The role of CD81 in hepatoma biology and hepatitis C virus infection

By

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Abstract

Hepatitis C Virus (HCV) is a global health problem, with over 170 million infected individuals worldwide. 70-80% of infected individuals develop progressive disease, and approximately 2% of these acquire hepatocellular carcinoma (HCC). HCV entry is dependent on tetraspanin CD81, scavenger receptor BI, and tight junction proteins claudin-1 and occludin.

Tetraspanins are involved in multiple biological functions including cell-ECM adhesion and motility. An actin polymerization-dependent cell spread was observed upon ligation of CD81 on hepatoma cells. Importantly, HCV infection perturbed CD81-dependent cell spread, suggesting HCV infection may modulate CD81 function in hepatoma cells.

Functional assays demonstrated that CD81 expression and HCV infection promote hepatoma cell motility. These findings allude to a link between HCV infection and associated HCC development.

Establishment of a chronic infection demonstrates that HCV can escape from the host adaptive immune responses. We developed an *in vitro* cell culture system to monitor viral transmission in the presence of neutralizing antibodies (nAb). Separation of producer and target cells ablated nAb resistant transmission, suggesting that cell-cell contact was essential. Furthermore nAb resistant transmission was dependent upon all four co-receptors. These observations confirm HCV immune evasion by cell-to-cell transfer and have major implications for anti-glycoprotein targeted therapies.

Dedication

I would like to dedicate this thesis to my parents John and Julia Brimacombe.

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Publications

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Abbreviations

AP-1:	Activator protein -1
BSA:	Bovine Serum Albumin
CMFDA:	5-chloromethlfluorescein diacetate
DAPI:	4'6-daimidino-2-phenylindole
DMEM:	Dulbecco's modified medium
DMSO:	Dimethyl sulfoxide
DNA:	Deoxyribonucleic acid
EC1:	Extra-cellular loop 1
EC2:	Extra-cellular loop 2
ECM:	Extra-cellular matrix
EGFR:	Epidermal growth factor receptor
ELISA:	Enzyme linked immunosorbent assay
EMT:	Epithelial mesenchymal transition
ER:	Endoplasmic reticulum
ERM:	Ezrin Radoxin Moesin
Ep:	Electroporation
ESCRT:	Endosomal sorting complex required for transport
F-actin:	Filamentous actin
FFU:	Focus forming units
FRET:	Fluorescence resonance energy transfer
GFP:	Green fluorescent protein
HBV:	Hepatitis B virus
HCC:	Hepatocellular carcinoma
HCV:	Hepatitis C virus
HCVcc:	Hepatitis C virus propagated *in vitro*
HCVpp:	Hepatitis C virus pseudo particles
HDL:	High density lipoproteins
HGF:	Hepatocyte growth factor
HIF:	Hypoxic inducible factor
HIV:	Human Immunodeficiency virus
Hr(s):	Hour(s)
HSV:	Herpes Simplex Virus
HTLV:	Human T cell leukemia virus type 1
HVR:	Hyper variable region
IFN:	Interferon
IGF-1:	Insulin like growth factor
IU:	Infectious Units
IRF:	Interferon regulatory factor
ISGs:	Interferon stimulated genes
JFH:	Virus isolated from a Japanese patient with fulminant hepatitis
Jak-STAT:	Janus Kinase-Signal transducer and activator of transcription
KD:	Knock down
LDL:	Low density lipoproteins
mAbs:	Monoclonal antibodies
MβCD:	Methyl-beta-cyclodextrin
MFI:	Median fluorescent intensity
Min(s):	Minute(s)
MLV:	Murine leukemia virus

MMP:	Matrix metalloproteinase
mRNA:	messenger RNA
MTS:	3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (Cell proliferation assay)
MTP:	Microsomal transfer protein
MVB:	Multivesicular body
nAb:	Neutralizing antibody
NASH:	Non alcoholic steatohepatitis
NK:	Natural Killer cells
OAS:	2'-5' oligoadenylate synthetase
ORF:	Open reading frame
PAMPs:	Pathogen Associated Molecular Patterns
PBS:	Phosphate buffered saline
PKC:	Protein Kinase C
PKR:	Protein Kinase receptor
PI4K:	Phosphatidylinositol 4-kinase
P-ERM:	Phosphorylated Ezrin Radoxin Moesin
PRR:	Pathogen Recognition Receptors
RIG-I:	Retinoic acid inducible gene I
RNA:	Ribonucleic acid
SGR:	Sub-genomic replicon
siRNA:	small interfering RNA
shRNA:	small hairpin RNA
SOCS:	Suppressor of cytokine signaling
SR-BI:	Scavenger receptor BI
TEM:	Tetraspanin enriched microdomain
TIRF:	Total internal reflection fluorescence
TLR:	Toll-like receptor
UTR:	Un-translated region
VEGF:	Vascular endothelial growth factor
WT:	Wild type

1. Introduction

1.1 History and Epidemiology of HCV

During the 1970's scientific advances were made enabling serological detection of hepatitis A and B virus infection (35, 136), it quickly became apparent that at least one other agent was responsible for hepatitis arising from blood transfusion, this was commonly referred to as non-A non-B hepatitis (NANBH) (137). Despite a large amount of research it wasn't until over a decade later that Hepatitis C Virus (HCV) was formerly identified. Choo and colleagues constructed a cDNA library from serum containing the NANBH agent isolated from infected chimpanzee plasma and successfully isolated a clone that specifically hybridized with RNA found only in NANBH infected chimpanzees and encoded a protein that bound antibodies from NANBH infected patients (206, 259). These findings enabled the establishment of assays to screen for HCV in blood, and since introducing these tests in the early 90's the risk of transmission through blood transfusion in the developed world is now extremely low (62).

HCV is endemic worldwide and according to figures from the WHO there are an estimated 170 million people infected making up approximately 3% of the worlds population. Prevalence varies greatly depending on geographical location. Areas with the highest recorded prevalence are in Africa and Asia; Egypt for example has a prevalence of 22% (148). Prevalence in North America, Japan and Western Europe are lower ranging from 0.6% in Germany (363) to 2.2% in Italy (11, 430). It is likely that these values under estimate the burden especially in the developing world where

there is less data available. In some areas the true burden may not yet be realized due to the asymptomatic nature of the disease during both the acute and early chronic phases of infection, preventing detection until late in disease progression (11, 430). Interestingly in different geographical locations the range in prevalence determined by age varies considerably. In North America for example the highest prevalence is among people between the age of 30 and 49 (12), this differs from Japan and Italy where the highest prevalence is among the over 50's (11). These differences are consistent with young adults in North America being at greatest risk around 20 years ago whilst in Japan the greatest risk was many years before this. Like Japan, in Egypt the highest prevalence is in the over 50's age group, however unlike Japan the prevalence in the younger age groups is also extremely high (1, 11).

HCV is the sole member of the genus *Hepacivirus* within the *Flaviridae* family. This family includes yellow fever and classical swine fever viruses, all of which are enveloped viruses containing a single stranded positive sense RNA genome. HCV is genetically very diverse with 7 major genotypes and many different subtypes (166, 442). Subtypes vary between 20 and 25% whilst genotypes can vary by 30% at the nucleotide level (440, 443). The geographical prevalence and diversity of the genotypes differ giving clues as to the origin of the virus (441). Genotype 1a, 1b and 3a are the most prevalent genotypes in the western world (440). Genotypes found in Africa and South-East Asia associate with specific geographical areas and are much more diverse. The genetic diversity of these viral strains suggest that the virus has been present in human populations in these areas for a long time and that it is only in recent history that the virus has transmitted to the western world (323). Genotypes 1, 2 and 4 are found specifically in sub Saharan Africa and genotypes 3 and 6 found in

South-East Asia (445). As for the origin of the 6 genotypes there is no evidence of HCV or HCV-like virus in old world ape or monkey species as observed for HBV (301). Interestingly though a distantly related virus named GB virus B has been reported to infect tamarins and other new world primate species (441, 447). Further studies are needed to understand the origin of HCV and its relation to GB virus B.

Risk factors of infection vary geographically and have altered over time. In the developed world a major source of transmission was through increased use of blood products in medical practices, as previously explained this is no longer a risk due to routine screening of donated blood (62). Since the 1960's injection drug abuse has become the primary route of transmission (12). Other lower risk factors associated with HCV transmission include perinatal, sexual and occupational transmission. There is little evidence for risk associated with sexual transmission and the data from occupational transmission suggests a low risk (11). Perinatal transmission occurs in approximately 2.7-8.4% of cases, interestingly this value increases significantly in mothers co-infected with HIV (139, 477). However in the developing world the picture is very different with major risk associated with contaminated blood products a result of financial constraints limiting screening (11). Another major risk factor in the developing world is through vaccination(s) with contaminated needles. Two studies in India demonstrated an association between HCV infection and visits to unlicensed medical practitioners (86, 305). In Egypt the majority of HCV infections are associated with a nationwide vaccination program against Schistosomiasis that was carried out between 1960 and 1987 (148). Contaminated needles in vaccination programs are likely to be the cause of infection in some developed countries where prevalence is highest in older populations (430). As for transmission of the virus in

Africa and Southern East Asia before the use of modern medicine it is hypothesized that transmission was and may still occur through tribal scarification practices or through insect vectors such as mosquito's or ticks. However, to date there is no substantial evidence to support these theories (430).

1.2 Disease progression and current treatment

1.2.1 Disease progression

HCV is unusual compared to other flaviviruses due to its capability to persist in infected individuals. Between 75 and 85% of individuals infected with HCV develop a persistent infection (204).

Due to the asymptomatic nature of early infection there is little data on acute infection, most of our current knowledge has been acquired from prospective studies of transfusion patients, chimpanzee studies or though a number of acute cohorts largely consisiting of injection drug users (IDUs) or health care workers (HCWs) (361, 392, 413, 492). Acute resolving infection typically lasts between 10 and 12 weeks. RNA can be detected within the first two weeks post exposure and rises to a peak of between 10^5 and 10^7 IU/ml at 6 to 10 weeks post infection, this often comes shortly before a peak in serum alanine aminotransferase (ALT) levels, a marker for liver injury (8, 135, 300, 476). The production of anti-HCV antibodies is more variable, with most patients developing anti-HCV between 7 and 8 weeks post infection (349, 369). The presence of neutralizing antibodies (nAbs) is critical in ensuring successful clearance of many viruses (61). Although there are a few examples where a nAb response during acute HCV infection has associated with

viral clearance (29, 268, 326, 377), the majority of studies show nAbs appearing after acute infection indicating a more dominant role for nAbs in controlling chronic infection (290). In contrast a cellular immune response is essential to ensure viral clearance. HCV specific T-cell responses are detectable between 4 to 8 weeks post infection. A correlation has been demonstrated on many occasions between a robust CD8+ and CD4+ T-cell response and viral clearance (97, 242, 271, 437, 476). In non-resolvers the response is often weak or not detected at all. Although most patients do not exhibit any symptoms, evidence suggests that when symptoms do occur there is an increased likelihood for successful viral clearance (9, 159).

The acute stage of a chronic infection with respect to antibody response, RNA level and ALT levels cannot be distinguished from the acute stage of resolving patients (204). If after 6 months HCV RNA levels are still detectable the patient is reported to have developed a chronic infection (204). RNA levels in the blood remain stable although they can vary greatly between donors and are not predictive of disease outcome (544). ALT levels decrease after the initial boost during acute infection and in the majority of cases levels remain elevated above normal and fluctuate during the course of chronic infection, again ALT was found not to be a good predictor of liver disease status (178). Some patients with a chronic infection have a mild non-progressive disease whilst 20-30% develop complications including cirrhosis and end stage liver disease, 2.5% of these patients develop hepatocellular carcinoma (HCC) (6, 10, 71). HCV associated liver damage is explored in more depth in chapter 4. Given the diversity of HCV it is surprising that large variations in clinical outcomes do not occur, in fact all genotypes are capable of initiating a chronic infection that can eventually lead to the development of liver disease and HCC. Although, as more

clinical data is collected differences between genotypes are becoming apparent. A number of studies looking at European cohorts have revealed that genotype 1 is more likely than genotypes 2 and 3 to firstly establish a persistent infection and secondly once a persistent infection is established to cause a greater degree of liver disease (311, 395, 542). A strong association between development of liver steatosis and genotype 3 infection has also been demonstrated (2, 405). It is believed that a block in lipoprotein secretion from hepatocytes causes this phenotype (426). More work is needed to validate and determine the mechanism of these observed differences.

Although the primary disease symptoms are liver related, chronic infection is also associated with other extra-hepatic conditions suggesting the liver is not the only reservoir for infection. Examples of which include auto-immune diseases such as cryoglobulineimia and glomerulonephritis (129) as well as B-cell non-Hodgkin lymphoma (562) and neurological conditions including cognitive disorders (193). Our laboratory has previously demonstrated HCV association with B-lymphocytes aiding infectivity *in vitro* (455) and we are currently investigating brain tissue as a further reservoir for HCV (142)(Nicola Fletcher, submitted).

1.2.2 HCV immune escape

HCV employs a number of mechanisms to hinder both the innate and adaptive immune responses allowing a persistent chronic infection within the host (48, 182). Briefly the intracellular innate immune response to viral infection is as follows; upon viral entry into host cells Pathogen Recognition Receptors (PRR) present in the cytoplasm identify signatures of viral infection such as single and double stranded RNA, these are termed Pathogen Associated Molecular Patterns (PAMPs) (409). In

hepatocytes the predominant PRRs associated with HCV infection are Toll-like receptor 3 (TLR-3) and RIG-I (276, 461, 548). Both of these PRR's recognize HCV double stranded RNA and induce a signaling cascade leading to the activation of latent cellular transcription factors Interferon regulatory factor IRF (3) and nuclear factor NFκB (280, 397). These transcription factors induce Interferon-beta (IFNβ) gene transcription. IFNβ is then secreted from the infected cells and functions in an autocrine and paracrine manner by engaging type I IFN receptors on the cell surface eliciting the Jak-STAT pathway (412). This pathway results in the formation of Interferon stimulated gene factor-3 (ISGF3) that enhances transcription of Interferon stimulated genes (ISGs). ISGs are responsible for antiviral actions within the cell (113, 153). Predominant anti-viral effecter genes include Protein kinase receptor (PKR) that inhibits translation of viral RNA (512) and 2'-5' oligoadenylate synthetase (OAS) that cleaves HCV genomic RNA into non-functional products, inhibiting viral replication (185). Other ISGs include p56, IRF7 and IRF3 (48). IRF7 and 3 induce transcription of IFNα gene leading to a positive feedback loop increasing IFN response (20). IFNα is also responsible for influencing the adaptive immune response (412). For HCV to successfully persist in its host it needs to be able to overcome these innate immune response. A number of HCV proteins including Core, NS3/4A protease and NS5A, are implicated in hindering stages of this immune response. NS3/4A for example blocks the RIG-I signaling pathway and completely ablate TLR-3 signaling (147, 276). Core protein has been shown to induce SOCS1 and SOCS3 expression; that are negative regulators of the Jak-STAT pathway (49). NS5A protein and glycoprotein E2 have both been implicated in inhibiting anti-viral effecter genes. For example E2 can bind PKR inhibiting its function (367, 472) and NS5A has been shown to inhibit PKR function (378) whilst also repressing OAS

functions (464). NS5A expression has also been shown to induce IL-8 expression; an inhibitor of IFNα induced ISG expression (387).

HCV has also evolved to escape from cellular and adaptive immune responses. For example HCV core protein has been implicated as an agent capable of inhibiting immune cells directly. An *in vitro* study reported that HCV core protein inhibits T-cell activation *by* interacting with complement factor gG1qR (244, 509). Of note core protein from genotype1a virus was used in this study and the results could not be repeated in a separate study with core from genotype 1b, indicating a possible genotype specific function (182, 289). As yet this effect has not been confirmed *in vivo*. As will be explained later HCV glycoprotein E2 binds co-receptor tetraspanin CD81 (384). NK cell cytopathic function is inhibited upon engagement of cell surface expressed CD81 with recombinant E2 protein, suggesting a possible mechanism for HCV to perturb the host immune response (105, 486). Controversy over whether these studies can be re-produced when E2 is expressed on a viral particle is currently under debate (549). A further example is the induction of escape mutants to nAb's and T cell epitopes. A single infected host contains a quasispecies (closely related species of virus subjected to genetic mutations, competition and selection) population of HCV (117). A quasispecies population develops for two main reasons; firstly HCV replicates rapidly allowing many opportunities for mutations to occur and secondly HCV has a single strand positive sense genome that is replicated by an RNA dependent RNA polymerase (RdRp). RdRp does not have proof reading ability and permits rapid evolution of the virus (37). Neutralizing antibodies have been shown to target the hypervariable region 1 (HVR-1) of glycoprotein E2 and mutations have been detected in this region in human and chimpanzee studies that confer

escape from immune pressures (434, 450, 497). Mutations in regions inhibiting CTL CD8+ T cell responses have also been detected in both chimpanzees and humans suggesting another possible mechanism of viral escape (73, 131, 500, 505). As will be discussed later HCV particles associate with lipoproteins (14, 351). This has been suggested as an additional mechanism employed by HCV to disguise the virion from the immune system reducing the effectiveness of nAb's. Lastly recent work published from our laboratory demonstrates that HCV can transmit in co-culture in the presence of nAbs capable of neutralizing cell free virus, suggesting that HCV may transmit from cell-to-cell via a novel route reducing exposure to the host immune system (480). This is discussed in Chapter 5.

1.2.3 Treatments

Standard treatment for HCV infection is a course of pegylated Interferon (IFN) and Ribavarin. This can be extremely effective (up to 90%) if treatment is started within the acute phase of infection (221, 510). After onset of chronic infection efficacy drops and varies considerably depending on the infecting genotype. 70-80% of individuals with genotypes 2 and 3 respond to Interferon based therapy compared to only 40-50% of patients infected with genotype 1 (181, 267, 303). The mechanism of action of IFN and Ribavarin based therapy is not fully understood. IFN is likely to reduce infection at both the viral level by inducing Interferon stimulated genes (ISGs) that have direct anti-viral functions as well as promoting the innate and adaptive immune response (38, 425, 479). Ribavarin is a synthesized guanosine that has previously been demonstrated to inhibit other RNA and DNA virus infections (138). It is thought that its main mechanism of action against HCV may be through increasing mutagenesis of viral RNA and thereby reducing specific infectivity (550). Limitations

in efficacy and severity of side effects associated with the current therapy make it increasingly important to find new more effective therapies.

Many alternative therapies are being explored and some of which have/are currently undergoing clinical trials with promising results (109, 428). Examples of these include drugs targeting viral enzymes including the NS3-4A viral protease and NS5B RNA polymerase (109, 261, 265, 394), these became promising targets after there structures were defined in 1996 and 1999 respectively (4, 56, 243). NS3-4A is a particularly attractive target not only because of its important role in the HCV lifecycle but also because of its role as an inhibitor of the innate immune response as previously described (147, 276). Our laboratory is currently working with a small molecular inhibitor of SR-BI, a co-receptor for HCV entry, this compound is soon to be trialed here in Birmingham. More information on this inhibitor is presented later in chapter 5. Further immuno-modulating agents have also been considered as therapeutics including TLR-7 and TLR-9 antagonists. Toll-like receptors are present on a number of immune cells and recognize microbial agents inducing an immune response primarily involving the induction of IFN whilst also priming an adaptive immune response (465). TLR-7 and TLR-9 in particular recognize single stranded RNA (50). Antagonists to TLR-7 have been trialed against HCV infection with promising results (520). Complications have arisen with many of the more potent anti-replicase agents including a rapid induction of viral escape mutants (258, 279, 329). It is therefore hypothesized that a combination of therapies targeting both virus and host will be required to overcome this problem in the future (109).

As yet there is no effective vaccine available against HCV, this has been due predominantly to the lack of knowledge on mechanisms of viral clearance, the evidence for re-infection of both humans and chimpanzees as well as lack of definitive evidence on the efficacy of neutralizing antibodies *in vivo*. Recent studies have given more hope into the eventual production of an effective vaccine. Firstly over time increasingly more information has become available on patients with resolving infection increasing our understanding of what constitutes an effective immune response (101, 271, 437). Secondly patients that are re-infected with HCV are often protected against the development of a chronic infection (266, 322). Lastly neutralizing antibodies have been detected that can cross-neutralize across different genotypes suggesting that a broad spectrum vaccine may be possible and furthermore a small number of studies have reported a correlation between the induction of neutralizing antibodies in the early stages of infection and viral clearance (29, 268, 326, 377). Supporting observations in humans, chimpanzee studies using an E1E2 peptide vaccine, although unable to stop re-infection, were able to prevent progression to a chronic infection in the majority of subjects tested (84). Chronic infections create the greatest burden of HCV disease both financially and to the host therefore the ability to reduce this occurrence would be extremely beneficial. More recent studies are looking at methods to develop a vaccine that will elicit both the production of cross neutralizing antibodies as well as inducing a broad cellular response, responses that have previously been associated with viral clearance (65, 207, 282). Although trials in animal models have been relatively successful, human trials are needed to determine their true efficacy. The use of vaccines as a therapeutic treatment is also being investigated in the field, the theory behind this is that a vaccine may be able to boost the immune system and therefore increase

efficacy of IFN based therapies (207). This theory is based on evidence that increased immune response prior to treatment increases the likelihood of success (34, 103, 347).

The absence of an effective prophylactic or vaccine for HCV together with the virus's propensity to cause considerable liver damage after long term infection makes it absolutely crucial that more research is done to fully understand HCV infection.

1.3 Tools available to study HCV in vitro

An *in vitro* system to study the full viral life cycle, from entry through to release of infectious viral particles has only recently become available. Although a partial sequence of HCV was identified in 1989 (85), it wasn't until 1996 that the full HCV genome sequence was completed using sequences isolated from patient H (Huchinson 1977), a patient infected with a genotype 1a virus (249). With this knowledge a model cDNA template was constructed (H77), and delivery of the transcribed RNA into the liver of chimpanzee's resulted in viral replication (248, 526). This became a model system allowing the study of immune response and viral evolution during infection (58). Unfortunately these clones were unable to replicate and assemble infectious particles in cell culture.

Although a number of techniques have been used to study HCV entry the most effective and widely used system is the retroviral pseudoparticle system (HCVpp). Pseudoparticles comprise of an HIV capsid with HCV glycoproteins. HCVpp allow glycoprotein dependent entry to be studied and have allowed considerable advances to be made in determining host cell entry receptors (30, 121, 208). These

pseudoparticles can only undergo one round of replication and cannot transmit to other cells.

A critical development in the race to find an *in vitro* culture system to study the full viral lifecycle was made by Lohmann et al., in 1999. Lohman and colleagues demonstrated replication of sub-genomic replicons in cell culture (292). The development of the replicon system has been a critical tool for increasing our understanding of HCV genome replication and a screening tool for the discovery of new anti-viral agents (26, 47, 381). Although full-length replicons replicated efficiently no infectious viral particles were produced and it was feared that Huh-7 cells may be unable to assemble or release virus particles (382). Cell culture adapted mutations were identified allowing increased replication efficacy *in vitro* but unfortunately the mutations severely reduced infection *in vivo* (283, 291).

It wasn't until 2005 that a full-length virus with no cell culture adaptive mutations was identified and demonstrated to replicate and assemble virus particles in cell culture (283, 499, 560). This was termed HCVcc, cc representing cell culture. JFH-1 (genotype 2a) was isolated from a Japanese patient with fulminant hepatitis and had previously been shown to replicate in the form of a sub-genomic replicon without any amino acid changes (237). Wakita and colleagues were the first to demonstrate that the full length JFH-1 genome could replicate in Huh-7 hepatoma cells and importantly also in the chimpanzee model (499). Chimera's of JFH-1 consisting of core to NS2 of J6 (genotype 2b) or H77 (genotype 1a) with the remaining non-structural proteins of JFH-1 were developed and were reported to be infectious in chimpanzees and uPA-SCID mice containing human liver grafts. Importantly the virus remained infectious in

cell culture after *in vivo* propagation (284, 545). The JFH-1 HCVcc system is the basis for much of our research today and recent advances have led to the production of numerous JFH-1 chimeric viruses representating the 7 major HCV genotypes allowing genotype specific comparisons to be made (166).

HCVcc replicates most efficiently in the Huh-7 hepatoma cell line (47). Hepatocytes in the liver are highly polarized and tight junction proteins claudin-1 and occludin have been identified as critical co-receptors for HCV entry (133, 385). Huh-7 cells do not polarize and therefore may not be the most appropriate representative model to use. Our laboratory is currently working with a polarised hepatoblastoma cell line HepG2, these cells exhibit hepatocyte polarity and support viral replication but have a low permissivity to HCV infection making them challenging to use (319, 320). To further *in vitro* studies a highly permissive cell line that exhibits hepatocyte polarity is needed. A model system utilizing primary hepatocytes would be ideal but because of difficulties in accessing and processing human liver tissue very little work has been reported with primary cells. Recent developments in this area have involved the establishment of new techniques to propagate primary hepatocytes for longer periods of time before de-differentiation (229, 241). This is an area of research that is still in its preliminary stages and needs to be developed in the future to allow greater accessibility for more laboratories to use these techniques.

1.4 HCV lifecycle

1.4.1 Genome and replication

HCV contains a 9.6kb genome consisting of a long Open Reading Frame (ORF) flanked by Non Translational Regions (NTR's) (285). A highly conserved region of the 5'NTR represents the internal Ribosome Entry Site (IRES) and mediates a CAP independent translation of the ORF (57, 357, 488). The ORF encodes a polyprotein of 3011 amino acids long consisting of a structural and a non-structural region (Fig.1.1). Host cell signal peptidases cleave the structural proteins and also p7/NS2 junction, whereas the non-structural proteins (NS2 to NS5B) are cleaved by two viral enzymes, NS2-3 and NS3-4A (285).

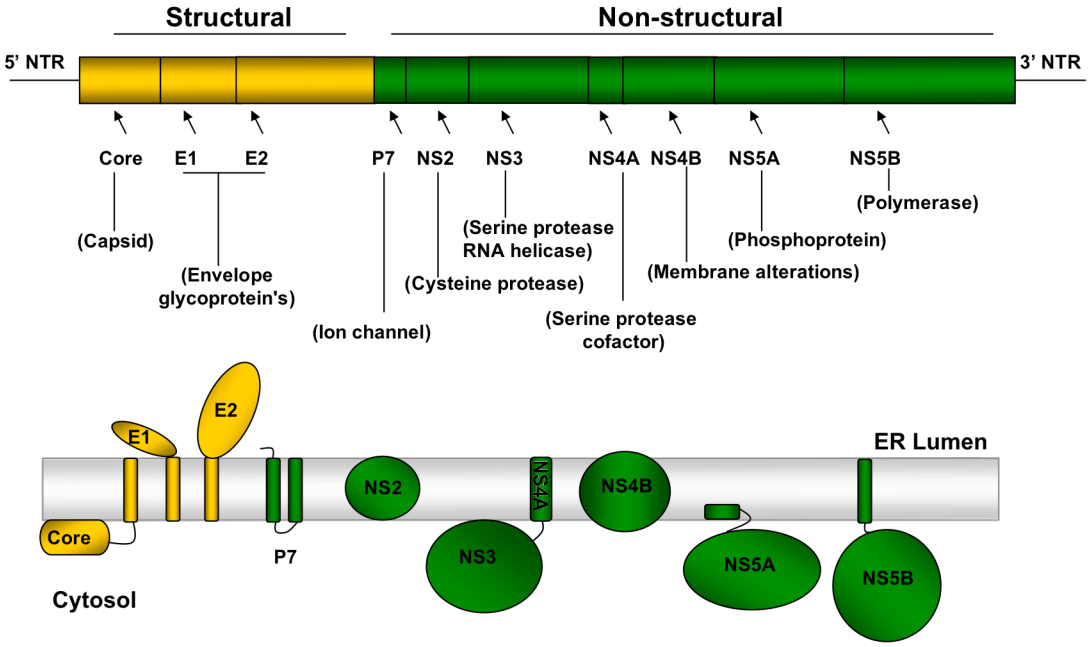


Figure 1.1: HCV genome and gene products.
The top image depicts the genome organization. Translation of the polyprotein is dependent on the IRES located in the 5'UTR. The polyprotein contains structural (gold) and non-structural (green) proteins. Host and viral proteases cleave the polyprotein producing 10 viral proteins. The lower image depicts the proteins within a membrane in the Endoplasmic reticulum (ER). Image based on (285, 374).

The structural region comprises the highly basic core protein and two glycoproteins E1 and E2 (285). Core protein has a hydrophilic N terminal (Domain I) that is able to bind RNA and is believed to be important in the packaging of the nucleocapsid, this domain is also responsible for homotypic interactions between core proteins allowing the formation of larger multimeric structures of core resembling nucleocapsid like structures *in vitro* (257, 316). Regions of the hydrophobic C terminal (Domain II) anchor core into the ER and allow interactions with lipid droplets (LD) (128, 165, 395, 404). This region also contains a cystein (Cys 128) that has been identified to have a role in particle production and has most recently been shown to form a disulphide bond between core monomers, allowing stabilization of the capsid (260, 339). As well as being structurally important for the virus, core protein also functions in many other aspects of the viral lifecycle including immune evasion strategies as previously described and viral assembly through the modulation of lipid droplets as will be described in more detail later on (49, 263).

Post translation, the E1E2 glycoproteins (consisting of c-terminal transmembrane and N-terminal ectodomains) are trans-located to the ER where they are cleaved from the poly-protein by host signal proteases and retained in the ER by restriction signals located in their transmembrane domains (92, 94, 123, 143). The transmembrane domains also function to initiate formation of stable non-covalent heterodimers (94, 110). A number of N-linked glycosylation sites have been identified on the E1E2 proteins. Glycosylation is thought to occur post translation to insure correct protein folding (124, 125, 162, 327). Viral glycoproteins have two major functions in the viral lifecycle. Firstly they are involved in viral attachment and have been shown to directly interact with co-receptors CD81 (384) and SR-BI (418).

Secondly they are thought to function as membrane fusion proteins coordinating pH dependent fusion of the viral and cell membranes in the early endosome permitting release of the viral genome into the cytoplasm of the host cells (208, 485, 496). Sequence comparison with other flaviviruses revealed that HCV E2 glycoprotein was likely to contain a class II fusion peptide (522) and a number of laboratories have since identified the C-terminus of E2 as being important for cell fusion (144, 286). Furthermore a number of regions in E1 have been discovered that are important for cell fusion (156, 275). A recent study showed evidence for a functional role of E1 in membrane fusion by measuring biophysical changes in membranes after interaction with an E1 peptide representative of a previously identified fusion domain (375). It is now widely believed that a number of regions are required for HCV-cell fusion events and this is likely to involve both E1 and E2 proteins, although the mechanism is still under debate (120, 130, 269, 371).

p7 is an integral membrane protein that is primarily located in the ER (67), it consists of two hydrophobic transmembrane domains connected by a short basic loop that is highly conserved between genotypes (173, 456). p7 forms oligomers and electron microscopy studies have revealed the structure of p7 hexamers (172, 296) and heptamers (91). These oligomeric structures form cation selective ion channels in lipid bilayers (172, 368). As such p7 has been categorized as a member of a group of viral permeability altering proteins called viroproteins; other examples include M2 from Influenza, vPu from HIV-1 and M from dengue virus (90). A number of *in vitro* studies have demonstrated an essential role for p7 in virus assembly and release this is supported by work showing that p7 is essential for infection in chimpanzees (230, 410, 456). Woznik and colleagues demonstrated that intracellular virus particles have

greater acid sensitivity compared to extra-cellular virions, suggesting a role for p7 in protecting maturing virions from acidic conditions by removing H+ ions from intracellular membranes (518). Inhibitors for p7 ion channel have been effective in *in vitro* studies making p7 an attractive therapeutic target for the future (170, 171, 457).

NS2 has been shown to have multiple functions in the viral life cycle and like p7 has been demonstrated to be critical for viral assembly (112, 225, 230). NS2 is a hydrophobic protein with several transmembrane domains in the N-terminal region (414, 525). NS2 is stimulated by cofactor NS3 and NS2-3 cysteine protease (Comprising of the C-terminal of NS2 and the N terminal of NS3 (364, 475)) cleaves the NS2/3 junction allowing NS3-4A serine protease to cleave all the downstream sites (364, 475). The cleavage of the NS2/3 junction is required for replication of full-length replicons (506) and also replication in chimpanzees (250). The N-terminal transmembrane domain of NS2 is necessary for virus assembly (225) and the C terminal protease domain but not its catalytic ability has also been demonstrated to be necessary for virus assembly (230).

NS3 to NS5B proteins are all required for viral replication although due to the small size of the viral genome it is likely that most viral proteins are multifunctional (338). RNA viruses replicate their genome associated with altered cytoplasmic membranes or membrane webs. Replication of HCV has been demonstrated to occur in association with cytoplasmic membranous webs in a distinct subcellular replication complex (RC) compartment (5, 165, 332). The positive sense RNA is translated to make new viral proteins as well as acting as a template for an intermediate negative

RNA strand that forms the template for the production of multiple sense genomes that are packaged into new virus particles (285).

NS4A is a cofactor for NS3, the presence of NS4A increases the stability of NS3 as well as allowing the cleavage of NS4B-NS5A junction (516). The C terminal region of NS4A is required for cleavage of the NS3-NS4A and NS5A-NS5B junctions (134). Membrane anchorage of NS3-NS4A has been shown to occur through the N terminus of NS4A (516). As previously discussed the NS3-NS4A protease also functions in immune evasion by blocking the RIG-I and TLR-3 signaling pathways (147, 276). Recent work has discovered that NS4A is also involved in recruiting creatine kinase B (ATP generating enzyme) to the replication complex (186). The initiation of the membrane web formation is down to NS4B protein (128). NS4B is an integral membrane protein that is thought to contain four transmembrane domains and interacts with other non-structural proteins as well as viral RNA (167). Recent work implies that NS4B may also play a role in viral assembly (231).

NS5A is a three domain protein that is found in hypo and hyper phosphorylated forms (469). This protein is involved in a number of stages of the viral lifecycle, the multifunctional capacity of NS5A is most likely down to its ability to interact with numerous host proteins (81, 82, 473, 474). NS5A is a critical member of the replication complex and all three domains are able to bind viral RNA, it has been suggested that part of its role in replication is to enhance NS5B activity (82, 146). As previously discussed NS5A is also important for HCV immune evasion strategies by effecting IFN sensitivity (378, 387, 464). Recently NS5A has also been shown to be

involved in viral particle assembly as discussed later on (473). The remaining nonstructural domain, NS5B codes for the RNA dependent RNA polymerase (393).

1.4.2 Particle assembly and egress.

The exact mechanism by which viral particles are formed and released from the cell is currently not well understood. Clues into the assembly process of HCV came from observations that virus isolated from either *in vitro* studies or from serum of infected patients contained particles of varying densities (74, 234, 285, 351). Andre et al., proposed that low density HCV particles associate with lipoproteins, he termed these lipoviral particles (LVP) and likened them to very low density lipoprotein (VLDL) (14). LVPs are enriched with high levels of triglycerides and both apolipoprotein B (Apo B) and apolipoprotien E (Apo E) have been detected in the viral RNA containing low density fractions (14, 215, 351). Recently Merz et al., established a high affinity purifaction assay using flag tagged viral particles, permiting further investigation of cell culture derived viral particle compostion. The lipid compositon of the viral particles was found to be distinct from the host cell membrane, predominantly consisting of Cholesteryl esters as well as high levels of Apo E and detectable levels of cholesterol (324).

Particle assembly is believed to occur on/near lipid droplet associated membranes that are derived from the ER (27, 331, 429). Nucleic acid association with core is vital for nucleo-capsid formation and this association may initiate viral particle formation (257). The C terminal region of HCV core protein is responsible for core proteins association with lipid droplets and ER membranes (128, 165, 404). Extensive work by McLauchlan and colleagues has demonstrated that core is released from the ER and loaded onto lipid droplets during HCV infection and

importantly this correlates with virion production (53, 315, 317). Furthermore, core protein induces the accumulation of lipid droplets and modulates their distribution in infected cells (52). Lipid droplets move through the cytoplasm and interact with the ER facilitating lipid and protein transport between organelles. Miyanari et al., demonstrated that core recruits non-structural proteins and replication complexes to lipid droplets associated with the ER, facilitating particle assembly (331).

We have previously acknowledged that non-structural proteins p7, NS2 and NS5A are involved in viral assembly. Work on p7 predominantly focused on protein structure (91, 172, 296) however, recent research has demonstrated that p7 modulates the pH of membrane structures involved in viral production, most likely providing protection for the immature virus from acidic conditions (518). As yet the mechanism of NS2 function in assembly is not well understood (225, 230).

NS5A like core can be found on lipid droplets in infected cells. NS5A and core are reported to interact with one another; importantly elimination of this association by mutations in domain III of NS5A reduces particle assembly (16, 331). Furthermore NS5A domain III also associates with Annexin A2 (phospholipid binding protein with multiple functions including endosome trafficking), this interaction has been shown to be important for viral assembly (21).

Proteins involved in the VLDL (Very low-density lipoproteins) secretion pathway have been identified in membranous web compartments containing the HCV replication complex, these include Apo E and ApoB (209). VLDLs are found only in the liver and inhibition of VLDL secretion limits HCV release from Huh-7 cells suggesting this

process is closely linked to the VLDL assembly and secretion pathway (209). Dependence on the VLDL secretion pathway for viral assembly and egress has been proposed as an explanation for hepatic tropism (157, 209). There is controversy over this data set as Jaing et al., were not able to repeat these observations and found only the VLDL associated protein Apo-E and not Apo-B or microsomal transfer protein (MTP) was important for viral assembly (223).

The important role of Apo E in viral assembly has been confirmed in a number of studies. Recent studies have demonstrated that silencing Apo E reduces viral assembly and egress, interestingly partial silencing was reported to permit viral assembly but inhibit release resulting in an accumulation of vius particles in the cytoplasm, indicating Apo E may be involved in two separate steps of viral assembly and release (40). Furthermore Apo E associates with NS5A (40). Cun et al., has recently confirmed that a specific alpha helix domain in the C terminal third of Apo E is responsible for this association and most importantly blocking this specific interaction using an ApoE deletion mutant protein inhibited viral production (108). It has been hypothesized that the interaction of NS5A with Apo E may provide a link between virus production and secretion (40).

In summary HCV assembly is likely to occur near lipid droplets that are associated with membranous vesicles containing the replication complex, key viral proteins involved in this process comprise NS5A and core protein and key host cell factors include lipid droplets and Apo-E protein.

The area of research covering HCV egress is to date quite limiting. As previously discussed the host cell protein Apo E is likely to be involved in egress as well as viral assembly (40) and the specifics of the involvement of the VLDL secretion pathway in viral egress is currently under debate (209, 223). Processing from high mannose to complex-type sugars of N-linked glycans on envelope proteins of HCVpp indicates post -translational modification in the Golgi a step that may support a link with VLDL secretion pathway (355). Recent work has implicated the involvement of late endosomes in viral release, a process completely separate from the endosomal pathway utilsed by the virus for cell entry (263). Lai et al., demonstrated that viral egress was dependent on the motility of early to late endosomes and hypothesized that following assembly, virus particles are transported through early and late endosomes to the plasma membrane where they are released (98, 263). This is supported by recent findings that the endosomal sorting complex required for transport (ESCRT) machinery in particular ESCRTIII and VSP4 permits HCV release (98). Briefly ESCRT machinery is required by the cell to incorporate ubiquitinated proteins into intra luminal vesicles within a multivesicular body (MVB) / late endosome and to traffic these vesicles for degradation by the lysosome, it is also used in cytokinesis and budding of enveloped viruses (517). ESCRT III and VSP4 are the most highly conserved of the ESCRT machinery; VSP4 is an AAA ATP that enables recycling of ESCRT III protein (517). It is hoped that a greater understanding of HCV viral egress will increase the availability of possible drug targets for the future.

1.4.3 Attachment and entry

HCV initially attaches to the host cell membrane through non-specific interactions with low affinity receptors (3, 28, 31, 155). Successful entry into the cell requires four essential co-receptors or entry factors. These are Tetraspanin CD81, Scavenger receptor BI (SR-BI), and tight junction proteins Claudin-1 and Occludin (Fig.1.2) (133, 384, 385, 418). HCV is thought to enter the host cell through a clathrin-mediated endocytosis followed by a low pH dependent fusion event within the early endosome allowing release of the genome into the cytoplasm. Indeed inhibitors of both clathrin endocytosis and early endosome acidification block entry of HCVcc and HCVpp *in vitro* (45, 208).

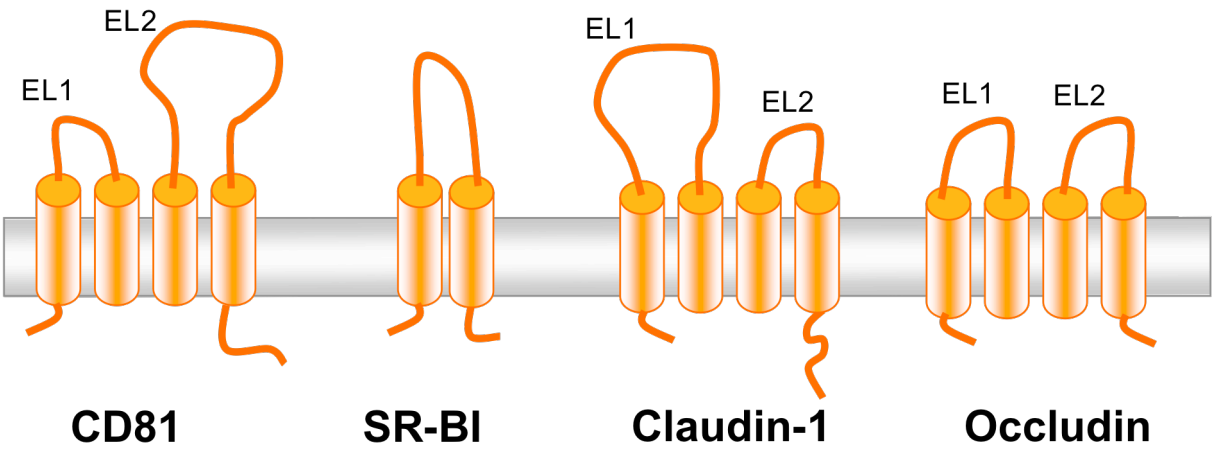


Figure 1.2: Schematic drawing of HCV co-receptors
SR-BI consists of a large extra-cellular loop and two transmembrane domains as opposed to CD81, Claudin-1 and Occludin that have four transmembrane domains and two extra-cellular loops.

Attachment factors

Low affinity receptors are thought to be important in the initial binding of the virion to the host cell prior to interaction with high affinity receptors. Glycosaminoglycan's (GAG's) are linear polysaccharides ubiquitously expressed on eukaryotic cell membranes; highly sulfated GAG's such as heparin sulphate (HS) are implicated in the attachment and subsequent entry of a number of viruse for example other flaviviridae including Dengue virus type 2 utlise HS as a viral receptor (80, 199). In 2003 Barth et al., demonstrated that sE2 binds HS suggesting it could be involved in attachment of HCV (28). Furthermore treatment with heparinase an enzyme that degrades heparin sulfate or heparin an analogue of HS, reduces HCVcc infection *in vitro* indicating that HS is indeed important for initial attachment of HCV to the host cell (31, 252). Interestingly upon formation of the E1E2 heterodimer E2 is no longer able to bind to HS, indicating the binding site is no longer visible and that the lipoproteins associated with HCV may be the factor mediating HCV attachment to HS and not the glycoproteins (60, 63).

Other low affinity receptors implicated in HCV attachment include the C-type lectin DC-SIGN (Dendritic cell-specific intercellular adhesion molecule-3-grabbing nonintegrin) and the liver related molecule L-SIGN (Liver and Lymph node specific DC-SIGN). DC-SIGN regulates the interaction between T-cells and dendritic cells and is important for the binding, uptake and antigen processing of multiple pathogens via the recognition of high mannose residues on the pathogens surface. L-SIGN has also been identified as a capture receptor for pathogens and was proposed as a capture receptor that could transmit virus to neighbouring cells (264, 293, 386). Both DC-SIGN and L-SIGN have been demonstrated to interact with HCV envelope

glycoproteins (155). HCVpp and sE2 have been shown to bind to both molecules on sinusoidal endothelial cells (264), and sE2 has also been found to bind DC-SIGN on mature human monocyte-derived dendritic cells (386). This led to the proposition that DC-SIGN and L-SIGN may capture and deliver HCV via dendritic cells to the liver, in fact Lozach et al., demonstrated that HCVpp bound to L-SIGN and DC-SIGN positive cells could be transmitted to Huh-7 cells in co-culture (293).

As previously described HCV has been isolated in low density fractions of plasma and is believed to associate with low-density lipoproteins, indeed recent research has identified Apo E, a component of low density lipoproteins, to be associated with virions exhibiting peak infectivity (14, 358). It is therefore no surprise that low-density lipoprotein receptor (LDL-R) was an obvious choice for an HCV receptor (3, 60). LDL-R is the most important receptor for LDL in plasma. LDL binds to the receptor and is endocytosed via clathrin dependent endocytosis. LDL-R is recycled to the cell surface whilst LDL is released from the endosome via a pH dependent process followed by degradation in lysosomes resulting in the release of cholesterol into the cytoplasm (406). The first indirect evidence of HCV entry via LDL was shown by Agnello et al., in 1999, where HCV uptake into cells correlated with low density lipoprotein receptor activity and a reduction in uptake was observed in the presence of LDL receptor antibodies (3). More recent work has demonstrated that antibodies specific for LDL-R inhibit serum derived virus replication in primary hepatocyte cultures, suggesting a role for LDL-R in HCV replication (333). Owen et al., silenced LDL-R expression and demonstrated a reduction in HCV entry, these results were substantiated by a rescue of infection following re-expression of LDL-R (358). Further to this Owen et al., demonstrated the specific involvement of Apo E in LDL-R

mediated HCV entry by firstly inhibiting entry using Apo E specific antibodies and secondly identifying that Apo E is associated with highly infectious virions that have a high dependence on LDL-R (358). As yet there has been no direct evidence of an association between HCVpp/HCVcc with LDL-Rs however this may reflect the differential lipid association of particles propagated *in vitro* (60).

Receptors

CD81 is a member of the tetraspanin superfamily. It has two small intracellular domains, four transmembrane domains and two extra cellular loops, a large extra cellular loop (EC1) and a small extra cellular loop (EC2) (Fig.1.2). Tetraspanins form networks at the cell surface and are reported to be involved in cell adhesion, motility, cell activation, metastasis, and signal transduction (274). In the liver CD81 has been demonstrated to regulate cell proliferation and more recently to regulate hepatocellular carcinoma cell migration (42, 66, 312, 313). Chapter 3 and 4 of this study further investigate the function of CD81 in hepatoma cells.

Pileri et al. demonstrated an interaction between HCV E2 glycoprotein and CD81 in 1999 (384). The large extra cellular loop of CD81 was demonstrated to bind soluble E2 and subsequent experiments with both HCVpp and HCVcc validated the essential role of CD81 in viral entry (99, 145, 314, 555). Zhang et al., 2004 reported that monoclonal antibodies to CD81 and small interfering RNA's to silence CD81 inhibited HCVpp infection *in vitro*. McKeating et al., 2004 demonstrated the infection of diverse HCV genotypes was dependent on CD81 expression. HCVcc and HCVpp only infect the hepatoma cell line, HepG2 when engineered to express CD81, confirming the critical role of CD81 in viral entry (555). Recently an *in vivo* study showed that

treatment with anti-CD81 antibodies prior to HCV infection of liver-uPA-SCID mice provided complete protection from HCVcc infection but treatment after infection had no effect (325). A number of studies have demonstrated that CD81 is a co-receptor for HCV entry and functions post attachment of HCV to the cell membrane (5, 99, 335). Tan et al., 2003 showed that CD81 is involved in HCV internalisation and that HCV particles can bind to the cell surface in the absence of CD81 but could only be internalised when cell surface CD81 was present. Cormier et al., 2004 demonstrated that an anti-CD81 monoclonal antibody inhibits HCV entry post attachment, further supporting this conclusion.

Regions of E2 important for E2/CD81 binding have been identified by antibody blocking experiments of E2 and structural modeling of E1E2 (360, 401). Owsianka et al., 2006 demonstrated conserved regions of E2 that were critical for CD81 binding for all genotypes. Specific residues on CD81 have been identified as critical for E2 binding. Four amino acid residues on the large extracellular loop of human CD81 were identified to differ from that of African green monkey CD81 that does not bind E2. One of these residues 186 was subsequently identified as being critical for E2 binding by mutagenesis studies (197). Drummer et al., 2002 used random mutagenesis to determine specific residues 182, 186, 184 and 162 on CD81 large extracellular loop that were critical for E2 binding (122). The above studies used E2 to assess binding specific residues on CD81 important for virus entry. Since then Flint et al., 2006 successfully infected HepG2 cells that expressed full length CD81 derived from a broad range of species and showed that sE2 binding and recombinant CD81 blocking of HCVpp infection were not good predictors for successful HCVcc

infection (145). Implying that some of the previous work that identified residues critical for E2 binding may not predict successful entry of HCV.

CD81 is closely associated with immunoglobulin like proteins EWI-2 and EWI-F. These proteins are members of a novel family of immunoglobulin proteins that are directly linked to actin linking ezrin-radixin-moesin (ERM) (411, 458). A cleavage product of EWI-2, EWI-2 wint (without its N terminus) that is not present in hepatocytes and was recently shown to inhibit HCV-CD81 interaction and reduce HCV infection in Huh-7 cells (402), suggesting that inhibitor proteins may contribute to the non-permissive nature of some cell types (194).

SR-BI is a lipoprotein receptor that binds HDL and oxidized LDL (oxLDL). It functions by mediating cholesteryl ester uptake from HDL and controlling cholesterol efflux (222). It is expressed highly in the liver although it can also be found in steroidogenic tissue and macrophages (235). SR-BI is a cell membrane protein that has two cytoplasmic terminal domains and a large extra cellular domain (Fig.1.2). The SR-BI gene gives rise to at least three isoforms, SR-BI, SR-BII and SR-BIII (127) The mechanism of SR-BI internalisation is unknown, however, SR-BII has been shown to endocytose via a clathrin dependent pathway (126).

Scarselli et al., 2002 was the first to identify that SR-BI could interact with HCV E2. Since this discovery further work has been carried out to characterize the role of SR-BI as a HCV receptor (70, 174, 175). Initial work focused on the effect of lipoproteins (SR-BI ligands) on HCV entry. Lavillette et al., 2005 found that human sera could increase HCVpp infectivity and hypothesised that an agent in the serum was

responsible for the increased infectivity (268). As previously described lipoproteins in serum have been found to associate with HCV *in vivo* (14, 351, 478) and to increase HCV glycoprotein E2 cell binding (519). Treatment of cells with high density lipoproteins (HDL) increased HCVpp infectivity, this was abrogated in the presence of inhibitors of SR-BI selective cholesterol uptake (BLT-2, BLT-4) suggesting that HCV entry may be dependent on the cholesterol uptake by SR-BI (495).

Grove et al., reported that both plasma and cell culture derived J6/JFH had increased infectivity for Huh-7.5 cells transduced to over-express SR-BI and SR-BII (174). Antibodies specific for SR-BI inhibited HCVcc infectivity, demonstrating a specific role for this receptor in HCV entry and replication (70). Kinetic studies have shown that SR-BI may have a role post viral attachment, as antibodies neutralized cell bound virus (553). However, work by Catanese et al., 2009 contradicted these findings, demonstrating that anti-SR-BI antibodies blocked vius attachment.

It is only very recently that the direct interaction of E2 with SR-BI has been demonstrated to be important for HCV entry (69). Catanese et al., produced mutant SR-BI proteins containing specific mutations required for sE2 binding and used them to show that firstly they were unable to restore infectivity after SR-BI knockdown treatment, suggesting that direct SR-BI E2 binding is important for virus infection. Secondly they demonstrated that HDL binding and cholesterol efflux was maintained in the mutant SR-BI expressing cells indicating that HDL and HCV E2 binding are distinct (69).

Dreux et al., 2009 expressed SR-BI in SR-BI negative cell lines, SK-hep1 (human liver endothelial cell) and BRL3A (rat hepatocarcinoma cell line), and demonstrated permisivity to HCVpp and HCVcc infection. These results for the first time defined SR-BI as an essential co-receptor. By expressing mutant SR-BI proteins in the SR-BI negatie cell lines Dreux et al., concluded that intracellular domains of SR-BI were important for HCV infection (118).

Kapadia et al., demonstrated the potential cooperation of CD81 and SR-BI in HCVcc infection with monoclonal antibodies to CD81 and SR-B1 (235). Huh-7 cells were incubated with both CD81 and SR-BI antibodies alone or in combination and screened for their ability to support JFH-1 infection. Treatment of cells with antibodies to both co-receptors simultaneously had a synergistic effect suggesting cooperation between the two receptors. Grove et al., demonstrated that both CD81 and SR-BI dependency could be altered by a single amino acid change in glycoprotein E2. Mutation at amino acid 451 reduced SR-BI dependency and rendered the particle more sensitive to neutralisation by glycoprotein antibodies and soluble CD81 as well as altering particle density: infectivity relationship (175). These findings suggest a co-coperation between HCV receptors.

