MODELLING HEPATITIS C VIRAL HOST INTERACTION AND CO-INFECTION

by

SAMANTHA MARY LISSAUER

A thesis submitted to
The University of Birmingham
for the degree of
DOCTOR OF PHILOSOPHY

Institute of Immunology and Immunotherapy
College of Medical and Dental Sciences
The University of Birmingham

January 2018

Abstract

Hepatitis C Virus (HCV) is a clinically important infection that leads to chronic liver disease and Human Immunodeficiency Virus (HIV) co-infected patients have more rapid progression to severe liver disease and show higher rates of HCV vertical transmission.

Hepatocytes are a highly differentiated cell type and support low level HCV replication. Most studies of the viral life cycle use de-differentiated hepatoma cell lines, which are highly permissive. The mechanism behind this difference is poorly understood. We show that dimethylsulfoxide (DMSO) differentiated Huh-7 cells have a 100-fold reduction in permissivity to HCV infection. We confirm that these cells are differentiated and upregulate key liver specific markers including miR122. They are metabolically active and have intact innate signaling pathways in response to infection. We observed a 10-fold reduction in the initiation of replication and a 10-fold loss in extra-cellular particle infectivity. In contrast cell-to-cell dissemination rates were comparable and cell-contact dependent infection of differentiated cells can overcome the restrictions seen in cell-free infection.

HCV cell-to-cell transmission can also be mediated by other cell types. T cells are the primary cell supporting HIV-1 infection. We have shown that HCV can bind primary and immortalized T cells and trans-infect hepatoma cells. This requires replicating HIV but is independent of co-receptor engagement. HIV-1 infection of CD4+ T cells induces a significant increase in HCV trans-infection by increased viral binding. T cells provide a vehicle for HIV-1 to promote HCV infectivity, transmission and persistence.

Dedication

For Dave, Jacob, Hannah and Reuben

– my world.

Acknowledgements

I would like to thank my supervisors, Professor Jane McKeating and Professor Deirdre Kelly for their huge amount of guidance and support throughout my PhD.

I would also like to thank all members of the McKeating laboratory throughout the time of my PhD. I would particularly like to thank Dr Luke Meredith who 'taught me everything' in the lab. I will forever be grateful for the time he spent helping me plan, execute and analyse experiments. I would also like to thank the other PhD students in the lab who helped with the highs and lows of doing a PhD. The other members of the team made me feel welcome and immediately included and contributed to making the whole experience enjoyable, and educational.

I would also like to thank Dave, who is great and encourages me to grab opportunities in life.

Table of contents

List of figures

List of tables

List of abbreviations

2CMC	2'-C-methylcytidine
AFP	Alpha-Fetoprotein
CMFDA	5-chloromethylfluorescein diacetate
CYP3A4	Cytochrome P450 3A4
DAA	Direct acting antivirals
DMSO	Dimethylsulfoxide
FFU	Focus Forming Unit
G6P	Glucose-6-Phosphatase
HBV	Hepatitis B Virus
HCV	Hepatitis C Virus
HCVcc	HCV cell culture
HCVpp	HCV pseudoparticle
HIF	Hypoxia-inducible factors
HIV	Human Immunodeficiency virus
HNF4	Hepatocyte Nuclear Factor 4
IL-2	Interleukin-2
ISG	Interferon Stimulated Gene
JFH	Japanese Fulminant Hepatitis
LDL	Low Density Lipoprotein
MDA5	Melanoma Differentiation-Associated protein 5
MiR	Micro-RNA
MOI	Multiplicity of Infection
PAMPS	Pathogen Associated Molecular Patterns
PBMC	Peripheral Blood Mononuclear Cells
PEPCK	Phosphoenolpyruvate carboxykinase
PRR	Pattern Recognition Receptors
RIG-I	Retinoic acid-inducible gene I
SNPs	Single Nucleotide Polymorphisms
TCR	T Cell Receptor
TLR	Toll like Receptors
TNF	Tumour necrosis factor
UTR	Untranslated Region
VLDL	Very Low Density Lipoprotein
VSV	Vesicular Stomatitis Virus
VX950	Telaprevir

Chapter 1: Introduction

1.1 : Hepatitis C – the clinical picture

1.1.1 Epidemiology

Hepatitis C virus is a significant global health problem. Acute HCV infection is often asymptomatic and between 15-45% of people can spontaneously clear the infection (WHO, 2017). Thus much HCV burden remains undiagnosed. The remaining 60-80% of people develop chronic infection; there are currently thought to be around 71 million people with chronic HCV infection worldwide. It is likely that only about 20% of these individuals know they are infected. Over time, chronic HCV infection causes ongoing liver inflammation, fibrosis and cirrhosis. The risk of liver cirrhosis is 15-30% within 20 years (WHO, 2017). A significant proportion of chronically infected patients develop hepatocellular carcinoma (1-5%). Liver disease resulting from infection with HCV is the seventh most common cause of mortality worldwide (Stanaway et al, 2016). **Figure 1.1** shows the leading causes of mortality worldwide from 1990-2013. Notably, unlike other communicable disease, mortality rankings for viral hepatitis (A, B, C and E) have increased over time. **Figure 1.2** shows a map of viral-related hepatitis mortality rates by country and relative contributions of the hepatitis virus to mortality rates. Most deaths were due to liver cirrhosis and carcinoma secondary to infection with HBV and HCV (Stanaway, et al, 2016).

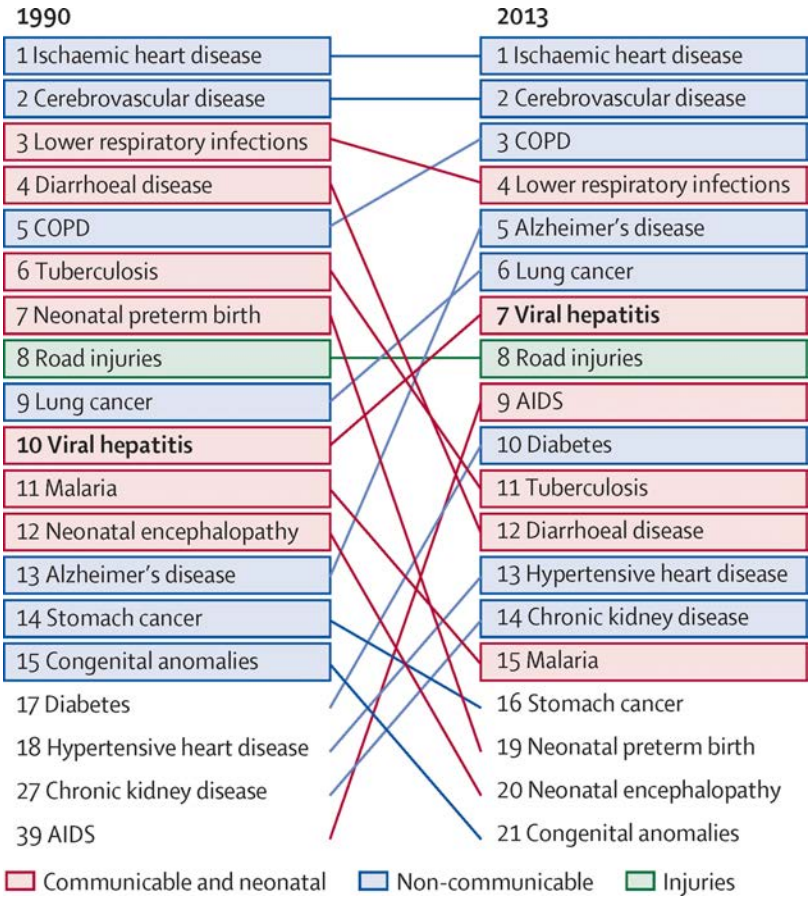


Figure 1.1 Leading causes of global mortality and trends 1990-2013

From Stanaway et al (2016) Reproduced with permission

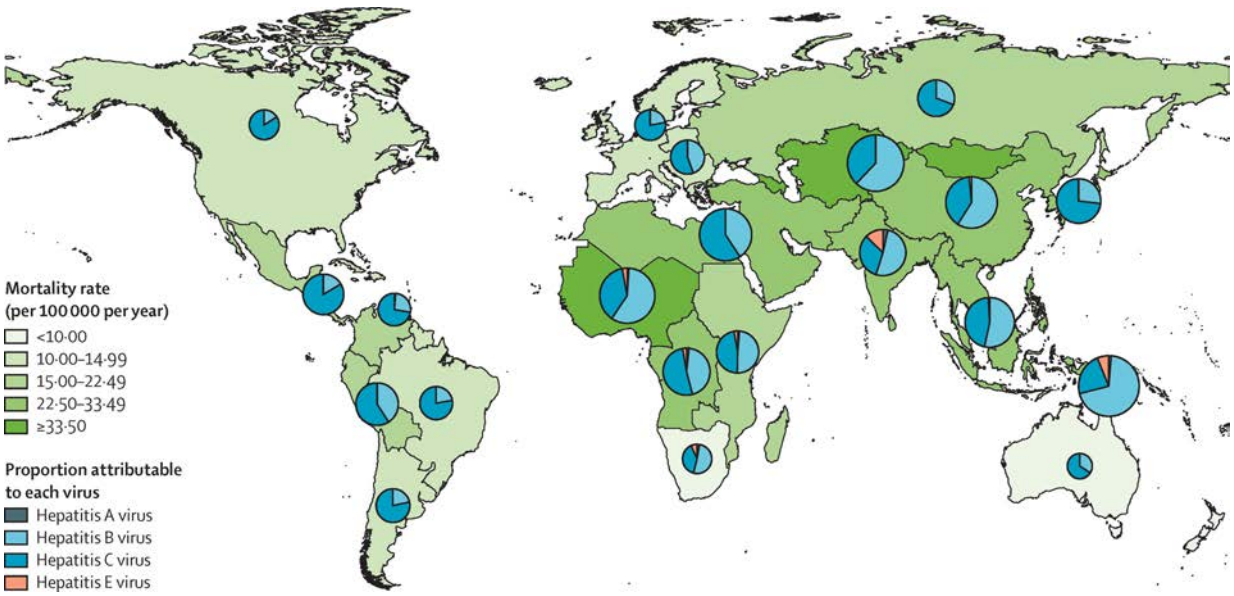


Figure 1.2 Viral hepatitis-related, age-standardised mortality rates by region.

Pie charts indicate the proportion of deaths attributable to each virus. From Stanaway et al (2016), reproduced with permission.

Despite concerning global statistics, it is likely that these figures will improve, as recent developments in treating HCV with direct acting antivirals have transformed outcomes for patient with chronic HCV. However, the challenge remains to identify these individuals globally and provide access to treatment and adequate follow up (WHO, 2017). The World Health Organisation has set ambitious targets to treat 80% of eligible patients with chronic HCV by 2030. If this target is achieved it will change the landscape of global HCV, however the burden of liver damage will remain. Liver transplants secondary to chronic HCV and associated liver damage remain as the second most common cause for transplantation in the UK (Neuberger, 2016).

1.1.2 Genotypes and geographical distribution

To date there have been seven genotypes of HCV classified (Simmonds, Holmes et al, 1993; Simmonds, McOrmish et al, 1993; reviewed in Simmonds, 2004) with significant differences in geographical prevalence. There is about 30% sequence divergence in the genotypes, however despite this they remain very similar in transmission, viral kinetics and pathogenesis (reviewed in Simmonds, 2004). Genotype 1 is the most commonly found in America (70%), Japan (75%) and Europe (50-75%), although genotype 2 and 3 are also found in these regions. Genotype 3 and 6 are found predominantly in Asia and South Asia and genotypes 4 and 5 predominate in Africa. A recently identified genotype 7 (Murphy et al, 2014) was identified in the democratic republic of Congo, in central Africa, but does not appear to have large clinical significance to date.

HCV genotypes were predictive of response to traditional interferon based treatment regimes, with genotypes 2 and 3 consistently showing higher rates of viral clearance than genotypes 1 and 4 (Manns et al, 2006). In contrast, most direct acting antivirals (DAAs) show pan-genotypic efficacy to cure infection. In fact, the first DAA were used to treat those with genotype 1 infection with very promising rates of viral response.

1.1.3 Co-infection

There are some populations who are at increased risk of HCV infection and complications. Patients co-infected with HIV have significantly accelerated progression of liver cirrhosis, decompensated liver disease and hepatocellular carcinoma (Chew and Bhattacharya, 2016). In some areas, HCV related liver disease is the leading cause of death in co-infected patients (Mandorfer et al, 2016; Peters et al, 2014). HIV co-infection increases perinatal transmission risk of HCV (reviewed in Sulkowski, 2008). It is estimated that there are around 2.3 million people co-infected with HIV and HCV (Platt et al, 2016). Pockets of high levels of co-infection exist, such as in Eastern Europe, where 80% of HIV infected patients are seropositive for HCV (Peters et al, 2014). In these areas intravenous drug use is the most common transmission route and co-infected subjects are a high risk group to develop HCV related complications.

1.1.4 Treatment of chronic HCV

Significant advances have been made in the treatment of HCV in the last 5 years. The first-generation direct acting antiviral drugs were the protease inhibitors Telapravir and Bocepravir, that were administered alongside interferon and ribavirin. They were only effective in genotype 1 infection, were associated with significant side effects and the virus rapidly developed resistance (Kieffer et al, 2007). Since then, the rapid development of next generation DAA's have revolutionized treatment options and outcomes for patients with chronic HCV, even with advanced liver disease or co-infection with HIV. Treatment algorithms are available (European Association for the Study of the Liver, 2017), but broadly, interferon free oral treatment regimens are now available for all genotypes (1-6) achieving high viral response rates. Sofosbuvir, a NS5B protease inhibitor was the first drug approved by the FDA for treatment of HCV. Initial studies including Sofosbuvir for the treatment of chronic HCV in genotype 1patients (alongside PEG-Interferon and ribavirin) were successful in achieving sustained virological response rate (SVR) of more than 90% versus 60% for standard treatment (Lawitz et al, 2013). A further significant step forward was the development of 'Harvoni' a combination of Sofosbuvir and Ledipasvir, an NS5A inhibitor. Genotype 1 infections, with or without cirrhosis can be treated with 8-12 weeks of an interferon free, oral fixed drug combination regimen, achieving a SVR of over 90% (NICE guidance, UK). Furthermore, a host of new drugs continue to become available, acting on various targets in the HCV viral lifecycle (European Association for the Study of the Liver, 2017).

Remarkably, treatment response rates are comparable between HIV/HCV co-infected patients and mono-infected patients.

Despite significant advances in HCV treatment there remains some challenges. Drug combinations are very expensive, and despite tiered pricing strategies and some generic drugs becoming available there is still significant difficulties in making these drugs widely available (WHO Guidelines, 2016). Furthermore, there are problems identifying those with chronic HCV, and organisational barriers exist to implementing drug treatment. In addition there are some patient groups that do not have access to these drugs, including pregnant women, despite significant clinical need. The residual burden of liver disease post HCV cure is also challenging and patients require ongoing monitoring for development of HCC (Ringelhahn et al, 2017).

HCV has a high genetic diversity through it's high replicative activity, producing an estimated 10^{12} new virions each day (Neumann et al, 1998) and the lack of proof reading ability of the RNA-dependent RNA polymerase (RdRp). Consequently, it is possible that quasi-species exist that already harbour resistance mutations against DAA in drug treatment naïve individuals (Rong et al, 2010). Raj et al recently described the pre-existence of mutations present in the liver of patients with chronic HCV that conferred resistance to NS5B inhibitors (Raj et al, 2017). Although this has not been seen as clinically relevant yet, the pre-existence of resistance mutations raises the possibility that suppression of wild type virus may function as selection pressure allowing growth of resistant virus (Sarazzin et al, 2007). It is therefore crucially important

that we continue to study the virus and pathogen/host interactions, even in the new DAA era.

1.2 : Hepatitis C – the virus

HCV is a positive strand RNA virus of the genus Hepacivirus in the Flaviridae family (Fields Virology). It was first identified as the cause of non-A non-B hepatitis in 1989, and since then huge strides have been made in understanding the virus, it's life cycle and host interactions.

The HCV 9.6-kb genome is an uncapped positive single RNA strand. The 5' and 3' untranslated regions (UTR) contain control elements essential for translation and replication. The 5'UTR, contains an internal ribosomal entry site (IRES) which initiates translation. The two UTR's are separated by a single open reading frame which is encodes a single polyprotein. (Hoffman and Liu, 2011, Moradpour et al, 2007, Lindenbach and Rice, 2005). The resulting polyprotein is cleaved into 10 different products (**Figure 1.3**): the structural proteins that form the viral particle include core and envelope glycoproteins E1 and E2; the non-structural proteins include the p7 ion channel, NS2-3 protease, the NS3 serine protease and RNA helicase, the NS4A polypeptide, the NS4B and NS5A proteins and the NS5B RNA-dependent RNA polymerase (Scheel and Rice, 2013). The polyprotein and function of viral encoded proteins is summarized in **Figure 1.3.**

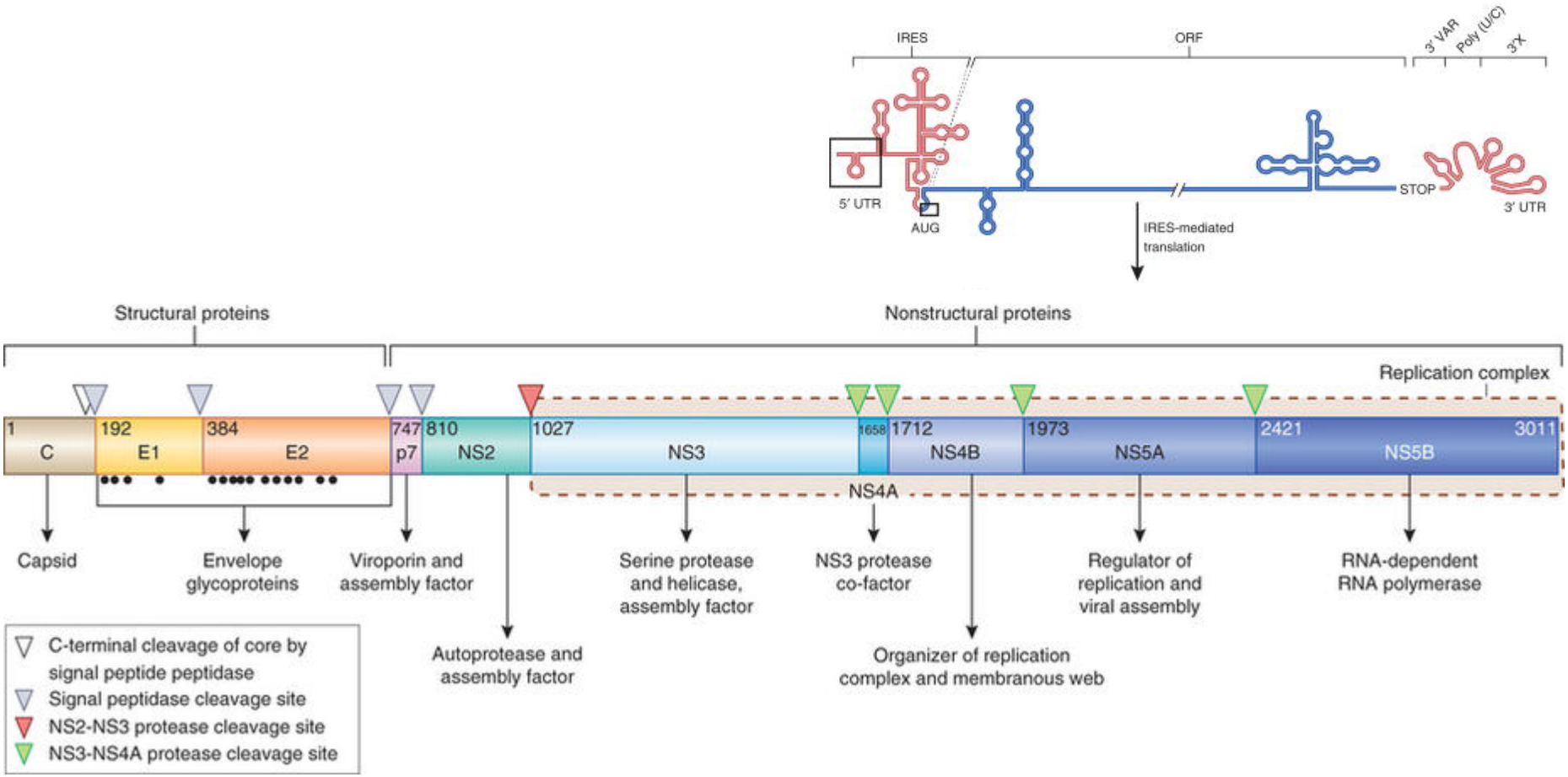


Figure 1.3 The HCV genome and polyprotein

The HCV genome encodes a single polyprotein, which is trafficked to the ER membrane where the maturation process includes cleavage at indicated sites (triangles). The C terminal of core is cleaved by a signal peptide peptidase (Penin et al, 2004). E1/E2 and p7 are cleaved from the polyprotein via signal peptidases. E1 and E2 undergo several further maturation steps, including glycosylation (indicated by black dots), conformation and assembly of heterodimers. The NS2/NS3 protease cleaves itself in an autocleavage mechanism (indicated by the red triangle). The protease in NS3 then acts with NS4 to cleave the remaining proteins. The arrows indicate the function of the individual envelope proteins

1.2.1 The virion

The single RNA HCV strand is surrounded by an icosahedral capsid and envelope derived from a host cell lipid bilayer (Bartosch et al, 2006). E1 and E2 glycoproteins form heterodimers that are embedded in the lipid bilayer (Gastaminza et al, 2008). The virion associates with low density (LDL) and very low density lipoproteins (VLDL), resulting in heterogeneity of the viral particle in culture and a low buoyant density (Popescu et al, 2014; Merz et al, 2011, Lindenbach and Rice, 2013). Human lipoprotein Apolipoprotein E and C (ApoE/C) associate with the viral particle *in vitro* and *in vivo* and are essential for viral replication (Chang et al, 2007). The heterogeneity of HCV particles makes recognition by E1E2-specific neutralizing antibodies challenging (Scheel and Rice, 2013).

1.2.2 Viral entry

The initial stage of the HCV life cycle starts with the virus binding to hepatocytes, which are the primary target cell. HCV entry is a multi-step process and an overview of the important virus-cellular interactions are shown in **Figure 1.4**. The two surface glycoproteins E1 and E2 play an important role in receptor recognition and binding. Due to the association of the virion with VLDL (André et al, 2002) the LDL receptor, alongside glycosaminoglycans, are thought to be an important capture molecule for initial low affinity binding to hepatocytes (Barth et al, 2003; Agnello et al, 1999). Scavenger receptor BI (SRBI), expressed on hepatocytes, interacts with HCV proteins E1 and E2 on the surface of the

lipoviral particle. It is likely that this acts as the primary attachment factor for HCV (Scarselli et al, 2002; Grove et al, 2007; Catanese et al, 2010; Syder et al, 2010). The CD81 tetraspanin interacts with E1 and E2 and most likely acts as a post attachment co-receptor (Pileri et al, 1998, Bartosch et al, 2003; Flint et al, 2006, Meuleman et al, 2008). There is evidence to suggest that CD81 forms complexes with the tight junction protein Claudin-1 (Harris et al, 2008; Harris et al, 2010). Trafficking of CD81 and Claudin-1 to tight junctions is promoted by the virion itself (Farquhar et al, 2012) and both receptors are endocytosed with the viral particle. Hepatocellular polarisation limits CD81 trafficking and may explain the limited infection of these primary cells in vivo (Harris et al, 2013; Mee et al, 2009). The tight junction proteins Claudin 1 (Evan et al, 2007; Meertens et al, 2008) and Occludin (Ploss et al, 2009) are essential factors defining HCV entry. Claudin 6 and 9 have been reported to replace Claudin 1 as an entry factor, however they are expressed at very low levels in the human liver (Zheng et al, 2007). The tyrosine kinases, epidermal growth factor receptor (EGFR) and ephrin receptor A2 (EphA2) are also important for entry as they regulate CD81-claudin-1 associations (Lupberger et al, 2011). More recently the transferrin receptor (TfR1) has been identified as an HCV entry factor (Martin and Uprichard, 2013). TfR1 is thought to act after the CD81 binding step and is likely to be involved in particle internalisation. Late in the entry process the Niemann-Pick C1-like 1 cholesterol absorption receptor mediates virion-cell membrane fusion via a cholesterol dependent step (Sainz et al, 2012).

Final uptake of the viral particle occurs through a clarithrin-mediated pH dependant process of internalisation, probably in endosomes (Tscherne et al,

2006; Farquhar et al, 2012). Acidification of the endosome induces HCV glycoprotein membrane fusion. It is not clear which protein mediates the process of membrane fusion, with recent evidence that E1 is important for this final step in HCV entry (Omari et al, 2013; Li et al, 2014).

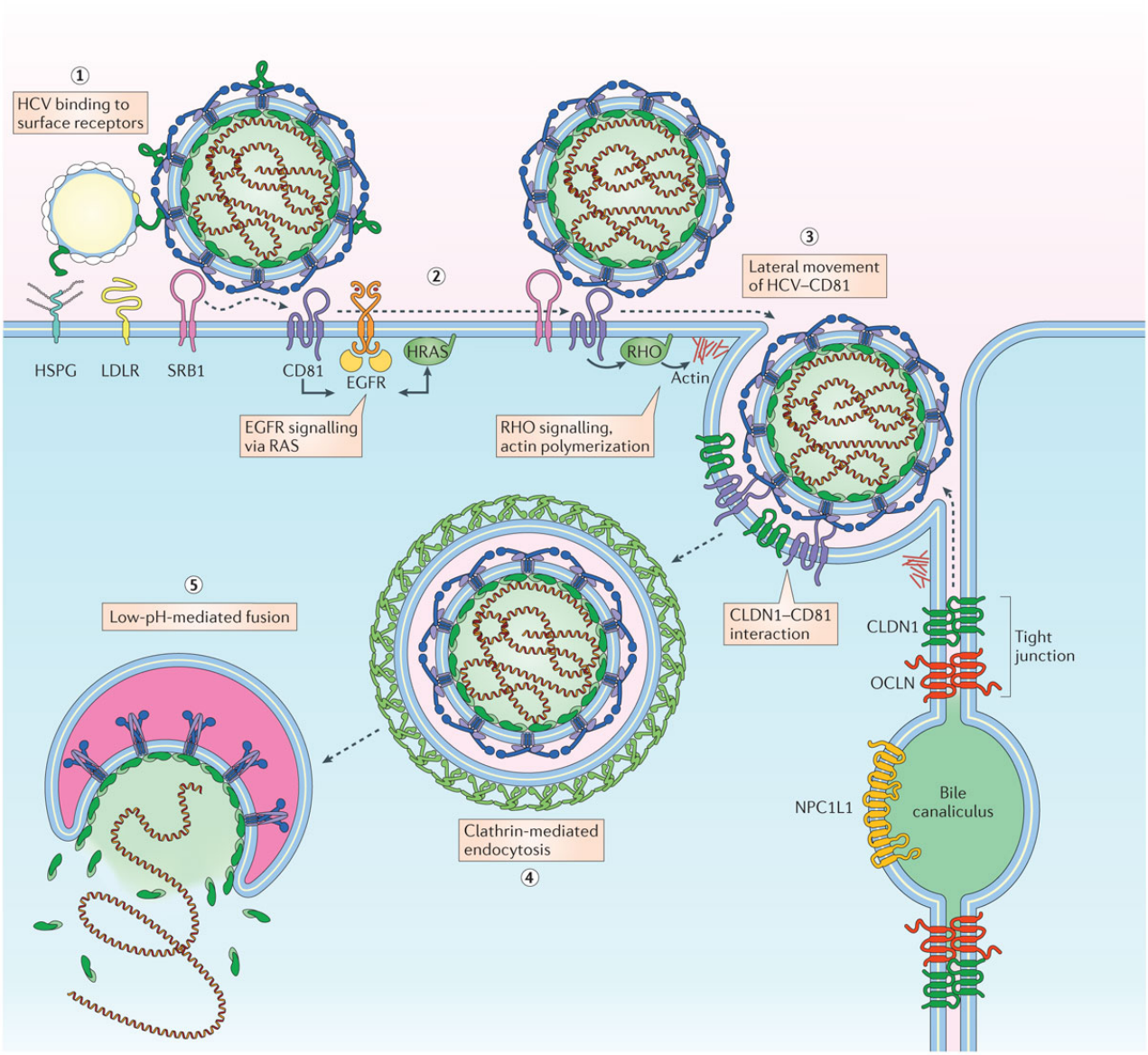


Figure 1.4 An overview of the steps involved in hepatitis C viral entry

(from Lindenbach and Rice, 2013, reproduced with permission)
Step 1 involves HCV lipoviral particle interacting with heparin sulphate proteoglycans (HSPGs), low density lipoprotein receptor (LDLR) and SRB1.
Step 2 involves interaction of E2 and CD81 which activates signal transduction through epidermal growth factors, RAS and RHO GTP-ases
Step 3 There is lateral movement of the CD81/HCV complex to tight junctions
Step 4 Interaction of virus with tight junction proteins mediate endocytosis
Step 5 Low pH inside the endosomes induces membrane fusion and uncoating of the viral particle, possibly mediated by E1.

1.2.3 Translation and Replication

Once internalised, the genome is trafficked to the endoplasmic reticulum where translation of the genome is initiated by an IRES located in the 5'UTR (reviewed in Hoffman and Liu, 2011). Translation initiation begins with recruitment of the 40S ribosomal subunit to the HCV IRES (Pestova et al, 1998). Correct positioning of the ribosomal subunit is required for translation (Wang, et al, 1995). Following ribosomal binding the cellular initiation factors eIF2 (eukaryotic initiation factor), eIF3 and eIF5 interact with the IRES/40S complex to form a translationally competent ribosomal unit that progresses with elongation and termination resulting in viral protein translation (Hellen et al, 2009; Hoffman and Liu, 2011; Fraser et al, 2007). In addition to eIF there are a number of other cellular factors identified that interact with the IRES or 3'UTR that can modulate translation. These are reviewed in Hoffman and Liu (2011) however include the micro-RNA's miR122 and miR196a (see section 1.3.6). Following translation the resulting polyprotein is cleaved by cellular and viral encoded proteases to produced 10 HCV proteins (**see Figure 1.3**).

Replication of the virus is accompanied by cellular ER membrane rearrangement to form the 'membranous web' mediated by NS4B (Gretton et al, 2005; Bartenschlager et al, 2004), which forms the membrane anchor for the replication complex. The replication stage of the HCV life cycle is closely related to host lipid metabolism. RNA replication occurs in membranes rich in cholesterol and sphingolipids, which are likely transported as part of the replication complex to the ER (Grassi et al, 2016). HCV also induces de novo lipid

metabolism within hepatocytes, likely mediated by the sterol regulatory membrane binding protein (SREBP) (Waris et al, 2007). Positive and negative strand RNA is transcribed and initiated by the NS5B RNA dependent RNA Polymerase (RdRp) (Zhong et al, 2000). The NS5A is important to HCV replication (Scheel and Rice, 2013), although it's exact mechanisms are not completely understood. NS5A associates with lipid rafts, which is essential for the HCV replication complex (Gao et al, 2004). NS5A phosphorylation state is important in regulating viral assembly (Appel et al, 2005), and regulates the balance between downstream processing and RNA replication (Neddermann et al, 2004). NS5A is also shown to bind Cyclophilin A, a cellular based peptidyl-prolyl cis-trans peptidase, that catalyses a conformational change in NS5A that is necessary for replication to occur (Yang, 2008).

1.2.4 Viral assembly and release

Less is known about the process of viral assembly than other steps in the viral lifecycle but it is closely linked with cellular lipid metabolism. Virion assembly is coordinated between synthesis of new RNA strands, encapsidation and acquisition of a lipid envelope via budding into the ER (Lindenbach and Rice, 2013). After initial cleavage the core protein relocates to lipid droplets in the cytoplasm (McLauchlan et l, 2002; Lindenbach and Rice, 2013). This relocation is an essential step as it coordinates the transfer of the viral replication site and E1/E2 from the membranous web to sites of particle assembly (Barba et al, 1997; Miyanari et al, 2007). Virus secretion is closely linked to the VLDL/LDL export pathway (Grassi et al, 2016; Scheer and Rice, 2013).

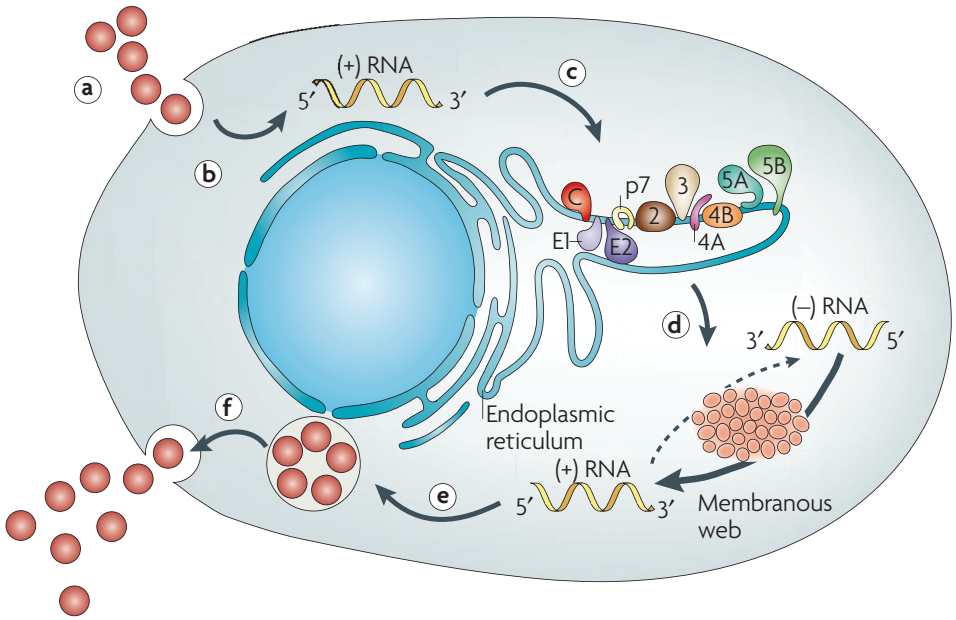


Figure 1.5 Schematic and simplified HCV life cycle
(from Moradpour et al, 2007. Reproduced with permission).
A Virus binding and internalisation
B pH dependent release of virus into cytoplasm and uncoating
C Internal Ribosomal Entry Site (IRES)-mediated translation and polyprotein processing in the rough ER
D RNA replication occurring in the membranous web; the nonstructural proteins together with the viral RNA and host factors form the replication complex. Mir-122 is a co-factor for viral replication (Jopling et al, 2005). The replication complex catalyses RNA amplification
E Packaging and virus assembly
F Virion maturation and release

1.2.5 Viral dissemination and spread

Once a virion is released from an infected cell it can infect other naive cells as described. In addition to cell-free virus transmission HCV can transmit directly cell-to-cell between hepatocytes (Timpe et al, 2008; Brimacombe et al, 2011; Witteveld et al, 2009). This allows the virus to evade host immune responses including neutralizing antibody (Timpe et al, 2008) and is an efficient route of dissemination route (Meredith et al, 2013). Although the exact mechanisms of cell-to-cell transmission have not been fully elucidated it is thought that the entry molecules: CD81, SR-BI, Claudin1 and Occludin are important (Grove et al, 2008; Ciesek et al, 2011; Brimacombe et al, 2011). The visualization of infected cells in foci within liver biopsy samples also suggests that this is an important route of viral dissemination in vivo (Liang et al, 2009). Furthermore, cell-to-cell dissemination can spread virus harboring resistance mutations to DAAs efficiently throughout the liver (Xiao et al, 2015). In addition to direct cell delivery between two hepatocytes there is increasing evidence that HCV can associate with other cells, such as B cells (Stamataki et al, 2009), which allow the possibility of cell-to-cell delivery of virus to distant sites in the liver.

1.2.6 Studying the HCV life cycle

10 years after the discovery of HCV a successful replicon system was developed allowing study of subgenomic viral replication in hepatoma cell lines (Lohman et al, 1999) (**Figure 1.6**). Since then many more full length and subgenomic replicons, of differing genotypes have been constructed. This has

allowed the study of viral replication and facilitated drug discovery. Despite the benefits of replicons it was still not possible to study viral entry or the full virus life cycle as replicons do not produce infectious virus. Retroviral pseudoparticles bearing the HCV glycoproteins E1 and E2 were developed in 2003, allowing the study of HCV viral entry (Hsu et al, 2003, Drummer et al 2003, Bartosch et al, 2003). Although important in the investigation of viral entry mechanism HCVpp models are single cycle, modeling HCV entry, but retroviral proteins direct other stages of replication. In 2005 a genotype 2a HCV strain was isolated from a patient with Japanese fulminant hepatitis (JFH-1) (Wakita et al, 2005; Lindenbach et al, 2005; Zhong et al, 2005). This strain replicated and released infectious virus particles in culture. This allowed the study of the complete viral life cycle in hepatoma cells in culture for the very first time.

