infection. To address this it was necessary to carry out work using the recently developed HCVcc system.

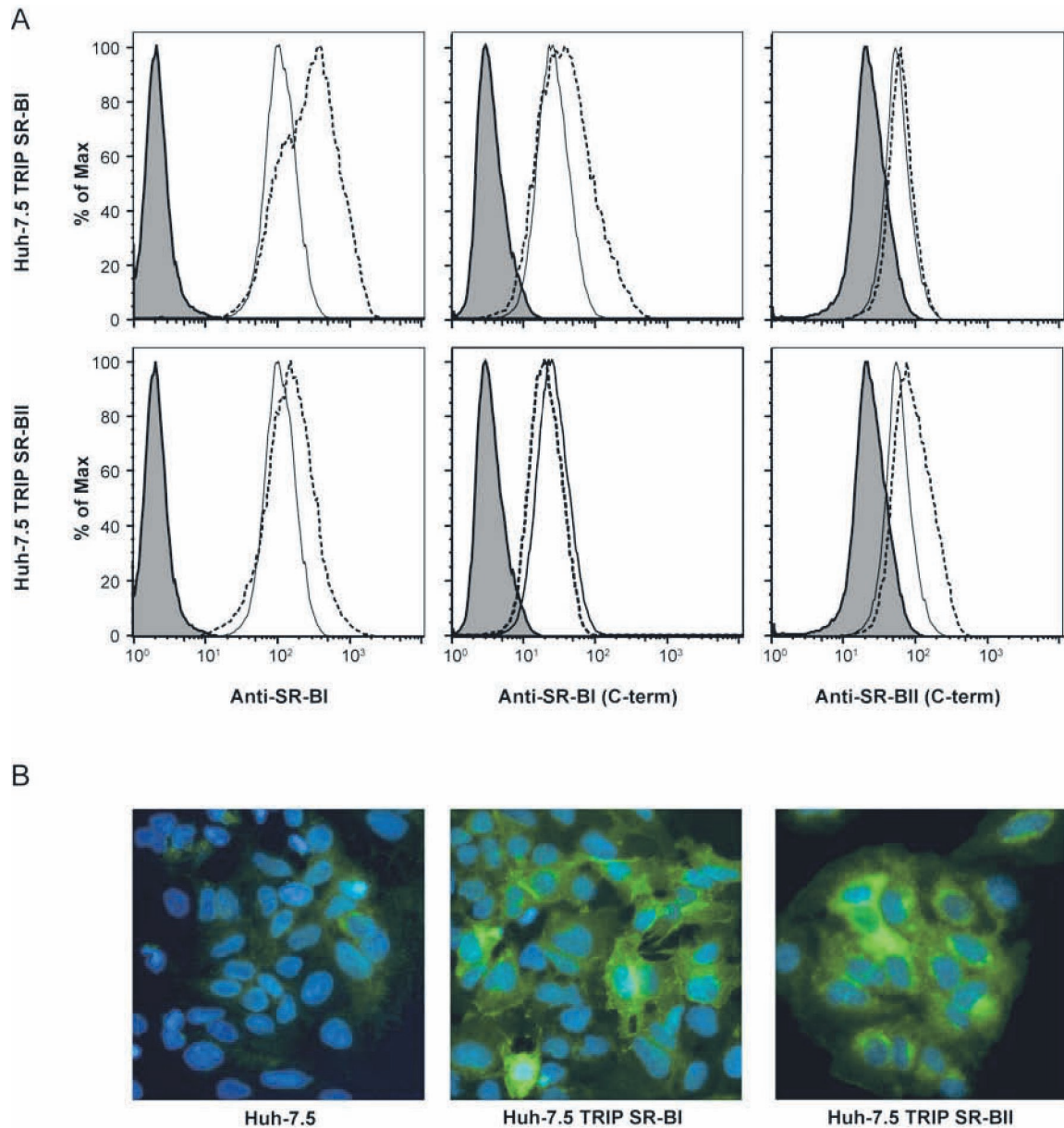
### **4.1 *Over expression of SR-BI/II in Huh-7.5 cells.***

Huh-7.5 cells are a human hepatoma cell line isolated by Keril Blight and co-workers that are highly permissive to HCV replication (37). They contain a mutation in the retinoic acid-inducible gene I, which encodes a protein responsible for detection of intracellular viral RNA, an important component in the Interferon induction pathway (299, 345). As a result HCVcc can establish a robust and prolonged infection of these cells.

Koutsoudakis et. al. established that HCV infection of Huh-7.5 cells is dependent on a critical level of CD81 expression and that susceptibility to HCV increased at higher CD81 density (159). We aimed to investigate whether this was the case for SR-BI. However, at the present time there are no human hepatoma cell lines that do not express SR-BI. Moreover, our attempts to silence SR-BI using siRNA targeting have been unsuccessful (data not shown) and previous studies suggest that it is an inappropriate tool for investigating HCV entry (171, 326). Therefore to study HCVcc dependence on SR-BI/II expression levels we employed the TRIP lentivirus system, validated earlier (page 105), to over express SR-BI and II in Huh-7.5 cells.

Figure 4-1A displays endogenous and exogenous expression of SR-BI in Huh-7.5 cells. Surface SR-BI/II expression was determined on live cells using anti-serum directed against their common extracellular loop; transduction with TRIP SR-BI increased plasma membrane expression by ~3 fold, lentivirus encoding SR-BII led to only ~2 fold increase (MFIs; Huh-7.5 =115, TRIP SR-BI = 350, TRIP SR-BII = 213). Staining of fixed and permeabilised cells with antibodies directed against the differential C-terminal domains of SR-BI and II demonstrated that transduction by either respective TRIP vector was specific. These anti-sera indicate that total SR-BI and II expression were increased two fold in either cell line, suggesting comparable levels of transduction, therefore the lower SR-BII surface levels probably reflect a more intracellular expression pattern, as would be expected in a hepatoma cell line. This is supported by images shown in Figure 4-1B; Huh-7.5 TRIP SR-BI cells display cell junctional staining indicating surface expression, whereas the TRIP SR-BII cells show a diffuse staining throughout the cytoplasm.

As a control Huh-7.5 cells were transduced to over express CD9, a tetraspanin thought to play no role in HCV infection, these cells display a ~13 fold increase in CD9 expression (data not shown). CD81 levels, cell viability and proliferation remained unaltered in each of the Huh-7.5 TRIP cell lines (data not shown).

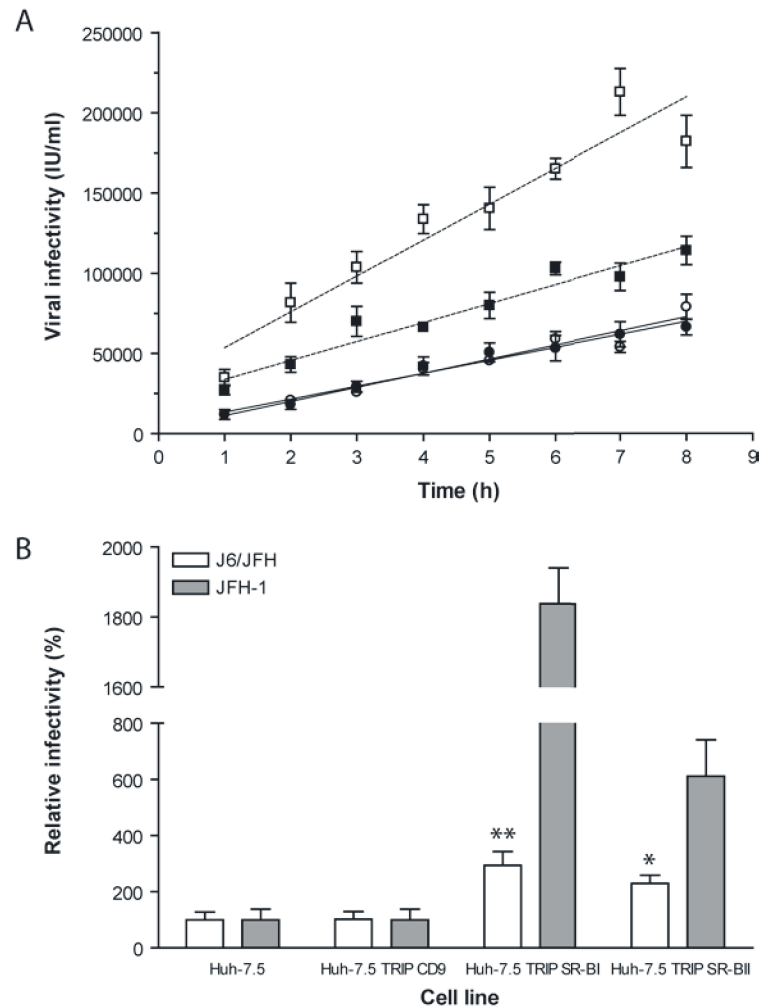


**Figure 4-1 Over expression of human SR-BI/II in Huh-7.5 cells.**

**A.** SR-BI/II expression was determined by flow cytometry using extracellular (EC) domain specific anti-SR-BI serum (on live cells) and C-terminus specific antisera capable of discriminating between SR-BI and SR-BII (on fixed and permeabilized cells). Each panel displays the isotype control (filled), parental (solid line), and transduced (dashed line) Huh-7.5 cells. **B.** Huh-7.5 TRIP cell lines were methanol fixed and stained with an SR-BI/II EC domain specific mAb suitable for immunofluorescence (mAb anti-Cla-1), images were taken at X200 magnification.

#### **4.2 Exogenous expression of SR-BI/II in Huh-7.5 cells enhances HCVcc infection.**

To investigate the effect of increased SR-BI/II surface expression on HCV infection, the Huh-7.5 TRIP cells were inoculated with J6/JFH virus (179) for 1-8 hrs followed by 72 hrs infection (Figure 4-2A). Infection of Huh-7.5 TRIP SR-BI and BII was enhanced by ~3 fold and ~2 fold respectively at all inoculation time points, suggesting that SR-BI/II surface expression limits HCVcc infection. To study intra-genotype dependence on SR-BI/II, parallel infections with J6/JFH and JFH-1 were carried out (Figure 4-2B). In contrast to J6/JFH, JFH-1 infection increased ~18 and ~6 fold in Huh-7.5 cells over expressing SR-BI and II, respectively. This implies that JFH-1 has a higher dependence on SR-BI/II than J6/JFH chimeric virus. Over-expression of CD9 did not enhance the infection of either genotype.



**Figure 4-2 Over expression of SR-BI and SR-BII in Huh-7.5 cells enhances HCVcc infection.**

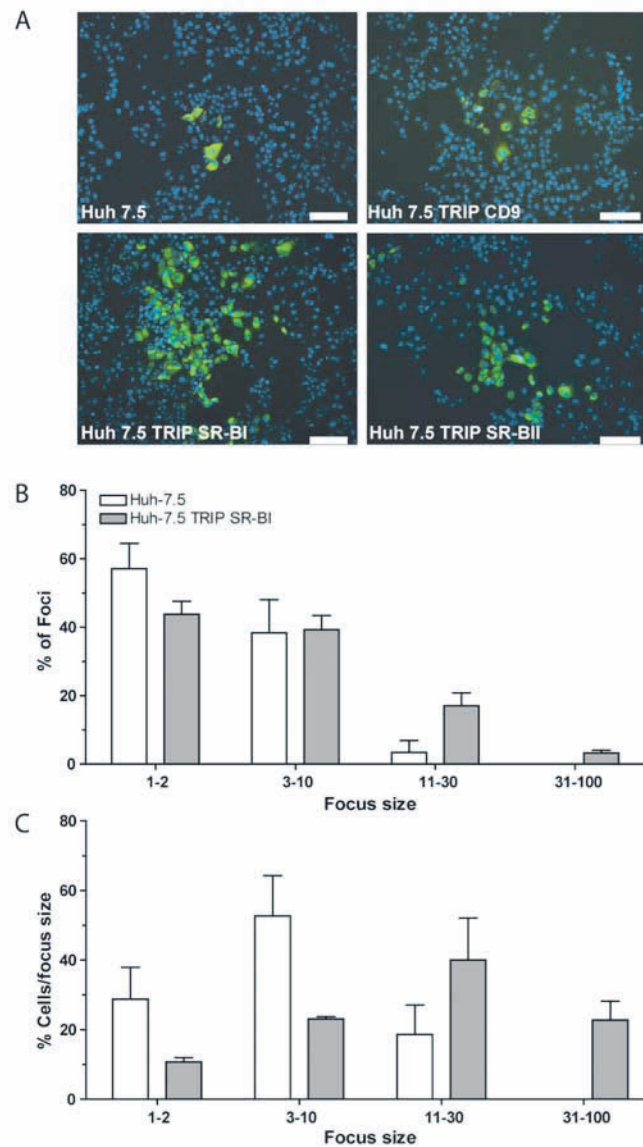
**A.** Parental (open circles) and transduced Huh-7.5 cells over expressing human CD9 (filled circles), SR-BI (open squares), or SR-BII (filled squares) were incubated with J6/JFH for various times between 1 and 8 hrs; unbound virus was removed by washing, and the infection allowed to proceed for 72 hrs. Both SR-BI and SR-BII transduced cells show elevated levels of infection. Virus infectivity is expressed as the number of NS5A-positive cells, or infected units (IU)/ml. **B.** Parental and transduced cells were incubated with J6/JFH (white bars) or JFH-1 (grey bars) for 1 hr, followed by a 72 hr infection. Infected cells were visualized by staining for intracellular NS5A, and infectivity for transduced cells expressed relative to the parental cells. J6/JFH and JFH-1 infectivities for parental Huh-7.5 cells were 11,000 +/- 3,000 IU/ml and 2,400 +/- 900 IU/ml, respectively. \*\* p=0.001 \* p=0.024 (unpaired t-test). The error bars indicate standard deviation from the mean (n=5).