Some cell lines that express CD81, SR-BI and LDL receptors are non-permissive cells to HCV infection, implying that at least one other liver specific receptor was needed. In 2007 Evans et al., screened a complementary DNA library from Huh-7.5 cells for genes that were able to confer HCVpp entry into non-permissive 293T cells. These studies identified the tight junction protein claudin-1 (CLDN-1) as a critical factor defining HCV entry (133). Claudin-1 is highly expressed within the liver and

recent evidence from our laboratory suggests that the protein localises to junctional and non-junctional regions of the plasma membrane (396). Claudin-1 consists of four transmembrane regions with two extra cellular loops (Fig. 1.2). The first extracellular loop (EL1) of claudin-1 was found to be essential for HCV entry, however, there is so far no evidence for a direct association between claudin-1 and HCV E1E2. Evans et al., reported that claudin-1 plays a role downstream of CD81 and is required for HCV glycoprotein mediated cell fusion (133). Furthermore Evans et al., identified two residues in EL1 essential for virus entry, I32 and E48 (133). Cukierman et al., identified by mutational studies a further 7 residues (6 highly conserved resdues, W30, GLW-51, C54 and C64 as well as a further residue D38) in EL1 that were critical for HCV entry (107). Claudin-1 is part of a family of claudin proteins. It has recently been shown that further family members, claudin-6 and claudin-9 can also permit HCV entry into the claudin-1 negative hepatocellular carcinoma cell line Bel7402 (321, 559).

A further tight junction protein occludin was recently implicated as an important entry factor(288, 385). Ploss et al., showed that expression of human occludin in murine cells rendered the cells permissive for infection with HCVpp and HCVcc. It is hoped that this knowledge may help to provide a mouse model for HCV infection by expressing human occludin. Occludin like claudin-1 has two extra-cellular loops EL1 and EL2 (Fig.1.2). Work by Benedicto et al., using shRNA to silence occludin expression confirmed a role for occludin in virus entry and eliminated the possibility of a role in replication. Furthermore Benedicto et al., established that occludin was not involved in attachment of the virion and demonstrated a possible role in glycoprotein dependent cell fusion, a later step in entry (39). Since then occludin EL2

has been identified by a number of studies to be important for mediating HCV entry (286, 328). Liu et al., expressed deletion mutant occludin proteins in 786-0 cells, (human renal carcinoma cells that are naturally occludin deficient) and demonstrated that deletion of EL2 abolished HCVpp and HCVcc infection whilst still exhibiting comparable cell surface expression to wild type (287). Mitcha et al., further confirmed the importance of EL2 by expressing occludin from different species in 786-0 cells and comparing permissivity to HCVpp, they were able to further define the area of importance to the second half of EL2 (328). A number of different splice variants of occludin have been identified (302). Kohaar et al., investigated the diversity of occludin present in normal human liver and compared activity when expressed in 786-0 cells (245). Some of the splice variants were not expressed at the cell surface and were unable to support infection, suggesting that the variability in splice variants identified across different human livers may contribute towards partly explaining the differences in disease outcome between patients and may also help to explain tissue tropism (245). Importantly expression of CD81, SR-BI, claudin-1 and occludin in non-liver derived cells (786-0(renal carcinoma cell line), TZM (HeLa cell-derived cervical carinoma cell line) and NIH3T3 (Mouse embroyo fibroblast cell line)) renders the cells permissive to HCV infection indicating that these four entry factors are the minimal requirement for viral entry (385).

Receptor complex and endocytosis

The involvement of many receptors in HCV entry suggests a complex process that is only now beginning to be investigated. Preliminary studies addressed receptor localization in liver tissue and investigated protein co-localisation by confocal imaging. To fully interpret this data it is important to appreciate the liver architecture (Figure 1.4A). The liver is a complex organ that is fed by both the portal vein and the hepatic artery. Blood is discharged from the portal vein and hepatic artery into sinusoids. Blood then travels through the sinusoids that weave around the hepatocytes until it reaches the central vein where it leaves the liver (433). Bile ducts are integrated throughout the structure and act to remove bile from hepatocytes and take it to the duodenum via the common hepatic bile duct (490). The liver is made up primarily of polarised parenchyma hepatocytes (493, 502) and other non parenchymal cells, examples of which include; Kupffer cells (resident macrophages), stellate / fat storing cells, and liver sinusoidal endothelial cells (LSEC) that form a permeated barrier between hepatocytes and the sinusoidal blood flow (493) (Fig.1.4B). The gap between the sinusoidal endothelium and the hepatocytes is termed the space of Disse and is where tissue fluid flows outward towards the lymphatics and is also where stellate cells can be found (433). Hepatocytes are highly polarised and their membrane can be split into three different surfaces: the sinusoidal / basolateral membrane faces the sinusoids and space of Disse and has irregular sized and spaced microvilli; the apical / canalicular membrane faces the bile and is separated from the other surfaces by the presence of tight junctions and desmosomes; finally, the third side faces other hepatocytes (433).

Reynolds et al., demonstrated the co-localisation of CD81 and claudin-1 at the sinusoidal (basolateral) and canalicular (apical) domains of polarized hepatocytes and the co-localisation of SR-BI and claudin-1 only at the sinusoidal domain (396). It is the sinusoidal domain of the hepatocytes that will be exposed to the sinusoidal blood and the site that we presume will come into contact with the virus. Evidence as to occludin localization in the liver is limited; investigations in our laboratory suggest localization predominantly at the bile canalicular, this is significantly different to expression in diseased livers where it is expressed at both the sinusoidal and canalicular membranes (Wilson, unpublished data).

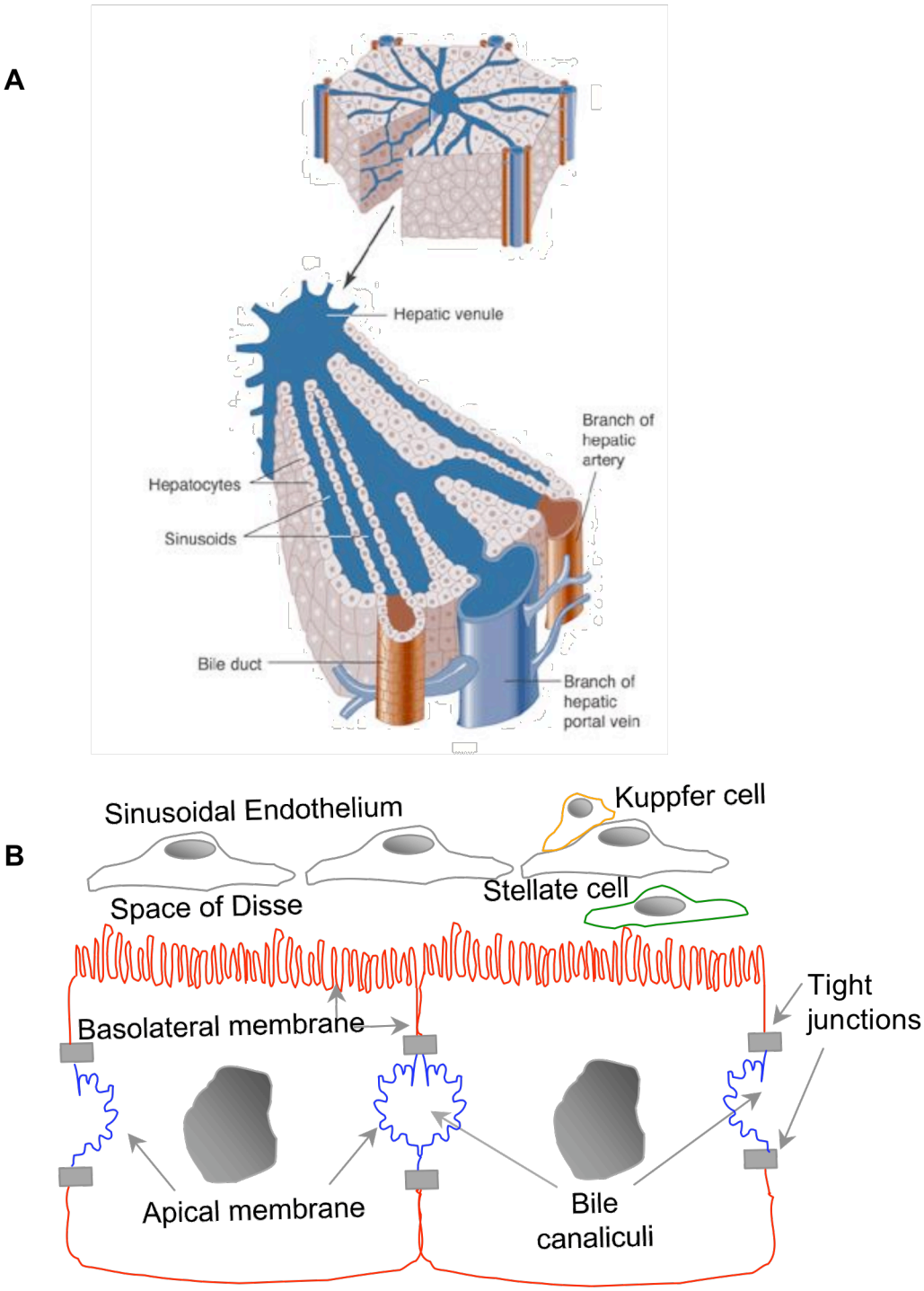


Figure 1.3: Liver organisation and hepatic polarity

A) The top image represents a liver lobule whilst the lower image represents a more detailed illustration of a segment of a liver lobule. **B**) Cartoon of polarized hepatocytes illustrating the sinusoidal/basolateral (red) and canalicular/apical (blue) membrane surfaces.

To understand whether the receptors form a complex, specific interactions between the co-receptors need to be determined. To date interactions between CD81, CLDN and occludin have been investigated (188, 189). Work from our laboratory has investigated these associations using FRET (Fluorescence resonance energy transfer), a technique that allows distances of very closely associated fluorescently tagged proteins to be measured (less than 10nm). FRET occurred between fluorescent tagged CD81 and claudin-1 in permissive and non-permissive cells suggesting co-localization and formation of a co-receptor complex (189). Further work by Harris & Davis et al., demonstrated that receptor active claudins (Claudin-1, 6 and 9) exhibit a 1 to 1 stoichiometry with co-receptor CD81. Furthermore disruption of claudin-1-CD81 association ablated HCV entry demonstrating that this complex defines HCV entry. Of equal importance claudin-1 - occludin interactions were not found to define HCV entry (188).

Since the identification of tight junction proteins claudin-1 and occludin as receptors for HCV entry there has been much speculation as to whether HCV may utilize a cellular receptor to traffic to tight junctions where claudin-1 and occludin reside to permit entry. A similar mechanism has previously been observed for group B coxsackievirus (CVB) infection that utilzes a cellular receptor, decay accelerating factor (DAF) to co-ordinate the movement of virus particles from the apical surface of polarized epithelial cells to reach a further receptor, cosackievirus and adenovirus receptor (CAR) that is a component of the tight junction (102). These speculations were hightened by research carried out by Brazzoli et al., showing a re-organisation of CD81 to tight junctions following sE2 engagement (54). Importantly our laboratory has been unable to repeat these results (Michelle Farquar, unpublished data).

Benedicto et al., 2009 suggested that occludin may be responsible for re-localisation of the virus to sites of entry, as yet there is no evidence for a direct association between extra-cellular occludin and HCV glycoprotein's making this hypothesis unlikely (39, 287). Work in our laboratory would argue against the need for re-localization of the virion to tight junctions as the CD81-claudin-1 complexes are predominantly at the basolateral membrane of polarised HepG2 cells (Cell line that exhibits hepatic polarity (319) and not tight junctions (188). Furthermore Coller et al., recently published data showing the movement of fluorescent labeled HCV particles along filopodia and across the cell body followed by internalization at sites that were predominantly not cell junctions (96).

Liu et al., recently demonstrated that dynamin is important for HCV entry, this is no surprise as dynamin is a large GTPase that functions in the scission of vesicles from the cell membrane including clathrin mediated endocytosis, which has previously been shown to be involved in HCV entry *in vitro* (45, 208). Indeed our laboratory has recently shown that dynamin is important for HCV entry (Farquar, submitted). Liu et al., demonstrated that occludin association with dynamin is important for entry, suggesting that this association may be involved in coordinating HCV endocytosis HCV (287). This is particularly interesting as recent work in our laboratory has shown that CD81 engagement by either anti-CD81 or sE2 promotes a clathrin and dynamin dependent co-endocytosis of CD81 and claudin-1 to early endosomes, arguing that HCV glycoprotein engagement with CD81 may mediate endocytosis of the virus-receptor complex. Our data supports previous observations by Coller et al., that visualized internalization of fluorescently labeled virions after association with claudin-1 and CD81 (96). The lack of an endocytic motif on CD81 and the knowledge

that CD81 lacking the N or C terminus is still endocytosed suggests that this process may be coordinated by an associated protein and that the previously identified CD81-claudin-1 interaction may be important in facilitating this process (Farquar, submitted), (188, 189).

The final stages of virus entry into the cell involve viral-cell fusion inside the early endosome allowing the release of the viral genome into the cytoplasm. Our laboratory has shown that antibodies to CD81 are able to block entry at late time points post infection suggesting that CD81 might be involved in these later stages of entry as well as attachment (Farquar, submitted). Claudin-1 (133) and occludin (39, 287) have also been implicated to function in these later stages of entry. Taken together these studies indicate that CD81, claudin-1 and occludin will need to be present inside the early endosome for the final stages of viral entry, whether they get there in association with one another and / or in association with the virus remains unknown. Evidently further work is needed to understand the complex nature of HCV entry. A cartoon depicting a possible route for HCV entry is shown in Figure 1.4.

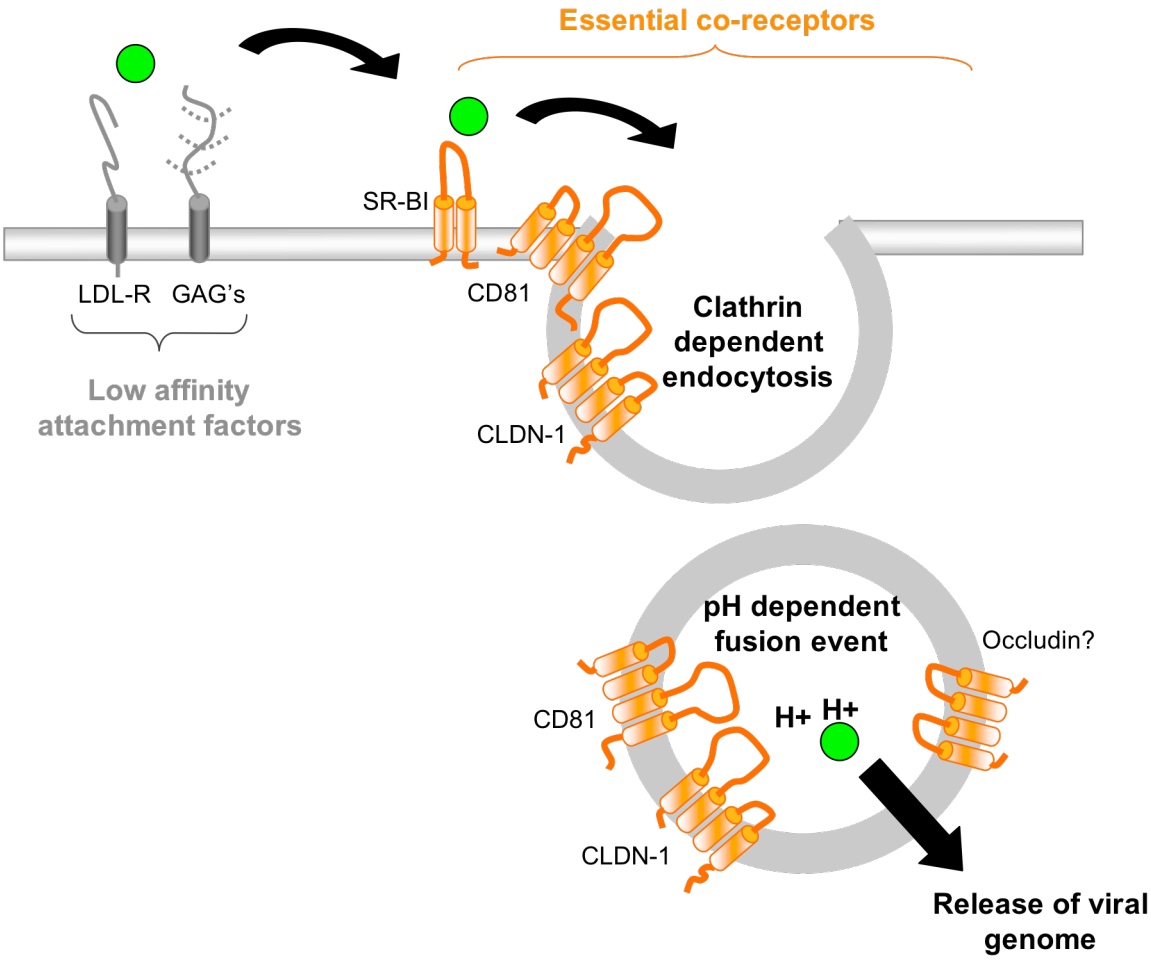


Figure 1.4: A possible pathway for HCV entry.

1.4.4 Viral transmission

An estimated 10^12 HCV particles are released into the serum of infected individuals everyday (350) and new viral hosts become infected through exposure to contaminated blood (11) suggesting that viral transmission between hosts is dependent on the release of infectious viral particles from infected hepatocytes.

Transmission of virions within the host is not so easy to decipher. A humoral and cellular response to infection is observed following HCV infection and the existence of escape mutants directed against these responses demonstrates that selective

pressure occurs *in vivo* (73, 131, 434, 450, 497, 500, 505). However, HCV infection is able to persist in the presence of neutralizing antibodies suggesting a direct cell-to-cell route of transmission may also be occurring (290). The ability to transmit directly from cell-to-cell without the release of infectious viral particles is often an advantage for the virus, it enables a more efficient mode of transmission and furthermore helps to protect the virion from the host immune system. Our laboratory has recently developed an assay to determine *in vitro* transmission. Timpe et al., 2008 used this assay to demonstrate that HCV can transmit efficiently in the presence of nAbs suggesting the presence of a novel direct cell-to-cell route of HCV transmission (480). This is investigated further in chapter 5 of this study.

1.5 Project aims

This project focuses on three areas. HCV entry requires glycoprotein engagement of tetraspanin CD81 (384), and a number of studies using immune cells have demonstrated that engagement of CD81 by antibody or recombinant sE2 protein induces a rearrangement of the actin cytoskeleton and alters cell function(s) (95, 104, 486). Our first goal focuses on the role of CD81 engagement on hepatoma cell morphology using a panel of anti-CD81 antibodies that recognize novel CD81 epitopes.

In a small but significant proportion of patients, HCV infection leads to the development of hepatocellular carcinoma (6, 10, 71). Tetraspanins have been reported to alter the metastatic potential of a number of different cancers (561). Therefore our second goal investigates a role for tetraspanin CD81 in hepatoma-ECM adhesion and migration and whether HCV infection modulates CD81 dependent function and/or CD81 independent hepatoma adhesion and migration potential.

Recent work in the HCV field suggests that HCV infection may transmit within a host in a neutralizing antibody resistant manner involving a direct cell-to-cell route of transmission (480). In the final chapter we characterized the receptor dependency of neutralizing antibody resistant transmission of HCV *in vitro*.

2. Materials and Methods

2.1 Basic techniques

2.1.1 Tissue culture

All cell lines used in this study are detailed in Table 2.1. All cells were propagated in Dulbecco's modified medium (DMEM) (Gibco) with 10% fetal bovine serum (FBS) (Gibco, CA, USA), 1% non-essential amino acids (Gibco), 1% L-Glutamine (Gibco) and 50units/ml penicillin and 50µg/ml streptomycin (P/S) (Gibco), Cells were maintained in tissue culture flasks (Becton Dickinson, NJ, USA) and grown at 37°C in 5%CO_2.

Cell lines			
Name	**Species:Tissue**	**Growth media**	**Source**
Huh-7.5	Human hepatoma	DMEM	Dr. R.C Rice, rockerfeller University, New York
Huh-7.5/SR-BI	Human hepatoma	DMEM	In house
Huh-7 Lunet parental	Human hepatoma	DMEM	Dr. T. Pietschmann, TWINCORE, Hanover
Huh-7 Lunet/CD81	Human hepatoma	DMEM	Dr. T. Pietschmann, TWINCORE, Hanover
Huh-7 Lunet/CD81 WT	Human hepatoma	DMEM	In house (See Retrovirus Phoenix cell Delivery)
Huh-7 Lunet/CD81 delta N	Human hepatoma	DMEM	In house (See Retrovirus phoenix cell Delivery)
Huh-7 Lunet/CD81 delta C	Human hepatoma	DMEM	In house (See Retrovirus phoenix cell Delivery)
Huh-7 CD81 nul	Human hepatoma	DMEM	Dr. Y.T Tan, Singapore
Huh-7 CD81	Human hepatoma	DMEM	In house (see Retrovirus/Trip Delivery)
HepG2	Human hepatocellular carcinoma	DMEM	American type culture collection
HepG2/CD81	Human hepatocellular carcinoma	DMEM	In house (See Retrovirus/TRIP Delivery)
HepG2/CD81 WT	Human hepatocellular carcinoma	DMEM	In house(See Retrovirus Phoenix cell Delivery)
HepG2/CD81 delta N	Human hepatocellular carcinoma	DMEM	In house(See Retrovirus Phoenix cell Delivery)
HepG2/CD81 delta C	Human hepatocellular carcinoma	DMEM	In house(See Retrovirus Phoenix cell Delivery)
293T	Human embryonic kidney	DMEM	American type culture collection
Phoenix cells	Human embryonic kidney	DMEM	American type culture

Table 2.1: Cell lines used

2.1.2 Antibodies

All antibodies used in this study detailed in Table 2.2.

Antibody name	Antigen	Type	Specificity	Species	Source
Primary antibody					
9E10	HCV NS5A	Hybridoma SN	Monoclonal	Mouse	Dr. C. M. Rice, The Rockefeller University, NY
9/27	HCV E2	Purified IgG	Monoclonal	Rat	In house
10/76b	HIV gp 120	Purified IgG	Monoclonal	Rat	In house
3/11	HCV E2	Purified IgG	Monoclonal	Rat	In house
11/20	HCV E2	Purified IgG	Monoclonal	Rat	In house
CBH2	HCV E2	Purified IgG	Monoclonal	Human	Dr. S. Foung, University of Stanford, California
CBH4G	HCV E2	Purified IgG	Monoclonal	Human	Dr. S. Foung, University of Stanford, California
2.s131	Human CD81	Purified IgG	Monoclonal	Mouse	In house, K.Hu and M.Goodall (See Table 3.1 for all other anti-CD81 in house antibodies)
PF72	Human SR-BI	Purified IgG	Monoclonal	Human	Pfizer Ltd.
Anti-Claudin	Human Claudin-1	Purified IgG	Monoclonal	Rabbit	Invitrogen
Anti-Claudin-1	Human Claudin-1	Hybridoma SN	Polyclonal	Rat	Dr. T. Baumert, University hospital Strasbourg, France
Anti-Occludin	Human Occludin	Purified IgG	Monoclonal	Rabbit	Invitrogen
VG67e	Human VEGF	Purified IgG	Monoclonal	Mouse	Prof.R.Bicknell, University of Birmingham, Birmingham
Anti-P-ERM	Human P-ERM	Purified IgG	Monoclonal	Rabbit	Cell signalling
Anti-CD9 (IAA2)	Human CD9	Purified IgG	Monoclonal	Mouse	Dr. M. Tomlinson, Univeristy of Birmingham, Birmingham
Anti-CD151 (11B1)	Human CD151	Ascites	Polyclonal	Mouse	Dr. M. Tomlinson, Univeristy of Birmingham, Birmingham
Anti-EWI-2 (8A12)	Human EWI-2	Purified IgG	Monoclonal	Mouse	Dr. M. Tomlinson, Univeristy of Birmingham, Birmingham
Anti-Beta1	Human Beta-1	Purified IgG	Monoclonal	Mouse	R&D systems
Anti-Beta2	Human Beta-2	Purified IgG	Monoclonal	Mouse	R&D systems
Anti-Beta3	Human Beta-3	Purified IgG	Monoclonal	Mouse	BD-pharmaceuticals
Anti-Beta4	Human Beta-d	Purified IgG	Monoclonal	Mouse	Chemicon
Anti-alpha1	Human alpha-1	Purified IgG	Monoclonal	Mouse	Chemicon
Anti-alpha2	Human alpha-2	Purified IgG	Monoclonal	Mouse	Immunotech
Anti-alpha4	Human alpha-4	Purified IgG	Monoclonal	Mouse	Chemicon
Anti-alpha5	Human alpha-5	Purified IgG	Monoclonal	Mouse	BD-pharmaceuticals
Anti-alpha6	Human alpha-6	Purified IgG	Monoclonal	Mouse	Serotech
Anti-alphaVBeta3	Human alphaVBeta3	Purified IgG	Monoclonal	Mouse	Chemicon
Anti-alphaV-Beta5	Human alphaVBeta5	Purified IgG	Monoclonal	Mouse	Chemicon
Anti-alphaVBeta6	Human alphaVBeta6	Purified IgG	Monoclonal	Mouse	Chemicon
Anti-Fibronectin	Human Fibronectin	Ascites	Monoclonal	Mouse	Sigma-Aldrich,MO
Anti-Collagen I	Human Collagen I	Ascites	Monoclonal	Mouse	Sigma-Aldrich,MO
Anti-Collagen IV	Human Collagen IV	Ascites	Monoclonal	Mouse	Sigma-Aldrich,MO
Anti-Beta-actin (AC-15)	Human Beta-actin	Purified IgG	Monoclonal	Mouse	Sigma-Aldrich,MO
Secondary antibody					
Anti-Mouse Alexa fluor -488	Mouse (IgG2a)	Purified IgG	Polyclonal	Goat	Molecular Probes, Invitrogen, CA
Anti-Mouse Alexa fluor-488	Mouse (H+L)	Purified IgG	Polyclonal	Goat	Molecular Probes, Invitrogen, CA
Anti-Mouse Alexa fluor-RPE	Mouse (IgG2a)	Purified IgG	Polyclonal	Goat	Molecular Probes, Invitrogen, CA
Anti-Mouse Alexa fluor-594	Mouse (IgG2a)	Purified IgG	Polyclonal	Goat	Molecular Probes, Invitrogen, CA
Anti-Rabbit Alexa fluor-594	Rabbit (H+L)	Purified IgG	Polyclonal	Goat	Molecular Probes, Invitrogen, CA
Anti-Mouse-HRP	Mouse	Purified IgG	Polyclonal	Donkey	Jackson ImmunoResearch laboratories
Anti-Human-HRP	Human	Purified IgG	Polyclonal	Donkey	Jackson ImmunoResearch laboratories
Anti-Mouse-HRP	Mouse	Purified IgG	Polyclonal	Sheep	Amersham, Biosciences, PA
Anti-Rabbit-HRP	Rabbit	Purified IgG	Polyclonal	Sheep	Amersham, Biosciences, PA

Table 2.2: Antibodies used

2.1.3 Plasmids and proteins

All plasmids and proteins used in this study detailed in Table 2.3

Plasmids	
Name	**Source**
HCVcc JFH-1	Dr. T. Wakita, National Institute of infectious diseases, Tokyo
HCVcc J6/JFH-1	Dr. C.M Rice, The Rockefeller University, New York
HCVcc JFH-1 G451R	Dr, F. Chisari, Dr. J Zhong, The Scripps Research Institute, La Jolia
HCVcc JFH-1 W529A	Dr. A.H. Patel,University of Glasgow, Glasgow
HCVcc J6/JFH del B	Dr. T Tellinghuissen, The Scripps Research Institue, Florida
HCVcc H77/JFH-1	Dr. J. BuKh, Copenhagen University hospital, Denmark
HCVcc J4/JFH-1	Dr. J. BuKh, Copenhagen University hospital, Denmark
HCVcc J6/JFH-1	Dr. J. BuKh, Copenhagen University hospital, Denmark
HCVcc J8/JFH-1	Dr. J. BuKh, Copenhagen University hospital, Denmark
HCVcc S52/JFH-1	Dr. J. BuKh, Copenhagen University hospital, Denmark
HCVcc ED43/JFH-1	Dr. J. BuKh, Copenhagen University hospital, Denmark
HCVcc SA13/JFH-1	Dr. J. BuKh, Copenhagen University hospital, Denmark
HCVcc HK6a/JFH-1	Dr. J. BuKh, Copenhagen University hospital, Denmark
HCVcc QC69/JFH-1	Dr. J. BuKh, Copenhagen University hospital, Denmark
Lifeact-Ruby	Dr. R Wedlich-Söldner, Max Planck Institute of Biochemistry, Germany
Moesin-GFP	Dr. M. Yanez-Mo, University hospital La Princesa, Madrid
N-Moesin-GFP	Dr. M. Yanez-Mo, University hospital La Princesa, Madrid
CD81 WT	Dr. M. Hemler, Dana-Farber Cancer Institute, Boston
CD81 Delta N	Dr. M. Hemler, Dana-Farber Cancer Institute, Boston
CD81 Delta C	Dr. M. Hemler, Dana-Farber Cancer Institute, Boston
TRIP SR-BI	In house
TRIP CD81.GFP	In house
HIV gagpol 8.2	Aaron Diamond AIDS Research Centre
VSV-G	Aaron Diamond AIDS Research Centre
shRNA Occludin	RHS4533-NM_002538 Open Biosystems
shRNA SR-BI	RHS4533-NM_005505 Open Biosytems
JFH HCV sE2-10/76b	In house
Proteins	
VEGF-165	Peprotech

Table 2.3 Plasmids and proteins used

2.1.4 HCVcc generation

All HCVcc viruses used in this study were constructed around the non-structural regions of JFH-1, a unique genome that was identified to assemble HCV particles in cell culture and is predominantly used throughout the HCV field of research (166, 283, 499, 560). RNA was transcribed from a plasmid containing the RNA genome (Table 2.3) and introduced into Huh-7.5 cells by electroporation.

RNA synthesis

- 5μg of the plasmid containing a cDNA clone of the HCV genome was linearized using Xba-1 digest (Promega)
- 1μg of the RNA was used as a template for RNA transcription using the megascript T7 kit (Ambion, Austin, TX), the reaction mixture was incubated at 37°C for 2hrs after which the RNA was purified using the RNeasy MinElute Kit (Qiagen, NL) according to manufacturer's instructions.
- RNA quality was assessed by gel electrophoresis on a 1% agarose gel (Bioline, UK). Typical yields as measured by UV spectrophotometry (Amersham), were 250-1500ng/μl.

Electroporation

- Huh-7.5 cells were grown to 60-80% confluence. Trypsin was used to remove the cells from the tissue culture plastic.
- Cells were washed twice by centrifugation at 1200rpm for 3min at 1°C and re-suspension in 50ml ice-cold phosphate buffered saline (PBS).
- $1.5x10^7$ cells were re-suspended in 1ml of ice-cold PBS and placed on ice.
- 400μl of cell suspension was then mixed with 4ng of transcribed RNA in a cuvette and electroporated under the following conditions; 5x100uS(820V) pulses with 1.1 second delay between pulses.

- Electroporated cells were then left for 5min at room temperature to rest prior to suspension in 10ml Iscove's modified dulbecco's medium (IMDM) (plus10% human serum, 1% non essential amino acids, 1% pen strep). 8ml of re-suspended cells were placed into a T75 flask, and the remainder put into 2 wells of a 24 well tissue culture plate to allow the monitoring of HCV protein expression. Cells incubated at 37°C at category three containment level. Media was replaced with DMEM (3% FBS) the following day.
- 72hrs post electroporation cells in the 24 well plates were fixed with ice-cold methanol and stained for HCV non-structural protein, NS5A (As described in section 2.1.7).
- Electroporated cells were used in assays at 72hrs or 96hrs post electroporation. In this scenario no viral particles were harvested.
- Where HCV particles were harvested to use in cell free infection assays the following procedure was followed; 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% FBS) and media containing secreted virions collected every 8-14hrs. Harvested virus was frozen prior to titration using the standard infection assay.

Standard infection assay

All infection assays in this study were performed as follows.

- $0.75x10^4$ cells were seeded per well into a 96 well tissue culture plate.
- The following day cells were infected with virus diluted in DMEM (3% FBS) in a total volume of 100μl per well.
- The virus was left on overnight and media replaced the following day.

- 48hrs post infection cells were fixed with ice-cold methanol and stained for HCV non-structural protein, NS5A (As described in section 2.1.7).
- Infected cells were counted as individual cells or foci of cells using a fluorescent microscope, Nikon TE2000. Infectious units (IU) or foci forming units (FFU) per ml were then determined.

2.1.5 Retrovirus delivery

TRIP retrovirus gene delivery

The TRIP system is a retrovirus gene expression vector developed by Zennou *et. al.* (554). 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 plasmids were delivered by TRIP retrovirus delivery CD81.GFP / SR-BI / shRNA Occludin and shRNA SR-BI.

- TRIP particles were produced by Fugene (Roche, Switzerland) transfection of 293T cells.
- Fugene/plasmid complex was made up as follows;
    - Following amounts of plasmids (400ng VSVG envelope, 600ng TRIP gag-pol and 600ng target gene) added to 6μl Fugene dissolved in 100μl Optimem (Gibco).
    - This was incubated at room temperature for 20min to allow formation of complex.

- Cells were transfected in 6 well tissue culture plates for 8hrs, after which the culture media was changed to DMEM (3% FBS + P/S).
- Transfection efficiency was monitored by the inclusion of a TRIP plasmid encoding enhanced green fluorescent protein as a target gene (TRIP EGFP).
- Culture media containing TRIP particles was harvested at 48 and 72hrs post transfection and passed through a 0.2μM filter to remove any contaminating 293T cells. Transduction of target cells was performed immediately.
- Target cells were seeded at $4x10^5$cells/well in a 6 well tissue culture plate 24hrs prior to transduction. To transduce, cells were incubated overnight with harvested TRIP culture media diluted 1:1 in DMEM (3% FBS + P/S). After which the media was changed to DMEM (10% FBS + P/S).
- Transduction efficiency was assessed after 48hrs by monitoring expression of TRIP EGFP and flow cytometric detection of target gene(s).

Phoenix cell retrovirus gene delivery

Pheonix cells are 293T cells that have been engineered to express gag-pol and envelope protein. Upon transient transfection with a plasmid containing LTR's (Long terminal repeats), cells produce retrovirus containing the required DNA. This system was used in this study in the production of CD81 mutant expressing Huh-7 Lunet and HepG2 cells. Like the TRIP retroviral system, subsequent transduction of a cell line with the retrovirus results in reverse transcription of the target gene and its insertion into the genomic DNA. Transduced cells were maintained under Zeosin selection (Invitrogen). HepG2 cells under 400μg/ml and Huh-7 Lunet cells under 600μg/ml.

- Retroviral particles were produced by Fugene transfection (Roche, Switzerland) of phoenix cells.
- Briefly, phoenix cells were seeded at $7x10^5$ cells per well into a 6 well culture dish

coated with poly-L-lysine hydro bromide (Sigma, Ca, USA) in DMEM (10% FBS) 24hrs prior to transfection. 1hr prior to transfection media refreshed with P/S free DMEM (3%FBS), 1.5ml per well.

- Fugene/plasmid complex was made up as follows;
    - 1μg DNA added to 6μl Fugene dissolved in 100μl Optimem (Gibco).
    - This was incubated at room temperature for 20min to allow formation of complex.
- Cells were transfected in 6 well tissue culture plates overnight, after which the culture media was changed to DMEM (3% FBS + P/S).
- Culture media containing retroviral particles was harvested at 48 and 72hrs post transfection and passed through a 0.2μM filter to remove any contaminating phoenix cells. Transduction of target cells was performed immediately.
- Target cells were seeded at $1.5x10^5$cells/well in a 6 well tissue culture plate 24hrs prior to transduction. To transduce, cells were incubated overnight with harvested retrovirus culture media diluted 1:1 in DMEM (3% FBS + P/S). After which the media was changed to DMEM (10% FBS + P/S).
- 72hrs post transduction cells were put under selection, control non-transduced cells were used to gauge how effective the transduction was and to verify selection efficacy.

2.1.6: Transient transfection of Huh-7.5 cells.

Plasmid transfection

Moesin-GFP, N-Moesin-GFP and LifeAct-Ruby DNA plasmids were all transefected into Huh-7.5 cells using Lipofectamine transfection.

- Transfections were carried out using the Lipofectamine (Invitrogen, CA, USA) kits according to the manufacturer's guidelines.

- Briefly, Huh-7.5 cells were seeded at $6x10^5$ cells per well in a 6 well culture dish in P/S free media 24hrs prior to transfection.
- Plasmid/lipofectamine complex made up as follows;
    - 4μg DNA added to 200μl of Optimem (Gibco) and mixed gently.
    - 8μl lipofectamine reagent added to 200μl of Optimen and mixed gently.
    - Diluted DNA and diluted lipofectamine reagent combined and incubated at room temperature for 15min to allow formation of complex.
- Complex added to well containing 1000μl of P/S free DMEM.
- Transfections were carried out at 37°C for 8hrs.
- After which the transfection mixture was replaced with DMEM (3% FBS + P/S).
- Expression efficiency was typically monitored 48 hrs post transfection.

siRNA transfection

Claudin-1 and CD81 siRNA (Invitrogen) were transfected into Huh-7.5 cells using Lipofectamine transfection.

- Transfections were carried out using the Lipofectamine RNAiMAX (Invitrogen, CA, USA) kits according to the manufacturer's guidelines.
- Briefly, Huh-7.5 cells were seeded at $6x10^5$ cells per well into a 6 well tissue culture dish in P/S free media 24hrs prior to transfection.
- siRNA-RNAiMax complex was made up as follows;
    - 100pmol siRNA added to 200μl of Optimem (Gibco) and mixed gently.
    - 4μl RNAiMAX added to 200μl of Optimen and mixed gently.
    - Diluted siRNA and diluted RNAiMAX combined and incubated at room temperature for 15min to allow formation of complex.
- Complex added to well containing 1000μl of P/S free DMEM.
- Transfections were carried out at 37°C for 8hrs.

- After which the transfection mixture was replaced with DMEM (3% FBS + P/S).
- Expression efficiency was typically monitored 48hrs post transfection.

2.1.7: Cytotoxicity testing

The cytotoxicity of compounds used in this study was tested using the Cell Titer One Solution Cell Proliferation (MTS) Assay (Promega) according to the manufacturer's guidelines.

- Briefly, cells were seeded at $1.5x10^4$ cells per well of a 48 well tissue culture plate.
- Cells then treated with compound at a defined concentration and cultured for a defined period of time, (24 or 48hrs) as stated in figure legends.
- Cells then washed with PBS followed by incubation with 200μl MTS (tetrazolium compound) at working concentrations and incubated for 2hrs at 37°C
- 100μl of supernatant transferred to a 96 well plate and absorbance read at 490nm. Final absorbance was determined by deleting absorbance recorded for an empty control well.

2.1.8: Cholesterol quantification.

The Amplex Red Cholesterol Assay Kit (Invitrogen, CA, USA) was used to quantify total cellular cholesterol according to the manufacturer's guidelines.

- Briefly cells were enumerated using a haemocytometer and $25x10^4$ cells were pelleted by centrifugation (12,000rpm for 5min) and lysed in 250μls of 1x Reaction buffer.
- 50μl of cholesterol samples diluted in reaction buffer plus cholesterol reference controls were added to separate wells of a 96 well plate.
- 50μl of Amplex Red Reagent was then added to samples.

- Wells then incubated at 37°C for 30min.
- Fluorescence was read in a Multi detector microplate reader (Biotech Synergy HT), excitation 530 and emission detection at 590.
- A no cholesterol control was used as a background. Final fluorescence reading deduced by subtracting the background.
- In each assay a standard curve was produced using the cholesterol reference standard (Fig.2.1) allowing cholesterol levels to be expressed as μM cholesterol per $1x10^4$ cells.

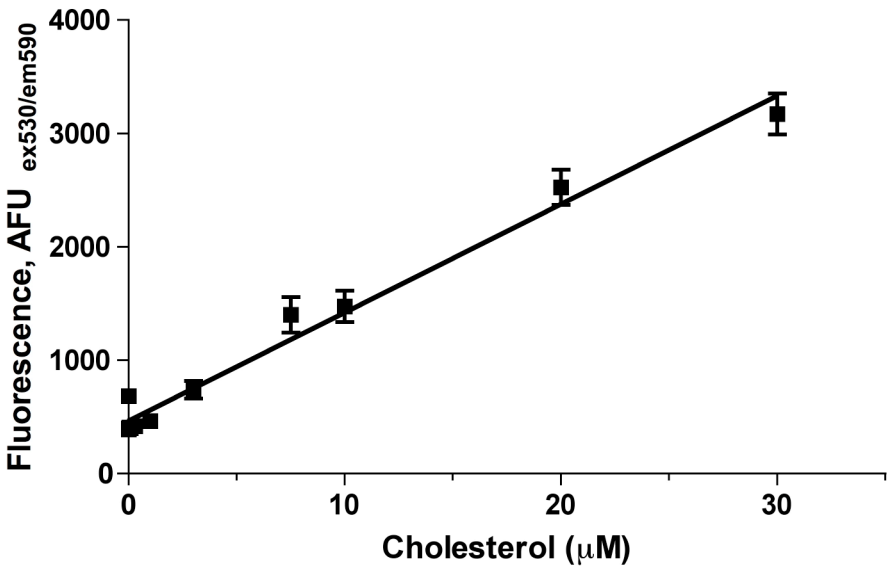


Figure 2.1: Cholesterol standard curve

2.1.9: Flow cytometry

HCV infection level

Throughout this study HCV infection level was determined using a primary antibody directed at the non-structural region 5A (NS5A) of HCV (9E10).

- Cells were removed from tissue culture plastic with trypsin.
- $25x10^4$ cells were seeded per well into a 96 well round bottom plate and fixed with 1% PFA for 5min at 4°C.
- Fixed cells were incubated at room temperature in PBS plus 1% BSA (blocking) and 0.1% saponin (permeabilisation) for 20min.

Cells were now removed from containment level 3

- Cells centrifuged at 1200rpm and the pellet re-suspended in primary antibody 9E10 (diluted in PBS + 1% BSA, 0.1% saponin) for 30min followed by 2x PBS washes.
- This was followed by incubation with secondary antibody Alexa-fluor 488 or RPE conjugated anti-mouse IgG 2a (Invitrogen) (diluted in PBS + 1% BSA, 0.1% saponin) for 30min at room temperature and 2x PBS washes.
- Percentage NS5A positive cells were determined by running the cells through a FACScalibur flow cytometer (BD Biosciences) and analysis on FLowJo software (Tree Star, San Carlos, CA).

Cell surface protein expression

- Cell surface expression was determined on live cells. Trypsin or cell dissociation buffer (as stated in figure legend) was used to remove the cells from tissue culture plastic.
- Cells were blocked with FACS buffer (PBS + 2% BSA, 0.01% Azide) for 20min at room temperature.
- $25x10^4$ cells were then seeded per well into a 96 well round bottom plate and centrifuged at 12,000rpm to pellet the cells.
- Cells were then re-suspended in 100μl of primary antibody diluted in FACS buffer, (concentrations in Table 2.4), and incubated for 30min at room temperature.

- This was followed by 2x PBS washes and then incubation with 100μl of appropriate secondary antibody (concentrations in Table 2.2) for 30min at room temperature.
- Cells were then washed again (2xPBS) and fixed with 1% Para formaldehyde (PFA) for 5min at 4°C.
- Protein surface expression was determined by running cells through a FACScalibur flow cytometer (BD Biosciences) and analysis using FlowJo software (Tree Star, San Carlos, CA).

2.1.10: Immuno-fluorescence

HCV infection level

Throughout this study to visualize HCV infectivity we stained the cells using a primary antibody directed at HCV non-structural region NS5A (9E10).

- Cells were fixed with ice-cold methanol for 5min.
- Fixed cells were then incubated with PBS plus 1% BSA (blocking) and 0.1% saponin (permeabilisation) for 20min.
- Primary antibody (9E10) was added to the cells at 1:200 dilution in PBS + 1%BSA + 0.1% saponin and incubated at room temperature for 30min followed by 2x PBS washes.
- Alexa fluor 488 or 594 conjugated anti-mouse IgG2a secondary antibody was then added at 1:1000 dilution in PBS + 1%BSA + 0.1% saponin for a further 30min at room temperature, followed by 2x PBS washes
- NS5A positive cells visualized on Nikon TE2000 fluorescence microscope on a Zeiss META head confocal microscope.

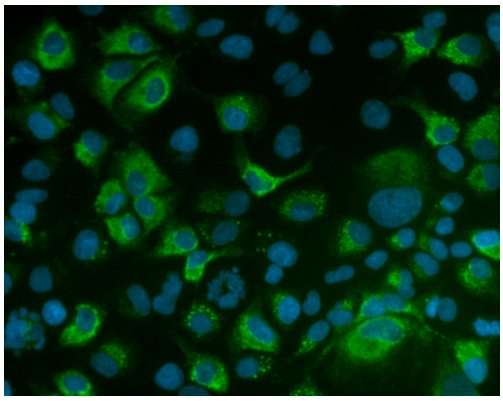


Figure 2.2: NS5A stain of HCVcc J6/JFH infected cells.
Huh-7.5 cells 72hrs post EP with J6/JFH RNA. NS5A stained with 9E10 primary antibody and Alexa-flour 488 conjugated secondary antibody (Green), cell nuclei counterstained with DAPI (Blue), visualized on Nikon TE2000 fluorescence microscope.

Cellular protein expression

To visualize cellular protein expression by confocal microscopy cells were seeded onto 24 well plates containing 13μm glass cover slips. For fluorescent microscopy analysis only, glass cover slips were not used.

- Cells were fixed with appropriate fixative (See Table 2.4) or stained live (SR-BI expression only).
- Fixed cells were then incubated with PBS/1% BSA (blocking) and 0.1% saponin (permeabilisation) or Triton-X-100 (permeabilisation) (P-ERM only) for 20min. This step not included for SR-BI expression.
- Primary antibody was added to the cells at appropriate dilution in PBS + 1%BSA + 0.1% saponin, PBS/1%BSA (P-ERM only) or serum free DMEM (SR-BI only) and incubated at room temperature for 30min followed by 2x PBS washes. See table 2.4 for antibody concentrations.
- Appropriate Alexa-fluor 488 or 594 conjugated secondary antibody was then added at 1:1000 dilution in PBS/1%BSA/0.1%saponin, PBS/1%BSA (P-ERM only) or serum free DMEM (SR-BI only) for a further 30min at room temperature,

followed by 2x PBS washes. Cells stained for SR-BI expression then fixed with 3% EM grade formaldehyde.

- Cells counter stained with DAPI (sigma) diluted in PBS.
- Stained cells visualized and imaged on Nikon TE2000 fluorescence microscope or cover slips mounted onto glass slides using ProLong Antifade mounting agent (Invitrogen, CA, USA) and imaged on a Zeiss META head confocal microscope with a 63x water immersion objective. Background fluorescence of samples was corrected based on control samples stained with species-matched IgG and secondary antibody only.

Antibody	Application	Dilution	Fixative for IF
Primary antibodies			
9E10 (Anti-HCV NS5A)	IF	1/200	Methanol
2.s131 (Anti-CD81)	IF/FC	1ug/ml	3% EM grade formaldehyde
PF72 (Anti-SR-BI)	IF/FC	1ug/ml	Live cell stain followed by 3% EM grade formaldehyde fixation
Anti-Claudin	IF	1ug/ml	Methanol
Anti-Occludin	IF	1ug/ml	Methanol
Anti-P-ERM	IF	1/200	3% EM grade Formaldehyde
Anti-P-ERM	WB	1/1000	
Anti-Beta1	FC	1ug/ml	
Anti-Beta2	FC	1ug/ml	
Anti-Beta3	FC	1ug/ml	
Anti-Beta4	FC	1ug/ml	
Anti-alpha1	FC	1ug/ml	
Anti-alpha2	FC	1ug/ml	
Anti-alpha4	FC	1ug/ml	
Anti-alpha5	FC	1ug/ml	
Anti-alpha6	FC	1ug/ml	
Anti-alphaVBeta3	FC	1ug/ml	
Anti-alphaVBeta5	FC	1ug/ml	
Anti-alphaVBeta6	FC	1ug/ml	
Anti-Fibronectin	ELISA	10ng/ml	
Anti-Collagen I	ELISA	10ng/ml	
Anti-collagen IV	ELISA	10ng/ml	
Anti-Beta-actin (AC-15)	WB	1/2500	
Secondary antibodies			
Anti-Mouse Alexa fluor-488	IF/FC	1/1000	
Anti-Mouse Alexa fluor-RPE	FC	1/1000	
Anti-Mouse Alexa fluor-594	IF/FC	1/1000	
Anti-Rabbit Alexa fluor-488	IF	1/1000	
Anti-human Alexa fluor-488	IF/FC	1/1000	
Anti-mouse-HRP	ELISA	1/5000	
Anti-human-HRP	ELISA	1/400	
Anti-Mouse-HRP	WB	1/2500	
Anti-Rabbit-HRP	WB	1/2500	

Table 2.4: List of antibody concentrations.
IF: Immuno-fluorescence, FC: Flow cytometry, ELISA: Enzyme linked immunosorbent assay, WB: Western blot

2.2: Specific assays

Chapter 3

2.2.1: Antibody engagement cell spread assay

For all cell spread assays cells were serum starved overnight prior to assay. Specific treatments were carried out on the morning of the assay as specified in table 2.5. For the cholesterol modulation assays the cells were removed from tissue culture plastic with trypsin and treated at 37°C in solution in universals ($2x10^6$ cells in 1ml of DMEM). For the remaining spread assays adherent cells were treated in a T25 flask or well of a 6 well tissue culture dish.

- 96 well ELISA plates (Thermo electron corporation) were prepared by coating with specified antibody at 5μg/ml diluted in 100μl of PBS and incubated at 4°C overnight.
- Antibody coated plates were then blocked with PBS/1%BSA for 30min and washed 2x with PBS.
- With the exception of the cholesterol modulation assay where cells are already in solution, the cells were removed from tissue culture plastic using trypsin. $3x10^4$ cells were then seeded per well in 100μl of serum free DMEM.
- Phase images were taken of cells at specified time points post seeding. For assays carried out in category three containment level, cells were fixed by adding EM grade formaldehyde directly to the wells (final concentration 3.4%) prior to removing cells for imaging.
- A Nikon TE2000 fluorescence microscope was used to image the cells. The total number of spreading and non-spread cells were enumerated in a field of view and percentage cell spread determined (See Fig.3.2 for assay set up).

TIRF microscopic analysis of cell spread assay

For TIRF microscopy spread assays we used Huh-7.5 cells expressing TRIP CD81.GFP (See section 2.1.5) and Lifeact-Ruby (See section 2.1.6). Cells were used 24hrs post transfection with Lifeact-Ruby.

- 35mm glass bottom dishes (World Precision Instruments Inc., FL, USA) were coated with specified antibody at 5μg/ml diluted in PBS and incubated at 4°C overnight.
- Antibody coated plates were then blocked with PBS/1%BSA for 30min and washed 2x with PBS.
- Cells were removed from tissue culture plastic using trypsin and $3.5x10^5$ cells were then re-suspended in 2500μl DMEM (No phenol red) (Gibco) + 20mM Hepes + 10% FBS and seeded into the coated 35mm dishes.
- 3 Fields of view per well were randomly selected and imaged by TIRF microscopy every 10 min up to 80 min using a Nikon A1R microscope.

Treatment	Source	Concentration	Pre-treatment
C3 transferase (Rho inhibitor)	Cytoskeleton	5ug/ml	4hr at 37degC
Rac-1 inhibitor (Rac inhibitor)	Calbiochem	100uM	1hr at 37degC
Y27632 (ROCK inhibitor)	Sigma	2.5uM	4hr at 37degC
Dynasore	Sigma	80uM	30min at 37degC
Bisindolymaleimide (PKC inhibitor)	Calbiochem	100nm	1hr at 37degC
U0126 (MAPK inhibitor)	Calbiochem	100uM	1hr at 37degC
PD98059 (MAPK inhibitor)	Calbiochem	100uM	1hr at 37degC
SB203580 (MAPK inhibitor)	Calbiochem	100uM	1hr at 37degC
Cytocholasin D	Sigma	5uM	1hr at 37degC
Latrunculin B	Sigma	0.1uM	1hr at 37degC

Table 2.5: Treatment summary for spread assays.

2.2.2: Recombinant sE2 engagement cell spread assay

Plate preparation

- 96 well ELISA plates (Thermo electron corporation) were prepared by coating with 10/76b capture antibody at 1μg/ml diluted in 100μl of PBS and incubated at 4°C overnight.
- Well then blocked with PBS/1%BSA for 1hr.
- Recombinant soluble E2 (JFH-10/76b) was then added to the wells at 1 in 3 dilution in PBS and incubated at 37°C for 2hrs.
- sE2 coated plates were then blocked with PBS/1%BSA for 30min and washed 2x with PBS.
- Control wells contained 10/76b only or empty wells blocked with PBS/1%BSA.

Spread assay

- Huh-7.5 cells were serum starved overnight and then removed from tissue culture plastic using trypsin. $3x10^4$ cells were then seeded per well in 100μl of serum free DMEM.
- Phase images were taken of cells at 1hr post seeding using a Nikon TE2000 fluorescence microscope.

ELISA for detection of bound sE2.