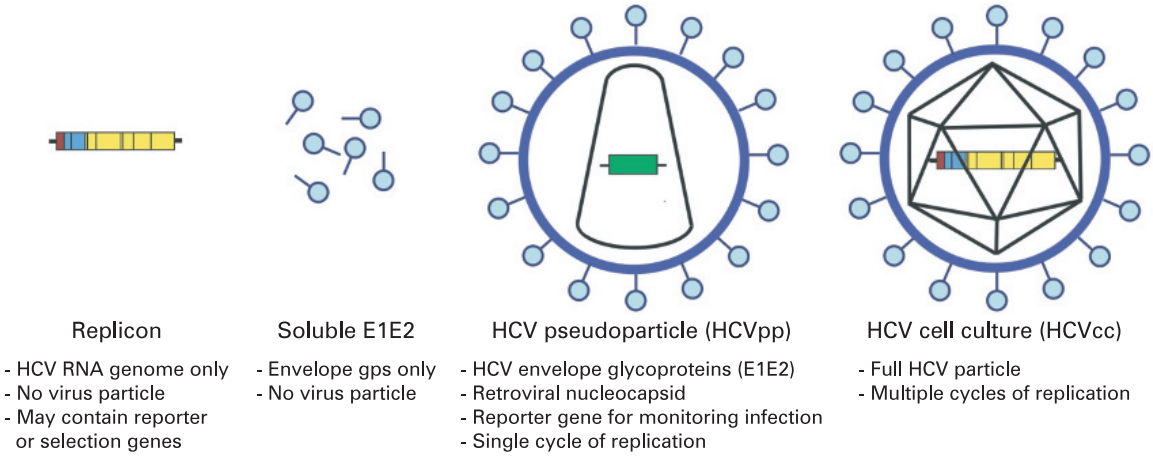


Figure 1.6 Model systems available to study hepatitis C
(from Timpe, Gut, 2008)

1.3 The host

HCV is a natural human pathogen and hepatocytes are the host cell. This narrow cellular tropism is due to a number of specific factors including proteins required for viral entry, lipid pathways important for viral assembly and molecules needed for replication, such as miR122 (Jopling et al, 2005). It is estimated that up to 30-50% of hepatocytes are infected in patients with hepatitis C (Wieland et al, 2014) and HCV infects in clusters with differential innate immune response to the virus between infected and uninfected cells (Wieland et al, 2014; Kandathil et al, 2013). Key viral/host interactions are important to study as these are likely to affect viral pathogenicity and outcome from infection.

1.3.1 The Liver

The liver is a large organ with a broad range of functions. It is made up largely of hepatocytes (75-80%), which support the main roles of the liver in secretory, metabolic and endocrine functions. The liver also has many other cell types; Kupffer cells are specialist macrophages, which line the sinusoids, hepatic stellate cells (HSC) are contractile cells within the space of Disse, and liver sinusoidal endothelial cells (LSECs) line the sinusoid. There are also intrahepatic lymphocytes present within the sinusoidal space (Krishna, 2013).

The cells are arranged within lobules: hexagonal structures with plates of hepatocytes radiating from a central vein. The portal triad, which has a branch of the hepatic artery and portal vein, supplies oxygenated blood to the lobule. Blood

from the portal vein/hepatic artery drains to the central vein via the liver sinusoids. The triad also contains a bile duct, which collects bile generated by hepatocytes and drains via the common bile duct to the gall bladder (Krishna, 2013). **Figure 1.7** and **1.8** show the macroscopic view of the liver and a detailed view of the portal triad.

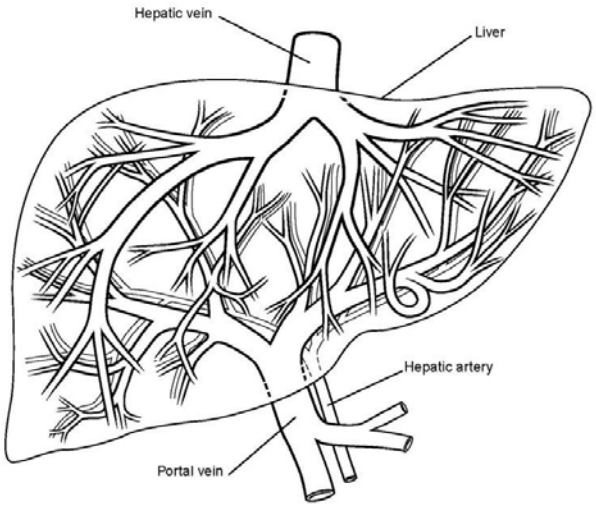
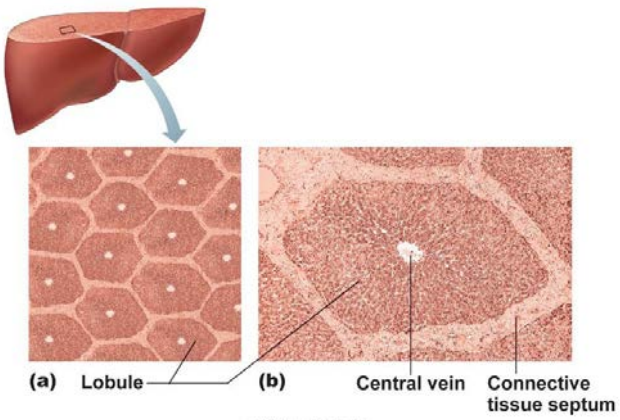


Figure 1.7 Macroscopic drawing of the Liver
The liver has a dual blood supply from the portal vein and heaptic artery. These branch and supply oxygenated blood to the liver lobules via the portal triad
Adapted from Kapoor, 2017 available at
URL:https://emedicine.medscape.com/article/1900159

A



B

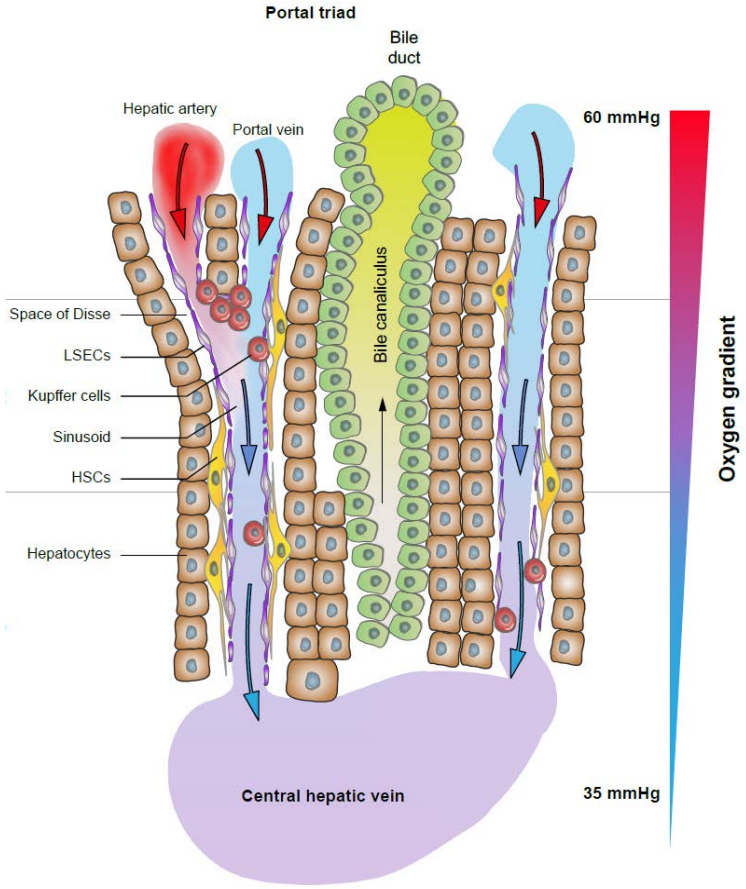


Figure 1.8 The liver lobule

(A) The liver is divided into hexagonal lobules of hepatocyte arranged around the central vein. Adapted from Chapter 22, The Digestive System at URL: https://cms.webstudy.com/WebstudyFileSystem/testovaci/GetFile/293875/Ch%2022/Ch22b/Ch22b_print.html

(B) A liver lobule showing plates of hepatocytes surrounding sinusoids which carry oxygenated blood from the portal triad to the central hepatic vein. Kupffer cells line the sinusoids and hepatic stellate cells (HSC) are found in the space of Disse. There is an oxygen gradient across the lobule with cells in the peri-portal triad existing in oxygen levels of around 8%, and cells in the peri-venous exposed to oxygen levels of around 4%. Adapted from Wilson et al (2014)

The liver has a range of known physiological functions, including the formation and secretion of bile and the metabolism of protein, lipid and carbohydrate. Some of the main functions of the liver are detailed below (Sendenburg and Dufour, 2011):

- Glucose homeostasis and glycogen storage
- De novo synthesis of proteins such as albumin and clotting factors
- Hematopoiesis, particularly in the fetal period
- Lipid metabolism including cholesterol synthesis, lipogenesis and triglyceride formation
- Production of IGF-1, an important anabolic hormone, particularly in childhood.
- Breakdown of insulin and other hormones
- Detoxification and excretion of metabolites, such as ammonia
- Drug metabolism via the cytochrome P450 system
- Iron metabolism

1.3.2 Hepatic Polarity

Hepatocytes, like many other epithelial cells, are polarized. This contributes to their function, and also their ability to maintain a barrier between two interfaces or body compartments, such as air/blood in lung epithelium or blood/bile in hepatocytes. Epithelial cells maintain distinct apical and basolateral domains that are mediated by tight junctions (Treyer et al, 2013; Shin et al, 2006). Most polarized epithelial cells in the body such as gut or lung epithelium maintain simple polarity, where there are single opposing apical and

basal layers (Mee et al, 2009). Lateral cell walls associate with other cells (**Figure 1.9**) and mediate cell:cell interactions. Hepatocytes, however, show a more complex polarity and have several apical and basal poles. The apical poles of adjacent hepatocytes form a continuous system of bile cannuliculi, into which bile is secreted. The basal poles are in contact with the sinusoids, and are important in uptake of nutrients and secretion of metabolized proteins and drugs (Decaens et al, 2008; Shinn et al, 2006). Tight junctions form the border between apical and basal domains in polarized cells (**see Figure 1.9**).

Tight junctions consist of transmembrane proteins and peripheral membrane proteins that interact with one another to form a complex network (Shin et al, 2006). Transmembrane proteins include Occludin (Feldman et al, 2005), Claudin (Tsukita et al, 2001) and Junctional Adhesion Molecules (JAM) (Mandell et al, 2005). They reach across the junction, connecting adjacent cell membranes to make a seal between cells. Transmembrane proteins bind intracellularly to peripheral proteins allowing cellular cytoskeleton arrangement and downstream signaling. Peripheral proteins include Zona Occludens (ZO proteins), Cingulin and Rab 13, amongst others (reviewed in Decaens et al, 2008). Adherens junctions containing proteins such as cadherin, nectin and catenin act to initiate and maintain cell:cell contact (Hartsock et al, 2008).

1.3.3 Hepatocyte Polarization and HCV

The establishment of cellular polarity has been reported to limit the entry for a number of viruses, as many use junctional receptors for entry (Schultze et al, 1998; Roberts et al, 1999; Chodosh et al, 2000; Mee et al, 2009). The HCV receptors Claudin, CD81 and SRB1 are distinctly located within the polarized hepatocyte. Claudin-1 is located at the apical-cannalicular tight junction region and is also found at the basal sinusoidal region in polarized hepatocytes. It is co-located with CD81. SR-B1 co-localises with Claudin-1 at the baso-lateral surface (Reynolds et al, 2008). Mee et al showed that there was limited HCV entry into a polarized hepatocyte derived cell line, HepG2. Perturbation of cellular polarity by activation of protein Kinase C (Mee et al, 2009) or by vascular-endothelial derived growth factor (VEFGF) (Mee et al, 2010) increased HCV infection and transmission. Furthermore, Fletcher et al (2014 and 2017) showed that long-term treatment with TNF promotes HCV entry by disrupting tight junctions. These studies indicate a key role for hepatocyte polarity in restricting HCV entry.

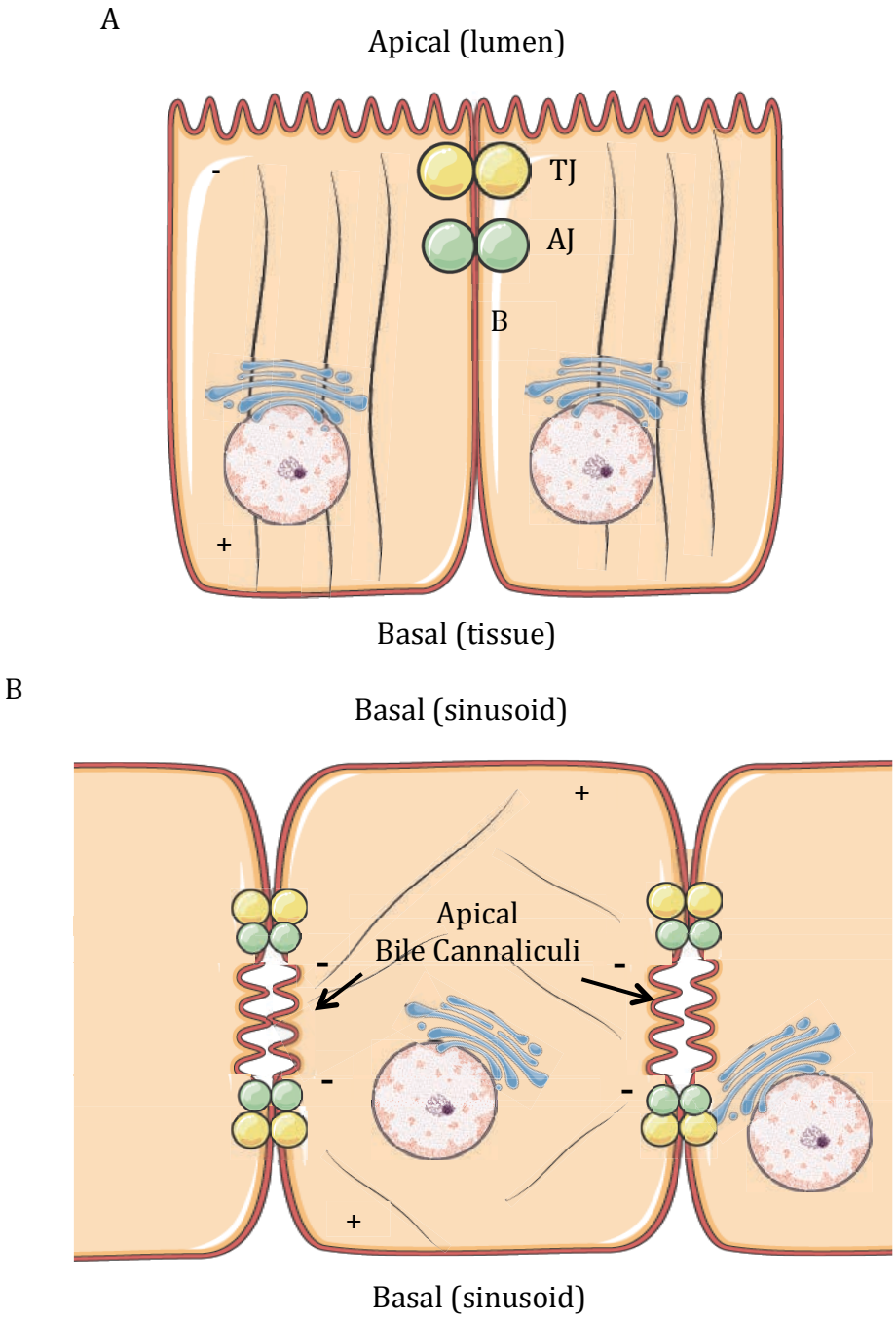


Figure 1.9 Simple and complex hepatic polarity
Adapted from Decaens et al (2008)
(A) Structure of a simple polarized epithelial cell. The apical surface facing the lumen is separated from the basal surface facing the tissue by tight junctions (TJ) and adherens junctions (AJ). Microtubules are orientated with the minus end at the apical pole.
(B) Structure of polarized hepatocyte with the apical lumen forming the bile cannaliculi between adjacent cells. These are separated by tight and adherens junctions from basal membranes, which are in contact with the liver sinusoids.

1.3.4 Oxygen levels and hepatocytes

Hepatocytes in the liver are exposed to varying amount of nutrients and oxygen; cells in the periportal region are exposed to nutrient rich, oxygenated blood, whereas cells in the peri-venous region are exposed to relative hypoxia (**see Figure 1.8**). This oxygen gradient across the liver results in liver zonation, where hepatocytes have distinct structural and functional properties (Braeuning et al, 2006; Jungermann et al, 2000). Modest changes to oxygen levels during viral hepatitis can induce a hypoxic response in hepatocytes via hypoxia inducible factors (HiFs). Hypoxia indubible factors in turn regulate transcription of a large number of genes involved in cellular functioning including cell metabolism and inflammation (reviewed in Wilson et al, 2014). Hifs have been implicated in altering the liver microenvironment contributing to liver damage and promoting oncogenesis (Wilson et al, 2014).

Hepatitis C virus can also directly stabilize Hifα, causing a 'pseudohypoxic' response in the liver, even under normal oxygen tension, which is mediated by the oxidative stress induced by HCV gene expression (Nasimuzzaman et al, 2007). Hifα is a transcriptional activator of VEGF, which can alter hepatocyte polarity and promote HCV entry (Mee et al, 2010). Additionally, exposing cells to low oxygen levels has been shown to increase HCV replication (Vassilaki et al, 2013) and inhibiting Hif can, in turn, reduce HCV replication (Wilson et al, 2012). There may be a number of pathways involved in hypoxia and HCV replication, as Vassilaki et al (2013) reported that exposing cells to low oxygen tension increased HCV replication, but this was independent of Hif. In this study

replication directly correlated with an increase in anaerobic glycolysis, suggesting that metabolic pathways affected by hypoxia are likely to alter viral/host interactions (Ripoli et al, 2010).

1.3.5 Hepatocyte metabolism

The liver is crucial to maintaining normal glucose homeostasis by balancing hepatic glucose production (gluconeogenesis) and utilization (glycolysis). These pathways are coordinated so that one is always more active in the cells than the other at any point in time (Shoji et al, 2012). **Figure 1.10** gives an overview of the main enzymes involved in glucose metabolism within hepatocytes. There is much clinical evidence that HCV infection leads to aberrant glucose metabolism and there is association between chronic HCV and Type 2 Diabetes (Mason et al, 1999; Negro et al, 2009; Negro et al, 2011). Although the precise mechanisms behind this are not known, it is likely that there is a combination of altered metabolism of glucose (Ripoli et al, 2010), reduced hepatic glucose uptake (Kasai et al, 2009) and the development of insulin resistance (Koike et al, 2007; Miyamato et al, 2007; Pazienza et al, 2007). There are conflicting data on whether HCV causes cells to switch to gluconeogenesis (Shoji et al, 2012) or glycolysis (Ripoli et al, 2010) when acutely infected. However, long-term HCV infection damages oxidative phosphorylation causing cells to switch to glycolysis as a means of maintaining cell survival and this is mediated by Hif1α (Cuninghame et al, 2014). Cells then utilize pyruvate as a main form of energy and produce lactate as a by-product. This phenomenon, termed the 'Warburg effect' is the metabolic process that de-differentiated

cancer cells effect in order to immortalize (Warburg et al, 1927; Shaw, 2006). Therefore there is evidence for a significant virus host interaction between glucose metabolism and HCV in both healthy hepatocytes and cancerous, de-differentiated hepatocytes. Additionally lipid metabolism within hepatocytes and the HCV viral lifecycle are closely linked as cellular lipoproteins are associated with several steps in the HCV lifecycle (reviewed in Popescu et al, 2014).

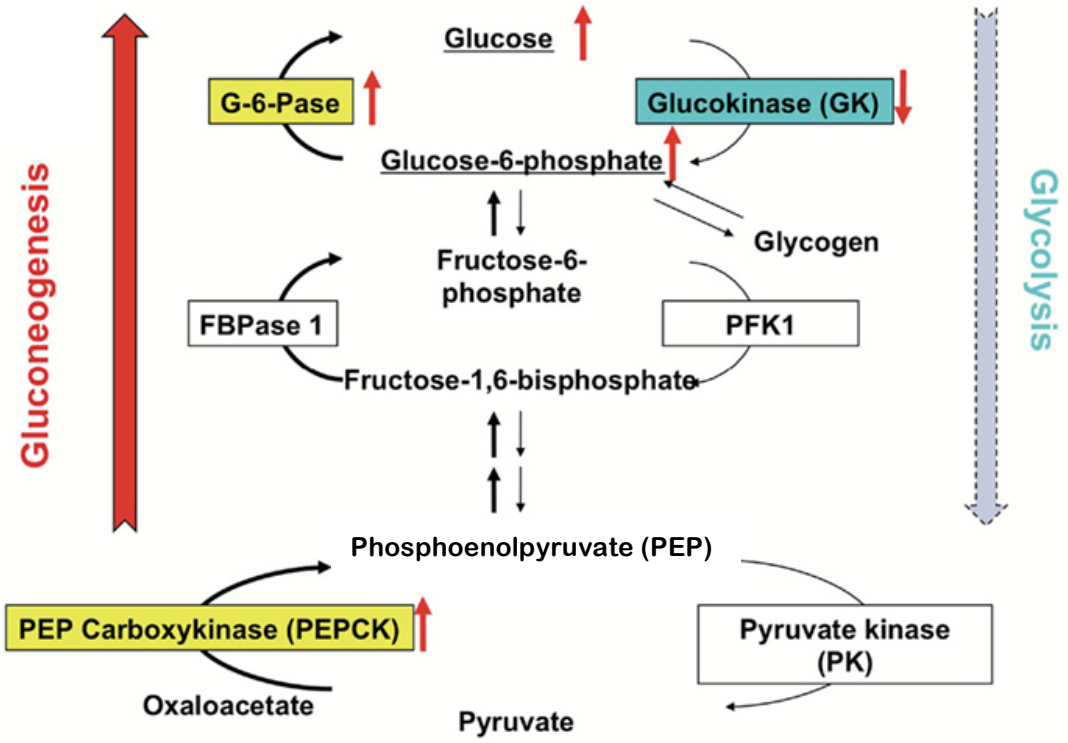


Figure 1.10 Maintenance of glucose homeostasis via gluconeogenesis and glycolysis.

Glucose-6-phosphate (G6P) and PEP Carboxykinase (PEPCK) are the rate limiting enzymatic steps in the conversion of pyruvate to glucose. Glucokinase (GK) is the rate limiting step in the utilisation of glucose in the glycolytic pathway. Adapted from Shoji et al (2012)

1.3.6 Liver Micro-RNAs and HCV

Hepatocytes express a huge number of micro-RNAs, many of which have been shown to interact with HCV. Micro-RNAs (miR) are endogenous short (about 22 nucleotides) non-coding RNAs that play a key role in regulating gene expression, by sequence specific binding to nucleic acids (reviewed in Filipowicz et al, 2008). There is increasing evidence that miRs are important in liver development, regeneration and metabolism, and some host miRs are known to directly impact the HCV lifecycle. Interestingly, miRs require a minimum of just 7 nucleotides to pair, meaning a single miR may target a number of different mRNA. Up to 60% of all human protein coding genes are predicted to be subject to miR modification (Friedman et al, 2009) and several miRs can act together along a pathway (Tsang et al, 2010). Consequently, there is a complex interaction between a huge range of miRs and the liver (Chen et al, 2014; Yang et al, 2015; Chen et al, 2016; Wei et al,, 2016; Vienberg et al, 2017).

Despite this complex interaction there are some important miRs that are known to interact with HCV; miR122 is the most studied of these and is the most abundantly expressed miR in the human liver (Lagos-Quintana et al, 2002; Girard et al, 2008). It's expression is driven by liver-specific transcription factors, including HNF4α and is very closely linked to hepatocyte differentiation (Deng et al, 2014). miR122 is unusual amongst miRs as being shown to positively regulate HCV (Jopling et al, 2005), by binding to a highly conserved region in the 5'UTR (Jopling et al,, 2008; Jangra et al, 2010; Machlin et al, 2011). This enhances viral translation and stabilizes the genome by a number of mechanisms. miR122

improves translation efficiency by enhancing ribosomal association with the viral RNA (Henke et al, 2008; Georgen et al, 2012). Additionally it protects the viral RNA from nucleolytic degradation (Wilson et al, 2011; Conrad et al, 2013; Li et al, 2013). Introduction of miR122 into cell lines that do not support HCV replication, such as HeLa cells, renders them permissive to support HCV replication (Chang et al, 2008; Costa et al, 2012; Fukahara et al, 2012;). Furthermore, pluripotent stem cells have been reported to support HCV replication in a miR122 dependent manner (Wu et al, 2012). There is also some evidence that miR122 can interact with Occludin and reduce viral entry (Sendi et al, 2015). Overall, miR122 is crucial to supporting HCV translation and replication and it's abundance is closely related to differentiation status of the liver.

Given its importance as a key regulator of HCV replication, miR-122 has been identified as a therapeutic target and Miravirsen was developed as a miR-122 inhibitor (Ottosen, Antimic Agent Chemo, 2015), although it's current use in the DAA landscape is likely to be limited. There is also a role for other micro-RNAs in HCV replication and key miRs that are known to regulate HCV replication are included in **Figure 1.11.**

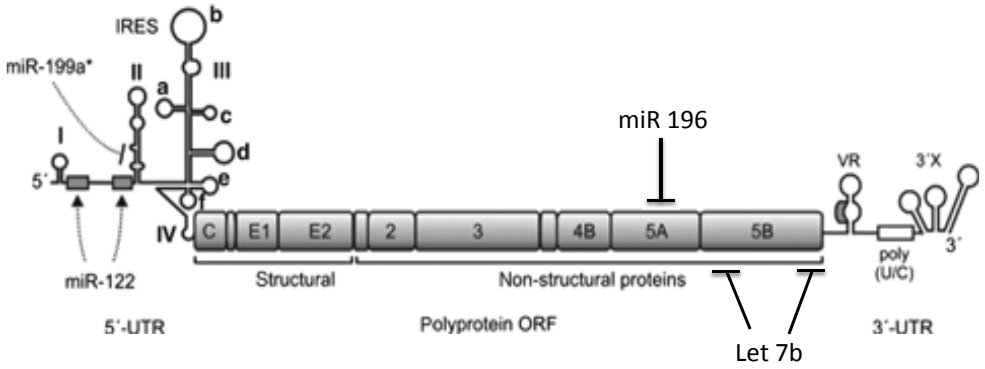


Figure 1.11 Cellular miRNAs targeting the viral genome.
miR122 binds to the 5'UTR promoting HCV replication. miR199a binds at the 5'UTR and inhibits viral replication. miR196 targets the NS5A. Let7b binds at two separate sites and inhibits replication. Adapted from Shrivastava et al (2015). and Hoffman (2011).

1.3.7 Iron

The human liver is important in maintaining homeostasis for a number of micronutrients and metabolites, including iron. Cellular iron is crucial for many processes, such as DNA generation and ATP synthesis. Given that HCV replication is dependent on intracellular machinery, it is feasible that altering cellular iron, may affect viral replication. Furthermore, there is a clinical correlation between diseases in which iron metabolism is altered, such as haemachromatosis or β-thalassaemia, and increased morbidity and mortality from HCV infection probably secondary to generation of free radicals and potentiating oxidative stress (reviewed in Drakesmith, Nature Reviews Microbiology, 2008).

Cellular iron has been shown to directly impact viral replication; increasing cellular iron is associated with an increase in HCV replication via stimulation of translation initiation factor eIF3 (Kakizaki et al, 2000; Theurl et al, 2004). However, other authors have shown that cellular iron can inhibit HCV replication by specifically binding to the Mg2+ binding pocket of the RNA polymerase and inhibiting enzymatic activity (Fillebeen et al, 2005). Furthermore, Huh-7 cells supporting HCV subgenomic replicon show an altered iron metabolism with reduced uptake and increased release (Fillebeen et al, 2007), possibly allowing viral escape from the effect of iron on established replication. Although the molecular interactions between cellular iron in the liver and HCV replication require further clarification, there is good evidence that there is interplay between virus and host.

1.4 : Innate and Adaptive Immunity

1.4.1 Innate signaling

The human innate immune response is the first line of defence against pathogens and interferons are a key feature of the chronic HCV infected liver. Hepatocytes can produce and respond to Type 1 and Type 3 interferons. Pathogen associated molecular patterns (PAMPS), such as double stranded RNA, are recognized by extra or intracellular pattern recognition receptors (PRRs), such as Toll-like receptors or RIG-I. **Table 1** gives an overview of PAMPS and PRRs involved in innate signalling.

	Receptor	*Localisation*	*Pathogen Associated Molecular Patterns (PAMPS)*
TLR	TLR1	Cell surface	Triacyl lipopeptides
	TLR2	Cell surface	Lipopeptides, Peptidoglycan
	TLR3	**Endosomes**	**Double stranded RNA (Viruses)**
	TLR4	Cell surface	Lipopolysaccharide
	TLR5	Cell Surface	Flagelin
	TLR6	Cell surface	Diacyl lipopeptides, Lipoteichoic acid
	TLR7	Endosomes	Single Stranded RNA (Viruses)
	TLR8	Endosomes	Single Stranded RNA (Viruses)
	TLR9	Endosomes	CpG containing DNA (Viruses)
	TLR10	Endosomes	Profilin-like proteins
RLR	**RIGI**	**Cytosol**	**RNA (double stranded regions and 5'triphosphates)**
	MDA5	**Cytosol**	**RNA (long with structural features)**
	LPG2	**Cytosol**	**dsRNA**

Table 1.1 An overview of human innate signalling molecules important in pathogen recognition.

Those in bold are important in HCV infection (adapted from Satoh and Akira, 2016).

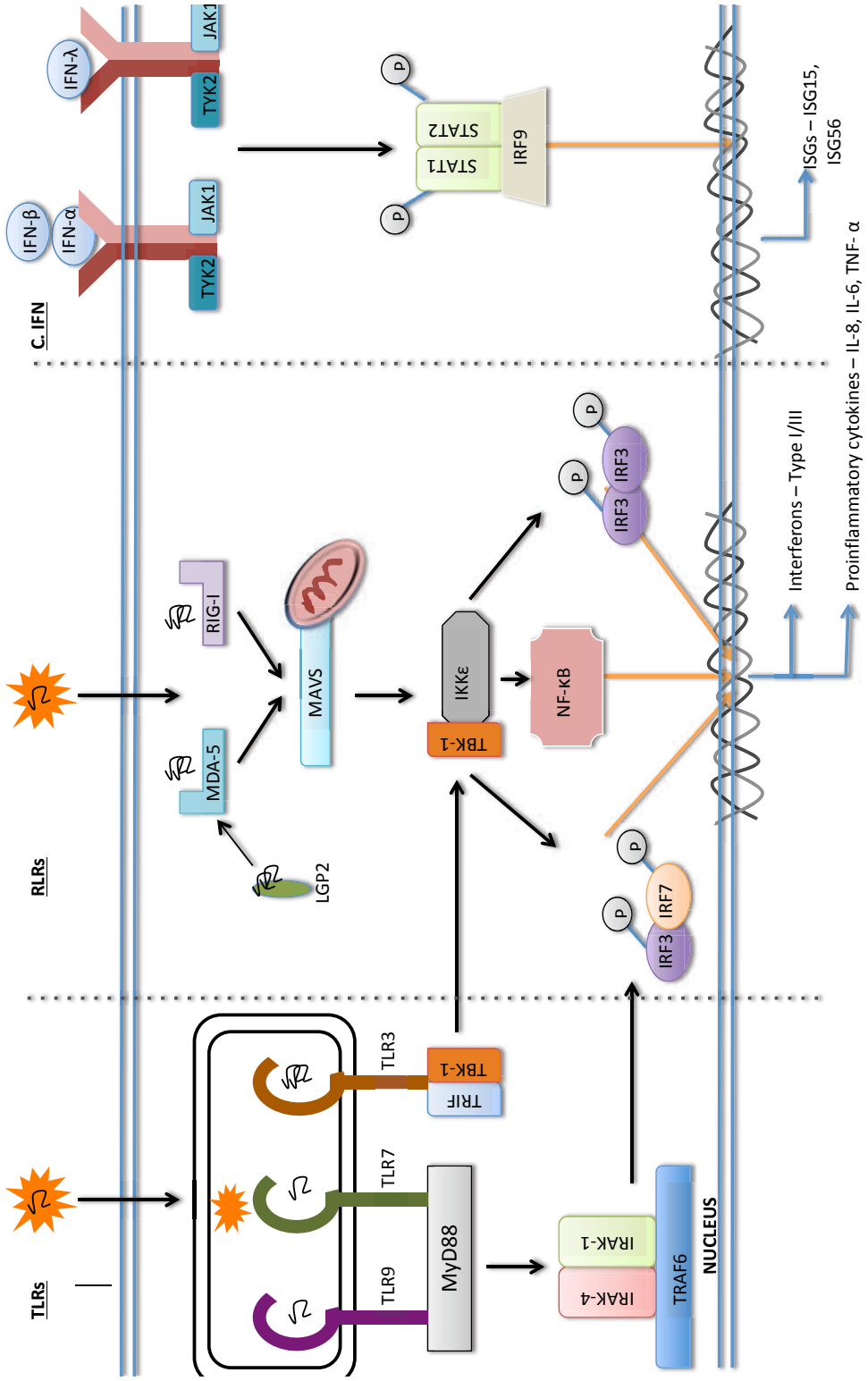

TLRs
RLRs
C. IFN
NUCLEUS
IFN-λ
IFN-β
IFN-α
JAK1
TYK2
JAK1
TYK2
STAT2
STAT1
IRF9
P
ISGs – ISG15, ISG56
Interferons – Type I/III
Proinflammatory cytokines – IL-8, IL-6, TNF- α
RIG-I
MDA-5
LGP2
MAVS
IKKε
TBK-1
NF-kB
IRF3
IRF3
IRF7
IRF3
TLR3
TLR7
TLR9
TBK-1
TRIF
MyD88
IRAK-1
IRAK-4
TRAF6

Figure 1.12 An overview of hepatocellular innate signalling HCV

Human hepatocytes express TLR3 on endosomes that can recognize dsRNA. Extracellular dsRNA can be taken up into uninfected cells by scavenger receptors (Dansako et al, 2013) and brought to endosomes, where it is bound by TLR3. HCV infected cells can also generate dsRNA. This activates the TIR domain-containing adapter-inducing IFN-β (TRIF). Additionally dsRNA intermediates intracellularly are recognized by retinoic acid-induced gene I (RIG-I) (Sumpter et al, 2005) and melanoma differentiation-associated gene 5 (MDA-5) (Israelow et al, 2014; Du et al, 2016). In addition LGP2 (laboratory of genetics and physiology 2), present in the cytoplasm is likely to predominantly synergise with MDA5 to promote efficient signal downstream (Satoh et al, 2010). LGP2 may also have a negative regulatory effect, by mopping up excess ds RNA in the cytoplasm (Komuro et al, 2006). MAVS and TRIF trigger a signaling cascade that results in IκB kinases (IKK) and TANK-binding kinase 1 (TBK1) activation leading ultimately to activation of NF-κB and IRF3 (Fitzgerald et al, 2003; Seth et al, 2005). These proteins bind to the promoter elements of IFNI and IFNIII in the nucleus leading to interferon and cytokine production. Interferons can act locally or at a more distant sites via binding cell surface receptors (Bruening et al, 2017). IFN receptors activate the JAK-STAT pathway that regulates a wide number of interferon stimulated genes (ISG) that can limit HCV replication. In chronic infection, HCV is able to persist and replicate within cells, due to the ability of the viral encoded NS3/4A protease to cleave the adaptor proteins MAVS (Li XD et al, 2005) and TRIF (Li K et al. 2005), that blunts the interferon response (Bellecave et al, 2010).

1.4.2 Interferon production and Interferon Stimulating Genes (ISGs)

Prior to the advent of novel direct acting antiviral drugs (DAAs) for treating HCV, exogenous interferon-α was the only treatment option available. This binds the interferon receptor leading to JAK-STAT activation and ISG upregulation within the cell. ISGs interfere with different stages of the viral lifecycle (reviewed in Schneider et al, 2014). **Table 1.2** provides an overview of ISGs produced in response to viral infection and their broad mechanism of action. The specific pattern and level of ISGs upregulated in response to interferon can predict outcome from acute infection and treatment response to interferons, independent of other factors. In chronic HCV infection the endogenous interferon response fails to clear the infection; the robust ISG upregulation in response to infection is thought to reflect a refractory state within the liver (Sarasin-Filipowicz et al, 2008; Chen, et al 2005; Asselah et al, 2008). Specific patterns of ISG upregulation can predict outcome to HCV infection, with a 4-gene classifier (IFI27, ISG15, RSAD2 and the tumour marker HTATIP2) showing accurate correlation with treatment response (Dill et al, , 2011).

Antiviral effectors Exert an antiviral effect	Viral Entry	Myxovirus resistance: **Mx1**	- Traps viral components e.g. nucleocapsid when entering cell
		Cholesterol-25-Hydoxylase: **CH25H**	- Probable interference with viral host membrane fusion - Can also directly inhibit HCV replication by altering protein synthesis
		Interferon Inducible Transmembrane: **IFITM**	- Inhibit viral entry in endosomal compartment
	Viral Translation and Replication	Tripartite motif: **TRIM**	Large family that exert multiple effects - TRIM14 degrades NS5A - TRIM22 ubiquitinates NS5A
		ISG15	Complex signaling via covalently attaching to target proteins (ISGylation) - ISGylation of host protein 4EHP blocks translation of HCV
		Virus inhibitory protein, endoplasmic reticulum-associated, IFN inducible: **Viperin (RSAD2)**	Inhibits RNA replication by disrupting host (VAP-1) and virus (NS5A) interaction within lipid droplets
		IFI27	Inhibits HCV replication in replicon system
	Viral egress	**Tetherin (BST2)**	Inhibits viral budding. Probably not a major player in HCV infection.
IFN desensitization		**SOCS** proteins	Inhibit JAK-STAT signaling
		USP18	Interacts with IFNAR protein preventing JAK binding and downstream signaling. Only inhibits Type 1 IFN (not Type III)

Table 1.2 Important Interferon Stimulated Genes modulating HCV infection.
Adapted from Schneider et al (2014)

Another important correlate of outcome in HCV infection is IFNλ gene polymorphisms. These were identified by genome wide association studies investigating the differences in response rate to interferon treatment between those of African and European descent (Thomas et al, 2009; Ge et al, 2009). Further work identified three major SNPs near the INFλ3 and IFNλ4 genes, which correlate with HCV treatment response (Rauch et al, 2010; Duggal et al, 2013) and raised the importance of IFNλ in treatment and innate response to HCV infection in hepatocytes. IFNλ levels are increased in humans infected with HCV (Dolganiuc et al, 2012), leading to increased ISG levels (Thomas et al, 2012; Duong et al, 2012), and hepatocytes infected with HCV express abundant IFNλR (Dolganuic et al, 2012). Primary human liver cultures also express IFNλ (predominantly IL29 IFNλ1) and HCV infection induces ISGs (Marukian et al, 2011), that can inhibit viral replication. Indeed, IL29 is the major Type III interferon produced by hepatocytes during acute HCV infection (Park et al, 2012), and there is very little Type I interferon expressed. Although Type I interferons were initially thought to provide the first line of defence against HCV, there is very little IFN α/β production in chronic HCV infection. HCV has been reported to interfere with TLR-3, RIG-I and JAK-STAT signalling (**see Figure 1.12**) downstream of the α/β receptor (Horner et al, 2009. In contrast, Type III interferon signaling pathways remain functional and intact (Park et al, 2012).