### ***4.3 Evidence of enhanced JFH-1 transmission in cells over expressing SR-BI.***

By allowing an infection to proceed for 72 hrs it is possible to observe both primary entry events and secondary rounds of infection following virus replication and de novo virion production. A characteristic of secondary infection is the appearance of HCV antigen positive cell foci, in JFH-1 infected Huh-7.5 cells the foci typically contain between 4 and 20 infected cells. We observed an increase in the size of infected foci upon over expression of SR-BI, although apparent in J6/JFH infected cells, this phenotype was particularly pronounced in JFH-1 infection (Figure 4-3A).

We quantified the differences in focus size by enumerating the number of infected cells per focus in JFH-1 infected Huh-7.5 and TRIP SR-BI cells. The foci were categorised as either small (1-2 cells/focus), medium (3-10), large (11-30) or very large (31-100). Figure 4-3B displays the percentage of foci that fall into each category, over expression of SR-BI increased the frequency of large and very large foci. However, presenting the data in this manner under-represents the contribution of large and very large foci to overall infected cell number. Therefore, Figure 4-3C presents the same data as the percentage of total infection occurring within each focus category. In this representation the effect of SR-BI over expression is more distinct; ~70% of infected Huh-7.5 TRIP SR-BI cells reside in large or very large foci, compared to only 20% of infected parental cells. These data suggest that increased focal size is a major contributor to enhanced infection of Huh-7.5 TRIP SR-BI cells by JFH-1.

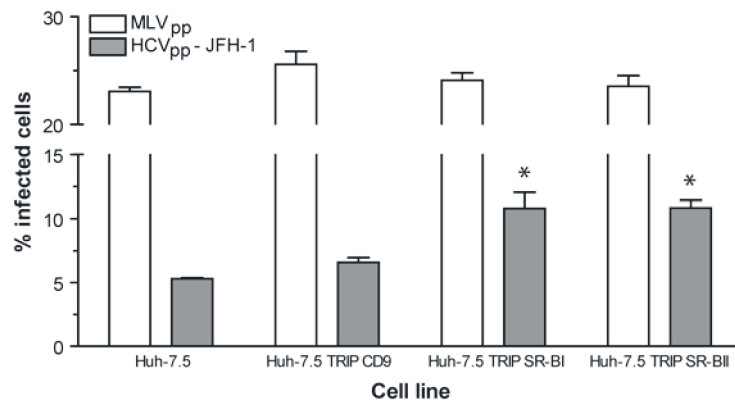


**Figure 4-3 Over expression of SR-BI and SR-BII in Huh-7.5 cells increases infected cell focus size.**

**A.** Parental and Huh-7.5 cells over expressing human CD9, SR-BI, or SR-BII were incubated with JFH-1 for 1 hr, and the infection allowed to proceed for 72 hrs. Infected cells were visualized by staining for NS5A (green), and the nuclei counterstained with DAPI. The images were taken at X100 magnification, scale bars represent 60 $\mu$ m. Quantification of focus size in parental (white bars) and Huh-7.5 TRIP SR-BI cells (grey bars): the number of infected cells within each focus was counted allowing foci frequency distribution across 4 size categories (1-2, 3-10, 11-30 and 30-100 cells/focus). The data is expressed in **B.** as the % of foci within each category and in **C.** as the % of total infected cells that reside within each category. In Huh-7.5 TRIP SR-BI cells ~70% of infected cells resided in large or very large foci compared to only ~20% for parental Huh-7.5 cells. Error bars indicate standard deviation from the mean (n=3).

#### ***4.4 SR-BI expression levels limit the entry of HCV pseudo-particles.***

HCVpp comprise a replication deficient retrovirus core and as such are only capable of a single round of infection (24, 130), allowing the investigation of primary HCV entry in isolation from downstream replication events. Huh-7.5 TRIP cell lines were infected with JFH-1 or control murine leukaemia virus (MLV) pseudo-particles and infection analysed by flow cytometric detection of a GFP reporter gene (Figure 4-4). MLVpp infection was unaltered in the Huh-7.5 TRIP cells, HCVpp – JFH-1 was enhanced two fold by over expression of SR-BI and II, indicating that SR-BI/II surface expression is limiting for HCV entry. However, this increase in infection is much smaller than that seen in JFH-1 HCVcc (Figure 4-2B), this is probably due to the lack of secondary infection in the HCVpp system and the fact that pseudo-particles may be a relatively poor mimic of authentic HCV virions.

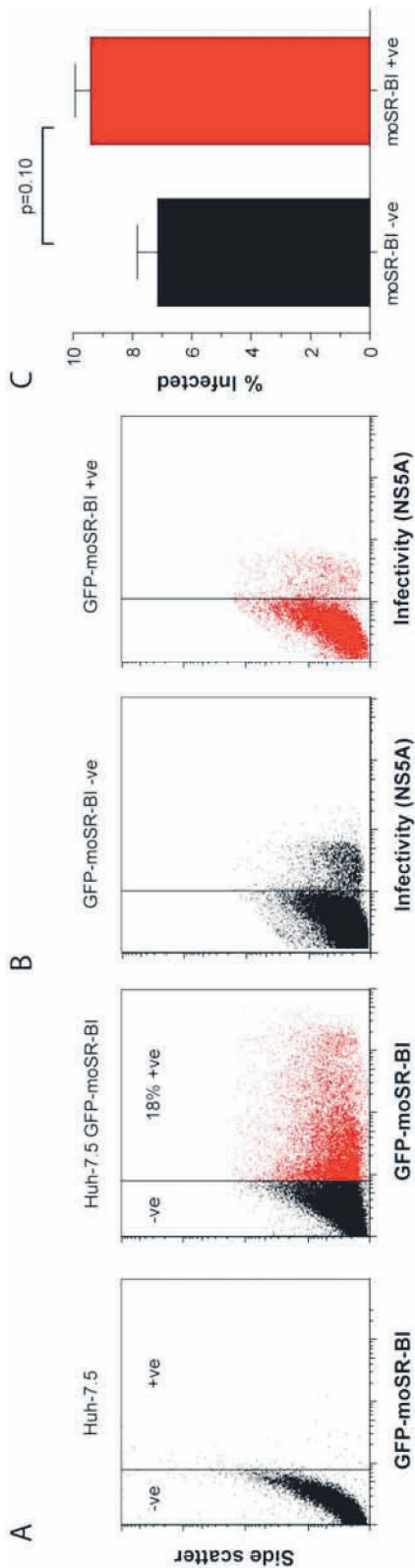


**Figure 4-4 Over expression of SR-BI and SR-BII in Huh-7.5 cells enhances HCVpp-JFH infection.**

Parental and Huh-7.5 cells over expressing human CD9, SR-BI, or SR-BII were incubated with murine leukaemia virus pseudo-particles (MLVpp) (white bars) or HCVpp - JFH-1 (grey bars), for 8 hrs, followed by a 72 hr infection. Infection was realised by flow cytometric detection of a pseudo-particle encoded GFP reporter gene. Over-expression of SR-BI/II enhanced HCVpp - JFH-1 infection by ~2 fold, MLVpp infection remained unaltered. \* = <0.04 (unpaired t-test) Errors bars indicate standard deviation from the mean (n=3).

#### **4.5 Murine SR-BI does not enhance HCVcc infection.**

Murine SR-BI is believed to be indistinguishable from human in regards to ligand binding and metabolism, indeed a number of studies have used murine SR-BI expressed in human hepatoma cell lines to study cholesterol uptake and trafficking (279, 300). HCV sE2 does not bind to murine SR-BI (274), however if HCV interaction with SR-BI occurs via apoproteins associated with the particle (190) both human and murine forms of SR-BI may act as co-receptors. To investigate this we challenged Huh-7.5 cells expressing murine SR-BI with JFH-1. The anti-SR-BI serum used in previous experiments is reactive against both species, therefore an N-terminal GFP tagged form of murine SR-BI (GFP-moSR-BI) was used, allowing flow cytometric detection of positive cells. Importantly, the GFP-SR-BI construct has previously been shown to be functional in regards to lipid uptake and metabolism (279). Figure 4-4A displays un-transfected Huh-7.5 cells and those expressing GFP-moSR-BI, ~18 % are positive for exogenous murine SR-BI. Gating of GFP-moSR-BI positive and negative Huh-7.5 cells allowed quantification of JFH-1 infection within each population (Figure 4-4B). Expression of murine SR-BI did not enhance JFH-1 infection of Huh-7.5 cells (Figure 4-4B & C), suggesting that HCV dependence on SR-BI is driven by a specific HCV E2-receptor interaction and not by apoprotein association.



**Figure 4-4 Expression of murine SR-BI in Huh-7.5 cells does not enhance JFH-1 infection.**

Huh-7.5 cells were transiently transfected to express murine GFP-SR-BI chimera 48 hrs prior to challenge with JFH-1, the infection was allowed to proceed for 72 h. Infected cells were realised by staining for intracellular NS5A followed by an alexaflour 633 conjugated secondary. Flow cytometric analysis allowed quantification of infected cells within moSR-BI -ve and +ve populations. **A.** Gating of GFP-moSR-BI -ve (black) and GFP-moSR-BI +ve (red) populations, the left hand panel displays untransfected Huh-7.5 cells. In transfected Huh-7.5 cells 18% were GFP-moSR-BI +ve (Cell counts: -ve = 45,000 +ve = 10,000) **B.** NS5A +ve cells within each population; GFP-moSR-BI -ve = 7.1% (~3000 cells), GFP-moSR-BI +ve = 9.1% (~1000 cells) **C.** percentage infected cells within each population, difference does not reach significance (unpaired t-test). Errors bars indicate standard deviation from the mean (n=3).

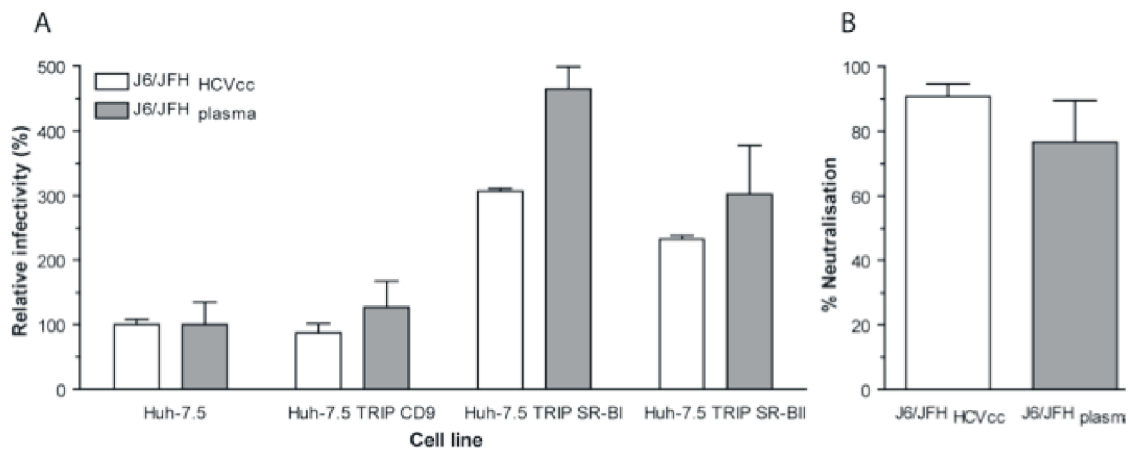
#### ***4.6 SR-BI/II expression levels modulate plasma-derived J6/JFH infectivity.***

The production of HCV virions is believed to be dependent on the assembly and secretion of VLDL, indeed Huh-7.5 are thought to produce HCV lipo-viro-particles (63, 109, 131, 224). However, it is accepted that the HCVcc system is only a surrogate model of in vivo virion production and as such may not support authentic HCV-lipoprotein associations. Numerous studies have shown that HCVcc is infectious in animal models such as the chimpanzee (144, 180), virus recovered from infected subjects have a lower buoyant density than cell culture virus (180), suggesting increased lipoprotein association. We therefore used J6/JFH virus recovered from the plasma of infected uPA-SCID mice with transplanted human livers (208, 209), to investigate the relationship between plasma derived virus and SR-BI/II.