- A cocktail of human anti-E2 antibodies (CBH2 and CBH4B) at 4ug/ml were added to each well and incubated at room temperature for 45min followed by 3x washes with PBS.
- Wells then incubated with appropriate HRP conjugated secondary antibody (Jackson ImmunoResearch laboratories) for 45min.

- Bound antibodies were visualized using TMB substrate (BioFX laboratories) and reaction was terminated after 5min incubation with stop reagent (BioFX laboratories).
- Absorbance was read on plate reader at 490nm (Multiskin ascent, Thermo Electron Corporation).

2.2.3: P-ERM western blot for anti-CD81 engagement time course assay.

Lysate preparation

- lysates were prepared by seeding $1x10^6$ Huh-7.5 cells per well into wells of a 6 well tissue culture dish. Cells were then serum starved overnight.
- Cells stimulated with anti-CD81 mAb (1.s262) diluted at 5μg/ml in serum free DMEM for 0,1,5,10,15 and 30min.
- Tissue culture media was removed and the cell monolayer rinsed with PBS at room temperature. All subsequent steps were carried out on ice using fresh, ice-cold buffers to prevent protein degradation.
- 2ml of ice cold PBS were added per well and adherent cells removed using a cell scraper. The cell suspension was transferred to universal and pelleted by centrifugation at 1200 rpm for 5 min at 4°C.

  The cell pellet was re-suspended in 500μl of NP40 lysis buffer (10mM Tris-HCL; pH 7.5, 2mM $CaCl_2$, 2mM $MgCl_2$, 1%NP40 plus1x Complete Mini Protease Inhibitor Cocktail Tablet + 1x Complete PhosStop Phosphatase Inhibitor Cocktail Tablet (Roche)) and incubated on ice for 30 min.
- The lysate was centrifuged at 15 000 rpm in a Biofuge primo R centrifuge (Heraeus) for 15 min at 4°C to separate nuclei and unsolubilized cell membranes from protein, after which the supernatant was collected and

frozen at -20°C.

- Protein was quantified using the BCA Protein Assay kit (Thermo Scientific, IL, USA) according to the manufacturer's instructions.
    - Briefly, 100μl of each sample or BSA standard were mixed with 200 μl of BCA Working Reagent in a 96-well microtiter plate in triplicates and incubated at 37°C for 30 min.
    - The plate was allowed to cool to RT and the absorbance at 490 nm measured using ELISA plate reader (Multiskin ascent, Thermo Electron Corporation).
    - To determine the protein concentration of each sample, a standard curve was prepared by plotting the average Blank-corrected 490 nm measurement for each BSA standard versus its concentration in μg/ml.

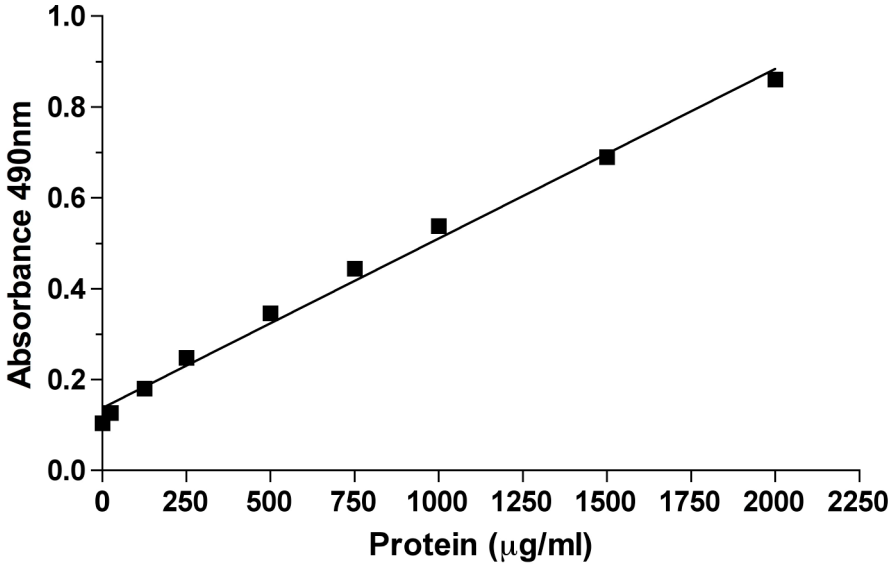


Figure 2.3: BCA protein assay standard curve.

SDS Polyacrylmide Gel Electrophoresis (SDS-PAGE).

Proteins were separated on 12% SDS gel.

- To prepare samples, defined amounts of protein were mixed with 3x Laemmli loading dye (H2O + 30% v/v Glycerol + 6% w/v SDS + 0.02% v/v Bromophenol Blue + 0.2M Tris-HCl; pH 6.8 plus 10% 2-mercaptoethanol) (Reducing conditions) and the total volume adjusted to 25μl with H2O. Samples were heat-denatured at 95°C for 5min and allowed to cool to room temperature prior to loading.
- Proteins were separated by electrophoresis using the Mini Protean 3 System (Bio-Rad Laboratories, CA, USA) according to the manufacturer's instructions.
    - Briefly, 20μl of protein sample were loaded per lane and gels run at 200 volts for 30-45 min.
    - Proteins were transferred onto PVDF membranes (Millipore, MA, USA) using a Mini Trans-Blot Electrophoretic Transfer Cell System (Bio-Rad) according to the manufacturer's recommendations.
    - Briefly, PVDF membranes were cut to the appropriate size and pre-treated with methanol for 1-2 min, rinsed with ultra pure water, and incubated in transfer buffer (25mM Trizma base + 0.2M Glycine + 200 ml MeOH + 0.5 ml 10% SDS) at room temperature for 5-20 min. Gels were equilibrated in transfer buffer for 5min to prevent shrinking and incomplete transfer.
    - Transfer was carried out at 350 A for 60min at room temperature.

Immunoprobing and chemiluminescent detection of proteins.

- All subsequent steps were carried out in 50 ml Falcon tubes with gentle agitation on a tube roller (Barloworld Scientific, UK) at RT.
- To block unspecific binding of antibodies, membranes were incubated in 2.5 ml of TBST (10mMTrizma base + 0.1M Sodium Chloride + 10% v/v Tween 20) + 5%

dry milk for 1hr.

- After which the blocking buffer was removed and the membranes were incubated with primary antibody, Anti-P-ERM, diluted in TBST + 5% BSA overnight followed by 5 x 5min washes, then Anti-β-actin diluted in TBST + 5% dry milk for 1hr followed by 5 x 5min washes with excess TBST.
- Membrane then Incubated with appropriate HRP conjugated secondary antibody (Amersham, Biosciences, PA) diluted in 2ml TBST + 5% dry milk followed by 5 x 5min washes with excess TBST.
- Chemiluminescent detection of HRP-conjugated antibodies was performed using the ECL Western Blotting Detection System (Amersham, UK) according to the manufacturer's instructions. Briefly, membranes were immersed in ECL detection reagent for 1 min, wrapped in cling film, and exposed to CL- XPosure Blue X-Ray Film (Thermo Scientific) for 1-5 min.

Antibody concentrations detailed in Table 2.4

Chapter 4

2.2.4: ECM adhesion assay.

Plate preparation

- 96 well plates (Falcon, Becton Dickinson) were coated with human extra-cellular matrix (ECM) proteins; Fibronectin (Sigma), Collagen type I (Sigma), Collagen type VI (BD Biosciences), and laminin (Sigma) at 10μg/ml (50μl/well) and incubated at room temperature for 1hr.
- Wells were then blocked with PBS/1% BSA for 30min followed by 3x PBS washes.

Adhesion assay

- Hepatoma cells were serum starved overnight and then removed from tissue culture plastic with trypsin.
- Cells were counted using a haemocytometer and $4x10^4$ cells were seeded per well in 50μl serum free DMEM. For crystal violet calibration curve 0.5, 1, 2, 3, 4 and $5x10^4$ cells were seeded per well.
- Cells were incubated at 37°C for a defined period of time.
- Non-adhered cells were removed with PBS wash.
- Adhered cells were then fixed with ice-cold methanol for 5min.
- Adhered cells were then stained with crystal violet to determine adhesion or stained for HCV non-structural protein NS5A to determine percentage infectivity of adhered cells.

Crystal violet stain

- Fixed cells were incubated with 0.1% crystal violet for 1hr
- Cells were then washed 3x with PBS and crystal violet solubalised in 36% acetic acid.
- Absorbance was read at 600nm on plate reader (Multiskin ascent, Thermo Electron Corporation).
- Control wells with no cells were used to determine background. Specific adhesion was determined by subtracting the absorbance read on PBS/1% BSA only coated plates from ECM coated plates that had been blocked with PBS/1% BSA.

Quantification of HCV infected cells

- Fixed cells were stained for HCV non-structural protein NS5A as described in section 2.1.10 and then cell nuclei counterstained with DAPI (Sigma).

- To determine the proportion of infected cells, three fields of view were imaged using the Nikon TE2000 fluorescence microscope. The number of NS5A positive cells and total nuclei per field of view were enumerated allowing calculation of percentage-infected cells.

2.2.5: ECM ELISA

Plate preparations

- For control assays 96 well plates were coated with gelatin for 1hr followed by specific concentrations of human fibronectin, collagen I and collagen IV for a further 1hr incubation (100μl/well).
- To determine ECM production of Huh-7.5 cells, $0.5x10^4$ cells in 200μl DMEM (3 or 10% FBS) were seeded per well into gelatin coated 96 well plates. The cells were then incubated at 37°C for a specified time.
- Cells lysed with cell lysis buffer (20mM NH4OH, 0.5% Triton X-100). The lysis step allowed the removal of plates from the level three containment laboratory.
- A titration of human fibronectin was carried out alongside Huh-7.5 fibronectin production assays enabling the concentration of fibronectin produced in μg/ml to be determined (See Fig.2.4)
- Plates were analyzed immediately by ELISA or stored at 4°C prior to analysis.

ELISA

- Wells were blocked with 1% gelofusine in 0.5% Tween20/PBS for 1hr, followed by 3x washes with wash buffer (0.5%Tween20/PBS).
- Wells were incubated with ECM specific primary antibodies at a concentration of 10ng/ml for 45min followed by 3x washes with wash buffer.
- Wells then incubated with appropriate HRP conjugated secondary antibody (Jackson ImmunoResearch laboratories) for 45min.

- Bound antibodies were visualized using TMB substrate (BioFX laboratories) and reaction was terminated after 5min incubation with stop reagent (BioFX laboratories).
- Absorbance was read on plate reader at 450nm (Multiskin ascent, Thermo Electron Corporation).

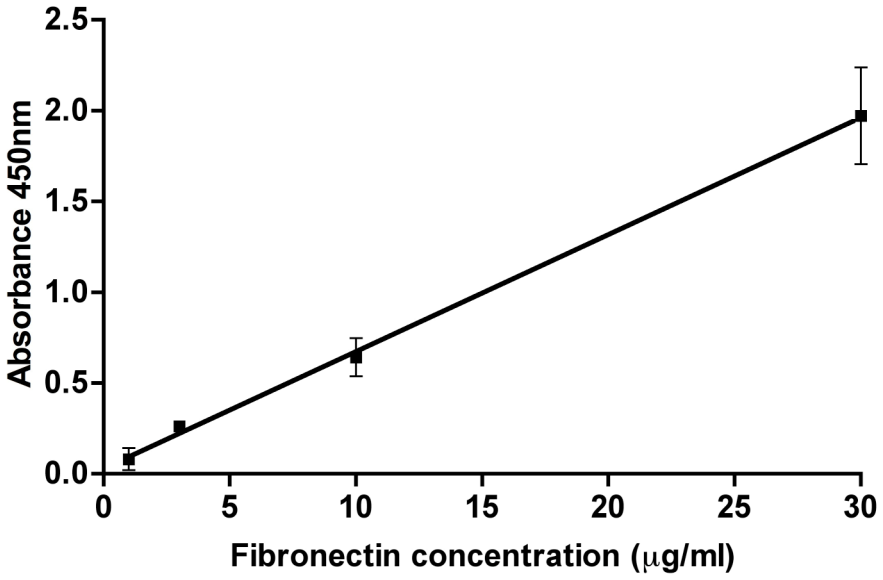


Figure 2.4:Fibronectin standard curve

2.2.6: Anti-CD81 engagement filopodia induction assay

- Huh-7.5 cells were seeded into wells of 24 well plates containing 13μm glass cover slips ($5x10^4$ cells per well) and cultured in serum free DMEM overnight at 37°C.
- Cells incubated with specific antibody for 0, 10 or 240min before fixation with 3.6% EM grade formaldehyde.

- For cells fixed at zero hours (prior to addition of anti-CD81 mAb), Anti-CD81 mAb diluted in 0.5%BSA/PBS was added to the wells for 1hr at 37°C
- All cells were then incubated with Alexa fluor 488 conjugated secondary antibody diluted in 0.5%BSA/PBS for 30min at room temperature followed by 2xPBS washes.
- Phalloidin-594 (Sigma) diluted 1 in 200 in 0.5%BSA/PBS was then added and incubated at room temperature for 30min followed by 2xPBS washes.
- Cells then counterstained with DAPI (Sigma) for 1min at room temperature.
- Stained cells visualized mounted onto glass slides using ProLong Antifade mounting agent (Invitrogen, CA, USA) and imaged on a Zeiss META head confocal microscope with a 63x water immersion objective. Background fluorescence of samples was corrected based on control samples stained with species-matched IgG and secondary antibody only.

2.2.7: Wound healing assay

Huh-7.5 cells ($7x10^5$ cells per well) were seeded into wells of a 6 well dish 24hrs prior to the start of the assay and serum starved overnight. For assays using CD81 knock down cells (See section 2.1.6), the cells were seeded 24hrs prior to transfection and 48hrs prior to the start of the assay. In this scenario cells were serum starved for 3hrs on the morning of the assay.

- A P200 tip was used to form a wound in the cell monolayer.
- Media was then refreshed with serum free DMEM containing 15µg/ml mitomycin C (SIGMA-ALDRICH, MO USA) and images of wound taken immediately (0hrs)
- Wells then incubated at 37°C for 24hrs and then further images of the wound were then taken. A black marker pen was used to mark the area of the wound

being imaged; this ensured the same area was imaged at each time point (See Fig.2.5).

- Images taken by phase on a Nikon TE2000 fluorescence microscope and area of wound determined using IPLab 4.0 (BD Biosciences) software.

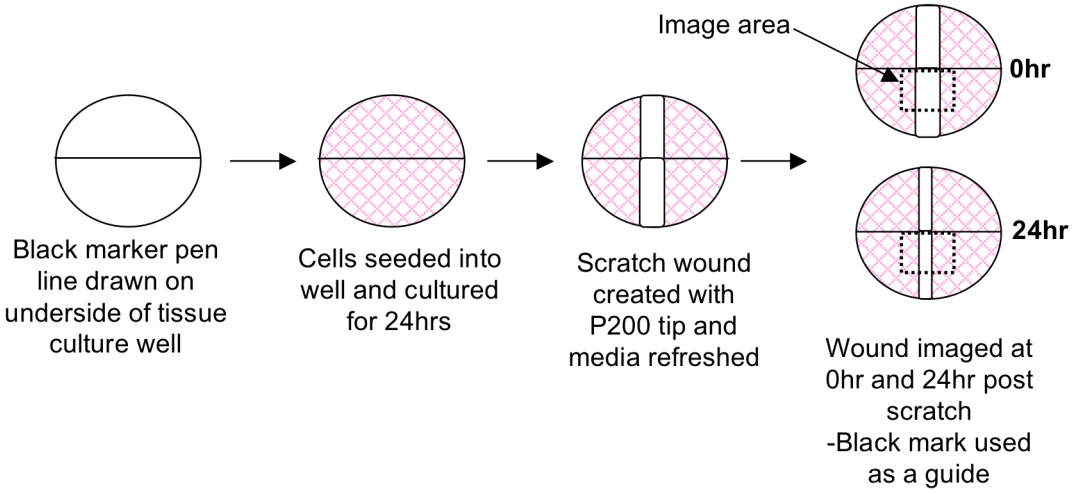


Figure 2.5: Schematic of wound healing assay.

2.2.8: Invasion assay

Mock vs. HCV infected cell invasion assays

Huh-7.5 cells were electroporated with *in vitro* transcribed full length HCV RNA or mock RNA (see section 2.1.4). Cells were cultured in DMEM (3% FBS) and then serum starved overnight prior to assay. Invasion assays were set up 96hr post electroporation.

- Cells were labeled with CMFDA (Invitrogen, CA USA) by incubating the cells at 37°C with 5μM CMFDA (DMEM + 3% FBS) for 30min.
- Labeled cells were then washed with PBS and removed from tissue culture plastic with trypsin.

- Cells were pelleted by centrifugation at 12,000rpm for 5min and the pellet re-suspended in serum free DMEM.
- A viable cell count was performed using trypan blue (Invitogen, CA USA) and a heamocytometer. $4x10^4$ cells (200μl) were seeded into the top well of a collagen coated (calf collagen, Sigma) 8μm pore transwell (BD Falcon, CA USA) in a 24 well tissue culture plate. Cells were also seeded onto a 24 well plate as a control.
- The bottom chamber was filled with 400μl serum free DMEM. Cells were cultured for 24hr at 37°C.
- Non-migratory cells were mechanically removed with a cotton bud and confirmed under light microscope.
- Migratory and control cells were fixed with ice cold methanol for 5min and then stained for HCV non-structural protein NS5A (See sectin 2.1.4) using 9E10 primary antibody and Alexa-fluor 594 conjugated secondary antibody.
- Three fields of view per well were captured on a Nikon TE2000 fluorescence microscope. The number of invaded cells was enumerated per field of view. The proportion of infected invaded cells and those in the control wells were also determined.

All other invasion assays

For the majority of assays hepatoma cells were serum starved overnight prior to assay set up and any specified pretreatment steps were carried out in serum free conditions (See Table 2.6). For assays using CD81 knock down cells, the cells were used 24hr post transfection and serum starved for 3hr prior to the assay.

Cells were removed from tissue culture plastic with trypsin. Cells were pelleted by centrifugation at 12,000rpm for 5min. The pellet was then re-suspended in serum free DMEM.

- A viable cell count was performed using trypan blue and a heamocytometer. $4x10^4$ cells (200μl) were seeded into the top well of a collagen coated (calf collagen, Sigma) transwell (Falcon 8μM pore, 24 well plate).
- The bottom chamber was filled with 400μl serum free DMEM. Where compounds were used cells were seeded in the presence of the compound and the compound was also present in the bottom chamber of the transwell.
- Cells were cultured for 24hr at 37°C 5% 0_2. Where specified cells were cultured in a hypoxic incubator (New Brunswick Galaxy 48R, Eppendorf) at 1% 0_2.
- Non-migratory cells were mechanically removed with a cotton bud and confirmed under light microscope.
- Migrated cells were fixed with ice-cold methanol for 5min and then stained with 0.1% crystal violet for 1hr followed by 3x PBS washes.
- Three fields of view per well were captured on a Nikon TE2000 fluorescence microscope. The numbers of invaded cells were enumerated for each field of view.

Name	Source	Concentration	Pre-treatment
HIF NSC-134754	Dr. M. Ashcroft, University College London, London	1uM	3hrs at 37degC
Anti-VEGF (VG67e)	Prof.R.Bicknell, University of Birmingham, Birmingham	400ng/ml	
VEGF-165	Peprotech	10ng/ml	
Anti-CD81 (1.s262)	In house, K.Hu and M.Goodall	0.1,1,10ug/ml	

Table 2.6: Treatment summary for invasion assays.

Antibody concentrations detailed in Table 2.4

2.2.9: Infectious co-culture assay

Standard assay for flow cytometry analysis

- Huh-7.5 cells were electroporated with *in vitro* transcribed full length HCV RNA (See section 2.1.4) 72hrs prior to use in assay.
- Naïve unlabelled target cells were seeded into collagen coated (calf collagen, Sigma) 12 well plates at a seeding density of $12.5x10^4$ cells per well. To determine the role of receptors in co-culture transmission target cells were seeded in the presence of control or receptor antagonists (Antibody and concentration specified in the figure legend) and allowed to rest for 1hr at 37°C prior to addition of producer cells.
- Infected producer cells were labeled with CMFDA (Invitrogen, CA USA) by incubating the cells at 37°C with 5μM CMFDA (DMEM + 3% FBS) for 30min.
- Labeled producer cells were then washed with PBS and removed from the tissue culture plastic with trypsin. $12.5x10^4$ producer cells were then seeded into co-culture with the naïve target cells. To enable distinction between nAb resistant and nAb sensitive transmission producer cells were seeded in the presence or absence of control or anti-glycoprotein antibody (Antibody and concentration specified in the figure legend). Final co-culture contained a 1:1 ratio of producer: target cells, each well totaling $25x10^4$ cells in 1ml of DMEM (3% FBS).
- 48hr post co-culture supernatants were collected and used in a standard infectious assay to allow quantification of cell-free virus and to determine efficacy of anti-glycoprotein antibody (See section 2.1.4). Trypsin was used to harvest the cells from each well.

- Harvested cells were seeded into a 96 well round bottom dish where they were centrifuged at 12,000rpm and fixed with 1%PFA for 5min at 4°C.
- Cells were then stained for HCV non-structural protein NS5A and analyzed by flow cytometry as described in section 2.1.9. This allowed determination of de novo transmission events (See Fig.5.2).

Co-culture assays to determine requirement for cell contact

All assays were performed in collagen coated 6 well dishes and in the presence or absence of control or anti-glycoprotein antibody.

- For standard seeding density: A total of $50x10^4$ cells were seeded per well at a 1:1 ratio of producer: target cells and at a final volume of 2ml per well.
- For 0.25x seeding density: A total of $12.5x10^4$ cells were seeded per well, again at a ratio of 1:1 producer: target cells and at a final volume of 2ml per well.
- For indirect co-culture: $25x10^4$ target cells were seeded into a collagen coated 6 well dish and $25x10^4$ infected producer cells were seeded onto the underside of an inverted 0.1μM transwell insert (BD Falcon, CA USA) and incubated at 37°C for 30min. The transwell inserts were then carefully added to the 6 well dishes containing the target cells.
- 48hrs post co-culture supernatants were collected. For in-direct co-culture supernatant was collected from the top and bottom chambers. Supernatants were then used in a standard infectious assay (See section 2.1.4).
- For standard and 0.25x seeding density cells were harvested, fixed, stained and analyzed as previously described for standard assay. For in-direct co-culture producer and target cells were analyzed independently.

Optimized co-culture assay for confocal microscopy analysis

To enable optimum visualization of foci of infection a number of preliminary experiments were carried whereby different producer: target ratios and cell seeding densities were explored. The optimized assay was as follows:

- The co-culture was performed on collagen coated 13μm glass cover slips in 24 well plates.
- Producer and target cells were seeded at a 1:20 ratio at 0.75x standard seeding density ($9.4x10^4$ cells per well) in a total volume of 500μl.
- 48hr post co-culture the cells were fixed with ice-cold methanol and stained for non-structural protein NS5A (See section 2.1.10) using 9E10 primary antibody and anti-mouse IgG2a Alexa-fluor 594 secondary antibody.
- Cells nuclei were counterstained with (DAPI) (Sigma) and mounted onto glass slides using ProLong Antifade mounting agent (Invitrogen, CA, USA).
- Stained cells were imaged on a Zeiss META head confocal microscope with a 40x water immersion objective. Background fluorescence of samples was corrected based on control samples stained with species-matched IgG and secondary antibody only.

3. Results: The role of CD81 in hepatoma cell spread.

3.0 Introduction

Tetraspanins are widely expressed small membrane proteins that share common structural features: four transmembrane domains, a small intracellular loop, two short intracytoplasmic termini, and two extra-cellular loops (EC1 and EC2). They associate with each other and non-tetraspanin partner proteins, CD81 is reported to interact with tetraspanin CD9, immunoglobulin like proteins EWI-2 and EWI-F as well as integrins $\alpha 3\beta 1$, $\alpha 4\beta 1$ and $\alpha v\beta 5$, and signaling proteins including protein kinase C (PKC) (42, 74, 411, 427, 458, 481, 528, 556). Posttranslational palmitoylation of juxta-membrane cysteine residues are essential to stabilize protein-protein interactions and to permit a link to cholesterol and gangliosides, allowing the formation of higher ordered networks at the cell surface termed tetraspanin enriched microdomains (TEMS) (43, 76, 77, 536). However, the role of palmitoylation may vary depending on the tetraspanin in question (77). TEMS are distinct from lipid rafts since they do not contain glycosylphosphatidylinositol-anchored proteins or caveolin (196, 270).

Tetraspanins are described to function as 'molecular organizers' by regulating associated signaling proteins via lateral protein-protein interactions. At the present time there is no data to support the existence of tetraspanin-specific agonist ligands (299, 561). Their role(s) can vary depending on both the cell type and tetraspanin in question and include cell adhesion, proliferation, motility, activation and signal transduction, processes often associated with cancer progression (561).

All anti-tetraspanin antibodies published to date bind EC2 that is implicated in tetraspanin-tetraspanin and tetraspanin-partner protein interactions (459). Anti-tetraspanin antibodies are thought to function by blocking tetraspanin – partner protein interactions (177, 352, 427) or by activating tetraspanin intracellular signaling through cross-linking and aggregation of cell surface proteins (449). Tetraspanin CD81 was originally identified as the target protein for an antibody that inhibited the proliferation of a number of lymphoid cell lines (356). Since then studies with anti-CD81 antibodies have suggested a role for CD81 in: Human T cell leukemia virus type I (HTLV) (218) and Human immunodeficiency virus (HIV) (164) induced syncytium formation; rat astrocyte morphology (158), T-cell maturation (51), and hepatic stellate cell migration. These studies have highlighted the diversity of role(s) for CD81 in different cell types and yet the mechanisms underlying these cellular processes are poorly defined.

Several studies have reported that recombinant forms of HCV E2 glycoprotein and anti-CD81 engagement of immune cells promote actin cytoskeletal rearrangement and altered cellular functions (104, 105, 253, 486, 498) including: T cell stimulation via the activation of Lck a Src family protein kinase (104, 498); inhibition of NK cytolytic activity (105, 486) and B-cell activation and proliferation (93, 403). These findings suggest that HCV engagement of CD81 on immune cells may promote liver inflammation and implicate a role for HCV in B-lymphocyte disorders including cryoglobulinemia and B cell non-Hodgkin lymphoma (93, 104, 403). However a recent study by Yoon and colleagues demonstrated that HCVcc had no effect on NK cell activity, suggesting that earlier reports may be an artefact of using recombinant HCV E2 (549).

Coffey et al., reported ezrin phosphorylation following CD81 engagement of B lymphocytes and hypothesized that ezrin and F-actin rearrangement(s) following CD81 engagement may be an underlying mechanism of CD81 function in other cell types (95). Anti-CD81 induced phosphorylation of Ezrin Radoxin Moesin (ERM) proteins on NK cells promotes their migration (253). Ligation of CD81 expressed on hepatoma cells (Huh-7) has been reported to activate MAPK-ERK and Rho-family GTPase signaling pathways, promoting cellular proliferation, and facilitating HCV entry (54, 66, 558).

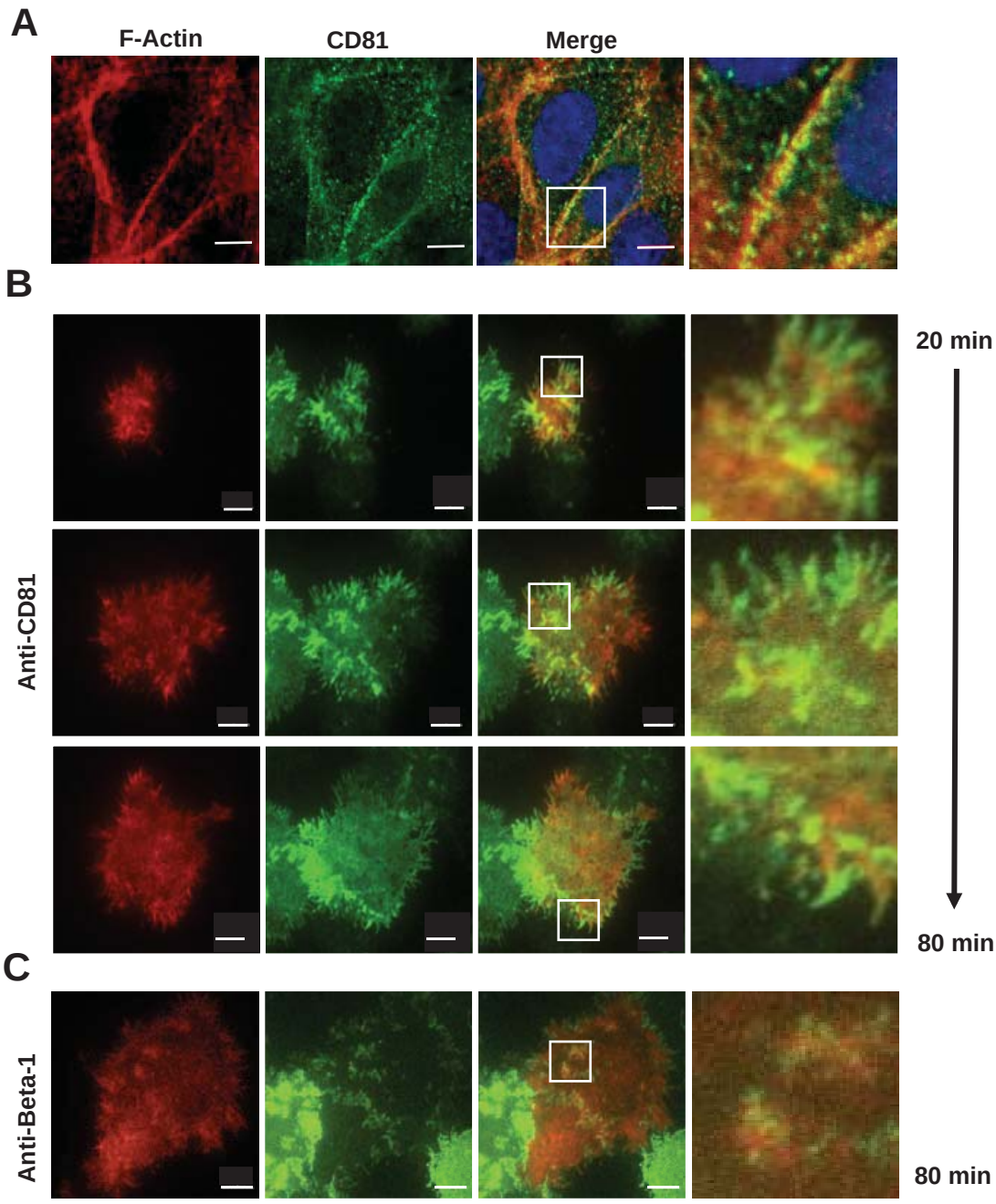
The aim of this chapter is to ascertain the effect(s) of CD81 engagement using a panel of antibodies recognizing novel epitopes for their effect(s) on hepatoma morphology and to ascertain whether HCV infection perturbs CD81-dependent cellular processes.

3.1 CD81 engagement promotes actin polymerisation dependent hepatoma cell spread.

To ascertain the distribution of CD81 and actin in the hepatoma cell line Huh-7.5 (47) the cells were fixed and stained with anti-CD81 mAb 2.s131 and phaloidin (F-actin stain) and imaged by confocal microscopy. CD81 localized at the cell membrane and in intracellular punctate regions, and co-localized with F-actin specifically at areas of cell contacts (Pearson's co-localization co-efficient Rr = 0.4210 (+/- 0.1121)) (Fig.3.1A). To determine whether anti-CD81 engagement of hepatoma cells promotes actin polymerization and subsequent changes in cellular morphology the cells were engineered to express GFP tagged CD81 and Ruby tagged Lifeact. The latter is a fluorophore tagged 17aa peptide that binds to filamentous actin (F-actin) without inhibiting its function allowing live cell imaging of the actin cytoskeleton (399). AcGFP.CD81 and Lifeact-Ruby expressing Huh-7.5 cells were seeded onto glass bottom 35mm dishes that had been coated with anti-CD81mAb 1.s262, anti-Beta-1 Integrin or irrelevant IgG control and blocked with 1% BSA to prevent non-specific binding. The cells were imaged by total internal reflection microscopy (TIRF). TIRF microscopy is an imaging technique that utilizes an evanescent wave to excite only the first 100nm of sample enabling highly sensitive images to be taken of the cell membrane (310). Images were collected every 10 mins over the course of 80 mins. Both anti-CD81 and anti-Beta-1 integrin promoted hepatoma cell spread, whereas the cells failed to attach and spread on control irrelevant IgG coated plates and could not be imaged (Fig.3.2B&C). Specific areas of actin - CD81 co-localization were observed in both anti-CD81 and anti-Beta-1 treated cells, indicating that their co-localization is not dependent on anti-CD81 engagement.

Figure 3.1: CD81 and F-actin expression in Huh-7.5 hepatoma cells.

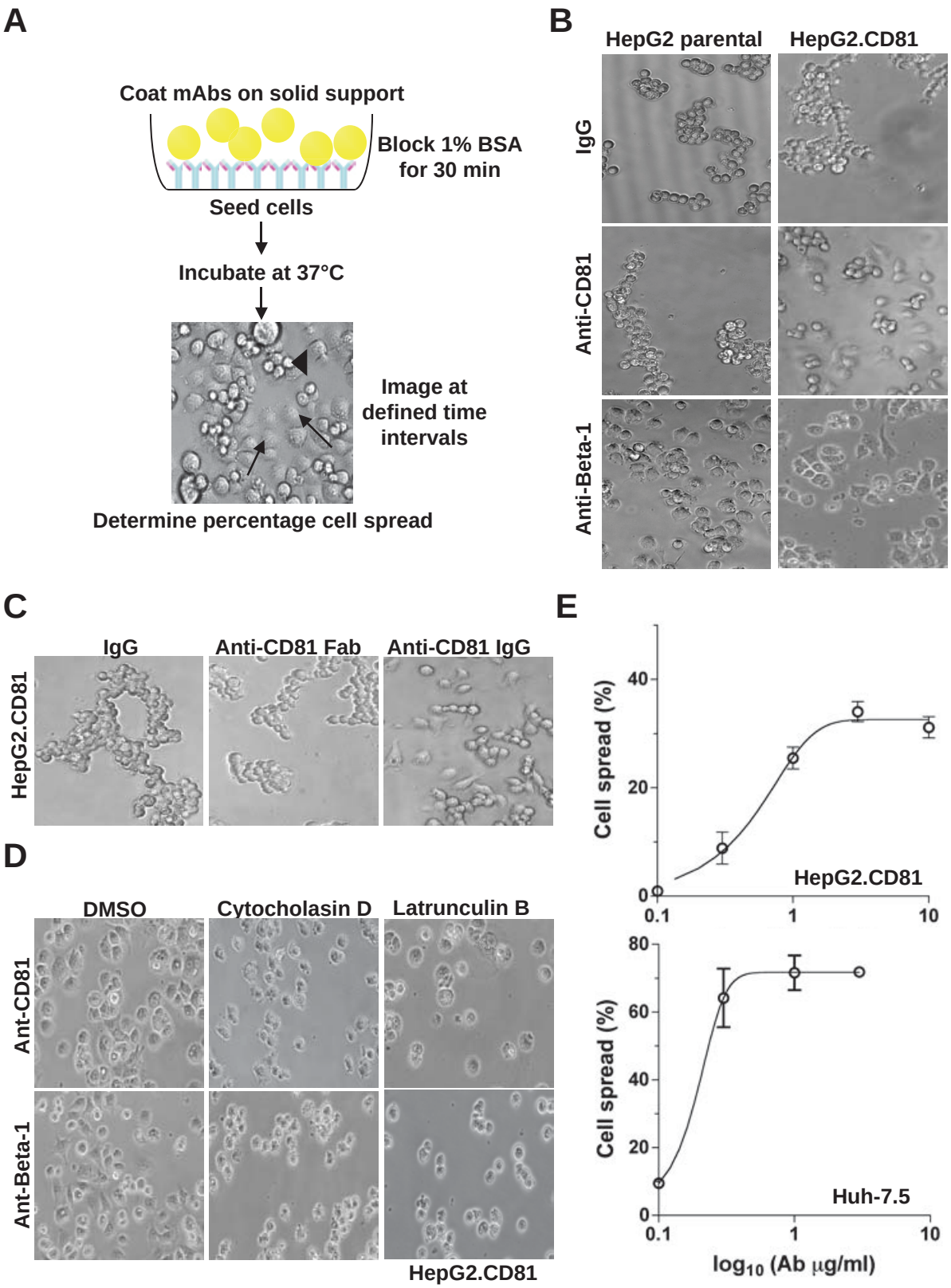
(A) Huh-7.5 cells were cultured on glass cover slips for 24hrs prior to formaldehyde fixation and saponin permeabilisation. CD81 was visualized using 2.s131 mAb and alexa-fluor 488 secondary antibody (green), F-actin visualized using phalloidin-594 (Red). Cell nuclei were counter stained with DAPI (Blue) and the cells imaged with a Zeiss META head confocal microscope with a 60x water immersion objective. White scale bar equal to 10μm. CD81 and F-actin co-localization was quantified ((Pearsons co-localization coefficient (Rr) = 0.42 (+/-0.1)) for 10 representative images using ImageJ co-localization software. Huh-7.5 cells were engineered to express GFP tagged CD81 (Green) and Ruby tagged Lifeact (Red) to visualize F-actin. Cells were seeded onto glass bottom 35mm dishes coated with (**B**) anti-CD81 (1.s262) or (**C**) anti-Beta-1 and images collected by TIRF microscopy (Nikon A1R) every 10min for a total of 80min. The data set is representative of two independent experiments.


A
F-Actin
CD81
Merge
B
20 min
Anti-CD81
80 min
C
Anti-Beta-1
80 min

To study the processes involved in anti-CD81 induced cell spread we developed an assay to quantify antibody-induced changes in cell morphology. Hepatoma cells were seeded onto antibody coated 96 well plates for defined periods of time before imaging by phase microscopy. This method allowed us to enumerate the percentage of spreading cells (Fig.3.2A). To confirm that anti-CD81 induced cell spread was dependent on CD81 expression we compared parental HepG2 cells that lack CD81 to those transduced to express CD81. HepG2.CD81 cells spread on both anti-CD81 and anti-Beta-1 coated wells in contrast to HepG2 parental cells that only spread on anti-Beta-1 coated wells (Fig.3.2B). Furthermore anti-CD81 IgG and not FAb fragments induced HepG2.CD81 cell spread, demonstrating that anti-CD81 induced cell spread is dependent on antibody bivalency (Fig.3.2C). To ascertain whether anti-CD81 induced changes in hepatoma shape were dependent on actin polymerization the cells were treated with Latrunculin B and Cytocholasin D, and assayed for their response to anti-CD81 and anti-Beta-1 integrin ligation. Latrunculin B and Cytocholasin D treated HepG2.CD81 cells showed no detectable spread in response to anti-CD81 and anti-Beta-1, demonstrating an actin polymerization dependent process (Fig.3.2D). Similar results were observed with Huh-7.5 cells and both hepatoma cell lines demonstrated a dose-dependent cell spread in response to anti-CD81 mAb 1.s262 (Fig.3.2E). Huh-7.5 and HepG2.CD81 cells showed different spread kinetics with maximum spread noted after 1hr and 6hrs, respectively (data not shown).

Figure 3.2: CD81 engagement promotes actin-polymerization dependent hepatoma cell spread.

(**A**) Cartoon of antibody induced cell spread assay. mAbs are immobilized onto ELISA plates (5μg/ml, 4°C overnight incubation). Wells blocked with PBS/1%BSA for 30min at room temperature. Cells were seeded at $3x10^4$ cells/well and incubated at 37°C for a defined period of time and imaged by phase microscopy. (**B**) Phase images of HepG2 and HepG2.CD81 spread on anti-CD81 1.s262, anti-Beta-1 integrin and irrelevant IgG control coated plates at 6hrs post seeding. (**C**) Phase images of HepG2.CD81 spread on anti-CD81 2.s66 IgG and 2.s66 Fab and irrelevant IgG control coated plates at 6hrs post seeding (**D**) Phase images of HepG2.CD81 spread on anti-CD81 1.s262 and anti-Beta-1 integrin coated plates after pre-treating cells with Cytocholasin D, Latrunculin B or DMSO control. (**E**) Dose dependent spread of HepG2.CD81 (6hrs post seeding) and Huh-7.5 cells (1hr post seeding) in response to CD81 ligation with mAb1.s262 at 0.1, 0.3, 1, 3 and 10μg/ml. Results are representative of three independent experiments.


A
Coat mAbs on solid support
Block 1% BSA for 30 min
Seed cells
Incubate at 37°C
Image at defined time intervals
Determine percentage cell spread
B
HepG2 parental
HepG2.CD81
IgG
Anti-CD81
Anti-Beta-1
C
IgG
Anti-CD81 Fab
Anti-CD81 IgG
HepG2.CD81
D
DMSO
Cytocholasin D
Latrunculin B
Ant-CD81
Ant-Beta-1
HepG2.CD81
E
Cell spread (%)
HepG2.CD81
Huh-7.5
log10 (Ab µg/ml)

Our laboratory developed a panel of anti-CD81 mAbs that have been mapped to a number of EC2 epitopes. The antibodies were screened for their reactivity with mutant CD81 EC2 proteins described by Drummer et al., 2002 & 2005 (Screen carried out by Ke Hu), enabling the mAbs to be classified into 6 epitope groups (Table 3.1). To evaluate the epitope specificity of antibody induced cell spread a selection of antibodies were screened for their ability induce HepG2.CD81 cell spread (Fig.3.3). All antibodies induced between 30% and 55% of HepG2.CD81 cells to spread. The high frequency of cell spread induced by anti-CD81 mAbs from different epitope groups suggest that specific EC2 epitope binding is not required to induce cell spread.

Table 3.1: CD81 antibody epitope grouping.

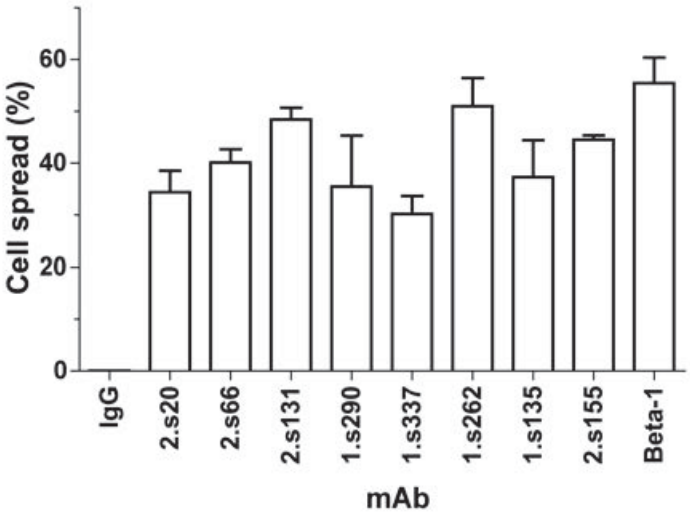
		Dimer									Monomer					
	HCV E2	**WT**	**V123A**	**T149A**	**L162P**	**T167I**	**P176S**	**I182F**	**N184Y**	**F186S**	**K124T**	**V146E**	**F150S**	**C157S**	**T166I**	**C190R**
	Antibody	-	A helix	B helix	3-10 helix	C helix	CD loop	D helix	D helix	D helix	A helix	B helix	B helix	BC loop	C helix	E helix
I	2.s20													↓↓↓	↓↓↓	↓↓↓
	2.s131													↓↓↓	↓↓↓	↓↓↓
	2.s63													↓↓↓	↓↓↓	↓↓↓
	2.s66													↓↓↓	↓↓↓	↓↓↓
	2.s48										↓			↓↓↓	↓↓↓	↓↓↓
	2.s139										↓			↓↓↓	↓↓↓	↓↓↓
II	1.s201				↓↓↓									↓↓↓	↓↓↓	↓↓↓
	1.s290				↓↓↓								↓	↓↓↓	↓↓↓	↓↓↓
III	1.s337				↓↓↓	↓↓↓							↓	↓↓↓	↓↓↓	↓↓↓
IV	1.s262		↓↓	↓↓	↓↓↓	↓↓↓						↓↓	↓↓↓	↓↓↓	↓↓↓	↓↓↓
V	1.s135		↓		↓↓↓								↓↓↓	↓↓↓	↓↓↓	↓↓↓
	1.s73		↓↓	↓↓		↓						↓↓	↓↓↓	↓↓↓	↓↓↓	↓↓↓
VI	1.s141		↓		↓								↓↓	↓↓↓	↓↓↓	↓↓↓
	2.s155		↓		↓								↓↓	↓↓↓	↓↓↓	↓↓↓
	2.s169												↓↓	↓↓↓	↓↓↓	↓↓↓

Table 3.1: CD81 antibody epitope grouping

Arrows depict reduction in binding of antibody to mutant CD81 EC2 protein compared to WT EC2 protein
↓ - 25 – 75% reduction in binding, ↓↓ - 75 – 90% reduction, ↓↓↓ >90% reduction in binding

Figure 3.3: Diverse panel of anti-CD81 mAbs induce HepG2.CD81 cell spread.

A panel of anti-CD81 mAbs plus anti-Beta-1 integrin and irrelevant control IgG were immobilized onto ELISA plates (5μg/ml, 4°C overnight incubation). Wells were then blocked with PBS/1%BSA for 30 min at room temperature prior to seeding HepG2.CD81 ($3x10^4$ cells/well) cells. Cells were incubated at 37°C for 6hrs and imaged by phase microscopy. The frequencies of spreading cells were enumerated and the results are representative of three separate experiments.

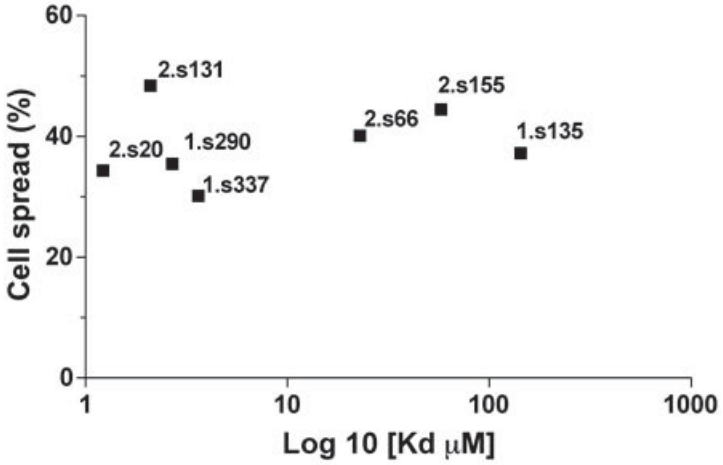

Cell spread (%)
0
20
40
60
IgG
2.s20
2.s66
2.s131
1.s290
1.s337
1.s262
1.s135
2.s155
Beta-1
mAb

To explore whether antibody affinity for CD81 associates with cell spread the percentage of cell spread was compared to binding affinity (Kd) (Fig.3.4A). Kd was determined for a selected number of mAbs using a technique called surface plasmon resonance. Briefly soluble CD81 EC2 was immobilized onto the surface of a biosensor chip and anti-CD81 binding was determined by flowing over increasing concentrations of antibody (Christopher Davis performed the surface plasmon resonance assays and determined the Kd value for each antibody). No association was observed between the Kd and the frequency of cell spread. Since the Kd values were determined using a recombinant protein that may not be representative of cellular expressed forms of CD81 we also compared cell spread to cell surface bound antibody. Cell surface bound antibody was determined by flow cytometry analysis at antibody saturating concentrations and expressed as median fluorescence intensity (MFI) (Fig.3.4B) (flow cytometry analysis performed by Ke Hu). Again no association was observed suggesting that antibody binding affinity is not indicative of anti-CD81 induced cell spread.

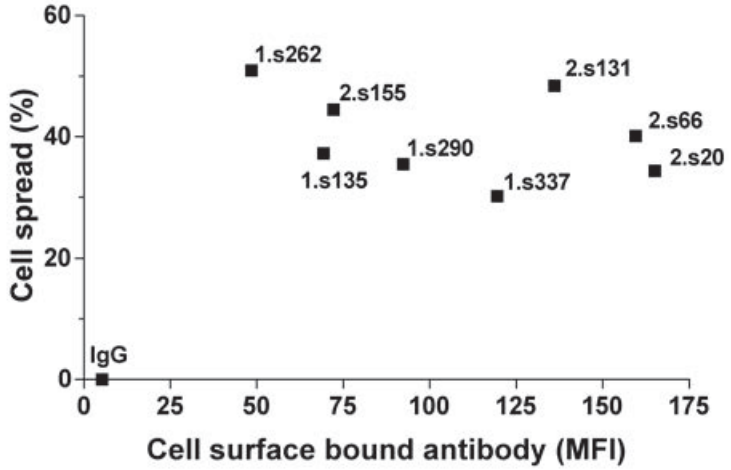
Figure 3.4: Antibody binding affinity is not indicative of anti-CD81 induced hepatoma cell spread.

Cell spread displayed in Fig.3.3 plotted against (**A**) antibody affinity (Kd) or (**B**) cell surface bound antibody expressed as median fluorescent intensity (MFI). No discernable association was observed (Spearman, n.s.).

A


60
40
20
0
Cell spread (%)
2.s131
2.s155
2.s66
1.s135
2.s20
1.s290
1.s337
1
10
100
1000
Log 10 [Kd μM]

B

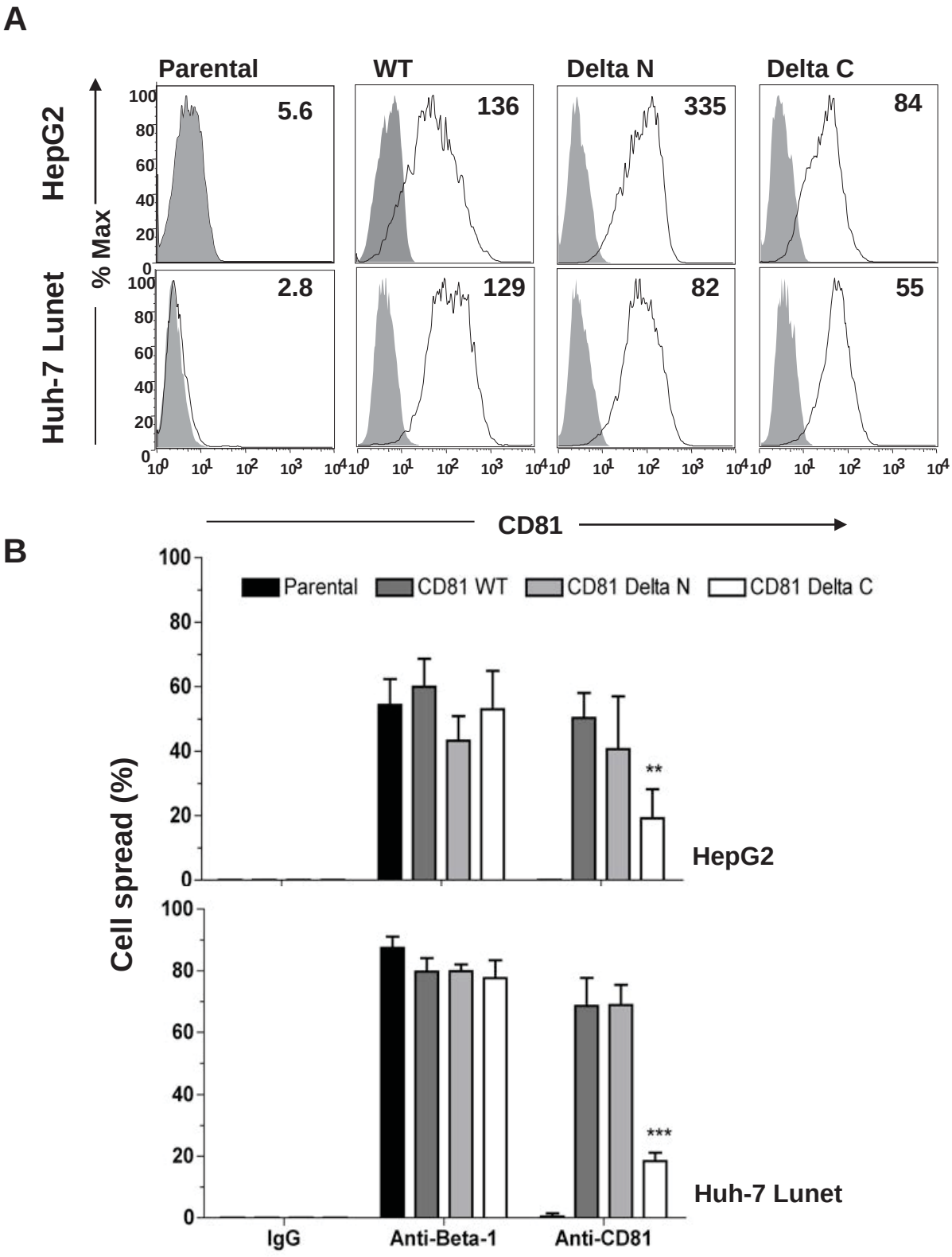

60
40
20
0
Cell spread (%)
1.s262
2.s131
2.s155
2.s66
1.s290
2.s20
1.s135
1.s337
IgG
0
25
50
75
100
125
150
175
Cell surface bound antibody (MFI)

3.2 The CD81 C terminus links to the actin cytoskeleton through association with actin-associated proteins Ezrin Radoxin Moesin.