There is likely to be variable response across the liver to infection with HCV. Kandathil et al (2013) showed, using laser capture micro-dissection, that HCV clusters in cells, away from areas of high IFITM expression. Similarly

Sheahan et al (2014), using the same method, was able to show that infected cells expressed a unique innate antiviral signature, which differed from uninfected cells and was variable, depending on IFNλ genotype. These data suggest that early viral host interactions, particularly innate responses, determine the outcome of HCV infection.

1.4.3 The cellular innate response

In addition to interferon production, cells of the innate immune system are also important in controlling HCV infection. The human liver contains many cells in addition to hepatocytes, such as NK cells and dendritic cells that can augment the response to acute infection. However, this is difficult to recapitulate in vitro and therefore studying innate signalling in hepatocytes is not a true reflection of the whole physiological innate response.

Intrahepatic NK cells and NK T cells are activated in response to acute HCV infection and produce IFNγ, which leads to cytotoxic killing of infected hepatocytes (Amadei et al, 2010). The production of IFNγ leads to activation of intra-hepatic Kupffer cells and the release of pro-inflammatory molecules including TNFα, galectin-9 and IL-18 (Mengshol et al, 2010; Chattergoon et al, 2011). The role of these molecules is not wholly defined but they promote inflammation and enhance maturation of dendritic cells (Mengshol et al, 2010) that can express IFNα (Takahashi et al, 2012; Zhang et al,,2013). Dendritic cells produce IFN in response to HCV infection in hepatocytes, a response that requires replicating HCV and direct cell contact (Takahashi et al, 2012). Myeloid

derived dendritic cells are also involved with the adaptive response by activating naïve T cells present in lymphoid tissues (Terilli and Cox, 2013).

When the innate and adaptive response to infection fails to clear the virus, ongoing inflammation and cell activation in the liver leads to a gradual loss of NK cell number and function (Amadei et al, 2010; Ahlenstiel et al, 2010). The presence of pro-inflammatory molecules is detrimental over time resulting in hepatocyte damage, inflammation and cirrhosis.

1.4.4 HIV/HCV Co-infection and the innate response

There is evidence that the innate response to HCV is altered when a patient is co-infected with HIV (Hou et al, 2009; Parczewski et al, 2012; Chen et al, 2016; Hullegie et al. 2012). Interestingly, the polymorphisms in the IL28B gene that are associated with improved outcome and response to treatment in HCV, are associated with increase in all-cause mortality in HIV infected patients on antiretroviral treatment. This suggests that HIV infection alters interferon λ expression, altering the ability of the innate system to respond to HCV (Parczewski et al, 2012). Furthermore interferon λ induced by HCV infection reduces macrophage infection by HIV by activating the JAK-STAT pathway (Hou at al, 2009; Liu et al, 2012). These studies suggest there is significant interplay between Type III interferon responses in HIV and HCV infection.

HIV can also affect cellular responses to HCV. HIV can infect dendritic cells, thereby altering their numbers and response to HCV (reviewed by

Lambotin et al, 2010). Similarly, chronic HIV can alter NK cell functioning (reviewed by Iannello et al, 2008). There is evidence for significant interplay between HIV and HCV and innate signalling in co-infection, which may limit the host's ability to respond to infection.

1.4.5 Adaptive immune response to HCV

The adaptive immune response to HCV shows a delayed antibody and T cell response that appear no earlier than 8-12 weeks following acute self-limiting or chronic infection (Chen et al, 1999; Park and Rehermann, 2014). There is limited evidence that the humoral antibody response is important, and in chronically infected patients there is generally low titre and late evolving antibody (Logvinoff et al, 2004).

By contrast, T cell responses to HCV infection are crucially related to outcome; the development of a robust CD4+ and CD8+ T cell response is associated with viral clearance, with both cells being important (reviewed by Neumann-Haefelin and Thimme, 2011; Klenerman et al, 2012). The CD8 repertoire is developed early in the disease and requires CD4+ T cell help to maintain the response (Cox et al, 2005). Despite this early response, the majority of patients go on to develop chronic HCV. CD4+ T cells and CD8+ T cells show rapid exhaustion and deletion in the context of persistent HCV infection (Bengsch et al, 2010). Viral mutations can lead to escape, meaning viral epitopes are not recognized by T cells. Furthermore, upregulation of markers of

exhaustion inhibit T cell function in chronic HCV infection (Rutebemberwa et al, 2008).

1.4.6 Co-infection and the adaptive immune response

HIV also alters the adaptive response to HCV. CD4 T cell depletion in HIV, alongside B cell exhaustion, reduced number of memory B cells and increased B cell turnover leads to a reduction of HCV specific antibodies (Lambotin et al, 2012; Netski et al, 2007). Patients can lose previously generated immunity against HCV when co-infected with HIV. The immune dysregulation that occurs with HIV therefore leads to lower rates of HCV clearance and increased rates of disease progression (Terili,and Cox, 2013).

There is a complex interplay of innate, cellular and adaptive cellular response to HCV infection that is altered by the presence of other viruses, such as HIV. HCV has evolved mechanisms of subverting the immune response, allowing the virus to establish chronic infection in the majority of cases. It is likely that innate responses, particularly IFNλ signalling and downstream effects are predominant in early acute infection. Studying the host/viral response requires a system that in vitro is able to produce Type III interferon and associated ISGs.

1.5 : Models for studying Hepatitis C virus-host interactions

There exist a number of model systems for studying HCV, the viral life cycle and viral-host interactions. The ideal system is one that is physiological, accessible, easy to use and supports the replication of primary patient derived viral strains. However, this does not currently exist. There are a number of advantages and disadvantages to the current model systems available which will be considered below.

1.5.1 Hepatoma cell lines

Huh-7 cells, derived from a patient with hepatocellular carcinoma, are a permissive immortalized cell line. This cell line supports high level HCV replication in vitro and has revolutionized HCV research. Blight et al (2002) were able to identify a clone of Huh-7 that supported high levels of HCV replication. They transfected cells with a HCV subgenomic replicon, then cured the cells with prolonged interferon α treatment. Cured cells were transfected with the replicon and screened for clones that were able to support increased replication (Blight et al, 2002). The Huh-7.5 clone was identified and has been key in HCV research since then, allowing study into viral entry pathways, replication and viral egress. Huh-7.5 clones have a defective RIG-I, which is thought to be central to their increased permissivity (Sumpter et al, 2005). Recently, Huh-7.5 were reported to support replication of patient derived virus, something that has long evaded HCV researchers. Saeed et al (2015) was able to show that expressing SEC14L2, a cytosolic lipid binding protein involved in cholesterol biosynthesis, allowed Huh-7.5 to support HCV replication after inoculation with patient sera. This is likely to

lead to advances in understanding the biology of patient derived virus. Despite these advances, Huh-7 and derivatives are carcinoma derived cells and suboptimal for the study of viral-host interaction, particularly studies investigating viral/cell interactions leading to cell damage and oncogenesis.

Sainz et al described a method to differentiate Huh-7 cells towards a more 'liver-like' state, using dimethylsulfoxide (DMSO), which is commonly used to enhance cellular differentiation (Sainz and Chisari, 2006). Huh-7 upregulated key markers of differentiation and supported HCV replication. Given their ease of use and ability to support HCV infection in vitro these cells offer great promise in providing a differentiated cell model for understanding virus/host interactions.

Huh-7.5 have also been differentiated by propagation in human serum, showing increased expression of cellular differentiation markers and HCV replication (Steenbergen et al, 2013). Although they do not appear to be a physiological system that recapitulates the viral lifecycle *in vivo,* given the high titre virus that is produced from these cells, the use of human serum in combination with other model systems may offer advantages.

Other hepatoma cell lines, such as Hep3B, Huh-6 and PLC/PRF/5 are derived from immortalized liver cancers, and support some aspects of the HCV viral lifecycle. However, none support the entire lifecycle, and their use is limited (reviewed in Wilson and Stamataki, 2012).

1.5.2 Polarised models

Hepatocytes exhibit a complex polarity in vivo (see **figure 1.9**). Cell systems that are able to polarize *in vitro* are important in order to understand the impact this has on HCV entry and replication. Huh-7 cells fail to polarize and even primary human hepatocytes fail to form hepatic polarization when cultured, and rapidly de-differentiate (Wilson et al, 2012). There have been attempts to grow hepatoma cell lines on scaffolding to allow a 3-D differentiated polarized structure to develop, however these are difficult to establish and are not commonly used (Molina-Jimenez et al, 2012; Sainz et al, 2009; Tran et al, 2013).

HepG2 cells are derived from a human hepatoblastoma and over time in culture, with the development of pseudo bile cannaliculi. When engineered to express CD81 and miR122, these cells can support low level HCV infection (Narbus et al, 2011). Despite this, studies using HepG2 cells have allowed us to understand the consequences of polarization on HCV entry (Mee et al,2009). Additionally, when HepG2 cells are infected with HCV they lose polarity and undergo cellular differentiation, allowing study of the effects of the virus on aspects of carcinogenesis (Wilson et al, 2012).

HepaRG cells are an interesting cell line derived from a patient with HCV associated hepatocellular carcinoma. They are a bi-potent progenitor cell that once plated and differentiated over time will form two cell types; hepatocyte like cells, which are surrounded by biliary like epithelial cells (Gripon et al, 2002).

They appear to also polarize and form bile cannaliculi structures within the culture (**Figure 1.13**). HepaRG cells (unlike HepG2) also express many enzymes involved in drug metabolism and are particularly useful for investigating xenobiotic metabolism (Guillouzo et al, 2007). As a highly differentiated and polarized system they support infection of Hepatitis B virus, however have not been shown to support the whole viral lifecycle in HCV (Gripon et al, 2002; Hantz et al, 2009; Ndongo-Thiam et al, 2011; Fletcher et al, 2017). Additionally, they are difficult to grow and maintain in culture and need to be plated for several weeks before they can be used in their differentiated form (Gripon et al, 2002).

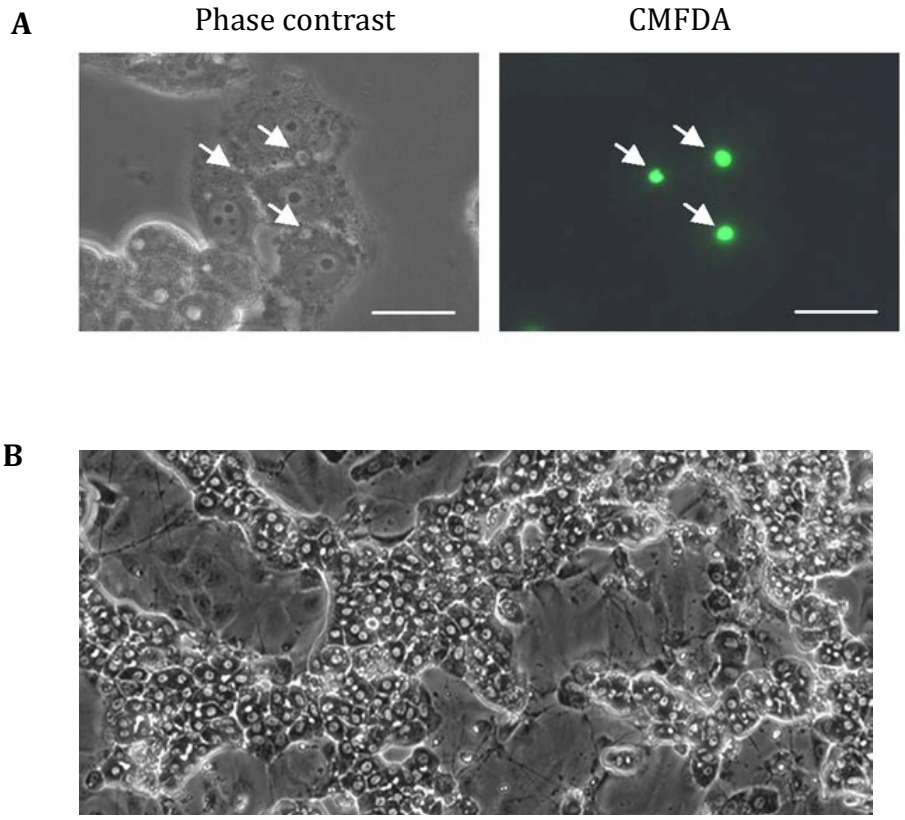


Figure 1.13 Polarised cell models

(**A**) HepG2 cells with phase contrast and CMFDA showing bile cannaliculi (from Mee et al, 2009)
(**B**) HepaRG cell morphology showing islands of hepatocytes surrounded by epithelial biliary-like cells. Small circular structures are seen within the hepatocyte pools that represent bile cannaliculi.

1.5.3 Primary cell culture

Primary Human Hepatocytes

The gold standard for in vitro HCV studies are primary human hepatocytes. These support HCV entry and replication although this is at a low level and there is significant donor variability (Wilson et al, 2015). Furthermore, these cells are well known to de-differentiate rapidly ex-vivo and are difficult to maintain in culture. This limits their ability to be used as a long-term model for HCV or other hepatotropic pathogens.

Recent studies have attempted to adapt primary human hepatocytes in culture in order to increase their utility in vitro. Micropatterned co-cultures have been developed where primary human hepatocytes are grown in micro-cultures supported by surrounding stroma and fibroblasts, in a model similar to HepaRG cells (March et al, 2015; Ploss et al, 2010). These can be maintained for weeks in culture and support the entire HCV life cycle in vitro.

Primary human hepatocytes can also be immortalized by transfection of HCV core protein (Ray et al, 2000; Basu et al, 2002). HCV core protein in these cells transcriptionally alters cellular genes involved in growth, apoptosis and innate signaling and may provide a useful model for understanding viral replication and assembly (Kanda et al, 2006).

Human Fetal Liver Cells

Human fetal liver derived cells have been used as a robust model system, particularly to study innate signalling responses to HCV (Marukian et al, 2011; Laidlaw et al, 2017; Andrus et al, 2011; Lázaro et al, 2007). They are derived from pools of human fetal livers at 16-22 weeks gestation. They support HCV infection with laboratory viral strains, however levels of viral replication differ significantly between cultures. Furthermore the robust innate response clears infection quickly if measures to dampen this response are not used (Andrus et al, 2011). Recently Guo et al described the development of a cell culture system based on human fetal liver stem cells that are able to support the entire viral lifecycle of patient derived virus (Guo et al, 2017).

Induced pluripotent stem cells

Hepatocyte like cells have also been generated from human induced pluripotent stem cells (methodology in Si-Tayeb et al, 2010). As these cells differentiate into hepatocyte like cells they produce albumin and express miR122 and HCV entry factors. They simultaneously lose foetal-specific markers suggesting they are differentiating towards adult hepatocytes, although culture methods have not yet driven these cells to terminal differentiation (Si-Taybe, et al 2009; Schwartz et al, 2011). These cells support the entire HCV lifecycle, including replication and production of infectious virus (Schwartz et al, 2011) and have been used to study HCV and host response. The particular benefit of

these cells is allowing a personalized approach to studying host genetics and response to virus infection.

1.5.4 Animal Models

Until recently, animal models to study HCV, which are the 'holy grail' in studying biological systems and viral infection, have eluded HCV researchers. Chimpanzees, which are the only known animal host for hepatitis C are not amenable to research, due to heavy ethical restrictions, however small primate models, based on GB-virus infection do exist (Marnata et al, 2015;Li et al, 2014). Mouse models using chimeric humanised mouse models and grafted humanised immune systems also exist and have allowed the study of HCV pathophysiology and therapeutic targets (reviewed in Kremsdorf and Strick-Marchland, 2017). Billerbeck et al (2017) have developed a small animal model system using rats that are natural hosts for Norway rat hepacivirus (NrHV) which is a flavivirus very closely related to HCV. This is a very exciting development in the field of HCV research and will allow study of viral host interaction, particularly host immune response and allow significant steps forward with regard to vaccine development.

There has been significant advancement in the development of model systems to study HCV, each with their own advantages. However, most of our understanding about the viral lifecycle has come from using the easily available and robust systems of hepatoma cells, particularly Huh-7 and Huh-7.5. Using a more differentiated version of these cells is likely to represent a 'middle ground'

and allow significant strides forward understanding how the virus replicates *in vivo* and host cellular response.

1.6: Co-infection

HCV can co-infect alongside other viruses, particularly other hepatitis virus, and HIV. Globally, HIV and HCV co-infection is a major concern, with overlapping modes of transmission and at-risk populations. The following section briefly considers the HIV lifecycle and models to study the interplay between HIV and HCV infections.

1.6.1 HIV virus

Human Immune deficiency virus, a single stranded RNA virus, is a lentivirus, a subgroup of retroviridae. There are two subtypes of HIV; HIV-1 and HIV-2. HIV-1 is the most virulent and geographically widespread and consequently is responsible for most of the HIV infections worldwide (Gilbert et al, 2003 Reeves and Doms, 2002). The virus causes HIV infection in humans and, if left untreated, develops into the acquired immune deficiency syndrome (AIDS) (Levy,1993).

Figure 1.14 gives an overview of the HIV viral lifecycle and major host cell factors that interact with the virus (Barré-Sinoussi et al, 2013). Briefly, the HIV virion contains two copies of single stranded RNA, surrounded by a lipid-bilayer envelope. The surface glycoproteins, gp120 and gp41, which are products of the 'env' gene are embedded within this layer, and form the outermost surface of the

virion (Lusic and Siliciano, 2017). These bind to the cell surface CD4 receptor, the main receptor for HIV (Maddon et al, 1986; Klatzmann et al, 1984; Dalgleish et al, 1984). Binding to the co-receptors CXCR4 (X4 tropic virus) or CCR5 (R5 tropic virus) allows conformational change in the cell necessary for HIV entry. HIV tends to be R5 tropic early in infection (Joshi et al, 2017). Over time mutations in the gp120 allow the virus to become dual tropic (Naif, 2013). Eventually, in about half of individuals, HIV becomes predominantly X4 tropic, a change that correlates with rapid disease progression clinically (reviewed in Naif, 2013). Following fusion of the viral capsule with the cell membrane protein core containing the viral genome is released into the cytoplasm (Thenin-Houssier and Valente, 2016). Reverse transcriptase makes a double stranded DNA copy from the viral RNA (Hu and Hughes, 2012). The double stranded DNA then moves to the nucleus of the cell and incorporates itself into host cell DNA (Craigie and Bushman, 2012). This integration of the viral DNA allows the cell to become latently infected evading immune recognition (reviewed in Lusic and Siliciano, 2017). In the presence of cellular transcription factors, such as NF-κB, (reviewed in Hiscott et al, 2001) nuclear DNA is transcribed and translated into viral RNA and viral proteins translocate to the cell surface to become packaged and released as new infectious virus (Freed, 2015). The new virions bud from the cell membrane and are released. During viral maturation the structural polyprotein is cleaved by protease to form gag proteins. These are essential in maturation of infectious virions (Freed, 2015).

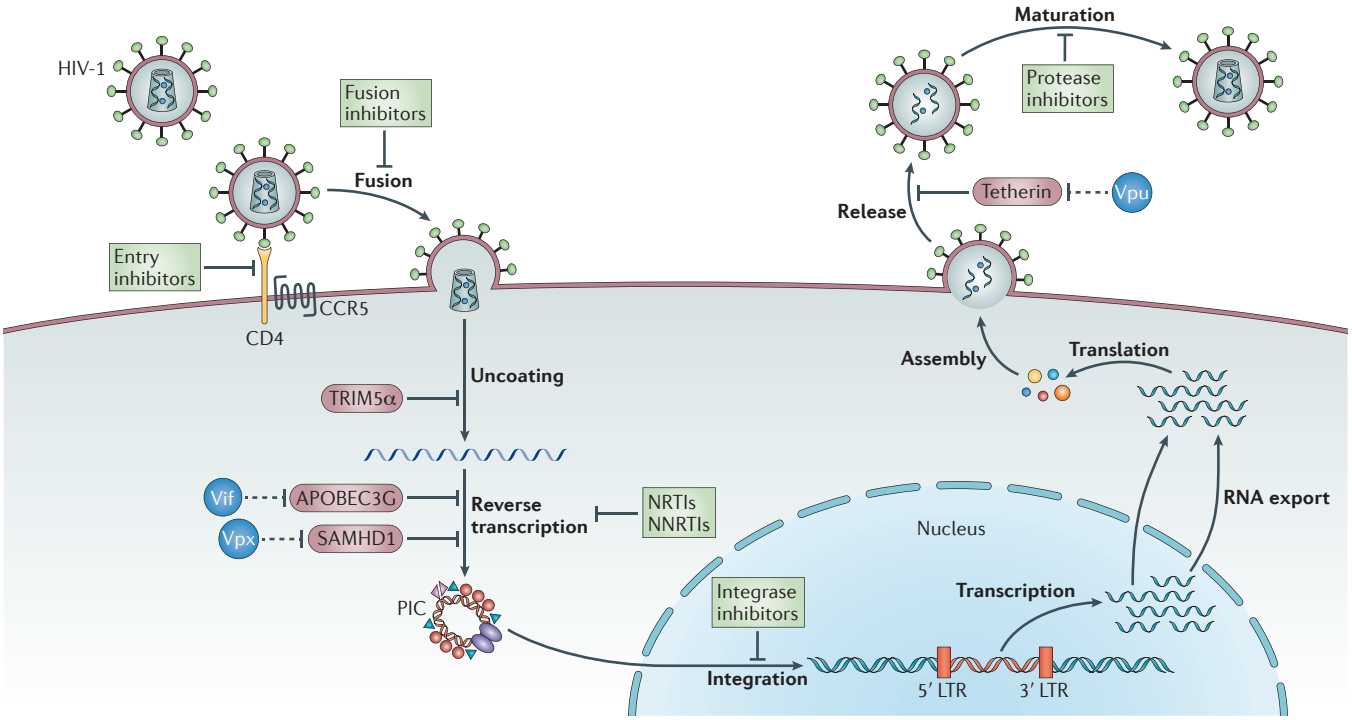


Figure 1.14 An overview of the HIV lifecycle

From Barré-Sinoussi et al (2013) Reproduced with permission.
Important cellular factors that interact with the virus (for exmple APOBEC3G and tetherin) are included in red boxes. Targets for antiviral drugs are included in green boxes.
There are a number of host cell factors that interact with the virus and alter replication (red boxes). APOBEC3G is a cytidine deaminase that inhibits viral replication, but is antagonized by the viral protein Vif (Sheehy et al 2002). Similarly Tetherin (BST2) inhibits virion release from the cell and is inhibited by the viral Vpu protein (Neil et al 2008; Van Damme and Guatelli, 2008). Other examples of host-viral interaction include SAMHD1 and it's viral inhibitor vpx (Laguette et al, 2011) and TRIM5α (Stremlau,et al 2004). The green boxes show the major antiretroviral drugs and the steps in the lifecycle that they block. Antiretroviral treatment is very successful in limiting disease from HIV for those patients who have access to it and are compliant with therapy (Mayer and Krakower, 2016).

During the process of viral replication HIV produces a number of regulatory proteins, other than vpu, vif and vpx (described in **Figure 1.14**). Tat accelerates the availability of the viral RNA for virus production (Fields Virology, 2013). Rev controls the splicing length of the mRNA, allowing regulatory proteins to be available early in replication, boosting the production of virus (Li and De Clercq, 2016). Nef downregulates CD4 expression on the cell surface, thereby reducing the immune response to an infected cell (Malim and Emerman, 2008). HIV has devised a huge number of mechanisms to evade host mechanisms of viral control and allow viral persistence and pathogenicity.

Human cells that support HIV entry and replication include CD4+ T cells, monocytes, macrophages and dendritic cells (Fields Virology, 2013). Following initial infection the majority of CD4 positive T cells that are infected immediately start producing new virus. Dendritic cells are particularly important at mucosal surfaces where they can capture virus and become infected using the C-type lectin DC-SIGN for attachment (reviewed in Wu and KewalRemani, 2006). HIV, like many other viruses can be transmitted cell free or directly cell-to-cell. Cell–to-cell transmission in HIV between and within cell types at the 'virological synapse' is highly efficient and contributes significantly to its virulence (reviewed in Costiniuk and Janabian, 2014; Sattentau, 2008).

1.6.2 Viral Interplay

The interaction between HIV and HCV affects the transmission and natural history of HCV infection, although the impact on HIV progression is much less clear.

Impact of HIV on HCV transmission

The largest burden of HIV/HCV co-infection currently exists within high-risk groups, such as intravenous drug users (IVDU) and men who have sex with men (MSM) (Nishijima et al, 2014). There is little evidence to suggest that HIV infection per se alters HCV transmission in IVDU, although these individuals are more likely to develop chronic HCV than non-HIV infected IVDU (Chew and Bhattacharya, 2016). There have also been reports of epidemics of HCV transmission in HIV-infected MSM, suggesting that the risk of sexually transmitted HCV is altered in the presence of HIV infection (Nishijima et al, 2014; Sánchez et al, 2013, Wandeler et al, 2012). There is inconclusive evidence as to why there is increased transmission risk in these individuals. HCV RNA levels are higher in the semen and blood of men co-infected with HIV and this may increase the likelihood that co-infected subjects will transmit infection (Briat et al, 2005; Hsieh et al, 2014).

HIV infection also significantly impacts HCV perinatal transmission. In developed countries the majority of children with HCV are vertically infected (Mohan et al, 2010). Children infected with HCV represents a small group

compared to adults infected with HCV; it is estimated that between 0.05 and 5% of children worldwide are infected with HCV (Arshad et al, 2011). They are an important group in whom infection is potentially preventable, but understanding of vertical transmission is limited. Interestingly, children infected vertically with HCV are less likely to clear infection, suggesting that pathways of viral persistence are altered by the route of HCV transmission (Abdel-Hady et al, 2011). Mother to child HCV transmission rates are around 5-10% (Yeung et al, 2010) and this increases fourfold when mothers are co-infected with HIV (Gibb et al, 2000; European Paediatric Hepatitis C virus network, 2001; Marine-Barjoan et al, 2007). This was thought to be related to the higher HCV viral load in co-infected mothers who transmitted virus, although this has not been consistently reported (Conte et al, 2000; Zanetti et al, 1995). Furthermore, it is feasible that altered immunity caused by HIV infection may alter innate or cellular responses at the fetal/maternal interface (Le Campion et al, 2012). As HIV infects trophoblasts (Vidricaire et al, 2007) it is also possible that the placental barrier function is affected by co-infection leading to increased HCV transmission (Kwiek et al, 2006). Interestingly, Azzari et al reported that vertical transmission of HCV is related to maternal peripheral blood mononuclear cell (PBMC) infection (Azzari et al, 2009), therefore raising the hypothesis that PBMC could act as a viral vector for HCV in the new host (Le Campion et al, 2012). Virus associated with cells that are permissive to HIV, such as T cells, raises an important hypothesis for altered HCV transmission in the presence of HIV. **Figure 1.15** outlines potential pathways of HCV transmission to the foetus.

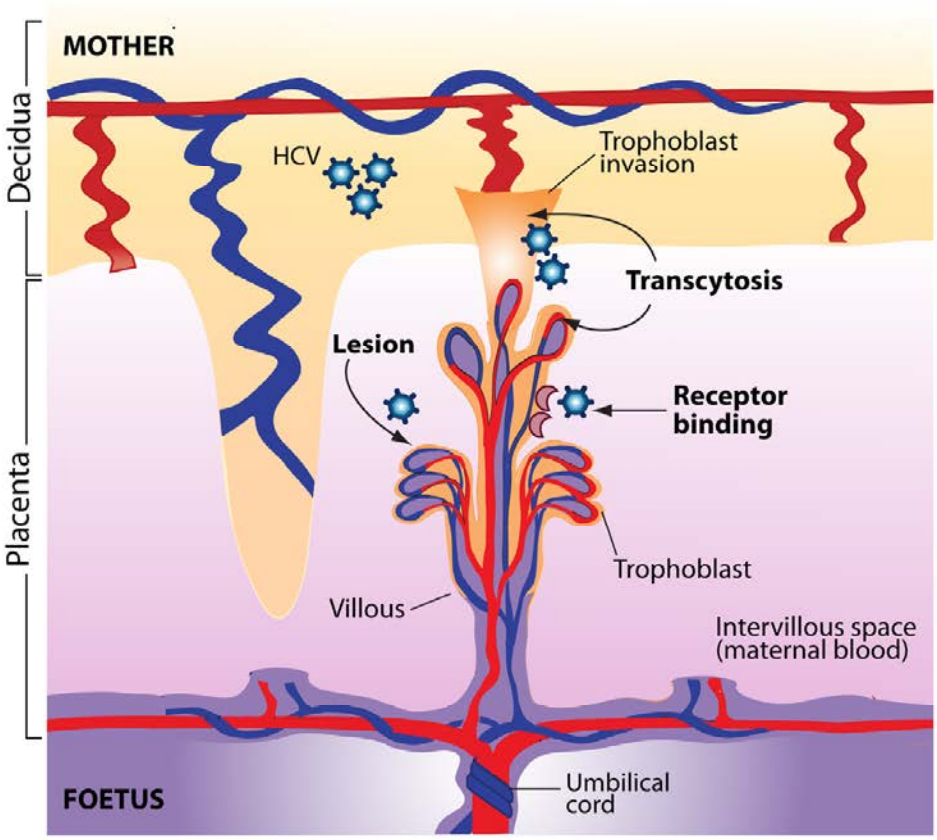


Figure 1.15 Models of HCV vertical transmission

From Le Campion, 2012 and reproduced with permission.
The placenta consists of fetally derived chorionic villi bathed in maternal blood. These have a stromal core with blood vessels and an outer layer of cytotrophoblasts. The fetal/maternal interface is formed by syncytiotrophoblasts and these mediate exchange between the mother and foetus. HCV infection could occur via; direct trancytosis through the cytotrophoblast layer, or mediated by HCV surface receptors, or blood mixing by micro-transfusions. Given that there is no HCV RNA found in amniotic fluid (Delorme-Axford et al, 2013), it is likely that HCV is transferred across the placenta, rather than infecting it directly.

Impact of HIV on HCV disease

HIV is also known to influence the progression of HCV disease, particularly liver disease, which prior to the DAA-era, was the leading cause of death in HIV infected individuals (Chew and Bhattacharya, 2016). It has been shown that HIV co-infection is associated with higher levels of HCV viraemia (Ghany et al, 1996; Thomas et al, 2000; Matthews-Greer et al, 2001), which is associated with lowered CD4+ T cells (Thomas et al, 1996, Martinez-Sierra et al, 2003) suggesting cellular responses are important in controlling HCV replication during chronic HCV infection. Furthermore, co-infection has been associated with reduced ability for spontaneous HCV clearance (Seal et al, 2007) resulting from impaired innate and cellular immune responses (reviewed in Chew and Bhattacharya, 2016). Furthermore there is accelerated hepatic fibrosis in co-infected, as compared to mono-infected patients. This is as a result of complex interplay between immune dysregulation, microbial translocation and infection and interaction of HIV with intra-hepatic cells (Chew and Bhattacharya, 2006).

Impact of HCV on HIV disease

The effect of HIV co-infection on HCV transmission and disease is well studied and described. However, the impact of HCV on HIV-related disease is much less clear. Much of the initial data on this, from the pre- and post-antiretroviral (ART) era is inconclusive in its findings. There is some evidence that HIV clinical progression is accelerated in co-infected patients (Greub et al, 2000; Piroth et al, 1998), but this is not associated with immunological decline

(CD4 T cell count) (Piroth et al, 1998). There may be some impact of HCV on CD4 T cell recovery after initiation of ART (Law et al, 2004; Greub et al, 2000), possibly mediated by chronic immune activation driven by HCV (Sajadi et al, 2012).

The interplay between HIV and HCV is complex. Overall HIV has a significant effect on HCV transmission and clinical progression, particularly accelerated liver disease and immune dysregulation. Developing models of co-infection will allow further studies into the mechanisms of viral interaction and provide areas for future investigation.

1.6.3 Studying HIV/HCV co-infection

Many published studies have used clinical material derived from patients in order to study the effect of viral co-infection on cellular immune response *in vivo*. However, there are limited mechanistic studies investigating the interplay between these viruses and their respective life cycles.

Studies of viral interplay *in vitro* are limited by the lack of cell systems that support both HIV and HCV. There are studies looking at HIV infection in hepatoma cells and the impact on HCV replication. Kong et al (2014) showed that HIV infection of hepatocytes is associated with an increase in HCV RNA. In addition Peng et al (2015) showed the HIV vpr can upregulate miR122 and promote HCV replication by hepatocytes. These studies may provide insight into HCV associated liver disease in HIV infection. In addition to hepatoma cells,

which appear to support very low level HIV replication (Xiao et al, 2008), there has been much debate within HCV research whether PBMCs can support HCV infection. Early studies reported detectable negative strand HCV RNA (the replicative intermediate) in PMBC of infected patients (Qian et al, 1992; Zignego et al, 1992; Wang et al 1992; Muller et al, 1993) but the specificity of these assays have since been questioned (McGuinness et al, 1994; Lanford et al, 1995). Studies using highly sensitive assays detecting HCV RNA intermediates have failed to consistently show evidence of replicative HCV in PBMC in mono or co-infected patients (Boisvert et al, 2001; Lin, et al, 2002; Laskus et al, 2007, Blackard et al, 2007). Marukian et al (2008) showed robustly, that PBMCs and specific cell subsets do not support ongoing HCV replication, are not permissive to HCVpp and that even if entry is bypassed are unable to translate virus. Azzari et al (2000) showed HCV positive and negative strand RNA detectable in PMBC in maternal blood associated with increased HCV maternal to child transmission and suggested that PBMC may act as a cell vector for virus as maternal cells cross into the fetal circulation (Petit et al 1995). Taken together this would suggest that virus is able to associate with immune cells, but fails to replicate.

Stamataki et al described an elegant model in which B cells can associate with and internalize HCV particles. These are transmitted to permissive cells in the liver allowing cell-mediated trans-infection of virus. Furthermore HCV infection increased the ability of B cells to adhere to hepatoma cells in vitro (Stamataki, Blood 2009). This raises the possibility of interactions between HCV and cells that support HIV infection as a model system for investigating transmission in co-infection.

1.7: Project Aims

The overall aims of this project were to develop and characterize a differentiated model system to study the HCV lifecycle and key viral host interactions. Utilizing the methodology developed by Sainz (2006) to differentiate hepatoma cells into a more physiological cell system, we aimed to

- Characterize differentiated cells with respect to important host factors that are known to interact with HCV
- Study the viral lifecycle in these cells. Hepatoma cells support high levels of viral replication compared to primary cells, but the mechanism behind this is not fully known.
- Study viral dissemination routes in differentiated cells given that HCV is known to infect hepatocytes efficiently via cell-to-cell transmission.

Furthermore, we aimed to develop a model to study novel routes of viral dissemination that may occur *in vivo* including lymphocyte-associated. We aimed to use this model to allow us to study HIV/HCV co-infection and viral interplay.

Chapter 2: Materials and Methods

2.1: Cell lines and virus

2.1.1 Cell lines and primary cells

Huh-7, Huh 7.5, 293T cells, HepG2 cells and the H77 subgenomic replicon (Blight, 2002) were provided by C. Rice (Rockefeller University, New York, NY). H77 replicon in Huh-7 cells (Ikeda et al, 2002) were provided by S Lemon (University of Texas Medical Branch at Galveston, Texas). Huh-7 cells containing the replicating subgenomic JFH1 HCV strain and expressing an HLA-A2 receptor and luciferase reporter (Jo et al, 2009) were provided by R Thimme (University of Freiburg, Germany). JW cells were a gift from J Witteveld and P Simmonds (University of Edinburgh, UK). All cells were propagated in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% foetal bovine serum (FBS), 1% nonessential amino acids and 1% penicillin/streptomycin (Invitrogen, Carlsbad, CA) at 37°C in 5% CO_2. G418 (Invivogen) at 1mg/ml was added to the media of the replicon cell lines. The LucA2 cells required additional Blasticidin (Invivogen) 6 micrograms/ml added to the media.

1.5% (vol/vol) dimethylsulfoxide (DMSO) was added to the media of Huh 7 and Huh 7.5 cells for 7 days prior to use and replenished every 3-4 days as previously described (Sainz and Chisari, 2006). DMSO treated cells were seeded at 3 x $10^4/cm^2$ and non-DMSO treated cells at 6 x $10^4/cm^2$ for all experiments other than the co-culture.

Primary human hepatocytes (PHH) used in this study were a kind gift from Dr Ragai Mitry (King's College, London). They were isolated according to

protocol (Mitry, 2009) and RNA was extracted by G Wilson (University of Birmingham, UK).

T cells lines Jurkat, SupT1 and MOLT4 (ATCC) were propogated in RPMI 1640 medium with 10% Fetal Bovine Serum, 1% penicillin/streptomycin, 1% non-essential amino acids and 1% L-Glutamine.

Primary Blood Lymphocytes were isolated from whole blood from healthy anonymous donors using Lympholyte-H as per manufacturers instructions (Cedarlane Laboratories, Ontaria, Canada). Briefly, whole blood was layered over Lympholyte-H in a 2:1 ratio using a 50ml centrifuge tube and then centrifuged at room temperature for 20 minutes at 800g. Post centrifugation the lymphocyte layer was removed by pipette and transferred to a new 50ml centrifuge tube. The cells were then pelleted and washed three times in serum free RPMI. The resultant cell pellet was then re-suspended in RPMI/10% FCS and CD4+ T cells were extracted.