J6/JFH<sub>plasma</sub> infection of Huh-7.5 cells was enhanced by over expression of SR-BI/II in a comparable manner to J6/JFH<sub>HCVcc</sub> (Figure 4-5 A) suggesting that J6/JFH<sub>plasma</sub> virus has a similar dependence for SR-BI to virus produced in vitro. However, Maillard et. al. reported that human sera derived HCV may interact with SR-BI via associated apoproteins and not virion envelope proteins (190). To address the importance of E2 glycoproteins in J6/JFH<sub>plasma</sub> infection we pre-incubated particles with ant-E2 mAb C1 prior to inoculation of Huh-7.5 cells (Figure 4-5 B). Both plasma and cell culture derived J6/JFH are neutralised by mAb C1, this implies that E2 is essential for infection by either virus. mAb C1 is reported to inhibit E2-CD81 interaction (172), however its

effect on SR-BI-E2 binding is not known and therefore it is difficult to speculate on the exact mode of neutralisation.



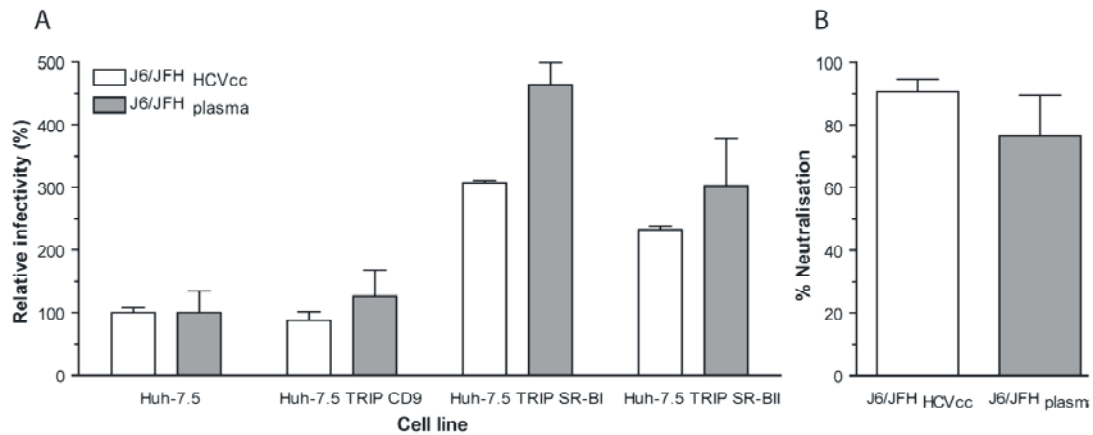


**Figure 4-5 Over expression of SR-BI and SR-BII in Huh-7.5 cells enhances the infectivity of cell culture- and plasma derived J6/JFH.**

**A.** Parental and Huh-7.5 cells over expressing human CD9, SR-BI, or SR-BII were incubated with J6/JFH derived from cell culture (J6/JFH<sub>HCVcc</sub>; white bars) or from the plasma of infected chimeric mice (J6/JFH<sub>plasma</sub>; grey bars) for 6 h, followed by a 72 hrs infection. Infected cells were visualized by staining for intracellular NS5A, and infectivity for transduced cells expressed relative to the parental cells. J6/JFH<sub>cc</sub> and J6/JFH<sub>plasma</sub> infectivities for parental Huh-7.5 cells were 35,000 (+/- 1,300) IU/ml and 2,250 (+/- 700) IU/ml, respectively. **B.** Comparable levels of infectious virus from the two sources were incubated with anti-E2 mAb C1 and a control anti-dengue virus mAb at 10µg/ml for 1 hr prior to infecting Huh-7.5 cells. Infectivity was measured by quantifying NS5A-positive cells, and the percent neutralisation of C1 defined by comparing infectivity in the presence of an anti-dengue virus mAb. Error bars indicate the standard deviation from the mean (n=2).

#### ***4.7 Anti-SR-BI serum inhibits cell culture and plasma derived J6/JFH infection.***

We have used cells over expressing SR-BI to demonstrate that SR-BI levels modulate HCV<sub>cc</sub> and HCV<sub>plasma</sub> infection. In the absence of robust siRNA silencing of SR-BI we employed anti-SR-BI sera to limit receptor availability during J6/JFH<sub>HCVcc/plasma</sub> infection (Figure 4-6A). Pre-incubation of Huh-7.5 cells with anti-SR-BI inhibits cell culture and plasma derived J6/JFH infection equally (Figure 4-6B), an anti-CD81 antibody was used as a positive control. To investigate whether the anti-serum is capable of preventing HCV E2-SR-BI interactions, we treated CHO SR-BI cells prior to performing a sE2 binding assay (Figure 4-6C). A known sub saturating concentration of anti-serum reduced sE2 binding to CHO-SR-BI cells by ~3 fold. Taken together these data support a model where HCV infection of Huh-7.5 cells is limited by the expression of SR-BI/II.

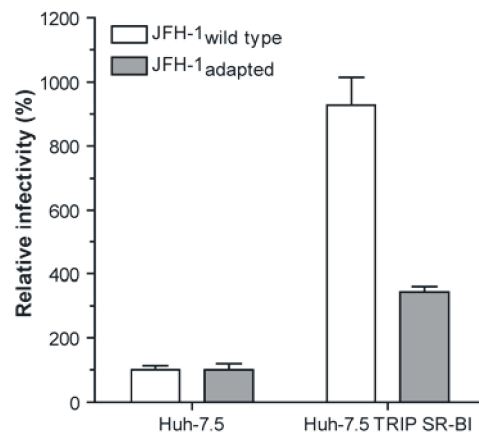


**Figure 4-6 Anti-SR-BI serum inhibits cell culture- and plasma-derived J6/JFH infectivity.**

**A.** Anti-SR-BI reactivity for Huh-7.5 cells (open circles), control pre-immune rabbit serum was tested at the highest dilution (filled circles). The data are expressed as the mean fluorescence intensity (MFI). **B.** Huh-7.5 cells were incubated with anti-SR-BI and control pre-immune sera at 1:500 or anti-CD81 MAb M38 and control isotype matched IgG at 1 $\mu$ g/ml for 1 hr prior to infection with cell culture (white bars) or plasma derived (grey bars) J6/JFH. Infectivity was measured by quantifying NS5A-positive cells, and the percent neutralisation of the receptor-specific antibodies determined by comparing infectivity in the presence of the control antibodies. Anti SR-BI and anti-CD81 inhibit the infectivity of both viruses. **C.** Parental CHO cells or those expressing SR-BI were pre-incubated with control rabbit serum (white bars) or anti-SR-BI (grey bars) at 1/400 prior to incubation with HCV-1 sE2 at 5 $\mu$ g/ml, cell-bound E2 was detected with 1 $\mu$ g/ml mAb 9/75. Data are expressed as mean fluorescence intensity (MFI). Anti-SR-BI inhibits HCV sE2 interaction with SR-BI expressed on CHO cells. Error bars indicate standard deviation from the mean (n=3).

#### ***4.8 Cell culture adaptation of JFH-1 HCVcc reduces SR-BI dependency.***

Numerous studies have documented cell culture adaptation of HCVcc during prolonged infection of human hepatoma cell lines (37, 144, 269, 352). We investigated the effect of adaptation on SR-BI dependence using a JFH-1 strain that had undergone multiple passages in Huh-7.5 cells. Although the occurrence of mutations has not been confirmed by sequencing, JFH-1<sub>adapted</sub> virus is more infectious than wild type (wt) virus (data not shown) suggesting an accumulation of selective mutations. In this study JFH-1 wt infection of Huh-7.5 TRIP SR-BI cells was enhanced ~10 fold, in contrast JFH-1<sub>adapted</sub> infection increased only ~3 fold (Figure 4-7). This data suggests that adaptation of JFH-1 HCVcc to Huh-7.5 cells involves an alteration in SR-BI dependence. This is consistent with SR-BI availability being a rate limiting factor during wt HCV infection of Huh-7.5 cells.



**Figure 4-7 Infection of Huh-7.5 over expression SR-BI by cell culture adapted JFH-1 HCVcc.**

Parental and Huh-7.5 over-expressing SR-BI were inoculated for 1 hr with wt JFH-1 or virus that had been selected for high extracellular virus infectivity. The infection was allowed to continue for 72h, the data is expressed relative to infection of parental Huh-7.5 cells. wt virus infection is enhanced by ~10 fold whereas adapted JFH-1 infection increases only ~3 fold. Errors bars represent standard deviation (n=3).

#### **4.9 Discussion.**

Over expression of SR-BI in Huh-7.5 cells increases their susceptibility to HCVcc (Figure 4-2), indicating that infection is limited by SR-BI expression levels. We made similar, though less pronounced, observations using cells over expressing the splice variant SR-BII, suggesting that it is a functionally active receptor for HCV attachment and/or entry (Figure 4-2). Comparable findings were reported for CD4 and chemokine receptor expression levels influencing human immunodeficiency virus cell entry (13, 249).

Over expression of SR-BI enhanced JFH-1 infectivity to a much greater extent than J6/JFH; 18-fold and 3-fold, respectively (Figure 4-2), suggesting strain specific variation within genotypic clades. HCVpp expressing diverse glycoproteins vary in their ability to infect HepG2 cells expressing CD81, supporting a model of genotypic variants with different dependencies for the viral co-receptors (101, 202).

HCVpp-JFH-1 infection of Huh-7.5 cells over expressing SR-BI was enhanced 2 fold indicating that the restriction occurs at the point of virus entry (Figure 4-4). However, this increase in HCVpp infection, was much less pronounced than that seen with JFH-1 HCVcc, suggesting a disparity between the two model systems. This discrepancy may be accounted for by the increase in JFH-1 infected cell focus size in cells over expressing SR-BI, as foci occur following multiple rounds of replication and HCVpp do not mimic this process. The fact that a large proportion of JFH-1 infected Huh-7.5 TRIP SR-BI cells reside in large foci (Figure 4-3) suggests that the phenotype seen in these

cells is largely manifested in secondary and local transmission. It has been recently shown that HCV is able to transfer infectivity between cells, resulting in the formation of large infected cell foci (314). Our data suggest that SR-BI promotes this route of transmission.

J6/JFH is infectious in vivo and virus recovered from infected animal models can be recultured in vitro. These HCV<sub>plasma</sub> particles are of a lower buoyant density and are thought to represent the particle-lipoprotein associations found in human plasma (180). SR-BI over expression enhanced plasma derived virus in a comparable manner to cell culture virus and anti-SR-BI serum inhibited the infection of both viruses (Figure 4-5 & Figure 4-6). The infectivity of both viruses was neutralised by anti-E2 mAb C1 (Figure 4-5), indicating that HCV<sub>plasma</sub> particle entry is dependent on viral envelope proteins in an analogous manner to HCVcc. These data suggest that particle associated lipoproteins do not alter the relationship with SR-BI, a view supported by our findings using murine SR-BI (Figure 4-4). This implies that HCVcc particles do not interact non-specifically with murine-SR-BI via associated apoproteins and that a specific interaction between HCV E2 and human SR-BI is necessary for enhanced infection in over expressing cells.

Interestingly, infection of Huh-7.5 cells over expressing SR-BI by a cell culture adapted JFH-1 variant was only enhanced ~3 fold (Figure 4-7). This observation suggests that adaptation to Huh-7.5 leads to a lower dependency on SR-BI and consequently its expression levels do not limit infection to the same extent. The adaptation of viruses to cell culture is a well documented

phenomenon, but the characteristics of these viruses differ from wt making it difficult to reconcile findings in vitro with the situation within a natural host (14, 136, 263, 270). However, when used appropriately adapted mutants are tools to examine the complex interactions between a virus particle and its receptor(s) (315, 335).

The work covered in this chapter has been published in the Journal of Virology - Grove, J., T. Huby, Z. Stamataki, T. Vanwolleghem, P. Meuleman, M. Farquhar, A. Schwarz, M. Moreau, J. S. Owen, G. Leroux-Roels, P. Balfe, and J. A. McKeating. 2007. Scavenger receptor BI and BII expression levels modulate hepatitis C virus infectivity. *J Virol* 81:3162-9.



## **5 Results: Identification of a residue in hepatitis C virus E2 glycoprotein that determines scavenger receptor BI and CD81 receptor dependency and particle sensitivity to neutralising antibodies.**

In the previous chapter we established that SR-BI expression levels limit HCVcc infection of Huh-7.5 cells (Figure 4-2), furthermore a cell culture adapted strain of JFH-1 exhibited an altered dependence on SR-BI (Figure 4-7). To investigate this further we obtained a characterised JFH-1 cell culture adapted virus from Jin Zhong and Frank Chisari of the Scripps Institute in La Jolla. This mutant virus carries a single amino acid change of glycine to arginine within the E2 glycoprotein; at position 451 of the polyprotein. Zhong et. al. have previously reported that JFH-1 G451R has a higher specific infectivity than JFH-1 wt and an altered relationship between particle density and infectivity, suggesting changes in particle-lipoprotein interactions.