Recent reports have identified the C terminus of CD81 as an essential regulator of CD81 induced effects (95, 411). To investigate whether anti-CD81 induced hepatoma cell spread is dependent on either the N or C termini, two independent cell lines that express low or negligible endogenous CD81 (HepG2 cells and Huh-7 Lunet cells) were transduced to express wild type (WT) or mutant CD81 proteins lacking the N-terminal ($CD81_{\Delta N}$) or C-terminal ($CD81_{\Delta C}$) regions. Cell surface CD81 expression was determined by flow cytometry and the Median fluorescence intensity (MFI) recorded (Fig.3.5A). HepG2 and Huh-7 Lunet cells expressing $CD81_{\Delta C}$ demonstrated a significantly reduced level of anti-CD81 induced cell spread compared to cells expressing wild type CD81, whilst no difference was observed in anti-Beta-1 induced cell spread. In contrast deletion of the N terminus had no detectable effect on anti-CD81 or ant-Beta-1 induced cell spread compared to wild type control in either cell line (Fig.3.5B).

Figure 3.5: A role for CD81 C terminus in actin polymerization dependent hepatoma cell spread.

HepG2 and Huh-7 Lunet cells were transduced to express wild type CD81 (WT), $CD81_{\Delta N}$ and $CD81_{\Delta C}$ proteins. (**A**) Cell surface expression of CD81 was analyzed by flow cytometry (anti-CD81 2.s131mAb used). CD81 expression (Black line) IgG control (Solid grey). Median fluorescent intensity (MFI) was determined and is stated in the top right corner of each graph. (**B**) Parental and transduced cells were seeded onto anti-CD81 1.s262, anti-Beta-1 integrin, and irrelevant control IgG coated wells (All coated at 5μg/ml). Phase images were taken and the frequency of spreading cells determined. Results are representative of three independent experiments. Error bars indicate standard deviation from the mean. A significant difference between percentage cell spread determined for $CD81_{\Delta C}$ compared to WT expressing cells in response to anti-CD81 mAb ligation was observed in both a HepG2 and Huh-7 Lunet cell background ($p= 0.0020$ and <0.0001 respectively, Two tailed unpaired T-test).


A
Parental
WT
Delta N
Delta C
HepG2
Huh-7 Lunet
% Max
5.6
136
335
84
2.8
129
82
55
CD81
B
Parental
CD81 WT
CD81 Delta N
CD81 Delta C
Cell spread (%)
HepG2
Huh-7 Lunet
**
***
IgG
Anti-Beta-1
Anti-CD81

Ezrin Radoxin Moesin (ERM) proteins bind actin and recent studies have highlighted a role for CD81-ERM association in a number of different cell types including Retinal pigment epithelium (RPE), Human umbilical vein endothelial cells (HUVEC), HeLa cells and lymphocytes (74, 365). Intramolecular associations between the N and C termini of ERM proteins hold the proteins in an inactive (closed) conformation. Phosphorylation of threonine residues in the actin binding domain(s) (ezrin Thr567, radixin Thr564, moesin Thr558) promote an active (open) conformation that allows the C terminus to interact with actin and the N terminal (FERM) domain to bind membrane proteins (140, 308, 448). Recent reports have demonstrated an essential role for the CD81 C terminus to associate with ERM in a number of cell lines (95, 411).

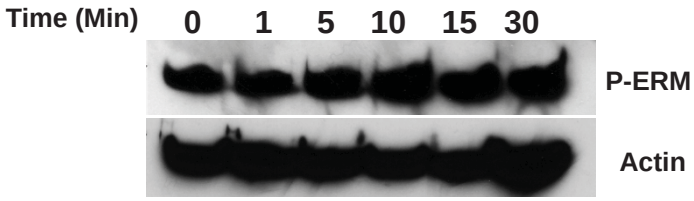
Given our earlier observation showing a role for the CD81 C terminus in hepatoma cell spread we were interested to investigate the role of ERM proteins in CD81 dependent hepatoma spread. To determine whether CD81 engagement promotes ERM phosphorylation, Huh-7.5 hepatoma cells were incubated with anti-CD81 mAb 1.s262 for defined periods of time before lysis and western blot analysis with antibodies specific for phosphorylated ERM (P-ERM) and actin. P-ERM was detected in the untreated cell lysates and showed no detectable increase following antibody treatment (Fig.3.6A). Additional experiments confirmed P-ERM expression in Huh-7.5 cells that co-localized with CD81 at the cell surface (Pearson's co-localization coefficient, Rr = 0.662 +/- 0.047) (Fig.3.6B). Finally, to determine whether ERM proteins are functionally relevant in anti-CD81 induced cell spread Huh-7.5 cells were transduced to express a GFP tagged dominant negative Moesin, N-Moesin-GFP, and control, Moesin-GFP (13) (Fig.3.6C). N-moesin-GFP was previously reported to alter

cell morphology by interfering with ERM dependent processes (13). Huh-7.5 cells expressing the dominant negative N-Moesin-GFP showed significantly reduced anti-CD81 induced spread compared to control Moesin-GFP expressing cells, demonstrating a role for ERM proteins in anti-CD81 induced cell spread (Fig.3.6D). Deletion of CD81 C terminus or use of a dominant negative ERM protein significantly reduced but did not eradicate anti-CD81 induced cell spread, suggesting that CD81 may be linked to the actin cytoskeleton through a number of indirect interactions consistent with CD81 being part of a TEM.

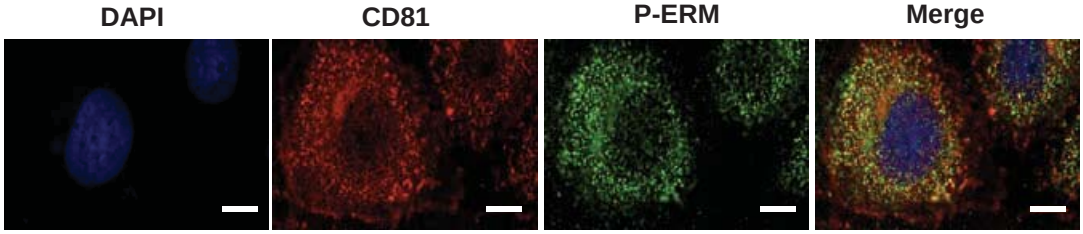
Figure 3.6: Actin associated proteins Ezrin Radoxin Moesin (ERM) facilitate ant-CD81 induced hepatoma spread.

(**A**) Huh-7.5 cells were exposed to anti-CD81 mAb 1.s262 for defined time periods prior to cell lysis and western blot analysis. Blots were probed for P-ERM and β-actin (loading control). (**B**) Huh-7.5 cells were cultured on glass cover slips for 24hrs prior to formaldehyde fixation and Triton-X 100 permeabilisation. CD81 was visualized using mAb 2.s131 and alexa-fluor 594 secondary antibody (Red), P-ERM visualized using anti-P-ERM (Cell signaling) and alexa-fluor 488 secondary antibody (Green). Cell nuclei were counter stained with DAPI (Blue) and the cells imaged with a Zeiss META head confocal microscope with a 60x water immersion objective. White scale bars represents 10μm. Pearsons co-localization coefficient Rr = 0.66 (+/-0.04) (ImageJ co-localization software). Mean and standard deviation of Rr determined from 10 images. (**C**) Cartoon of the structure of Moesin, Moesin-GFP, and N-Moesin-GFP proteins based on work by Amieva et al., 1999. (**D**) Huh-7.5 cells were transfected to express Moesin-GFP and dominant negative N-Moesin-GFP (13) 24hrs prior to seeding onto anti-CD81 1.s262, anti-Beta-1 integrin, and irrelevant control IgG coated wells (All coated at 5μg/ml). Frequency of GFP+ve cell spread was determined. Results expressed as percentage cell spread and are representative of three independent experiments. Error bars indicate standard deviation from the mean. A significant difference between percentage cell spread determined for Moesin-GFP compared to N-Moesin-GFP positive cells in response to anti-CD81 mAb ligation was observed ($p<0.0001$, Two tailed unpaired T-test).

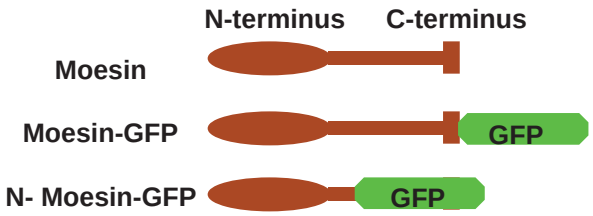
A


Time (Min)
0
1
5
10
15
30
P-ERM
Actin

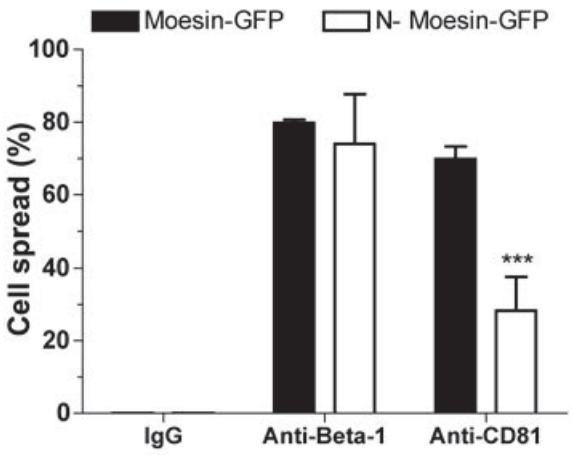
B


DAPI
CD81
P-ERM
Merge

C


N-terminus
C-terminus
Moesin
Moesin-GFP
GFP
N- Moesin-GFP
GFP

D

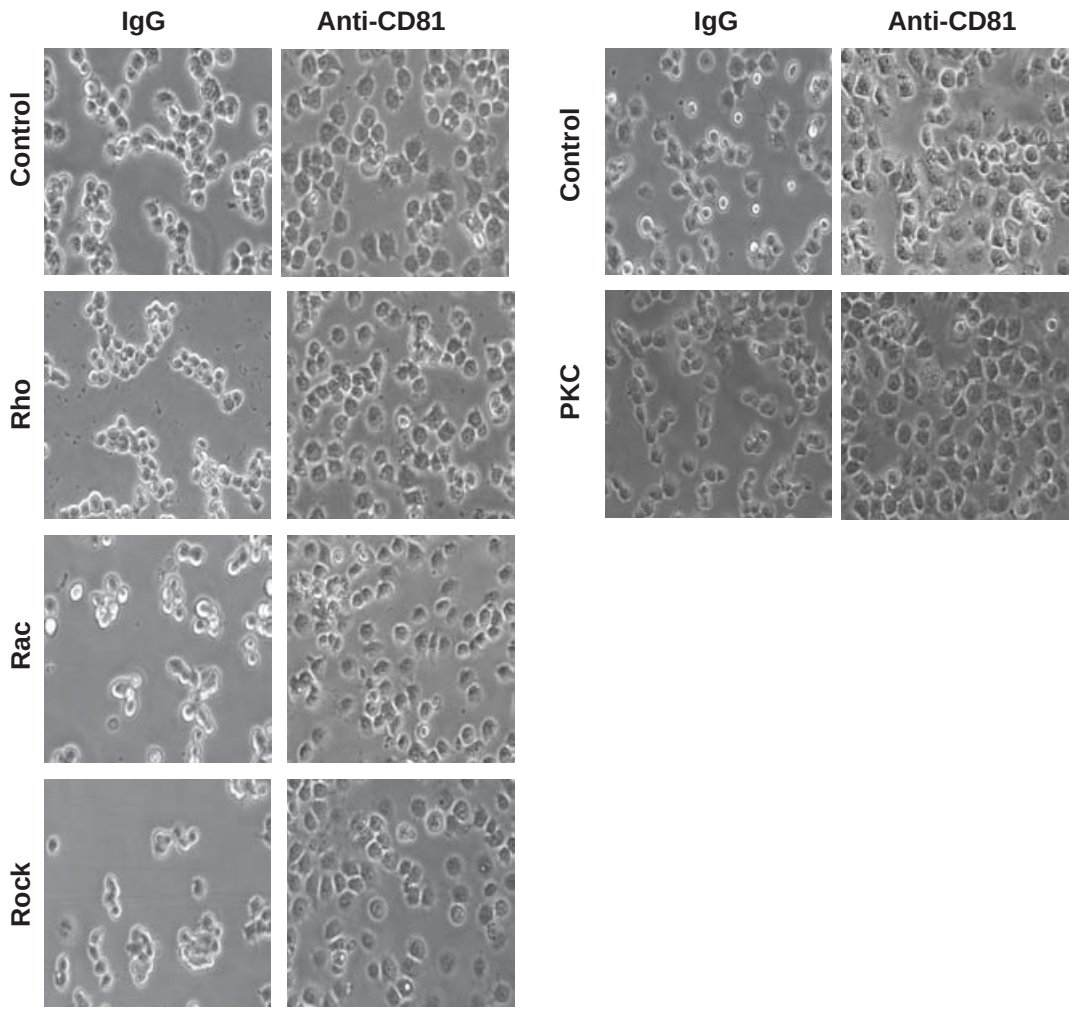

Moesin-GFP
N- Moesin-GFP
Cell spread (%)
100
80
60
40
20
0
***
IgG
Anti-Beta-1
Anti-CD81

3.3 Investigation into the role of different signaling pathways involved in anti-CD81 induced cell spread.

Signaling proteins including Rho-family GTPases, and several Ser/Thr kinases including ROCK (Rho associated kinase) have been shown to be stimulated following CD81 engagement (54) or to associate with CD81 (PKC) (556). Furthermore, all of these pathways have been implicated with either the activation of ERM proteins (308, 448, 547) or the regulation of the actin cytoskeleton in different cell types (88, 253, 272, 277, 366). To determine whether any of these signaling proteins play a role in anti-CD81 induced hepatoma cell spread, Huh-7.5 cells were pretreated with specific inhibitors against Rho (C3 transferase, 5μg/ml), Rac (Rac 1 inhibitor, 100μM), ROCK (Y276232, 2.5μM) and PKC (Bisindymaleimide, 100nM) prior to seeding the cells onto anti-CD81 coated wells. All inhibitors were present throughout the entire assay (Fig.3.7). All inhibitors had minimal effects on anti-CD81 induced cell spread.

Figure 3.7: Effect of inhibitors to Rho family GTPases (Rho and Rac) and Ser/Thr kinases (ROCK and PKC) on anti-CD81 induced hepatoma cell spread.

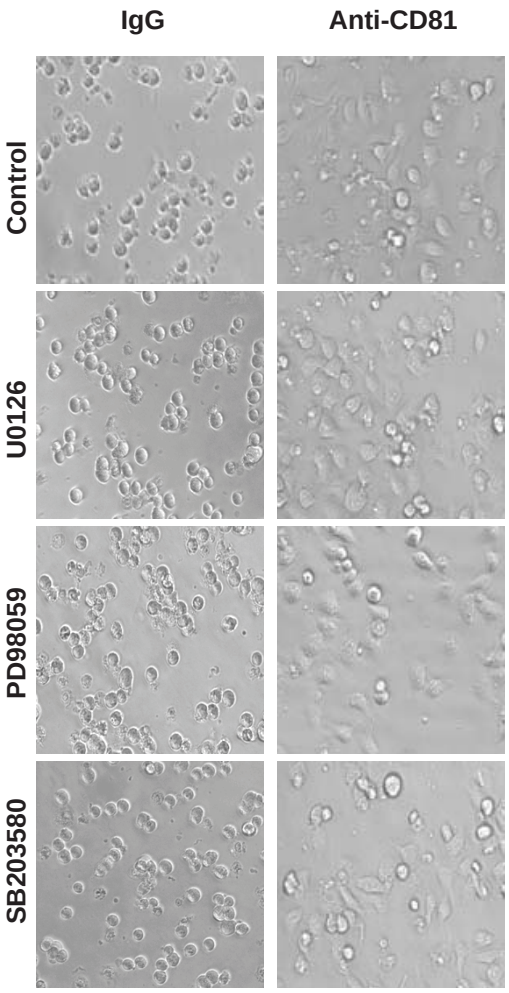
Huh-7.5 cells were treated with inhibitors to Rho (C3 transferase, 5μg/ml), Rac (Rac 1 inhibitor, 100μM), ROCK (Y276232, 2.5μM) and PKC (Bisindymaleimide, 100nM) as well as untreated or DMSO control. Concentrations used had no cytotoxic effects determined using an MTS assay (data not shown). Treated cells were seeded onto anti-CD81 1.s262 and irrelevant IgG control coated wells in the continued presence of the inhibitors for 1hr. Cells were imaged by phase microscopy. Images representative of three independent experiments.


IgG
Anti-CD81
IgG
Anti-CD81
Control
Control
Rho
PKC
Rac
Rock

Ab engagement of CD81 has been reported to activate mitogen activated protein kinase (MAPK) in a number of cell types resulting in different cellular responses, including the activation of T cells (104) and increased proliferation of hepatoma cell lines (54, 66, 498). MAPK signaling has been associated with the modulation of cell spread, including the regulation of macrophage, fibroblast and endothelial cell spread (115, 451, 491). AP-1 (Activator protein-1) is a transcription factor regulated by MAPK (489, 514). Our laboratory has previously used an AP-1-luciferase reporter assay to monitor MAPK signaling in Huh-7.5 cells. Huh-7.5 cells have a high basal MAPK activity that is reduced following treatment with MAPK inhibitors U0126, PD98059, and SB203580 (All AP-1 reporter work was carried out by Michelle Farqhuar). To ascertain whether MAPK signaling is involved in anti-CD81 induced hepatoma spread, Huh-7.5 cells were treated with the inhibitors and seeded onto anti-CD81 and monitored for spread after 1hr. None of the inhibitors modulated anti-CD81 induced cell spread implying that this process is MAPK independent (Fig.3.8).

Figure 3-8: MAP Kinase independent cell spread

Huh-7.5 cells were treated with MAPK inhibitors (SB203580, PD98059, and U0126) at 100μM and DMSO control. Treated cells were then seeded onto anti-CD81 1.s262 and irrelevant IgG control coated wells in the continued presence of the inhibitors for 1hr. Concentrations used had no cytotoxic effects determined using an MTS assay (data not shown). Cells were imaged by phase microscopy and representative images presented.

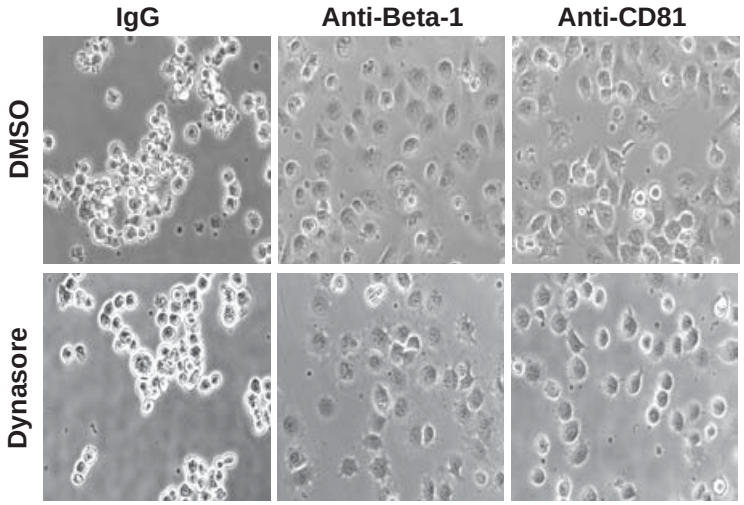

IgG
Anti-CD81
Control
U0126
PD98059
SB203580

Dynamin is a large GTPase and primary research focused predominantly on its role in clathrin mediated endocytosis. Interestingly dynamin has been found to localize with actin filaments and recent evidence suggests that dynamin coordinates actin cytoskeleton dynamics associated with cell spread (24, 176, 318, 420, 422, 524). To investigate a role for dynamin in anti-CD81 induced hepatoma cell spread, cells were incubated with the dynamin specific inhibitor dynasore prior to and during the cell spread assay (298). Dynasore significantly reduced anti-CD81 induced hepatoma spread and yet had no detectable effect on anti-Beta-1 induced cell spread (Fig.3.9) demonstrating a role for dynamin in anti-CD81 induced hepatoma cell spread.

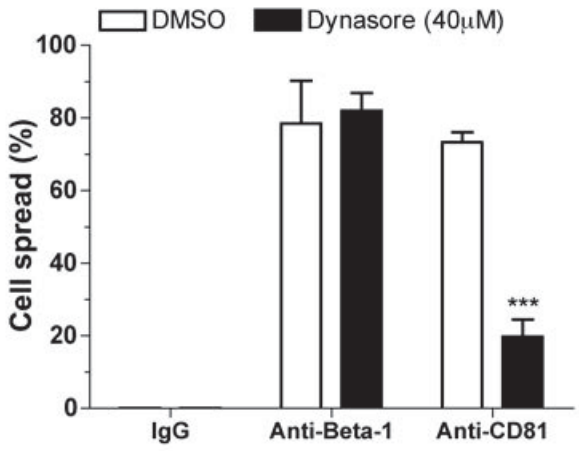
Figure 3.9: Anti-CD81 induced hepatoma cell spread is dependent on large GTPase Dynamin.

Huh-7.5 cells were pretreated with dynamin inhibitor dynasore (40μM) and DMSO control. Pretreated cells were then seeded onto anti-CD81 1.s262, anti-Beta-1 integrin and irrelevant IgG control coated wells for 1hr in the continued presence of inhibitor. (**A**) Cells were imaged by phase microscopy. (**B**) The frequency of spreading cells was determined. Error bars indicate standard deviation from the mean. A significant difference between percentage cell spread for control compared to dynasore treated cells in response to anti-CD81 mAb ligation was observed, ($p < 0.0001$, Two tailed unpaired T-test used). Results are representative of three separate experiments.

A


IgG
Anti-Beta-1
Anti-CD81
DMSO
Dynasore

B


DMSO
Dynasore (40μM)
Cell spread (%)
100
80
60
40
20
0
***
IgG
Anti-Beta-1
Anti-CD81

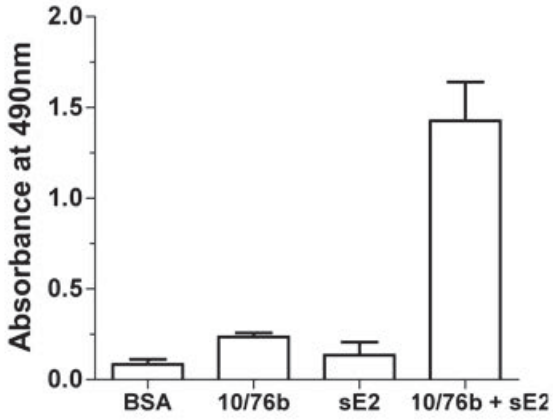
3.4 HCV E2 glycoprotein does not induce hepatoma cell spread

HCV E2 glycoproteins have been reported to bind to CD81 (384) and we were interested to know whether sE2 would induce hepatoma spread. Given our earlier observation that bivalent anti-CD81 was required to promote cell spread, we captured sE2 (JFH-1) at 1 in 3 dilution onto 96 well plates via an antibody specific for an epitope tag expressed at the C-terminus of E2. sE2 was bound by the specific antibody and yet failed to promote hepatoma spread (Fig.3.10). Previous research has reported modulation of intracellular signaling upon sE2-CD81 binding and HCV infection (54, 66) making it important to determine the effects of HCV infection on anti-CD81 induced cell spread.

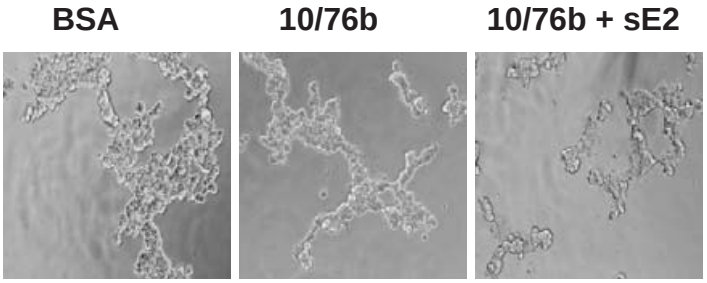
Figure 3.10 HCV E2 glycoprotein engagement of CD81 did not induce hepatoma cell spread.

(**A**) sE2 was immobilized onto ELISA plates by 10/76b capture; bound glycoprotein was detected with 4μg/ml cocktail of human anti-E2 mAb (CBH2/CBH4G). Absorbance displayed for BSA only, 10/7b (capture) only, sE2 only and 10/76b plus sE2 coated wells. sE2 used at 1 in 3 dilution. (**B**) Huh-7.5 cells were seeded onto captured sE2-coated plates (1 in 3 dilution) or control BSA or 10/76b (capture) only coated wells for 1hr. Cells were imaged by phase microscopy. Images are representative of two separate experiments.

A


Absorbance at 490nm
2.0
1.5
1.0
0.5
0.0
BSA
10/76b
sE2
10/76b + sE2

B


BSA
10/76b
10/76b + sE2

3.5 HCV infection perturbs anti-CD81 induced cell spread.

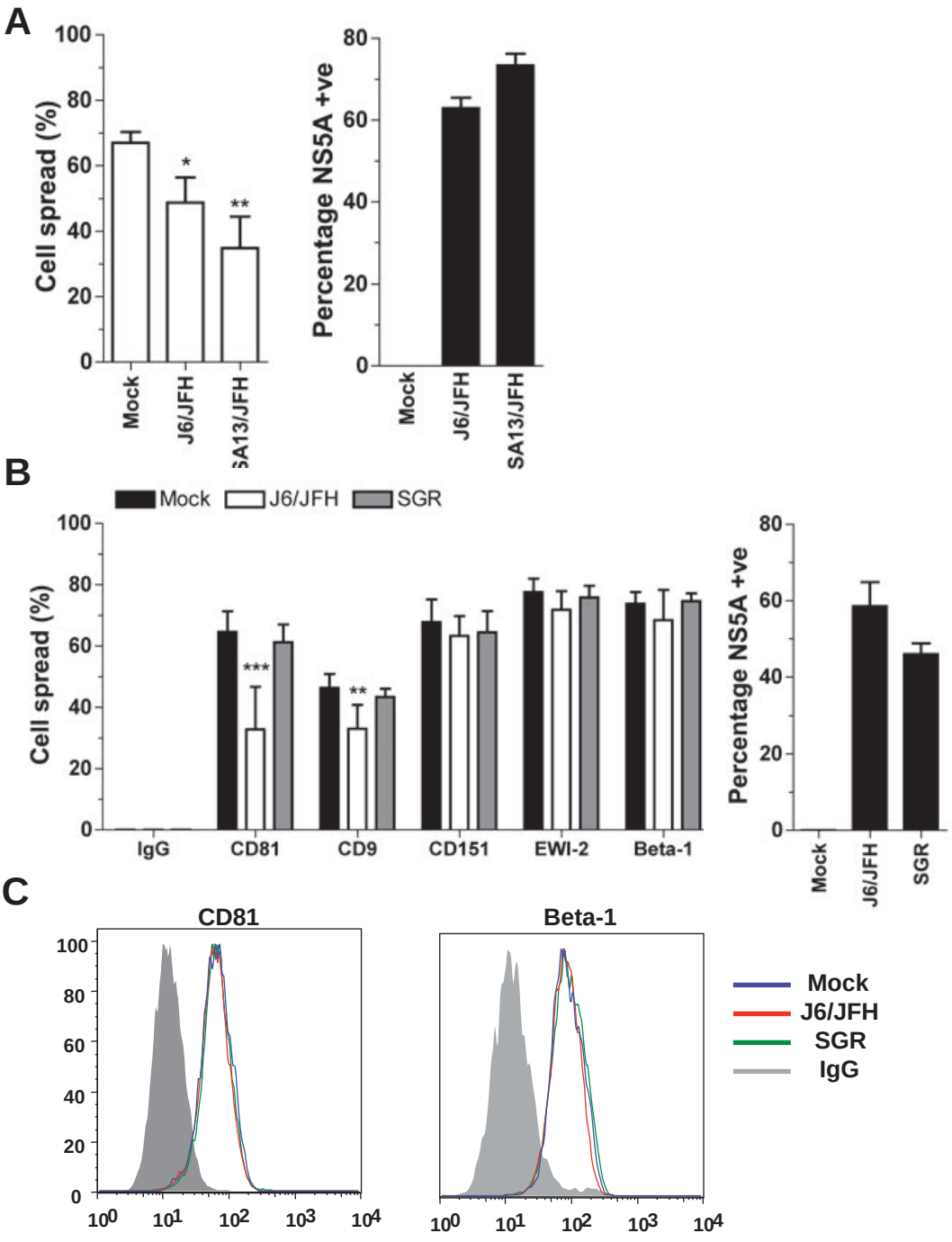
Huh-7.5 cells were electroporated with genomic HCV RNA of different JFH-1 chimeric strains (J6/JFH genotype 1b and SA13/JFH genotype 5a) and 96hr post electroporation mock and HCV infected cells were assessed for their ability to spread in response to anti-CD81 ligation. The frequency of cells expressing NS5A was determined by flow cytometry. HCV infected cells showed a reduced spread in response to anti-CD81 stimulation (Fig.3.11A). It is important to note that the frequency of infected cells varied between electroporations and this in turn reflected the defect in anti-CD81 induced hepatoma cell spread observed, despite this HCV infection repeatedly perturbed anti-CD81 induced cell spread.

To determine whether this phenomenon was common for other anti-tetraspanin and associated proteins, Huh-7.5 cells supporting full-length HCVcc (J6/JFH) or control mock infected Huh-7.5 cells were seeded onto plates coated with antibodies specific for tetraspanins and tetraspanin associated proteins. Furthermore to ascertain whether this perturbation was due to HCV replication the assay was performed with Huh-7.5 cells supporting JFH-1 sub genomic replicon (SGR). J6/JFH infected cells showed reduced spread compared to mock cells in response to anti-CD81 and anti-CD9 induced spread. In contrast J6/JFH infected cells spread in a comparable manner to mock cells in response to other anti-tetraspanin and associated protein specific antibodies. Hepatoma cells supporting SGR RNA spread in a comparable manner to mock infected cells in response to all antibodies tested (Fig.3.11B), suggesting that virus particle genesis or the viral structural proteins perturb anti-CD81 induced changes in hepatoma morphology.

Many viruses down regulate their receptors to prevent super infection (55, 348, 511). To investigate whether virus infection modulated CD81 expression, uninfected and infected cells were stained for CD81 and Beta-1 integrin expression (Fig.11C). No differences were noted in cell surface CD81 or Beta-1 integrin expression, demonstrating that reduced cell surface CD81 expression does not account for the observed changes in anti-CD81 induced cell spread.

Figure 3.11: HCV infection reduces anti-CD81 induced hepatoma spread.

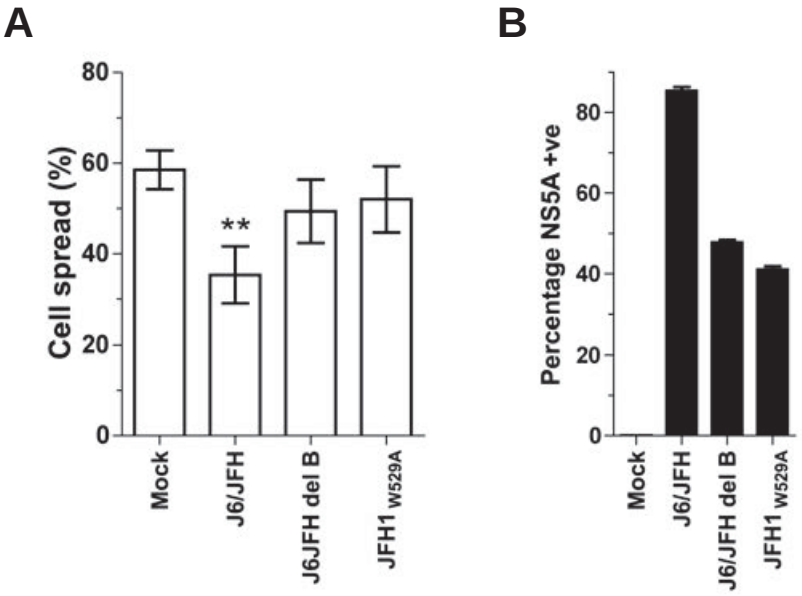
(**A**) HCV strain J6/JFH and SA13/JFH RNA were delivered into Huh-7.5 cells by electroporation (ep) and 96hr later seeded onto anti-CD81mAb 1.s262 coated wells for 1hr. Cells were imaged by phase microscopy and the frequency of spreading cells determined. A significant difference between percentage cell spread determined for J6/JFH and SA13/JFH infected cells compared to mock cells in response to anti-CD81 mAb ligation was observed, ($p = 0.0195$ and 0.0053 respectively, Two tailed unpaired T-test). HCV NS5A expression was determined for each cell population by flow cytometry. (**B**) J6/JFH full length and JFH-1 sub-genomic (SGR) RNA were delivered into Huh-7.5 cells by ep and 96hrs later seeded onto anti-CD81 1.s262, anti-CD9, anti-CD51, anti-EWI-2, anti-Beta-1 integrin and irrelevant IgG control coated plates. Phase images were taken at 1hr post seeding and the frequency of spread cells determined. HCV NS5A protein expression was determined for each cell population by flow cytometry. A significant difference between percentage cell spread determined for J6/JFH compared to mock cells in response to anti-CD81 and anti-CD9 mAb ligation was observed, ($p=0.0005$ and 0.0046 respectively, Two tailed unpaired T-test). (**C**) Cell surface CD81 and Beta-1 integrin expression were determined for J6/JFH, SGR and mock infected Huh-7.5 cells using flow cytometry. Error bars indicate standard deviation from the mean. Results are representative of three indepent experiments.


A
Cell spread (%)
Mock
J6/JFH
SA13/JFH
Percentage NS5A +ve
B
Mock
J6/JFH
SGR
IgG
CD81
CD9
CD151
EWI-2
Beta-1
C
CD81
Beta-1
Mock
J6/JFH
SGR
IgG

To investigate whether HCV particles or virus binding to CD81 are required to perturb anti-CD81 induced cell spread two mutant viruses were employed: J6/JFH del B contained a mutation in domain III of NS5A that prevents a phosphorylation event that is critical for particle assembly whilst permitting genome replication and protein translation (473); $JFH1_{W529A}$ contains a mutation that prevents E2-CD81 binding (515). Both viruses had no significant effect on anti-CD81 induced cell spread suggesting that HCV particle genesis and/or CD81 engagement is essential to modulate anti-CD81 induced cell spread (Fig.3.12A). The frequencies of NS5A positive cells were determined by flow cytometry (Fig.3.12B). For the mutant viruses, J6/JFH del B and $JFH1_{W529A}$, the frequency of NS5A positive cells were low, 42 and 48 % respectively, compared to full length J6/JFH, 85%, suggesting a possible alternative explanation for theminimal effect(s) on anti-CD81 induced hepatoma spread.

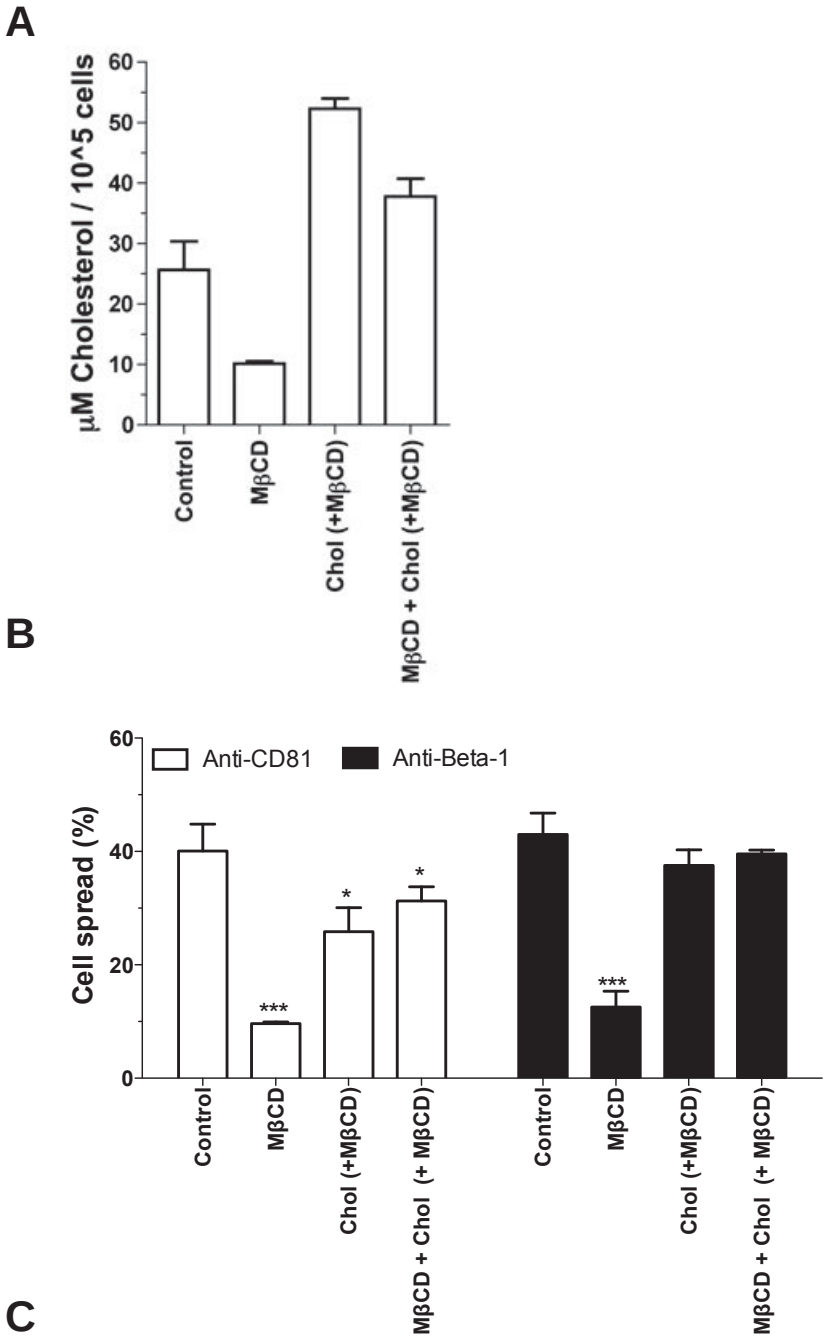
Figure 3.12: Anti-CD81 induced spread of J6/JFH del B virus and JFH-1 CD81 mutant virus expressing hepatoma cells.

J6/JFH, J6/JFH del B (473) and $JFH1_{W529A}$ mutant (515) RNA were delivered into Huh-7.5 cells by electroporation (ep) and 96hrs later seeded onto anti-CD81mAb 1.s262 coated plates for 1hr. Cells were imaged by phase microscopy and the frequency of cell spread determined. HCV NS5A protein expression was determined for each cell population by flow cytometry. Error bars indicate standard deviation from the mean. Experiments were performed in triplicate and results are representative of three independent experiments. A significant difference between percentage cell spread determined for J6/JFH compared to mock infected cells in response to anti-CD81 mAb ligation was observed, ($p=0.0062$, Two tailed unpaired T-test). No significant difference between cell spread determined for J6/JFH del B and $JFH1_{W529A}$ expressing cells compared to mock infected cells in response to anti-CD81 mAb ligation was observed ($p=0.1266$ and 0.2546 respectively, Two tailed unpaired T-test).


A
B
Cell spread (%)
Percentage NS5A +ve
80
60
40
20
0
**
Mock
J6/JFH
J6JFH del B
JFH1 W529A
J6/JFH del B

Cholesterol metabolism is important for many aspects of the HCV lifecycle (235, 540) and has been reported to play a role in tetraspanin membrane organization (78, 402, 439, 521). To investigate whether changes in cholesterol modulate anti-CD81 induced spread Huh-7.5 hepatoma cells were treated with methyl-beta-cyclodextrin (MβCD) to selectively remove cholesterol or with MβCD-cholesterol complexes to promote cholesterol uptake in naïve cells or cells treated with MβCD (87). All treatments had their expected effect(s) on total cholesterol levels (Fig.3.13A). Reducing total cellular cholesterol decreased hepatoma cell spread in response to anti-CD81 or anti-Beta-1 integrin ligation (Fig.3.13B). In contrast, increasing total cholesterol perturbed anti-CD81 induced cell spread and had no significant effect on anti-Beta-1 integrin induced effects (Fig.3.13B). Importantly, we noted that HCV infection promotes total cellular cholesterol levels (Fig.3.13C), providing an explanation for the observed perturbation of anti-CD81 induced spread in HCV infected cells.

Figure 3.13: CD81 dependent cell spread is sensitive to changes in cholesterol.
Huh-7.5 cells were treated in suspension with MβCD (5mM), Cholesterol (+MβCD) or MβCD followed by cholesterol(+MβCD) or left untreated (Cholesterol used at 5mM). (**A**) Cells were counted and lysed for total cell cholesterol quantification (Amplex Red Cholesterol Assay Kit, Invitrogen) or (**B**) seeded onto anti-CD81 mAb 1.s262, anti-Beta-1 integrin or irrelevant IgG control coated wells for 1hr. Cells were imaged by phase microscopy and the frequency of spreading cells determined. A significant difference between percentage cell spread determined for MβCD treated compared to untreated cells in response to anti-CD81 and anti-Beta-1 ligation was observed ($p=0.0004$ and $P=0.004$ respectively, Two tailed unpaired T-test). A significant difference was observed between cholesterol(+MβCD) treatment of naïve or MβCD treated cells compared to untreated cells in response to anti-CD81 mAb ligation but not anti-Beta-1 integrin ligation, $p=0.0179$, 0.0467, 0.1968 and 0.1134 respectively. (**C**) J6/JFH RNA was delivered into Huh-7.5 cells by electroporation (ep) 96hrs later cells were counted and lysed for total cell cholesterol quantification. A significant difference between total cell cholesterol in J6/JFH infected cells compared to mock infected cells was observed, ($p<0.0001$, Two tailed unpaired T-test). Error bars indicate standard deviation from the mean. Experiments were performed in triplicate and results are representative of three separate experiments.


A
µM Cholesterol / 10^5 cells
60
50
40
30
20
10
0
Control
MβCD
Chol (+MβCD)
MβCD + Chol (+MβCD)
B
Anti-CD81
Anti-Beta-1
Cell spread (%)
60
40
20
0
***
*
*
***
Control
MβCD
Chol (+MβCD)
MβCD + Chol (+ MβCD)
Control
MβCD
Chol (+MβCD)
MβCD + Chol (+ MβCD)
C
µM Cholesterol / 10^5 cells
60
50
40
30
20
10
0
***
Mock
J6/JFH

3.6 Discussion

The function of tetraspanin CD81 in hepatoma cell biology is largely unknown. In this study we used a diverse panel of novel CD81 EC2 binding antibodies to investigate the effect(s) of ligation on hepatoma cell morphology. Antibodies can be useful tools for investigating tetraspanin function(s), they are reported to disrupt partner protein interactions or promote intracellular signaling through protein aggregation at the cell surface (177, 352, 427, 449). Importantly anti-CD81 mAbs may also mimic ligation of CD81 by HCV glycoproteins. Ligation of CD81 on immune cells by anti-CD81 mAb or HCV sE2 glycoprotein activates numerous signaling cascades and induces actin dependent morphological changes (95, 104, 253, 342, 486, 498). We observed hepatoma spread upon engagement of a diverse panel of immobilized anti-CD81 mAbs but not recombinant protein sE2. Importantly HCV infection was found to perturb this response. Our observations point to an indirect mechanism of inhibition through perturbation of intracellular events possibly the result of increased total cellular cholesterol levels. Tetraspanins are often associated with cancer progression and hence these observations may have important consequences in HCV associated liver disease.

To investigate the effect(s) of CD81 ligation on hepatoma cells, the cells were seeded onto anti-CD81 mAb immobilized onto ELISA plates and cultured for defined periods of time before imaging by phase microscopy (Fig.3.2A). Huh-7.5 and HepG2.CD81 cells were observed to spread in a dose dependent manner in response to IgG whilst they did not spread after binding corresponding Fab fragments, indicating this process is dependent on antibody bivalency and cross-

linking CD81 is most likely required (Fig.3.2C). Furthermore actin polymerization inhibitors Latrunculin B (Lat B) and Cytochalasin D (Cyt D) successfully blocked cell spread revealing the process to be dependent on actin polymerization (Fig.3.2.D). The actin polymerization inhibitors have different modes of action; Lat B binds actin monomers preventing polymerization (100, 336) whilst Cyt D binds F-actin high affinity binding sites inhibiting filament elongation (141).

CD81 and F-Actin were observed to co-localize at hepatoma cell contacts and further analysis using TIRF microscopy demonstrated that during anti-CD81 and anti-Beta-1 integrin induced hepatoma cell spread, actin and CD81 co-localized in specific regions on the lower surface of the cell (Fig.3.1). These observations demonstrate that CD81-F-actin co-localization is not dependent on CD81 engagement as previously observed on B cells (95). An explanation for this could be due to differences in cell type, resting lymphocytes used in the previous studies are cultured in suspension as opposed to hepatoma cells that adhere to cell culture plastic and grow in monolayers. They are also quiescent, held in the G_0 stage of the cellular lifecycle, differing again from hepatoma cells that are continually dividing (151).

The regions of high CD81 expression observed on the cell surface of Huh-7.5 cells by TIRF microscopy may represent tetraspanin-enriched microdomains (TEMS). Tetraspanins are reported to continually interchange between confined (TEM) and randomly diffused states (132), whether tetraspanins interact with partner proteins and function outside of TEMS is relatively unknown and currently under debate (530). Interestingly CD81 is reported to function independently of TEMS for HCV entry

(402). New live cell imaging techniques, for example single particle tracking are only now becoming available to properly address these questions (132, 530).

A high frequency of cell spread in response to CD81 ligation by antibodies targeting different epitopes of CD81 (Fig.3.3), suggesting that antibody-induced cell spread is independent of EC2 epitope. Cell spread did not correlate with antibody affinity (Kd) determined using recombinant CD81 EC2 (Fig.3.4A). Since recombinant EC2 may not be truly representative of *in vitro* CD81, cell spread was also compared to cell surface bound antibody (MFI), again no correlation was observed (Fig.3.4B). These results suggest that cell spread is independent of antibody binding affinity. To determine whether ligation of other cell surface proteins could induce hepatoma cell spread we compared cell spread to antibodies specific for different cell surface proteins (data not shown). All tetraspanin and integrin antibodies tested induced cell spread to varying degrees, whereas antibodies to HCV co-receptor claudin-1 induced cell spread to a much lower degree than anti-CD81. Antibodies targeting a further HCV co-receptor, SR-BI, did not induce cell spread. As well as differences in the frequency of cell spread observed, the morphology of the spreading cells varied depending on the cell surface protein engaged.

CD81 induced hepatoma cell spread was repeatedly perturbed by HCV infection. It is not uncommon for viruses to down regulate cell surface receptors to prevent super-infection (55, 348, 511), we therefore compared CD81 expression of naïve and infected Huh-7.5 cells (Fig.3.11C). No change in CD81 expression was observed eliminating this as a possible mechanism. Infection only perturbed anti-CD9 induced cell spread and had no effect on antibodies to other other tetraspanin (CD63, CD82)

or associated proteins (EWI-2, Beta-1 integrin) (Fig.3.11B). CD81 and CD9 have been reported to work in conjunction with one another, *in vitro* studies have shown a strong phenotype only upon silencing both CD81 and CD9 (72, 262, 466) and single tetraspanin knock out mice are reported to have a mild phenotype compared to double knock outs (466, 467). The observed inhibition of both CD81 and CD9 induced spread suggests these tetraspanins may be working together in this process. sE2 ligation of CD81 did not induce hepatoma cell spread (Fig.310), this differs from previous observations where antibody and sE2 engagement of CD81 have induced similar effects (54, 95, 104, 105, 253). Yoon et al., 2009 reported that HCV infection had no effect on NK cell function suggesting that earlier results with sE2 may be incorrect, demonstrating that recombinant E2 is not always representative of E2 on viral particles most likely due to configuration and concentration differences.

To ascertain whether HCV infection inhibits anti-CD81 induced spread directly through HCV envelope binding to CD81 or indirectly through HCV replication and/or protein expression, experiments were performed using Huh-7.5 cells expressing mutant viruses and a JFH-1 sub genomic replicon (Fig.3.11&12). Mutant virus J6/JFH del B has a mutation in domain III of NS5A that prevents assembly of viral particles (473) and $JFH1_{W529A}$ mutant virus was previously demonstrated to be deficient in CD81 binding (515). No reduction in anti-CD81 induced spread of cells expressing these mutant viruses or JFH-1 sub-genomic replicon was observed compared to controls. These results suggest firstly that HCV genome replication is not enough to drive this perturbation and secondly that engagement of CD81 by HCV is essential in this process. In all assays cells were cultured for 96hrs post delivery of viral RNA to ensure a high level of infection, unfortunately due to variations in

electroporation efficiency and the fact that sub-genomic RNA and mutant viruses are unable to transmit infection (See chapter 5) we were unable to achieve a comparable level of infection compared to the full length viruses. Less than 50% of J6/JFH del B, $JFH1_{W529A}$ and sub-genomic replicon expressing cells were NS5A positive compared to greater than 60% of the full-length virus infected cells. Therefore the lower NS5A expression levels may be an alternative explanation for these results, thus it is still un-resolved as to whether CD81 engagement is needed for this phenotype.

The C terminus of CD81 has previously been reported to be important for eliciting anti-CD81 induced affects on immune cells (95, 411). Hepatoma cells expressing a CD81 protein lacking its C terminus ($CD81_{\Delta C}$) had a significantly reduced spread compared to cells expressing CD81 WT and $CD81_{\Delta N}$ protein upon CD81 ligation, implying that the C terminus is also important for CD81 function in hepatoma cells (Fig.3.5). A PDZ domain located on the C terminus of CD81 is reported to bind actin-associated proteins Ezrin, Radoxin, Moesin (ERM) (411). Threonine phosphorylation of all ERM proteins (ezrin Thr567, radixin Thr564, and moesin Thr558) induces a conformational change leading to activation (140, 308, 448). We failed to observe any change in P-ERM levels following antibody ligation of CD81 on hepatoma cells (Fig.3.6A) as previously reported for NK and B cells respectively (95, 253). A high basal level of P-ERM was observed in our hepatoma cells that may have masked any small changes in P-ERM levels. Despite this CD81 co-localized with P-ERM at the surface of hepatoma cells, and cells expressing a dominant negative N-moesin protein had significantly reduced anti-CD81 induced cell spread (Fig.3.6). These observations suggest that ERM proteins do indeed play a role in anti-CD81 induced hepatoma cell spread and we hypothesize this is through interactions with the C

terminus of CD81. The $CD81_{\Delta C}$ and N-moesin-GFP expressing cells only exhibited partial inhibition of anti-CD81 induced cell spread suggesting that this is not the only means by which CD81 can link to the actin cytoskeleton consistent with CD81 functioning as part of a TEM in this process.

Of note HCV infection was not inhibited in cells expressing the dominant negative N-moesin-GFP protein compared to control (data not shown) suggesting firstly that HCV infection is independent of ERM proteins and secondly that the mechanism underlying anti-CD81 induced spread is independent of the mechanism involved in HCV entry. Coffey et al., 2009 observed a Syk dependent Tyrosine phosphorylation of Ezrin upon CD81 ligation in B cells. Syk is a non-receptor protein kinase and was originally thought to only be present in heamatopoetic cells but has since been identified in a number of non-haematopoietic cell lineages including endothelium, epithelium and importantly hepatocytes (487, 527). Interestingly HCV NS5A N-terminus interacts directly with Syk and NS5A expression has been reported to inhibit Syk kinase activity (220), this could be a possible explanation for HCV perturbation of anti-CD81 induced cell spread. Further work is necessary to determine whether Syk dependent tyrosine phosphorylation of Ezrin upon CD81 ligation occurs in hepatocytes.

CD81 ligation has been associated with regulating a number of signaling cascades. MAPK signaling for example was previously demonstrated to increase upon CD81 ligation on hepatoma cells (54, 66, 558). Work in our laboratory using an AP-1 reporter system demonstrated that MAPK inhibitors (U0126, PD98059, and SB203580) inhibit MAPK signaling in Huh-7.5 cells (Michelle Farhquar, unpublished

data). However none of these inhibitors were found to have any observable effect on hepatoma cell spread (Fig.3.8) suggesting CD81 induced hepatoma cell spread is MAPK independent. A further kinase Protein Kinase C (PKC) is also associated with CD81 (556), and inhibion of PKC again had no effect on anti-CD81 induced hepatoma cell spread. Rho GTPases are primarily involved in modulation of actin dynamics and have also been associated with CD81 function (54, 272, 366, 398). We investigated the effect(s) of a number of Rho GTPase family inhibitors; again none of the inhibitors had any observable effect(s) on cell spread (Fig.3.7). The inhibitors used all have limitations in specificity that may explain their inability to inhibit spread. (438, 494) (154, 438). Another Rho family GTPase member that is not tested in this study is cdc42 again this GTPase has been implicated in modulating cell spread (389). The involvement of Rho GTPases in cancer progression is fueling ongoing research to identify more specific inhibitors (295), these may be useful for the future work needed to dismiss the involvement of Rho GTPase family members in anti-CD81 induced hepatoma cell spread.