T cells were purified manually by negative selection using the Easysep CD4+ T cell Enrichment Kit and "The Big Easy" Silver EasySep magnet according to manufacturer's instructions (StemCell Technologies, Vancouver, Canada). Flow cytometry analysis showed 99% of cells were CD4+ T cells within the isolate. Isolated CD4+ T cells were stimulated using anti-CD3 (OKT3) + anti-CD28 (Invitrogen) at 1mcg/ml and cultured in complete RPMI 1640 medium in the presence of 200 IU/ml of IL-2 (resulting in "stimulated T cell blasts"). Cells were used 5-7 days post isolation.

The HIV indicator cell line GHOST CXCR4+ CCR5+ cells were purchased from NIH AIDS Reagent programme and maintained in DMEM with 10% foetal bovine serum and high glucose DMEM, 90% and supplemented with 500 µg/ml G418, 100 µg/ml hygromycin, pen/strep, and 1 µg/ml puromycin.

ACH2 cells are an HIV-1 latent T cell clone and were obtained through the NIH AIDS Reagent Program, from Dr. Thomas Folks (Clouse, 1989; Folks, 1989). Cells were maintained in RPMI 1640 supplemented with 10 mM HEPES, 2 mM L-glutamine, 90%; heat inactivated fetal bovine serum, 10%.

2.1.2 Antibodies

The primary antibodies were anti-NS5A 9E10 (C. Rice, Rockefeller University), anti-CD81 (2.131), anti–claudin-1 (Abnova, Taipei, Taiwan and R&D, Minneapolis, MN), anti-occludin (Invitrogen), anti–ZO-1 (Invitrogen) and anti-MRP2 (Sigma Aldrich). Intracellular p24 staining used LIVE/DEAD fixable violet (Invitrogen), anti-CD4-APC antibody (BD Biosciences) and KC57-RD1 P24 specific antibody (Beckman Coulter). Immunoglobulin (Ig), used in the cell-to-cell experiments was derived from healthy volunteers (HCV(-)IgG) and chronically HCV-infected donors. This was then purified by protein G affinity chromatography (HCV(+)IgG) as previously reported (Grove, 2008).

Fluorescent secondary antibodies Alexa-Fluor 488 anti-mouse, anti-rat and anti-rabbit IgG and RPE IgG2a anti-mouse were obtained from Invitrogen. 7

aminoactinomycin (7AAD) for analysis of the cell cycle was obtained from Sigma Aldrich.

Table 2.1: Cell lines used, tissue type, growth media and source

Cell Type	Tissue type	Growth media	Source
Huh-7	Human Hepatoma	DMEM	C Rice, Rockefeller University, NY, USA
Huh-7.5	Human Hepatoma	DMEM	C Rice, Rockefeller University, NY, USA
Primary Human Hepatocytes	Human Liver	Williams E	Ragai Mitry, Kings College London, UK
HepG2-CD81	Human Hepatoblastoma	DMEM	In house
HepG2-NTCP-IOV	Human Hepatoblastoma	DMEM	U Protzer, TUM, München, Germany
A2-HCV replicon-luciferase Huh-7 cells	Human Hepatoma	DMEM	Robert Thimme, University of Freiburg
H77 replicon Huh-7 cells	Human Hepatoma	DMEM	S Lemon, Texas University, USA
JW cells	Human Hepatoma stably expressing SEC14L2	DMEM	J Witteld, University of Edinburgh, UK
Caco-2	Human Colonic Adenocarcinoma	DMEM	ATCC
293T	Human Embryonic Kidney	DMEM	ATCC
Jurkat	Human T cell leukaemia	RPMI-1640	ATCC
MOLT-4	Human T cell lymphoblastic leukaemia	RPMI-1640	ATCC
Sup-T1	Human T cell lymphoblastic lymphoma	RPMI-1640	ATCC
ACH2	Subclone A3.01 of CEM, a human T cell lymphoblastic leukaemia line. Harbours a single copy HIV-1 proviral DNA	RPMI-1640	W Paxton, University of Liverpool, UK
Primary CD4+ T cells	PBMC from healthy anonymous donors	RPMI-1640	Queen Elizabeth Hospital, Birmingham, UK
GHOST CXCR4+ CCR5+	Human Osteosarcoma cells. Contains hGFP under transcriptional control of HIV-2 LTR promoter and expresses HIV-1 co-receptors CXCR4 and CCR5		NIH AIDS Reagant Programme, Germantown, US

Table 2.2: Primary Antibodies used

Antibody	Antigen	Type	Specificity	Species	Conc. (µg/ml)	Application	Source
Anti-NS5A (NE10A)	HCV NS5A	Hybridoma Supernatant	Mono clonal	Mouse	2	IF, Flow	Rockefeller University, NY, US
Anti-CD81 (2s131)	Human CD81	Purified IgG	Mono clonal	Mouse	1	IF	In house
Anti-Claudin1	Human Claudin1	Purified IgG	Poly clonal	Rabbit	1	IF	Zymed, CA, US
Anti-ZO1	Human ZO-1	Purified IgG	Poly clonal	Rabbit	1	IF	Zymed, CA, US
Anti-MRP2	Human MRP2	Purified	Mono clonal	Mouse	2	IF	Abcam, UK
Anti-Occludin	Human Occludin	Purified IgG	Poly clonal	Rabbit	1	IF	Zymed, CA, US
Anti-CD4-APC	Human CD4	Purified IgG	Mono clonal	Mouse	3	Flow	BDBiosciences,
Anti-p24-FITC (KC57 RD1)	HIV-1 55, 39, 33 and 24 kDa proteins	Purified IgG	Mono clonal	Mouse	0.5	Flow	Beckman Coulter

Table 2.3 Secondary Antibodies used

Antibody	Antigen	Type	Specificity	Species	Working dilution	Application	Source
Mouse Alexa-Fluor 488	Mouse IgG	Purified IgG (H+L)	Polyclonal	Goat	1:500	IF, Flow	Molecular Probes, Invitrogen, CA
Rabbit Alexa-Flour 488	Rabbit IgG	Purified IgG (H+L)	Polyclonal	Goat	1:500	IF	Molecular Probes, Invitrogen, CA

2.1.3 Virus

HCVcc

Plasmids encoding chimeric SA13/JFH (Jensen J Infect Dis 2008) and J6/JFH were used to generate RNA as previously described (Lindenbach, 2005). Briefly, RNA was electroporated into Huh-7.5 cells, and supernatants were collected at 72 and 96 hours and stored at −80°C. The SA13 virus was then passaged serially in infected Huh 7.5 cells and the supernatant collected and stored at -80°C. Infected cells were fixed with ice-cold methanol and stained for NS5A with monoclonal antibody 9E10 and isotype-matched Alexa 488–conjugated anti-mouse IgG (H+L). NS5A-positive foci were counted, and infectivity was expressed as focus-forming units per milliliter. The SA13 used in all experiments had an infectivity of 2.5 million FFU/ml. For experiments using DMSO, the viral inoculum was added to cells without DMSO. Cells were incubated with virus for 6-8 hours and normal media +/- DMSO was added with no washing step.

HCV and HIV Pseudoparticles

Luciferase reporter pseudoparticles expressing a panel of HCV envelope glycoproteins (HCVpp), vesicular stomatitis virus G glycoprotein (VSV-Gpp), HIV envelopes BAL and LAI (gift from Bill Paxton, University of Liverpool, UK) or a no-envelope control were generated as previously reported (Hsu M, 2003). A panel of patient derived HCV pseudoparticles was gifted from Stuart Ray (Johns Hopkins University, Baltimore, USA).

Supernatants were harvested at 48h post transfection, clarified, and filtered through a 0.45-µm membrane. Virus-containing medium was added to target cells plated in 96 well plates seeded at 7.5 x 10^3 cells/cm^2 and incubated for 72h. After 72h, cells were lysed with cell lysis buffer (Promega, Madison, WI) and luciferase activity measured for 10s in a luminometer (Lumat LB 9507). Specific infectivity was calculated by expressing the HCV signal relative to the VSV-G luciferase signal (relative light units, RLU).

gLUC virus

Huh-7 cells were electroporated with in vitro-transcribed RNA of Jc1, containing the Gaussia luciferase reporter gene (Jc1gLuc) (Gottwein, 2011) or replicase-negative JC1gLuc-GNN. For infection gLuc assays, HCVgLuc were added to Huh-7 cells seeded the day before in 48-well plates and incubated for 2 hours at 37°C. The supernatants were then removed, and the cells were incubated in DMEM with 10% FBS at 37°C. At 4, 24, 48, 72 or 144 h post infection Gaussia luciferase assays were performed as indicated by the manufacturer (Promega). Results plotted are the luciferase signal of the Jc1gLuc virus over the GNN control.

HIV

The full-length HIV plasmids HXb2, NL4.3, NL4.3 GFP and BAL were gifted from W Paxman (University of Liverpool, UK). 293 T cells were plated on Poly-L-lysine (Sigma-Aldrich) coated plates and the plasmids were transfected using Fugene-6 (Promega, Canada) and optimem. Cells were transfected for 6 hours at 37° and the media replaced with 3% FBS/DMEM without

Penicillin/Streptomycin. Subsequently cells were incubated and virus collected at 48-72 hours post-transfection. Virus stocks were pooled, clarified by centrifugation at 2000 rpm for 5 minutes and stored in aliquots at -80°C. HIV titers were determined using infection of Jurkat cells and analysis of p24 in the media using ELISA, or using the HIV indicator cell line GHOST CXCR4+ CCR5+.

GHOST cells were seeded at $1.5x10^4/cm^2$ in a 48 well plate in DMEM supplemented with 10% FCS, 500 µg/ml G418, 100 µg/ml hygromycin, pen/strep, and 1 µg/ml puromycin. HIV-1 virus was added in serial dilutions for 48 hours prior to being fixed with paraformaldehyde (PFA) (TAAB, UK) at 1 in 10 dilution for 20 minutes. GFP positive cells, as a marker of HIV infectivity, were visualized using a fluorescent UV microscope (Nikon eclipse TE2000-5 inverted) and imaged with a digital camera (Hammatsu, Japan) or quantified by flow cytometry.

Sendai and EMCV

Infection of Huh-7 and Huh-7^{diff} with Sendai virus and encephalomyocarditis virus (EMCV) for the IL29 experiments were kindly carried out by A Mayer, University of Oxford. Briefly, differentiated cells were infected on Day 10 post plating. Cells were infected with Sendai virus (from LGC standards) by the addition of virus to the culture medium at $5.3x10^4$ pfu/well in a 24 well plate. EMCV (Rehwinkel, University of Oxford) was added to the media at $5.3x10^4$ pfu/well in a 24 well plate. 4 hours post infection virus was removed and normal media (+/-) DMSO was added to the cells with no washing step.

Supernatants were then collected 24 hours post infection. Cell numbers per well were enumerated on the day of infection and cell viability was assessed visually by light microscopy 24 hours post infection. Cells were also lysed in 1% NP40 lysis buffer and stored at -80°C. Supernatants and cell lysates were then sent from Oxford and IL-29 in the supernatant was quantified using ELISA as described. Results were normalized for cell count.

2.1.4 Drugs and cytokines

Huh-7 and Huh-7^{diff} were plated and infected with HCVcc SA13 as described. 24 hours post infection VX950 or 2CMC was added to the media at varying concentrations. VX950 and 2CMC were given as gifts from J Neyts (Rega Institute, Belgium). 72 hours post infection cells were pelleted, stained for NS5A and the number of NS5A positive cells quantified using flow cytometry.

For experiments with exogenously added nucleotides, the nucleotides were added at day 0, which is the day when the cells first became confluent (1 day after cell seeding). Uridine, and Cytidine were purchased from Sigma and used as previously described (Nelson, HV 2008).

TNF-α was used to disrupt tight junctions prior to infecting with HCVpp. Cells were treated with TNF- α (Sigma Aldrich) at 100ng/ml^{-1} for 15 minutes prior to infecting with HCVpp as decribed (Fletcher, 2017). TNF- α was also used to activate ACH2 cells. Cells were treated with 10ng/ml (Duh, 1989; Gallastegui, 2012) of TNF-α added directly to the media for 12 hours. PMA (Sigma Aldrich)

was also used to activate ACH2 cells at 10nM (Folks et al, 1989) added to the media for 12 hours.

Zidovudine (AZT from NIH Aids Reagant) was used to treat HIV infected T cells. AZT was added at 10mM to HIV infected T cells for 48 hours prior to use in a trans-infection. HIV cure was confirmed by quantifying p24 in the supernatant by ELISA.

2.2: Routine techniques

2.2.1 Cell differentiation

For differentiation of Huh-7 and Huh-7.5 with dimethylsulfoxide, cells were plated at $5x10^4$/ml in a 48 well plate, unless otherwise stated. DMSO (1.5% vol/vol) was added directly to the media and maintained throughout the cell culture, except for during viral inoculation, where DMSO was removed for up to 8 hours and them immediately replaced. Cells could be maintained for up to 6 weeks using this method of differentiation, but used 7-10 days post differentiation for all experiments unless otherwise stated. DMSO was stored at -20°C in 1 ml aliquots in order to prevent loss of efficacy if stored at room temperature for long periods of time (verbal communication, David Durantel).

Huh-7 were also differentiated using 2% human serum (to replace FBS) in DMEM supplemented with 1% nonessential amino acids and 1% penicillin/streptomycin (Invitrogen, Carlsbad, CA) as described (Steenbergen, 2013).

To compare cells using alternative methods of differentiation Huh-7 were propogated for 4 weeks (by N Frampton) in Williams E media (Gibco) supplemented with 5% FCS, 40 IU/ml Insulin, 1µl/ml Iosine, 22µl/ml Hydrocortisone, 1% nonessential amino acids and 1% penicillin/streptomycin (Invitrogen, Carlsbad, CA) (J Graf, University Munich, Germany).

2.2.2 Indirect Immunofluorescence

HCV infection was detected by immunofluorescence. Infected cells were fixed using either ice-cold methanol (Fisher Scientific, UK) for 5 minutes or 3.6% paraformaldehyde (PFA) (TAAB, UK) for 20 minutes, at room temperature. Cells were blocked and permeabilised in PBS containing 1% BSA and 0.1% saponin for 30 minutes at room temperature. Cells were washed x2 (PBS) prior to the addition of primary antibody (anti-NS5A 9E10) for 60 minutes at room temperature. Cells were then washed (PBS) and the secondary fluorescent conjugated antibody was added. Cells were incubated in the dark for 60 minutes at room temperature. Staining was visualized using fluorescent UV microscope (Nikon eclipse TE2000-5 inverted) and imaged with a digital camera (Hammatsu, Japan) at magnification x10, unless otherwise stated.

2.2.3 Flow cytometry

For detecting HCV infection cells were fixed using PFA (TAAB, UK) for 20 minutes, at room temperature. Primary and secondary antibodies were added as above. Cells were then acquired using a FacsCalibur flow cytometer (Becton

Dickinson, Europe). The intact live cell population was identified by size and density and analysed using FlowJo software (Tree Star, OR, USA).

Intracellular HIV infection was detected using p24 staining. Cells in a 48 well plate were washed (PBS) before adding 0.2 µl/well of the reconstituted LIVE/DEAD fixable Dead Cell Stain dye with violet fluorescent reactive (Invitrogen, Carlsbad, CA). After incubating on ice, at darkness for 30 minutes, cells were then washed with 200µl/well of FACS washing buffer (PBS/0.5%BSA/2mM EDTA) and stained with 2µl/well of anti-CD4-APC antibody (BD Biosciences, San Jose, CA) on ice for 20 minutes. Samples were washed prior to being fixed by 100µl/well perm/fix buffer (BD Biosciences). After 20 minutes fixation at 4°C degree, samples were then washed twice with perm/washing buffer (BD Biosciences) before 3µl/well of KC57-RD1 P24 specific antibody (Beckman Coulter) being added to each sample. Samples were then incubated on ice for 30 minutes before a final wash step. Cells were fixed in 200µl of cell fix (BD Biosciences) prior to being acquired on a FacsCalibur flow cytometer (Becton Dickinson, Europe) as above.

For analysis of the cell cycle, cells were fixed in ice-cold methanol for 10 minutes. Cells were washed x 2 in PBS and then permeabilised with PBS containing 1% BSA and 0.1% saponin. 7AAD (Sigma-Aldrich), which binds to double stranded DNA, was added to the blocking/permeabilisation buffer at 1 in 100 dilution. Cells were incubated at room temperature for 20-30 minutes prior to being washed x2 PBS and acquired on a FacsCalibur flow cytometer. Analysis of the cell cycle was done using the Dean/Jett/Fox algorithm on FlowJo.

2.2.4 Real-Time Reverse-Transcription Polymerase Chain Reaction

For cells used in quantitative PCR assays, purified cellular or tissue RNA samples were extracted (RNEasy MiniKit, Qiagen) according to the manufacturers instructions. The RNA was eluted in 35µl of RNA free water as the end step. Purified cellular RNA samples were amplified for HCV RNA (Primer Design Ltd) or specific primers in a single tube RT-PCR in accordance with the manufacturer's guidelines (CellsDirect One Step qPCR Kit, Invitrogen). Fluorescence was monitored in an ABI 7500 PCR machine (Applied Biosystems). The housekeeping gene GAPDH was included as an internal endogenous control for amplification efficiency and RNA quantification (ABI). The assay cutoff for HCV RNA was 100 copies and the number of copies was calculated using a standard curve. The relative expression of all target genes were evaluated by normalizing the Ct-values with the internal GAPDH expression (2^Δ ct). Specific primers were all commercially available Taqman primers (Applied Biosystems).

Total miRNA for mir-122 and other micro-RNA expression was prepared by using the using the miRNeasy minikit (Qiagen) according to manufacturers instructions. miR-122 and other miR expression was determined using the specific RT and PCR primers provided in the Taqman microRNA assays according to the manufacturers instructions (Life Technologies, ThermoFischer). The micro-RNA miR-130 was used as the endogenous control as this was expressed stably across cell lines used (Schaefer, 2010).

2.2.5 Enzyme Linked Immunosorbent Assay (ELISA)

HIV-1-p24, as a marker of HIV infectivity was measured by ELISA. Supernatant was collected from infected cells and stored at -20°C. p24 was measured as per manufacturers instructions (Aalto Bio Reagants Ltd, Dublin, Ireland) and absorbance measured at 450nm using a Multiskan Ascent platereader.

IL-29 was measured using ELISA. Supernatant was collected from infected cells at various time points post infection for quantification of extracellular IL-29 and stored at -20°C. For intracellular IL-29 duplicate wells of a 12 well plate were treated with trypsin and cell pellets re-suspended in 200µl of 10% DMEM. Cells were then rapidly freeze thawed three times prior to be centrifuged at 2000rpm to clarify. Samples were stored at -20°C. IL-29 was measured as per manufacturers instructions (eBioscience, ThermoFisher Scientific) and absorbance measured at 450nm using a Multiskan Ascent platereader.

2.3: Specific techniques

2.3.1 Confocal microscopy

Huh-7.5 were plated onto collagen-coated coverslips (Fisher Scientific, Leicester, United Kingdom) and maintained in the presence of DMSO for up to 21 days. Non-DMSO treated controls were plated and used 24 hours later. Cells were fixed with ice-cold methanol (claudin-1, occludin, ZO-1) or 3%

paraformaldehyde (CD81). After incubation with primary antibodies cells were incubated with Alexa 488 secondary antibodies, counterstained with 4′,6-diamidino-2-phenylindole, and viewed by laser scanning confocal microscopy on a Zeiss (Dublin, CA) MetaHead microscope with a 100× oil immersion objective.

2.3.2 Measurements of polarity

To determine the functionality of tight junctions and whether they restricted paracellular diffusion of solutes from circular structures, presumed to be bile cannaliculi (BC) to the basolateral medium, Huh-7^{diff} were incubated with 5 mM CMFDA (Invitrogen) at 37 °C for 10 min to allow translocation to the BC lumen. After washing extensively with PBS, the capacity of BC to retain CMFDA was enumerated using a fluorescence microscope.

Huh-7.5 cells were seeded onto transwells and grown in normal media for up to 7 days. Caco-2 cells were used as a positive control. FITC-labelled dextran FD-4 (4 kDa) (Sigma Chemical Co., St. Louis, MO, USA) was added to the apical side of the filters at a concentration of 250 µg/ml. The media in the basolateral compartment were sampled every 15 min for 2 h. The concentration of FD-4 was determined using a fluorimeter (Spectra Max Gemini, Molecular Devices, Sunnyvale, CA, USA) with an excitation wavelength of 490 nm and an emission wavelength of 515 nm. Apparent permeability (cm/s) was calculated as previously described (Artursson P, J Pharm Sci, 1990).

2.3.3 Viral entry timeline

Virus containing medium was added to cells in 48 well plates and every 30 minutes (up to 150 minutes post infection) triplicate wells were washed three times with phosphate buffered saline and normal media replaced. Cells were incubated for 72 hours. Cells were lysed, and luciferase activity was measured (Lumat LB9507 luminometer, Bertholt, Bad Weilbad, Germany). Infectivity is expressed as relative light units, where the no-envelope signal is subtracted from HCVpp or VSVpp signals.

2.3.4 Iron assay

Cell pellets from DMSO treated Huh-7.5 and Huh-7 were collected after 7 and 14 days post plating in 6 well plates and re-suspended in 100µl HEPES saline (10mM HEPES in 0.9% (w/v) sodium chloride at pH 7.4). Ferrozine assay was carried out as previously described by E Shawcross, University of Birmingham, UK (Riemer J, Anal Biochem 2004).

2.3.5 Glucose assay

Huh-7^{diff} were propagated in DMEM with varying concentrations of glucose as indicated. Standard DMEM (glucose 4.5g/L) was added to glucose free DMEM in addition to 10% FCS, 1% nonessential amino acids and 1% penicillin/streptomycin (Invitrogen, Carlsbad, CA) and 1.5% DMSO (vol/vol) in order to achieve glucose concentrations as indicated. 24 hours later cells were infected with HCVcc. 72 hours post infection cells were fixed and stained for NS5A as a marker of infectivity. Duplicate wells were also treated with RLT lysis

buffer and expression of enzymes associated with glucose metabolism was quantified by qPCR as indicated.

2.3.6 miR122 assay

Huh 7 cells were seeded in 24 well plates and 1.5% DMSO added as described. Cells were transfected with a reporter firefly luciferase RNA, a capped renilla luciferase control RNA and either a 2'-O-methylated antisense oligonucleotide to sequester miR122 (122-2'OMe) or a randomized control 2'O-methylated nucleotide (Rand-2'OMe) as described (Roberts, Nucleic Acid Research, 2011). Cells were lysed at 6h post transfection in 50 µl Passive Lysis Buffer (Promega) per well. Firefly and *Renilla* luciferase activity was determined in 5 µl of each sample using the Dual Luciferase Assay Kit (Promega), with 25 µl of each reagent, using a Glomax 96 microplate luminometer (Promega). Firefly/*Renilla* luciferase ratios were determined and plotted as a percentage of the value obtained in untreated Huh7 cells containing Rand-2'OMe. miR122 assays were kindly carried out by C Joplin (University of Nottingham, UK).

2.3.7 HCV dissemination and spread

Huh-7 and Huh-7^{diff} were plated in 24 well plates. Cells were infected with HCV SA13 at a comparable MOI to achieve matched number of infected cells 48 hours post infection. 12 hours post infection HCV(+)IgG and HCV(-)IgG were added to duplicate wells. Cells were then incubated for 2, 3 and 4 days post infection. Cells were trypsinised, fixed with 1% paraformaldehyde and stained using anti-NS5A NE10 at 1:1000 in Phosphate Buffered Saline/0.5% Bovine Serum Albumin/0.1% Saponin. Bound antibody was detected with Alexa-Fluor

488 secondary and analysed by flow cytometry using a FACSCalibur (BD Biosciences) and FlowJo software (TreeStar, Ashland, OR).

2.3.8 Cell-to-cell viral dissemination

A co-culture method was used to assess cell-to-cell and cell-free spread of HCV within a culture of Huh-7^{diff} and control Huh-7 (Meredith et al, 2013). 'Producer' cells were electroporated with SA13/JFH (Jensen) or J6/JFH (Lindenbach et al, 2005) and then labeled with CMFDA Cell-tracker Green (Invitrogen). 'Producer' cells were co- cultured with an equal number of naïve Huh-7^{diff} cells (used at 7-10 days post differentiation) or Huh-7. Extracellular virus was neutralized by treating cultures with anti-HCV Ig or control Ig (150 µg/ml) and antibody efficacy confirmed by analysing the culture media after 24 h co-culture for the levels of infectious virus. Cells were harvested at defined time points. Infected target cells were identified by staining for HCV NS5A and flow cytometry and can be differentiated from producer cells, or producer cell division, as these were CMFDA labeled. Cell-to-cell transmission is defined as the frequency of infection events in the presence of neutralizing anti-HCV-Ig, and subtraction of this value from the total number of infected cells (from duplicate wells in the presence of control Ig) provides an estimate of cell-free infection.

2.3.9 Innate signaling assays

Huh-7 (+/- DMSO) or HepG2 NTCP IOV cells at 1 x 10^5/ml were seeded in 12 well plates. In duplicate wells HCV J6 RNA and PolyIC (low or high MW) (SigmaAldrich) were transfected using Fugene 6 (Promega, Canada) and Optimem. Cells were transfected for 4 hours prior to washing with PBS and

normal media +/- 1.5% DMSO replaced. Wells were also infected with HCVcc at 1.5 FFU/ml. Huh-7 'producer' cells electroporated with SA13/JFH were added at equal cell numbers. 4 hours post infection cells were washed and the anti-viral drug VX950 (1µg/ml) was added to duplicate wells to prevent HCV replication. Sendai virus ($5.3x10^4$ pfu/well) and EMCV ($5.3x10^4$ pfu/well) were added to wells for 4 hours prior to being washed x 2 in PBS and normal media replaced. At indicated time points post infection (24,48 or 72 hours) the supernatant was collected and IL29 quantified by ELISA. Cells were trypsinised and the cell pellet freeze/thawed x 3 in order to release intracellular IL29. The pellet was resuspended in 200µl DMEM and clarified prior to IL29 quantification by ELISA. Cells were also collected for IL29 and IFNα qPCR as described in section 2.2.4.

2.3.10 HCV Trans-infection assay

Target cells (Huh-7 or LucA2 replicon cells) were plated at $2.5 \times 10^4/cm^2$ in 48 well plates for 24 hours prior to use. Lymphocytes (T cell lines or primary CD4+ T cells) in complete RPMI medium were infected with HIV-1 (at a concentration of 50ng p24/ml). At the indicated time points post infection cells were washed twice in RPMI. T cell +/- HIV were then incubated at 37°C with HCVcc (150µl neat virus per 1 million T cells) for 2 hours. After virus association, the lymphocytes were washed 5x with serum free RPMI and the last wash was tested to confirm removal of virus. Lymphocytes were then co-cultured at the indicated cell numbers per well with Huh-7or LucA2 replicon cells for 72 hours and trans-infection enumerated by NS5A staining of infected hepatomas as described (Stamataki et al., 2009). The effect of trans-infection on established replication in a LucA2 replicon was quantified by lysing cells and measuring

luciferase activity (Lumat LB9507 luminometer, Bertholt, Bad Weilbad, Germany). Replication is expressed as relative light units, where the media only control signal is subtracted from LucA2 signal. Supernatant collected from wells and pooled at the end of the trans-infection experiment was added in serial dilutions to LucA2 replicons. 48 hours later cells were lysed and replication measured by luciferase activity.

HIV infected T cells and control T cells were collected following incubation with HCVcc and the subsequent washing steps, pelleted and suspended in RLT lysis buffer for HCV RNA qPCR.

2.3.11 Statistical analysis

Statistical analyses were performed using Student t-test for bar charts or linear regression for XY graphs in Prism 6.0 (GraphPad, USA) with $P \leq 0.05$ being considered statistically significant.

Chapter 3: Developing a differentiated cell model system for the study of HCV

3.1: Introduction

The study of Hepatitis C Virus (HCV) in vitro is largely restricted to hepatoma cells, derived from a de-differentiated tumour cell line (Blight et al, 2000; Blight et al, 2002). These cells are highly permissive and allow study of the viral lifecycle, however they do not permit study of viral-host interactions in a differentiated system. The ideal model for the study of HCV is one that physiologically resembles the liver; cells that upregulate liver specific genes and are fully responsive to double stranded RNA (Sainz and Chisari, 2006). Primary human hepatocytes are the gold standard; they are differentiated and polarised. However, they support variable and low level HCV replication in vitro (Gondeau et al, 2014; Podevin et al, 2010; Wilson et al, 2015). Furthermore, they de-differentiate rapidly ex-vivo, are highly variable between individuals, and are difficult and expensive to obtain. Therefore there is the need for a differentiated cell system that is reproducible, easy to maintain and not costly.

There are various methods that have been used to differentiate hepatoma cells, including using human serum (Steenbergen et al, 2013), DMSO (Sainz and Chisari, 2006) and with media containing insulin, DMSO and other growth factors (verbal communication, J Graf). DMSO (dimethlysulfoxide) is widely available and is well known for its ability to induce differentiation of numerous cell lines. It can differentiate myeloid cell lines into mature granulocytes or macrophage-like cells (Makowske et al, 1987; Collins et al, 1978; Myers and Siegel, 1984). It has also been shown to differentiate osteoclasts (at 0.5% vol/vol) (Lemieux et al, 2001) and enhance differentiation of pluripotent stem

cells into multiple lineages (Chetty et al, 2013), including hepatic differentiation (Czysz et al, 2015). Furthermore, DMSO has been used to differentiate hepatocytes, including HepaRG cells (Gripon et al, 2002) and human hepatoma Huh-7.5 (Sainz and Chisari, 2006). It can also maintain the differentiated phenotype of rat hepatocytes (Isom et al, 1985) and primary human hepatocytes in culture (Zhou et al, 2014).

Several reports have shown that at low concentrations (1% vol/vol) DMSO can differentiate human hepatoma cells through upregulation of key hepatocyte genes, such as HNF4α, albumin and cytochrome P450 enzymes (Sainz and Chisari, 2006; Choi et al, 2009). These cells can be maintained in culture due to cell cycle growth arrest, and support HCV replication in vitro. They are increasingly used as a model for chronic HCV (Xiao et al, 2014) and are interferon responsive, expressing a wide array of interferon stimulated genes (ISGs) compared to untreated hepatoma cells (Bauhofer et al, 2012). We hypothesized that DMSO at low concentrations in the media (1-2%) would drive hepatoma cell differentiation, allow the differentiated cells to persist in culture and permit the study of HCV replication in a physiological system.

Studies assessing whether stem cell induced hepatocytes support HCV replication suggest a role for differentiation in viral permissivity, defined by miR122 (Wu et al, 2012). Furthermore, other hepatitis viruses, such as Hepatitis B virus (HBV), prior to the discovery of key entry factors, would only replicate in highly differentiated cells (Gripon et al, 2002). We therefore hypothesized that differentiation status of hepatoma cells would alter viral permissivity of HCV.

This chapter describes the development of differentiated cells for the study of the HCV lifecycle in a physiological system and the impact of cellular differentiation on viral permissivity.

Chapter Aims:

1. To characterize differentiated Huh-7 treated with DMSO, specifically looking at:
    a. Cell cycle arrest and maintenance of cells in culture.
    b. Hepatocyte specific genes and miR expression.
2. To define viral permissivity in differentiated Huh-7.

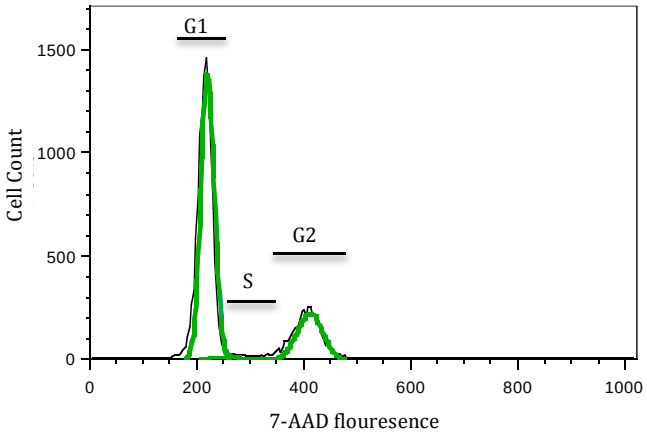
3.2: DMSO induces cell cycle arrest

Previous studies have shown that DMSO, at low concentrations, can induce cell cycle arrest in hepatoma cells (Sainz and Chisari, 2006) and promote differentiation. To define the optimal DMSO concentration for cell-cycle arrest, cells were treated with varying DMSO concentrations (from 0.5% to 2%) for 7 days with cell cycle progression assessed using 7-amino-actinomycin (7-AAD). 7-AAD is a single colour fluorochrome dye that interacts with double stranded DNA and is widely used to study the cell cycle (Vignon et al, 2013). Results showed that DMSO slows cell cycle progression compared to untreated cells with an increased percentage of cells in G1 phase compared to untreated cells (70% versus 40% in untreated cells) **(Figure 3.1)**. There was no observed difference in the percentage of cells undergoing active cell division, in 'S' phase, between the different DMSO concentrations, with the majority of cells in G1 phase. Given that cell cycle analysis appeared similar at all DMSO concentrations used, and previous publications had used 1-2% (Sainz and Chisari, 2006, Bauhofer et al, 2012) 1.5% DMSO was utilised for all future experiments. Morphological changes were observed in DMSO-treated cells, with results showing cells were more regular and compact, phenotypically resembling primary human hepatocytes (**Figure 3.2**).

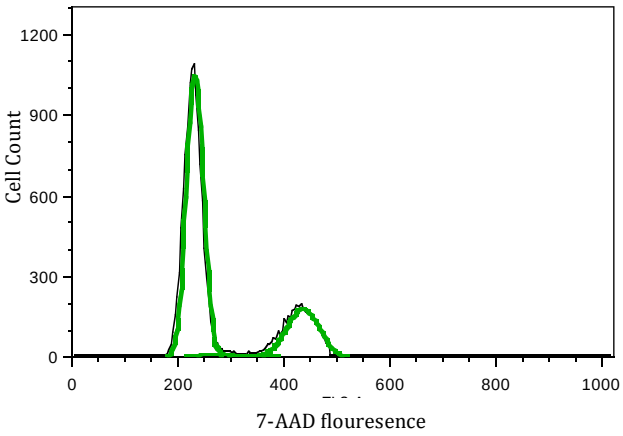
While DMSO-treated cells could be maintained in culture for up to 6 weeks post seeding, our results showed that DMSO must be continuously present to prevent cell cycle re-activation (**Figure 3.3**). Cells were treated with DMSO for 7 days prior to removal of DMSO from the media for 24 hours. Cell cycle

progression was assessed by 7AAD staining and results analysed by flow cytometry. Cells became cell cycle active after 24 hours of DMSO removal. Shortly afterwards these cells lifted off the plate and were no longer viable. Therefore during infection experiments DMSO was removed for the period of viral inoculation only (maximum 8 hours) and then replaced to maintain cell viability. There was no morphological change at 8 hours without DMSO and cells remained viable.

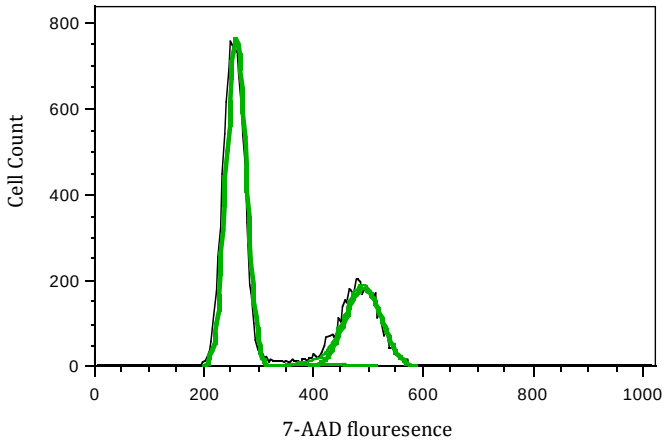
In summary, these results show that 1.5% DMSO (vol/vol) treatment can slow cell cycle progression in Huh-7 cells and allow the cells to become morphologically more 'hepatocyte like'. DMSO must be present throughout the culture period to maintain cell viability. Without DMSO cell were found to reactivate, begin dividing and are were no longer viable.



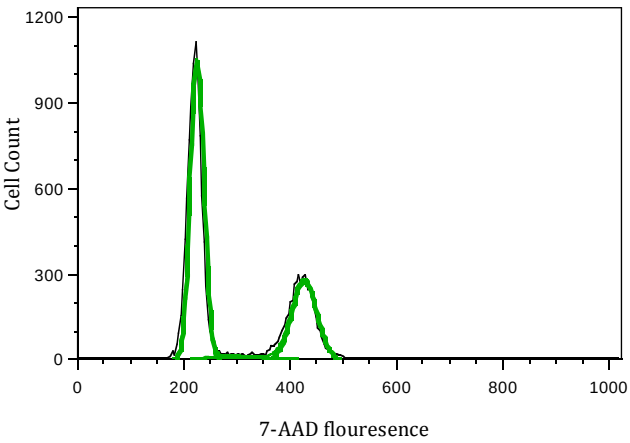
0.5% DMSO
G1 71% S 4.5% G2 23%



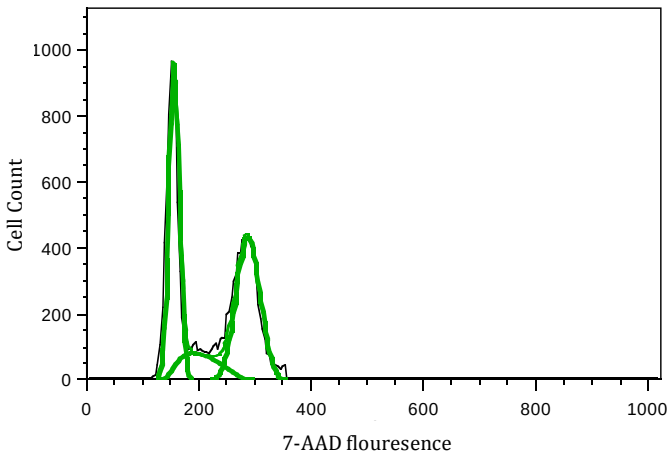
1% DMSO
G1 72% S 4.1% G2 25%



1.5% DMSO
G1 67% S 3.2% G2 30%



2% DMSO
G1 65% S 5% G2 30%



No DMSO
G1 40% S 15% G2 43%

Figure 3.1 DMSO treatment slows cell cycle progression in Huh-7 cells

Huh-7 cells were seeded in 48 well plates and treated with DMSO at indicated concentrations (vol/vol) for 7 days. Control cells were plated at comparable seeding density and used when confluent at 48-72 hours post seeding. Triplicate wells were stained using 7-aminoactinomycin (7AAD) and analysed by flow cytometry (FACSCalibur). Cell cycle stage was determined by FlowJo Dean Fox Jett algorithm, which calculates percentage of total cell count by stage of cell cycle, based on 7-AAD fluorescence. Results represent means of triplicate wells and two independent experiments.