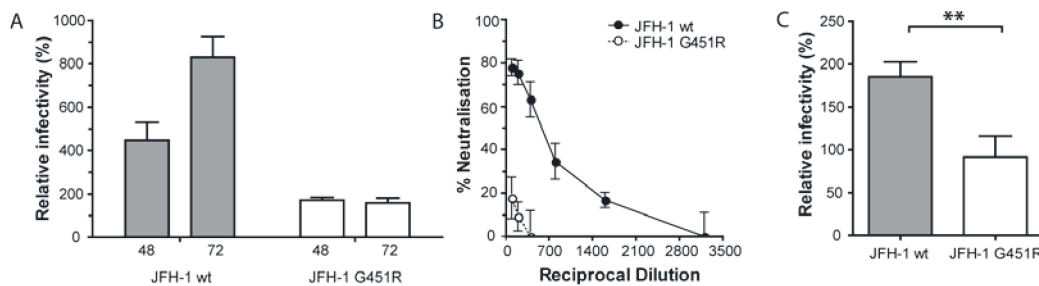
### ***5.1 JFH-1 G451R has a reduced dependence on SR-BI.***

To investigate whether the G451R mutation alters SR-BI dependence in a manner similar to that seen in Figure 4-7, parental Huh-7.5 and cells transduced to over express SR-BI were infected with JFH-1 wt and G451R. We previously reported that HCV can infect hepatoma cells via cell-free and cell-cell routes (314). To discriminate between cell-free particle primary infection and secondary transmission events, infection was allowed to proceed for 48 and 72 hrs, respectively (314). Over expression of SR-BI

enhanced the infectivity of JFH-1 4-fold at 48 hrs post infection and this increased to 8-fold by 72 hrs (Figure 5-1A). At 72 hrs the increased infectivity was associated with an increase in the size of infected cell foci comparable to that seen in Figure 4-3 (data not shown), suggesting increased cell-cell transfer of infection. In contrast, JFH-1 G451R showed 1.5-fold increase in infectivity at both time points with no change in focal size (Figure 5-1A).

To further investigate JFH-1 G451R interaction(s) with SR-BI we employed the neutralising rabbit anti-SR-BI serum characterised in Figure 4-6 (120, 190). Huh-7.5 cells were pre-incubated with the anti-SR-BI sera prior to challenging with JFH-1 wt or G451R. Whilst the infectivity of both viruses was inhibited, JFH-1 G451R is less sensitive to neutralisation; a 1/100 dilution of antiserum reduced JFH-1 G451R infectivity by 20%, compared to 80% for wt virus (Figure 5-1B).

It is widely reported that HDL enhances HCV infection via an SR-BI dependent mechanism requiring the transfer of lipids from HDL (25, 80, 324). To ascertain whether the altered relationship of G451R with SR-BI extends to lipoprotein enhancement, JFH-1 wt and G451R infections were supplemented with 10µg/ml HDL. HDL promoted JFH-1 wt infectivity 2-fold, consistent with previous reports, however G451R infectivity was unaltered (Figure 5-1C). Taken together, these data suggest that cell culture adaptation reduces the requirement for SR-BI during virus entry.

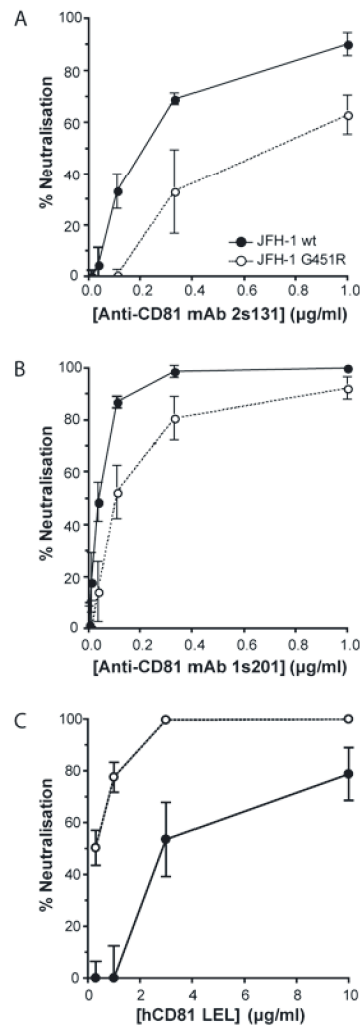


**Figure 5-1 JFH-1 G451R has an altered dependence on SR-BI.**

**A.** Huh-7.5 cells over expressing SR-BI were incubated with JFH-1 wt (grey bars) or JFH-1 G451R (white bars) for 8 hrs. Cells were fixed after 48 and 72 hrs, stained for NS5A and the mean number of infected cells per well determined. Infectivity is expressed relative to parental Huh-7.5 cells. At 72 hrs JFH-1 wt infection is enhanced ~8-fold whereas G451R remains largely unaltered (~1.5-fold) **B.** Huh-7.5 cells were incubated with a serial dilution of rabbit anti-SR-BI sera for 1 hr prior to challenge with JFH-1 wt (closed circles) or JFH-1 G451R (open circles). The data is expressed as percentage neutralisation relative to infection of Huh-7.5 cells treated with control rabbit sera. JFH-1 wt is inhibited by anti-SR-BI, G451R is not. **C.** Huh-7.5 cells were inoculated with JFH-1 wt or JFH-1 G451R in the presence of 10 $\mu$ g/ml high density lipoprotein (HDL). Infection is expressed relative to infection in the absence of HDL. HDL promotes JFH-1 wt infectivity 2-fold but has no significant effect on JFH-1 G451R, \*\*  $p=0.0069$  (unpaired t-test). Error bars indicate standard deviation from the mean ( $n=3$ ).

## ***5.2 Cell culture adapted JFH-1 G451R has an increased sensitivity to neutralisation by soluble CD81.***

Having established that the G451R mutation alters the relationship between HCV and SR-BI, we wanted to investigate the effects of this mutation on CD81-dependent routes of entry. Huh-7.5 cells were incubated with anti-CD81 mAbs prior to infecting with JFH-1 wt or G451R. The infectivity of both viruses was reduced, however JFH-1 G451R was less sensitive to treatment by anti-CD81 mAbs (Figure 5-2A & B). We and others have reported that a soluble form of human CD81 LEL (hCD81 LEL) interacts with the viral gps and inhibits HCV infectivity (24, 101, 130, 179). JFH-1 G451R demonstrated an increased sensitivity to neutralisation by hCD81 LEL, with 10-fold less protein required to reduce infectivity by 50% (Figure 5-2C), suggesting an increased exposure or affinity of CD81 binding residues on the mutant viral gps.



**Figure 5-2 CD81 dependence of JFH-1 wt and G451R infection.**

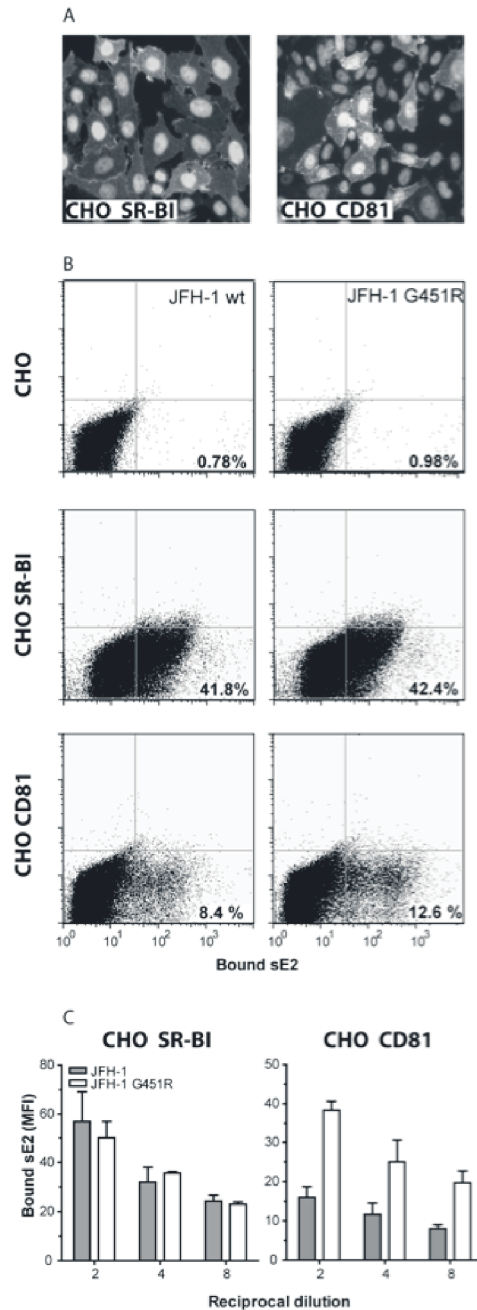
Huh-7.5 cells were incubated with a serial dilution of 2s131 (A.) or 1s201 (B.) mouse anti-CD81 mAbs for 1 hr prior to challenge with JFH-1 wt (closed circles) or JFH-1 G451R (open circles). Lab adapted virus is less sensitive to anti-CD81 mAb. JFH-1 wt or G451R viruses were incubated with human CD81 LEL for 1 hr prior to infecting Huh-7.5 cells (C.). JFH-1 G451R is more sensitive to neutralisation by hCD81 LEL. The data is expressed as percentage neutralisation relative to viral infection in the presence of an irrelevant mouse IgG or non-active mouse CD81 LEL, respectively. Error bars indicate standard deviation from the mean (n=3).

### ***5.3 sE2 glycoprotein bearing the G451R mutation demonstrates increased binding to CD81.***

To investigate whether the G451R mutation modulates E2 binding to SR-BI or CD81, soluble forms of JFH-1 wt and G451R E2 were expressed in 293T cells and used to quantify receptor interactions (as documented in section 2.7). sE2 was harvested from the media of cells supplemented with 3% delipidated FBS to eliminate the possibility of lipoprotein association(s). CHO cells expressing either human SR-BI or CD81 (Figure 5-3A) were incubated with comparable amounts of JFH-1 wt or G451R sE2, the bound gps were detected via a C-terminal tag recognised by mAb 10/76b and quantified by flow cytometry (Figure 5-3B). JFH-1 wt sE2 bound specifically to CHO cells expressing either SR-BI or CD81, as previously reported for genotype 1 sE2 (99, 120, 274). JFH-1 wt and G451R sE2 bound to CHO-SR-BI cells with comparable staining intensities, however the mutant protein showed enhanced binding to CD81 with 50% more CHO-CD81 cells binding G451R sE2 compared to JFH-1 wt (Figure 5-3B & C). These data are consistent with the increased sensitivity of mutant virus to hCD81 LEL neutralisation.

To further study the interaction of wt and G451R sE2 with CD81 we followed the binding of sE2 with hCD81 LEL by enzyme immunoassay. Previous studies have reported that the interaction of E2 with CD81 is dependent on the dimeric status of CD81 (83, 84). CD81 dimers bound approximately 3-fold more JFH-1 G451R than wt sE2 (Figure 5-4A), confirming our earlier studies with CHO cell expressed CD81. hCD81 LEL monomers failed to interact with wt or mutant sE2 (Figure 5-4B). To further characterize the interaction of

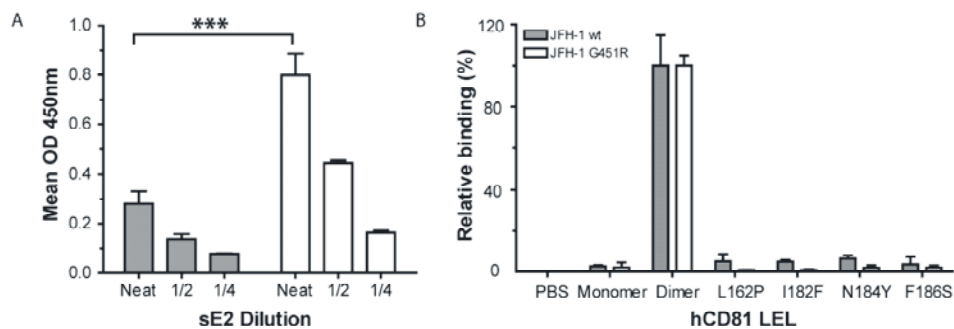
G451R sE2 with hCD81 LEL we compared mutant and wt sE2 interactions with a panel of CD81 variants with substitutions at amino acid residues reported to be critical for interacting with E2 (84). All mutations abrogated CD81 interaction with both wt and G451R sE2 proteins, suggesting that the G451R mutation does not alter the nature of the E2-CD81 interaction (Figure 5-4B).



**Figure 5-3 JFH-1 wt and G451R soluble E2 interaction with CHO cells expressing SR-BI and CD81.**

A. CHO cells expressing either human SR-BI (>80% positive) or CD81 (20-30% positive), were methanol fixed and stained with mAb anti-CLA1 or anti-CD81 2s139. B. Binding of recombinant JFH-1 wt and G451R sE2 to parental CHO, CHO-SR-BI and CHO-CD81 cells. Bound E2 was detected with mAb 10/76b. C. The mean fluorescent intensity (MFI) of JFH-1 wt (grey bars) and G451R (white bars) sE2 bound to CHO-SR-BI and CHO-CD81 cells is shown, the signal from sE2-CHO cell interaction was subtracted. JFH-1 G451R sE2 displays enhanced binding to CHO-CD81 cells. Error bars indicate standard deviation from the mean (n=3).