Dynasore, an inhibitor of the large GTPase dynamin, specifically perturbed anti-CD81 induced cell spread and not anti-Beta-1 integrin induced cell spread (Fig.3.9). These results suggest that dynamin is either directly or indirectly involved in CD81 function. Further experiments are necessary to determine whether dynamin specifically inhibits CD81 function or whether this perturbation is common to other tetraspanins. To date no literature exists on a link between tetraspanins and dynamin although dynamin is reported to associate with actin (176, 318, 419, 420). Dynamin has also been reported to regulate the localization of Rac a member of the Rho family GTPases (422). Although our data does not implicate Rac GTPase involvement in anti-CD81

induced hepatoma cell spread our analysis is not conclusive and reports have previously implicated Rho GTPases in anti-CD81 induced effects (54). It is therefore plausible that inhibition of dynamin may indirectly inhibit anti-CD81 induced cell spread through inhibition of Rac. Furthermore, a direct association between HCV co-receptor tight junction protein Occludin and dynamin has recently been reported (287) and research carried out by our laboratory has demonstrated that Occludin can be found associated with Claudin-1 and CD81 on the surface of hepatoma cells (188) linking CD81 indirectly with dynamin.

Cholesterol is important for correct tetraspanin localization and function (78, 402, 439, 521). In fact levels of membrane cholesterol can alter the function of numerous cell surface proteins effecting, cell signaling, adhesion and migration (390). Modulation of cholesterol levels have previously been shown to reduce CD81-Claudin-1 and CD81-CD81 association in hepatoma cells (188). Further to this a number of studies have demonstrated that cholesterol depletion inhibits HCV entry (188, 235, 402). To determine whether cholesterol levels modulate anti-CD81 induced hepatoma cell spread, Huh-7.5 cells were pre-treated with MβCD to deplete cholesterol or with MβCD-cholesterol complexes to increase cholesterol uptake. Anti-CD81 and anti-Beta-1 integrin induced hepatoma cell spread were reduced upon depletion of cellular cholesterol. Furthermore a significant decrease in anti-CD81 but not anti-Beta-1 integrin induced cell spread upon addition of cholesterol was observed (Fig.3.13). These findings suggest that cholesterol levels are important for CD81 function in hepatoma cells.

Numerous viruses rely on and modulate cholesterol metabolism for effective viral production and replication and HCV is no exception (111). Hepatocytes acquire cholesterol through two major processes, firstly through the mevalonate pathway that synthesizes cholesterol and other lipids from acetyl-CoA (163) and secondly by acquisition of cholesterol from low and high-density lipoproteins (LDL and HDL) in the plasma through LDL receptor and Scavenger receptor BI (SR-BI) respectively (254, 406). A number of studies have reported that inhibitors of steps in the mevalonate pathway can inhibit HCV replication demonstrating HCV reliance on lipid and cholesterol metabolism (86, 217, 236). HCV infection *in vivo* is often associated with reduced serum cholesterol levels (201, 344, 426). A number of mechanisms are thought to be responsible for this for example HCV infection induces oxidative stress through mitochondrial dysfunction resulting in increased levels of reactive oxygen species (ROS) that in turn reduce fatty acid oxidation and export (470). Peroxisome proliferator activated receptor alpha (PPRPα) expression is reduced (114), PPRPα is responsible for up-regulating genes involved in transport and β-oxidation of fatty acids (453). Microsomal triglyceride transfer protein (MTP) activity is also reported to be reduced upon HCV infection (376), MTP is involved in the assembly and secretion of VLDL (213). Furthermore HCV infection up regulates numerous genes associated with cholesterol and fatty acid metabolism by activating ER associated transcription factors SREBPs (sterol regulatory element binding protein) (504). The increase in production together with reduced release ultimately results in higher cholesterol/lipid levels inside the cell thought to provide an ideal environment for HCV replication and production (463). In support of the literature we observed a significant increase in cellular cholesterol levels of J6/JFH infected Huh-7.5 cells compared to mock infected cells (Fig.3.13C). The observed increase in cellular cholesterol levels

provides an explanation for the perturbation of anti-CD81 induced hepatoma cell spread by HCV infection.

The main function of the liver is to control cholesterol homeostasis and therefore HCV induced alteration(s) in cholesterol metabolism may have many pathological consequences. An example of which is Liver steatosis, caused by accumulation of lipid droplets, it occurs in approximately 73% of patients infected with genotype 3a and 50% of patients infected with other genotypes (343, 344). Until now changes in cholesterol levels induced by HCV infection have not been studied with respect to effect(s) on cell surface protein function(s), this is an area of research that will need further attention in the future.

4. Results: The role of CD81 in hepatoma migration and effects of HCV infection.

4.0 Introduction

The major causes of liver disease are viral hepatitis B and C, alcoholic liver disease and non alcoholic steatohepatitis (NASH). Fibrosis, a wound healing response to damage is a characteristic feature of liver disease and is associated with increased extra-cellular matrix (ECM) production (32). ECM comprises approximately 3% of the relative mass of a normal liver and has an important role in structure and cell proliferation, gene expression, migration and differentiation (36). In normal liver ECM is found in the portal tracts, sinusoidal walls and central veins (281) and comprises predominantly of collagens I, III, IV and V. Collagens I, III, and V are found in the portal tract and central vein whereas collagen IV is found with laminin in a low density basement-like membrane along the sinusoids. The other major ECM components of the liver include fibronectin, tenascin and nidogen. Increased ECM restricts intra-hepatic blood flow and promotes fibrotic scars that can promote the development of regenerating hepatocyte nodules, defined as liver cirrhosis (150). Intra-hepatic blood flow is restricted and hepatocyte function diminishes. Cirrhosis is a major clinical risk factor in the development of hepatocellular carcinoma (HCC) (273).

Unlike HBV, HCV does not integrate into the host genome and the role of HCV in HCC pathogenesis is unclear. The observation that HCC develops in HCV infected patients predominantly after liver cirrhosis (68, 200, 246, 273) supports an indirect mechanism for HCV induced pathology. In rare cases HCC has been reported to develop prior to cirrhosis and recent research has reported a link between HCV protein expression and oncogenic cellular pathways, including core (17, 33, 340),

NS3 (294) and NS5A (89, 330). In patients suffering from HCC, recurrence of a tumour following treatment is high leading to a poor long-term survival prognosis (537). In a study by Yang et al. 64% of patients (n=348) developed intrahepatic recurrences and 14% had extrahepatic metastasis after tumor resection. Recurrence after treatment is thought to occur because of multicentric HCC or local intrahepatic metastasis (537).

Metastasis is the process by which a tumor cell moves from the primary tumor to a secondary site, this process is complex and involves a number of steps including; detachment from the primary tumor, migration/invasion through surrounding tissue, and transport through blood or lymph vessels and growth at the secondary site (546). These steps involve transition from a non-motile, polarized epithelial cell to a non-polarized, motile, mesenchymal cell state, a reversible process termed epithelial mesenchymal transition (EMT) (546). EMT is not only associated with cancer progression but is a fundamental process during development and tissue regeneration (83, 233). Numerous factors are involved in the induction of EMT associated with cancer progression, including transforming growth factor beta (TGFβ) (551), hepatocyte growth factor (HGF) (417) and insulin like growth factor (IGF-1) (168). Furthermore, the ECM composition has been demonstrated to induce EMT (435). Interestingly, the stabilization of hypoxic inducible factor (HIF-1α) has also been implicated in the induction of EMT (198, 532, 534, 535) and recent research demonstrating that HCV infection stabilizes HIF-1α under normoxic conditions, highlights a novel mechanism for HCV associated HCC pathogenesis (191, 341, 400).

Changes in cell-cell and cell-ECM adhesion are mediated by an altered expression of adhesion proteins such as E-Cadherin (370), N-Cadherin (211) and specific integrins (187). Integrins are a large family of heterodimeric cell membrane proteins containing an alpha and beta subunit that interact with different ECM ligands and coordinate inside out and outside in signaling (212). Not surprisingly they have been implicated in regulating hepatocellular adhesion and migration (345, 346, 531, 538). The transcription factors Snail, Slug and TWIST are all implicated in controlling E-cadherin expression during EMT (64, 183, 532) and they can all be regulated by HIF-1α (534, 535). The modulation of actin cytoskeleton dynamics is mediated through the induction of complex signaling cascades. For example, TGFβ induced EMT engages the cytoskeletol modeling Rho GTPases (408) and different kinases including mitogen activated protein kinase MAPK (22) and phosphoinositol-3 kinase PI3K (23). It Is important to note that not all metastatic cancers possess the molecular signatures of EMT (471, 508), however current evidence demonstrates that hepatocellular carcinoma involves EMT of hepatocytes (33, 160, 169, 552).

As previously mentioned tetraspanins have been implicated in tumor progression (561). Tetraspanins are commonly expressed on filopodia and lamellipodia, a prime location to co-ordinate cell adhesion and invasion (372). A number of tetraspanins are directly or indirectly linked to integrins and have been demonstrated to modulate integrin signaling and localization (41, 42, 195, 427). For example, CD151 redistributes integrins α3β1 and α6β4 to the leading edge of a migrating cell (513). In addition, tetraspanins can recruit signaling proteins such as protein kinase C (PKC) and phosphatidylinositol 4-kinase (PI4K) to be in close proximity with integrins (539, 556).

Expression levels of certain tetraspanins have been reported to associate with patient outcome, for example high CD151 expression levels in lung, colon and prostate cancer predict poor patient outcome (15, 190, 482). In contrast, high CD82 expression levels are associated with good prognosis (484). CD9 expression has been associated with both tumor suppression and progression, depending on the cell type (203, 416, 501). Tetraspanin expression levels are hypothesized to modulate cell migration and adhesion by regulating the function or enzymatic activity of associated partner proteins (530). For example tetraspanin CD151 activates proMMP-7, a matrix metalloproteinase involved in ECM degradation, and increased CD151 expression promotes MMP-7 function and thus results in increased metastatic potential of the cell in question (436). A further example is the association of tetraspanin CD82 with integrin α6 and epidermal growth factor receptor (EGFR), where CD82 induces the internalization of these surface proteins and concomitant changes in cell-ECM adhesion and invasive potential (192, 353). In addition to investigating the effect(s) of tetraspanin expression on cellular processes *in vitro* studies have used anti-tetraspanin antibodies to elucidate the role of tetraspanin function as discussed in the previous chapter.

CD81 has been reported to interact with a number of integrins namely α3β1, α4β1 and αvβ5 (42, 75, 427, 481, 528) and in some instances these associations have been implicated in altering cell-ECM adhesion (481, 528). The role of CD81 as a coordinator of metastasis is not straightforward, with some studies reporting CD81 as a tumor suppressor (42, 481) and others suggesting a tumor promoter role (262, 312, 528). Mazzocca and colleagues reported that activation of CD81 with anti-CD81

mAbs in liver tumor cells increased their proliferation and CD81 expression decreased cell motility (66, 313).

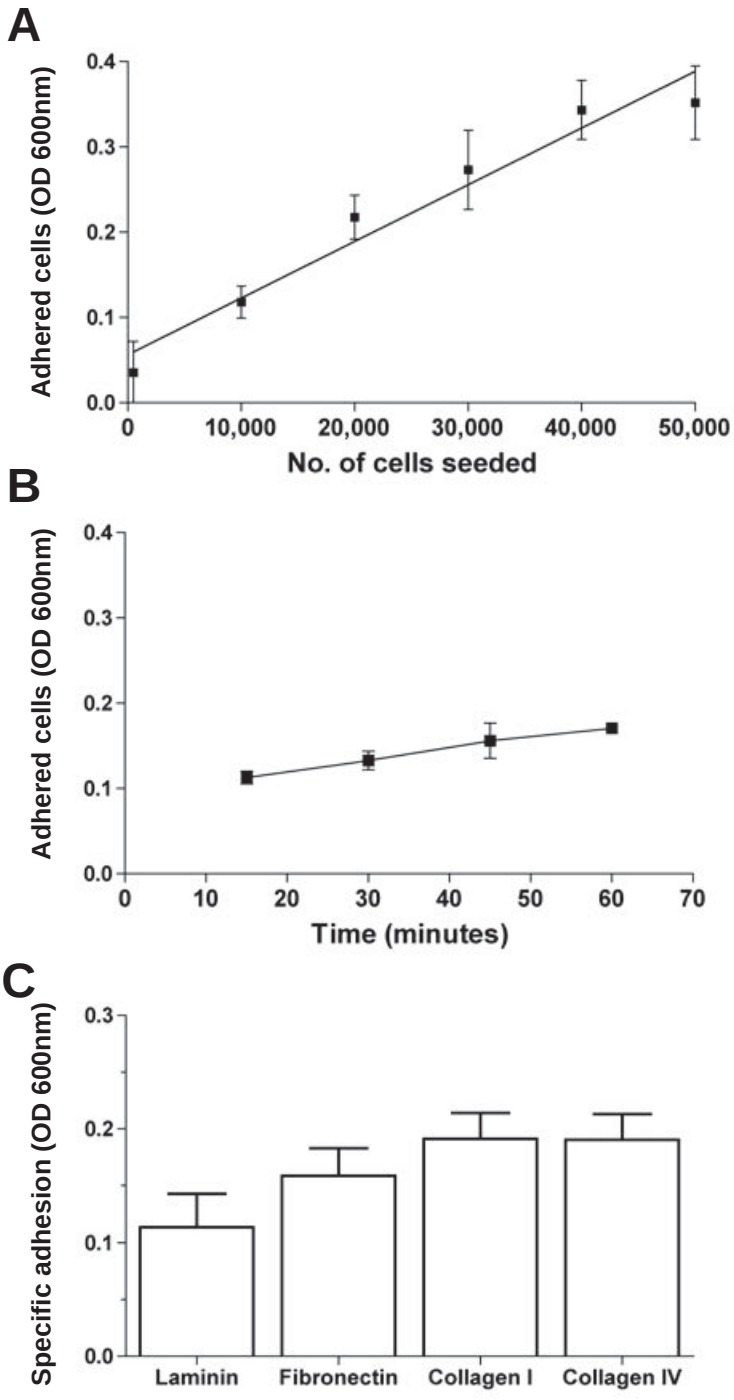
In the previous chapter we established that engagement of CD81 on hepatoma cells induced an actin polymerization dependent cell spread that was perturbed by HCV infection. In this chapter we aim to expand on these findings by investigating a possible role for CD81 in hepatoma migration and whether HCV infection perturbs these cellular processes.

4.1 Role of tetraspanin CD81 in hepatoma-ECM adhesion

To investigate whether CD81 is involved in hepatoma cell-ECM adhesion we established an assay to quantify cell adhesion. Cells were seeded in 96 well tissue culture plate and incubated for 1hr at 37°C. Non-adhered cells were removed by washing and the remaining cells fixed with methanol and stained with crystal violet. Adhered cells were quantified by measuring absorbance at 600nm. A linear relationship between the number of cells seeded and absorbance was observed (Fig.4.1A). A maximal number of hepatoma cells successfully adhered to the plate following 15min incubation (Fig.4.1B). To investigate the relative adhesion of Huh-7.5 cells to different ECM proteins, their association to laminin, fibronectin, collagen I and collagen IV coated wells was determined. ECM coated wells were incubated for 30min with 1% BSA to block non-specific binding prior to seeding the cells. Results are presented as specific adhesion to ECM by subtracting the optical density obtained for BSA only coated wells (Fig.4.1C).

Figure 4.1: Development of hepatoma-ECM adhesion assay.

Huh-7.5 cells were seeded in triplicate (**A**) at known concentrations into a 96 well tissue culture plastic plates and incubated at 37°C for 1hr or (**B**) at a concentration of $4x10^4$ cells per well and incubated for 15, 30, 45 or 60min at 37°C. Non-adhered cells were washed off with PBS and adhered cells fixed with ice-cold methanol. Attached cells were stained with 0.1% crystal violet and quantified by absorbance read at 600nm. (**C**) Huh-7.5 cells were seeded in triplicate at a concentration of $4x10^4$ cells per well into ECM coated wells (laminin, fibronecin, collagen I, collagen IV (10μg/ml) and uncoated control). All wells were blocked with 1% BSA/PBS prior to seeding the cells. Cells were then incubated for 15min at 37°C. Non-adhered cells were washed off with PBS and adhered cells fixed with ice-cold methanol. Attached cells were stained with 0.1% crystal violet and quantified by absorbance read at 600nm. Specific adhesion was determined by subtracting the absorbance reading of the BSA uncoated control. All results are representative of three independent experiments.

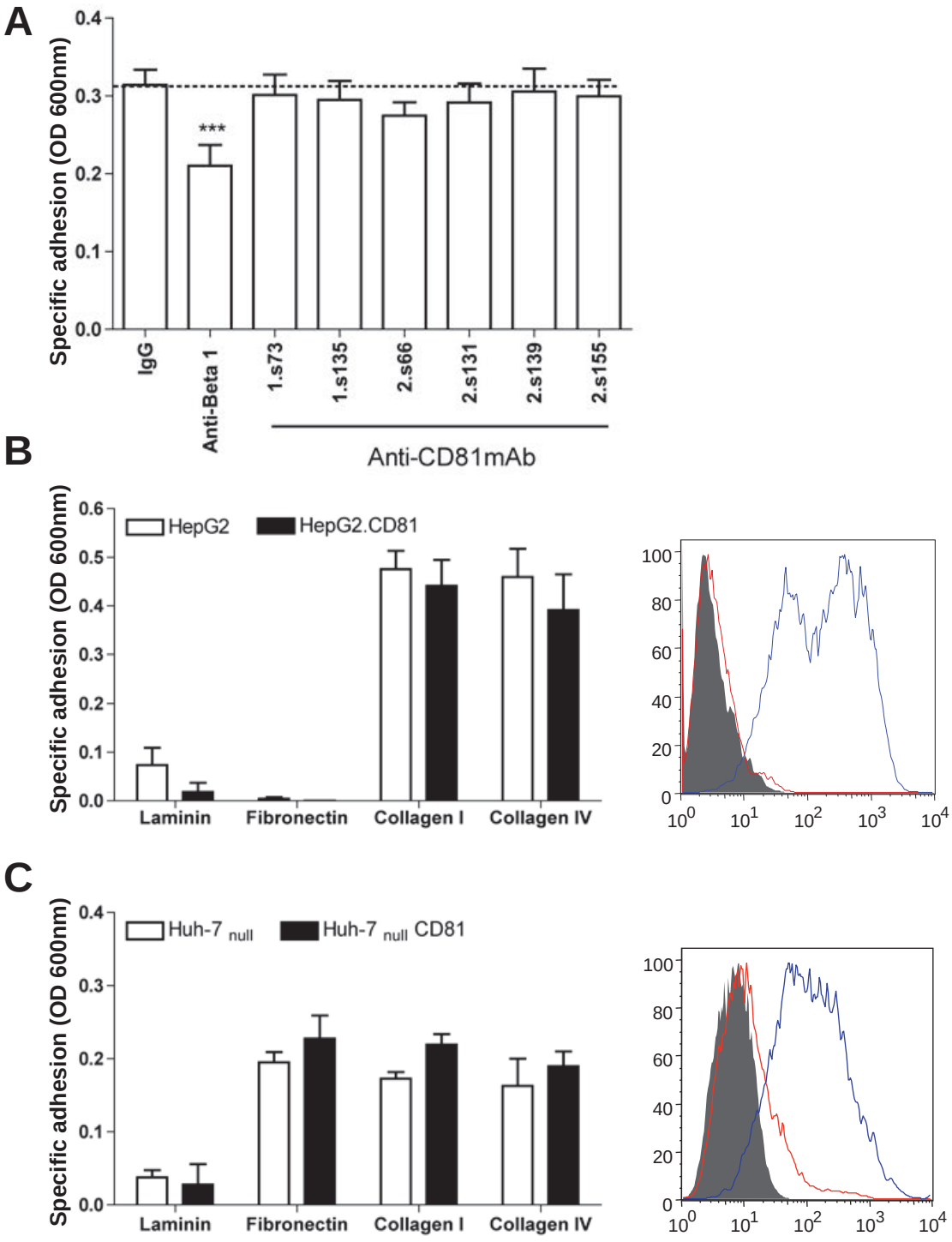

A
Adhered cells (OD 600nm)
0.4
0.3
0.2
0.1
0.0
0
10,000
20,000
30,000
40,000
50,000
No. of cells seeded
B
Adhered cells (OD 600nm)
0.4
0.3
0.2
0.1
0.0
0
10
20
30
40
50
60
70
Time (minutes)
C
Specific adhesion (OD 600nm)
0.3
0.2
0.1
0.0
Laminin
Fibronectin
Collagen I
Collagen IV

Initial experiments investigated whether CD81 engagement would alter Huh-7.5 adhesion to fibronectin. Huh-7.5 cell monolayers were removed from tissue culture plastic using trypsin and cell suspensions treated with a panel of anti-CD81mAbs (Described in section 3.1) prior to seeding onto fibronectin-coated wells. All mAbs were used at 1μg/ml, a concentration previously demonstrated to saturate the cell surface by flow cytometry (Flow cytometry analysis carried out by Ke Hu, data not shown). As a positive control we used an antibody against integrin β1 subunit, previously shown to inhibit cell-ECM adhesion (46). All of the anti-CD81 mAbs had a minimal effect on Huh-7.5 cell adhesion to fibronectin whilst the anti-Beta-1 (integrin) antibody significantly reduced cell adhesion (Fig.4.2A).

CD81 expression levels were previously reported to reduce epithelial cell adhesion to fibronectin (481). We therefore investigated the role of CD81 expression on hepatoma-ECM adhesion. To do this we engineered two hepatoma cell lines (CD81 negative), HepG2 and Huh-7 $CD81_{null}$ cells to express CD81 and compared their adhesion to different ECM proteins, laminin, collagen I, collagen IV and fibronectin. CD81 cell surface expression was confirmed by flow cytometry. No differences were noted in hepatoma-ECM adhesion between parental and CD81 expressing cells (Fig.4.2B&C).

Figure 4.2: CD81 is not involved in hepatoma - ECM adhesion.

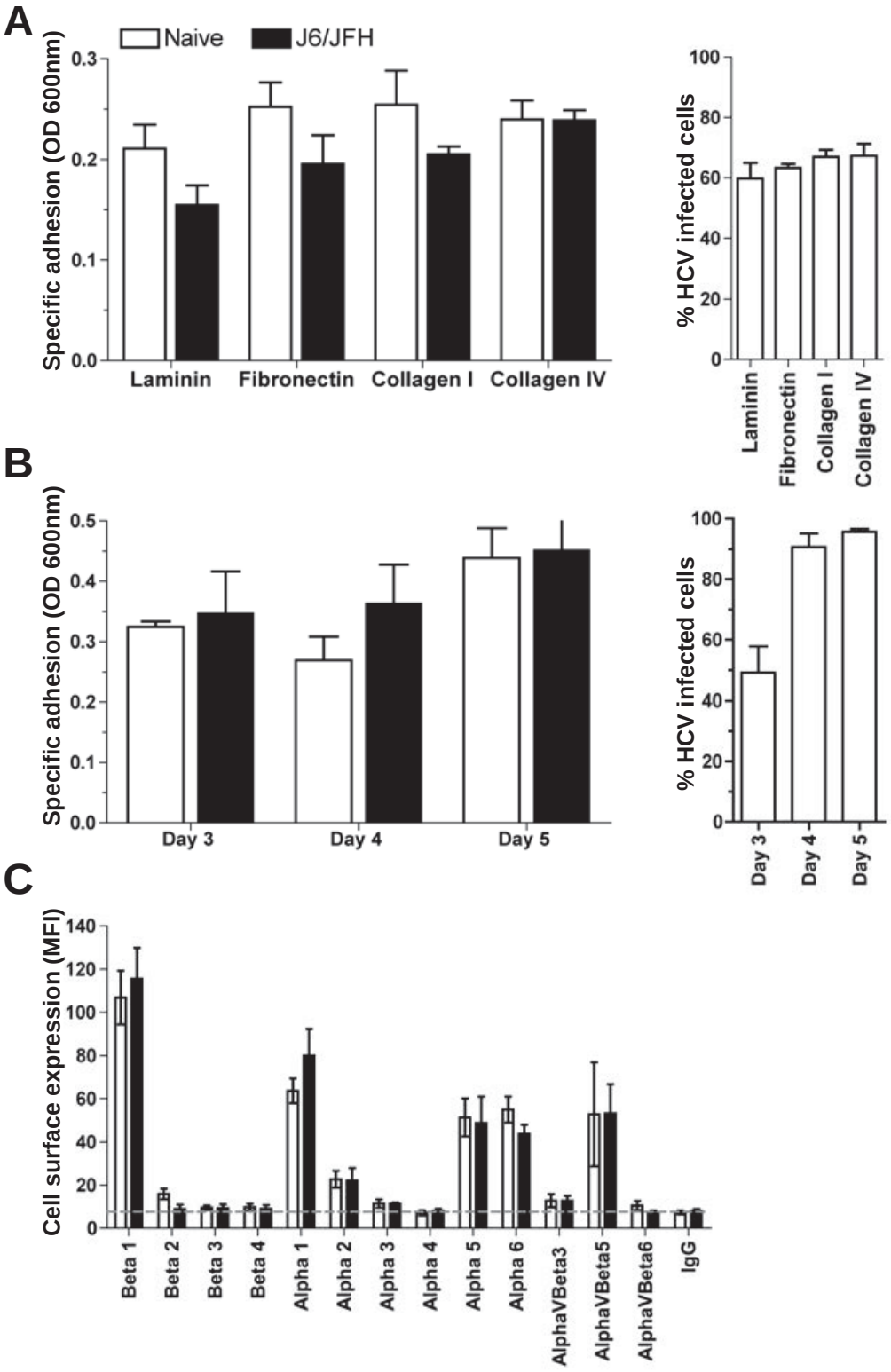
(**A**) 96 well tissue culture plates were coated with fibronectin (10μg/ml) or left uncoated (control), wells then blocked with 1% BSA/PBS. Huh-7.5 cells were incubated for 30min at 37°C in the presence of CD81mAbs (1μg/ml) in suspension prior to seeding onto coated plates ($4x10^4$ cells per well). Cells were allowed to adhere for 15min at 37°C. Non-adhered cells were washed off with PBS and adhered cells fixed with ice-cold methanol. Attached cells were stained with 0.1% crystal violet and quantified by absorbance read at 600nm. Specific adhesion was determined by subtracting absorbance reading from the BSA control. 96 well plates were coated with laminin, fibronecin, collagen I, collagen IV (10μg/ml) or left uncoated (control), wells then blocked with 1% BSA/PBS. HepG2 (**B**) and Huh-7_{null} (**C**) and corresponding cells engineered to express CD81 were seeded at a concentration of $4x10^4$ cells per well. Attached cells were quantified as in previous experiments. CD81 cell surface expression was detected using 2.s131 (anti-CD81mAb) primary antibody and Alexa-fluor 488 secondary antibody and analyzed by flow cytometry. Grey filled lines represent IgG control, Red lines represent parental cells and Blue lines represent cells engineered to express CD81. In all assays cells were serum starved overnight prior to the assay and the assay was performed in serum free DMEM. Error bars indicate standard deviation from the mean. Results are representative of three independent experiments.


A
Specific adhesion (OD 600nm)
0.4
0.3
0.2
0.1
0.0
***
IgG
Anti-Beta 1
1.s73
1.s135
2.s66
2.s131
2.s139
2.s155
Anti-CD81mAb
B
Specific adhesion (OD 600nm)
HepG2
HepG2.CD81
0.6
0.5
0.4
0.3
0.2
0.1
0.0
Laminin
Fibronectin
Collagen I
Collagen IV
100
80
60
40
20
0
10^0
10^1
10^2
10^3
10^4
C
Specific adhesion (OD 600nm)
Huh-7 null
Huh-7 null CD81
0.4
0.3
0.2
0.1
0.0
Laminin
Fibronectin
Collagen I
Collagen IV
100
80
60
40
20
0
10^0
10^1
10^2
10^3
10^4

Several viruses have been reported to modulate cell surface expression of adhesion molecules including integrins resulting in altered cell-ECM adhesion. Examples include: Ebola virus infection down-regulates β1 integrin expression and reduces the adhesion of infected cells to ECM (446); Rotavirus infection increases integrin expression of intestinal cells (184) and Epstein-Barr virus up-regulates α5 integrins in transformed B lymphocytes and promotes their invasion (210). To investigate whether HCV infection alters hepatoma-ECM adhesion, mock and HCV strain J6/JFH infected Huh-7.5 cells (3 days post infection) were seeded onto different ECM protein coated wells. Adhered cells were fixed with methanol, and stained with crystal violet to determine adhesion or with anti-NS5A antibody to determine the frequency of ECM-bound infected cells. No significant differences were noted between the adhesion of mock or J6/JFH infected cells to fibronectin (Fig.4.3A-B), demonstrating that HCV infection does not modulate hepatoma-ECM adhesion. In support of this conclusion HCV infection did not alter integrin expression (Fig.4.3C).

Figure 4.3 HCV infection does not alter cell-ECM adhesion

96 well plates were coated with fibronectin, laminin, collagen I, collagen IV (10μg/ml) or left uncoated, wells then blocked with 1% BSA/PBS. **(A)** Huh-7.5 cells were infected with J6/JFH HCVcc. At day 3 post infection J6/JFH infected and naïve Huh-7.5 cells were seeded at $4x10^4$ cells per well into the different ECM coated wells or (**B**) at days 3, 4 and 5 post infection cells were seeded onto fibronectin coated wells. Cells were allowed to adhere for 15min at 37°C and attached cells were then either stained with crystal violet to determine specific adhesion as in previous assays or anti-NS5A and DAPI to determine HCV infection level of adhered cells. For (**B**) a proportion of cells were PFA fixed each day, stained for NS5A and analyzed by flow cytometry to quantify infection level of cells prior to seeding (Approximately 65, 84 and 93% NS5A +ve at days 3,4 and 5 respectively). (**C**) Integrin cell surface expression of J6/JFH HCVcc infected or naïve Huh-7.5 cells was determined by flow cytometry using specific anti-integrin antibodies as detailed in Table 2.4. Cell surface expression expressed as median fluorescence intensity (MFI). Error bars indicate standard deviation from the mean. Experiments were performed in triplicate and results are representative of three independent experiments.

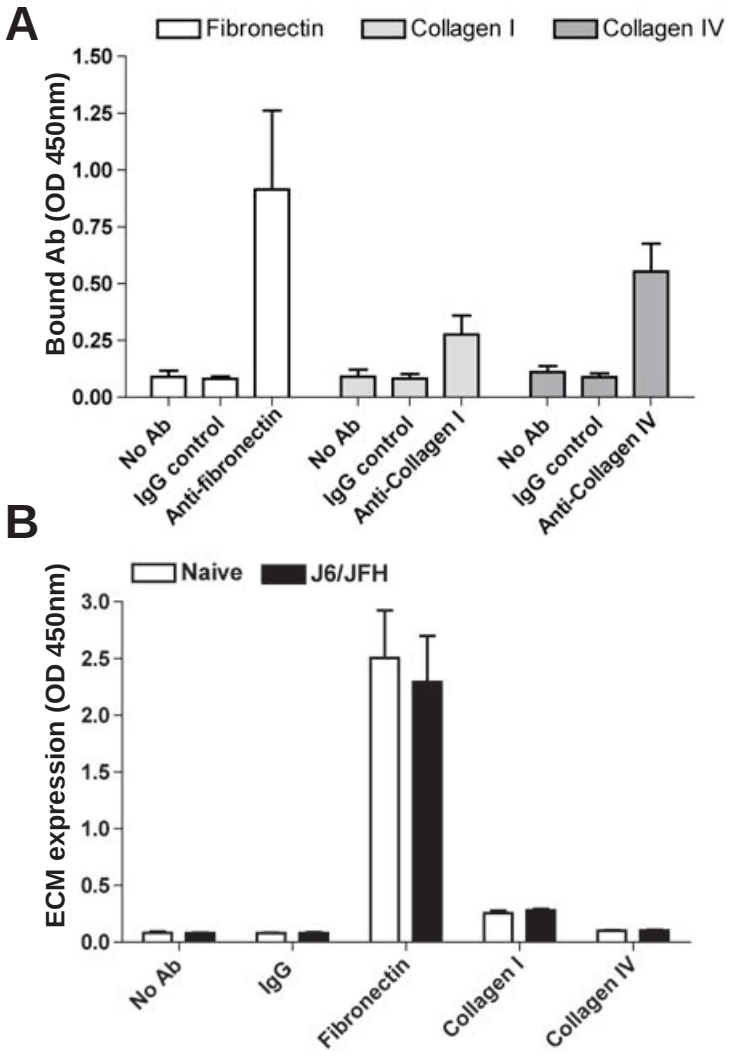

A
Naive
J6/JFH
Specific adhesion (OD 600nm)
0.0
0.1
0.2
0.3
Laminin
Fibronectin
Collagen I
Collagen IV
% HCV infected cells
0
20
40
60
80
100
Laminin
Fibronectin
Collagen I
Collagen IV
B
Specific adhesion (OD 600nm)
0.0
0.1
0.2
0.3
0.4
0.5
Day 3
Day 4
Day 5
% HCV infected cells
0
20
40
60
80
100
Day 3
Day 4
Day 5
C
Cell surface expression (MFI)
0
20
40
60
80
100
120
140
Beta 1
Beta 2
Beta 3
Beta 4
Alpha 1
Alpha 2
Alpha 3
Alpha 4
Alpha 5
Alpha 6
AlphaVBeta3
AlphaVBeta5
AlphaVBeta6
IgG

As previously mentioned ECM plays an essential role in liver structure and function (36) and increased ECM production is frequently associated with liver disease (32). Hepatic stelate cells are thought to be the most important cells in coordinating liver fibrosis and express collagens and basement membrane (149). Hepatocytes are the dominant source of plasma fibronectin in the body and although the role of plasma fibronectin in fibrosis is not known, mRNA for cellular fibronectin has also been reported in hepatocytes. Thus, the role of hepatocyte ECM expression in fibrosis is still under debate (161, 307, 334, 507). Recent reports demonstrating hepatocyte EMT argue for a more dominant role of hepatocytes in fibrosis than originally thought (33, 160, 552).

To ascertain whether HCV infection alters hepatoma ECM production we developed a quantitative ELISA to measure collagen I, collagen IV and fibronectin ECM proteins (Fig.4.4A). Huh-7.5 cells were seeded onto gelatin-coated plates and infected with mock or J6/JFH HCVcc and 96hr post infection cells were lysed and ECM expression quantified. A replicate plate was methanol fixed and stained for NS5A to determine the level of infected cells (Fig. 4.4B). Naïve and J6/JFH infected Huh-7.5 cells expressed comparable levels of fibronectin and collagen I, no detectable collagen IV.

Figure 4.4: HCV infection does not alter hepatoma ECM expression.

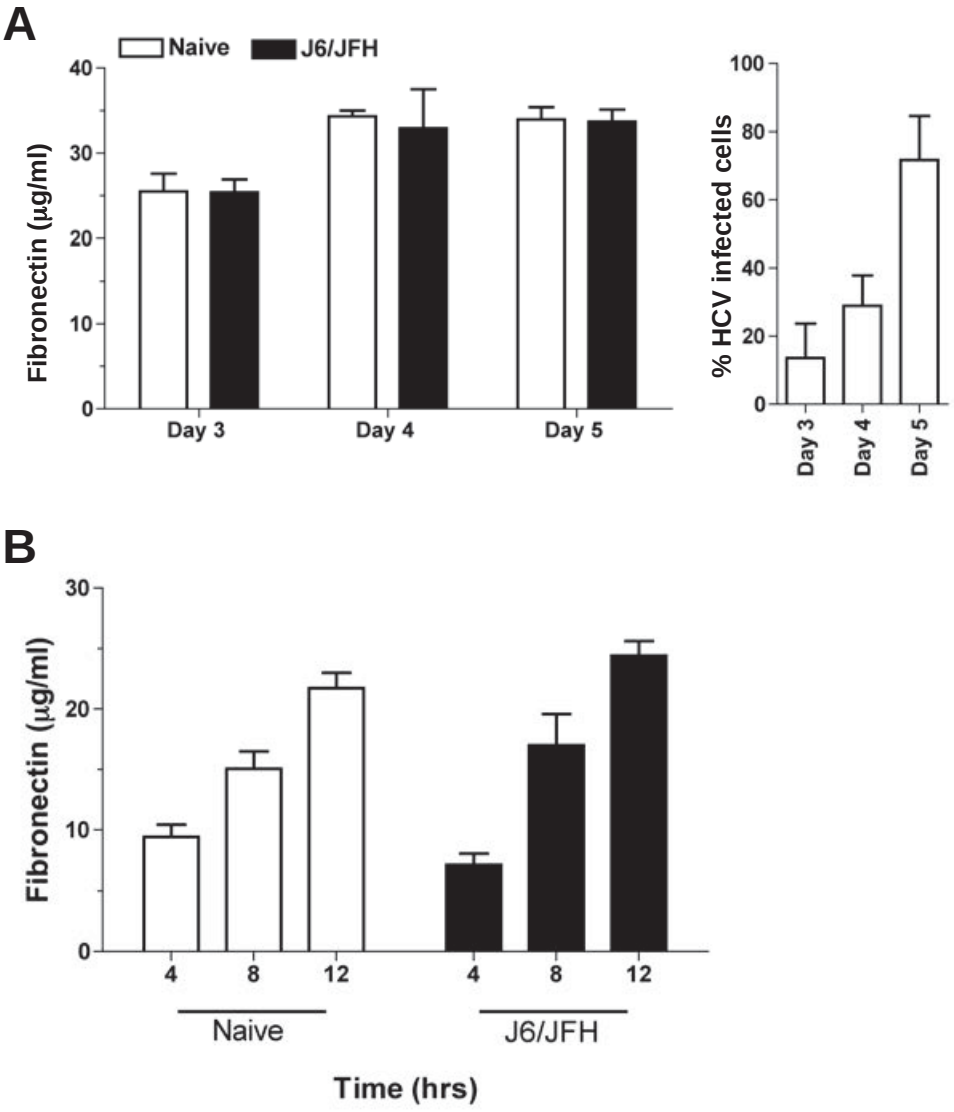
(**A**) 96 well plates were coated with gelatin followed by ECM proteins. Plates were then developed using ECM ELISA described in section 2.2.5. (**B**) Huh-7.5 cells were seeded onto gelatin coated plates. Cells were infected with J6/JFH HCVcc or mock infected one day post seeding. On day 5 post seeding one plate was fixed with ice cold methanol and stained with anti-NS5A and cell nuclei counter stained with DAPI to determine HCV infection level of adhered cells. Cells were lysed from the replicate plate and ECM expression determined using the ECM ELISA. Error bars indicate standard deviation from the mean. Experiments were performed in triplicate and results are representative of three independent experiments.


A
Fibronectin
Collagen I
Collagen IV
Bound Ab (OD 450nm)
1.50
1.25
1.00
0.75
0.50
0.25
0.00
No Ab
IgG control
Anti-fibronectin
No Ab
IgG control
Anti-Collagen I
No Ab
IgG control
Anti-Collagen IV
B
Naive
J6/JFH
ECM expression (OD 450nm)
3.0
2.5
2.0
1.5
1.0
0.5
0.0
No Ab
IgG
Fibronectin
Collagen I
Collagen IV

To investigate this further the production of fibronectin was determined over 5 days, and no significant differences were observed (Fig.4.5A). Finally we compared fibronectin expression during the first 12hrs post seeding. Mock and J6/JFH infected cells were seeded onto gelatin-coated plates and cells lysed 4, 8 and 12hrs later (Fig.4.5B). Both mock and J6/JFH infected cells expressed comparable levels of fibronectin over the first 12hrs post seeding.

Figure 4.5: HCV infection does not alter hepatoma ECM expression.

(**A**) Huh-7.5 cells were seeded onto gelatin coated 96 well plates. Cells were infected with J6/JFH HCVcc or mock infected one day post seeding. Cells were then lysed at days 3, 4 and 5 post infection and stored at 4°C. All plates were then developed using the ECM ELISA described in section 2.2.5 and expression displayed as μg/ml of fibronectin produced. On a replicate plate cells were fixed with ice-cold methanol and stained with anti-NS5A and cell nuclei counter stained with DAPI to determine HCV infection level. (**B**) J6/JFH (5 days post infection, approximately 80% NS5A positive determined by flow cytometry) or mock infected cells were seeded onto gelatin coated plates. Cells were then lysed at 4, 8 and 12hrs post seeding. The plate was developed using the ECM ELISA and expression displayed as μg/ml of fibronectin produced. Error bars indicate standard deviation from the mean. Experiments were performed in triplicate and results are representative of three independent experiments.


A
Naive
J6/JFH
Fibronectin (μg/ml)
40
30
20
10
0
Day 3
Day 4
Day 5
% HCV infected cells
100
80
60
40
20
0
Day 3
Day 4
Day 5
B
Fibronectin (μg/ml)
30
20
10
0
4
8
12
4
8
12
Naive
J6/JFH
Time (hrs)

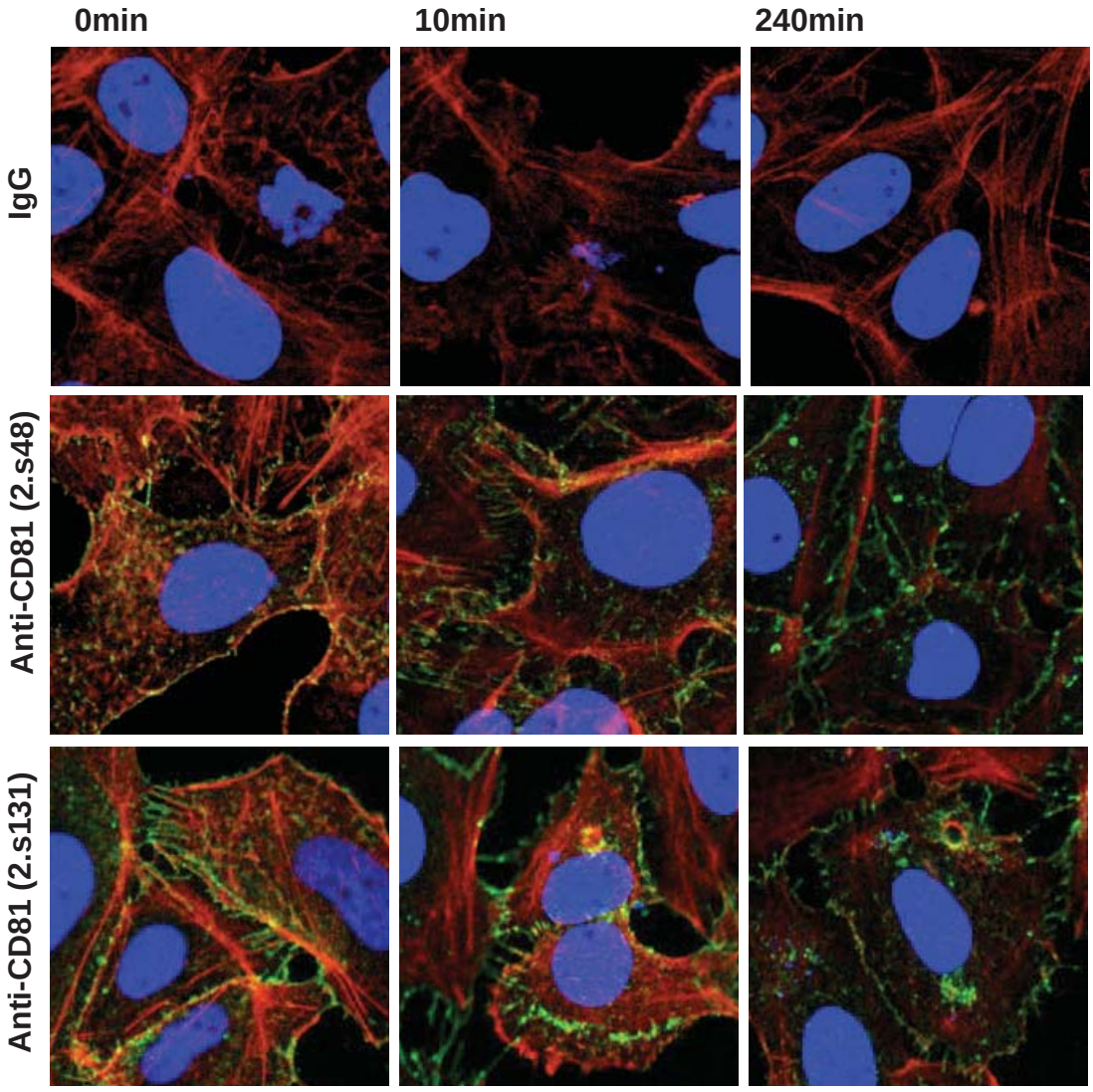
4.2 Role of CD81 in hepatoma invasion

The ability of a cell to migrate or invade though ECM is an important phenotypic feature that defines a cells metastatic potential. Invading cells induce membrane ruffles at their leading edge in a process involving the reduction of rigid actin stress fibers leaving only fine cortical actin (25, 256). This leads to the formation of flat or sheet like projections containing a branched actin network termed lamellipodia. Filopodia emerge from lamellipodia as rod like protrusions with tight bundled actin. Invadopodia is the term for a further cellular membrane protrusion, these are actin rich protrusions containing high expression levels of integrins and cell MT1-MMPs and enable remodeling and cell penetration into ECM (18, 546).

Tetraspanin-integrin complexes localize to microvilli like protrusions, including CD81-$\alpha 3\beta 1$ complexes on mammary carcinoma cells. Interestingly anti-CD81 mAbs have been reported to induce cellular protrusions and matrix metalloproteinase MMP-2 production and increased cell invasion (460). We previously observed that anti-CD81 ligation promoted hepatoma cell spread. To determine whether antibody engagement induced microvilli or filopodia like protrusions, Huh-7.5 cells were seeded onto glass cover slips and exposed to anti-CD81 mAbs or IgG control (2μg/ml). Wells were fixed with EM grade formaldehyde at 0, 10 and 240min post exposure to antibody. CD81 was visualized using a 488 conjugated secondary antibody and F-actin visualized with phalloidin-594, cells were imaged by confocal microscopy. Huh-7.5 cells had numerous microvilli or filopodia protruding from their cell body with CD81 expressed at the cell surface, however we failed to observe any increase in filopodia following anti-CD81 stimulation (Fig.4.6).

Figure 4.6: Anti-CD81 mAbs do not induce hepatoma filopodia

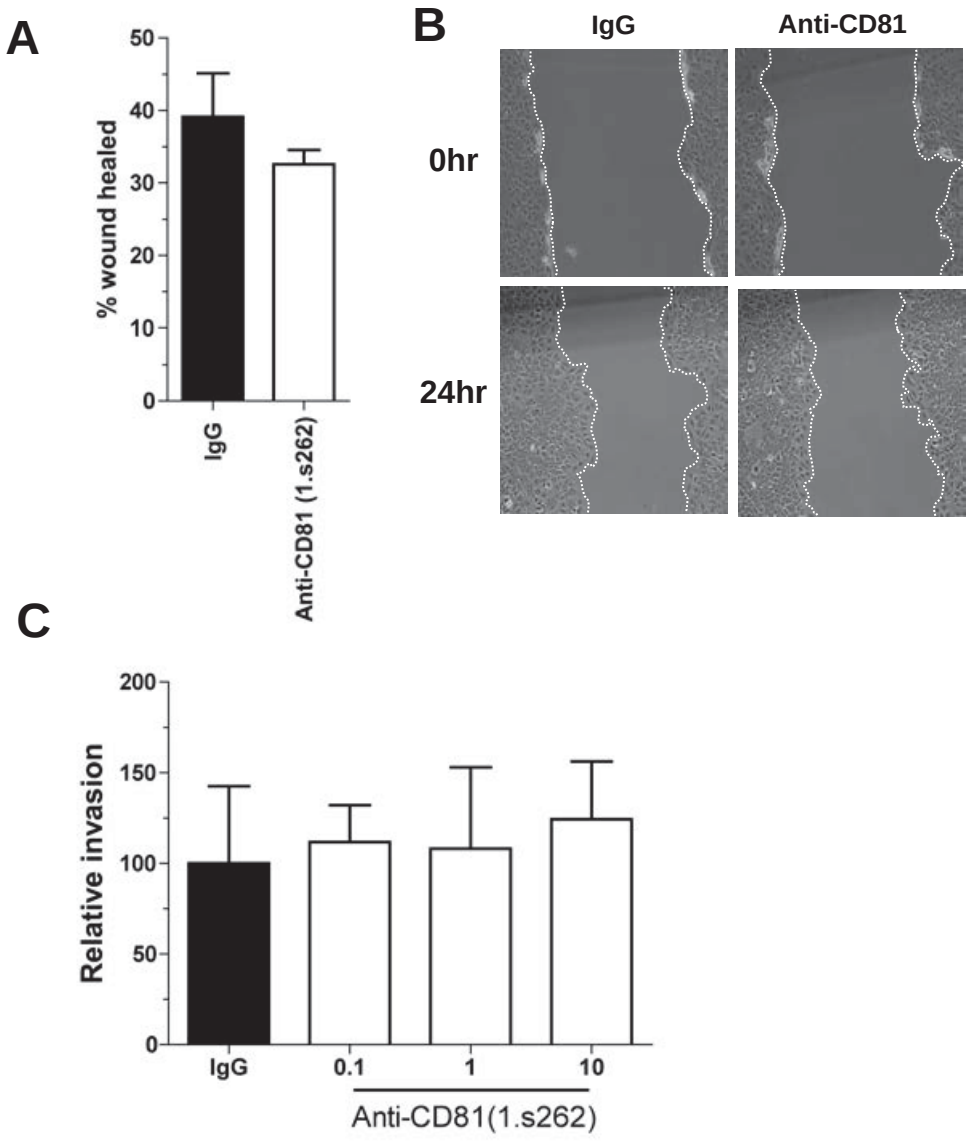
Huh-7.5 cells were cultured on glass cover slips and serum starved overnight. Cells were incubated at 37°C with anti-CD81 mAbs (2.s131 and 2.s48) or IgG control diluted in serum free DMEM for 0,10 or 240 mins. Cells were then fixed with EM grade formaldehyde and CD81 visualized using an Alexa-fluor-488 secondary antibody, actin was visualized with phalloidin-594 and cell nuclei counter stained with DAPI. Images were taken using a Zeiss META head confocal microscope with a 60x water immersion objective. Images are representative of three separate experiments.


0min
10min
240min
IgG
Anti-CD81 (2.s48)
Anti-CD81 (2.s131)

To investigate whether CD81 engagement has a role in hepatoma migration a confluent monolayer of Huh-7.5 cells were wounded using a p200 tip and wound closure measured over 24hrs in the presence of anti-CD81 mAb (1.s262) or irrelevant control IgG (Fig.4.7A). No differences were observed in wound closure in antibody treated or untreated cells.

As previously described the processes involved in cell migration and cell invasion differ and we therefore investigated the effect(s) of CD81 ligation on hepatoma invasion through a collagen coated 8μm pore-size transwell insert (Fig.4.7B). Cells were incubated for 24hrs at 37°C followed by mechanical removal of non-invaded cells and methanol fixation of invaded cells. The invaded cells were stained with crystal violet and counted. Anti-CD81 mAb had no effect on hepatoma invasion.

Figure 4.7: Anti-CD81 mAb has no effect on hepatoma migration or invasion.
(**A**) Huh-7.5 cells ($1x10^6$) were seeded into 6 well dishes and serum starved overnight. A wound was performed using a P200 tip and media replaced with serum free DMEM (plus mitomycin C at 15μg/ml) containing anti-CD81 mAb (1.s262) or IgG at 10μg/ml. Cells were incubated at 37°C and phase images taken at 0 and 24hrs. Percentage wound closure determined using IPLab 4.0 (BD Biosciences). (**B**) Huh-7.5 cells were serum starved overnight. Trypsin was used to remove the cells from the tissue culture plastic prior to re-suspension in serum free DMEM containing anti-CD81 mAb (1.s262) at 0.1,1, and 10μg/ml or irrelevant control IgG at 10μg/ml. Cells were seeded (4x104 cells/200ul/cell) into the top chamber of an 8μm pore collagen coated transwell insert. The lower chamber contained 300μl of DMEM containing the corresponding treatment. Cells were incubated at 37°C for 24hrs. Non-invaded cells were mechanically removed and invaded cells methanol fixed and stained with 0.1% crystal violet. 3x fields of view were imaged per transwell at 40x magnifcation on a fluorescent microscope (NikonTE2000, Japan). The number of invaded cells was determined and invasion expressed relative to control. Two tailed unpaired T-test's were performed to determine whether there was a significant difference in wound closure or invasion between untreated and anti-CD81 treated cells. No significant differences were observed $P>0.05$. Error bars indicate standard deviation from the mean. Assays were performed in duplicate and the results are representative of three separate experiments.


A
% wound healed
50
40
30
20
10
0
IgG
Anti-CD81 (1.s262)
B
IgG
Anti-CD81
0hr
24hr
C
Relative invasion
200
150
100
50
0
IgG
0.1
1
10
Anti-CD81(1.s262)

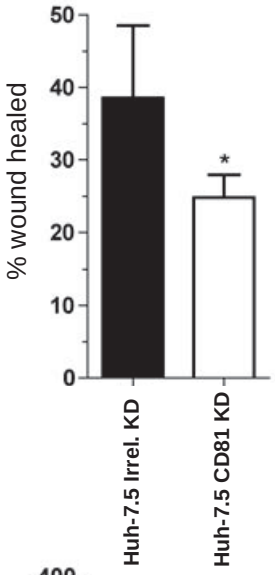
To determine whether CD81 expression modulates hepatoma cell migration, CD81 expression was silenced using CD81 siRNA treatment and wound closure compared to irrelevant control siRNA treated cells (Fig.4.8A). The assay was performed in the presence of mitomycin C, mitomycin C prevents cell proliferation and thus enables discrimination between cell proliferation and cell migration. Cells were fixed and stained for CD81 at the end of the assay to confirm reduction in CD81 expression. We observed a small but significant reduction in cell migration of siRNA CD81 treated cells compared to control.