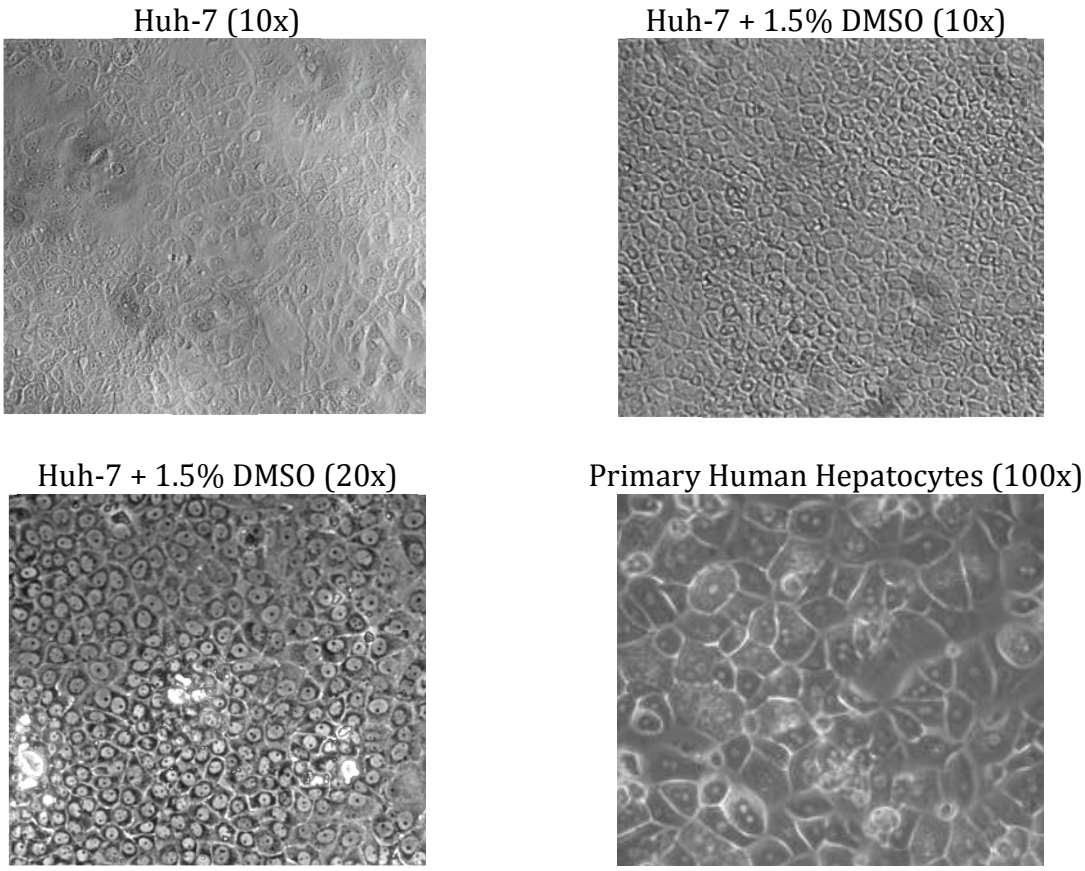
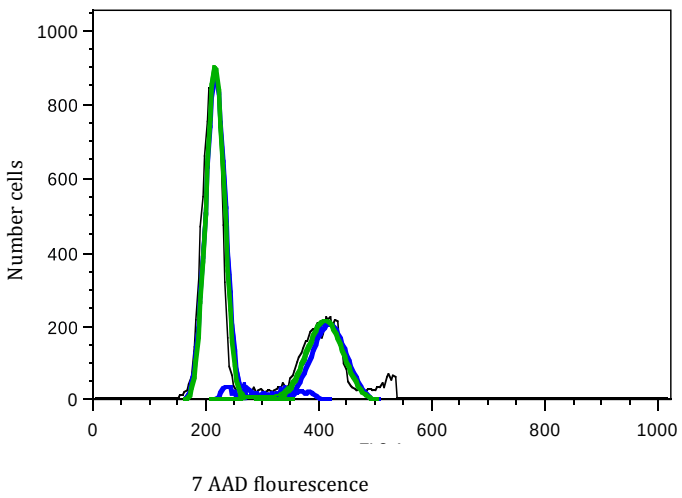
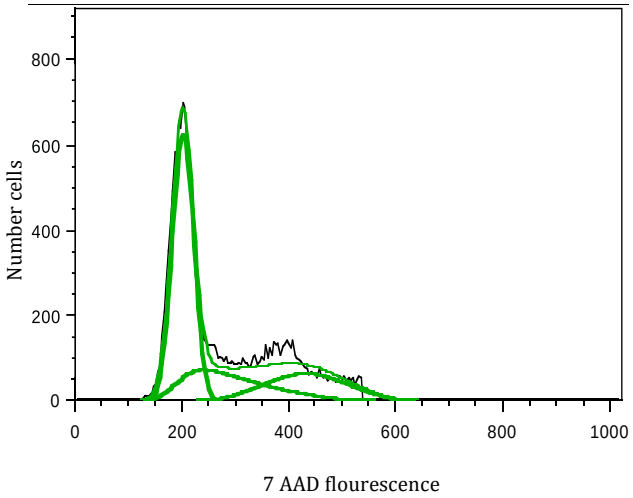


Figure 3.2 DMSO induces morphological changes in Huh-7 cells

Huh-7 cells were seeded in 48 well plates and treated with 1.5% DMSO (vol/vol) for 7 days. Cells were fixed and images taken by light microscopy. Images show cells from multiple experiments and are representative of the entire culture. Control Huh-7 cells were imaged at 48 hours post seeding at 100% confluency. Image of primary human hepatocytes were provided by Professor J McKeating



1.5% DMSO
G1 65% S 2%



Removal DMSO for 24 hours
G1 52% S 26%

Huh-7 DMSO removed 24 hours (100x)

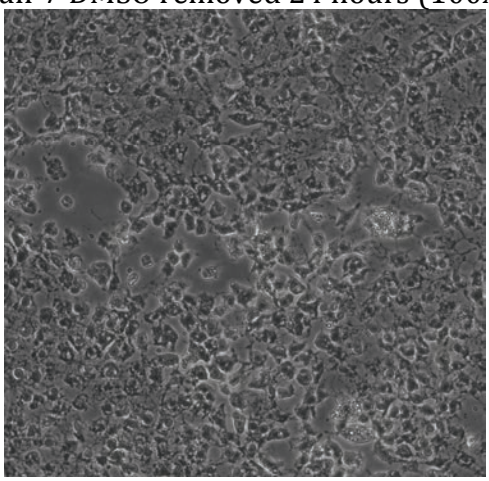


Figure 3.3 Cells require DMSO in the media to maintain viability

Huh-7 cells were seeded in duplicate 48 well plates and treated with 1.5% DMSO (vol/vol) for 7 days. DMSO was removed from the media for 24 hours and cells stained with 7-AAD. Results are the mean of triplicate wells and represent duplicate experiments. Image shows cells that have been fixed and image taken by light microscopy. Image is representative of the entire culture. Following 24 hours of DMSO removal cell viability is limited with considerable cell death.

3.3: DMSO induces hepatoma cell differentiation towards a hepatocyte-like phenotype

Previous results showed that hepatoma cells treated with DMSO have slowed cycle progression and can be maintained in culture over time, developing a hepatocyte-like morphology. Next, the expression of hepatocyte-specific gene markers was investigated to determine how "hepatocyte-like" the arrested cells were over time.

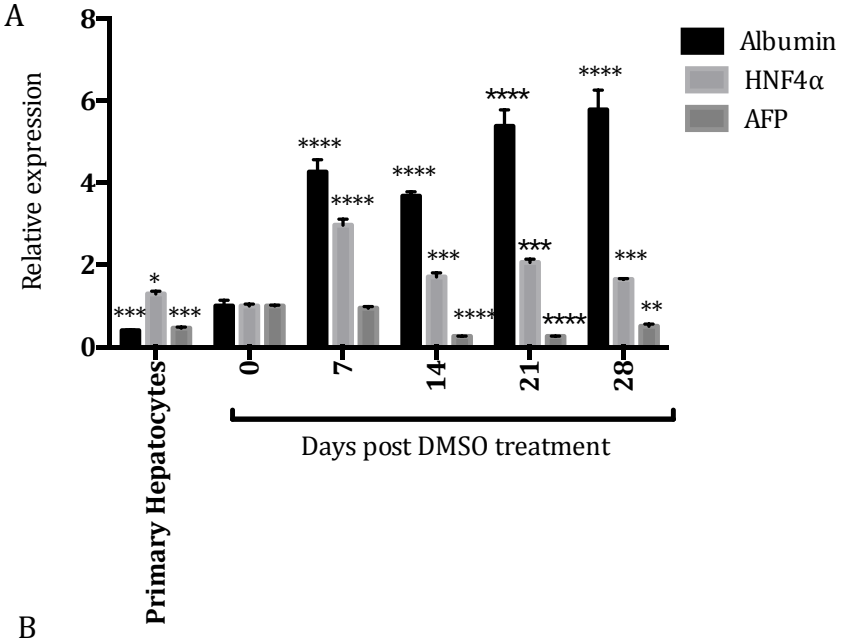
Hepatocytes express many liver-specific markers including HNF4α, the cytochrome P450 enzymes and albumin. Differentiated hepatocytes, in particular, also downregulate fetal markers such as alpha-fetoprotein (Klaunig et al, 1981; Sainz and Chisari, 2006). **Figure 3.4A** and **B** shows that DMSO treated Huh-7 significantly increase mRNA levels of several hepatocyte markers. These were at a level comparable to primary human hepatocytes (PHHs) cultured for 48 hours. Levels of liver-specific markers are increased up to 7 days post DMSO treatment and were then maintained throughout the course of the culture. Despite mRNA levels being comparable to PHH in culture for up to 48 hours, relative levels in DMSO treated cells did not reach that of liver biopsies taken on the day of liver explant (**Figure 3.4C**).

Hepatocytes are metabolically active and the liver is a major site for glucose metabolism. Hepatocytes maintain glucose homeostasis and subsequently modulate glycolysis (the breakdown of glucose to release energy) or gluconeogenesis (the generation of new glucose from substrates) depending

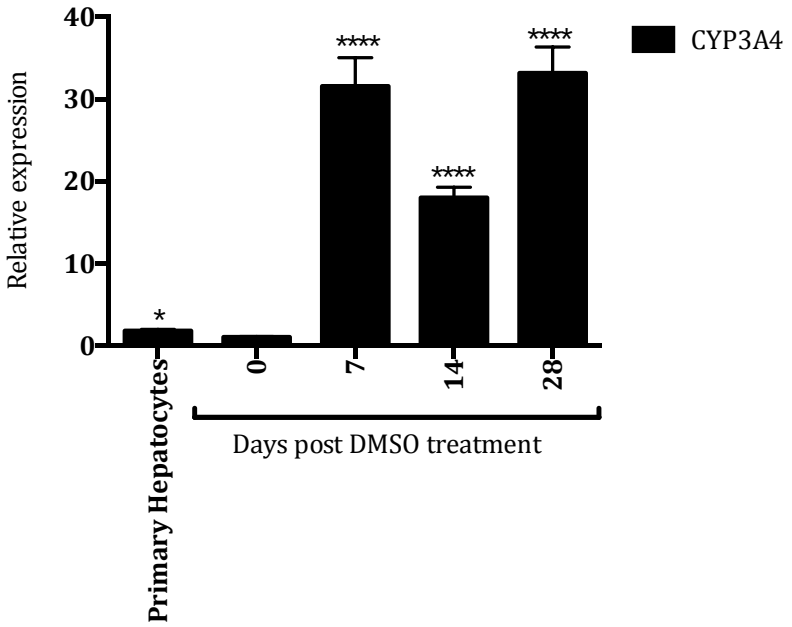
on glucose levels (Shoji et al, 2012). The major enzymes involved in these steps are outlined in **Figure 3.5A**, with phosphoenolpyruvate carboxykinase (PEPCK) and glucose-6-phosphatase (G6Pase) controlling key steps within the gluconeogenic pathway. To determine gene expression in DMSO-treated cells, differentiated Huh-7 were plated for up to 28 days in medium containing 1000mg/L glucose (5.5mM) that equates to normal blood sugar levels i*n vivo*. A panel of genes responsible for regulating gluconeogenesis, glycolysis and oxidative phosphorylation were quantified by qPCR. DMSO differentiation increased PEPCK mRNA levels over time to levels comparable with PHH in culture (**Figure 3.5B**) while G6Pase mRNA was also induced in hepatoma cells after 7 days treatment with DMSO, while it was absent completely from untreated cells. This suggests that cells have active metabolic pathways and are cycling glucose when differentiated.

In summary, we show that DMSO treated cells are increasingly differentiated over time, up-regulating key hepatocyte markers and metabolic features associated with hepatocytes. As such, these results suggest that DMSO treatment of Huh-7 differentiates these cells towards a hepatocyte-like phenotype and are a more physiological model system compared to non-differentiated hepatoma cells.

A



B



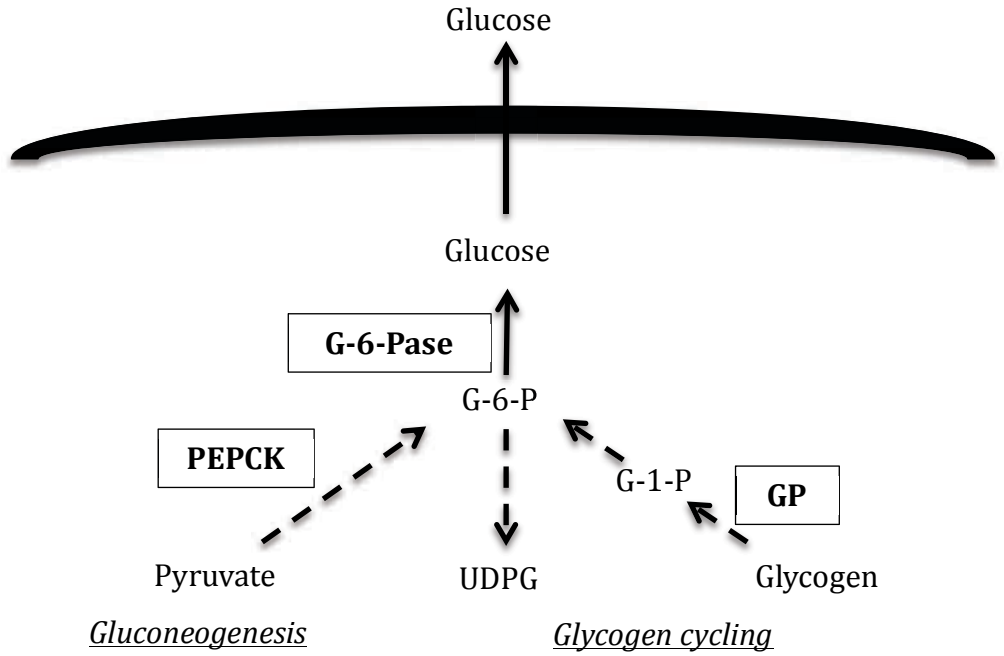
C

	Liver (relative expression)	DMSO treated Huh-7 (relative expression)
Albumin	89	5.7
CYP3A4	7350	26.7

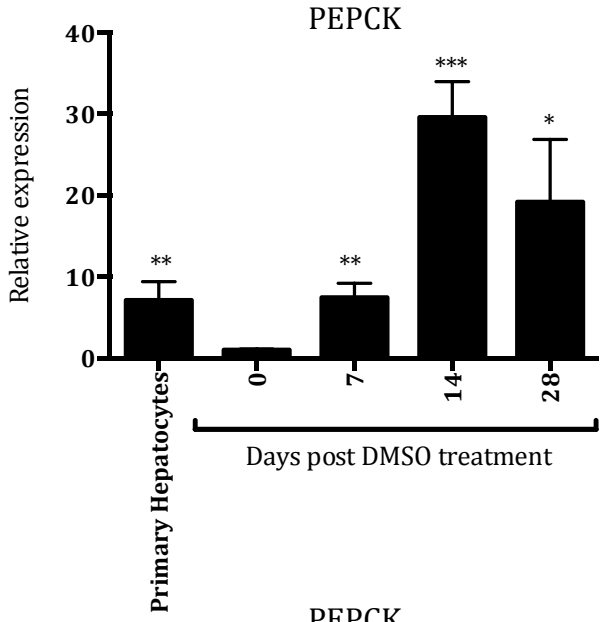
Figure 3.4 DMSO upregulates hepatocyte markers over time

Huh-7 cells were seeded as previously and treated with 1.5% DMSO for up to 28 days. Differentiation status was assessed by quantifying mRNA levels of (A) human albumin, HNF4α, α-fetoprotein (AFP) and (B) CYP3A4. These were compared to undifferentiated cells and primary human hepatocytes (from 3 donors) 48 hours post seeding, prior to de-differentiation. Differentiation markers were also compared to mRNA from normal human liver biopsies (C). RNA was extracted as described. Data was analysed by the 2-ΔΔct method, normalized for GAPDH and compared to undifferentiated Huh-7 cells. Results are pooled triplicate wells and representative of three independent experiments. Mean expression relative to Day 0 cells, with standard deviation are plotted. Significant differences were analysed using Students t test and compared to Day 0 cells. No marker = non significant. * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$

A


Glucose
Glucose
G-6-Pase
G-6-P
PEPCK
G-1-P
GP
Pyruvate
UDPG
Glycogen
Gluconeogenesis
Glycogen cycling

B


PEPCK
Relative expression
40
30
20
10
0
**
**
***
*
Primary Hepatocytes
0
7
14
28
Days post DMSO treatment

C

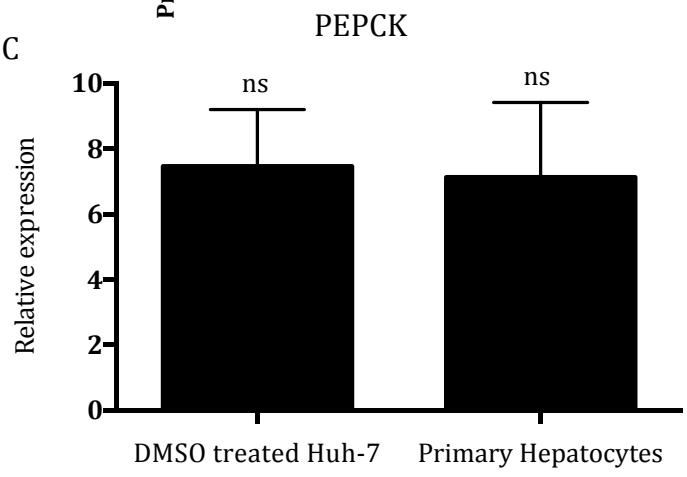

PEPCK
Relative expression
10
8
6
4
2
0
ns
ns
DMSO treated Huh-7
Primary Hepatocytes

Figure 3.5 DMSO upregulates important enzymes in hepatic glucose metabolism

A) A schematic diagram of glucose metabolism within a hepatocyte showing de-novo glucose synthesis (gluconeogenesis) and glycogen cycling (adapted from Greenberg et al, 2006) showing G-6-Pase (glucose-6-phosphatase), G-6-P,(glucose-6-phosphate) G-1-P (glucose-1-phosphate) UDPG (UDP glucose) GP (glycogen phosphorylase) and PEPCK (phosphoenolpyruvate carboxykinase). B) Huh-7 cells were seeded as previously and treated with 1.5% DMSO for 28 days. Cells were lysed, PEPCK mRNA was quantified by RT-qPCR, and compared to undifferentiated (Day 0) cells and primary human hepatocytes 48 hours post seeding. (C) PEPCK mRNA levels were compared in Day 7 DMSO treated cells with primary human hepatocytes 48 h post-seeding. Results plotted are means of relative expression compared to Day 0 cells. Means are from pooled triplicate wells representative of two independent experiments. Significant differences were analysed using Students t test and compared to Day 0 cells. ns = non significant. * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$

3.4: Huh-7^{diff} upregulate host cellular factors known to alter HCV replication

Previous results were aimed at ascertaining the consistent working conditions for subsequent experiments. Key liver and metabolic markers are upregulated by Day 7 in DMSO treated Huh-7 and cells have slowed cell cycle progression and can be maintained in culture over time. Therefore for all future experiments Huh-7 were treated for 7-10 days with DMSO; following this they will be considered differentiated and termed Huh-7^{diff} (Bauhofer et al, 2012).

Since Huh-7^{diff} upregulate a number of genes involved in hepatocyte function, the impact of DMSO treatment on host factors reported to play a role in HCV replication was assessed. miR122 is the most abundant liver-specific micro-RNA and has been shown to be important for HCV replication *in vivo* (Jopling et al, 2005; Machlin et al, 2011; Goergen and Niepmann, 2012). Furthermore, hepatocytes induced from stem cells show a key role for miR122 in permissivity to HCV (Wu et al, 2012). Additionally, several other micro-RNAs known to alter key cellular processes such as lipid metabolism have been implicated in regulating HCV replication (reviewed in Pfeffer and Baumert, 2010, Conrad and Niepman, 2014). Table 1 summarizes hepatocellular micro-RNAs (other than miR122) that have been reported to play a role in HCV replication at the time of investigation. Therefore candidate micro-RNA expression levels were measured by qPCR following differentiation. Results showed that miR122 (**Figure 3.6**) and other candidate miRs were increased in Huh-7^{diff} (**Figure 3.7**). Let-7b, which has been shown to independently alter HCV replication by acting on the HCV genome

(Cheng et al, 2012), was not present in undifferentiated cells, however was quantifiable (raw ct value mean 37) in Huh-7^{diff} 7 days post differentiation.

Additionally, TAP 1 (tocopherol-associated protein 1) or SEC14L2 was recently reported as an essential host cell factor required for the hepatocellular replication of primary HCV strains (Saeed et al, 2015). SEC14L2 is ubiquitously expressed in human tissues (Allen-Baume et al, 2002), despite being absent from hepatoma cell lines (Saeed et al, 2015) and is a cytosolic lipid binding protein family member. SEC14L2 mRNA levels were quantified in Huh-7^{diff} and showed gene expression in undifferentiated cells that was significantly enhanced following DMSO-dependent differentiation (**Figure 3.8**). In addition we confirmed that SEC14L2 was expressed at comparable levels in Huh-7 and Huh-7.5.

The expression in Huh-7^{diff} of key cellular factors that influence HCV replication via a number of mechanisms support the use of these cells as a more physiologically relevant model system for studying host/viral interaction.

Table 3.1 Micro-RNAs that are known to interact with HCV

Micro-RNA	Affect on HCV	Reference
130a	Inhibits HCV replication by upregulation of Type I interferon pathways. Decreases miR122	Li et al, 2014; Li et al, 2017
27a	Preferentially expressed in HCV infected livers. Involved in lipid metabolism and LDLR expression Silencing miR-27a increases cellular lipids, reduces viral buoyant density and promotes viral replication. Inducing 27a decreases viral replication	Shirasaki et al, 2013; Choi et al, 2014
29a	Decreased levels found in livers of HCV infected patients Regulates expression of ECM* and collagen, and lipid droplet formation Overexpression of miR-29 decreases HCV replication modestly	Bandyopadhyay et al, 2011; Mahdy et al, 2016
320a	Role in MAPK pathway Levels increased by anti-Claudin Antibody Reduction in 320a decreases HCV replication	Mailly et al, 2015
491	Can enhance HCV replication via PI3K/Akt pathway	Ishida et al, 2011
196a	IFN-β induced Directly targets the genomic RNA of HCV	Pederson et al, 2007
Let-7b	Decreases HCVcc by interfering directly with HCV genome and protein expression	Cheng et al, 2012

*ECM = extracellular matrix protein

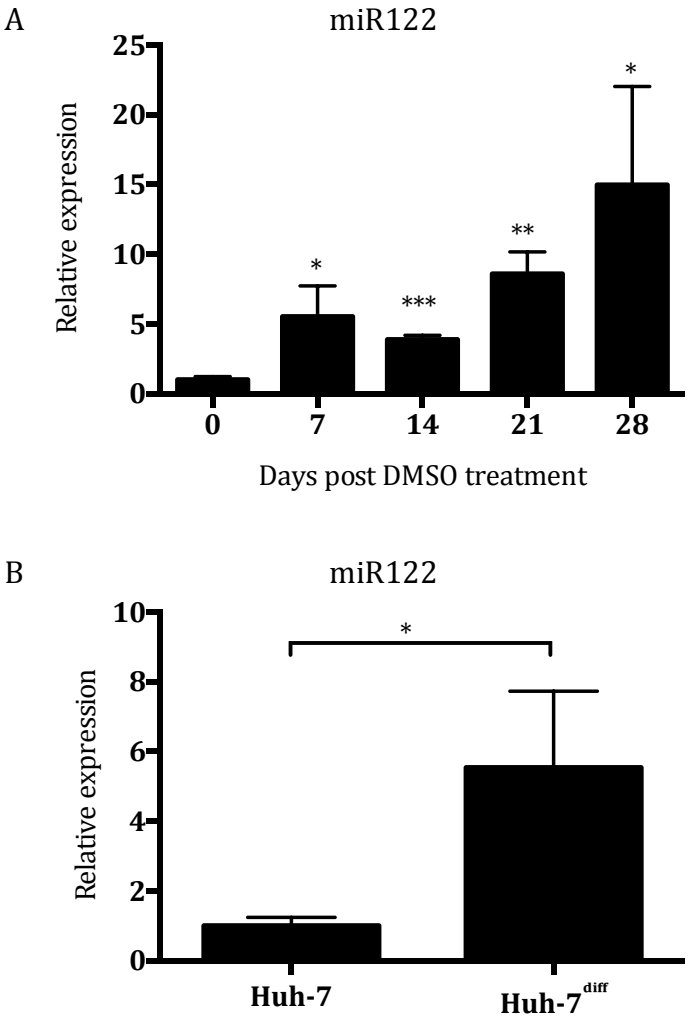


Figure 3.6 miR122 increases over time in Huh-7^{diff}.

Huh-7 cells were differentiated for up to 28 days with 1.5% DMSO (vol/vol). Cells were lysed, RNA extracted and the levels of miR122 quantified (A and B) using a using a two-step qPCR and normalized to an endogenous control (miR-130). Data is expressed relative to undifferentiated cells calculated by the ΔΔct method Results are means of triplicate wells and 2 independent experiments * p ≤0.05, ** p ≤0.01, ***p ≤0.001 (Students t test)

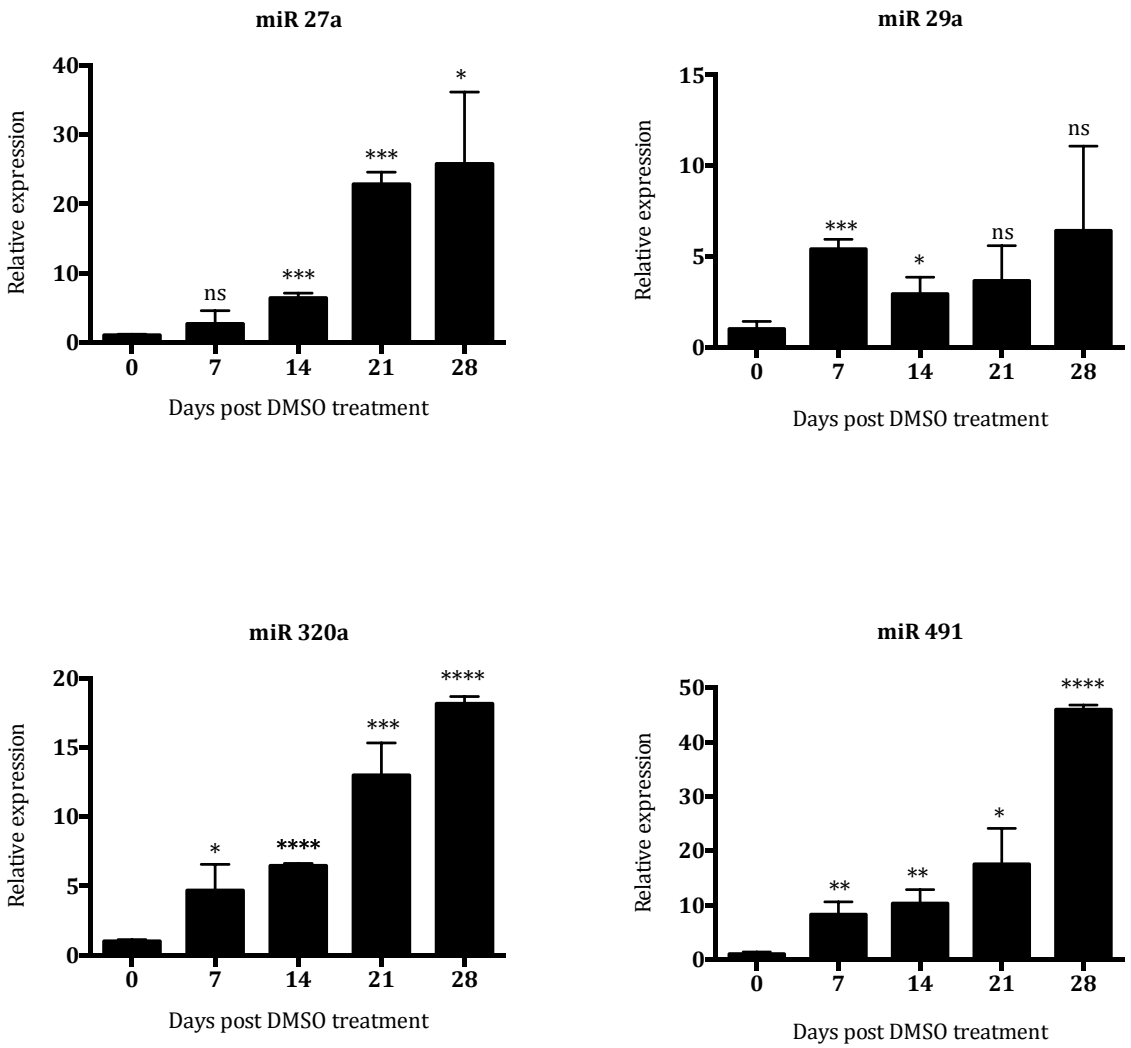


Figure 3.7 Differentiated cells upregulate key micro-RNAs over time.

Huh-7 cells were differentiated for up to 28 days with 1.5% DMSO (vol/vol). Cells were lysed, RNA extracted and levels of indicated miRs were quantified using a using a two-step qPCR and normalized to an endogenous control (miR-130). Relative expression is calculated by the ΔΔct method compared to Day 0 samples (untreated Huh-7). Results are means of triplicate wells and 2 independent experiments. Significant differences were analysed using Students t test and compared to Day 0 cells. ns = non significant. * p ≤0.05, ** p ≤0.01, ***p ≤0.001, **** p ≤0.0001

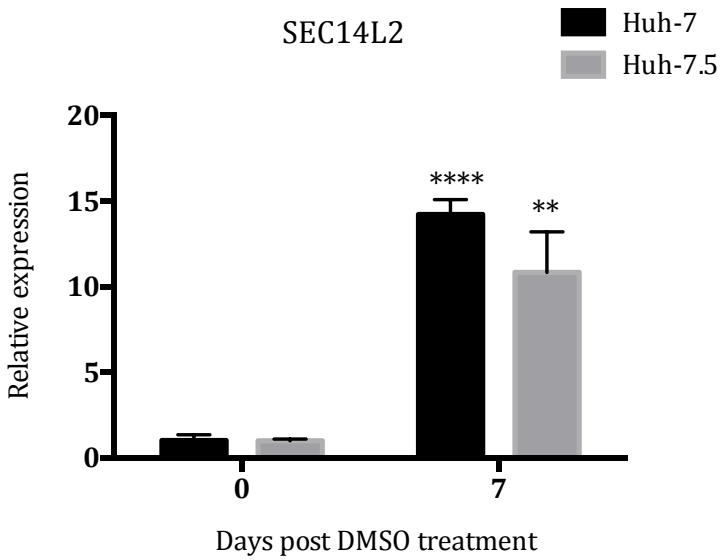


Figure 3.8 SEC14L2 is upregulated in Huh-7^{diff} over time

Huh-7 and Huh-7.5 cells were differentiated for up 7 days with 1.5% DMSO (vol/vol). Cells were lysed with RLT lysis buffer and RNA extracted as previously. Levels of SEC14L2 were quantified using a using qPCR and normalized to GAPDH. Relative expression is calculated by the ΔΔct method compared to Day 0 samples. Results are means of triplicate wells and 2 independent experiments. Significant changes were analysed using the Students t test and compared to Day 0 cells. ns = non significant ** $p \leq 0.01$, **** $p \leq 0.0001$

3.5: Huh-7 differentiation limits HCV replication.

While the results have shown that Huh-7^{diff} provide a more physiological system for studying HCV using metabolic and cellular markers, the ability of these cells to support HCV infection has yet to be assessed. It was hypothesised that the permissivity of these cells to HCV will be altered, as primary hepatic cells are generally resistant to HCV compared to hepatoma cell lines.

In order to investigate this, Huh-7^{diff} and non-differentiated cells were inoculated with HCV SA13/JFH and frequency of NS5A expressing cells enumerated after 72h. The results showed 4.5% NS5A expressing Huh-7^{diff} compared to 90% of non-differentiated cells (**Figure 3.9**), showing a clear and significant difference in permissivity. To more accurately quantify this, cells were infected with virus at various dilutions and 72h later the number of infected cells and viral titre enumerated. Both infectious titre (**Figure 3.10A**) and HCV RNA expression (**Figure 3.10B**) were reduced 125-fold in Huh-7^{diff}. Furthermore, NS5A expressing foci in Huh-7^{diff} were infrequent and small, consisting of 1-2 cells, compared to undifferentiated Huh-7 (**Figure 3.10C**), which had large multicellular foci, suggesting that differentiation limits HCV spread.

To investigate whether the time of DMSO-treatment affected HCV infection, cells were differentiated for 7-21 days, infected with HCV SA13/JFH and infection (HCV RNA) quantified by qPCR 72h later (**Figure 3.11**). Longer differentiation times resulted in less infection, confirming that differentiation of hepatoma cells significantly reduces permissivity to infection.

Previous reports (Nelson and Tang, 2006) have suggested that cells grown at high density also have reduced ability to support HCV infection due to cell contact-mediated inhibition. This can be overcome by the addition of nucleosides to the media. In order to test that the difference in permissivity was not due to cell-contact inhibition, media was supplemented with additional nucleosides as described in Nelson and Tang (2006). This failed to restore levels of infection compared to regular Huh-7 (**Figure 3.12**), suggesting that contact inhibition was not causing the reduction in permissivity seen in these cells.

It was also important to verify that DMSO was not affecting the viral particle itself. HCVcc was incubated with 1.5% DMSO for 4 hours. The virus was then pelleted by ultra-centrifugation and used to infect Huh-7 alongside control virus. There was no difference in the viral infectivity showing that the effect of DMSO was on the target cells and not on the viral particle itself (**Figure 3.13**).

In summary, differentiated Huh-7 cells support significantly lower levels of HCV replication.

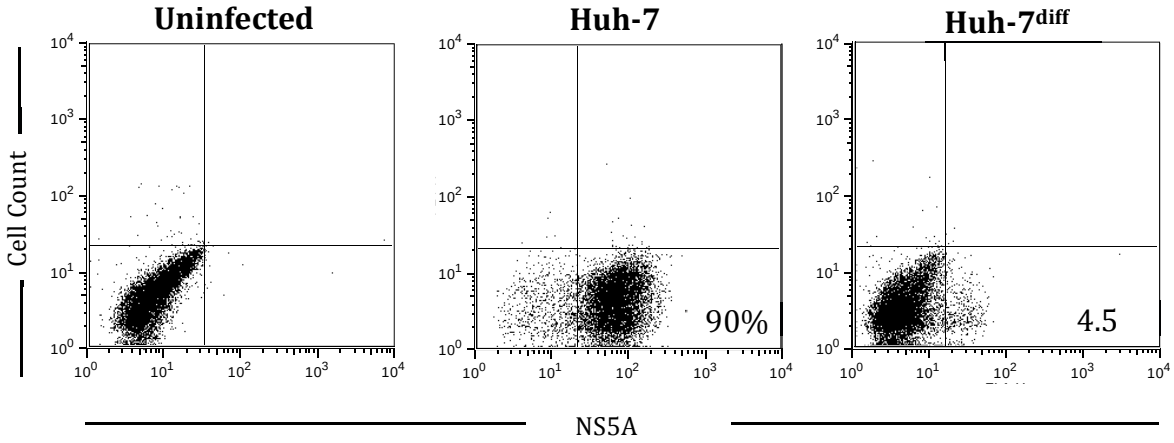
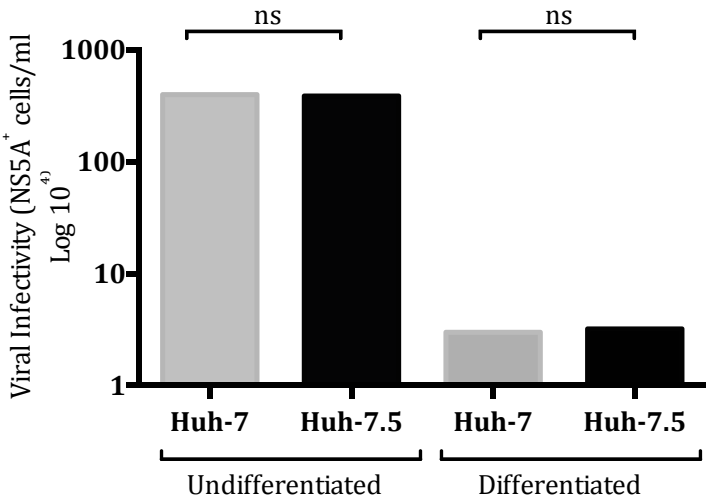


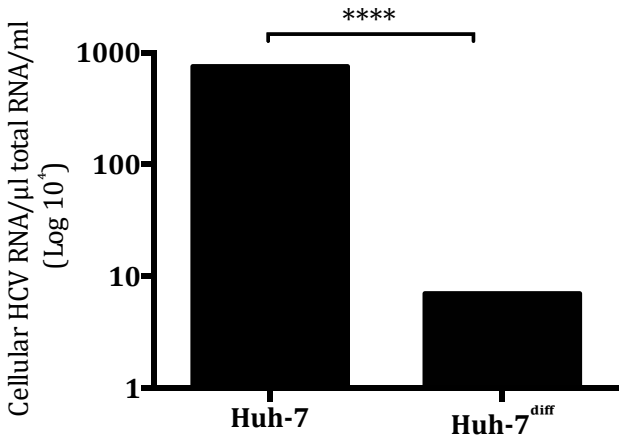
Figure 3.9 Huh-7^{diff} support reduced permissivity to HCV.