**Figure 5-4 JFH-1 wt and G451R soluble E2 interaction with recombinant CD81.**

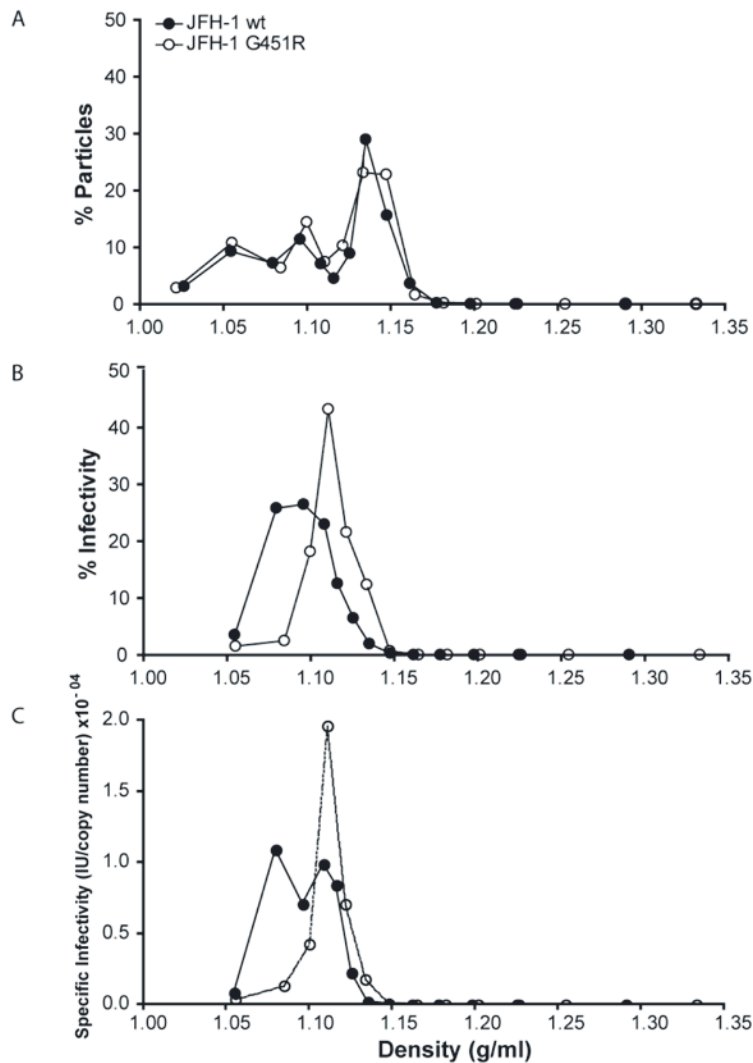
**A.** Dose dependent binding of JFH-1 wt (grey bars) or G451R (white bars) sE2 with human CD81 LEL (hCD81 LEL) dimer. Data is represented as the mean optical density (OD) at 450nm. G451R sE2 demonstrates increased binding to immobilised hCD81 LEL. \*\*\*  $p < 0.0001$  (unpaired t-test) **B.** JFH-1 wt or G451R sE2 association with PBS, monomeric and dimeric hCD81 LEL and mutants of hCD81 LEL that abrogate CD81 interaction with E2. All mutants were characterized for their effects on CD81 oligomerization and were shown to have minimal effect on dimerization. E2 binding to the mutants is expressed relative to CD81 LEL wt dimer. JFH-1 wt and G451R sE2 binding was ablated by each of the hCD81 LEL mutations. Error bars indicate standard deviation from the mean ( $n=3$ ).

#### ***5.4 Relationship between JFH-1 and G451R particle density, infectivity and co-receptor interactions.***

HCV particles associate with lipoproteins to form LVPs that can be fractionated according to their buoyant density. (8, 179, 180, 215, 233, 312). Initial experiments examined the distribution of wt and mutant particles across iodixanol gradients by quantifying genome copy numbers in each fraction. Wt and mutant viruses demonstrated a similar range of particle densities, suggesting comparable physical properties (Figure 5-5 A). Low density particles are reported to have the highest specific infectivity (179, 180), in support of this we observed peak JFH-1 wt infectivity at a density of 1.09g/ml (Figure 5-5 B). In contrast, the majority of JFH-1 G451R infectivity resided in the higher density fractions at 1.12g/ml (Figure 5-5B), suggesting an altered relationship between particle density and infectivity. The RNA copy numbers and infectivity data were used to calculate the specific infectivity of particles within each fraction (Figure 5-5 C); the G451R adaptation appears to have increased the infectivity of higher density particles, whilst perturbing the infectivity of those of a low density.

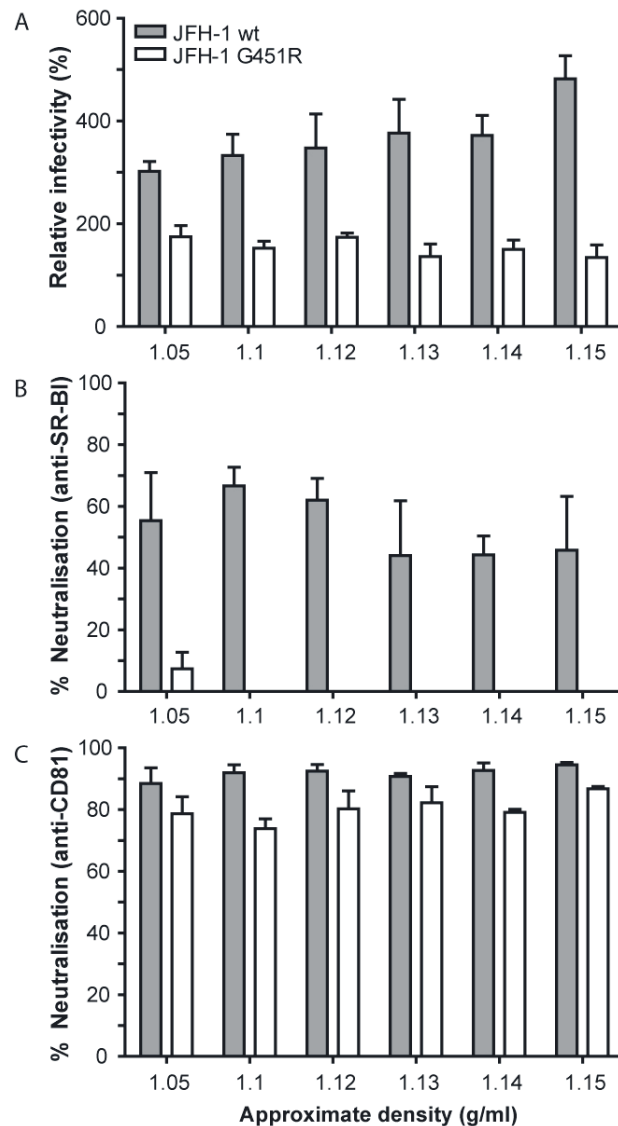
To study the relationship between particle density and receptor dependent infection, JFH-1 wt and G451R iodixanol gradient fractions were screened for infection of parental and Huh-7.5 cells transduced to over express SR-BI and for their sensitivity to neutralisation by SR-BI and CD81 specific antibodies (Figure 5-6). We failed to observe any association between wt or mutant virus density and SR-BI dependence, with all fractions showing comparable levels of infectivity under the respective conditions (Figure 5-6A & B). JFH-1 G451R

demonstrated a lower dependence on SR-BI, with minimal inhibition observed with the anti-SR-BI antibody (Figure 5-6B). Similar results were observed with the anti-CD81 mAb, with all fractions demonstrating a similar sensitivity to neutralisation (Figure 5-6C). The lack of correlation between JFH-1 wt and G451R particle density and sensitivity to anti-receptor antibodies suggests that the mutant phenotype of reduced SR-BI dependence and increased sensitivity to hCD81 LEL neutralisation may be largely attributable to an altered affinity or interaction of the viral gps with CD81 (Figure 5-3 & Figure 5-4).



**Figure 5-5 Analysis of JFH-1 wt and G451R buoyant density.**

Concentrated JFH-1 wt (closed circles) and G451R (open circles) were separated on an iodixanol gradient. **A.** The number of HCV particles per fraction was assessed by quantifying HCV RNA genomes by RT-PCR. The particle number in each fraction is expressed as a percentage of the total for either virus. JFH-1 wt and G451R exhibit comparable particle distribution throughout the gradient. **B.** The infectivity per fraction was assessed by inoculating Huh-7.5 cells. The infectivity within each fraction is expressed as a percentage of the total for either virus. The cell culture adaptation alters the relationship between particle density and infectivity. **C.** The number of infectious units (IU) per RNA genome copy were calculated to analyse the specific infectivity of particles within each fraction. The G451R mutation increases the infectivity of higher density particles whilst reducing the infectivity of low density particles.



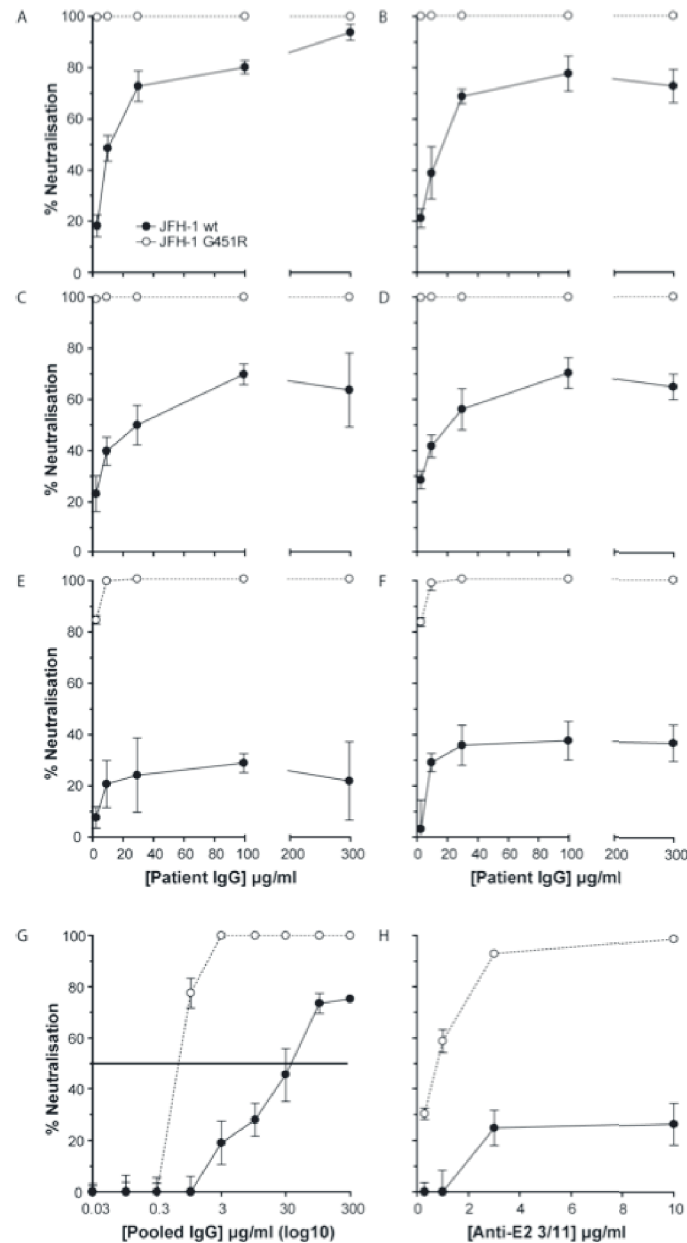
**Figure 5-6 Relationship between particle density and co-receptor dependency.**

The density fractions containing infectious JFH-1 wt (grey bars) or JFH-1 G451R (white bars) were used to assess the relationship between particle density and SR-BI and CD81 interaction(s). The approximate densities of each fraction are indicated on the x-axis. **A.** Huh-7.5 cells over expressing SR-BI were inoculated with JFH-1 wt and G451R virus and infectivity expressed relative to parental Huh-7.5 cells. Huh-7.5 cells were incubated with either **B.** anti-SR-BI sera at 1/300 or **C.** anti-CD81 1s201 at 0.1 µg/ml prior to challenge with infectious fractions of JFH-1 wt and G451R. There is no correlation between particle density and receptor dependence. Error bars indicate standard deviation from the mean (n=3).