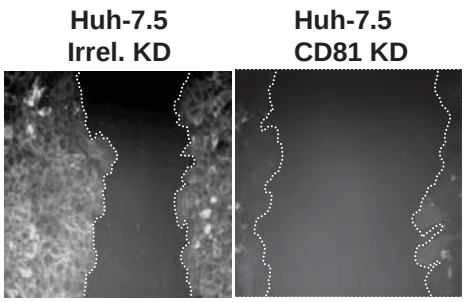
Two approaches were taken to investigate the effects of CD81 expression on hepatoma cell invasion through a collagen coated transwell insert. Firstly invasion of HepG2 parental cells and those engineered to express CD81 (HepG2.CD81) were compared. Secondly CD81 expression was silenced in Huh-7.5 cells using CD81 siRNA treatment and invasion compared to irrelevant control siRNA treated cells. A control well was stained for CD81 to confirm CD81 silencing. We observed increased invasion of HepG2 cells expressing CD81 compared to parental cells demonstrating that CD81 expression increases hepatoma invasion potential (Fig.4.8B). Confirming this phenomenon we observed a decrease in the invasion of the CD81 silenced Huh-7.5 cells compared to control (Fig.4.8C). These results demonsrate in two independent hepatoma cell lines that CD81 expression increases hepatoma cell invasion and implicates CD81 as a tumour promoter.

Figure 4.8: CD81 expression increases hepatoma cell invasion.

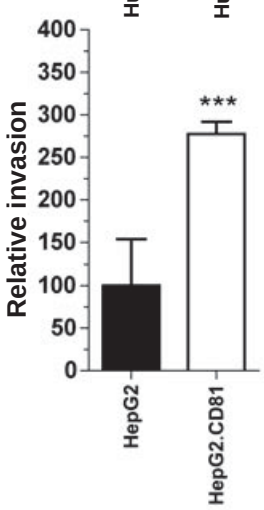
(**A**) Huh-7.5 cells were seeded into 6 well dishes and transfected with si CD81 RNA (CD81 KD) or irrelevant control siRNA. 24hrs post transfection a wound was performed using a P200 tip and media replaced with serum free DMEM, cells were incubated at 37°C and images taken at 0 and 24hrs post wound. Images display expression of CD81 KD cells and control cells taken 24hrs post wound on. CD81 expression visualized using anit-CD81 (2.s131) primary antibody and alexa-fluor 488 secondary antibody (green). A two tailed unpaired T-test was performed to determine whether there was a significant difference in wound closure between control and CD81 KD cells. A significant difference was observed P=0.0387. (**B**) HepG2 and HepG2 cells engineered to express CD81 (HepG2.CD81) or (**C**) Huh-7.5 control and Huh-7.5 CD81 KD cells were cultured in a tissue culture flask in serum free conditions overnight, cells were trypsinised and re-suspended in serum free DMEM. Cells then seeded ($4x10^4$ cells/200μl/well) in the top chamber of an 8μm pore collagen coated transwell insert. The lower chamber contained 300μl of serum free DMEM. Cells were incubated at 37°C for 24hrs. Non-invaded cells were mechanically removed and invaded cells methanol fixed and stained with 0.1% crystal violet. 3x fields of view were imaged per transwell at 40x magnification on a on a fluorescent microscope (NikonTE2000, Japan). The number of invaded cells was determined and invasion expressed relative to control. Images display CD81 expression of CD81 KD cells and control cells taken at the end of the assay in replicate wells. CD81 expression was visualized using anti-CD81 specific antibody (2.s131) and alexa-fluor 488 secondary antibody. Two tailed unpaired T-tests were performed to determine whether there was a significant difference in invasion between HepG2 and HepG2.CD81 cells and control and CD81 KD cells. A significant difference was observed, P=< 0.0001 and 0.004 respectively. Error bars indicate standard deviation from the mean. Assays were performed in duplicate and the results are representative of three separate experiments.

A

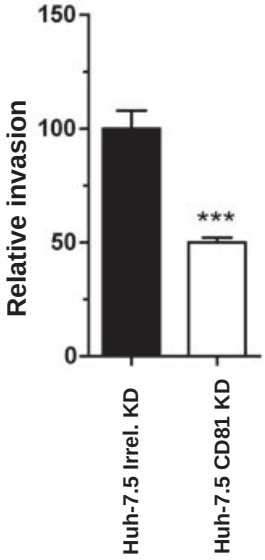

% wound healed
50
40
30
20
10
0
*
Huh-7.5 Irrel. KD
Huh-7.5 CD81 KD

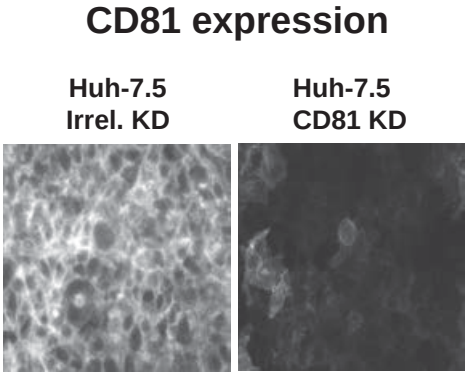

CD81 expression
Huh-7.5
Irrel. KD
Huh-7.5
CD81 KD

B


Relative invasion
400
350
300
250
200
150
100
50
0
***
HepG2
HepG2.CD81

C


Relative invasion
150
100
50
0
***
Huh-7.5 Irrel. KD
Huh-7.5 CD81 KD


CD81 expression
Huh-7.5
Irrel. KD
Huh-7.5
CD81 KD

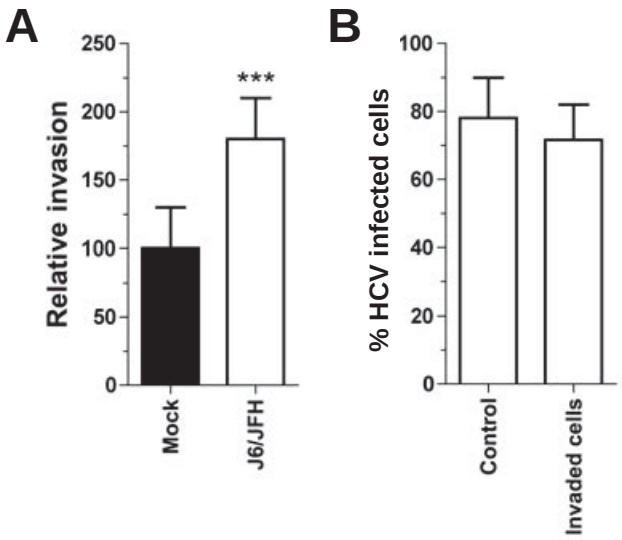
4.3 HCV infection modulates hepatoma cell invasion

We previously demonstrated that HCV infection modulates CD81 dependent hepatoma cell spread (Chapter 3) and were interested to assess the effect(s) of HCV infection on hepatoma invasion and migration. Unfortunately due to category three restrictions of imaging we were unable to study the effects of infection on hepatoma migration in a scratch wound assay.

Huh-7.5 cells were electroporated with J6/JFH 96hrs prior to the assay. The invasion assay was carried out as previously described with the following exceptions; (i) cells were labeled with CMFDA (cell tracker green) prior to the assay instead of a crystal violet stain post invasion, (ii) cells were seeded in replicate (control) 24 well plates not containing transwell inserts, the control and invaded cells were then stained for NS5A at the end of the assay to determine the frequency of infected invaded cells compared to non-invaded cells. A greater number of cells in the J6/JFH infected culture invaded compared to the uninfected cells (Fig.4.9A). However, no difference was observed in the frequency of NS5A expressing cells in the control and invaded cell population (Fig.4.9B) and thus we hypothesize that a soluble factor may be responsible for the increased invasive phenotype of the infected cell population.

Figure 4.9: Effect of HCV infection on invasion.

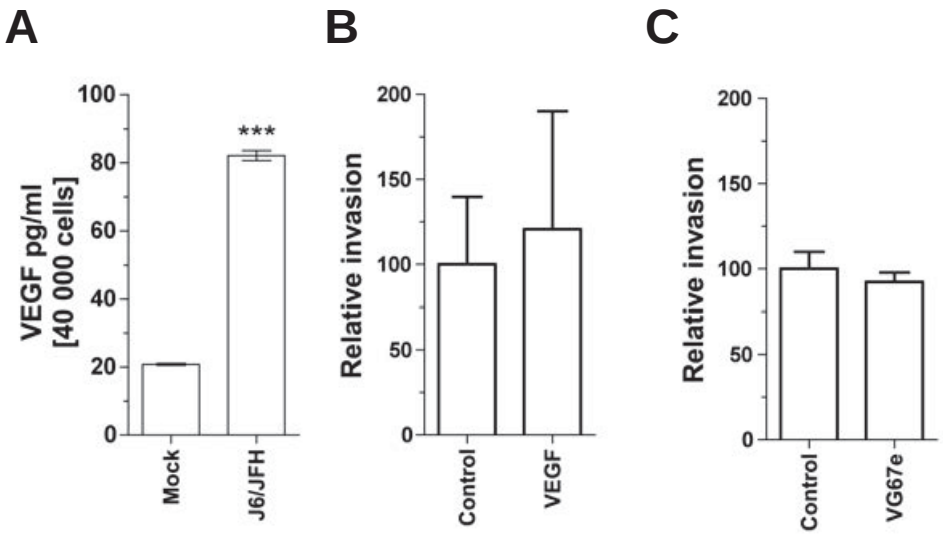
(**A**) HCV J6/JFH RNA was delivered into Huh-7.5 cells by electroporation (ep). 72hrs post ep cells were cultured in serum free conditions overnight. Cells labeled with CMFDA, removed from tissue culture plastic with trypsin and re-suspension in serum free DMEM. Cells seeded ($4x10^4$ cells/200μl/well) into the top chamber of an 8μm pore collagen coated transwell insert or control 24 well plates not containing a transwell insert. The lower chamber contained 300μl of serum free DMEM. Cells were incubated at 37°C for 24hrs. Non-invaded cells were mechanically removed and the invaded and control cells methanol fixed. 3x fields of view were imaged per transwell at 40x magnification on a fluorescent microscope (NikonTE2000, Japan). The number of invaded cells was determined and invasion expressed relative to control. A Two tailed unpaired T-test was performed to determine whether there was a significant difference in the invasion of naive and J6/JFH infected cells. A significant difference was observed P=0.0010. (**B**) Fixed cells were stained for NS5A and infection level determined for control and invaded cells. Error bars indicate standard deviation from the mean. Assays were performed in duplicate and the results are representative of three separate experiments.


A
Relative invasion
250
200
150
100
50
0
***
Mock
J6/JFH
B
% HCV infected cells
100
80
60
40
20
0
Control
Invaded cells

Previous research reported that HCV infection promotes Vascular endothelial growth factor (VEGF) expression (191, 341, 400). Chris Mee has since confirmed this in our laboratory (Fig.4.10A). VEGF promotes angiogenesis and elevated VEGF levels in patients with HCC are considered to be one of the most important indicators of increased tumor angiogenesis (214). Angiogenesis is the process by which new blood vessels are formed and is vital in tumor development. Without a blood supply tumors cannot grow above a certain size due to hypoxia and lack of essential nutrients. Blood supply is also essential for tumor cells to metastasize to other areas of the liver or to other organs, a common complication of HCC (537). Apart from its important role in angiogenesis VEGF has been demonstrated to reduce HepG2 hepatoma cell polarity (319, 423). VEGF has also been reported to increase the migration of HepG2 cells (531). We therefore investigated whether exogenous VEGF stimulation (Fig.4.10B) or neutralization of endogenous VEGF with an anti-VEGF mAb (VG67e) (Fig.4.10C) would modulate Huh-7.5 cell invasion. We observed no effect of exogenous VEGF or VG67e on Huh-7.5 cell invasion suggesting that VEGF does not promote Huh-7.5 cell invasion.

Figure 4.10: Huh-7.5 hepatoma cell invasion is independent of VEGF

(A) Levels of VEGF secretion from naïve and J6/JFH infected Huh-7.5 cells was quantified by ELISA (Peprotech), data provided by Chris Mee. A Two tailed unpaired T-test was performed to determine whether there was a significant difference in VEGF production between naive and J6/JFH infected cells. A significant difference was observed $P<0.0001$. Huh-7.5 cells were cultured in serum free DMEM overnight. Cells were removed from tissue culture plastic with trypsin and re-suspension in serum free DMEM containing (**B**) exogenous VEGF (10ng/ml) or untreated control and (**C**) anti-VEGF (VG67e) (400ng/ml) or untreated control. Cells were seeded ($4x10^4$ cells/200μl/well) into the top chamber of an 8μm pore collagen coated transwell insert. The lower chamber contained 300μl of serum free DMEM and relevant treatment. Cells were incubated at 37°C for 24hrs. Non-invaded cells were mechanically removed and invaded cells methanol fixed and stained with 0.1% crystal violet. 3x fields of view were imaged per transwell at 40x magnification on a fluorescent microscope (NikonTE2000, Japan). The number of invaded cells was determined and invasion expressed relative to control. Two tailed unpaired T-test's were performed to determine whether there was a significant difference in invasion between untreated and exogenous VEGF treated or anti-VEGF (VG67e) treated cells. No significant differences were observed $P>0.05$. Error bars indicate standard deviation from the mean. Assays were performed in duplicate and the results are representative of three separate experiments.

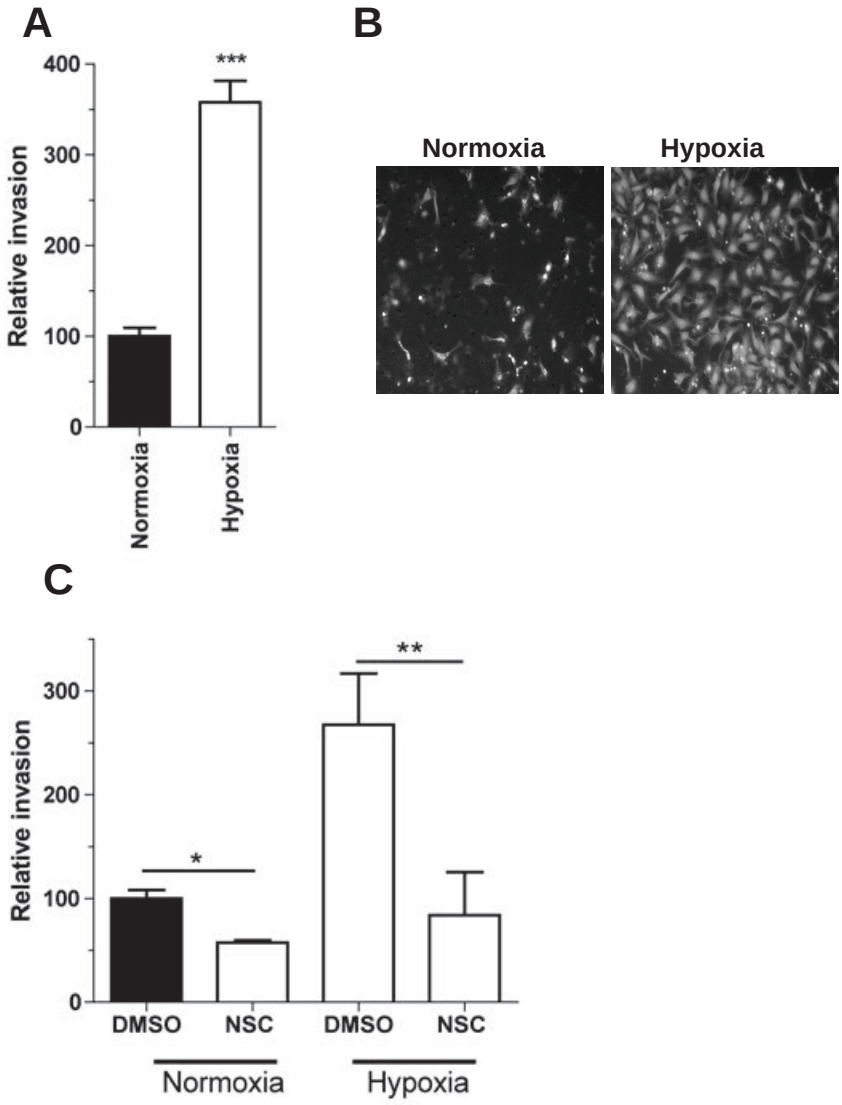

A
B
C
VEGF pg/ml
[40 000 cells]
100
80
60
40
20
0
***
Mock
J6/JFH
Relative invasion
200
150
100
50
0
Control
VEGF
Relative invasion
200
150
100
50
0
Control
VG67e

VEGF production is increased in hepatoma cells following HCV infection through stabilization of hypoxia inducible facor -1alpha (HIF-1α) (191, 341, 400). HIF-1α is a regulatory sub unit of the HIF transcription complex. Under normal cellular oxygen levels HIF-1α is continually expressed and degraded by the following oxygen dependent process; HIF-1α is hydroxylated by HIF prolylhydroxylases enabling it to be recognized by von Hippel-Lindau tumor suppressor protein (pVHL). pVHL is a recognition component of the E3 ubiquitin ligase complex and targets HIF-1α for proteasomal degradation (238). If HIF-1α becomes stably expressed it is translocated to the nucleus where it binds to ubiquitously expressed HIf-1β and recruits other transcriptional co-factors making up the HIF transcription complex (238). As well as VEGF the HIF transcription complex is responsible for the up-regulation of numerous genes responsible for proliferation, cell survival, metastasis and glycolysis (238). HIF-1α can be stabilized through a number of mechanisms including growth factor induction, genetic mutations of oxygen sensing genes, loss of tumor suppressor function, mitochondrial dysfunction as well as hypoxia (388). To determine whether hepatoma cell invasion increases upon stabilization of HIF-1α we compared invasion of Huh-7.5 cells under hypoxic (1%O_2) or normoxic (22% O_2) conditions. We observed a significant increase in cell invasion under hypoxic conditions compared to the normoxic control (Fig.4.12A&B). To establish whether the increased invasive potential is dependent on HIF-1α, hepatoma cells were treated with the HIF-1 translation inhibitor, NSC-134754 (1μM) (79, 388). We observed a significant decrease in cell invasion under hypoxic conditions in the presence of inhibitor whilst only a moderate inhibition was observed under normoxic conditions. We propose that the increased invasion of HCV infected hepatoma cells may be

mediated through a VEGF independent pathway induced by the stablization of HIF-1α.

Figure 4-11: Hypoxia promotes Huh-7.5 cell invasion.

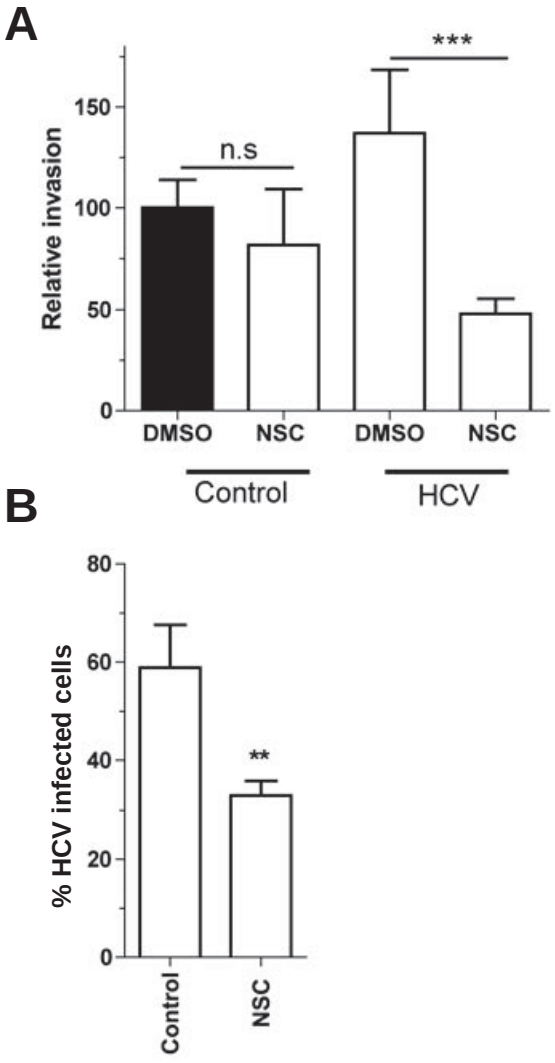
(A) Huh-7.5 cells were cultured in serum free DMEM overnight. Cells were removed from tissue culture plastic with trypsin and re-suspended in serum free DMEM or (**B**) DMEM containing NSC-134754 (1μM) or DMSO control. Cells seeded ($4x10^4$ cells/200μl/well) into the top chamber of an 8μm pore collagen coated transwell insert. The lower chamber contained 300μl of serum free DMEM +/- relevant treatment. Cells were incubated at 37°C for 24hrs in normoxic ($22\%0_2$) or hypoxic ($1\%0_2$) conditions. Non-invaded cells were mechanically removed and invaded cells methanol fixed and stained with 0.1% crystal violet. 3x fields of view were imaged per transwell at 40x magnification on a fluorescent microscope (NikonTE2000, Japan). A Two tailed unpaired T-test was performed to determine whether there was a significant difference in invasion between Huh-7.5 cells under normoxic and hypoxic conditions. A significant difference was observed $P<0.0001$. Further Two tailed unpaired T-tests were performed to determine whether there was a significant difference in invasion of Huh-7.5 cells under normoxic or hypoxic conditions in the presence and absence of HIF inhibitor. T tests were performed on raw data. Significant differences were observed $P=0.027$ and 0.0039 respectively. Images in (**A**) are representative of invaded cells in normoxic and hypoxic conditions. The number of invaded cells was determined and invasion expressed relative to control. Error bars indicate standard deviation from the mean. Assays were performed in duplicate and the results are representative of three independent experiments.


A
B
C
Relative invasion
400
300
200
100
0
***
Normoxia
Hypoxia
Normoxia
Hypoxia
Relative invasion
300
200
100
0
*
**
DMSO
NSC
DMSO
NSC
Normoxia
Hypoxia

To determine whether a HIF inducible factor is responsible for the increased invasive potential of HCV infected cells we quantified the invasion of uninfected and HCV infected Huh-7.5 cells in the presence of HIF inhibitor, NSC-134754 (79, Poon, 2009 #570). We observed a significant decrease in invasion of infected cells in the presence of NSC-134754 (1μM) (Fig.4.11A). Interestingly we also observed a significant decrease in the percentage of NS5A positive invaded cells in the presence of NSC-134754 (Fig.4.11B). The HIF inhibitor may specifically inhibit the invasion of HCV infected cells or may differentially regulate the proliferation of uninfected and HCV infected cells or block HCV replication.

Figure 4.12: HCV induced cell invasion is sensitive to HIF-1α inhibitor.

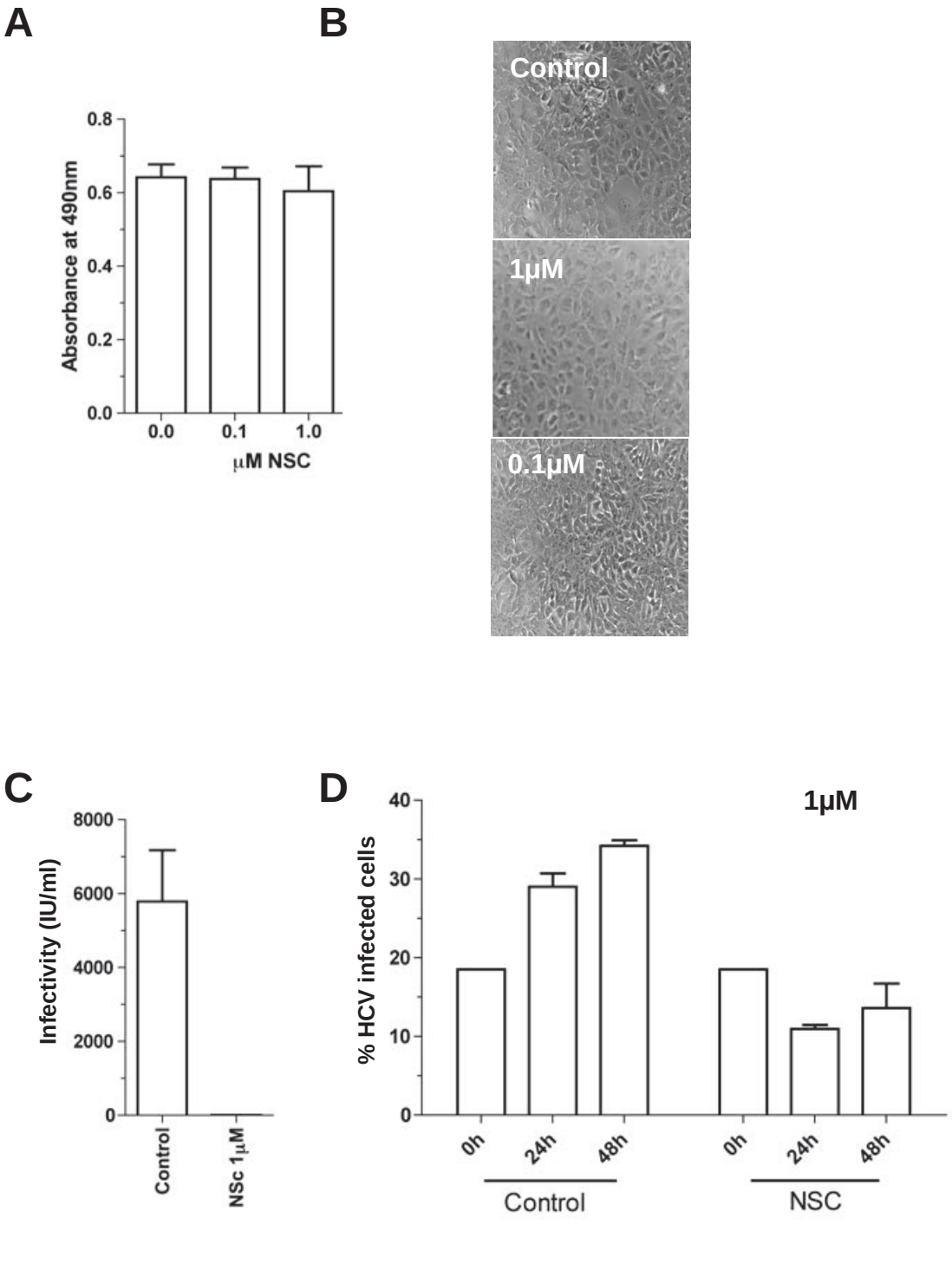
HCV J6/JFH RNA or control no RNA was delivered into Huh-7.5 cells by electroporation (ep). 72hrs post ep cells cultured in serum free conditions overnight. HCV infected and control cells were labeled with CMFDA, removed from tissue culture plastic with trypsin and re-suspended in serum free DMEM containing NSC-134754 (1μM) or DMSO control. Cells were seeded ($4x10^4$ cells/200μl/well) into the top chamber of an 8μm pore collagen coated transwell insert. The lower chamber contained 300μl of serum free DMEM plus relevant treatment. Cells were then incubated at 37°C for 24hrs. Non-invaded cells were mechanically removed and invaded cells methanol fixed. (**A**) 3x fields of view were imaged per transwell at 40x magnification on a fluorescent microscope (NikonTE2000, Japan). The number of invaded cells was determined and invasion expressed relative to control. No significant difference in invasion of naïve cells in the presence and absence of HIF inhibitor was observed (P=0.1726, two tailed unpaired T-test). A significant difference was observed in invasion of J6/JFH infected Huh-7.5 cells in the presence and absence of HIF inhibitor (P=0.0270, two tailed unpaired T-test). (**B**) Fixed cells were stained for NS5A and infection level determined for control and NSC-134754 treated cells. A significant difference was observed in infection level of invaded cells in the presence and absence of HIF inhibitor (P=0.0013, two tailed unpaired T-test). Error bars indicate standard deviation from the mean. Assays were performed in duplicate and the results are representative of three separate experiments.


A
***
n.s
150
100
50
0
Relative invasion
DMSO
NSC
DMSO
NSC
Control
HCV
B
80
60
40
20
0
% HCV infected cells
**
Control
NSC

To investigate this further we assessed the effects of the HIF inhibitor on HCV replication and hepatoma proliferation. At 1μM the HIF inhibitor successfully inhibited HCV infection (Fig.4.12A). To determine whether the HIF inhibitor affects HCV transmission J6/JFH infected cells were treated with NSC-134754 and viral spread quantified over a 48hr period by monitoring NS5A expression by flow cytometry (Fig.4.12B). A decrease in the percentage of infected cells in the presence of NSC-134754 compared to control was observed, demonstrating that NSC-134754 inhibits new rounds of infection. To determine whether the HIF inhibitor has a cytotoxic or anti-proliferative effect on Huh-7.5 cells we performed an MTS assay (colorimetric test that measures metabolic function). We observed a small decrease in MTS signal after a 24hr treatment with NSC-134754 compared to control (Fig.4.12C), this can be more clearly seen in the corresponding phase images (Fig.4.12D). Results suggest that HCV infection may be dependent on HIF-1α stabilization.

Figure 4.13: HIF-1α dependent modulation of proliferation and infection.

(A) Huh-7.5 cells were treated with NSC-134754 and control at 5,1,and 0.1 μM plus DMSO control. 24hrs post treatment an MTS assay was performed to determine metabolic function results presented as absorbance at 490nm (minus background). (**B**) Representative phase images of treated cells prior to MTS assay. (**C**) Huh-7.5 cells were seeded into 96 well tissue culture plate ($0.75x10^4$ cells/well), the following day cells were pre-treated for 1hr with DMSO control or NSC-134754 (1μM) containing DMEM (3% FBS). Cells were infected with J6/JFH HCVcc in the presence or absence of inhibitor. 48hrs post infection cells were fixed with ice-cold methanol and stained for NS5A. Infected cells enumerated and infection level represented as Infectious units (IU) per ml. (**D**) HCV J6/JFH RNA was delivered into Huh-7.5 cells by electroporation (ep). 48hrs post ep cells seeded into 12 well dishes ($25x10^4$cells/well) in the presence of DMSO control or NSC-134754 (1μM) containing DMEM (3% FBS). 0, 24 and 48hrs post treatment cells were harvested, fixed, stained for NS5A and quantified using flow cytometry. Results presented as frequency of NS5A positive cells. Error bars indicate standard deviation from the mean. Assays were performed in duplicate and the results are representative of three independent experiments.


A
B
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0.8
0.6
0.4
0.2
0.0
0.0
0.1
1.0
µM NSC
Control
1µM
0.1µM
C
D
Infectivity (IU/ml)
8000
6000
4000
2000
0
Control
NSc 1µM
% HCV infected cells
40
30
20
10
0
1µM
0h
24h
48h
0h
24h
48h
Control
NSC

4.4 Discussion

HCV infection is one of the leading causes of hepatocellular carcinoma and recent research suggests HCV protein expression may be directly responsible (17, 33, 89, 294, 330, 340). Liver cancer is very difficult to treat and is associated with a high frequency of relapse and metastasis (537). As such a greater understanding of the processes involved is desperately needed for the development of future anti-cancer drugs. In this chapter we demonstrate a role for HCV receptor tetraspanin CD81 in hepatoma invasion and furthermore report that HCVcc infection increases the invasive potential of hepatoma cells.

Tetraspanins are relatively small membrane proteins and are not believed to directly interact with extra-cellular matrix (ECM) proteins (530), however a number of tetraspanins namely CD151, CD82, CD9 and importantly CD81 are associated with integrins and may therefore be able to indirectly alter cell-ECM interactions (427, 42, 43, 195) . Major integrin-ligand binding combinations for the ECM proteins we used are as follows; Fibronectin binds αIIβ3, αVβ3, αVβ6, αVβ1, α5β1, α8β1, collagen binds αI0β1, α2β1, α1β1, α11β1, and finally laminin binds α10β1, α2β1, α1β1, same as collagen, and additionally α7β1, α6β4, α6β1 and α3β1 (212). A number of integrin subunits were detected on the cell surface of Huh-7.5 hepatoma cells (Fig.4.3.B). This included high expression levels of the most predominant beta subunit, β1, as well as detection of a number of alpha subunits that form dimers with the β1 subunit to bind fibronectin (α5), collagen, (α1, α2) and laminin (α1, α2, α6). We observed no effect of CD81 expression on hepatoma cell (Huh-7 and HepG2 cells) adhesion to different ECM proteins (Fig.4.2B&C). In addition to studying the effect(s) of tetraspanin expression on cell adhesion, tetraspanin targeting antibodies have also

been reported to alter cell-ECM adhesion by regulating tetraspanin – partner protein interactions (177, 352, 427). Adhesion of hepatoma cells to ECM protein, fibronectin, was not affected by ligation of cell surface CD81 with a diverse panel of anti-CD81 mAbs (previously described in chapter 3) (Fig.4.2A). In conclusion our findings demonstrate that CD81 is not involved in hepatoma cell-ECM adhesion.

Previous research reported the induction of microtubule like projections from mammary carcinoma cells embedded in matrigel after exposure to CD81 mAbs, thought to be coordinated through an association of CD81 with laminin binding integrin $\alpha 3\beta 1$ (460). We were unable to detect $\alpha 3$ integrin cell surface expression on our hepatoma cells using flow cytometry analysis. Numerous filopodia like protrusions were observed extending from the cell body of Huh-7.5 cells and CD81 was highly expressed along the cell membrane of these protrusions, ideally located to function in the modulation of filopodia and cell migration. However, CD81 engagement did not promote filopodia/microtubule like protrusions (Fig.4.6). Furthermore ligation of CD81 had no effect on hepatoma cell migration and invasion (Fig.4.7A&B).

However, CD81 expression was observed to increase hepatoma migration in two independent cell lines and in both the scratch wound and collagen invasion transwell assays (Fig.4.8). This is in contrast to an earlier publication by Mazzoca et al., 2008 (313) who reported decreased invasion of CD81 expressing HepG2 cells in response to Insulin-like growth factor (IGF-1) stimulation. CD81 was reported to coordinate this process through its association with type II phosphoinositide 4-kinase (PI4KII) leading to the re-localization of actinin-4 into CD81 enriched intracellular vesicles

preventing actin cytoskeleton remodeling. These findings were supported with an *in vivo* mouse model experiment whereby Huh-7 cells with un-detectable levels of CD81 were observed to metastasize more than those expressing CD81, of note the differences between the two cell types were small and the error bars large. The discrepancies between the two data sets are difficult to explain. IGF-1 is an inducer of epithelial mesenchymal transition (EMT) (168), a complex process often involved in tumor progression, it is therefore important to study cell invasion/migration in the absence of exogenous factors. In the absence of IGF-1 Mazzoca et al., (2008) (313) observed no differences in the invasion of HepG2 and HepG2 cells expressing CD81. Interestingly CD81 was observed to be highly expressed in intracellular vesicles in the HepG2 cells featured in Mazzoca et al., 2008 (313) this differs from our cells where CD81 is predominantly expressed at the plasma membrane. *In vivo* studies of hepatocytes in liver tissue also report that CD81 is expressed predominantly around the plasma membrane (396). This comparison suggests that CD81 may not be localized correctly in the HepG2 cells in Mazzoca's paper, and may explain the discrepancy between our results. In support of Mazzoca's findings a previous publication reported that a loss of CD81 expression was associated with differentiation and metastasis in HCC patients, however this report is limited having only a small sample number (219). Observations in our laboratory would suggest that CD81 expression is not altered in the diseased liver (396). It is clear that further *in vivo* research is needed to clarify whether CD81 expression is associated with liver cancer progression.

In support of our findings that CD81 promotes hepatoma invasion and migration CD81 expression in other cell types has been reported to increase their invasive

potential (72, 262, 312, 528). Recent reports are beginning to unravel the highly dynamic and complex relationship between CD81 and its partner proteins (72, 247, 262). CD81 is reported to associate with MT1-MMP (247, 262) along with other tetraspanins including CD151, CD9 and TSPN12 (262, 529). MT1-MMP is a membrane bound matrix metalloproteinase that enables cell penetration into ECM, high expression levels of MT1-MMP have so far been reported to associate with poor prognosis in breast cancer patients, no research to date has looked at its role in HCC (205, 224). Lafleur et al., 2009 (262) reported that CD81 expression increases MT1-MMP function by preventing lysosomal degradation, this may explain the increased migration observed in our hepatoma cells. Interestingly in the study by Lafeur et al., 2009 a double knock out of CD81 and CD9 was needed to observe this phenotype, in future studies it may be of interest to investigate whether a stronger phenotype is observed following knock down of both CD81 and CD9 in hepatoma cells. Other CD81 associated membrane proteins have also been reported to modulate the migratory phenotype of cells, including immunoglobulin-like proteins EWI-2 and EWI-F (72, 247, 411). Chambrion et al., 2010 (72) reported that CD9 and CD81 expression in HEK-293 cells negatively regulated EWI-F(CD9-P1) induced effects on cell migration. A further publication this time investigating EWI-2 function reported that expression of EWI-2 to glioblastoma cell lines reduced CD9 and CD81 association with MMP-2 and MT1-MMP leading to a decrease in invasion potential (247). These observations suggest that EWI-2 and EWI-F expression levels may regulate CD81 localization and function or vice versa and are worth considering in future experiments. Previous research into tetraspanin partner-protein interactions and function has predominantly centered on biochemical-immunoprecipitation studies. Current research utilizing new live cell imaging techniques in particular total

internal reflection microscopy (TIRF) and single particle tracking technologies suggest that tetraspanins are more dynamic than originally thought and diffuse between tetraspanin enriched microdomains as a single or a group of molecule(s) (132). Our laboratory has now started to use these techniques for *in vitro* study of CD81 dynamics in hepatoma cells (Helen Harris, unpublished data).

In the previous chapter we reported that HCV infection reduced CD81 induced cell spread, we therefore wanted to investigate the effect(s) of infection on CD81 function in hepatoma-ECM adhesion and invasion through collagen. We observed no effect(s) of infection on hepatoma integrin expression (Fig.4.3C), hepatoma–ECM adhesion (Fig 4.3A&B), or hepatoma ECM expression (Fig. 4.4 & 4.5). However infection promoted hepatoma invasion through collagen (Fig. 4.9).

Since antibody engagement of CD81 had no effect on hepatoma cell-ECM adhesion, migration or invasion we hypothesized that it would be unlikely for HCV particle association with cell surface expressed CD81 to account for the increased invasion of infected cells. Given our results that siRNA silencing of CD81 expression reduced hepatoma invasion, we hypothesize that CD81 expression levels may predict the invasive capacity of hepatocytes. Earlier results showing that HCV infection does not alter CD81 expression levels (Fig.3.11C) eliminate this as a possible explanation for their increased invasive capacity.

The complex nature of cellular migration and invasion means that HCV infection could increase hepatoma invasion through numerous mechanisms independent of CD81. If HCV infected cells have an increased invasive potential perse one might

expect the frequency of infected invaded cells to be greater than the control population. This was not the case and thus we postulated that a soluble factor secreted from the infected cells might have a paracrine effect on the entire infected cell population (Fig.4.9). Previous research reported that HCV infection causes mitochondrial deregulation resulting in stabilization of HIF-1α (191, 341, 380, 400). Nasimuzzaman et al., 2007 (341) identified that HIF-1α stabilization in hepatoma cells induced by HCV protein expression resulted in an up-regulation of VEGF expression. Increased expression of VEGF upon HCV infection was confirmed by work performed in our laboratory (Fig.4.10). We hypothesized that VEGF may regulate hepatoma migration as previously described (531). However, exogenous VEGF or neutralization of endogenous VEGF had no effect on Huh-7.5 cell invasion (Fig.4.10). These results suggest HCV infection induced increase in invasion potential is VEGF independent.

To investigate whether a HIF-1α inducible factor may be responsible for the increased invasion observed for HCV infected cells, uninfected Huh-7.5 cells were subjected to hypoxic conditions and their invasion compared to cells in replicate wells in normoxic conditions. A dramatic increase in hepatoma cell invasion was observed under hypoxic conditions compared to control, this increased invasion was inhibited by HIF-1α inhibitor NSC-134754 (Fig.4.12), demonstrating that HIF-1α stabilization promotes hepatoma invasion. The HIF-1α inhibitor inhibited the invasion of infected cells, and interestingly the frequency of infected invaded cells was reduced compared to control (Fig.4.13). There are several interpretations of this data; HIF-1α inhibitor (i) specifically reduced the invasion of HCV infected cells or (ii) differentially regulated the proliferation of uninfected and HCV infected cells or (iii) blocked HCV

replication. To ascertain whether the HIF-1α is essential for HCV replication we investigated cell free infection and transmission of HCV infection in the presence and absence of the HIF-1α inhibitor. The HIF-1α inhibitor completely blocked HCV infection and furthermore transmission of HCV infection during cell culture was perturbed (Fig.4.14). These results suggest that HCV infection is dependent on HIF-1α and explain why there was a reduced frequency of infected invaded cells in the presence of inhibitor. A modest reduction in Huh-7.5 cell proliferation was observed in the presence of the HIF-1α inhibitor at 1μM concentration, this was most clearly observed by phase microscopy (Fig.4.14). It is reported that Huh-7.5 cells under normal conditions do not stably express HIF-1α (341, 400), suggesting NSC-134754 exerts a degree of non-specific inhibition of hepatoma cell proliferation.

We can conclude that HCV infection increases hepatoma cell invasion through a VEGF independent pathway and postulate that this could be through an alternative protein induced by HIF-1α stabilization. We can also speculate that induction of HIF-1α may be an advantage to HCV infection whilst having a possible pathological consequence for the host.

5. Results: Neutralizing antibody resistant HCV transmission

5.0 Introduction

It is accepted that HCV persists *in vivo* predominantly through rapid sequence evolution. HCV replicates using an RNA dependent polymerase (RdRp) that has no proof reading ability, thus resulting in a highly varied viral population in an individual patient termed a quasispecies (closely related species of virus subjected to genetic mutations, competition and selection) (37, 117). In general the humoral immune response is one of the first lines of defense against microbial infections. Neutralizing antibodies (nAbs) are readily detected in chronically infected patients (29, 290). Although these antibodies have been shown to effectively neutralize infection in standard *in vitro* assays they are un-able to clear the virus *in vivo* (454). Studies in both humans and chimpanzees have shown that HCV evolves to escape from nAbs (290, 497). In particular sequence evolution in a highly variable region of E2 (HVR-1) is reported to occur in response to nAbs (434, 450, 497). HCV has also been shown to escape nAbs targeting functional domains of E2 for example the CD81 binding domain (7, 152, 180, 226, 240, 359). Escaping from these antibodies though comes at a great price to viral particle infectivity (239). It is therefore likely that HCV utilizes other mechanisms to escape the humoral immune response.

Many viruses have developed mechanisms to transmit directly from cell-to-cell within an infected host. These routes of transmission are less reliant on particle diffusion and are generally considered to be more efficient. In some cases cell-to-cell transmission can protect virions from nabs and are therefore often defined as neutralizing antibody resistant (337, 415).

The HCV retroviral pseudo-particle system (HCVpp) has been used to determine the efficacy of patient nAbs against native E1 and E2 glycoprotein's (30, 179, 208, 391). HCVpp has been invaluable to understand the humoral immune response to HCV although as previously explained this system has its limitations. HCVpp can only be used to measure viral cell entry and not secondary infection events and therefore cannot be used to understand cell-to-cell transmission of the virus. With the recent development of the cell culture system allowing efficient propagation of full length HCV virus genomes *in vitro* (HCVcc) it is now possible to investigate cell-to-cell transmission of HCV (283, 499, 560).

A recent study from our laboratory reported that HCVcc infection can spread in the presence of an agarose overlay, traditionally used to minimize cell free viral dissemination, and in the presence of nAbs, suggesting that HCV can transmit via cell free and cell-to-cell routes (480). A new technique using two-photon microscopy detected clusters of virus antigen expressing hepatocytes within infected liver tissue, again indicative of HCV cell-to-cell transmission (278).

In this study we aimed to establish an *in vitro* co-culture assay to investigate nAb resistant cell-to-cell HCV transmission.

5.1 Establishment of an *in vitro* co-culture system to study neutralizing antibody resistant transmission.

To quantify viral transmission *in vitro* it is essential to distinguish naïve uninfected target cells from infected producer cells. Timpe et al., 2008 previously published a co-culture assay in which target cells were labeled with a fluorescent dye, CMFDA ,and co-cultured with unlabelled producer cells. Following a 48hr incubation the cells were harvested, stained for viral antigen NS5A and analysed by flow cytometry. NS5A+ / CMFDA+ cells were identified as infected target cells. It has since become apparent that this method yields false positives, by the aggregation of unlabeled infected producer cells and labeled target cells. In contrast labeling infected producer cells instead of target cells and quantification of CMFDA-/NS5A+ cells eliminates false positives as demonstrated in Figure 5.1.

The revised co-culture assay used throughout this study is depicted in Figure 5.2. Briefly Huh-7.5 cells were electroporated with full length HCV RNA and incubated at 37°C for 72hrs. These infected producer cells were labeled with CMFDA and co-cultured at a 1:1 ratio with naïve unlabelled “target cells” in the presence of control or nAb for a defined period of time. Upon culmination of the assay both the supernatant and cells were harvested. The levels of infectious extra-cellular virus were measured by inoculating Huh-7.5 cells as listed in the materials and methods. Harvested cells were fixed, stained for NS5A, and analyzed by flow cytometry to determine the frequency of newly infected target cells. Non-structural protein NS5A is only expressed once replication has occurred making it a good marker for infectivity (383). Despite the elimination of all cell free virus, 14.6% of target cells still became infected (Fig.5.2), demonstrating the presence of nAb resistant HCV transmission.

Figure 5.1: Comparison of labeling method to enumerate HCV transmission.

Transmission from HCV infected Huh-7.5 producers to Huh-7.5 or to the relatively non-permissive Huh-7 Lunet cells was monitored by flow cytometry. (**A**) The lower two FACS plots represent transmission observed where target cells were labeled with CMFDA. The upper two FACS plots represent transmission observed in a parallel experiment where producer cells were labeled with CMFDA. The apparent infection of Huh-7.5 cells was higher when the target cells were labeled (lower left/Blue compared to upper left./Red). Approximately 25% of CMFDA positive Huh-7 Lunet cells were recorded as NS5A positive (lower right/Blue) in contrast to very few Huh-7 Lunet cells detected in the NS5A positive quadrant when the target cells were unlabeled (upper right/Blue). (**B**) Further analysis of forward vs. side scatter plots of the infected target cell population revealed that double CMFDA/NS5A positive cells (Red) were composed mainly of aggregates (high forward scatter), in contrast to the NS5A positive cells (Red) where the majority of cells appeared as a single cell population (low forward scatter)

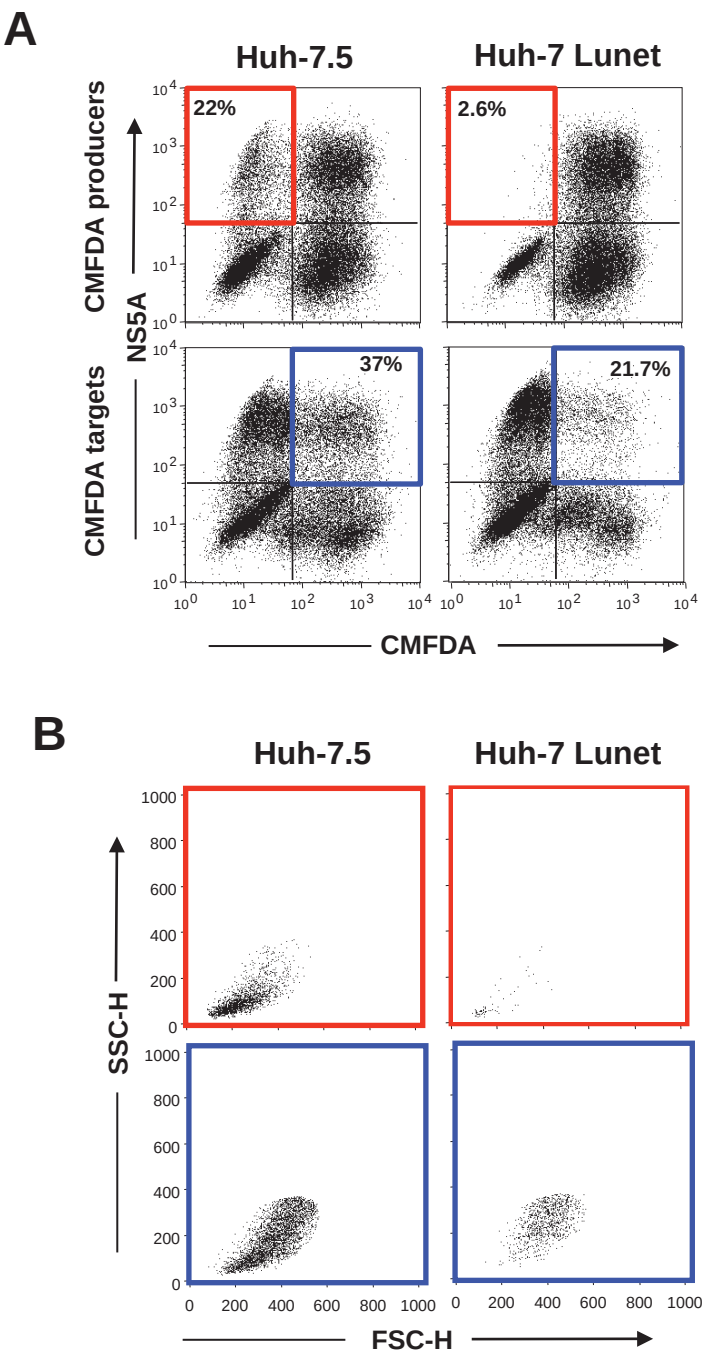
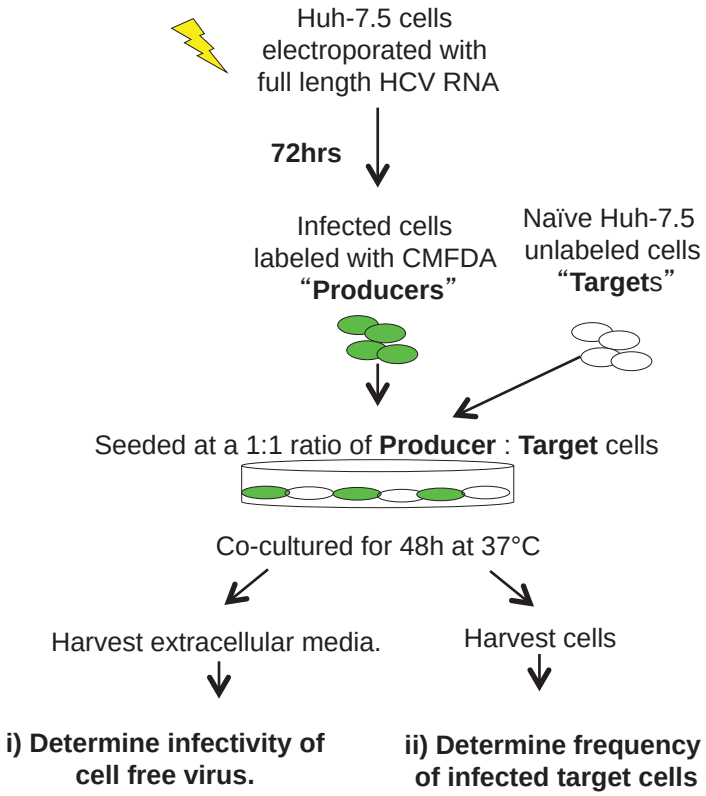

A
Huh-7.5
Huh-7 Lunet
CMFDA producers
NS5A
CMFDA targets
22%
2.6%
37%
21.7%
CMFDA
B
Huh-7.5
Huh-7 Lunet
SSC-H
FSC-H

Figure 5.2: The infectious co-culture assay.

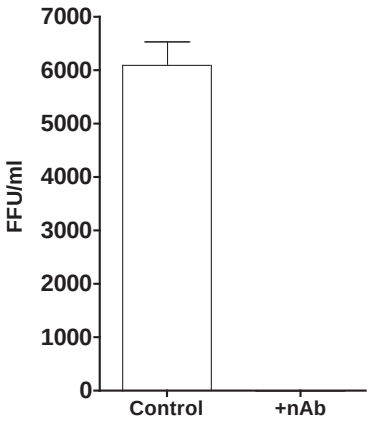
(**A**) HCV strain H77/JFH RNA was delivered into producer cells by electroporation (ep). 72h post ep producer cells were labeled with CMFDA and co-cultured with unlabeled naïve target cells at a 1:1 ratio in the presence of 10μg/ml irrelevant control IgG or anti-E2 nAb 9/27. (**B**) i) Supernatant was collected upon termination of the assay and used to infect naïve Huh-7.5 cells in a standard infectious assay, infectivity was readily detectable in the presence of control IgG, none was observed in culture media containing nAb, data represented as foci forming units per ml (FFU/ml). ii) Cells were harvested, fixed, stained for NS5A and quantified by flow cytometry. The cartoon is typical of the data obtained; events appearing within the top left hand quadrant represent (CMFDA - / NS5A +) infected target cells, representative dot plots are shown where the frequency of infected target cells is depicted.