Huh-7^{diff} and control Huh-7 cells of comparable density were infected with HCV SA13/JFH (MOI 10), incubated for 72h and the frequency of NS5A expressing cells assessed by flow cytometry (FACSCalibur). Raw FACS plots analysed by FlowJo are plotted and represent mean values from three independent experiments.

A


ns
ns
1000
100
10
1
Viral Infectivity (NS5A+ cells/ml Log 10 4)
Huh-7
Huh-7.5
Huh-7
Huh-7.5
Undifferentiated
Differentiated

B


****
1000
100
10
1
Cellular HCV RNA/µl total RNA/ml (Log 10 4)
Huh-7
Huh-7diff

C

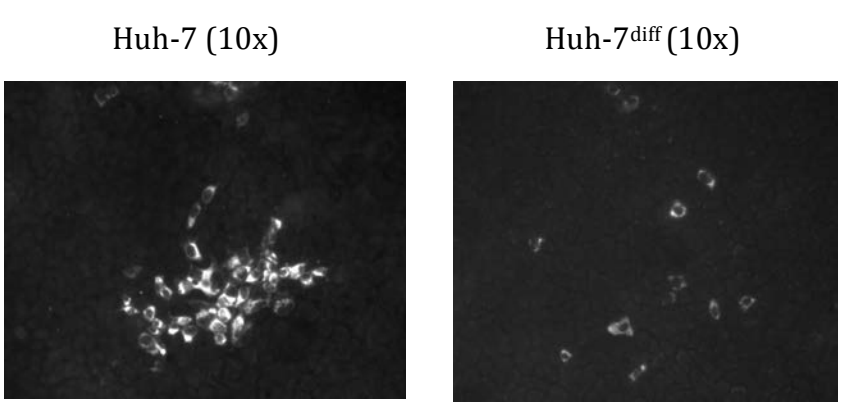

Huh-7 (10x)
Huh-7diff (10x)

Figure 3.10 Differentiation reduces Huh-7 permissivity to support HCV replication.

Relative infectivity was determined by titrating HCV SA13/JFH onto Huh-7^{diff} or Huh-7 cells. (A and B) Infection was determined at 72h post-infection by immunofluorescence for NS5A expression or by qPCR for viral RNA. Results are expressed as the number of NS5A positive cells per ml of virus (A) or HCV copies per microliter of RNA per ml virus (B). Results are representative mean and standard deviation of 3 separate experiments. ****p≤0.0001 (students t-test). (C) NS5A foci at 72 hours post infection. Images are representative of the whole plate and three independent experiments.

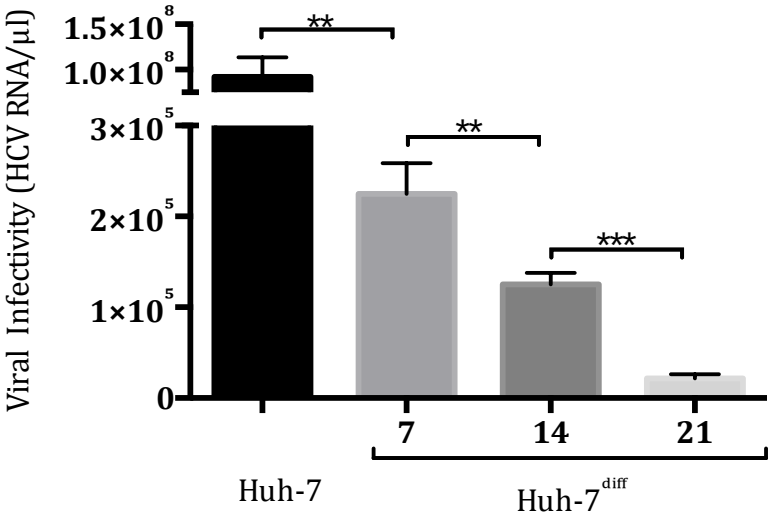


Figure 3.11 Extended time periods of Huh-7 differentiation reduces HCV replication.

Non-differentiated and Huh-7^{diff} cells were infected with HCV SA13/JFH at indicated time points post-differentiation. Cellular HCV RNA copies were quantified by RT-qPCR and normalized to GAPDH, and the mean and standard deviation of 3 experiments shown. ** $p \leq 0.01$ *** $p \leq 0.001$ (Students t-test).

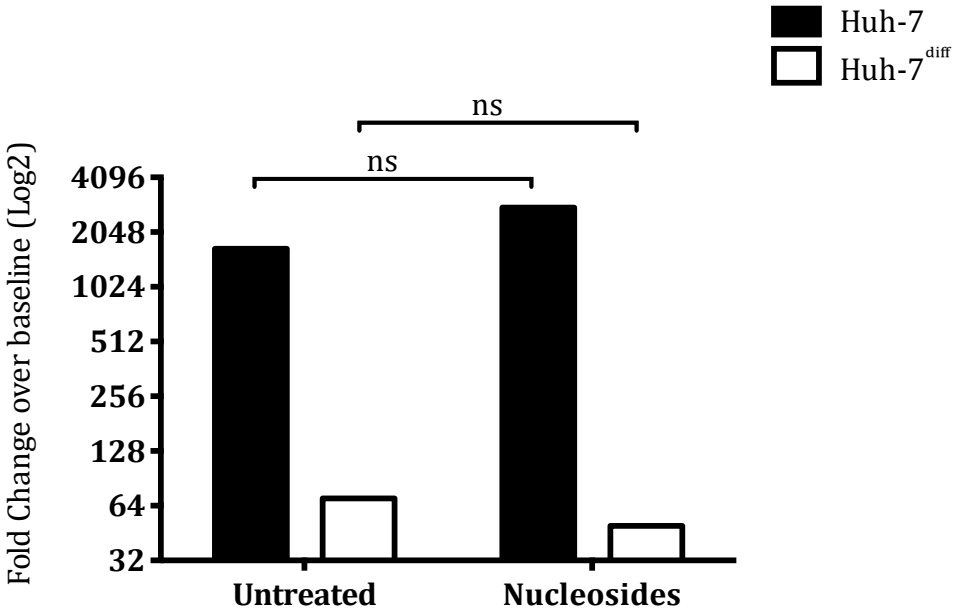


Figure 3.12 Nucleoside addition does not alter permissivity

Huh-7 and Huh-7^{diff} were seeded at comparable density and transfected with JC1gLuc HCV RNA for 8h, before transfection reagent was removed. Cells were then treated with uridine and cytidine (200 microMolar) (Nelson and Tang, 2006) or untreated. Supernatant was collected at 72 hours post transfection. Replication was assessed by measuring the luciferase signal in the supernatant and plotted as fold change over baseline (4 hours). Results are the mean and standard deviation of 3 experiments. ns = non-significant (Students t test).

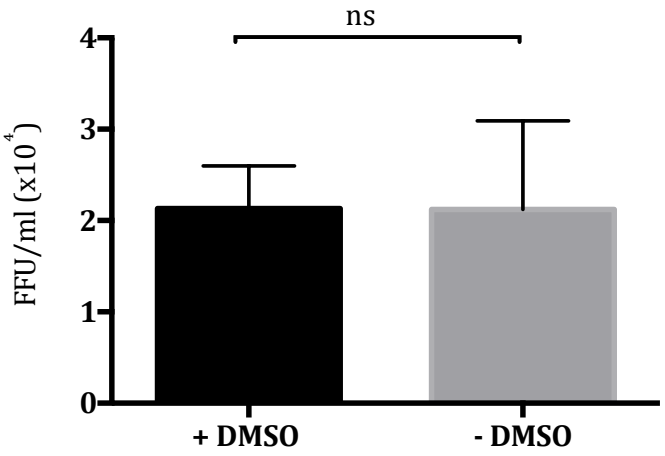


Figure 3.13 DMSO has no effect on HCV particle infectivity

HCV SA13/JFH was incubated in 1.5% DMSO vol/vol (or control 10% FCS/DMEM) for 4 hours prior to pelleting by ultra-centrifugation. The pelleted virus was then used to infect Huh-7 cells and infectivity calculated by counting NS5A positive cells at 48 hours post infection. Results plotted are focus forming units/ml and are the means of two independent experiments. ns = non significant (Students t test)

3.6: Differentiated cells are less sensitive to direct acting anti-viral drugs

Huh-7 become increasingly physiological and metabolically active when differentiated with DMSO. This includes upregulation of genes such as CYP3A4, which is one of the key enzymes involved in drug metabolism in the liver. It was therefore interesting to investigate whether the efficacy of direct acting antiviral drugs (DAAs) were altered in this system compared to standard hepatoma cells.

Huh-7^{diff} or parental cells were infected with HCV SA13/JFH at a comparable MOI in the presence of Telaprevir (VX950, protease inhibitor) or 2'CMC (polymerase inhibitor) and infection assessed 72h later. Results showed a reduction in the ability of both drugs to limit HCV replication in differentiated cells (**Figure 3.14 A** and **B**), with a 2-fold and 8-fold increase in the IC_{50} of VX950 and 2CMC respectively, although the difference in VX950 was not significant (**Table 3.2**).

This observation highlights the importance of assessing therapeutic interventions in differentiated cells where important cellular responses, including drug metabolism, are likely to be more effective *in vivo* than in non-differentiated cells.

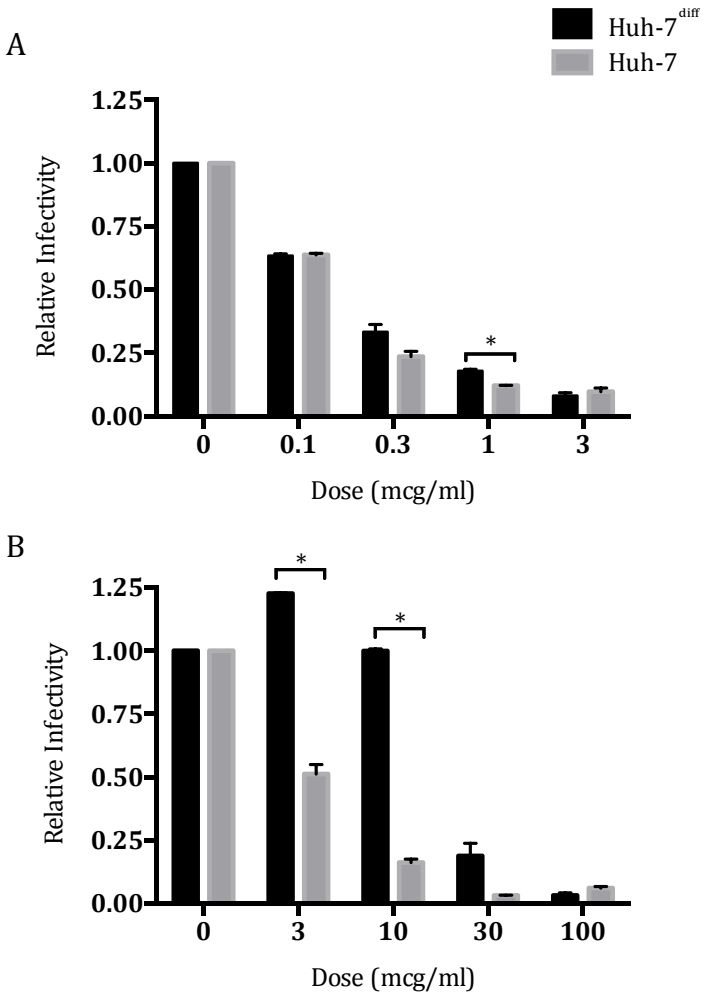


Table 3.2

Compound	Inhibitor	Huh-7 IC_{50} (mcg/mL)	Huh-7^{diff} IC_{50} (mcg/mL)	P value
VX950	Protease	0.05	0.1	0.1
2CMC	Polymerase (nucleoside)	3.1	24.5	<0.001

Figure 3.14 Huh-7^{diff} show reduced sensitivity to anti-viral drugs.

Sensitivity to direct acting antiviral drugs was assessed by infecting Huh7^{diff} and Huh7 with HCV SA13/JFH at comparable MOI. 24 hours post infection VX950 (A) or 2'CMC (B) were added at the indicated concentrations. 72 hours post infection the percentage of NS5A positive cells was quantified by flow cytometry (FACSCalibur). Relative infectivity was compared to untreated cells and the drug IC50 calculated (Table 3.2). Results represent the mean of triplicate wells and two independent experiments. *p=≤0.05 (t-test).

3.7: Discussion

In this chapter, we have described a model system for the study of HCV that is more physiological than standard hepatoma cells. The addition of 1.5% DMSO to Huh-7 cells for 7-10 days, results in cells that are arrested and are altered morphologically, physiologically and metabolically, becoming more 'liver like'. These cells are able to be maintained in culture for several weeks and are permissive to infection with HCVcc, although support 125 fold reduced levels of infection.

The use of DMSO has many advantages over other methods of cell differentiation; it is widely available, and the methodology of differentiation is very straightforward. Previously, DMSO has been used to differentiate cells including hepatoma cell lines (Sainz and Chisari, 2006; Choi et al, 2009; Nikolaou et al, 2016). When differentiation status of cells is important for viral permissivity, which is the case for hepatitis B *in vitro*, pre-treatment with DMSO is essential for viral entry and replication (Iwamoto et al, 2014; Gripon et al, 2002; Ni and Urban, 2017). The exact mechanism of action of DMSO in cell differentiation is unknown, although treatment with DMSO is known to alter a large number of host cell genes (N Frampton, verbal communication; Bauhofer et al, 2012) and has also been shown to; alter cellular membrane integrity (Melkonyan et al, 1996; Menorval et al, 2012) induce cell cycle arrest (Chetty et al, 2013; Sainz and Chisari, 2006), increase RNA and protein synthesis (Strätling, 1975, Liu, 2001) and alter intracellular signalling pathways, such as protein Kinase C (Makowske et al, 1988) or integrin expression(Fiore and Degrassi,

1999). Although we did not investigate further the mechanism, the results showed that DMSO upregulated liver specific genes (Figure 3.4), including those involved in glucose homeostasis and metabolism (Figure 3.5). It is well known that primary human hepatocytes, differentiate rapidly *ex vivo,* are highly heterogeneous, expensive and difficult to obtain. Other methods of differentiation, such as using human serum or DMSO in conjunction with insulin and other growth factors, are more complex and do not allow cells to be maintained over a long period of time. Given the restrictions of other cells and methods, Huh-7^{diff} offers a reproducible, physiological cell system for studying HCV replication.

Despite the significant advantages to Huh-7^{diff} over other cell systems for studying HCV, there are a number of limitations of the system. DMSO must be present throughout the culture for the cells to maintain viability (Figure 3.3) which limits the ability to carry out any experiments free from DMSO and, as outlined above, this may have significant effects on other cells. Cells cannot be split and re-seeded as they lose their viability (data not shown). We observed that cells were highly confluent after 5-7 days in culture and remained like this over several weeks. Chetty et al (2013) showed that stem cells, differentiated by DMSO treatment lost differentiation when cell contacts were impaired. Taken with our results this suggests that cell contacts are important in allowing the cells to maintain differentiation status.

Much of the data shown to support increased differentiation in these cells is transcript rather than functional data. Although this is a well-recognised way

of showing cellular differentiation (Sainz and Chisari, 2006; Nikolaou et al, 2016) it does mean more guarded conclusions should be drawn. Upregulation of the key enzymes involved in gluconeogenesis to levels similar to those in primary human hepatocytes in culture suggests that although Huh-7^{diff} are largely non-dividing they are not metabolically quiescent (Figure 3.5).

The results also showed that Huh-7^{diff} upregulates cytochrome P450 CYP3A4 RNA. CYP3A4 is a common and important oxidative enzyme and plays a crucial role in the metabolism of many drugs, including Telaprevir/VX950 (drug information sheet). Interestingly, the action of the polymerase inhibitor in this study, which has a similar mechanism of action to Sofosbuvir (drug information sheet), is significantly altered by hepatocyte differentiation. There are likely to be altered drug pathways, including enzymatic changes in differentiated cells which in part may explain the differences in the IC_{50} values seen between differentiated and standard hepatoma cells. It is important to consider these changes when evaluating drug activity in hepatoma cells, and we suggest that a differentiated model provides more translatable results.

We hypothesized differentiated cells would have altered permissivity to HCVcc. We then went on to show that Huh-7^{diff} are less permissive to HCVcc and this was defined as 125 fold difference (Figure 3.9-3.10). Given the low and variable infectivity *in vitro* of other differentiated cells, such as primary human hepatocytes (Wilson et al, 2015) the altered permissivity of Huh-7^{diff} provides further evidence that these cells provide an alternative model for the study of HCV replication. We were only able to infect these cells using a highly adapted

strain of SA13 that had been passaged and was a high MOI. The use of this virus, although not physiological, allowed sufficient viral replication to allow us to study this further.

The results showed that Huh-7^{diff} upregulate a number of key genes involved in regulating HCV replication within a cell. miR122 and a number of other candidate micro-RNAs alter expression over time (Figure 3.6). We demonstrated a 3-4 fold induction of HNF4α, a hepatocyte specific gene, in day-7 Huh-7^{diff} cells and this is a known regulator of miR122 *in vivo* (Li et al, 2011). In hepatocytes differentiated from hepatic progenitor cells, permissivity to HCV was notably correlated with detectable expression of miR122 (Wu et al, 2012). miR122 is an important cellular determinant of HCV replication whose induction or repression is caused by the hepatocyte specification process and requires further study. Additionally SEC14L2 (TAP) has recently been shown to be an essential host cell factor required for the hepatocellular replication of primary HCV strains (Saeed et al. 2015). Results showed that expression of SEC14L2 is also increased in differentiated hepatoma cells and offers an exciting possibility of utilising this model with patient derived virus in the future.

Summary

This chapter demonstrates that Huh-7^{diff} cells are a more physiological model for the study of HCV. The permissivity of these cells is altered by differentiation status. The next steps therefore concentrate on understanding the interaction between host cell and virus and will be explored in the next chapter.

Chapter 4: The HCV lifecycle in Huh-7^{diff}

4.1: Introduction

The previous chapter has outlined hepatoma cell differentiation and showed how these cells have significantly reduced permissivity to HCV infection. This is an important observation as it can offer insights into viral host interactions *in vivo*. This chapter will focus on exploring the reasons behind the alteration in viral permissivity.

The Hepatitis C virus lifecycle is well described and there are known factors that influence each step. Differentiated hepatoma cells have changes in many cellular factors that are known to impact viral permissivity. We therefore looked at each step of the viral lifecycle to identify how the virus is affected by host cellular differentiation and possible underlying mechanisms.

HCV entry into hepatocytes is a multi-step process that is dependent on host cell molecules; scavenger receptor B1 (SRB1) (Westhaus et al, 2017; Grove et al, 2007), CD81 (Pileri et al, 1998; Meulemann et al, 2008; Harris et al, 2010) the tight junction proteins claudin-1 (Evans et al, 2007; Harris et al, 2010; reviewed in Tawar et al, 2015) and occludin (Ploss et al, 2009; Liu et al, 2009), and Niemann-Pick C1-like 1 cholesterol absorption receptor (NPC1L1) (Sainz et al, 2012) prior to being internalized by clathrin-mediated endocytosis. **Figure 1.4** gives an overview of the key steps in HCV entry (reviewed in Lindenbach and Rice, 2013). Tight junctions play an important role in viral entry and exist between adjacent hepatocytes *in vivo*. Cells establish complex hepatic polarity by separating the apical and basolateral membranes of cells (**Figure 1.9**). The HCV

receptors localise distinctly in a polarised cell. Claudin is located at the apical tight-junction region and is also found in the basal-sinusoidal region co-located with CD81 (Reynolds et al, 2008). SR-B1 is found at the basolateral surface (Reynolds, Harris 2008). Claudin also associates with other proteins such as Zonula Occludens (ZO) to maintain structural integrity of the tight junctions between neighbouring hepatocytes (Shin et al, 2006). Hepatic polarisation, as well as being essential to physiological functioning of the liver, also restricts HCV entry (Mee et al, 2008; Mee et al; 2009; Harris et al, 2013) and consequently HCV permissivity. Huh-7 cells are not known to polarise and do not form tight junctions between cells. However, previous results have shown that differentiated cells alter morphologically. Therefore we investigated the localisation of HCV receptors, tight junction proteins and subsequent HCV entry into differentiated cells.

Following entry the viral capsid uncoats and the RNA is translated to generate the polyprotein (see **Figure 1.3**). The viral encoded non-structural proteins mediate replication of the viral RNA in membrane bound replication complexes (Lindenbach et al, 2005). Essential to this process is miR122, which binds to the 5'UTR enhancing viral translation and stabilizing the genome (Jopling et al, 2005; Henke et al, 2008; Georgen et al, 2012). Additionally, it protects viral RNA from degradation (Conrad et al, 2013; Li et al, 2013), can modulate the IFN response to virus (Li et al, 2013) and has also been implicated in viral entry (Sendi et al, 2015). Expression of miR122 in stem cell derived hepatocytes correlates with permissivity to HCV infection (Wu et al, 2012).

Previous results have shown that differentiation of Huh-7 cells increases miR122 levels and this may play an important role in viral replication in these cells. Furthermore, mir-122 is known to regulate iron homeostasis (Castoldi and Muckenthaler, 2012). Iron inhibits the HCV polymerase and suppresses subgenomic replication of HCV (Fillebeen et al, 2005). DMSO treatment of hepatoma cells has been reported to alter heam synthesis, a downstream pathway for the production of cellular iron (Galbraith et al, 1988). Changes in cellular iron represent a possible mechanism for reduction in HCV infectivity in differentiated hepatoma cells.

In addition to cellular iron, both lipid and glucose metabolism are essential to the normal function of a hepatocyte and are known to interact closely with HCV. Infected hepatocytes shift their glucose pathways to resemble glycolysis (Ripoli et al, 2010) via hypoxia-inducible factor (Hif)-1 stabilisation to promote energy conservation and survival of infected cells (Diamond et al, 2010). There is little known about whether altering glucose pathways within infected cells can influence viral replication, however this may be important to consider. We investigated whether these factors may play a role in the alteration in viral permissivity in differentiated cells.

One of the major restriction factors limiting HCV replication is cellular innate signalling. **Figure 1.12** gives an overview of innate signalling in hepatocytes in response to HCV. The host cell responds to HCV through the pattern recognition receptors, TLR3 on endosomal surfaces and cell membranes and cytosolic RIG-I (Satoh and Akira, 2016; Sumpter et al, 2005) and MDA-5 (Du

et al, 2016). These lead to a downstream cascade that results in interferon production. The predominant interferon produced by immune competent hepatocytes in response to HCV infection is IL29 (IFNλ1) a type III interferon (Park et al, 2012). This can act independently of type 1 interferons to inhibit HCV replication. Gene association studies have also shown the important roles for IFN λ alleles in predicting outcome of HCV infection and response to infection (Balagopal et al, 2010; Rauch et al, 2010). Once infection is established, the virus can evade immune signalling as the NS3/4A is capable of cleaving cellular adaptive proteins MAVS (also called IPS-1, VISA, Cardif) and TRIF (Li XD et al, 2005; Li K et al, 2005; reviewed in Heim and Thimme, 2014).

Interestingly, Huh-7 and Huh-7.5 have defective immune recognition; Huh-7 lack TLR3 expression but express RIG-I, and Huh-7.5 do not have TLR3 and have a defective RIG-I (Li K et al, 2005). This is thought to explain why hepatoma cells are highly permissive to infection *in vitro*. There is loss of TLR3 expression in Hepatocellular carcinoma (Wang et al, 2009; Kataki et al, 2017) suggesting that as cells become de-differentiated they lose the ability to recognise extracellular or endosomal HCV. Transiently expressed TLR3 in hepatoma cells allowed cells to re-establish an antiviral response and limited HCV replication (Wang et al, 2009). Bauhofer et al (2012) showed that Huh-7 cells differentiated with DMSO expressed a broader range of ISG mRNA than undifferentiated cells when stimulated with interferons. Changes in the immune response to HCV in differentiated cells, particularly production of IL29 and upregulation of key ISGs, would likely lead to significantly reduced permissivity.

We went on to investigate the role of innate signalling in response to viral infection within the DMSO differentiated system.

Recently the discovery of the host factor SEC14L2 has revolutionized the possibilities in HCV research. SEC14L2 is a cytosolic protein that stimulates an enzyme in the cholesterol synthesis pathway. Overexpression of SEC14L2 in Huh-7.5 allows the replication of a diverse range of HCV genotypes and plasma derived virus, which was previously difficult to achieve (Saeed et al, 2015). Previous results have shown that differentiation of Huh-7 causes upregulation of SEC14L2 mRNA. We went on to investigate whether this had any effect on viral permissivity.

Once the virus has entered the host cell and the genome has replicated, new particles assemble and are secreted from the infected cell, in a process that involves viral proteins associating with lipid droplets (Miyanari et al, 2007; Lindenbach, 2013) and apoplipoproteins (Benga et al, 2010). The final stages of viral maturation are closely linked with the VLDL secretion pathway (Gastaminza et al, 2008; Lindenbach, 2013). The mature particle can then infect naïve target cells via cell-free or cell-to-cell transmission routes (Timpe et al, 2008; Brimacombe et al, 2011). Some HCV strains (J6/JFH) are more efficient at spreading cell-to-cell than cell-free, whereas other strains (SA13/JFH and (HK6/JFH) show comparable rates in undifferentiated hepatoma cells (Meredith et al, 2013). The mechanism of HCV direct cell-cell spread is not fully understood, but may be less dependent on CD81 and SRB1 expression (Lindenbach and Rice, 2013). Studies of clusters of HCV infected cells within

livers of patients (Liang et al, 2009; Kandathil et al, 2013) suggest that cell-cell transmission is a potentially important route *in vivo* and may be influenced by cellular differentiation status.

Previous results showed that DMSO differentiates human hepatoma cells to become more 'hepatocyte like', whilst being easily accessible, cost effective and stable in culture over many days. They can provide an excellent model system for studying viral-host cell interactions. Driving cellular differentiation is associated with a significantly reduced permissivity to support HCV infection. This chapter will investigate the viral lifecycle and associated pathways aiming to understand how HCV enters, replicates and disseminates in differentiated cells.

Aims

- To understand how HCV enters, replicates and disseminates in differentiated Huh-7 cells.
- To identify steps in the viral lifecycle regulated by cellular differentiation status.
- To investigate the role of associated host pathways, such as interferon signalling and miR122 expression on viral replication in differentiated Huh-7 cells.

4.2: HCV receptor expression and localisation in Huh-7^{diff}

Hepatocytes in the liver are highly polarised. Currently available *in vitro* models to study hepatocyte polarity include HepG2 cells and HepaRG cells that differentiate over time into hepatocyte-like and biliary-like cells. Polarised cells support low level HCV replication with limited viral dissemination (Mee et al, 2009) much like the phenotype of HCV infected differentiated cells. Since receptor expression and localisation can define cellular susceptibility to HCV entry and viral permissivity we assessed whether DMSO treatment affects receptor localisation and cell polarisation.

Hepatoma cells were treated with DMSO for up to 21 days and stained for HCV receptors CD81, tight junction proteins Claudin-1, Occludin and ZO-1 along with bile cannaliculi marker MRP2 (multidrug-resistant-protein 2). Receptor localisation was analysed by confocal microscopy. Unlike hepatocytes that have multipolar organization, Claudin-1 in differentiated cells is expressed almost exclusively at the apical surface (**Figure 4.1**). This does not suggest that cells are developing complex hepatocyte polarity, rather that receptor localisation is more consistent with columnar epithelial-type polarisation (Singh and Harris, 2003; Treyer and Müsch, 2013) where the cell is polarised in one direction (i.e. from apical to basolateral surface) (**see Figure 1.9**). CD81 was ubiquitously expressed and there was no alteration with differentiation. There were several small circular structures staining for MRP2, a bile cannalicular marker, and ZO-1, a tight junction protein, in Day 21 differentiated cells. These were seen specifically in areas where the cells were highly compact (**Figure 4.2**). We utilised CMFDA

(cell tracker green), which enters hepatocytes and is then pumped into the bile cannaliculi where it accumulates and can be easily visualized. CMFDA staining shows fluorescence collecting in small circular areas between cells (**Figure 4.3**), suggesting that these areas have intact tight junctions allowing CMFDA accumulation. However, it is not clear whether these are true bile cannaliculi.

To investigate whether differentiation affects paracellular permeability as suggested by receptor localisation changes, Huh-7 were treated for 7 days with DMSO and a 70-kilodalton fluorescein isothiocynate/dextran flux measured (**Figure 4.4**) (Lambert et al, 2005). Highly confluent Huh-7 (Day 5 post seeding) were used as a comparator for cell density and Caco-2 cells, which are highly polarised and allow minimal dextran to pass through, were used as a positive control. There was a significant restriction in permeability in differentiated cells, although these were not as restricted as the positive control.

Differentiation alters receptor localisation and expression, particularly of Claudin-1 over time. Cells may be polarised, as suggested by reduced permeability to dextran/CMFDA and tight junction protein expression, however this is dynamic and heterogenous within the culture. These cells have potential to alter HCV entry (Mee et al, 2009), however polarisation does not appear to be complex hepatocyte polarity. In this context it is therefore important to consider whether viral entry is altered in these cells.

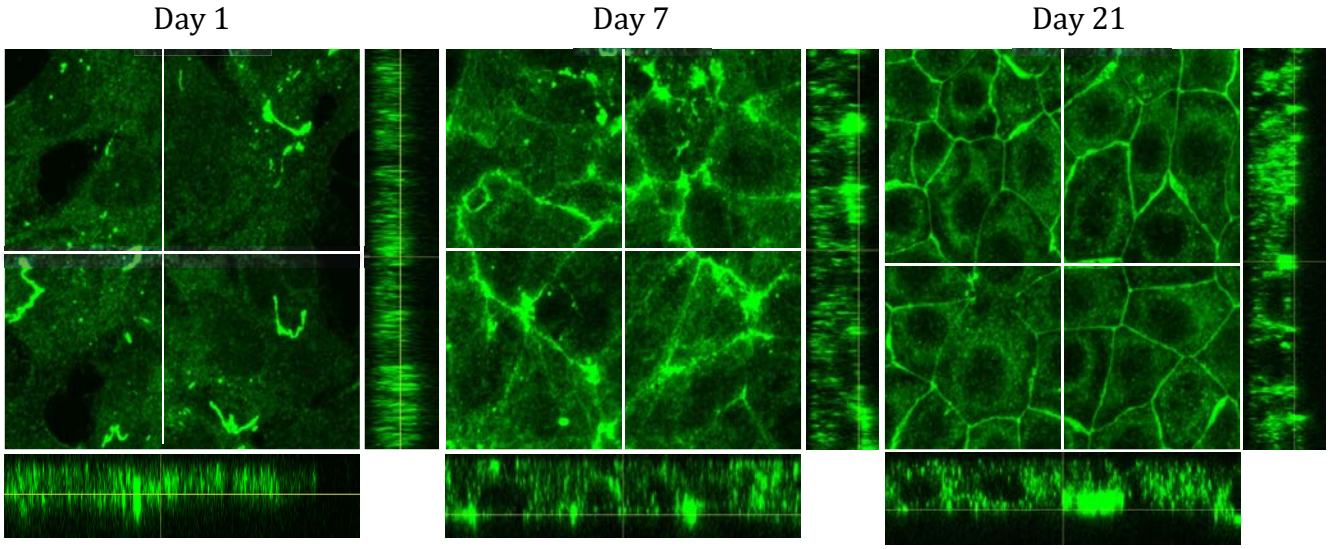


Figure 4.1 Claudin-1 expression and localisation in differentiated hepatoma cells

Huh-7.5 were plated onto glass coverslips and fixed at 1, 7, and 21 days post treatment with 1.5% DMSO (vol/vol). Cells were stained with anti-Claudin-1 antibody. Images were taken using Zeiss (Dublin, CA) MetaHead microscope with a 100× oil immersion objective and presented as a z-stack image showing the apical and basolateral cell surfaces. Results are representative images of two independent experiments and duplicate wells.

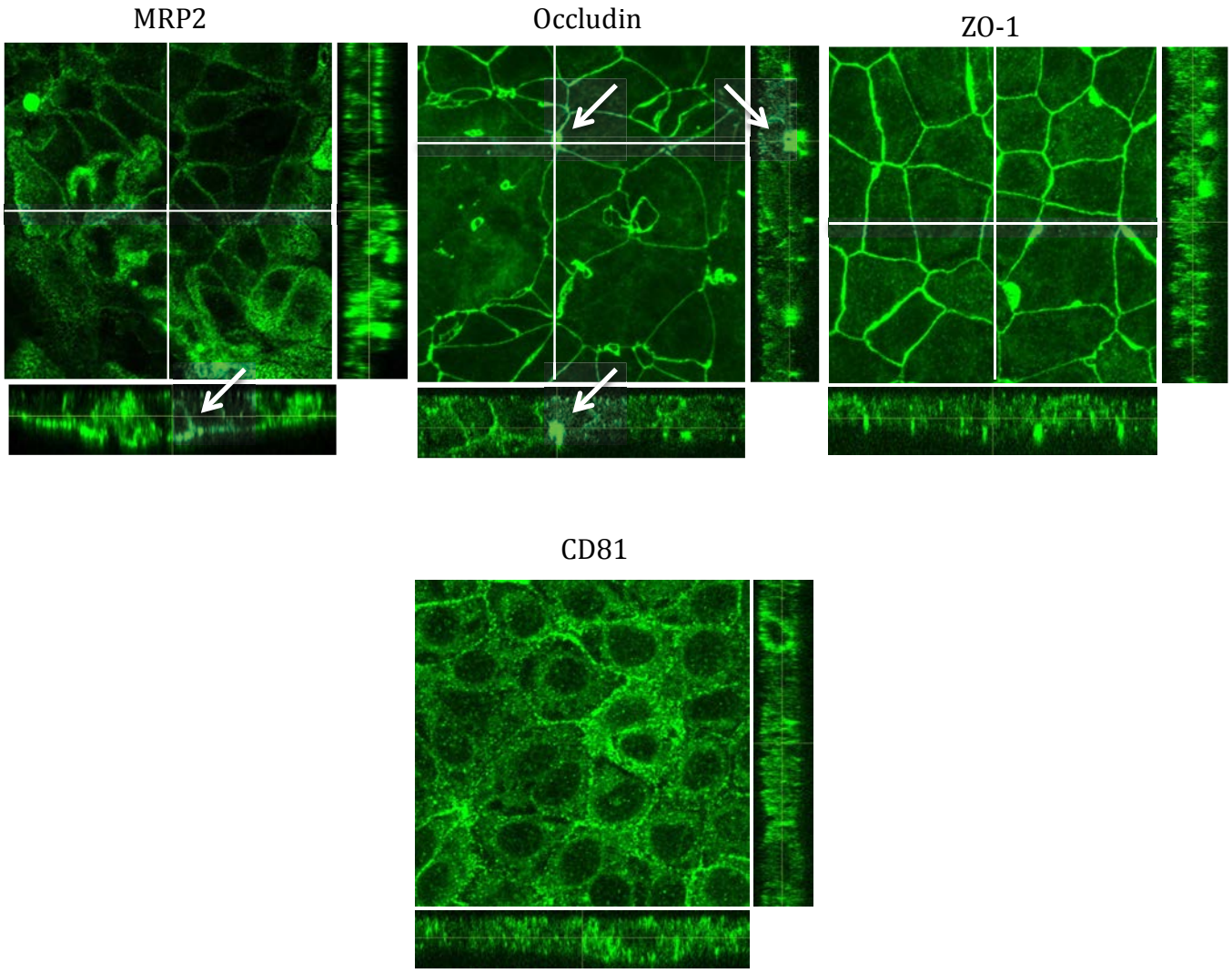


Figure 4.2 Tight junction proteins stain circular structures in Huh-7.5^{diff}
Huh-7.5 were plated onto glass coverslips and fixed at 14-21 days post treatment with 1.5% DMSO (vol/vol). Cells were stained for tight junction proteins MRP2, Occludin, ZO-1 and tetraspannin CD81. Images were taken using Zeiss (Dublin, CA) MetaHead microscope with a 100× oil immersion objective and presented as a z-stack image showing the apical and basolateral cell surfaces. Small circular structures between and within cells are marked with an arrow. Results are representative images of two independent experiments and duplicate wells.