### **5.5 Adapted JFH-1 demonstrates an increased sensitivity to nAbs.**

Several reports have suggested that antibodies specific for the HCV gpS neutralise viral infectivity by inhibiting HCV interaction(s) with CD81 (145, 147, 237)(reviewed in (295)). Given the increased binding of G451R E2 to CD81 we were interested to investigate the effects of this mutation on particle sensitivity to nAbs. We screened the sensitivity of JFH-1 wt and G451R virus to neutralisation by IgG purified from the sera of six HCV infected individuals. In each case G451R demonstrated an increased sensitivity to inhibition by patient IgG (Figure 5-7A-F). JFH-1 wt was inhibited to varying degrees by the patient IgG and in 5 of 6 cases the percentage neutralisation reached a plateau below 80%, suggesting that a population of particles were resistant to neutralisation. To quantify the increased sensitivity of G451R to antibody-dependent neutralisation, we determined the concentration of pooled HCV<sup>+</sup> patient IgG required to inhibit 50% of infectivity (inhibitory concentration, IC<sub>50</sub>). The IC<sub>50</sub> for the wt and mutant viruses are 40µg/ml and 0.75µg/ml respectively, indicating that G451R is 50-fold more sensitive to neutralisation (Figure 5-7G). Patient derived IgG is polyclonal in nature and likely to target multiple conformation-dependent epitopes. To study virus neutralisation via a defined epitope we screened the sensitivity of both viruses to mAb 3/11, specific for E2 amino acids 412–423. G451R showed an increased sensitivity to 3/11 neutralisation (Figure 5-7H). These data are consistent with an increased sensitivity of G451R particles to nAbs targeting diverse epitopes, suggesting an increased availability of epitopes on G451R compared to the parental virus. However we failed to detect any difference in HCV<sup>+</sup> patient IgG or 3/11 binding to immobilised JFH-1 wt or G451R sE2 or to infected cells by

flow cytometry (data not shown), indicating that differential epitope presentation may only occur in the context of a virus particle.



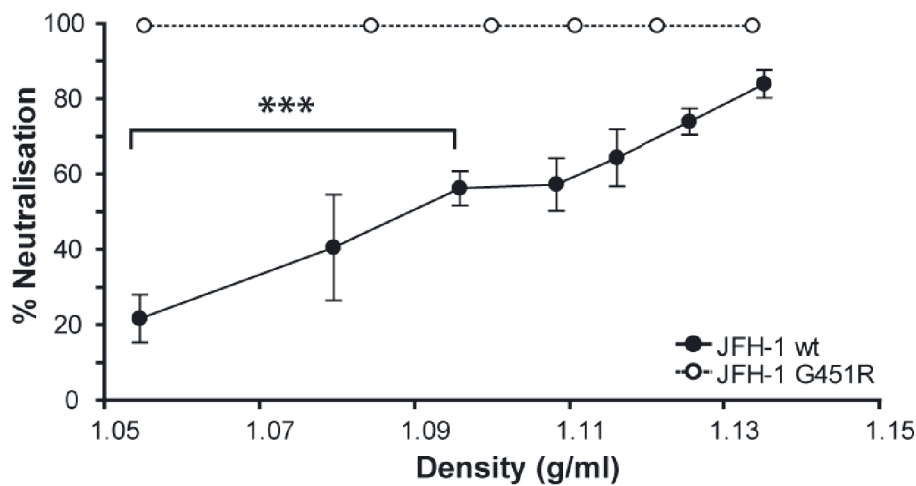
**Figure 5-7 JFH-1 G451R demonstrates an increased sensitivity to neutralisation by gp-specific antibodies.**

JFH-1 wt (closed circles) or G451R (open circles) were incubated with IgG purified from the sera of six HCV infected subjects (A-F) or anti-E2 mAb 3/11 (H), prior to infecting Huh-7.5 cells. Percent neutralisation was calculated by quantifying viral infectivity in the presence of anti-HCV specific antibodies relative to HCV negative IgG or irrelevant mAb, respectively. G. To determine the concentration of IgG required to neutralise 50% of JFH-1 and G451R infectivity (IC<sub>50</sub>), both viruses were incubated with a pool of the six patient derived IgG. The IC<sub>50</sub> is depicted as a horizontal line. JFH-1 G451R is 50-fold more sensitive to neutralisation by pooled patient IgG. Error bars indicate standard deviation from the mean (n=3).



### **5.6 Low density JFH-1 particles are less sensitive to nAbs.**

To investigate whether the increased sensitivity of G451R to nAbs was attributable to alterations in particle density, the iodixanol gradient fractions of wt and mutant viruses were normalized for infectivity and incubated with 10µg/ml pooled HCV<sup>+</sup> patient IgG. For JFH-1 wt, the sensitivity to neutralisation increased with particle density with the lower density fraction being neutralised by 20%, compared to 80% with the highest (Figure 5-8). In contrast, all G451R fractions were neutralised by 100%. Control experiments established that iodixanol concentration had minimal effects on JFH-1 infectivity and sensitivity to IgG neutralisation (data not shown). These data provide the first evidence that lipoprotein association of JFH-1 reduces the sensitivity of particles to nAbs, however, the increased infectivity of high density G451R viruses does not explain their heightened sensitivity to antibody-dependent neutralisation.



**Figure 5-8 Association between JFH-1 particle density and sensitivity to neutralising antibodies.**

JFH-1 wt (closed circles) and G451R (open circles) were separated on an iodixanol gradient as detailed in Figure 5-5 and the fractions tested for their sensitivity to neutralisation by pooled HCV infected patient IgG (10µg/ml). Data is expressed as percent neutralisation calculated by comparing infectivity in the presence of HCV negative IgG. A positive correlation was observed between JFH-1 particle density and neutralisation by pooled patient IgG, \*\*\*  $p < 0.0001$  (unpaired t-test). Error bars indicate standard deviation from the mean (n=4).

### **5.7 Discussion.**

We have demonstrated that a cell culture adaptive mutation in E2 has pleiotropic effects on HCV interaction(s) with SR-BI, CD81 and nAbs. JFH-1 G451R infectivity was not enhanced in Huh-7.5 cells transduced to over-express SR-BI and was insensitive to anti-SR-BI and HDL treatments (Figure 5-1), suggesting a reduced requirement for SR-BI during entry. Definitive evidence of SR-BI independence is hampered by the lack of SR-BI negative permissive cell lines. We failed to detect any effect(s) of the G451R mutation on sE2 interaction with CHO cells expressing SR-BI (Figure 5-3B & C), however, soluble forms of E2 may not recapitulate the interaction of virus particles with SR-BI.

As SR-BI over expression enhances JFH-1 focus size (Figure 4-3) we hypothesise that it may have a key involvement in direct cell-cell transmission (314). We were interested to study this route of transmission for JFH-1 wt and G451R using the recently developed 'infectious centre assay' (ICA) (314). However, the ICA is reliant on efficient antibody neutralisation of particles as they are released into the culture media, thereby eliminating cell-free transmission. In the absence of efficient neutralisation it is difficult to differentiate between cell-free and direct cell-cell routes of transmission. We observed an apparent 2 fold increase in cell-cell transmission from JFH-1 infected producer cells to naïve target cells over expressing SR-BI in the infectious centre assay (data not shown). However, as our current neutralising agents are relatively inefficient at perturbing JFH-1 wt infection (Figure 5-7) the data was difficult to interpret. To fully address the role of SR-BI in cell-cell

transmission we will need to overcome these technical challenges in the future.

CD81 is a critical co-receptor for HCV particle entry (179, 202), the mutant virus demonstrated a 10-fold increase in the sensitivity to neutralisation by hCD81 LEL, suggesting an increased affinity of the gps for CD81 (Figure 5-2C). Indeed, G451R sE2 demonstrated increased binding to CHO-CD81 and hCD81 LEL, supporting this conclusion (Figure 5-3 & Figure 5-4). The observation that G451R demonstrated a reduced sensitivity to neutralisation by anti-CD81 mAbs than wt virus, is consistent with an increased affinity of G451R glycoproteins being able to more effectively compete with sub-saturating levels of mAbs to interact with surface expressed CD81 (Figure 5-2A & B). Mutations in CD81 reported to prevent interaction with E2 (84, 99) abolished the binding of both JFH-1 wt and G451R sE2 (Figure 5-4B), suggesting that the interface between JFH-1 G451R E2 and CD81 is unaltered and the viral phenotype may simply reflect a greater affinity of the gp with the co-receptor. Studies with HCVpp suggest that the CD81 binding site on E2 involves three discontinuous regions (81, 237, 265) and G451R is located immediately downstream of one such region G436WLAGLFY (81). Thus, the adaptive mutation may directly modulate E2 affinity for CD81. Our data suggest an important role for position 451 in JFH-1 E2 coordination of particle interaction with SR-BI and CD81 and highlight the importance of studying mutant gp association with multiple viral co-receptors.

Numerous reports have used density gradient centrifugation to study the association of plasma or serum-derived HCV with the major VLDL apoproteins (ApoB-100 and ApoE) (8, 233, 313). Recent data implicates VLDL synthesis in HCV particle assembly and/or release, suggesting that HCVcc particles interact with lipoproteins in an analogous manner to blood-derived virus (63, 109, 131, 180, 224). Nielsen et. al. reported that iodixanol density gradient centrifugation preserved HCV-VLDL interactions (233) and we employed this technique to investigate the relationship between JFH-1 particle density and infectivity. Zhong et. al. reported an altered relationship between JFH-1 G451R buoyant density and infectivity, with high density mutant virus demonstrating greater infectivity than wt (352). Similar data was observed with iodixanol density gradient separation (Figure 5-5 B). Importantly, analysis of particle number(s) by quantitative RT-PCR demonstrated a comparable distribution of wt and mutant viruses (Figure 5-5 A), suggesting that both viruses associate with lipoproteins in a comparable manner. Analysis of the specific infectivity throughout the density gradient suggests that the G451R mutation increases the infectivity of higher density particles whilst reducing that of low density particles (Figure 5-5 C). Determining how the cell culture adaptation alters the relationship between particle density and infectivity, will require a better understanding of the contribution lipoprotein components make to particle infectivity.

Lipoproteins have been implicated in HCV entry and particle interaction(s) with SR-BI (9, 63, 190). However, we failed to observe any association between infectious particle density and responsiveness to SR-BI over-

expression or receptor 'neutralisation' for JFH-1 wt or G451R (Figure 5-6). These data do not discount the role of lipoproteins in the primary engagement between virus and receptors, however, it suggests that the 'functional outcome' i.e. entry, is principally driven by the viral gps. This interpretation is consistent with our observations using plasma derived J6/JFH virus and murine SR-BI (Figure 4-5 & Figure 4-4). HCVpp assembly is not thought to be dependent on host lipoproteins and offers a tool to dissect the role of the adaptive G451R mutation in viral entry. However, E1E2 gps with the G451R mutation failed to generate infectious HCVpp (Ke Hu, personal communication).

The heightened sensitivity of JFH-1 G451R to neutralisation is not confined to hCD81 LEL; IgG from six HCV infected individuals inhibited JFH-1 G451R 50-fold more effectively than wt (Figure 5-7A-G). The polyclonal IgG was isolated from patients infected with HCV genotypes 1 or 3 and most likely reflects a mixture of antibodies specific for diverse conformation-dependent epitopes. In addition, G451R was >10 fold more sensitive to neutralisation by anti-E2 mAb 3/11 (Figure 5-7H), which is specific for amino acids 412-423 (130). Experiments to assess the binding of polyclonal HCV<sup>+</sup> patient IgG and 3/11 to immobilised JFH-1 wt and G451R sE2 by enzyme immunoassay found no differences (data not shown). However, sE2 may not be an accurate mimic of epitope availability on a native virus particle (67, 101, 148).

We hypothesised that virus association with lipoproteins reduces the efficacy of nAbs. Taking particle density as a measure of lipoprotein interaction(s) we

demonstrate that low density JFH-1 has reduced sensitivity to HCV<sup>+</sup> patient IgG neutralisation (Figure 5-8), showing a range of neutralisation values from 20-80% between the densities of 1.04-1.14g/ml. The data support a model where lipoproteins obscure critical epitopes from nAbs and, since the majority of infectious JFH-1 particles are of low density (Figure 5-5), this may explain their insensitivity to nAbs (Figure 5-7). The mechanism by which this occurs is unknown, however lipoproteins may restrict the access of antibodies to E2 or promote the stabilization of virus particles. Our findings are consistent with observations reported with serum derived HCV (8, 180, 312), where Thommsen et. al. reported that apoprotein-B-100 associated virus failed to precipitate with polyclonal HCV<sup>+</sup> IgG (313). Similarly, Molina et. al. reported that serum virus infectivity was neutralised with anti-CD81 antibodies targeting the host cell, whereas infectivity was resistant to hCD81 LEL, suggesting a reduced exposure of CD81 binding epitopes on circulating particles (217).

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## 6 **Discussion and conclusions.**

### 6.1 ***SR-BI and HCV attachment and entry.***

It is becoming clear that the concept of a virus particle targeting and entering a host cell by interacting with a single receptor is outdated. Findings from across the field of virology suggest that a large proportion of viruses engage a series of co-receptors in directing their entry into a permissive environment. In many cases virus entry is dependent on a sequence of interactions with each step facilitating the next (184, 194, 291, 293). The attachment and entry of HCV is revealing itself to be one of the more intricate pathways currently under investigation, with at least three membrane proteins implicated. In this study we attempted to characterise the role of SR-BI in HCV entry, the following section discusses our findings in the context of what is already known about HCV attachment and entry.