A

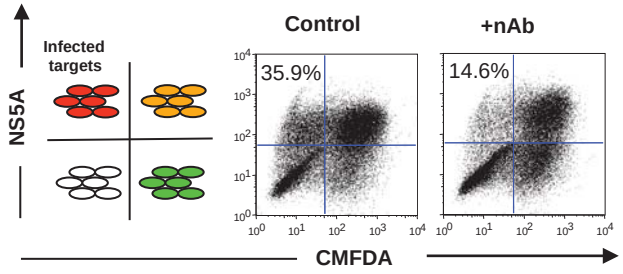


B

i) Determine infectivity of cell free virus



ii) Determine frequency of infected target cells



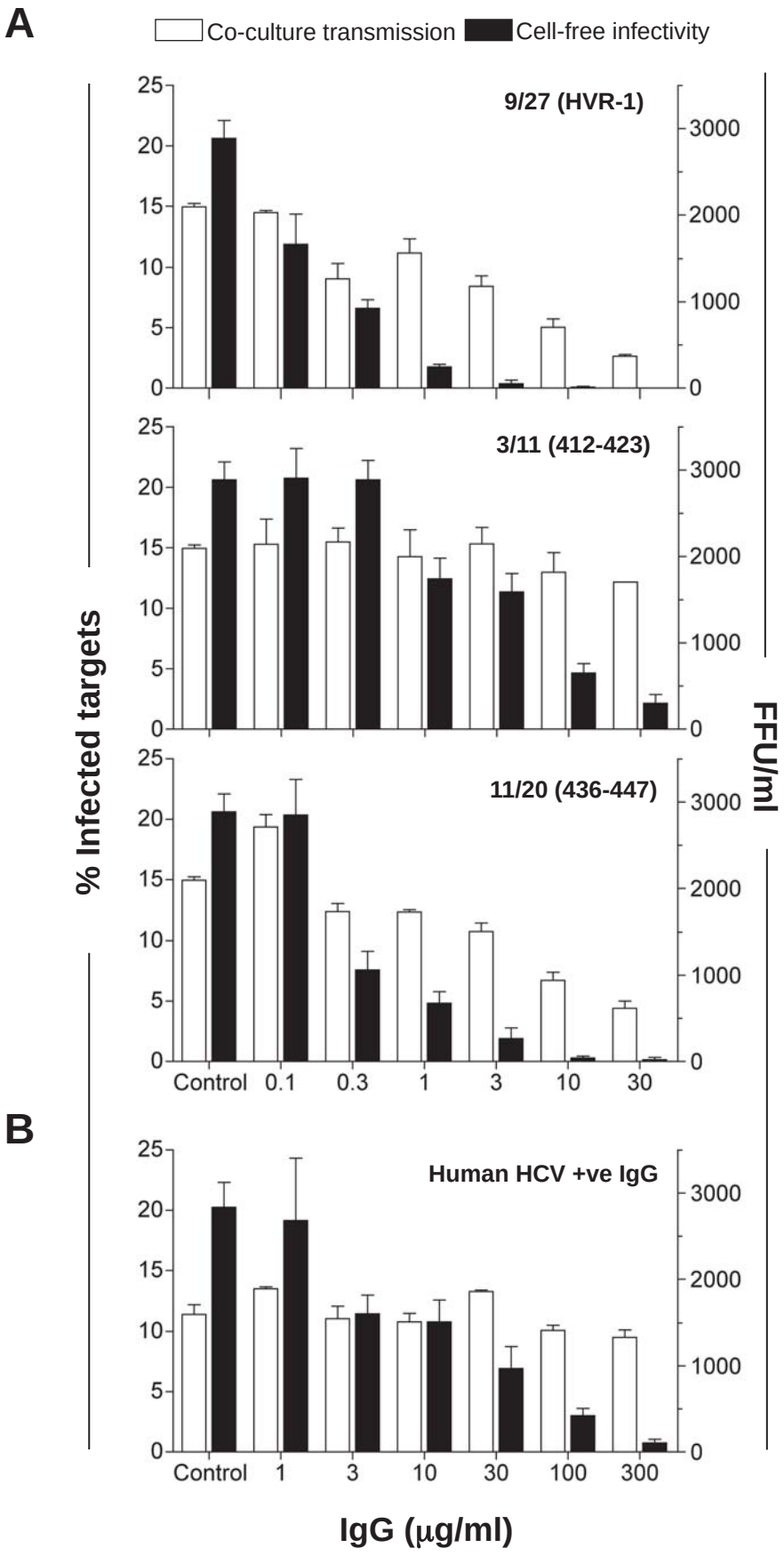
We next wanted to establish whether antibodies to different epitopes of E2 also fail to neutralize cell culture transmission. Three epitope specific antibodies were used 9/27, 3/11 and 11/20 (208). These antibodies target specific regions of H77 E2 glycoprotein. 9/27 targets the HVR-1 region (396-407) and 11/20 and 3/11 target different regions of the CD81 binding domain (412-423 and 436-447 respectively). Pooled patient IgG isolated from 6 HCV chronically infected patients was also used. All antibodies were evaluated for their effects on HCV strain H77/JFH chimera, transmission (Fig.5.3).

Patient IgG and mAbs 9/27 and 11/20 neutralized all detectable cell-free virus at the following concentrations; 3μg/ml for 9/27, 10μg/ml for 11/20 and 300μg/ml for patient sera. In the absence of detectable extra-cellular virus HCV transmission still occurred, interestingly the percentage of newly infected target cells varied depending on the antibody under study. Both 9/27 and 11/20 were the most effective Abs at reducing transmission and continued to neutralize infection once all cell-free virus had been eliminated, however they were unable to completely ablate transmission at a maximum concentration of 30μgml.

From these assays we were able to define optimal conditions to measure cell-to-cell transmission. For H77/JFH chimera we used mAb 9/27 at 4μg/ml. This was the most effective neutralizing antibody and at this concentration we could be confident that all cell free virus was neuralized. Where other JFH-1 chimeric viruses were used, cell free infectivity was neutralized using pooled patient IgG at 300μg/ml.

Figure 5.3: Effect of anti-glycoprotein antibodies on H77/JFH cell free infectivity and co-culture transmission.

(**A**) Anti-E2 monoclonal antibodies 9/27, 3/11, and 11/20 and (**B**) Pooled IgG isolated from 6 HCV infected individuals were titrated for their effect(s) on H77/JFH transmission. Co-culture transmission (white bars), expressed as the percentage of infected target cells, plotted against the left Y-axis. Cell-free infectivity (black bars) expressed as foci forming units per ml (FFU/ml), plotted against the right Y-axis. The treatments were performed in duplicate and the error bars indicate standard deviation from the mean. The data set is representative of three independent experiments.


A
Co-culture transmission
Cell-free infectivity
9/27 (HVR-1)
3/11 (412-423)
11/20 (436-447)
% Infected targets
FFU/ml
Control 0.1 0.3 1 3 10 30
B
Human HCV +ve IgG
Control 1 3 10 30 100 300
IgG (μg/ml)

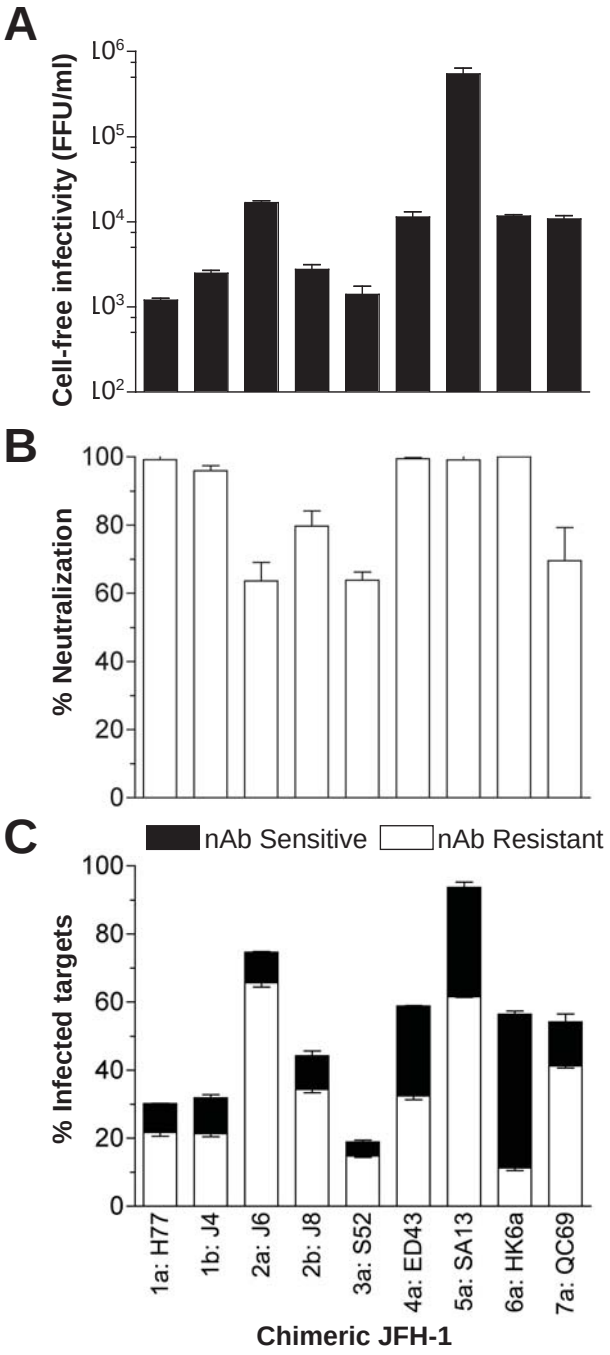
5.2 Transmission of diverse HCV genotypes in co-culture.

HCV can be grouped into 7 major genotypes and numerous sub-types (166, 444). To investigate whether all genotypes can transmit in co-culture in the presence of nAbs Huh-7.5 cells were electroporated with RNA from 11 chimeric JFH viruses, representative of all major genotypes and subtypes (166). Co-culture transmission was determined in the presence of pooled patient IgG at 300μg/ml and control IgG.

The levels of infectious cell free virus varied between the different genotypes and generally predicted efficiency of co-culture transmission (Fig.5.4A), for example genotype 5a virus (SA13/JFH) produced the highest level of cell-free virus and transmitted the most efficiently (Fig.5.4C). Cell-free virus of genotypes 1a, 1b, 3, 4, 5 and 6 were completely neutralized by pooled patient IgG (Fig.5.4B). We determined the proportion of nAb resistant (co-culture transmission in the presence of nAb) and nAb sensitive transmission (Total co-culture transmission minus nAb resistant transmission) for all viruses (Fig.5.4C). nAb resistant transmission constituted greater than 50% of the transmission events with the exception of genotype 6a virus (HK61/JFH) indicating that this is a preferred route of transmission in co-culture under the conditions used in this assay.

Figure 5.4: Genotype transmission in co-culture.

A panel of chimeric JFH-1 RNA was delivered into Huh-7.5 cells by ep. 72hrs post ep cells were labeled with CMFDA and co-cultured with unlabelled target cells in the presence of pooled patient IgG or control IgG (300μg/ml) for 48hrs. Supernatants were harvested at the end of the assay and evaluated for their levels of infectious virus, (**A**) cell free infectivity displayed as foci forming units per ml (FFU/ml) and (**B**) neutralization of cell-free infectivity presented as percentage neutralization. (**C**) Cells were harvested, stained for NS5A and analysed by flow cytometry to determine the frequency of newly infected target cells. Results displayed as a stacked histogram, the white bars represent nAb resistant (Percentage infected target cells in the presence of nAb) and black bars represent nAb sensitive (Total transmission – nAb resistant transmission) transmission. The treatments were performed in duplicate and the error bars indicate standard deviation from the mean. The data set is representative of two independent experiments.


A
Cell-free infectivity (FFU/ml)
10^6
10^5
10^4
10^3
10^2
B
% Neutralization
100
80
60
40
20
0
C
nAb Sensitive
nAb Resistant
% Infected targets
100
80
60
40
20
0
1a: H77
1b: J4
2a: J6
2b: J8
3a: S52
4a: ED43
5a: SA13
6a: HK6a
7a: QC69
Chimeric JFH-1

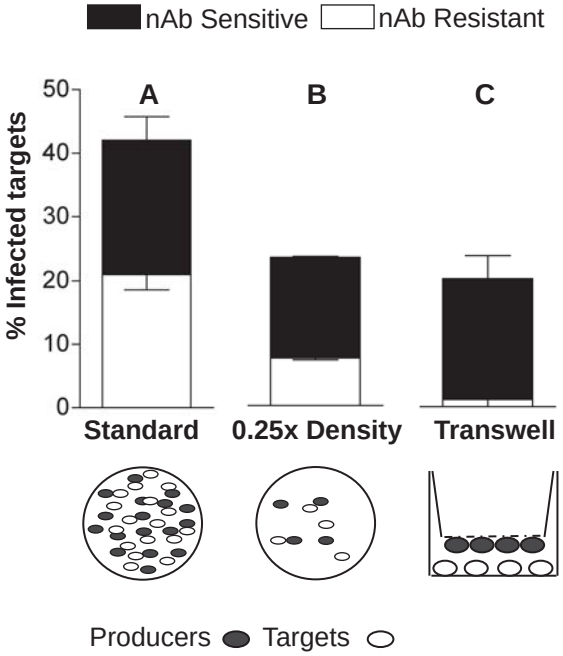
5.3 nAb resistant transmission is dependent on cell contact and particle assembly.

To determine the importance of cell contact for nAb resistant virus transmission two systems were employed, firstly the seeding density was reduced to 0.25x of the standard density to lower the frequency of cell contacts. Secondly the producer and target cells were completely separated using a transwell system, thereby eliminating all cell contacts. Producer cells were seeded on the underside of a transwell insert and target cells into the bottom well, allowing the two cell populations to reside in the same chamber and to reduce limitations of viral diffusion.

The proportion of nAb resistant transmission was greatly reduced at 0.25x standard cell density compared to control (Fig.5.4A&B), suggesting that cell contacts are important for efficient nAb resistant transmission. Upon separation of target and producer cells in the transwell system nAb resistant transmission was ablated confirming that nAb resistant transmission is dependent on cell contacts (Fig.5.4C), indicating that HCV may transmit via a direct cell-to-cell transfer of infection.

Figure 5.5: nAb resistant transmission requires cell contact.

H77/JFH RNA was delivered into Huh-7.5 cells by ep and used in the following assays set up in parallel (**A**) Standard assay. (**B**) 0.25x standard density. (**C**) Transwell assay; Producer cells were seeded on the underside of a transwell insert and target cells seeded into the bottom chamber. For each assay an irrelevant control IgG or nAb 9/27 was used at 4μg/ml. For (**A**) and (**B**) cells were harvest as normal and for (**C**) the target cells were harvested separately. All cells were stained for NS5A and analysed by flow cytometry. Results are represented as stacked histograms displaying nAb resistant (white bars) and nAb sensitive (black bars) transmission. For all assays cell free infection was determined in a standard infectious assay and >95% of cell free infection was neutralized in the presence of nAb 9/27. The treatments were performed in duplicate and the error bars indicate standard deviation from the mean. The data set is representative of three independent experiments.

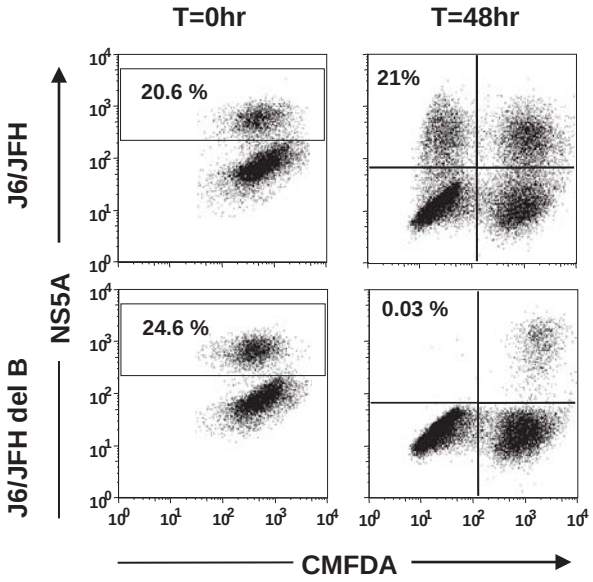

nAb Sensitive
nAb Resistant
% Infected targets
50
40
30
20
10
0
A
B
C
Standard
0.25x Density
Transwell
Producers
Targets

It is possible that HCV infection can transmit in a nAb resistant manner via direct transfer of RNA or incomplete viral particles through a cell fusion event similar to that reported for herpes viruses (483). Further to this it has been suggested that HCV RNA can be incorporated into exosomes and may transfer HCV infectivity (306, 424).

To investigate whether viral particles are required for nAb resistant transmission a mutant virus J6/JFH del B was employed that contains a mutation in the third domain of NS5A preventing a phosphorylation event that is critical for particle assembly whilst permitting wild type levels of replication and protein translation (473). To ensure that control (J6/JFH) and mutant (J6/JFH del B) producer cells supported comparable levels of replicating RNA at the start of the assay NS5A expression of the cells was monitored by flow cytometry prior to co-culture T=0hr (Fig.5.6). No nAbs were used in this assay. The mutant virus showed minimal evidence of transmission indicating that particle assembly is essential for viral transmission in co-culture.

Figure 5.6: Particle assembly is essential for co-culture transmission.

J6/JFH and J6/JFH del B RNA were delivered into Huh-7.5 cells by electroporation ep. 72hrs post ep a standard co-culture assay was performed. NS5A expression was determined at 72hrs post ep (0h) and after co-culture transmission (48hr). No nAb was used in this assay. Representative dot plots are shown displaying (**A**) the percentage of infected producer cells at 0hrs and (**B**) the percentage of infected target cells at the end of the assay +/- standard deviation. The assay was performed in duplicate. The data set is representative of two independent experiments.


T=0hr
T=48hr
J6/JFH
J6/JFH del B
NS5A
20.6 %
21%
24.6 %
0.03 %
CMFDA

5.4 Receptor dependency of nAb resistant transmission of HCVcc

Results so far demonstrate that HCV can transmit efficiently in co-culture in a nAb resistant manner, that is dependent on cell contact and particle assembly. These results suggest that HCV transmits via a direct cell-to-cell manner as well as through release of cell free viral particles. We next wished to determine whether direct cell-to-cell transmission is dependent on all four entry factors, CD81, SR-BI, Claudin-1 and Occludin, previously demonstrated to be essential for cell-free viral entry (39, 133, 288, 384, 385, 418).

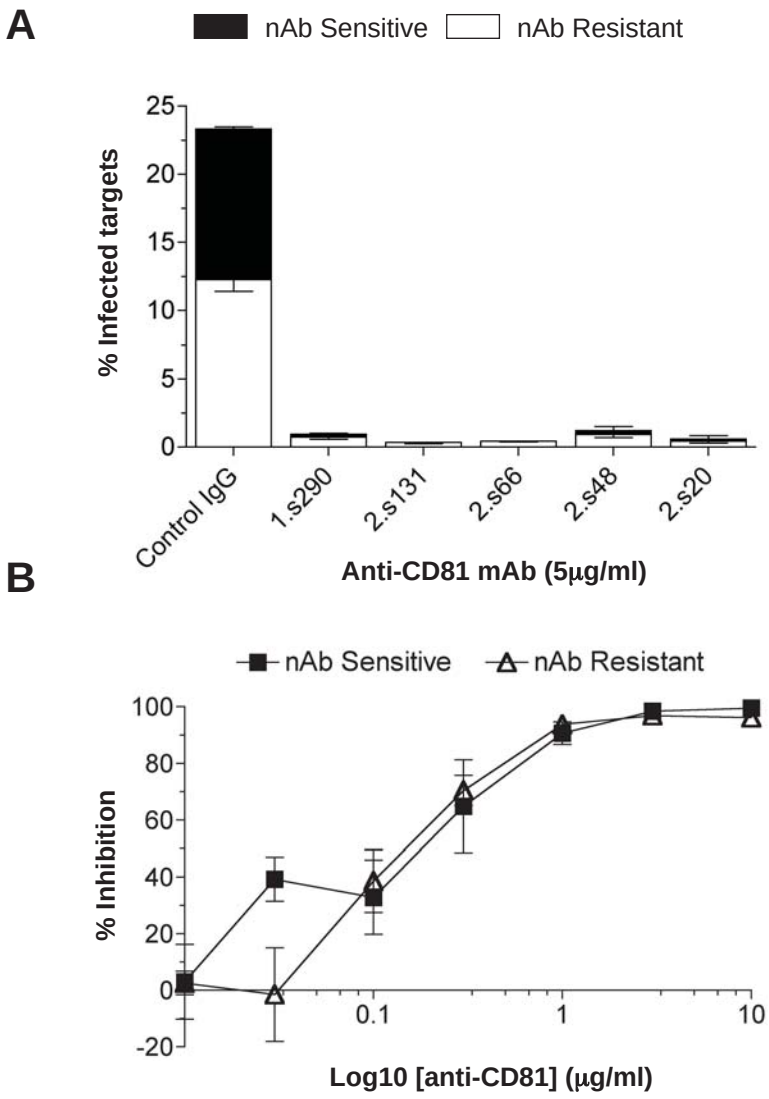
5.4.1 Role of tetraspanin CD81 in nAb resistant transmission.

HCV glycoprotein E2 binds to a defined domain on the large extra cellular loop of tetraspanin CD81, an essential co-receptor reported to be involved in the early steps of HCV entry (119, 384, 468). To date there has been contradicting evidence for the dependency of CD81 in cell-to-cell transmission. Timpe et al., 2008 and Witteveldt et al., 2009 have shown that HCV can transmit in co-culture in a CD81 independent manner, however both of these studies labelled the target cells, and we believe this may have resulted in false positives (Fig.5.1). A further study reported that CD81 was essential for HCV transmission (407). To confirm whether CD81 is indeed essential for nAb resistant co-culture transmission the effects of a panel of anti-CD81 monoclonal antibodies on HCV transmission were investigated. All of the anti-receptor antibodies were incubated with the target cells for 1hr at a concentration of 5μg/ml prior to culturing with H77/JFH infected producer cells and nAb 9/27. All anti-CD81 antibodies inhibited greater than 95% of nAb

resistant and nAb sensitive transmission, demonstrating that both routes of transmission are CD81 dependent (Fig.5.7A). To investigate this further a titration of one of the anti-CD81 monoclonal antibodies, 2.s131 was performed (Fig.5.7B). The antibody inhibited nAb resistant and nAb sensitive transmission to comparable levels.

Figure 5-7: Effect of anti-CD81 monoclonal antibodies on HCV transmission.

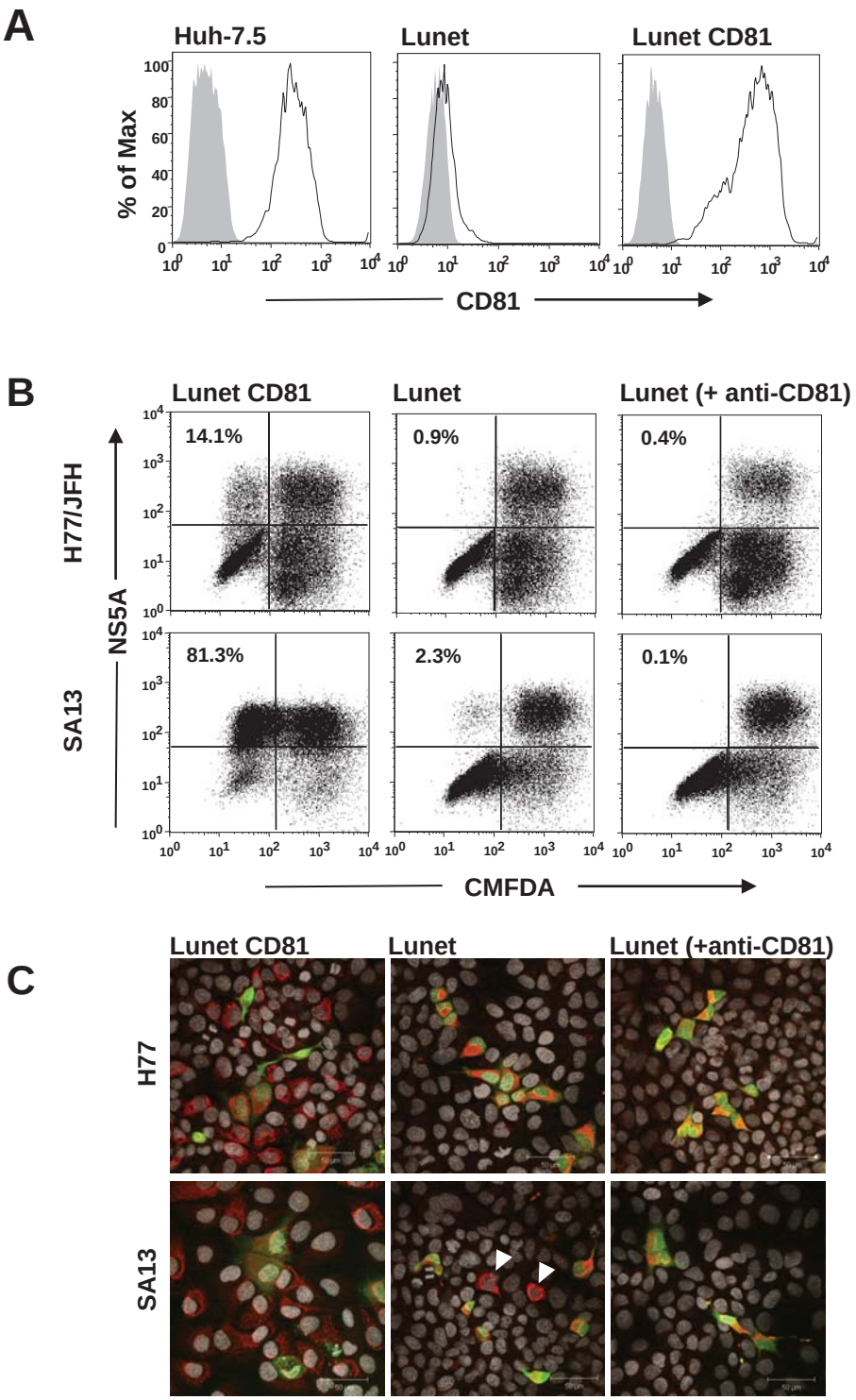
H77/JFH RNA was delivered into Huh-7.5 cells by electroporation. (**A**) 72hrs post ep a standard H77/JFH co-culture assay was performed. Target cells were incubated with a panel of anti-CD81 monoclonal antibodies or control IgG at 5μg/ml for 1hr prior to co-culture. Results are displayed as a stacked histogram, nAb resistant transmission (white bars) and nAb sensitive transmission (black bars). (**B**) Anti-CD81 mAb 2.s131 was titrated in a standard H77/JFH co-culture assay. The percentage inhibition of nAb sensitive (■) and nAb resistant (△) transmission plotted. For all assays cell free infection was determined in a standard infectious assay and >95% of cell free infection was neutralized in the presence of nAb 9/27 at 4μg/ml. The treatments were performed in duplicate and the error bars indicate standard deviation from the mean. The data set is representative of three independent experiments.


A
nAb Sensitive
nAb Resistant
% Infected targets
25
20
15
10
5
0
Control IgG
1.s290
2.s131
2.s66
2.s48
2.s20
Anti-CD81 mAb (5μg/ml)
B
nAb Sensitive
nAb Resistant
% Inhibition
100
80
60
40
20
0
-20
0.1
1
10
Log10 [anti-CD81] (μg/ml)

To confirm whether CD81 is essential for co-culture transmission we used a Huh-7 derived cell line (Lunet) that was reported to express low to undetectable levels of CD81 (251). CD81 cell surface expression was determined by flow cytometry and a small frequency of Lunet parental cells expressed low levels of CD81. Lunet parental cells transduced to stably express CD81 expressed similar levels to Huh-7.5 cells (Fig.5.8A). H77/JFH and SA13/JFH transmission to parental Lunet cells and those transduced to express CD81 was monitored (Fig.5.8B). No nAb was used during co-culture. A small amount of SA13/JFH NS5A expression could be detected in the Lunet parental cells after co-culture, suggesting a potential CD81 independent transmission route. Alternatively, this transmission could be attributed to the small population of cells expressing CD81. To investigate this anti-CD81 (2.s131) was added to the co-culture, the low level of transmission previously observed was eliminated, suggesting this was not the result of CD81 independent transmission (Fig.5.8B). These findings were confirmed in a further assay that was analysed instead by confocal microscopy (Fig.5.8C).

Figure 5.8: Transmission to a hepatoma cell line with low CD81 expression levels.

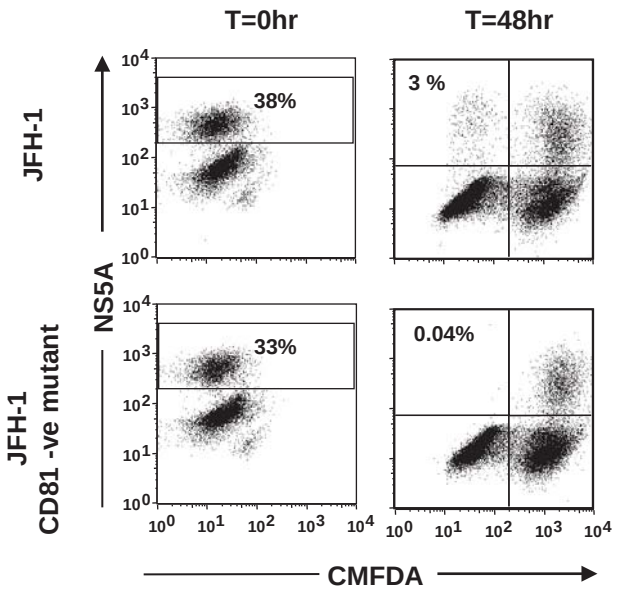
(**A**) Histograms display cell surface expression of CD81 on Huh-7.5 cells, Huh-7 Lunet cells and Huh-7 Lunet cells transduced to express CD81. (**B**) H77/JFH and SA13/JFH RNA was delivered into Huh-7.5 cells by ep. 72hrs post ep cells were labeled with CMFDA and incubated with the following unlabeled target cells; Huh-7 Lunet parental cells and those transduced to express CD81. Anti-CD81 mAb (2.s131 at 5 μg/ml) or irrelevant control IgG was incubated with the Lunet cells for 1hr prior to addition of producer cells. Representative dot plots are shown displaying the percentage of infected target cells. (**C**) Infectious co-culture assay was repeated, stained for NS5A and imaged on a Zeiss META head confocal microscope with a 40x water immersion objective. Cell nuclei (grey), producer cells (green), infected target cells (red), infected producer cells (orange), white arrows depict infected Lunet target cells. Scale bar represents 50μm.


A
Huh-7.5
Lunet
Lunet CD81
% of Max
CD81
B
Lunet CD81
Lunet
Lunet (+ anti-CD81)
H77/JFH
NS5A
SA13
14.1%
0.9%
0.4%
81.3%
2.3%
0.1%
CMFDA
C
Lunet CD81
Lunet
Lunet (+anti-CD81)
H77
SA13

A mutant virus defective in binding CD81 was previously reported to transmit *in vitro* using a CD81-independent route (515). To investigate this further transmission of the CD81 mutant virus ($JFH1_{W529A}$) was compared to transmission of parental JFH virus in our co-culture system. The assay was carried out as previously described for the J6/JFH del B mutant virus. Comparable levels of NS5A expression were noted in the JFH and $JFH1_{W529A}$ expressing producer cells prior to co-culture (T=0hr). However, the mutant virus showed minimal evidence of transmission, supporting our previous data that CD81 is essential for co-culture transmission (Fig.5.9). As previously discussed we suspect the small percentage of infected cells observed by Witteveldt et al., was possibly due to cell aggregates.

Figure 5.9: Transmission of a CD81 negative mutant virus.

JFH and JFH1$_{W529A}$ RNA was delivered into Huh-7.5 cells by ep. 72hrs post ep a standard co-culture assay was performed. NS5A expression was determined at 72hr post ep (0hr) and after co-culture transmission (48hr). No nAb was used in this assay. Representative dot plots are shown displaying (**A**) the percentage of infected producer cells at 0hr and (**B**) the percentage of infected target cells at the end of the assay. The assay was performed in duplicate. The data set is representative of two independent experiments.


T=0hr
T=48hr
JFH-1
JFH-1
CD81 -ve mutant
NS5A
38%
3 %
33%
0.04%
CMFDA

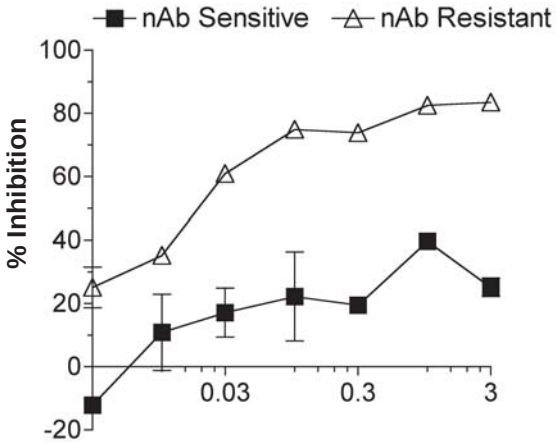
5.4.2 Role of SR-BI in nAb resistant co-culture transmission

SR-BI is a cell membrane protein involved in cholesterol metabolism; it functions as a receptor for high-density lipoproteins. It has also been shown to bind directly to HCV E2 glycoprotein and is believed to be important in the early stages of HCV attachment and entry (174, 418). To investigate the role of SR-BI in nAb resistant co-culture transmission the effect of anti-SR-BI antibody on transmission was investigated. As previously described the target cells were cultured in the presence of the anti-receptor antibody for 1hr prior to addition of H77/JFH infected producer cells and nAb 9/27. Titration of the anti-SR-BI antibody revealed that nAb resistant transmission is preferentially inhibited at all concentrations (Fig.5.10A). To investigate whether this phenomenon was due to a potential synergy between 9/27, a neutralizing antibody specific for E2 HVR-1, and the anti-SR-BI antibody transmission in the presence of another epitope specific antibody 11/20 was determined (Fig.5.10B). 11/20 targets part of the E2 CD81 binding domain (208). A significant difference between anti-SR-BI inhibition of nAb resistant and sensitive transmission was observed in the presence of both epitope specific nAbs indicating the synergy is not specific to the HVR-1 targeting antibody.

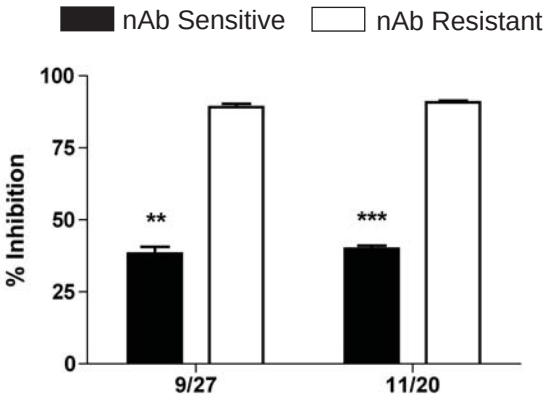
Figure 5.10: Effect of anti-SR-BI antibody on HCV transmission.

H77/JFH RNA was delivered into Huh-7.5 cells by ep. (**A**) 72hrs post ep target cells were incubated with titrated anti-SR-BI mAb (PF72) or control IgG for 1hr prior to addition of H77/JFH infected producer cells. The percentage inhibition of nAb sensitve (■) and nAb resistant (△) transmission plotted. (**B**) 72hrs post ep target cells were incubated with anti-SR-BI mAb at 1μg/ml or control IgG for 1hr. H77/JFH infected producer cells were then added in the presence of nAb 9/27 at 4μg/ml, nAb 11/20 at 10μg/ml or control IgG. Two tailed unpaired T-tests were performed to determine whether there was a significant difference between nAb resistant and sensitive transmission in the presence of nAb 9/27 or 11/20. A significant difference was determined P= 0.0013 and 0.0003 respectively. For both assays cell free infection was determined in a standard infectious assay and >95% of cell free infection was neutralized in the presence of nAb. The treatments were performed in duplicate and the error bars indicate standard deviation from the mean. The data set is representative of three independent experiments.

A


nAb Sensitive
nAb Resistant
% Inhibition
100
80
60
40
20
0
-20
0.03
0.3
3

B

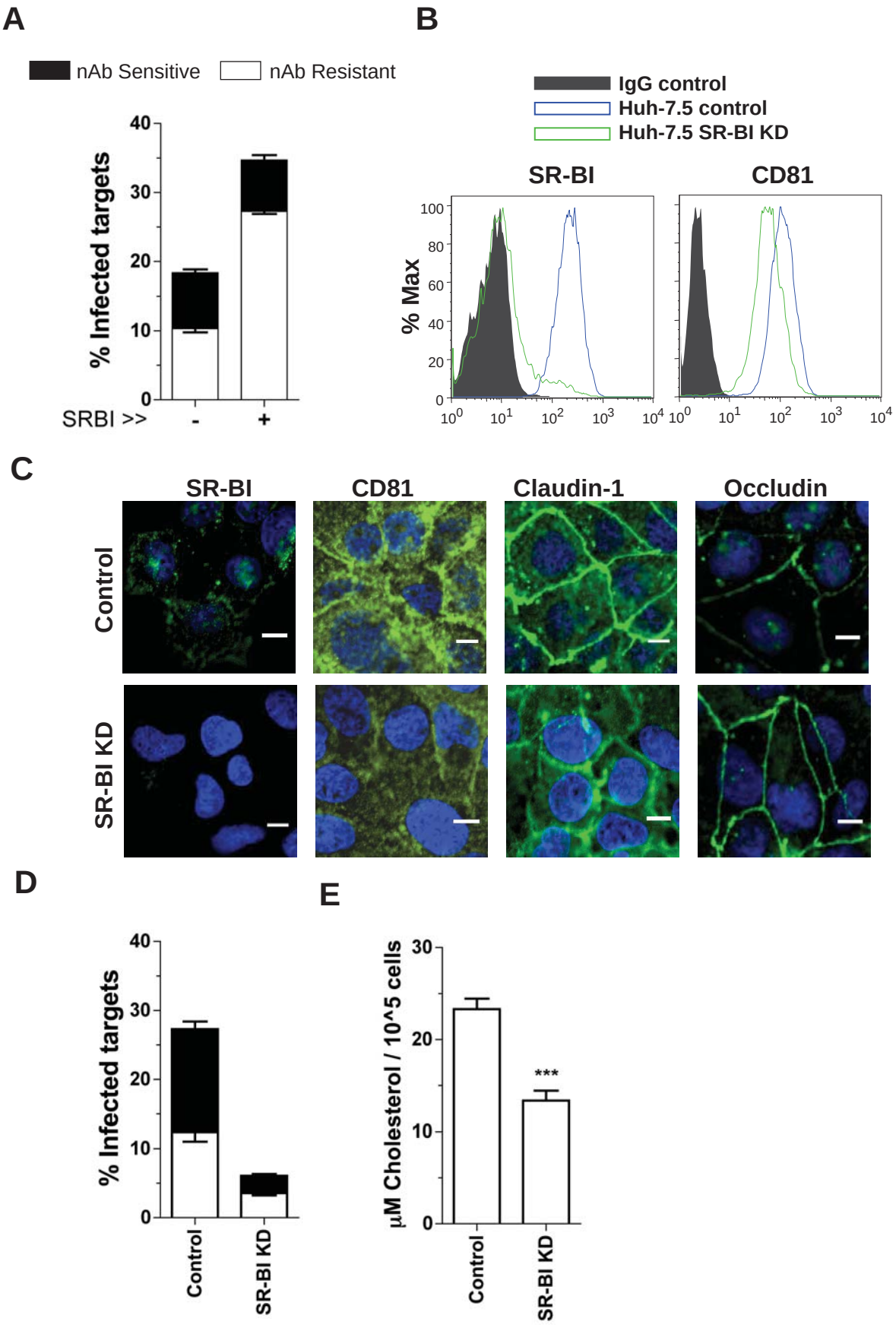

nAb Sensitive
nAb Resistant
% Inhibition
100
75
50
25
0
**
***
9/27
11/20

To further examine the role of SR-BI in nAb resistant transmission, virus dissemination was determined to Huh-7.5 cells and those transduced to over express SR-BI. Our laboratory previously published that cell-free virus infection of these cells induced greater foci size indicative of increased cell-to-cell transmission (175). nAb resistant transmission of virus to target cells over expressing SR-BI significantly increased however, SR-BI overexpression had no effect on nAb sensitive transmission (Fig.5.11A). This data indicates that SR-BI expression may be a limiting factor for efficient nAb resistant transmission.

To further investigate the role of SR-BI in nAb resistant infection, SR-BI was silenced in Huh-7.5 cells and transmission compared to parental Huh-7.5 cells. Receptor staining of parental and SR-BI silenced Huh-7.5 cells was carried out to determine efficacy of SR-BI silencing and to verify any off target effects (Fig.5.11B). SR-BI expression level was reduced and expression level of the other receptors remained with the exception of tetraspanin CD81 that was reduced; this was confirmed by flow cytometry (Fig.5.11C). Silencing SR-BI significantly inhibited both routes of viral transmission and did not preferentially inhibit nAb resistant transmission as expected from previous results (Fig.5.11D). It has recently been published that silencing SR-BI reduced cholesterol levels and inhibited plasmodium infection of hepatocytes (523). A sample of SR-BI silenced cells were lysed at the start of the assay to determined cholesterol levels. This confirmed that indeed SR-BI silenced cells had reduced levels of total cholesterol (Fig.5.11E).

Figure 5.11: Effect of SR-BI expression levels on HCV transmission.

H77/JFH RNA was delivered into Huh-7.5 cells by ep. (**A**) A standard co-culture assay was performed with control target cells and those transduced to over express SR-BI. A significant difference in nAb resistant transmission to control and SR-BI over expressing cells was observed, (P=0.0007, two tailed unpaired T-test). No significant difference was observed in nAb sensitive transmission, (P=0.2430, two tailed unpaired T-test). Huh-7.5 target cells were transduced to express sh SR-BI RNA (SR-BI KD). (**B**) Receptor expression of SR-BI KD and control cells. Images taken on a Zeiss META head confocal microscope with a 60x water immersion objective, receptor expression visualized using alexa-fluor 488 secondary antibody (green) and DAPI (blue) show cell nuclei. Scale bar represents 10μm. (**C**) SR-BI and CD81 expression also determined by flow cytometry (Anti-SR-BI (PF72) and anti-CD81 (2.s131mAb) used). (**D**) A standard H77/JFH co-culture assay was performed with control and SR-BI KD cells as targets. Two tailed unpaired T-tests were performed to determine whether there was a significant difference in nAb sensitive and resistant transmission between control and SR-BI KD cells. A significant difference was determined P= 0.0127 and 0.0045 respectively. For both co-culture assays supernatants were collected to determine cell free infection and >95% of cell free infection was shown to be neutralized in the presence of nAb 9/27 at 4μg/ml. (**E**) Total cholesterol levels were determined in control and SR-BI KD cells using Invitrogen Amplex Red Cholesterol Assay Kit. Assays were performed in duplicate and the error bars indicate standard deviation from the mean. The data sets are representative of two independent experiments.

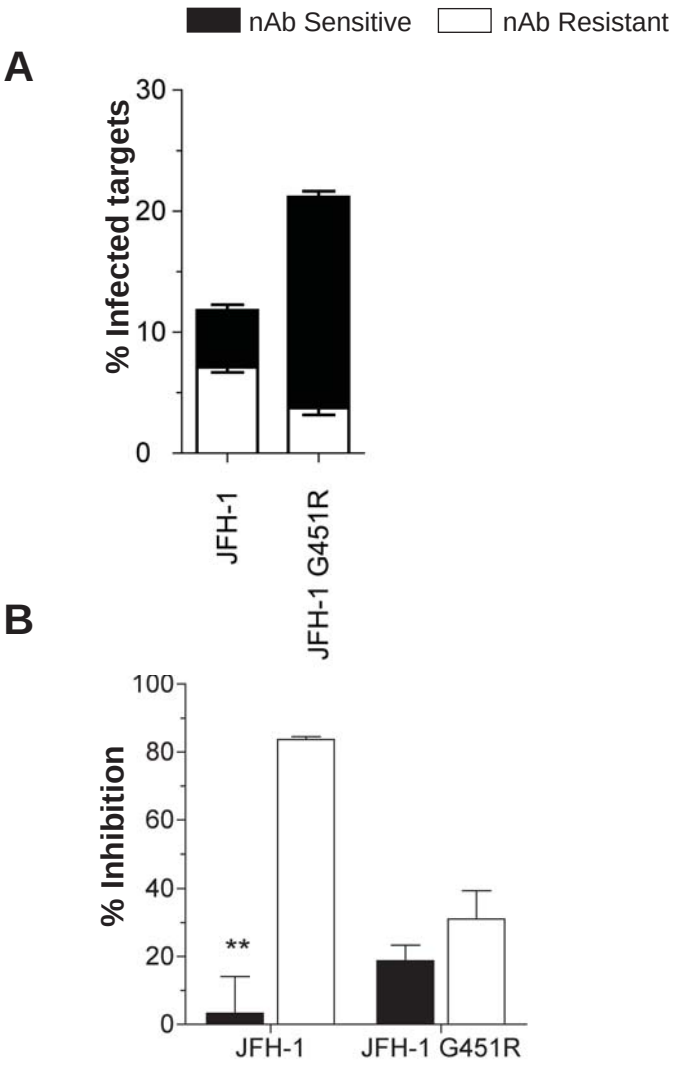

A
nAb Sensitive
nAb Resistant
% Infected targets
40
30
20
10
0
SRBI >>
-
+
B
IgG control
Huh-7.5 control
Huh-7.5 SR-BI KD
SR-BI
CD81
% Max
100
80
60
40
20
0
10^0
10^1
10^2
10^3
10^4
C
SR-BI
CD81
Claudin-1
Occludin
Control
SR-BI KD
D
% Infected targets
40
30
20
10
0
Control
SR-BI KD
E
µM Cholesterol / 10^5 cells
30
20
10
0
***
Control
SR-BI KD

Our laboratory previously published that a cell culture adapted virus containing a single mutation G451R within E2 of JFH has a reduced SR-BI dependency and increased infectivity (175). Transmission of parental and G451R adapted virus was compared in our co-culture system using HCV + sera at 300μg/ml to neutralize all cell free infection. JFH-1 G451R transmitted more efficiently in co-culture compared to control although only a small proportion of this transmission was nAb resistant (Fig.5.12A). Suggesting that nAb resistant transmission is SR-BI dependent.

To investigate SR-BI dependency of JFH-1 and JFH-1 G451R co-culture transmission we repeated the previous co-culture assay and included a pre-incubation step with an anti-SR-BI antibody. The Anti-SR-BI antibody inhibited JFH-1 nAb resistant transmission to a significantly greater extent than nAb sensitive transmission as previously shown with HCV strain H77/JFH-1 in Figure 5.11 (Fig.5.12B). The anti-SR-BI antibody reduced both routes of JFH-1 G451R co-culture transmission and there was no significant difference between the inhibition of nAb resistant and sensitive transmission. This data supports our previous findings that SR-BI is important for nAb resistant transmission and previous published data that JFH-1 G451R has reduced SR-BI dependency (175).

Figure 5.12: Transmission of a cell culture adapted virus with reduced SR-BI dependency.

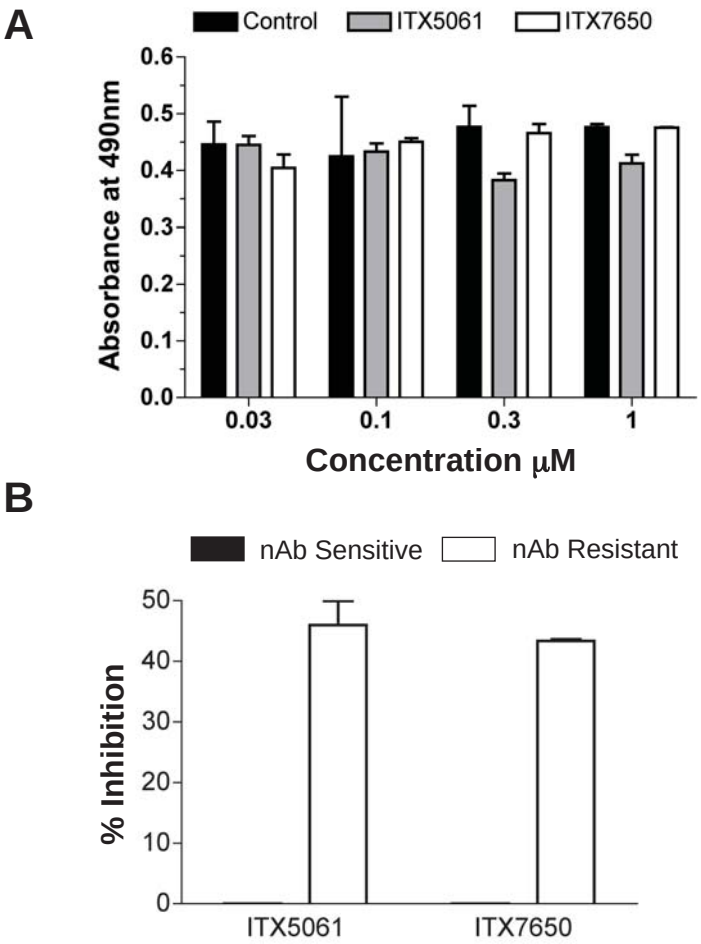
JFH-1 and JFH-1 G451R RNA were delivered into Huh-7.5 cells by ep. (**A**) 72hrs post ep a standard co-culture assay was performed. The stacked histogram displays nAb resistant (white bars) and nAb sensitive transmission (black bars). (**B**) Target cells were incubated with anti-SR-BI mAb (PF72) or IgG control at 1μg/ml for 1hr prior to addition of JFH-1 and JFH-1 G451R infected producer cells. The mAb was present throughout the duration of the assay. Results are represented as percentage inhibition of nAb resistant (white bars) and nAb sensitive (black bars) transmission. A significant difference between percentage inhibition of nAb resistant and sensitive transmission of JFH-1 virus was determined, (P=0.0092, two tailed unpaired T-test). No significant difference for JFH-1 G451R was observed, (P=0.2096, two tailed unpaired T-test). For all assays cell free infection was determined in a standard infectious assay and >95% of cell free infection was neutralized in the presence of pooled patient IgG (300μg/ml). Assays were performed in duplicate and the error bars indicate standard deviation from the mean. The data set is representative of three independent experiments.


nAb Sensitive
nAb Resistant
A
% Infected targets
30
20
10
0
JFH-1
JFH-1 G451R
B
% Inhibition
100
80
60
40
20
0
**
JFH-1
JFH-1 G451R

Syder and colleagues recently reported a number of small molecular inhibitors that target SR-BI as effective inhibitors of HCV entry (462). ITX5061 and ITX7650 have been reported to function by inhibiting HCV E2 glycoprotein-SR-BI interaction and not by affecting overall SR-BI expression levels. We wanted to investigate their efficacy to inhibit nAb resistant and nAb sensitive transmission. An MTS assay (colorimetric test that measures metabolic function) was performed to ascertain the effect(s) of ITX compounds on hepatoma cell proliferation (Fig.5.13A). The inhibitors were then used in our standard co-culture assay (Fig.5.13B). These inhibitors were relatively ineffective at targeting cell free infection but as with antibodies targeting SR-BI they were able to efficiently inhibit nAb resistant transmission.

Figure 5.13: Effect of small molecular inhibitors of SR-BI on HCV transmission.

(A) Huh-7.5 cells were treated with small molecular inhibitors of SR-BI ITX5061, ITX7650 and control at 3,1,0.3 and 0.1μM. 48hrs post treatment an MTS assay was performed to determine metabolic function, and the results presented as absorbance at 490nm (minus background). (B) H77/JFH RNA was delivered into Huh-7.5 cells by electroporation (ep). 72hrs post ep a standard H77/JFH co-culture assay was performed. Target cells were incubated with ITX5061 and ITX7650 at 0.1 μ1M for 1hr prior to addition of H77/JFH infected producer cells. Compounds were present throughout the duration of the assay. Results are displayed as percentage inhibition of nAb resistant (white bars) and nAb sensitive transmission (black bars). For all assays cell free infection was determined in a standard infectious assay and >95% of cell free infection was neutralized in the presence of nAb 9/27 at 4μg/ml. The treatments were performed in duplicate and the error bars indicate standard deviation from the mean. The data set is representative of three independent experiments.


A
Control
ITX5061
ITX7650
Absorbance at 490nm
0.6
0.5
0.4
0.3
0.2
0.1
0.0
0.03
0.1
0.3
1
Concentration μM
B
nAb Sensitive
nAb Resistant
% Inhibition
50
40
30
20
10
0
ITX5061
ITX7650

5.4.3. Role of tight junction proteins CLDN-1 and Occludin in nAb resistant HCV transmission.

The tight junction proteins Claudin-1 and Occludin have only recently been identified as co-receptors for HCV, as such there are limited tools available to study these receptors. Recent studies indicate that they are both important in the latter stages of HCV entry and as yet there is no evidence for a direct interaction of these proteins with the HCV glycoproteins (39, 133, 288, 385).