Huh-7^{diff} Huh-7^{diff} + CMFDA

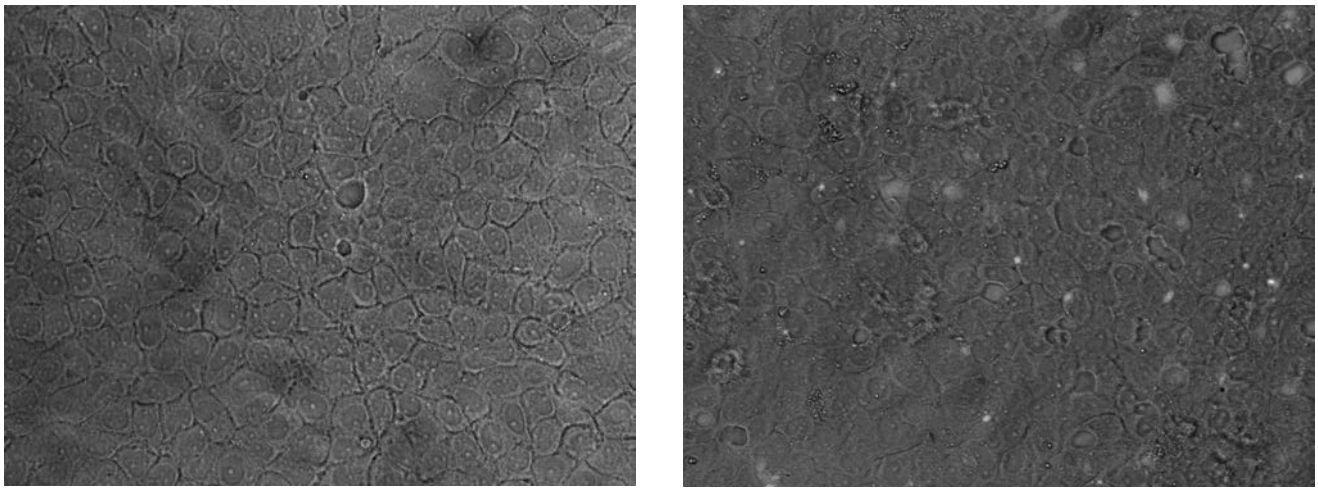


Figure 4.3 CMFDA accumulates in small round structures within and between cells.

Huh-7^{diff} at 7 days post differentiation were incubated with cell tracker green CMFDA (5-chloromethylfluorescein diacetate) prior to fluorescent images being taken (20x). CMFDA accumulates in areas where functional tight junctions are present between and within cells. Results are indicative of 3 independent experiments and duplicate wells.

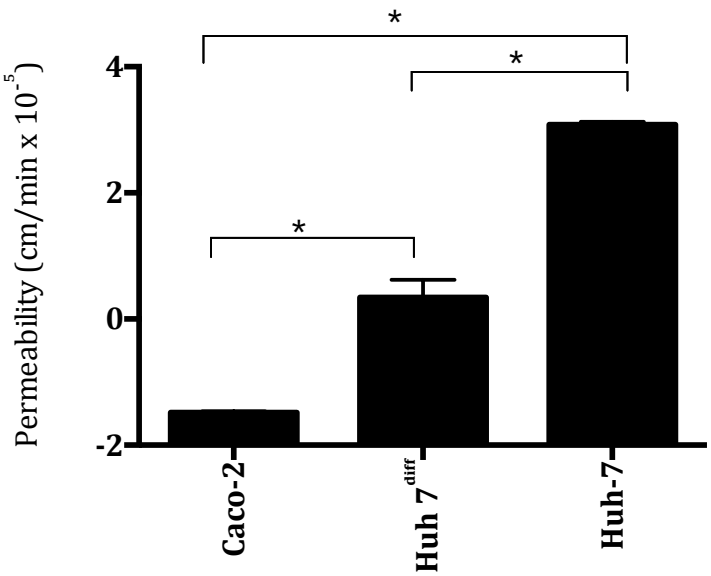


Figure 4.4 Differentiation alters Huh-7 para-cellular permeability
Huh-7/Caco-2 and Huh-7^{diff} were cultured on permeable filters as described. Para-cellular permeability to 70-kilodalton fluorescein isothiocyanate/dextran flux was measured. Results are representative of 2 independent experiments and duplicate wells. p=≤0.05 (Students t-test).

4.3: Differentiated cells do not restrict HCV entry

Results have shown that differentiated cells alter the localisation of receptors important for HCV entry into a permissive cell. We therefore hypothesised that differentiated cells may restrict viral entry resulting in reduced permissivity.

The HCV pseudoparticle system (HCVpp) was utilised to study viral entry kinetics. Differentiated or control cells were inoculated with H77 HCVpp and patient derived HCV strains (1a and 1b envelopes) and infection quantified by luminescence. We noted comparable rates of HCVpp entry regardless of the genotype of the pseudovirus used, suggesting that entry is not altered in these cells (**Figure 4.5** and **Table 4.1**). Given that differentiated cells appear to polarise, we utilised the ability of TNFα to disrupt tight junctions (Fletcher et al, 2014) and measured viral entry using a patient derived 1a envelope HCVpp. TNFα did not enhance viral entry and there was comparable entry between differentiated and control cells (**Figure 4.6**).

Despite change in HCV receptor expression and localisation in differentiated cells, there are comparable rates of viral entry, and tight junction perturbation did not alter this. Viral entry is not restricted in differentiated cells.

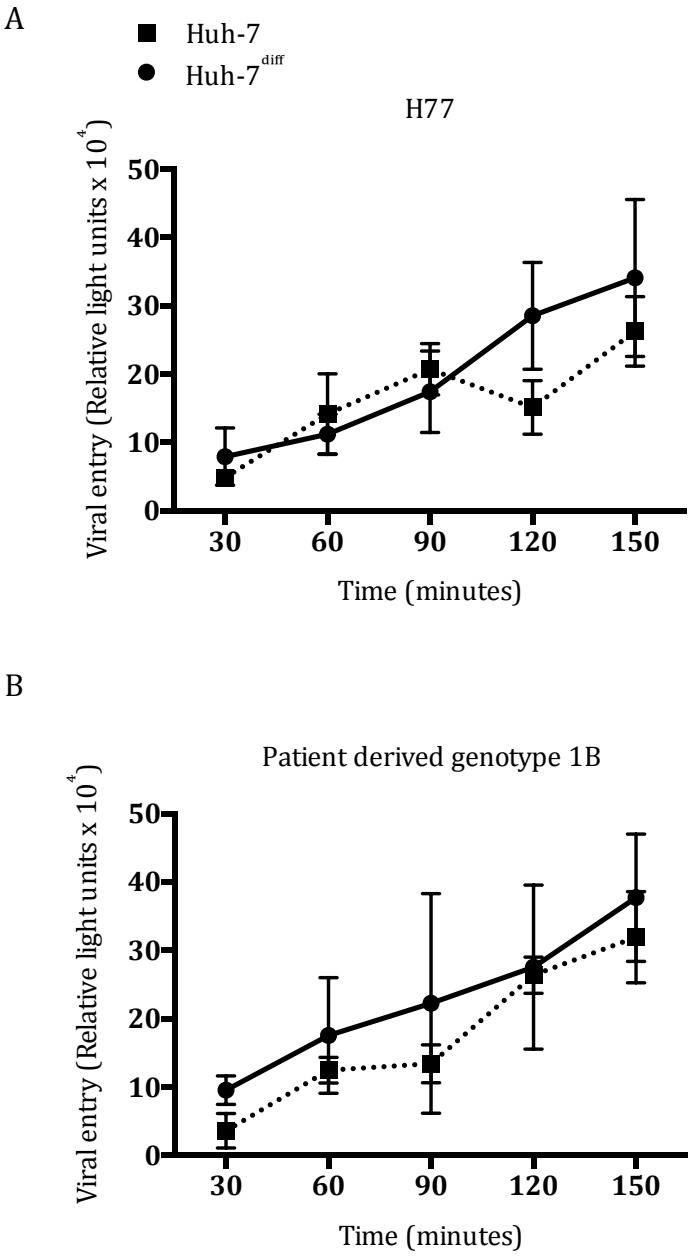


Figure 4.5 Viral entry is not affected by DMSO-differentiation

Huh-7^{diff} or Huh-7 cells of comparable density were infected with HCVpp expressing prototype laboratory strain H77 (A) or patient derived genotype 1B (B). Virus was removed at indicated time points post infection and cells incubated for 72 hours. Cells were lysed and luciferase activity measured by luminescence (Relative Light Units) minus 'No Envelope' control and corrected for cell number. Results represent the mean of triplicate wells and 4 independent experiments.

HCVpp	Huh-7 Rate entry (RLU x 10^4/min)	Huh-7^{diff} Rate entry (RLU x 10^4/min)	P value
H77pp	0.15 ± 0.05	0.23 ± 0.02	0.1
1A51002pp	0.09 ± 0.01	0.125 ± 0.02	0.24
1A80pp	0.07 ± 0.01	0.04 ± 0.01	0.23
1A46pp	0.17 ± 0.02	0.11 ± 0.01	0.6
1B51pp	0.23 ± 0.03	0.22 ± 0.02	0.9
1B46pp	0.04 ± 0.01	0.03 ± 0.01	0.8
1B49pp	0.13 ± 0.03	0.07 ± 0.02	0.4

Table 4.1 HCV entry rate was not affected by differentiation

Rate of viral entry for H77 and patient derived strains expressed as RLU/min. These were calculated from the slope of the line by linear regression and any differences analysed for significance by a two-way ANOVA. Results are expressed relative to uninfected controls minus 'No Envelope' values and corrected for cell number. Results represent the mean of triplicate wells and 4 independent experiments. p values = not significant.

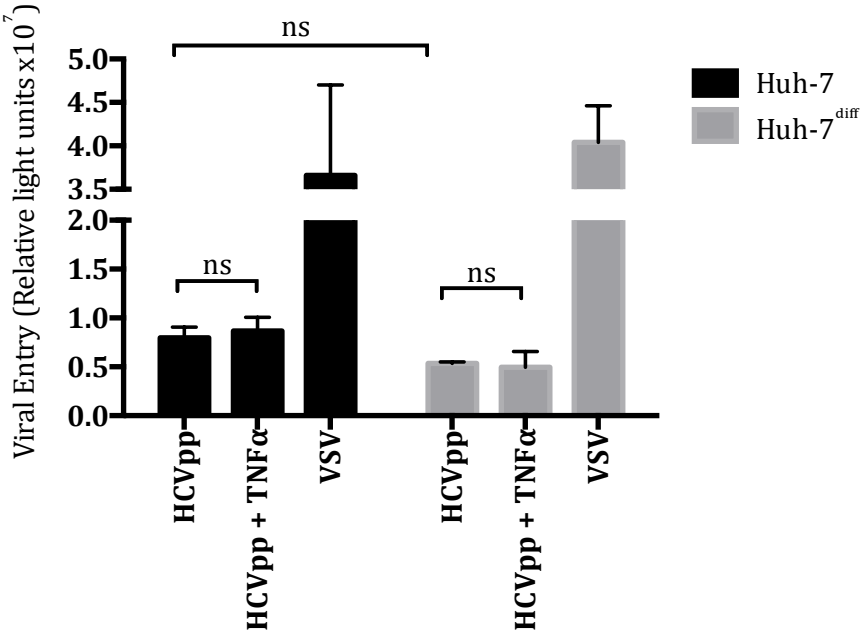


Figure 4.6 TNFα does not alter viral entry in differentiated cells

Huh-7^{diff} or Huh-7 cells of comparable density were infected with HCVpp expressing prototype laboratory strain H77. Duplicate wells were treated with TNFα (100ng/ml) for 1 hour prior to HCVpp infection. Cells were then incubated for 72 hours. Luciferase activity was measured by luminescence (Relative Light Units) minus 'No Envelope' control and corrected for cell number. Results represent the mean of triplicate wells and 2 independent experiments.

4.4: Differentiation of Huh-7 cells restricts HCV replication

Previous results have shown that restriction in permissivity in Huh-7^{diff} is not due to altered viral entry in differentiated cells. The next step was to assess subsequent steps in the HCV lifecycle in differentiated cells to try and identify the point at which viral replication and/or dissemination is altered.

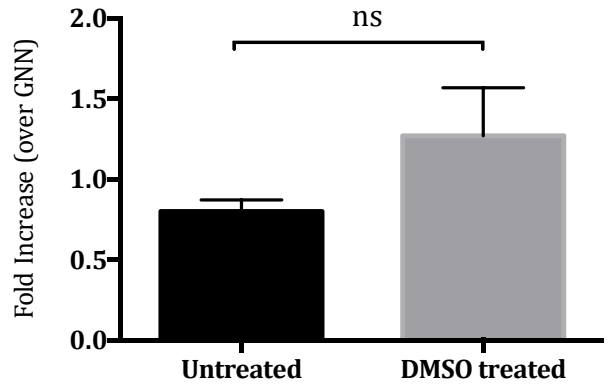
Viral translation and early replication was assessed by utilising the JC1gLuc reporter virus (Gottwein et al, 2011). JC1gLuc or the replicase-negative JC1gLuc-GNN RNA were transfected into Huh-7^{diff} or control cells and replication assessed by measuring secreted luciferase at indicated time points over the next 72h. Results show comparable transfection efficiency at 4h post-delivery (**Figure 4.7A**), however by 72h there was a 10-fold reduction in luciferase values in Huh-7^{diff} cells (**Figure 4.7 C** and **D**). The protease inhibitor VX950 was included to confirm authentic HCV RNA replication.

Replication is reduced in Huh-7^{diff} and a key cellular factor, miR122, is able to alter HCV replication. Initiation of replication of HCV is affected by miR122 expression, and we have previously shown that levels are increased with differentiation (**Figure 3.6**). Therefore, we assessed the effect of miR122 on HCV IRES driven translation. A firefly luciferase reporter RNA flanked by the HCV 5' and 3'UTRs (**Figure 4.8A**) was introduced into differentiated cells, with a miR122 inhibitor or oligonucleotide control (**Figure 4.8B**). Results indicate that differentiation decreases HCV replication in a miR122 independent manner.

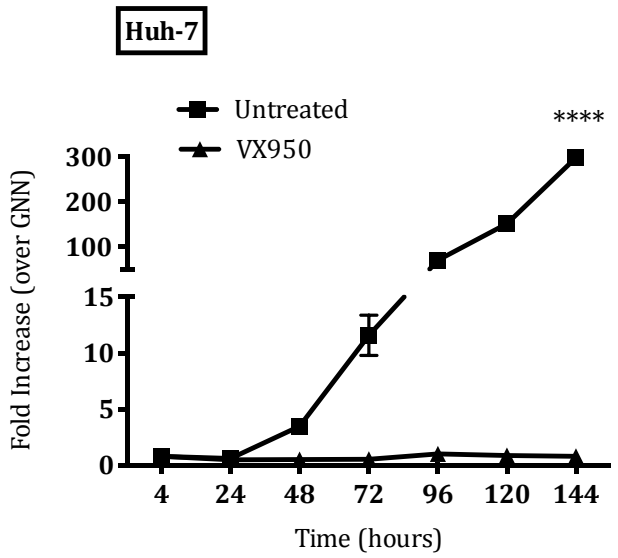
In order to test the impact of differentiation on established replication we used a replicon-based system. Huh-7 expressing full-length H77 replicon were treated with 1.5% DMSO for up to 14 days. HCV RNA copies were quantified by qPCR. Results indicate that differentiation has no impact on HCV RNA levels (**Figure 4.9**) in an established replicon line, suggesting, together with the gLuc results, that the defect is at the level of early replication.

These results indicate that differentiation limits HCV early replication but this is not dependent on miR122.

A


ns
2.0
1.5
1.0
0.5
0.0
Fold Increase (over GNN)
Untreated
DMSO treated

B


Huh-7
Untreated
VX950
****
300
200
100
15
10
5
0
Fold Increase (over GNN)
4
24
48
72
96
120
144
Time (hours)

C

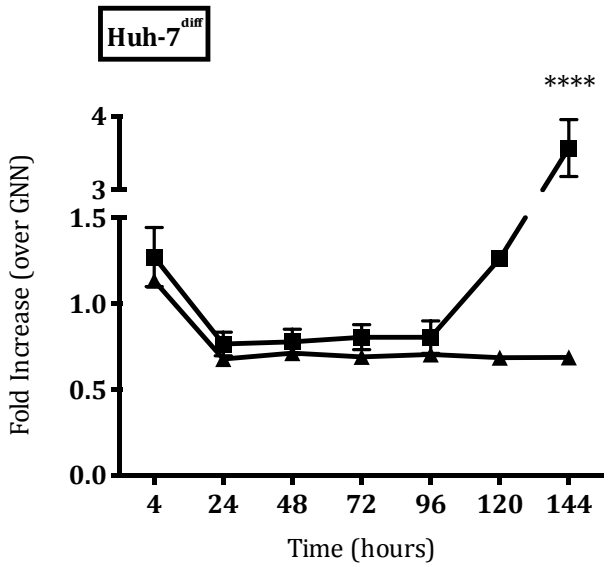
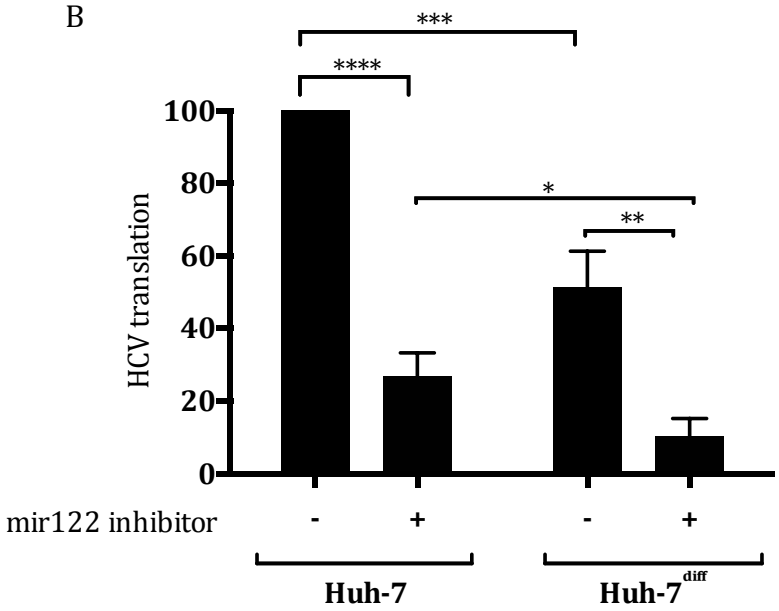

Huh-7diff
****
4
3
1.5
1.0
0.5
0.0
Fold Increase (over GNN)
4
24
48
72
96
120
144
Time (hours)

Figure 4.7 Huh-7^{diff} show a 10-fold reduction in initial HCV replication

Huh-7 or Huh-7^{diff} were plated at comparable densities and transfected with JC1gLuc HCV RNA for 8h, before transfection reagent was removed. (A) Supernatant was taken at 4 hours post transfection. The figure shows luciferase levels in the supernatant measured by luminescence in order to compare transfection efficiency. (B and C) Cells were treated with the protease inhibitor, VX950 (1ug/mL), or a DMSO control, and supernatant collected at indicated timepoints for Huh-7 (B) and Huh-7^{diff} (C). Replication was assessed by measuring luciferase in the supernatant by luminescence. Results are means and standard deviation of triplicate wells and represent 3 independent experiments. Differences between the gradients of the lines was assessed by a two-way ANOVA ****$p \leq 0.001$


A
IRES
5'
GCCAGCGGACACUCCACCAUGAAUCACUCC
CG
CG
CG
CG
CG
CG
U U
GA
miR122
seed match 1
miR122
seed match 2
G
AU
Firefly luciferase
HCV 3'UTR
5'LUC3' RNA


B
***
****
*
**
100
80
60
40
20
0
HCV translation
mir122 inhibitor
-
+
-
+
Huh-7
Huh-7diff

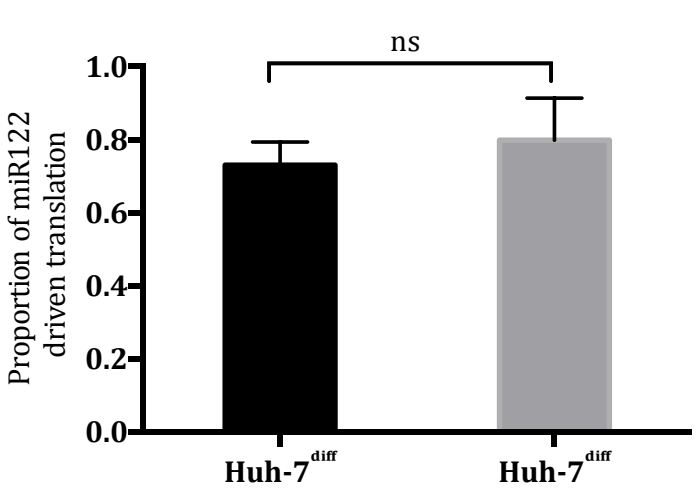

C
ns
1.0
0.8
0.6
0.4
0.2
0.0
Proportion of miR122
driven translation
Huh-7diff
Huh-7diff

Figure 4.8 Differentiation of Huh-7 cells reduces HCV RNA replication in a miR122 independent manner.

(A) A firefly luciferase reporter RNA flanked by the HCV 5' and 3'UTRs was introduced into Huh-7^{diff} or control, with either a control oligonucleotide or miR122 inhibitor. Cells were harvested at 6h post transfection. (B) Results show firefly luciferase activity relative to a Renilla luciferase transfection control as a percentage of the Huh-7 control level, showing a reduction in HCV IRES-driven translation with miR122 inhibition. (C) The proportion of total translation that is mir122 dependent is plotted and represents the difference between HCV translation in each cell type with or without the inhibitor. Data represent a mean and standard deviation of three independent experiments. ns = non significant. * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$ (Students t-test).

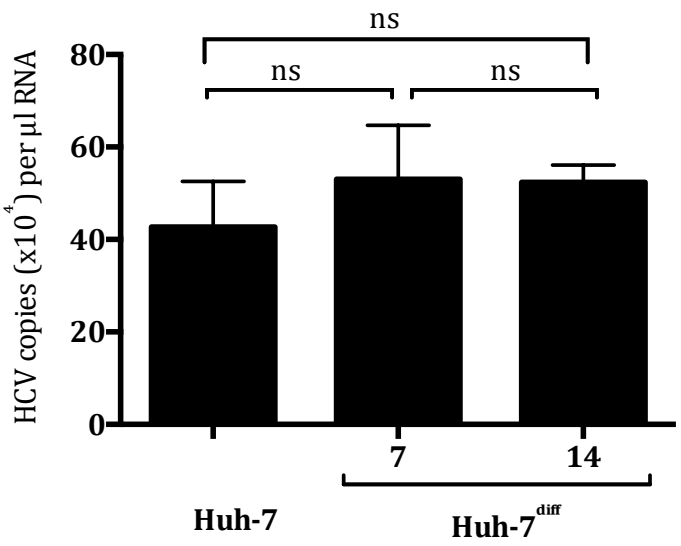


Figure 4.9 Differentiation does not impact established HCV replication

Huh-7 cells stably expressing full length H77 replicon were treated with 1.5% DMSO for 7-14 days. HCV RNA was quantified by qPCR and compared to untreated cells. Results represent means of triplicate wells in 3 independent experiments. ns = no significant difference (Students t-test).

4.5: Key cellular factors that alter HCV replication *in vivo* do not play a role in viral permissivity in Huh-7^{diff}

Differentiated Huh-7 cells are a more physiological system that becomes more 'hepatoctye-like' over time. Therefore, key cellular factors, that are altered by cellular differentiation and are known to impact HCV replication, were investigated.

The liver is a major regulator of iron metabolism and there is clinical and molecular evidence that iron can impact HCV replication (reviewed in Drakesmith and Prentice, 2008; Fillebeen et al, 2015). In addition to this known interaction between HCV and cellular iron, DMSO can alter hepatoma haem synthesis (Galbraith et al, 1986). Therefore, it was important to study the effect of DMSO on Huh-7 iron levels. A cell based ferrozine colorimetric assay was used to quantitate the amount of cellular iron within Huh-7^{diff} (Riemer et al, 2004). Results show that there was no appreciable induction of iron seen in DMSO treated cells (**Figure 4.10**). Given there were no changes in the amount of iron seen we did not go on to investigate the interaction between iron and HCV replication. Changes in cellular iron levels are unlikely to be playing a role in the reduced permissivity noted in Huh-7^{diff}.

Huh-7^{diff} upregulate key markers of gluconeogenesis (PEPCK and G-6-Pase) when grown in physiological amounts of glucose (**Figure 3.5**). Hepatocytes maintain glucose homeostasis. In relatively de-differentiated cells, such as those from hepatocellular carcinoma and in HCV-infected hepatocytes,

gluconeogenesis is downregulated and glycolytic pathways are increased (Koike, 2007; Ripoli et al, 2010). We therefore attempted to switch cells into a glycolytic state by reducing the amount of available glucose and assessing the effect on HCV replication. Cells were grown in reducing amounts of glucose (by diluting DMEM 4.5g/dl glucose with glucose free DMEM) and G-6-Pase levels measure by qPCR. G-6-Pase is the irreversible rate-limiting step for cells to generate new glucose. Results show G-6-Pase is downregulated in Huh-7^{diff} cultured in media with reduced amounts of glucose (**Figure 4.11A**) as a marker of cells metabolizing predominantly via glycolysis. Utilising LucA2 replicon cells to assess the effect of glycolysis on viral replication, cells were treated with DMSO for 7 days and then grown in reducing amounts of glucose. HCV replication was assessed by luciferase activity (**Figure 4.11B**). Similarly, Huh-7^{diff} infected with HCVcc after exposure to reducing concentrations of glucose (**Figure 4.11C**) showed a trend to reducing viral copies by qPCR although this was not significant. Importantly, altering metabolism in these cells failed to rescue the reduced permissivity in differentiated cells. Although glucose metabolism may play an important role *in vivo*, there was no direct effect of altering the metabolic profile of differentiated cells on HCV replication *in vitro*.

In addition to iron and glucose metabolism, Saeed et al (2015) have shown that SEC14L2 is an important cellular factor that allows hepatoma cells to support a wide range of non-JFH based genotypes. Cellular differentiation increases SEC14L2 mRNA expression (**Figure 3.8A**). We assessed whether overexpression of SEC14L2 altered HCV permissivity. Cells were infected with an early SA13 virus (containing no laboratory adaptation mutations) using Huh-7.5

clones overexpressing SEC14L2 (Witteveld et al, 2016). The level of SEC14L2 mRNA expression is outlined in **Figure 4.12A**. Multiple clones were selected in order to demonstrate a dose dependent effect and to control for off-target effects within a single clone. SEC14L2 overexpressing clones were not more permissive to HCVcc (SA13/JFH) (**Figure 4.12B**). Furthermore differentiated Huh-7.5^{diff} with an increased expression of SEC14L2 mRNA did not have an altered permissivity to HCVcc compared to parental Huh-7.5^{diff} (**Figure 4.12C**). Results show a non-significant difference in permissivity expressed as fold change in viral infectivity (FFU/ml) of undifferentiated cells compared to differentiated cells; Huh-7.5 show a mean 93 fold difference and JW cells show a mean 70 fold difference ($p=0.26$). In this experiment we used Huh7.5^{diff} rather than Huh-7^{diff} to allow direct comparison between the parental SEC14L2 over-expressing clones (parental cells Huh-7.5) and differentiated cells. Comparison between Huh-7 and Huh-7.5 shows a non-significant difference in the permissivity to HCVcc once DMSO differentiated (see **Figure 4.17**). We have concluded from these experiments that SEC14L2 expression does not alter viral permissivity in differentiated cells.

In summary, cellular factors such as iron and glucose metabolism, and the induction of SEC14L2, are not mediating the reduction in HCVcc permissivity seen in Huh-7^{diff}.

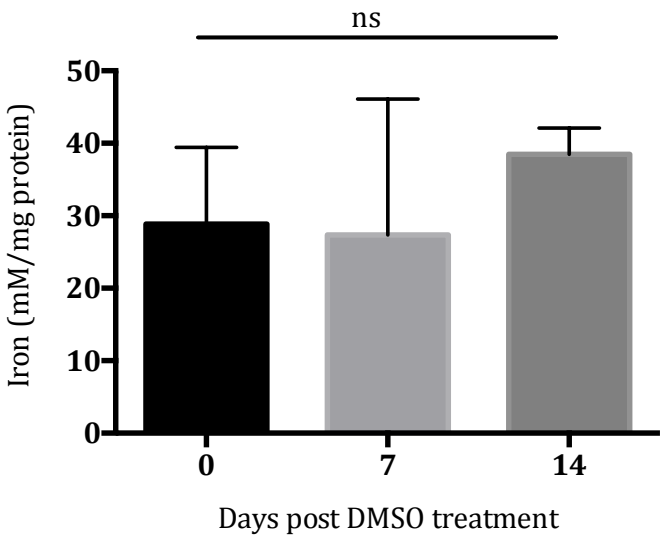
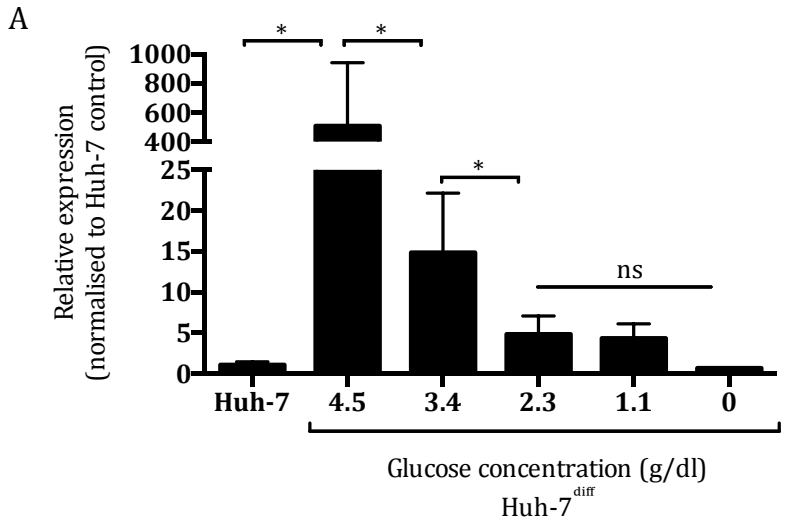
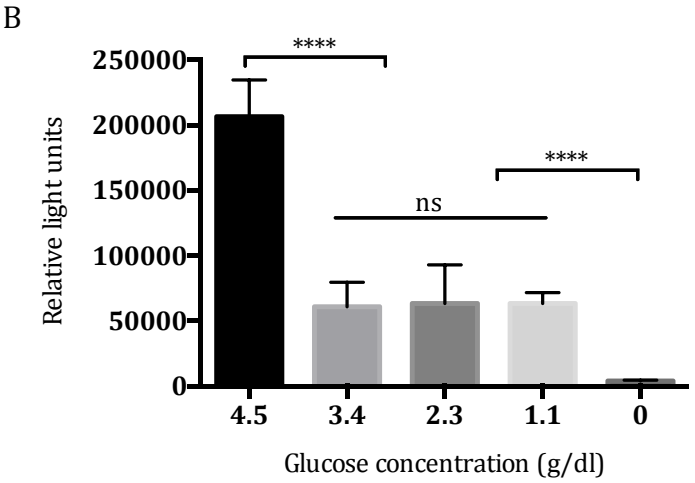


Figure 4.10 Differentiation of Huh-7 cells has a modest effect on iron expression.

Huh-7 cells were treated with DMSO for 7 and 14 days. Amount of iron relative to protein in the cell pellets was quantified using a colorimetric ferrozine based assay. Results are means of 4 wells and duplicate experiments. ns = not significant (Students t-test)


A
Relative expression
(normalised to Huh-7 control)
1000
800
600
400
25
20
15
10
5
0
*
*
*
ns
Huh-7
4.5
3.4
2.3
1.1
0
Glucose concentration (g/dl)
Huh-7diff


B
Relative light units
250000
200000
150000
100000
50000
0
****
ns
****
4.5
3.4
2.3
1.1
0
Glucose concentration (g/dl)

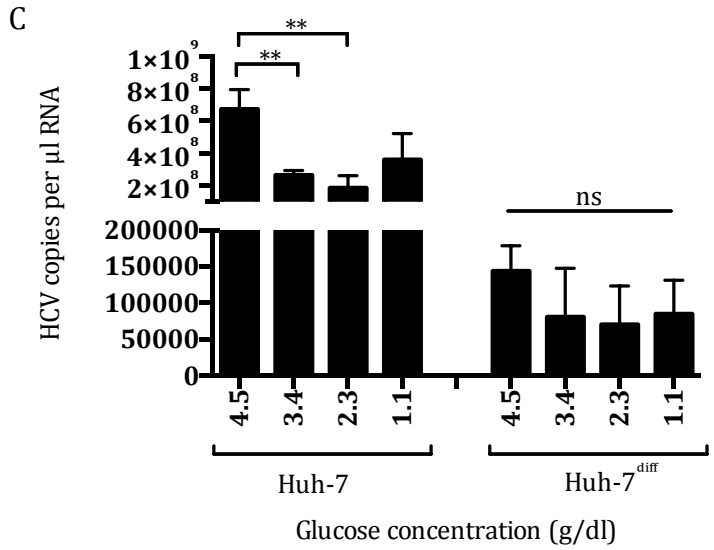
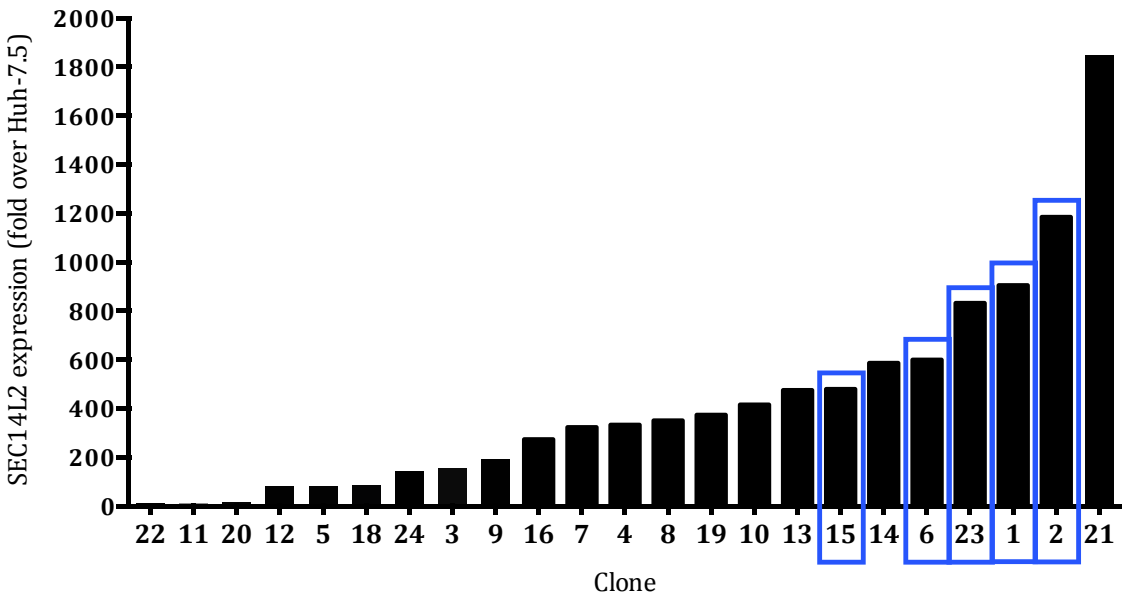

C
HCV copies per µl RNA
1×10^9
8×10^8
6×10^8
4×10^8
2×10^8
200000
150000
100000
50000
0
**
**
ns
4.5
3.4
2.3
1.1
4.5
3.4
2.3
1.1
Huh-7
Huh-7diff
Glucose concentration (g/dl)

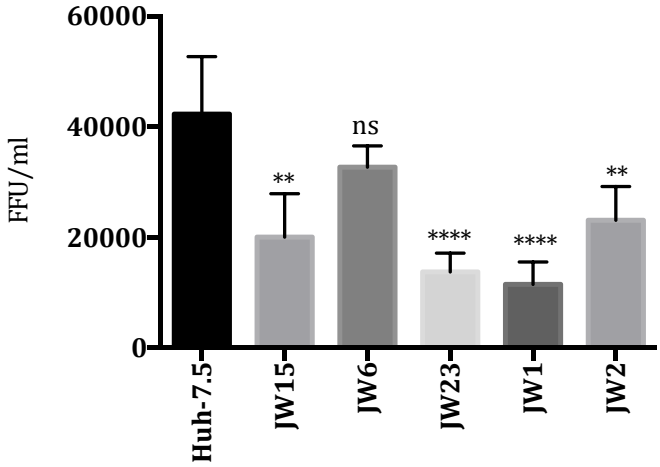
Figure 4.11 Changing glucose concentration does not alter HCV permissivity

(A) Huh-7^{diff} were cultured in DMEM containing variable amounts of glucose as indicated for 48 hours. Cells were lysed and G-6-Pase mRNA levels quantified by qRT PCR. (B) Huh-7 cells harbouring LucA2 subgenomic replicon were grown in 1.5% DMSO (vol/vol) for 7 days. Following this cells were cultured in DMEM containing variable amounts of glucose as indicated for 48 hours. Cell were lysed and luciferase signal quantified using a luminometer. (C) Huh-7^{diff} were infected with SA13/JFH(MOI 10) under conditions of reduced glucose for 48 hours. Cells were lysed and HCV copies quantified by qPCR as previously described. Results are representative of triplicate wells in duplicate experiments. ****= $p \leq 0.0001$ **= $p \leq 0.01$ *= $p \leq 0.05$ ns= not significant (Students t-test).