Investigating the role of CD81 and CLDN1 in HCV entry has been facilitated by the availability of cell lines that do not express either respective receptor (91, 101, 202, 314). Studies to address the role of SR-BI are hampered by the lack of any such cell line. We have reported that primary human sinusoidal endothelial cells do not express SR-BI, however exogenous expression of SR-BI does not confer permissivity to HCVpp infection (257). We and others have used SR-BI specific antibodies to inhibit HCV infection (Figure 4-6Figure 5-1) (60, 120, 139, 346), these findings are consistent with SR-BI having a role in HCV entry. It has also been reported that siRNA silencing of SR-BI reduces HCVcc infection (346). Furthermore, using Huh-7.5 cells transduced



to over express SR-BI we were able to demonstrate that SR-BI levels are a limiting determinant of HCV infection. SR-BI is predominantly expressed in the liver, indeed if there is a critical level of SR-BI expression required for efficient particle entry this may contribute to HCV hepatotropism (161, 257).

Particle associated apoproteins have been implicated in the binding of HCV to SR-BI, however we found no evidence to suggest that the relationship between HCV and SR-BI is defined by anything other than a specific interaction between particle and receptor. We observed no difference in SR-BI dependency between particles of a low and high density, or those derived from cell culture and infected animal plasma (Figure 5-6 & Figure 4-5). Moreover, murine SR-BI did not enhance HCVcc infection (Figure 4-4), despite its ability to recapitulate the interactions of human SR-BI with its natural ligands. Current data suggests that HCV envelope protein engages SR-BI via HVR-1 (hyper variable region-1) (25, 274). This N-terminal domain is believed to be under selection from neutralising antibodies resulting in high antigenic variance (320, 326). However, basic residues are maintained at specific positions within HVR-1 and removal of this globally basic characteristic significantly impairs the infectivity of HCVpp (57, 240). It has been reported that SR-BI/II has broad pattern recognition abilities and that this is important for the invasion of some bacterial pathogens (246, 323). Therefore it is possible that HCV exploits this function, allowing redundancy within the HVR-1 which in turn enables escape from neutralising humoral responses. There has yet to be a definitive study demonstrating which residues of HVR-1, or indeed elsewhere within E2, are critical for HCV-SR-BI

interactions and we are preparing to carry out a series of experiments to address this.

It has been previously shown that antibodies specific to SR-BI and CD81 block HCV infection in a synergistic manner, suggesting that they function co-operatively during HCV entry (139, 346). Our studies using JFH-1 G451R have for the first time shed light on an inter-dependence between HCV interaction with SR-BI and CD81 (Figure 5-1 & Figure 5-2). Taken together, these data point towards sequential receptor engagement by HCV, much like that seen with other viruses (184, 293, 315, 335). It is believed that HCV interaction with CD81 occurs after a particle has bound to the cell surface (31, 91, 346). Indeed Flint et. al. demonstrated that particle neutralisation by hCD81 LEL can only occur post binding (101), suggesting that HCV attachment primes particle envelope proteins for CD81 engagement. Evans et. al. reported that HCVcc particles bind to CHO-SR-BI cells whilst not to cells expressing CD81 or CLDN1 (91).

We hypothesise that HCV engagement of SR-BI precedes and facilitates further interactions with co-receptors such as CD81, indeed, many of the epitopes exposed on sE2 bound to SR-BI are critical for CD81 binding (

Figure 3-3). Building on this model, the G451R mutation may cause a conformational change that primes E2 to interaction with CD81 and therefore negates the requirement for prior attachment to SR-BI. In support of this we demonstrate that JFH-1 G451R infectivity is less dependent on SR-BI (Figure

5-1), better able to engage CD81 (Figure 5-2Figure 5-3Figure 5-4) and is highly susceptible to polyclonal neutralising IgG (Figure 5-7) suggesting an alteration in the conformation of E2.

Such a hypothesis is not unprecedented, primary attachment of HIV to CD4<sup>+</sup> lymphoid cells promotes a conformational change in the viral envelope protein, gp120, which exposes epitopes that are critical for later stages of entry (273, 315, 335). As a result, CD4 expression levels limit HIV entry and cell culture adaptation leads to viruses with reduced dependence on CD4 and a heightened sensitivity to nAbs (13, 38, 86, 129, 249). Similar observations have been made using mutant respiratory syncytial virus (196). Over expression of SR-BI enhanced JFH-1 infection to greater extent than J6/JFH, suggesting strain specific differences in SR-BI dependency. Given our findings using JFH-1 G451R it will be interesting to investigate how this phenotype correlates with particle CD81 engagement and sensitivity to neutralising antibodies.

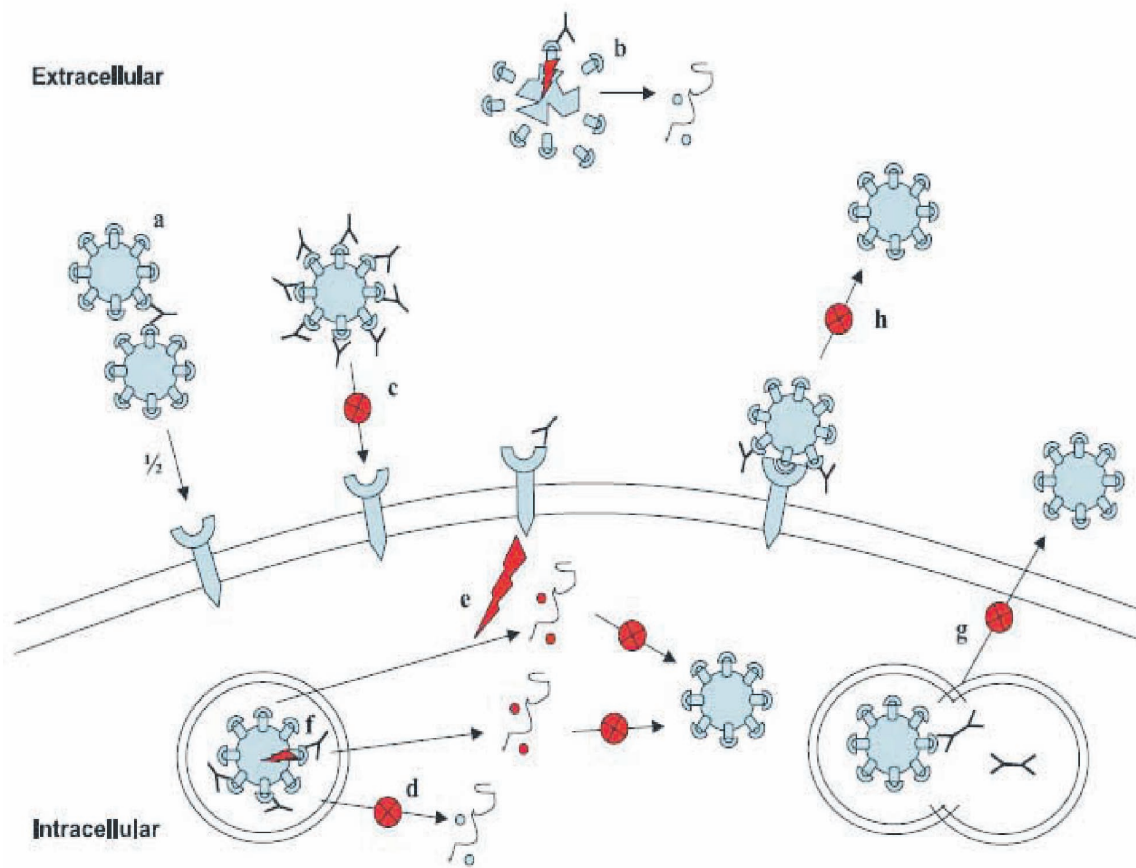
HDL (high density lipoprotein) enhances HCVcc infection, this process is thought to involve SR-BI mediated cholesterol exchange, followed by the liberation of apoC-I from HDL (25, 79, 324, 325). Recent reports suggest that apoC-I integrates into HCV particles and enhances attachment and fusion events (79, 210, 211). Notably, JFH-1 G451R did not respond to supplemental HDL (Figure 5-1), this is most likely linked to its lower dependence on SR-BI. Based on our current understanding, it is difficult to speculate on the mechanisms by which apoC-I promotes infection, however our data suggests

that E2 determines the relationship between apoC-I and HCV particles, and this is closely linked to SR-BI engagement.

## **6.2 *Antibody mediated neutralisation of HCV.***

A virus particle is considered to be 'neutralised' when its infectious potential falls beneath the threshold of detection (152). There are varied mechanisms by which antibodies may neutralise virus particles, including; the aggregation of virions, inhibition of virus-receptor interaction(s) and prevention of viral penetration events such as fusion (152, 256). The varied modes of neutralisation are summarised in Figure 6-1.

For each mechanism, neutralisation requires a specific interaction between an antibody paratope with its respective epitope and in most cases the efficiency of neutralisation by any given antibody is a function of its ability to bind a particle (54). The efficiency of this binding is determined by i) paratope-epitope affinity ii) antibody concentration and iii) epitope availability (152, 256). The most prevalent modes of antibody mediated neutralisation are believed to be inhibition of particle attachment and, in the case of enveloped viruses, prevention of membrane fusion (152, 256). Our findings with JFH-1 wt and G451R HCVcc will be discussed from this perspective.



**Figure 6-1 An overview of antibody dependent neutralisation of virus infectivity.**

“Virions are represented as spiked circles, cellular receptors as a blue semicircle mounted on an arrow, IgG as a Y, and blocked events as an arrow carrying an X mounted in a red circle. **A.** Antibody aggregates virions and reduces the number of infectious centres. The fraction shown ( $1/2$ ) represents the loss in infectivity. **B.** Antibody that mimics cell receptor ligation binds virions and leads to the disruption of the virion capsid (red lightning) and premature release of the genome. **C.** Antibody inhibits virion attachment by blocking receptor engagement. **D.** Antibody inhibits fusion occurring inside an endocytotic vesicle (illustrated) or at the cell membrane (not shown). **E.** Antibody binds to a cell-surface protein and results in the transduction of a signal into the cell (red lightning) that aborts the infection by modification of the replication complex. **F.** Post-entry neutralisation by transmission of an allosteric signal via the virus surface protein to the virion core (shown as red lightning). The core is released into the cytoplasm but is defective and unable to replicate. As in (E), this process is poorly understood. **G.** Transcytosing IgA antibodies (represented as a double-ended Y) neutralise virus when their respective vesicles fuse. **H.** Antibody binds nascent virions and blocks their budding or release from the cell surface” Taken from Reading et. al. (256) .

There are various models of how antibody binding prevents virus attachment, entry and penetration, the most simplistic view is of a particle coated with antibodies unable to function due to steric hindrance exerted by the antibody shell (54). However, neutralisation efficiency does not necessarily directly correlate with the degree of antibody coating, indeed in some cases particles saturated with mAb remain infectious (97). Therefore more sophisticated models have been developed to accommodate such experimental data. Multiple hit and occupancy models of neutralisation draw upon the concept that virions carry numerous copies of glycoprotein(s) required for virus entry, however particle entry may only require a fraction of the total number to be functional (151). Therefore, a particle can tolerate antibody mediated inactivation of multiple envelope proteins without any appreciable reduction in infectious potential. Experimentally, this phenomena manifests itself as a lag in neutralisation, whereby at lower antibody concentrations there is no detectable inhibition (105, 152).

The importance of epitopes targeted by humoral responses cannot be overlooked. Ligation of some epitopes may exert moderate to weak neutralisation by partial inhibition of envelope protein function. Other, so called critical epitopes, contribute directly to events such as receptor engagement; blocking of these will render an envelope protein completely inactive (152). Consequently, antibodies that target critical epitopes can exhibit strong neutralisation at low occupancy; that is to say relatively few epitopes are 'occupied' per particle (228). It has been reported that for some viruses the binding of a single antibody is sufficient to inactivate a particle, in these cases

it is believed that ligation of a critical epitope causes a catastrophic conformational change rendering the particle non-infectious (333). This single hit model results in a linear relationship between antibody concentration and neutralisation with no lag in efficiency (105, 152).

The shape of the neutralisation curve observed for JFH-1 G451R (Figure 5-7G), is consistent with a multiple hit model of neutralisation. No inhibition was detectable at IgG concentrations below 1µg/ml, whereas complete neutralisation was achieved at 3µg/ml. These data suggest a threshold in IgG concentration at which the number of functioning envelope proteins per particle drops below the level required for efficient infection. The shape of the curve is also consistent with a high degree of neutralising epitope availability, this supports our hypothesis of the adaptive mutation causing a conformational change within E2 (page 156). In contrast JFH-1 wt is relatively insensitive to neutralisation by HCV+ IgG, resisting inhibition over a 100µg/ml range, eventually reaching a plateau at 80% neutralisation (Figure 5-7). Assuming that JFH-1 wt and G451R particles carry the same number of envelope proteins, this observation suggests that JFH-1 wt limits epitope exposure such that even at high IgG concentrations a subset of virions possess sufficient functioning envelope proteins to perform entry.

A number of viruses are reported to use specific protein conformations to obscure critical epitopes (38, 73, 264), indeed another member of the Flaviviridae, West Nile Virus (WNV), was recently shown to limit epitope availability resulting in resistance to nAb. The envelope proteins on WNV

undergo proteolytic cleavage during egress, leading to a particle rearrangement and maturation, however this process is not essential for infectivity. Nelson et. al. demonstrated that mature WNV particles are up to 95-fold more resistant to anti-envelope mAb and that this could be attributed to reduced exposure of certain epitopes (228). However, describing HCV by analogy with other viruses only extends so far, our data suggests that HCV association with lipoproteins renders particles more resistant to neutralisation (Figure 5-8). We do not know whether lipoprotein components themselves obscure epitopes or if they simply stabilise particle structure, in either case HCV appears to have adopted a unique mechanism of evading host humoral responses.