To study the role of Claudin-1 in nAb resistant transmission a recently described polyclonal antiserum raised to Claudin-1 was used (255). Target cells were incubated with a pre-immune and a post-immune serum for 1hr prior to the addition of H77/JFH infected producer cells. A standard co-culture assay was then performed using nAb 9/27 at 4μg/ml to neutralize cell free infectivity. The polyclonal anti-Claudin-1 antibody inhibited approximately 50% of nAb sensitive transmission and approximately 30% of nAb resistant transmission, indicating both routes of transmission require Claudin-1 (Fig.5.14A).

Further to this we carried out a standard co-culture assay using Claudin-1 silenced cells as targets. Receptor staining of control and silenced cells were carried out to determine the efficacy of silencing and to confirm expression of other HCV co-receptors. Immuno-fluorescence results revealed that Occludin expression was also reduced upon Claudin-1 silencing (Fig.5.14B). Claudin-1 silencing reduced nAb sensitive and resistant transmission by 47 and 32 %

respectively supporting our previous data that both routes of transmission require Claudin-1 (Fig.5.14C).

No antibodies to extra-cellular Occludin epitopes are available; we therefore silenced Occludin in the target cells and performed a standard co-culture assay as previously carried out for Claudin-1. Receptor staining of control and Occludin silenced cells was carried out to determine efficacy of silencing and to confirm expression of other HCV co-receptors. Occludin was efficiently silenced and no differences in co-receptor expression were observed (Fig.5.14A). Occludin silencing reduced nAb sensitive and nAb resistant transmission by 80% and 60% respectively (Fig.5.14B). Results indicate that both tight junction proteins Claudin-1 and Occludin are essential for both routes of transmission.

Figure 5.14: Claudin-1 is essential for nAb resistant transmission.

H77/JFH RNA was delivered into Huh-7.5 cells by ep. (**A**) 72hrs post ep a standard co-culture assay was performed. Target cells were incubated with anti-Claudin-1 polyclonal antibody for 1hr prior to addition of producer cells. Percentage inhibition of nAb resistant (white bars) and nAb sensitive (black bars) transmission shown. A significant difference between inhibition of nAb sensitive and nAb resistant transmission by anti-Claudin-1 polyclonal antibody was determined, (P= 0.0062, two tailed unpaired T-test). Huh-7.5 target cells were transduced to express si Claudin-1 RNA. (**B**) Receptor expression of Claudin-1 silenced (Claudin-1 KD) and control cells. Images taken on a Zeiss META head confocal microscope with a 60x water immersion objective, receptor expression visualized using alexa-fluor 488 secondary antibody (green) and DAPI (blue) show cell nuclei. Scale bar represents 10μm. (**C**) Claudin-1 KD cells were used as targets in a standard H77/JFH co-culture assay and transmission was compared to control. Co-culture transmission displayed as stacked histograms, nAb resistant (white bars) and nAb sensitive (black bars) transmission. Two tailed unpaired T-tests were performed to determine whether there was a significant difference in nAb sensitive and resistant transmission between control and Claudin-1 KD cells. A significant difference was determined P= 0.0439 and 0.0079 respectively. For all assays cell free infection was determined in a standard infectious assay and >95% of cell free infection was neutralized in the presence of nAb 9/27 at 4μg/ml. The treatments were performed in duplicate and the error bars indicate standard deviation from the mean. The data set is representative of two independent experiments.

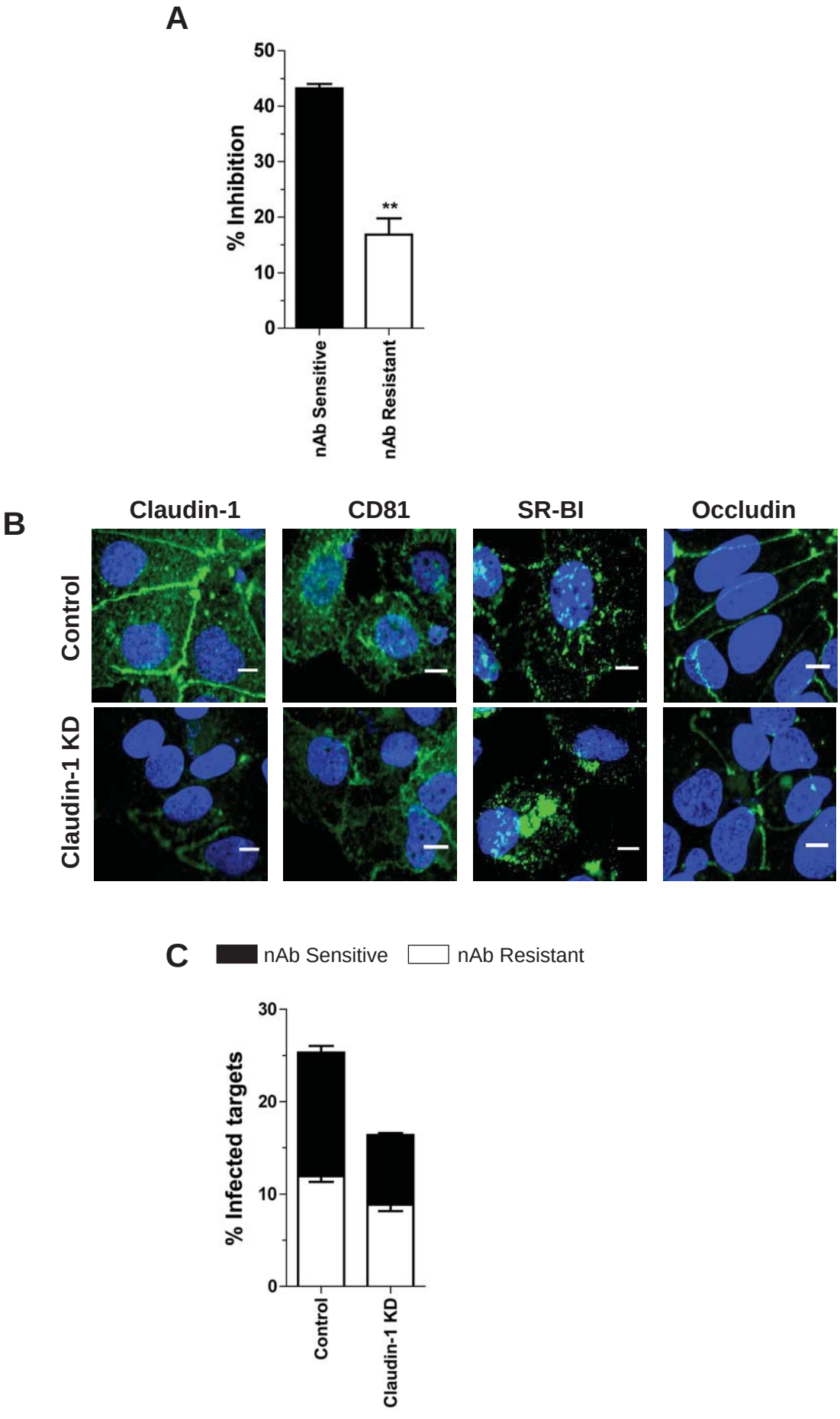
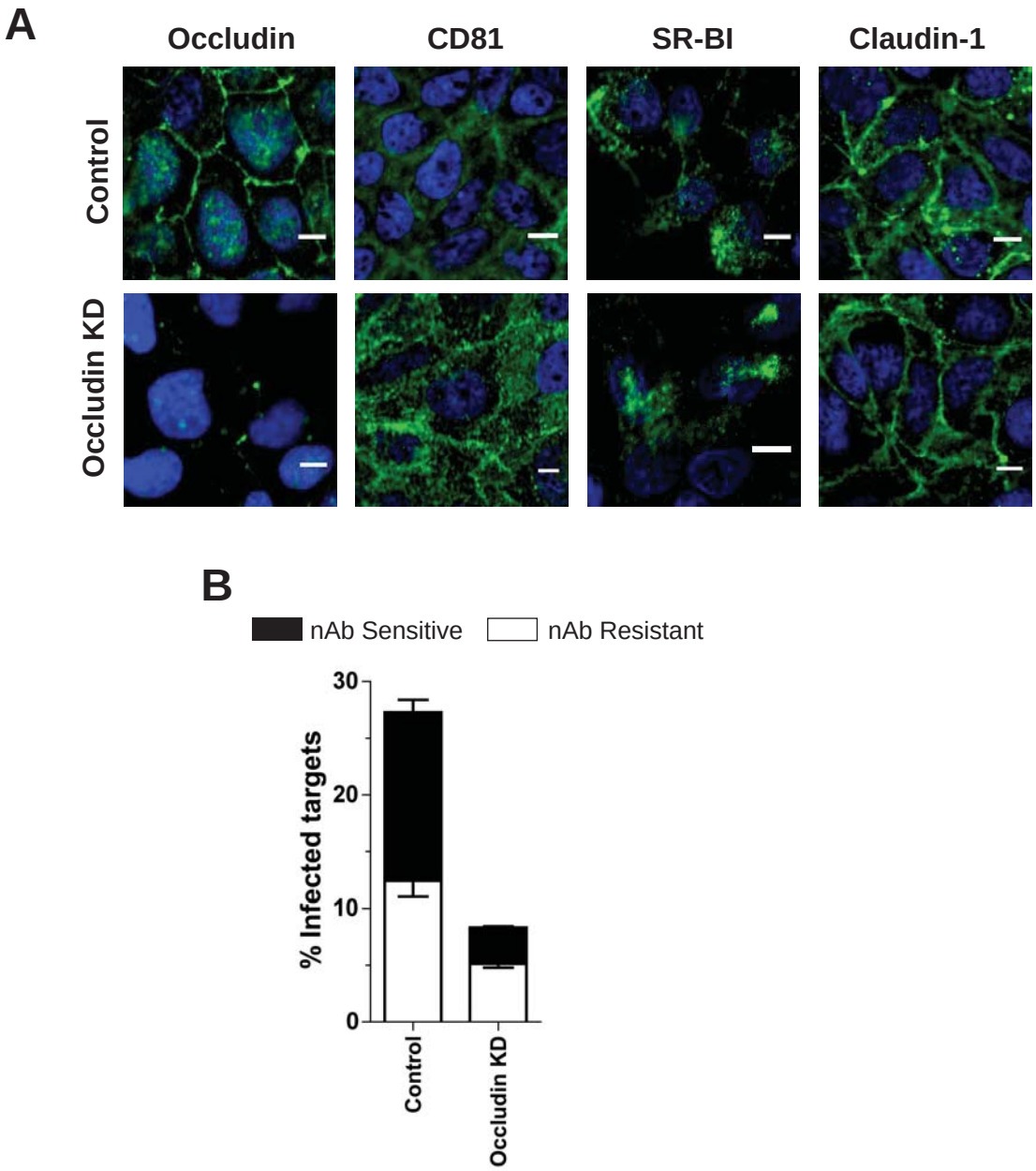

A
50
40
30
20
10
0
% Inhibition
**
nAb Sensitive
nAb Resistant
B
Claudin-1
CD81
SR-BI
Occludin
Control
Claudin-1 KD
C
nAb Sensitive
nAb Resistant
30
20
10
0
% Infected targets
Control
Claudin-1 KD

Figure 5.15: Occludin is essential for nAb resistant transmission.

Huh-7.5 target cells were transduced to express sh occludin RNA (Occludin KD). (**A**) Images display receptor expression of occludin KD cells and control cells. Images taken on a Zeiss META head confocal microscope with a 60x water immersion objective, receptor expression visualized using alexa-fluor 488 secondary antibody (green) and DAPI (blue) show cell nuclei. Scale bar represents 10μm. (**B**) H77/JFH RNA was delivered into Huh-7.5 cells by ep. 72h post ep a standard H77/JFH co-culture assay was performed with control and occludin KD cells as targets. Co-culture transmission displayed as stacked histograms, nAb resistant (white bars) and nAb sensitive (black bars) transmission. Two tailed unpaired T-tests were performed to determine whether there was a significant difference in nAb sensitive and resistant transmission between control and occludin KD cells. A significant difference was determined P= 0.0186 and 0.0051 respectively. For all assays cell free infection was determined in a standard infectious assay and >95% of cell free infection was neutralized in the presence of nAb 9/27 at 4μg/ml. The treatments were performed in duplicate and the error bars indicate standard deviation from the mean. The data set is representative of two independent experiments.


A
Occludin
CD81
SR-BI
Claudin-1
Control
Occludin KD
B
nAb Sensitive
nAb Resistant
% Infected targets
30
20
10
0
Control
Occludin KD

5.4.4 Receptor dependency of nAb resistant transmission of multiple HCV genotypes.

In this project so far, we have only studied the effect(s) of antibody antagonists on genotype 1a chimeric virus H77/JFH co-culture transmission. To strengthen our findings the effect(s) of these antagonists were determined for multiple HCV genotypes. A pooled patient sera at 300μg/ml was able to neutralize cell free infectivity of JFH chimera's representative of genotypes 1a, 1b, 3, 4, 5 and 6 in co-culture (Fig.5.4B). Therefore a standard co-culture assay was performed using patient IgG to neutralize cell free infectivity for all of the above genotypes. Target cells were incubated for 1hr with the following inhibitors prior to co-culture; anti-CD81 (2.s131 at 5μg/ml), anti-CLDN-1 (polyclonal antibody at 1in 100), anti-SR-BI (PF72 at 5μg/ml) and small molecular SR-BI inhibitors (ITX5061 and ITX7650 at 1μM) (Table 5.1). Sensitivity to anti-CD81 antibodies were similarly high for all genotypes and inhibited both nAb resistant and sensitive transmission equally with the exception of SA13/JFH. The anti-CLDN-1 polyclonal antibody was not efficient at blocking co-culture transmission and in the majority of cases blocked nAb sensitive to a greater extent than nAb resistant transmission. Treatment with the anti-SR-BI antibody and SR-BI small molecule inhibitor ITX5061 had comparable effects on transmission and both antagonists preferentially blocked the nAb resistant route.

		% INHIBITION							
		Anti-CD81		Anti-CLDN1		Anti-SR-BI		ITX5061	
Genotype	JFH chimera	Sensitive	Resistant	Sensitive	Resistant	Sensitive	Resistant	Sensitive	Resistant
1a	H77	88 (±1)	92 (±2)	43 (±1)	17 (±3)	7 (±6)	87 (±1)	7 (±12)	81 (±1)
1b	J4	82 (±1)	88 (±1)	55 (±5)	32 (±1)	7 (±14)	79 (±1)	8 (±2)	73 (±1)
4a	ED43	97 (±1)	94 (±2)	54 (±5)	36 (±4)	7 (±2)	75 (±2)	4 (±5)	69 (±1)
5a	SA13	46 (±4)	92 (±1)	8 (±5)	22 (±2)	0 (±4)	44 (±2)	0 (±0)	41 (±2)
6a	HK6a	94 (±1)	93 (±1)	10 (±1)	1 (±1)	10 (±2)	34 (±7)	15 (±1)	40 (±3)

Table 5.1: Receptor dependency of multiple genotypes.

5.5 Discussion

In this study we developed a co-culture assay that allowed us to examine HCV transmission *in vitro* (Fig.5.2). Our results support previous observations made using standard infectious assays; cell free HCV particles are relatively sensitive to neutralizing antibodies *in vitro* (29, 359, 560). Importantly our results demonstrate that a proportion of co-culture transmission is highly insensitive to neutralization by antibodies targeting the viral glycoproteins. We envisage that the co-culture system used in this study demonstrates neutralizing antibody sensitivities that are more akin to the *in vivo* situation than standard infection assays.

To investigate the sensitivity of HCV transmission to different antibodies targeting the viral glycoproteins, H77/JFH chimera (genotype 1a virus) was studied. Patient IgG isolated from HCV infected patients and eptiope specifc antibodies 11/20 and 9/27 targeting the CD81 binding domain and the HVR-1 of E2 respectively neutralized > 95% of the infectivity of cell-free virus whilst still allowing new rounds of virus transmission to occur (Fig.5.3). Interestingly different levels of co-culture transmission were observed depending on the antibody studied. Importantly, once all cell free virus infectivity was neutralized, mAbs 11/20 and 9/27 were able to limit co-culture transmission although they were unable to completely abrogate all transmission at a final concentration of 30μg/ml. These results suggest that nAb resistant transmission does not occur through a sealed junction, that is impermeable to HCV glycoprotein targeting antibodies. Rather susceptibility to neutralization

is variable and dependent on the antibody in question, this maybe due to differences in antibody affinities or target epitope availability. The majority of HCV RNA is reported to associate with lipoproteins *in vitro* and *in vivo*, these are termed lipo-viro-particles (LVP) (14). It has previously been reported that lipoprotein association increases specific infectivity and decreases neutralization efficacy (175, 215). It is therefore interesting to postulate whether the associated lipoproteins may aid nAb resistant viral transmission.

To further characterize co-culture transmission we defined nAb resistant cell-to-cell transmission as the proportion of transmission that occurred in the presence of a anti-glycoprotein specific Ab capable of inhibiting between 90 and 100% of all cell-free virus infectivity. For the majority of experiments we investigated H77/JFH transmission and used the most proficient epitope specific antibody 9/27 at 4μg/ml, a concentration able to effectively neutralize cell free infectivity. When it was not possible to use H77/JFH we used patient IgG isolated from HCV infected patients that was able to neutralize cell free infectivity at 300μg/ml.

The proportion of nAb resistant transmission decreased when target and producer cell contacts were reduced (Fig.5.5A) and was ablated upon separation of the cells (Fig.5.5B). These findings suggest that cell contacts are essential for nAb resistant transmission and HCV can transmit via direct cell-to-cell route(s) as well as release of cell-free virus. A mutant virus (J6/JFH del B) that is un-able to assemble viral particles did not transmit infection in co-culture (Fig.5.6), demonstrating that viral particles are required for co-

culture transmission. Furthermore, comparison of the levels of infectious virus released from the chimeric JFH viruses and the percentage of target cells infected in co-culture revealed an association between infectious cell-free virus and transmission, suggesting that particles are required to initiate both nAb resistant and sensitive forms of transmission (Fig 5.6). These findings do not support a recent hypothesis that HCV RNA directly transmits through cell-to-cell fusion events via the exosomal excretory pathway (424).

Importantly we demonstrated that diverse HCV genotypes can transmit via nAb resistant cell-to-cell route(s) using a panel of chimeric JFH viruses expressing the structural proteins that represent the major genotypes (166) (Fig.5.4). For 7 out of the 11 strains studied > 95% of cell-free infectivity was neutralized by IgG purified from the sera of HCV infected subjects (Fig.5.4B). We can be confident that these genotypes transmit in a nAb resistant manner. However the remaining genotypes were only partially neutralized by the patient IgG, therefore we cannot conclusively determine whether these viruses transmit in a nAb resistant manner.

Four co-receptors are known to be essential for entry of cell-free virus, we were interested to investigate the receptor dependency nAb resistant cell-to-cell transmission. Contradicting evidence exists as to whether the tetraspanin CD81 is essential for HCV co-culture transmission (229, 407, 480, 515). Timpe et al., 2008 reported that HCV could transmit to a CD81 negative HepG2 cell line. Witteveldt et al., 2009 supported these findings by showing that a mutant virus ($JFH1_{W529A}$) that was un-able to interact with CD81 was

able to transmit *in vitro*. We believe these findings were a result of misinterpretation of flow cytometry data due to the formation of aggregates between labeled target cells and unlabelled infected producer cells (Fig. 5.1). We failed to observe any detectable transmission of infectivity of the $JFH1_{W529A}$ virus in our co-culture system (Fig.5.9), suggesting that HCV interaction with CD81 is indeed essential, as previously reported by Russel et al., 2008. A panel of anti-CD81 monoclonal antibodies were able to block greater than 85% of both routes of transmission for all HCV genotypes with the exception of genotype 5a virus SA13/JFH (Table 5.1). Suggesting that for the majority of genotypes CD81 is equally important for all routes of co-culture transmission. Transmission of H77/JFH and SA13/JFH was monitored to Lunet cells that express very low levels of CD81 (Fig.5.8B&C). Negligible transmission of H77/JFH was recorded to Lunet cells and this was rescued by ectopic CD81 expression, supporting our previous data that CD81 is essential for co-culture transmission. Notably a small population of SA13/JFH infected Lunet cells was observed, importantly both flow cytometry analysis and confocal imaging detected this transmission. To investigate whether this was evidence supporting CD81 independent transmission an anti-CD81 mAb was added to the co-culture. Anti-CD81 mAb completely abrogated SA13/JFH transmission to lunet cells demonstrating that the previous transmission observed was not evidence of CD81 independent transmission rather it was showing the efficiency of SA13/JFH transmission. Recent data from our laboratory revealed that SA13/JFH RNA level per infected cell is comparable to other genotypes indicating the difference in infectivity is unlikely to be due to an increase in replication efficacy (Un published data by Dr. P. Balfe and

Dr. N. Fletcher). These results suggest that SA13/JFH is able to utilize a lower threshold of CD81 compared to other viruses. Koutsoudakis et al., reported that HCVcc needs a threshold level of CD81 to infect hepatoma cells (251). It would be interesting to see if the same threshold holds true for SA13/JFH. In summary we have demonstrated that CD81 is indispensable for HCV infection and transmission in co-culture.

Our observations demonstrate that both tight junction proteins Claudin-1 and Occludin are required for HCV co-culture transmission. Previous studies using parental 293T cells and those transduced to express Claudin-1 reported that claudin-1 is essential for co-culture transmission of HCVcc but did not discriminate between nAb resistant and nAb sensitive transmission (480). We observed that nAb sensitive transmission was blocked to a greater extent than nAb resistant transmission by an anti-Claudin-1 polyclonal antibody for all genotypes tested (Table 5.1). Supporting this, nAb sensitive transmission was more sensitive to Claudin-1 expression than nAb resistant transmission (Fig.5.14C). Of note silencing Claudin-1 expression also reduced Occludin expression (Fig.5.14B). Therefore the observed reduction in transmission may have resulted from a reduction in both Claudin-1 and Occludin expression. To date no research has previously looked at the role of Occludin in HCV co-culture transmission. No antibodies are available to block Occludin function in HCV entry; therefore to investigate the role of Occludin in co-culture transmission Occludin expression was silenced in the target cells. nAb resistant and nAb sensitive transmission to occludin silenced target cells

compared to parental cells was equally reduced, suggesting that Occludin is important for both routes of transmission (Fig.5.15).

Our data suggests that SR-BI expression may be a limiting factor for nAb resistant cell-to-cell HCV transmission. A mutant virus, JFH-1 G451R, that was previously reported to be relatively SR-BI independent (175), demonstrated minimal nAb resistant compared to nAb sensitive transmission (Fig.5.12). Furthermore small molecular inhibitors targeting SR-BI and an anti-SR-BI antibody blocked significantly greater levels of nAb resistant compared to nAb sensitive transmission (Table 5.1). This was demonstrated with a large panel of HCV genotypes and occurred in the presence of antibodies targeting different epitopes and polyclonal IgG purified from HCV infected patient sera. Suggesting these observations were not a result of a synergy between a specific anti-E2 antibody and a specific SR-BI targeting antibody or molecule. Further to this over expressing SR-BI in target cells promoted viral infection and specifically nAb resistant transmission (Fig.5.11A). Our laboratory previously published that over expression of SR-BI increased foci size of infection compared to control, further supporting our findings that SR-BI expression may be particularly important for nAb resistant cell-to-cell transmission (175). We hypothesized that silencing SR-BI would specifically reduce nAb resistant transmission compared to nAb sensitive transmission. However silencing SR-BI expression in the target cell reduced both routes of transmission equally (Fig.5.11D). Silencing SR-BI has previously been shown to modulate membrane cholesterol levels and CD81 localisation reducing Plasmodium sporozite infection of hepatocytes (523). It is also reported that

cholesterol depletion reduces CD81 expression, perturbs CD81-Claudin-1 association and reduces HCVpp and HCVcc entry (188, 235, 402). CD81 directly associates with membrane cholesterol (78) it is therefore not surprising that cholesterol levels may modulate CD81 and its function in HCV entry. Cholesterol has also been shown to be important in other stages of the HCV life cycle including replication and particle assembly and release (209, 236, 541). We found that total cholesterol levels were significantly reduced compared to control hepatoma cells following SR-BI silencing and CD81 cell surface expression was reduced (Fig.11.B,C&E). The cholesterol depletion may have altered CD81 localization in the plasma membrane, reducing epitope availability for the antibody and in turn lowering the detection level of cell surface CD81. Reduced cellular cholesterol may also increase CD81 internalization as reported for tetraspanin CD82 (521). It is therefore likely that silencing SR-BI may have numerous effects that reduce HCV transmission. Our results emphasize the important role SR-BI plays in Huh-7.5 cells and that it is always important to consider indirect effects of silencing. We hypothesize that SR-BI may have an essential role in cell-to-cell spread of HCV *in vivo* and may be a good target for future therapeutics. Unlike other co-receptors SR-BI is predominantly expressed in the liver, therefore reducing the opportunity for off-target effects.

In conclusion we have observed *in vitro* the efficient transfer of HCV in the presence of neutralizing antibodies, indicating a possible mechanism by which HCV infection can persist *in vivo*. This has major consequences for the use of E2 specific antibodies as therapeutics and highlights receptors in particular

SR-BI as possible targets to inhibit nAb resistant transmission. Furthermore the co-culture system we have developed and used throughout this study may be a preferred system to use when determining efficacy of future therapeutics due to its high stringency compared to standard infectious assays. However it is important to remember that although HCV may transmit efficiently by a cell associated route within the host the release of cell free viral particles is essential for HCV to transmit from host to host.

6.0 Final Remarks

Tetraspanin CD81 function in hepatoma biology:

Actin polymerization dependent morphological changes were observed upon ligation of CD81 on the surface of hepatoma cells with immobilized IgG but not HCV sE2. This process was found to be highly dependent on cellular cholesterol levels, where both a decrease and an increase in total cholesterol perturbed anti-CD81 induced cell spread. Cholesterol interacts directly with CD81 and is reported to coordinate the localization of tetraspanins in TEMs (78, 402, 439, 521), suggesting that anti-CD81 induced spread is dependent on CD81 interactions with partner proteins. In chapter 3 we utilized the phenomenon of cell spread to investigate the involvement of different partner proteins and signaling pathways in CD81 function in hepatoma cells. Figure 6.1 illustrates reported partner proteins of CD81. In chapter 4 functional assays were performed determining that CD81 does not play a role in hepatoma-ECM adhesion, however CD81 expression increases hepatoma cell migration and invasion. We postulate that our findings from chapter 3 may be useful for predicting possible mechanisms involved in CD81 induced hepatoma cell migration.

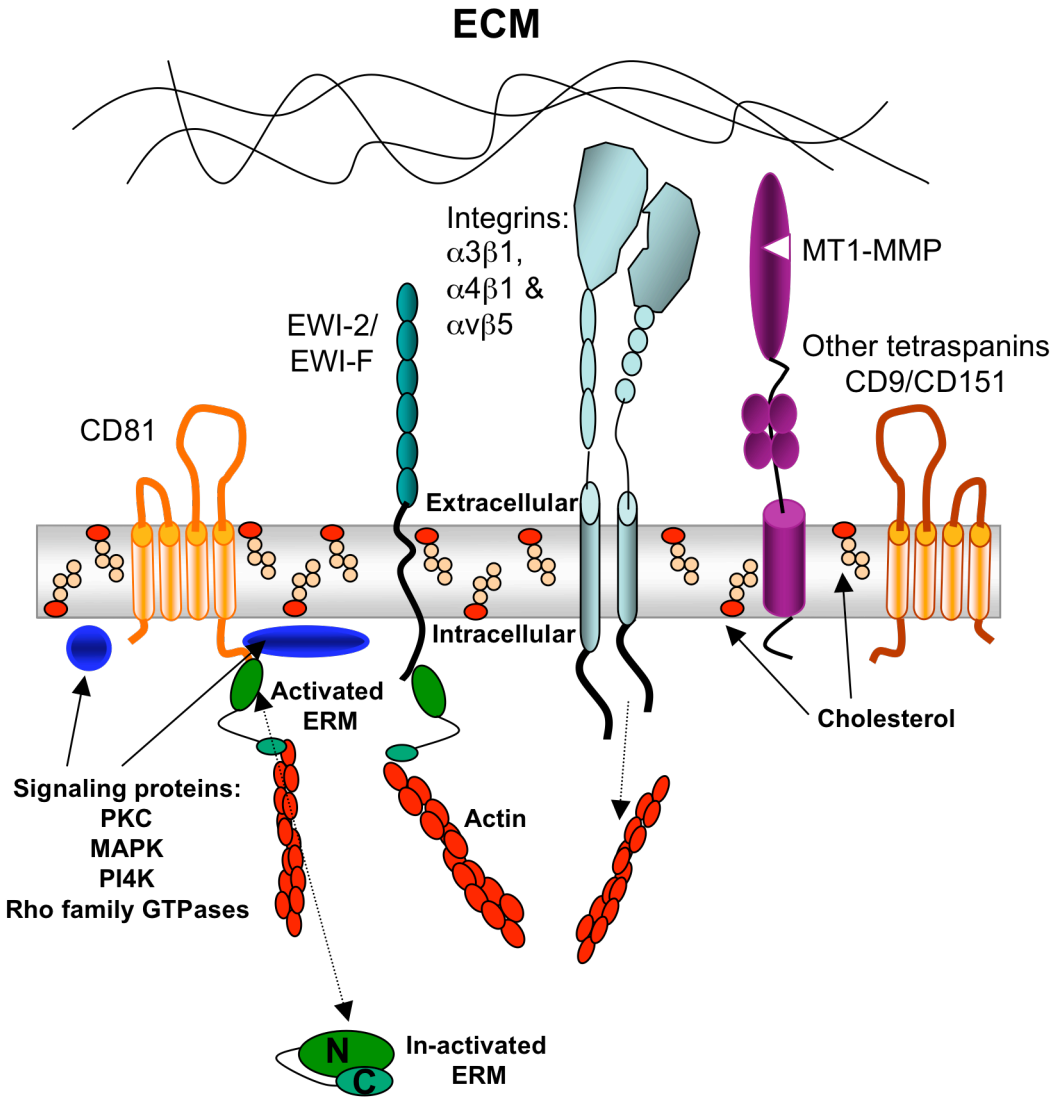


Figure 6.1: Schematic diagram of CD81 as part of a TEM.

Diagram represents reported partner protein interactions of CD81 that may be occurring in TEM(s) present in hepatoma cell membranes. Image not drawn to scale.

Notably anti-CD81 induced hepatoma cell spread was dependent on the C terminus of CD81 (Fig.3.5), previously reported to contain a PDZ domain believed to permit association with actin-associated proteins Ezrin Radoxin Moesin (ERM) (95, 411). Further to this CD81 co-localized with activated ERM proteins in hepatoma cells and anti-CD81 induced cell spread was dependent on ERM function (Fig.3.6). Our observations suggest the presence of a functional link between the CD81 C terminus and ERM proteins in hepatoma cells as illustrated in Figure 6.1. Importantly this association was not important for CD81 to function as an HCV co-receptor (data not shown). However we can speculate that the link between CD81 and the actin cytoskeleton through ERM proteins may be important for CD81 function in tumor cell migration. Indeed a number of reports have identified ERM protein Ezrin as an indicator of hepatoma cell metastasis potential (354, 543, 557). This has also been confirmed by *in vivo* studies of Ezrin expression in tumors of hepatitis B virus (HBV) infected HCC patients, where higher levels of Ezrin expression associate with a smaller tumour size and a higher frequency of metastasis (543).

Immunoglobulin like proteins EWI-2 and EWI-F associate with CD81 and are reported to associate with ERM proteins. Interestingly their association with ERM proteins has been reported to negatively regulate cell migration (411). Our observations together with these reports suggest that the relationship between CD81, EWI-2, EWI-F and ERM is important in determining cellular-migration potential.

Although previous reports have identified a number of different signaling cascades to be involved in CD81 function, inhibitors to these pathways had no observable effect on anti-CD81 induced cell spread (Fig.3.7-8). This may be explained by compensatory actions of different pathways. Interestingly inhibition of the large GTPase, dynamin, perturbed hepatoma cell spread suggesting an important role for dynamin in CD81 function (Fig.3.9). Dynamin is reported to play a role in HCV entry through its role in clathrin mediated endocytosis (287)(Farquar, submitted), however until now it has not been associated with tetraspanin function.

A number of different integrin sub-units were detected on the surface of hepatoma cells, some of which have previously been identified to associate either directly or indirectly with CD81 (42, 75, 427, 481, 528). CD81 expression or cell surface ligation did not modulate hepatma–ECM adhesion suggesting that CD81 is not involved in coordinating integrin function in hepatoma–ECM interactions (Fig.4.2). However, CD81 expression was observed to increase invasion of hepatoma cells (Fig.4.8), a process potentially coordinated through CD81's association with integrins. Additional partner proteins not investigated in this study that could be involved in hepatoma invasion include the matrix metalloproteinase MT1-MMP. Lafleur et al., reported MT1-MMP to be stabilized by CD81 expression, promoting invasive potential (262).

In summary, we have identified that CD81 expression increases hepatoma cell migration and invasion *in vitro*. We hypothesize that this may have

consequences for hepatoma carcinoma development *in vivo,* CD81 expression levels could prove to be a useful marker for tumor progression. This is an area of research that has been paid little attention to in the past, our findings help justify this as an important area of research in the future. CD81 is ubiquitously expressed making it an unattractive target for therapeutics for fear of off target effects, and importantly we observed no inhibition of migration in response to ligation with soluble IgG (Fig.4.7). The later observation also makes it unlikely that HCV E2 binding to CD81 will modulate CD81 function in migration and invasion. Cholesterol levels, dynamin, ERM and CD81 C terminal function were all observed to be important in coordinating anti-CD81 induced hepatoma cell spread (Chapter 3) and may also be involved in CD81 function in hepatoma migration and invasion.

HCV perturbation of anti-CD81 induced cell spread:

Recombinant forms of truncated HCV E2 glycoprotein directly bind CD81 (384), and HCV has been shown to bind sCD81 (30, 208) therefore we speculated as to whether HCV infection may modulate CD81 function in hepatoma cells. Interestingly HCV infection perturbed anti-CD81 induced hepatoma cell spread (Fig.3.11). CD81 expression levels were comparable for naïve and infected cells, eliminating this as a possible explanation for the attenuated spread (Fig.3.11). We hypothesized a number of possible mechanisms that could be responsible for this observation, illustrated in Figure 6.2. Firstly HCV engagement of CD81 may directly perturb CD81 partner protein interactions (Fig.6.2A). Supporting this hypothesis viruses unable to associate with CD81 had no effect on anti-CD81 induced spread

(Fig.3.12). However the infection levels recorded for the cell populations expressing these viruses were relatively low, providing an alternative explanation for these results. Secondly we hypothesized that modulation of CD81-ERM association/activation, or dynamin function including inhibition of down stream signaling pathways may be responsible (Fig.6.2B-D). For example HCV NS5A protein is reported to inhibit Syk kinase activity (220) and tyrosine phosphorylation of Ezrin upon CD81 ligation on B cells is dependent on Syk (95). Thus if this is also true for hepatoma cells, NS5A expression may be responsible for the observed perturbation. Lastly, we hypothesized that HCV modulation of cellular cholesterol levels may perturb anti-CD81 induced hepatoma cell spread (Fig.6.2E). Supporting this hypothesis an increase in total cellular cholesterol levels in HCV infected cells was recorded and elevated levels of cholesterol perturbed anti-CD81 induced cell spread (Fig.3.13).

Given our earlier results implicating CD81 as a promoter for hepatoma cell migration and invasion we postulate that these findings might have consequences for HCV associated hepatoma carcinoma.

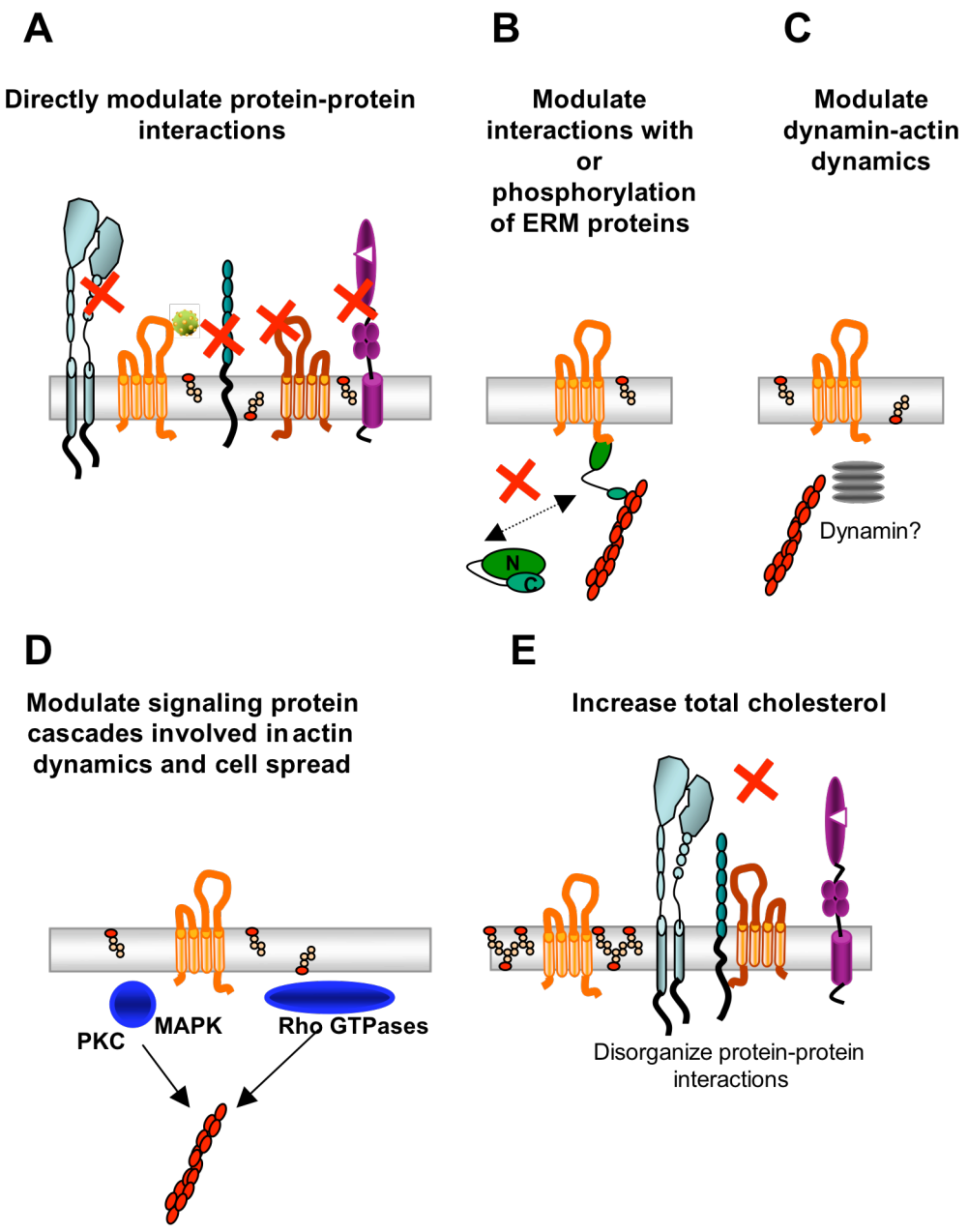


Figure 6.2: Possible mechanisms underlying HCV perturbation of CD81 function in hepatoma cell spread.

HCV as a tumor promoter:

An increase in hepatoma cell invasion was observed upon HCV infection *in vitro*, suggesting that HCV infection may directly increase the risk of developing HCC *in vivo*. As previously suggested this may be due to an alteration of CD81 function by HCV infection. However the processes involved in cell migration are extremely complex and are likely to be controlled through numerous mechanisms. Previous research in the HCV field has reported that expression of certain HCV proteins may be responsible for the development of HCC (17, 33, 89, 294, 330, 340), expression of HCV core protein for example was reported to increase HepG2 cell migration through modulation of E-cadherin levels (17). Until now no one has previously reported an increase in invasion potential of hepatoma cells upon expression of full-length replicating HCV virus.

Stabilization of HIF-1α following HCV infection has been reported by a number of laboratories (191, 341, 400). The HIF transcription complex formed upon stabilization of HIF-1α is responsible for the transcription of numerous proteins involved in tumor cell progression and has recently been linked with the development of epithelial mesenchymal transition (EMT) (198, 534, 535). We postulated that stabilization of HIF-lα might be responsible for the observed increase in hepatoma cell invasion (Fig. 6.3). This was supported by our observations that HCV induced migration as well as hypoxia induced migration of Huh-7.5 cells was perturbed by HIF-1α inhibitor NSC-134754 (Fig.4.11-12). A commonly known growth factor transcribed by the HIF transcription factor is VEGF. VEGF has previously been reported to be

responsible for increased angiogenesis, an extremely important process in tumor progression, as well as increased invasion potential of HepG2 cells (214, 423). However our findings suggest that VEGF was not responsible for the increased invasion of HCV infected Huh-7.5 cells (Fig.4.10) and speculate that another HIF target gene may be responsible. Other target genes of the HIF transcription factor associated with migration and metastasis include the transcriptional factors TWIST and Snail, both of which have been demonstrated to be responsible for repressing E-cadherin expression and increasing invasion potential of cancer cells (309, 532, 533, 535). Chemokine receptor CXCR4 and matrix metalloproteinase's including MMP2 are also reported to be targets of the HIF transcription factor and are implicated in mediating cancer cell invasion (19, 297, 379, 503). CXCR4 in particular is associated with hepatocellular carcinoma (44).

We also observed inhibition of infection upon incubation with NSC-134754 suggesting that HIF-1α stabilization may be fundamental for efficient HCV infection (Fig.3.13). In summary our research suggests that a HIF-1α inhibitor could be advantageous not only to prevent development of HCC but to also inhibit HCV infection.

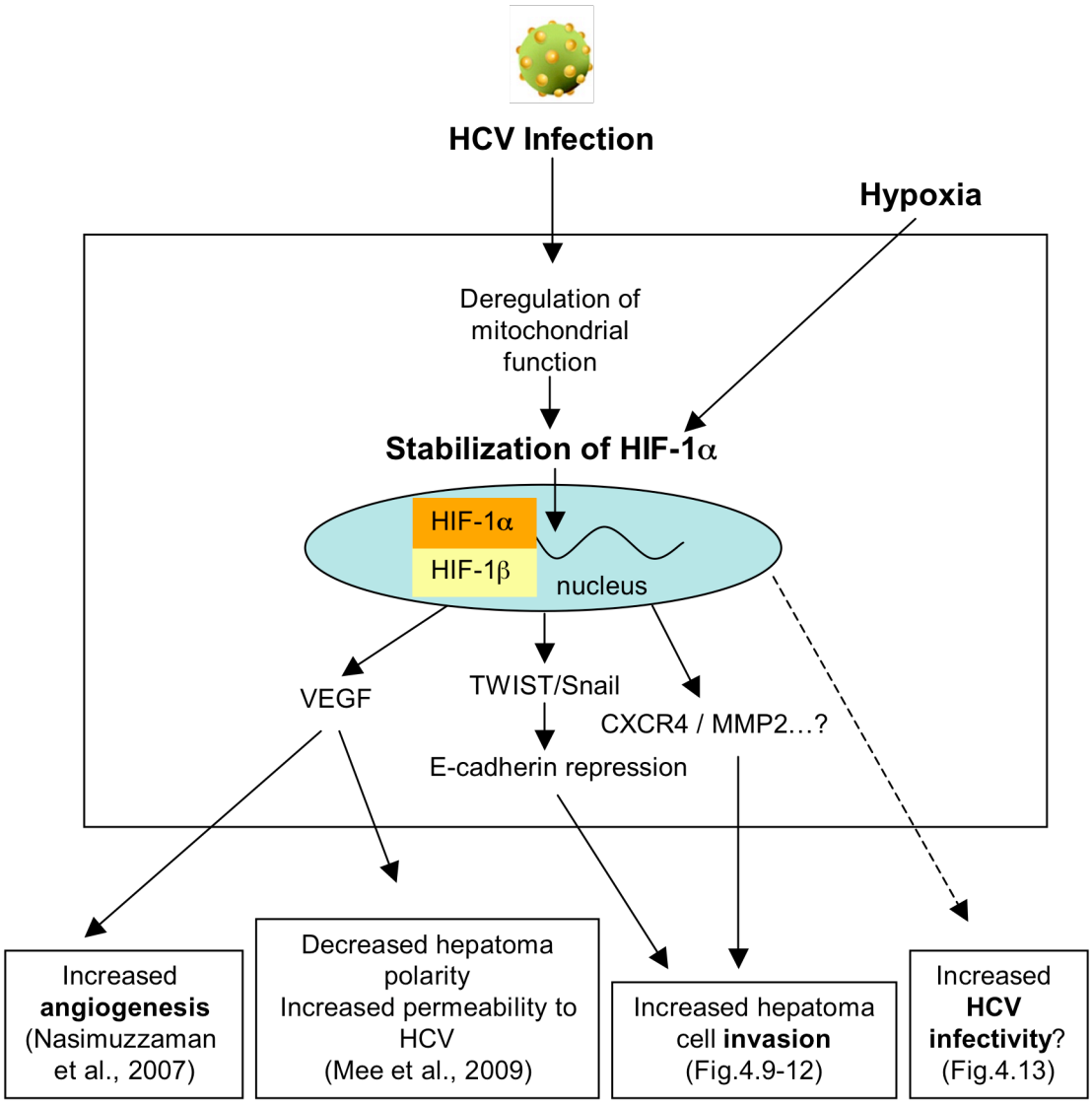


Figure 6.3: Possible mechanism(s) for HCV induced hepatoma migration and tumor progression through stabilization of HIF-1α.

Neutralizing antibody resistant transmission of HCV:

Finally we investigated nAb resistant transmission of HCV *in vitro* (Chapter 5). The results we obtained suggest that HCV transmits efficiently in co-culture via a cell contact and particle-dependent means of transmission that is highly insensitive to nAbs. This nAb resistant transmission is dependent on all co-receptors previously shown to be essential for cell-free entry indicating the mechanism of entry is likely to be the same. Here we postulate possible mechanisms for this highly efficient neutralizing antibody resistant transmission.

It is unlikely that HCV transmits through cell fusion events or formation of synctia, as demonstrated by many herpes viruses since this would negate the need for correct viral assembly and usage of viral co-receptors (415, 483). Huh-7.5 cells use in this study do not polarize (319). Consequently we were unable to determine whether tight junctions may be used to transmit virus as observed for other viruses such as Herpes Simplex Virus (HSV). HSV has been demonstrated to target virions to cell junctions of epithelial cells to promote transmission of infection (227). The liver is highly ordered and made up predominantly of hepatocytes that are highly polarized. HepG2 cells provide a model system to study hepatocyte polarity (319, 320). Unfortunately HepG2 cells have reduced permissivity to support HCV replication and are unsuitable for use in the co-culture assay (320). Future work is needed to investigate cell-to-cell transmission in a polarized cell line. In the current system with Huh-7.5 cells it is unlikely that HCV exploits cell junctions that are

impermeable to HCV glycoprotein targeting antibodies since we found differences in efficacy of antibodies able to inhibit co-culture HCV transmission. It is more likely that HCV transmits either through a virological synapse similar to those induced by HIV between T cells or through utilization of cellular protrusions for example filopodia, microvillus or nanotubes (337, 415). See Figure 6.4 for illustrations of plausible routes of transmission.

HIV and HTLV induce virological synapes between T-cells (216, 228, 362). These allow efficient transfer of virus but are still permeable to neutralizing antibodies (304). New techniques allowing visualization of virions have resulted in an increase in the number of viruses known to utilize cellular protrusions for intercellular transmission. Examples include HIV that has recently been shown to travel in nanotubes connecting T-cells allowing efficient transmission (452). This differs from murine leukemia virus (MLV) that attaches to receptors on filopodia of target cells where it then proceeds to travel along the surface using F-actin retrograde flow to reach the target cell body (432). A number of viruses including MLV remain associated with the cell surface once released and are directed to adjacent cells in a process called cell “surfing”, MLV has recently been shown to be assembled at sites of cell contact further increasing the efficiency of transmission (59, 304, 421, 431). African Swine Fever virus and vaccinia viruses utilize cellular protrusions in a completely different way, actin tails propel the virions to neighbouring cells (106, 232). Interestingly vaccinia viruses use this system to propel themselves across infected cells until they reach naïve cells further increasing efficacy of transmission (116).

Filopodia are easily visible between our Huh-7.5 cells in co-culture (Fig.4.6) and have been shown to exist *in vivo* (202). A recent paper visualized HCV traveling along filopodia prior to endocytosis upon reaching the cell body (96). These findings support the theory that HCV may utilise a cell associated means of transmission including the use of filopodia to transmit effectively in co-culture and suggest that this may also be possible *in vivo*. We observed an apparent increase in dependence on co-receptor SR-BI during nAb resistant transmission (Fig.5.12-15). If filopodia or microvilli were indeed important for mediating nAb resistant transmission one would envisage that expression of SR-BI at these locations would be important. In support of this Peng et al., reported that SR-BI is found in clusters on microvili on a number of different cultured cell lines, and furthermore it is at these locations that SR-BI predominantly functions as a mediator for cholesterol trafficking between the cell and HDL particles (373).

Transmission of viruses along cellular protrusions allows directed transfer of virions to neighbouring cells negating the rate limiting step of diffusion and in turn reducing exposure and time of exposure to neutralizing antibodies. These cell-associated routes may allow the highly neutralizing antibody resistant transmission we have observed for HCV *in vitro*. The crucial next step to further understand HCV cell-to-cell transmission is to visualize viral transmission in *in vitro* and *in vivo* studies.

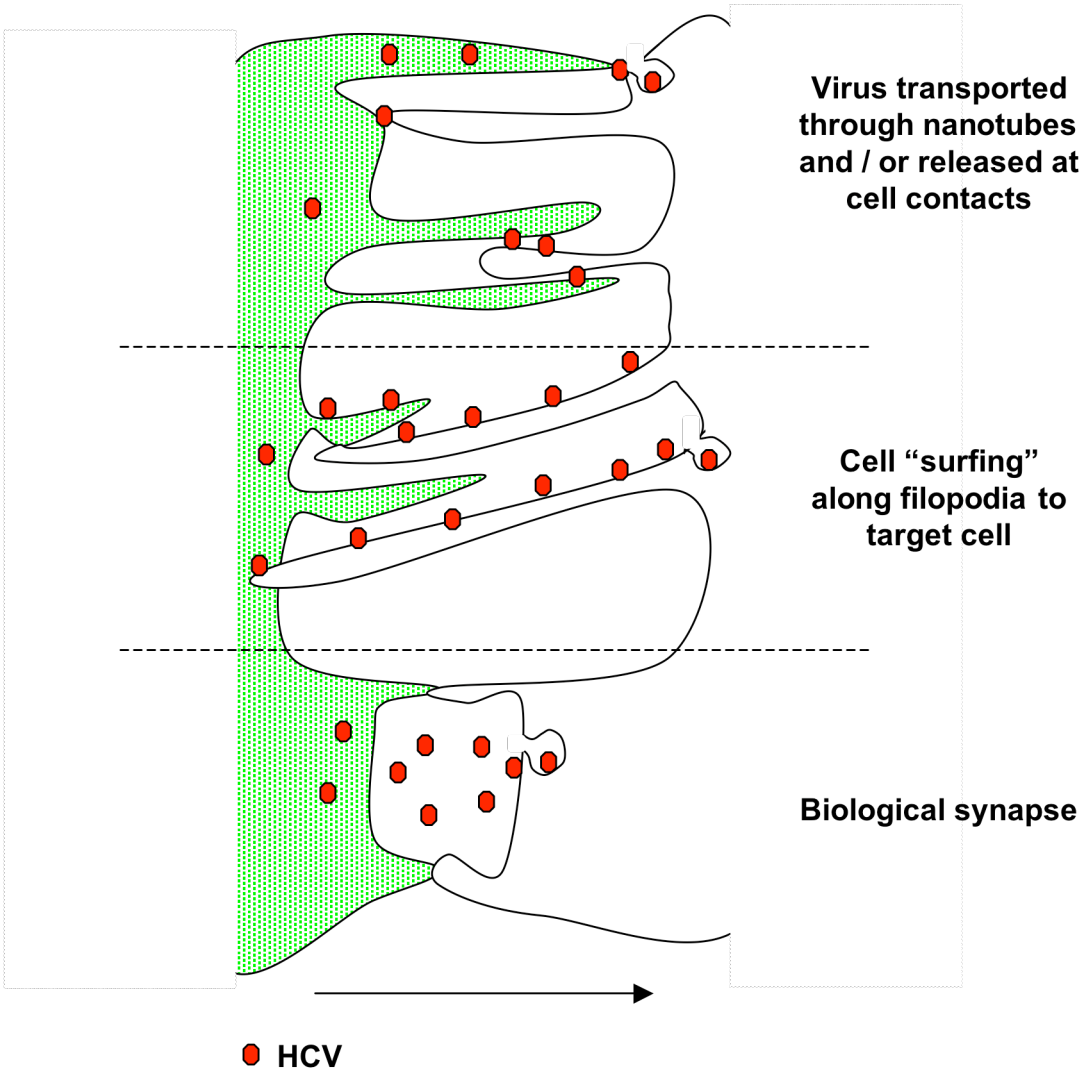


Figure 6.4: Possible routes of HCV cell-to-cell transmission in co-culture.

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