Lipoprotein association and epitope concealment, however, are not the only strategies employed by HCV to avoid nAbs. Recent reports indicate that B cells and dendritic cells are capable of uptake and cross-presentation of HCV, interestingly, SR-BI has been implicated in both cases (21, 23, 294). In B cells, internalised particles are shielded from nAbs and transfer of HCV from B cells to human hepatoma cells enhances infectivity by 30 fold (294), indicating that B cells may offer HCV safe and efficient passage to the liver. As discussed previously, HCV is also capable of direct cell-cell transmission (314), similar to that reported for other viruses (134, 137, 284). It is conceivable that in an organ such as the liver, cell-cell transmission may represent the major route of infection within a host, indeed if this is the case it will have major implications for those hoping to treat HCV with monoclonal antibody therapies (146).



Given our and other's findings regarding the ability of HCV to evade nAb responses, the lack of clear evidence supporting a role for humoral immunity in controlling infection is unsurprising (87, 140, 182, 295, 327). Indeed the relatively late development of anti-HCV antibodies (64), may be partly attributable to the low availability of critical epitopes on circulating particles. However, there remains a discrepancy between in vivo studies and experiments using the HCVpp and HCVcc models of virus entry. Antibodies isolated from patients have been shown to efficiently neutralise HCVpp (182, 245), moreover there have been numerous accounts of anti-E2 mAbs inhibiting both HCVpp and HCVcc infectivity (130, 145, 172, 236, 307, 308). However, observations in vivo (87, 140, 182, 295) and our findings using JFH-1 wt suggest that HCV may be relatively resistant to humoral immunity, indicating that more work is necessary to understand the relationship between authentic HCV particles and neutralising antibodies. Our findings using JFH-1 G451R demonstrate a link between particle sensitivity to neutralising antibodies and SR-BI dependency. Therefore, we are interested in examining the relationship between these two characteristics in different strains of HCVcc; J6/JFH exhibits a lower dependence for SR-BI (Figure 4-2), this phenotype may also reflect particle sensitivity to neutralisation.

HCVpp are unlikely to mimic the structure of lipo-viro-particles and recapitulate natural HCV envelope protein conformation(s). Consequently they may not be suitable for assessing antibody mediated neutralisation. Indeed, experiments using HCVpp have suggested that blocking epitopes necessary

for HCV-CD81 interaction is a common feature of neutralising antibodies (130, 145, 236, 237, 308); however our findings pose a question over the accessibility of these epitopes on HCVcc particles. Furthermore, we observed that cell culture adaptation of JFH-1 altered particle sensitivity to nAbs, therefore studies quantifying humoral immunity using HCVcc need to ensure that similar mutations have not occurred. However, a large proportion of cell culture adaptations reported so far fall within the non-structural genes of HCV (141, 144, 269) and therefore would not be expected to alter the relationship between particles and antibodies.

It is clear that the currently available in vitro models of HCV replication are limited in their ability to recapitulate the characteristics of particles circulating within an infected host. Therefore it is necessary to develop innovative systems to overcome the disparity between observations made in vitro and in vivo. A recent study by Vanwolleghem et. al. used immuno-compromised mice bearing humanised livers as a target for infection with patient serum derived HCV (322). Mice that had been passively immunised with polyclonal IgG isolated from a chronically infected patient were resistant to infection with HCV derived from the same source. This suggests that the infected patient had raised a neutralising antibody response, an observation that will encourage those hoping to develop a B-cell vaccine. In the future, infectious systems such as humanised mice will allow us to validate observations made using cell culture models, paving the way to a better understanding of how HCV establishes persistence in such a large proportion of cases.

### **6.3 HCV lipo-viro-particles.**

As discussed throughout this thesis, HCV virions exist as LVPs, containing viral structural proteins, dietary lipids and apoproteins (8, 63, 109, 131, 233, 312). It is unclear what contribution the host lipoprotein moieties make to HCV infection. However, several reports have demonstrated that apoproteins are essential for HCV particle assembly (63, 109, 131, 224) and may play an important role in virus attachment and entry (9, 63, 190, 211). In this way apoproteins may be thought of as functional structural components of HCV particles.

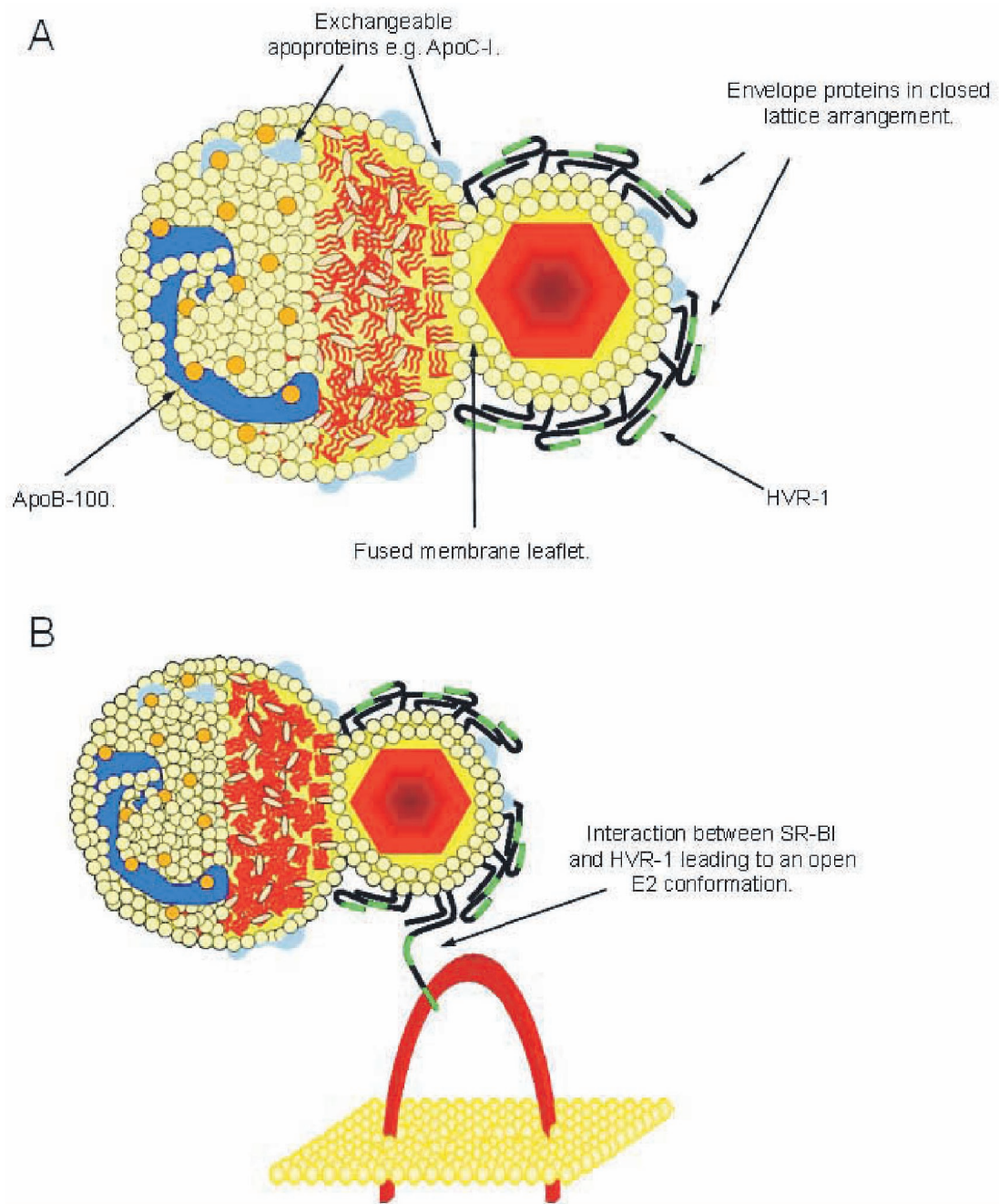
This does, however, present difficulties in modelling the structure of a HCV lipo-viro-particle. The particles of related Flaviviruses such as Dengue have highly ordered 'smooth' structures, with the viral envelope proteins lying flat across the membrane surface (222, 247); a classical Flavivirus particle is analogous to a golf ball. These structures are comprised of an ordered lattice of viral glycoproteins (222, 247), in the case of HCV it is difficult to imagine how apoproteins, some of which are large (>500kDa), could be incorporated into such an arrangement.

HCV LVPs act as metabolically functional lipid carriers (77), demonstrating lipoprotein like properties and structure. Additionally, reports suggest that HCV LVPs are not simply virions with lipoproteins attached to their surface (8, 77). Therefore the structure of HCV LVPs must reconcile these disparate characteristics, allowing them to function as virus particles and lipoproteins simultaneously.

Particle density is believed to be determined by lipid composition; lower density HCV virions have a higher lipid content and therefore may be regarded as the 'most lipoprotein like' form. Low density HCV have the highest specific infectivity (45, 179, 180) and we have shown that particle density correlates with sensitivity to neutralising antibodies (Figure 5-8). Taken together these observations suggest that adopting a lipoprotein like formation promotes HCV infection, and possibly stabilises the virion glycoproteins in a manner that reduces epitope availability. A number of apoprotein species can be found in HCV LVPs, indeed apoC-I is thought to enhance infection (79, 210, 211), however it is not known how apoproteins function within an infectious particle. Understanding the role of apoproteins within circulating HCV particles will be key to determining the relationship between particle density and infectivity.

Figure 6-2 displays a model structure of a HCV LVP; this particle has both virion and lipoprotein like characteristics. The envelope proteins lie flat within the membrane surrounding the capsid, however the outer leaflet of the virion envelope is fused with the lipoprotein lipid monolayer. This proposed structure would confine the viral glycoproteins to the virion envelope bilayer, whereas the very large structural apoprotein, apo-B100, resides within the triglyceride core of lipoprotein (321) and should not diffuse into the virion. However, the smaller exchangeable apoproteins reside within the lipoprotein lipid monolayer (321), allowing their diffusion throughout the particle and interaction with viral glycoproteins. The areas of the viral envelope proteins shaded in green

represent the hyper-variable regions, as discussed earlier these are under sustained immunological selection and are likely to be accessible. Other critical regions of E2 remain buried within the LVP, reducing epitope exposure, we hypothesise that a conformation change within E2 upon interaction with SR-BI leads to the exposure of these regions (Figure 6-2B).



**Figure 6-2 Model lipo-viro-particle.**

**A.** Cartoon representation of a LVP. **B.** Proposed model of LVP-SR-BI interactions.

The G451R mutation disrupts the relationship between particle density and infectivity, suggesting that E2 contributes to the relationship between HCV and lipoprotein components. However, HCV pseudo-particles are not thought to mimic lipoprotein association and yet remain infectious, therefore it is clear that envelope proteins function outside of the context of a LVP. Given JFH-1 G451R's reduced dependency for SR-BI it is tempting to speculate that lipoprotein association promotes viral entry events closely linked to SR-BI and that the adaptive mutation allows HCV to negate these processes. For instance, it is possible that associated lipoproteins define initial particle attachment, allowing specific interaction(s) between the viral glycoproteins and SR-BI.

It is becoming clear that to understand the interaction of HCV particles with cellular receptors and the immune system it will be necessary to unravel the relationship between HCV and host lipoproteins. In further characterising HCV attachment and entry the principal challenge will be to define the functional constituents of an infectious lipo-viro-particle.

#### **6.4 Closing remarks.**

It has been three years since the advent of the HCVcc system and much has been achieved in this short time. Work with this novel system has fallen to three areas: investigating HCV entry, understanding the viral proteins in the context of the entire viral life cycle and elucidating the events surrounding HCV assembly and release. In this study we have used the HCVcc system to characterise the role of SR-BI in virus attachment and entry. More importantly,

we have, for the first time, demonstrated an interplay between HCV particle interaction with co-receptors, neutralising antibodies and host lipoproteins. We hope to continue this line of investigation, focussing on how HCV LVP composition and structure defines envelope protein exposure, receptor engagement and ultimately infectivity.

In continuing our work it is important to continuously evaluate the techniques we use. Cell culture proficient HCV is a very powerful tool, however we must make efforts to understand how our findings relate to the situation within a HCV infected individual. Much of the observations documented in this thesis are virtue of a lab adapted virus and, although fascinating, JFH-1 G451R is emblematic of the problems faced by those using a cell culture proficient HCV. Additionally, some of the inherent limitations in the HCVcc system lie in the cells used to support infection; Huh-7.5 cells are a very good target for infection but doubts remain over how well they represent human hepatocytes. Therefore, the ongoing attempts to propagate patient derived HCV in primary human hepatocytes (51, 216, 217) are an important aspect of HCV research. Consolidating observations from cell culture, animal models and HCV infected individuals will pave the way towards more effective treatments in the future.



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