A


SEC14L2 expression (fold over Huh-7.5)
2000
1800
1600
1400
1200
1000
800
600
400
200
0
22 11 20 12 5 18 24 3 9 16 7 4 8 19 10 13 15 14 6 23 1 2 21
Clone

B


FFU/ml
60000
40000
20000
0
**
ns
****
****
**
Huh-7.5
JW15
JW6
JW23
JW1
JW2

C

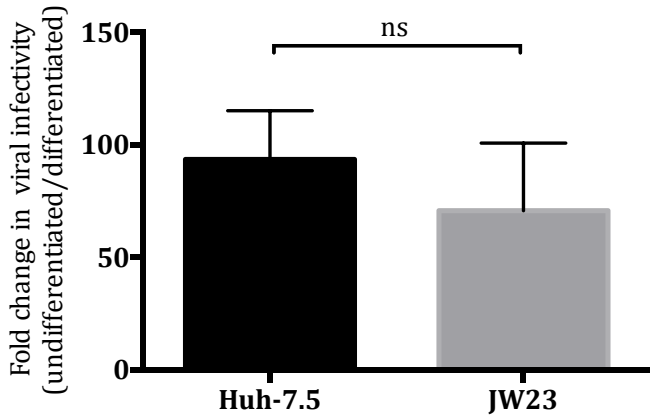

Fold change in viral infectivity (undifferentiated/differentiated)
150
100
50
0
ns
Huh-7.5
JW23

Figure 4.12 Overexpression of SEC14L2 does not restore permissivity of Huh-7.5^{diff}

(A) Huh-7.5 cells were transduced to over-express SEC14L2 by Jeroen Witteveld (University of Edinburgh) and selected clones for experiments are depicted. (B) Huh-7.5 and SEC14L2 overexpressing JW clones were infected with early passage HCV SA13/JFH. 48 hours post infection cells were fixed and stained for NS5A. Results are FFU/ml and are means of 3 wells in duplicate experiments. (C) Huh-7.5 and JW clone 23 cells were differentiated for 10 days as previously described and infected with HCVcc (early SA13/JFH MOI 10). 72 hours post infection cells were fixed, stained and NS5A positive foci counted. Viral infectivity represents FFU/ml and fold change was calculated by infectivity of undifferentiated cells/infectivity of differentiated cells. Results are means of 4 wells and represent two independent experiments ****$p \leq 0.0001$ **$p \leq 0.01$ *$p \leq 0.05$ ns= not significant (Students t-test).

4.6: Differentiated cells upregulate innate HCV sensors

One of the major factors defining human hepatocyte permissivity to HCV is innate cellular signalling. Huh-7 and derivatives support high level HCV replication and are known to have defects in innate signalling pathways (Sumpter et al, 2005) and do not produce interferons when infected with HCVcc. This allows permissivity to HCVcc in vitro, however, does not support the study of virus host immune interaction.

Primary human hepatocytes robustly upregulate an innate immune response when infected with HCV (Dill et al, 2012) and Huh-7^{diff} have been shown to express a broad range of ISGs in response to interferons *in vitro* (Bauhofer et al, 2012) suggesting that differentiation alters the anti-viral state of the cell. Furthermore, de-differentiated cells downregulate TLR3 (Wang e al, 2009; Li et al, 2005), losing the ability to recognise and respond to extracellular or endosomal HCV. To investigate the impact of differentiation on TLR3 expression Huh-7 and Huh-7.5 cells were differentiated for up to 10 days and TLR3 mRNA assessed by qPCR. Both Huh-7 and Huh-7.5 were included as they are known to have altered innate signalling pathways with Huh-7.5 having a single point mutation in RIG-I. Results show there was a robust upregulation of TLR3 mRNA over time, that was enhanced by ongoing differentiation (**Figure 4.13**) and this level was similar between Huh-7 and Huh-7.5.

Viral RNA is also recognised by RIG-I, which is known to be functionally present in Huh-7. RIG-I exists in various locations within the cell; mitochondrial membranes, peroxisomal membranes or the mitochondrial associated

membranes of the endoplasmic reticulum (Seth et al, 2005; Dixit et al, 2010; Horner et al, 2011). The location of the RIG-I engaged by dsRNA then signals a downstream cascade that results in numerous antiviral activities. RIG-I on peroxisomes, is known to induce widespread ISGs but not Type I interferon, consistent with the response seen in hepatoma cells to HCV (Odendall, et al, 2014). Interestingly, peroxisomal abundance increases during cellular differentiation and polarisation in intestinal cells (Odendall et al, 2014). We therefore assessed peroxisome abundance in Huh-7 during the process of differentiation using a surrogate marker Pex11β which is a master regulator of peroxisome proliferation (Odendall et al, 2014). Both Huh-7^{diff} and Huh-7.5^{diff} showed Pex11β mRNA increases over time (**Figure 4.14**), suggesting that differentiated hepatomas have increased peroxisome abundance. Although further investigation into localisation of RIG-I in these cells is required.

Despite possible changes in RIG-I localisation, we did not go on to fully investigate the role of differentiation status on function or downstream signalling of RIG-I in hepatoma cells. Permissivity of Huh-7^{diff} and Huh-7.5^{diff} is comparable, with both showing a significantly altered RNA replication compared to undifferentiated cells (**Figure 4.15**). Given the mutated RIG-I present in Huh-7.5, it is unlikely that RIG-I is key for restricting HCV replication and this was not investigated further.

Differentiated cells upregulate the innate sensor TLR-3 and potentially alter RIG-I localisation. Altered TLR-3 may be an important observation in the antiviral state of differentiated cells and requires further investigation.

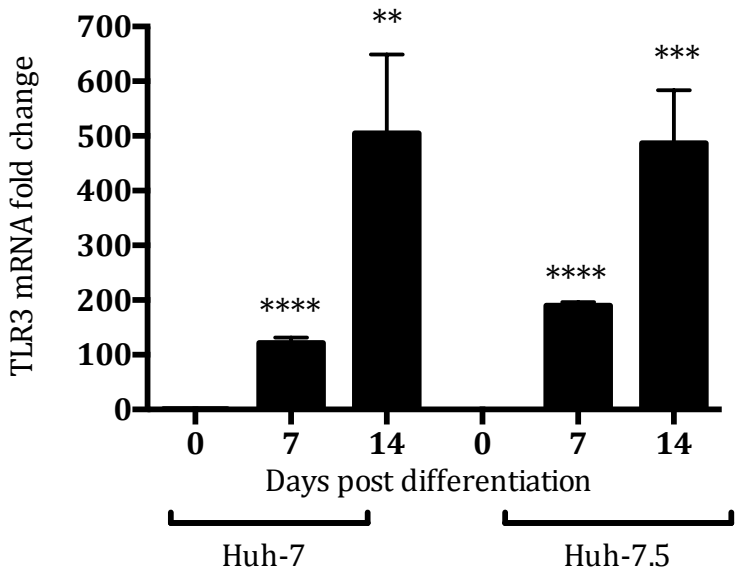


Figure 4.13 Differentiated hepatomas upregulate TLR3

Huh-7 and Huh-7.5 were treated with DMSO for up to 14 days, cellular RNA extracted and TLR3 quantified by qPCR. Fold induction in Huh-7.5^{diff} is plotted (over untreated controls). Results are means of triplicate wells and two independent experiments. Significant changes were analysed using the Students 't' test and compared to Day 0 cells. ****p≤000.1 *** p≤0.001**p≤0.01.

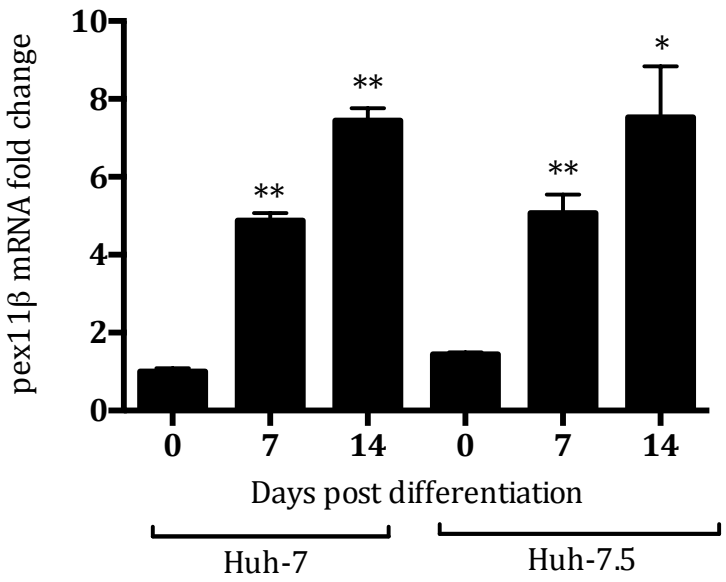


Figure 4.14 Differentiated hepatomas upregulate the peroxisome marker Pex-11β

(A) Huh-7 and Huh-7.5 were treated with DMSO for up to 14 days, cellular RNA extracted and Pex-11β quantified by qPCR. Raw delta ct values are plotted to allow direct comparison between quantification in Huh-7 and Huh-7.5. (B) Fold induction is plotted relative to untreated controls. Results are means of triplicate wells and two independent experiments. Significant differences in fold change were analysed using a students t-test and compared to Day 0 cells **p≤0.01 *p≤0.05.

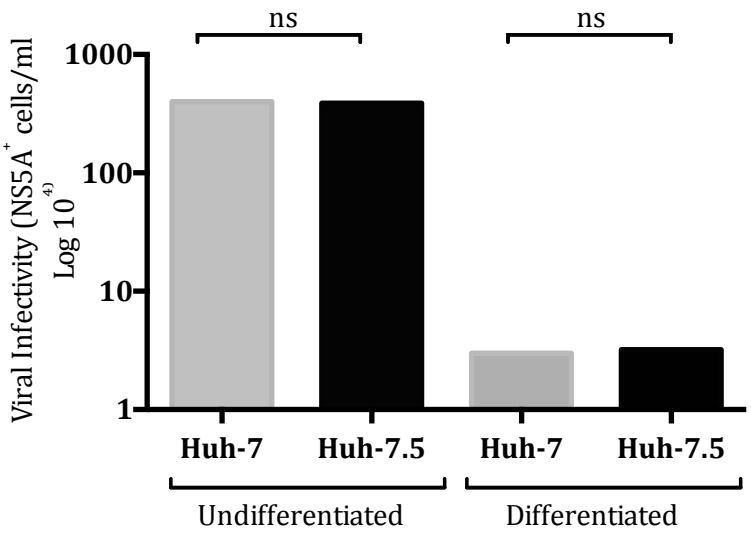


Figure 4.15. There is comparable viral permissivity between Huh-7^{diff} and Huh-7.5^{diff}

Relative infectivity was determined by titrating HCV SA13/JFH onto Huh-7^{diff} or Huh-7 cells. Infection was determined at 72h post-infection by immunofluorescence for NS5A expression or by qPCR for viral RNA. Results are expressed as the number of NS5A positive cells per ml of virus. Results are mean and standard deviation of triplicate wells and representative of 3 separate. Results were analysed using students t-test. ns= non significant

4.7: Differentiated cells exhibit an innate immune response to HCV infection

Results have shown that differentiated hepatoma cells have an increased abundance of TLR3 mRNA and are known to express a broad range of ISGs (Bauhofer et al, 2012). We investigated whether differentiated hepatoma cells respond to innate signalling by HCV and produce interferons.

The predominant interferon produced by primary human hepatocytes infected with HCV is IL-29 (IFN λ1) and this exerts an anti-viral effect in vitro (Park et al, 2012; Marukian et al, 2011). This response is not restricted to primary human hepatocytes, as HepG2 cells also produce Interferon λ in response to HCV which attenuates replication (Israelow et al, 2014). Mouse derived liver cells were able to produce both type 1 (IFN α) and type 3 (IFN λ) interferon when transfected with a HCV subgenomic replicon, which led to abrogation of infection (Anggakusuma et al, 2015). We investigated whether Huh-7^{diff} were able to produce IL29 (IFN λ1) and IFNα in response to HCV or synthetic analogues.

The early innate response of Huh-7^{diff} (6 hours) to stimulators of TLR3, RIG-I and MDA-5 was investigated. PolyIC was used to mimic dsRNA both transfected into the cell (RIG-I, MDA-5) and delivered extracellularly (TLR3). Specific viral activators of RIG-I and MDA5 were also used; Sendai virus is a RIG-I activator (Kell and Gale, 2015) and Encephalomyocarditis virus (EMCV) a specific MDA-5 activator (Rodriguez et al, 2014). Results show that there was no

IFNα mRNA detectable in either cell type. However, Huh-7^{diff} increased IFNλ mRNA in response to both extracellular PolyIC (TLR3) and transfected PolyIC (RIG-I) and EMCV (MDA-5) (**Figure 4.16**). Results confirm that innate signalling pathways via TLR3, RIG-I and MDA5 are active in differentiated cells and upregulate IL29 mRNA in response to ligand activation.

Previous authors have shown that the innate response in human fetal liver cells to HCV is maximal at 24 hours post stimulation (Park et al, 2012). We confirmed this, showing that the IFNλ mRNA level was greatest at 24 hours post stimulation both to PolyIC and to transfected HCV RNA (J6/JFH) (**Figure 4.17A**). We also looked at Mx1, a key ISG, which is induced within 6 hours of HCV infection in the livers of chimpanzees (Park et al, 2012). Results show comparable data, with Huh-7^{diff} showing an early Mx1 response (**Figure 4.17B**). There was minimal IFNα mRNA at 24 hours, confirming the predominance of Type 3 signalling in Huh-7^{diff} (**Figure 4.17C**) and an antiviral state within the cells.

Given the significant upregulation of IL29 mRNA we went on to investigate whether this was translated into IL29 protein. HepG2 cells were used as a positive control, as they are known to be responsive to PolyIC, producing and secreting detectable extracellular IL29 protein 24 hours post stimulation (Israelow et al, 2014). **Figure 4.18A** shows that despite significant mRNA levels, there is no detectable IL29 protein produced after PolyIC or HCV RNA transfection in Huh-7^{diff}. IL29 is known to have paracrine effects when secreted,

however it also has autocrine effects (Bruening et al, 2017) and may act locally without significant amounts being secreted into the media. Similarly, another closely related interferon, IFNλ4 is produced in response to HCV infection, in HepG2 and primary human hepatocytes, however is partially retained within the cytoplasm (Onabajo et al, 2015). Therefore we tested for the presence of IL29 intracellularly by stimulating the cells as previously, and lysing cells at 24 hours, looking for evidence of IL29 protein. Detectable IL29 was present in the lysed cells, suggesting that Huh-7^{diff} can produce protein in response to RIGI/MDA5 stimulation, although this was at a very low level (**Figure 4.18B**). In contrast to HepG2 cells, Huh-7^{diff} do not secrete significant amounts of protein. Furthermore, supernatant from Huh-7^{diff} stimulated with PolyIC and HCV RNA did not impact upon established HCV replication (**Figure 4.18C**), suggesting that there were not antiviral mediators secreted in the media.

Despite the robust upregulation of IL29 mRNA with synthetic stimulators a key questions was whether Huh-7^{diff} would respond to HCVcc in the same way, confirming their physiological response to infection compared to Huh-7. Huh-7^{diff} were infected with HCVcc (SA13/JFH) at high MOI and IL29 mRNA measured at 24, 48 and 72 hours post infection. Interestingly, Huh-7^{diff} showed detectable levels of IL29 mRNA, which was completely abrogated with the addition of VX950 (**Figure 4.19**).

In summary, we have shown that Huh-7^{diff} exhibit a robust innate immune response to HCV infection that was associated with an increase in IL29 and Mx1 levels in a pattern consistent with that found in primary human hepatocytes and

HepG2 cells. However, cells failed to secrete measurable IL29 protein, even though small amounts were found intracellularly, and supernatant was not able to inhibit established HCV replication.

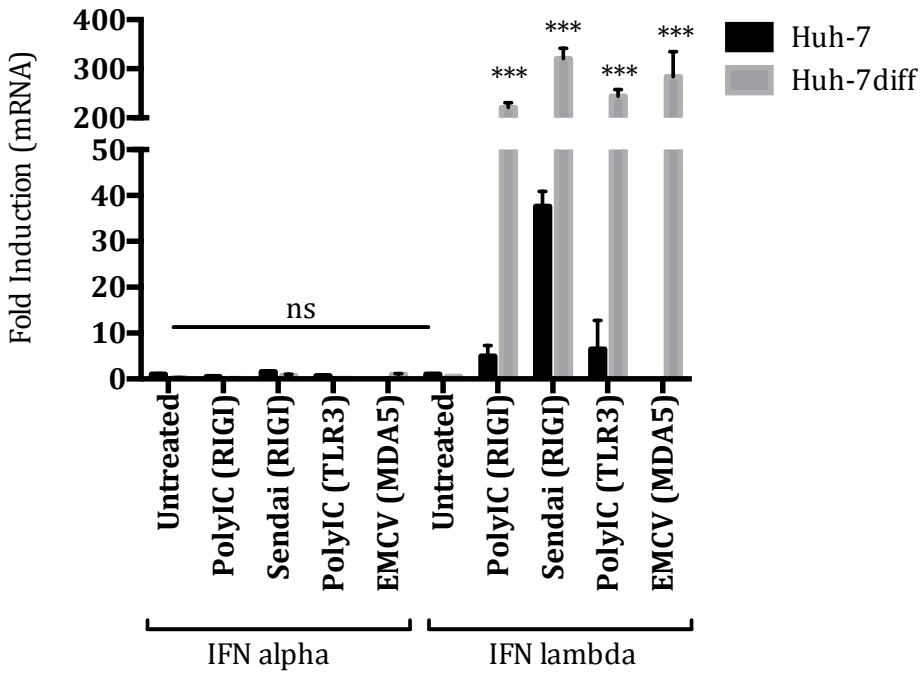


Figure 4.16 Differentiated cells upregulate Interferon lambda mRNA.

Huh-7 and Huh-7^{diff} were infected with Sendai virus, EMCV or transfected with PolyIC (LMW) at 1mcg/well. Additionally PolyIC was added to the media (1mcg/well) in duplicate wells. Cells were lysed and mRNA for IFNα (IFNA1) and IFNλ (IL29) were quantified by qPCR. Results are expressed as fold induction over untreated cells, means of triplicate wells and representative of three independent experiments. Analysis was by students t-test and compared to undifferentiated Huh-7 *** $p \leq 0.001$.

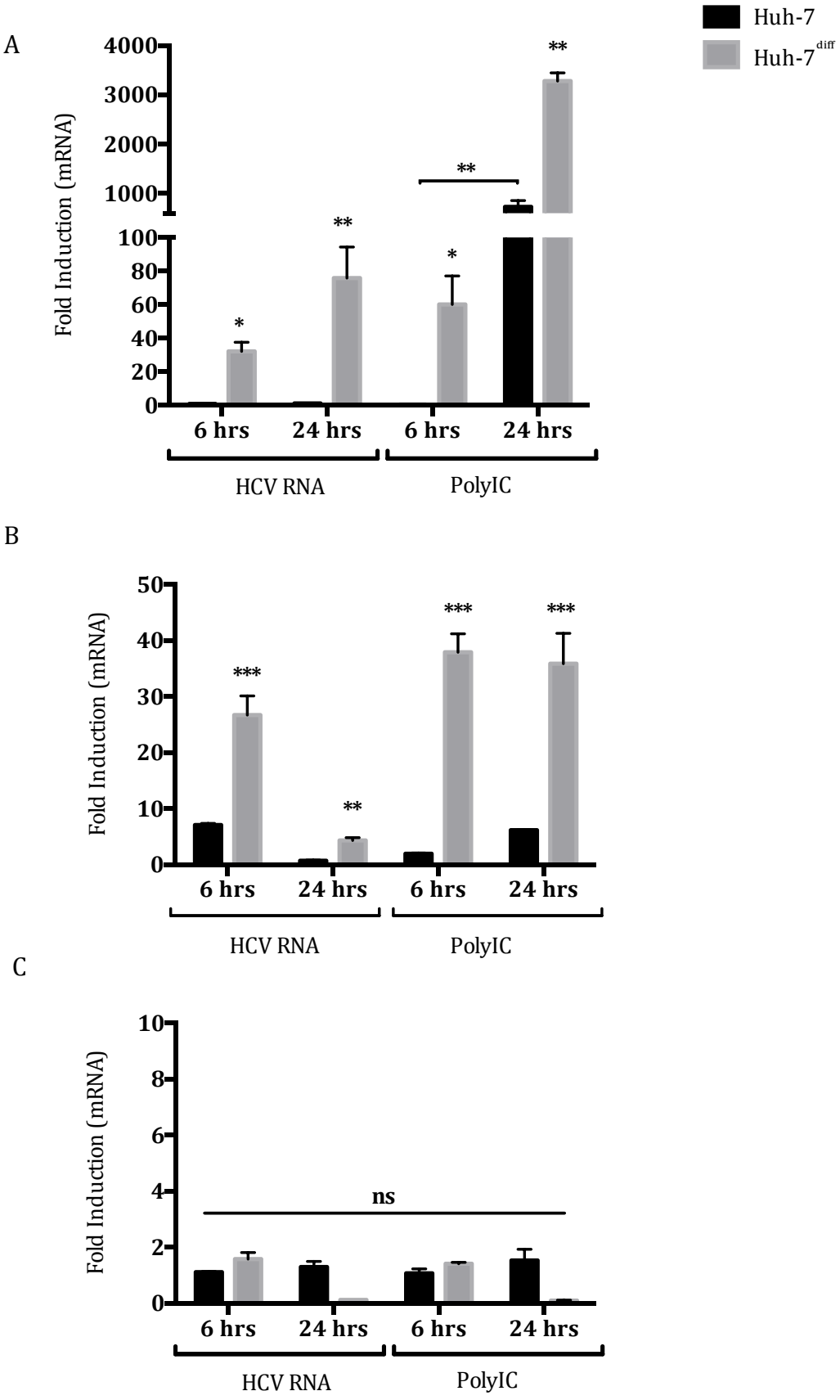
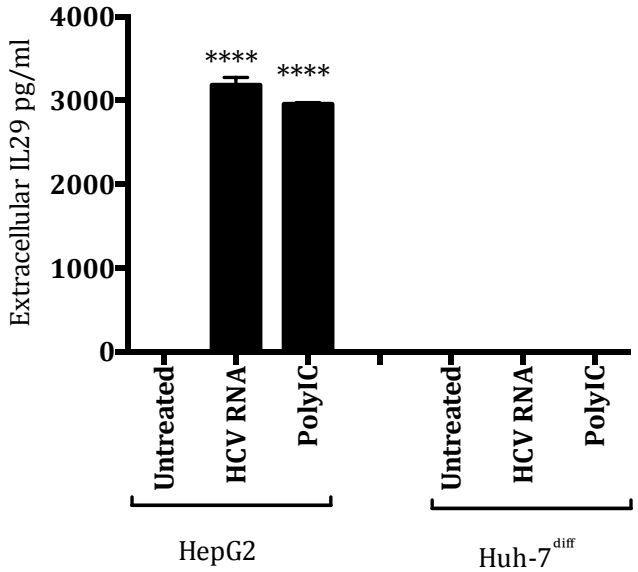

A
Huh-7
Huh-7diff
Fold Induction (mRNA)
4000
3000
2000
1000
100
80
60
40
20
0
*
**
*
**
**
6 hrs
24 hrs
6 hrs
24 hrs
HCV RNA
PolyIC
B
Fold Induction (mRNA)
50
40
30
20
10
0
***
**
***
***
6 hrs
24 hrs
6 hrs
24 hrs
HCV RNA
PolyIC
C
Fold Induction (mRNA)
10
8
6
4
2
0
ns
6 hrs
24 hrs
6 hrs
24 hrs
HCV RNA
PolyIC

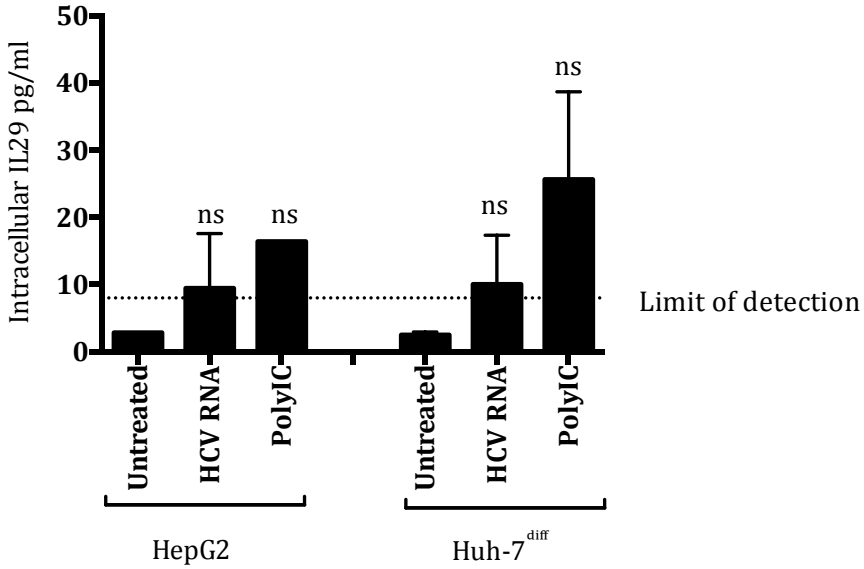
Figure 4.17 An Type 3 interferon response is seen in differentiated cells in response to RLR stimulation

Huh-7 and Huh-7^{diff} were transfected with HCV RNA (J6/JFH) and PolyIC (LMW). Cells were lysed at 6 or 24 hours post transfection. IL29 (A), Mx1 (B) and INFα1(C) mRNA quantified by qPCR. Results are means of duplicate wells (IFN) or triplicate wells (Mx1) and representative of three independent experiments. Results were analysed by students t-test and compared to undifferentiated cells unless indicated *** $p \leq 0.001$ ** $p \leq 0.01$ * $p \leq 0.05$ ns= not significant.

A


Extracellular IL29 pg/ml
4000
3000
2000
1000
0
****
****
Untreated
HCV RNA
PolyIC
Untreated
HCV RNA
PolyIC
HepG2
Huh-7diff

B


Intracellular IL29 pg/ml
50
40
30
20
10
0
ns
ns
ns
ns
Limit of detection
Untreated
HCV RNA
PolyIC
Untreated
HCV RNA
PolyIC
HepG2
Huh-7diff

C

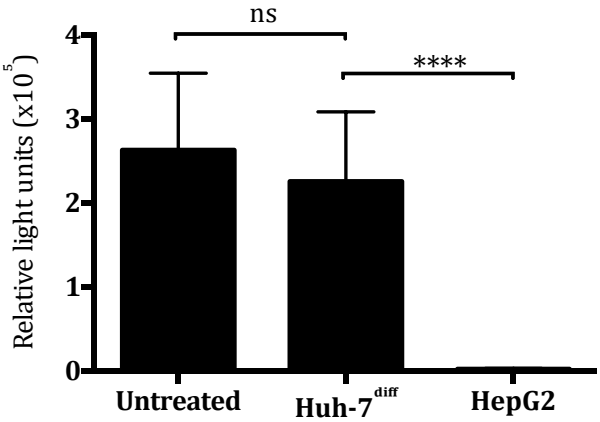

Relative light units (x10^5)
4
3
2
1
0
ns
****
Untreated
Huh-7diff
HepG2

Figure 4.18 RIG-I stimulation results in very low levels of IL29 protein in differentiated cells.

Huh-7^{diff} and HepG2 control were transfected with J6/JFH and PolyIC (LMW). (A) 24 hours post transfection cells supernatant was removed and IL29 was quantified by ELISA. (B) Cells were trypsinised and freeze thawed (3 times) in 200 µl media to release intracellular IL29. Protein was quantified by ELISA according to manufacturers instructions using a standard curve (lower limit of sensitivity 8pcg/ml indicated by dotted line). Results are means of triplicate wells and representative of 4 independent experiments. (C) Supernatant was added (1:1) to LucA2 replicons. 48 hours later cells were lysed and replication quantified by luciferase. Results were analysed by students t-test and compared to untreated cells ****p≤0.0001 ns=non significant.

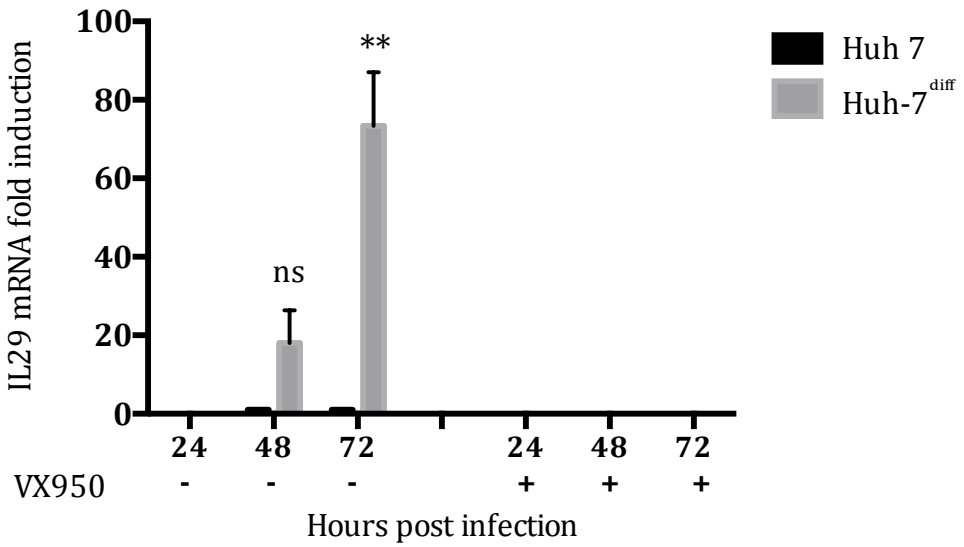


Figure 4.19 HCV infection can trigger an innate response in Huh-7^{diff}

Huh-7^{diff} were infected with HCVcc at MOI 10, with duplicate wells being treated with VX950 (1mcg/ml). At indicated time points post infection cells were lysed and IL29 mRNA levels quantified by qPCR. Results are IL29 fold induction over baseline (24 hours Huh-7^{diff}) and are means of two independent experiments and triplicate wells. Results were compared to 24 hours Huh-7^{diff} and analysed by students t-test **p≤0.01 ns= not significant.

4.8: Differentiation of Huh-7 cells limits the infectivity of secreted extracellular HCV particles

Differentiation of Huh-7 cells results in reduced HCV RNA replication and translation. There is upregulation of innate signalling within cells, however results have not shown that this has a significant impact on viral replication. The next steps in the viral lifecycle were investigated in order to understand how HCV replicates and disseminates in a differentiated host.

Huh-7^{diff} or non-differentiated cells were infected with HCV SA13/JFH at an equivalent MOI and after 72h HCV RNA and infectious virus measured. The results showed comparable levels of intracellular and extracellular HCV RNA expressed in Huh-7^{diff} and non-differentiated cells (**Figure 4.20A**), suggesting that virus release is not impaired. However there was a 10-fold reduction in the infectivity of extracellular virus collected from Huh-7^{diff} cells (**Figure 4.20B**), suggesting a loss of particle infectivity. We have previously shown that DMSO does not have an effect on the viral particle and therefore loss of infectivity is due to the differentiation process and not DMSO itself (**Figure 3.13**). Similarly, differentiation of cells using other methods (personal communication, N Frampton) results in a loss of permissivity, suggesting this is due to the differentiation process itself (**Figure 4.21**) rather than an off target effect of DMSO.

The reduction in specific infectivity of released virus suggests that there is likely to be a difference in viral spread. As HCV can spread via cell-to-cell and

cell-free routes (Brimacombe et al, 2011), we utilised HCV neutralizing antibodies that prevent cell-free infection to investigate whether differentiated cells support different viral transmission routes. Huh-7^{diff} or Huh-7 cells were infected with HCV SA13/JFH at an equivalent MOI in the presence of anti-HCV IgG or control IgG, to measure viral dissemination via cell-cell or cell-free routes over a 72h period. HCV infected 90% of the non-differentiated Huh-7 cells by 72h that was largely explained via cell-free infection (**Figure 4.22A**). In contrast, there were minimal new infection events in Huh-7^{diff} cells and the majority occurred via cell-cell transmission (**Figure 4.22B**). Given that new viral transmission events can be masked by cell division (representing infected cell division rather than true de-novo infection) we corrected for cell division in Huh-7 cells. Meredith et al (2013) showed that Huh-7 cells double in number every 24 hours, whereas Huh-7^{diff} are cell cycle arrested and do not alter cell number over time. Therefore we were able to estimate the number of cells infected by cell division over 2, 3 and 4 days post infection. We normalised this, so infection events were representative of de-novo infection and not cell division. When Huh-7 cell division is taken into account (**Figure 4.22C**), we observed comparable rates of estimated cell-cell HCV transmission suggesting that the reduced viral spread in Huh-7^{diff} cells is explained by the limited infectivity of extracellular virus.

In summary results show that virus released from differentiated cells has a 10 fold reduction in specific infectivity. Cell spread is reduced which is likely due to limited infectivity of extracellular virus and suggests that cell-to-cell spread may be equally as efficient in Huh-7^{diff} as standard hepatoma cells.

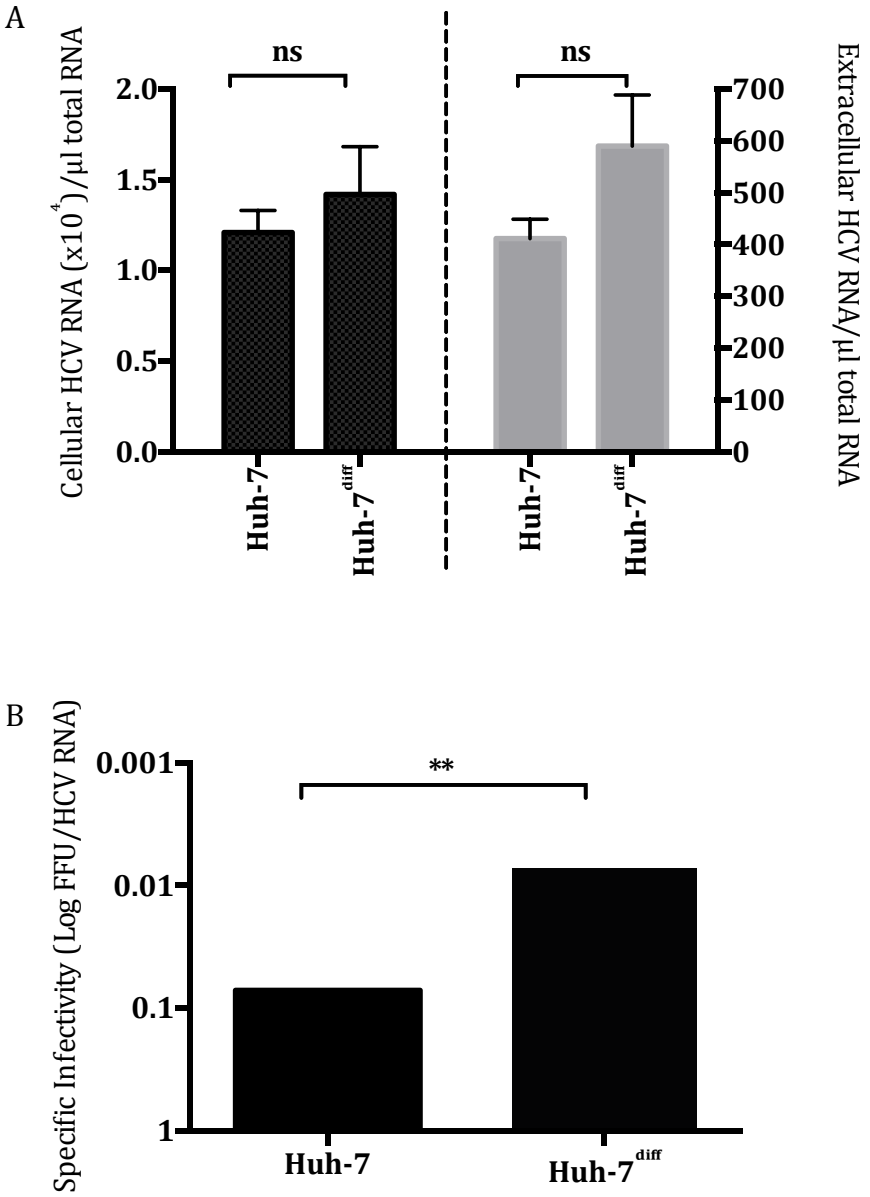


Figure 4.20 Virus released from differentiation has reduced specific infectivity

(A) Huh-7^{diff} or Huh-7 of comparable density were infected with SA13/JFH with an MOI sufficient to achieve an equivalent number of infected cells (10 and 0.1 respectively) at 48h post infection. Intracellular and extracellular virus was then quantified by RT-qPCR and results plotted as HCV RNA per microlitre of loaded total RNA. (B) Extracellular virus was then used to infect Huh-7 and the specific infectivity determined by calculating the number of HCV RNA infecting the target cells (FFU/HCV RNA). Results were anlaysed using the students t-test **p≤0.01 ns= not significant.

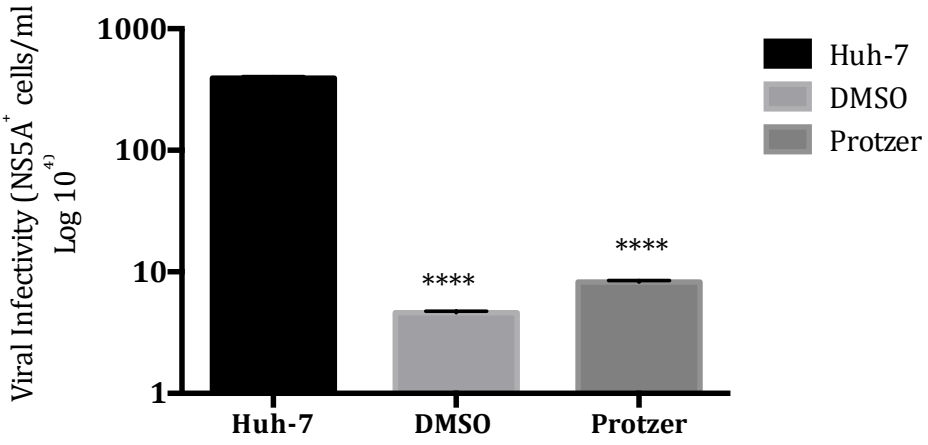
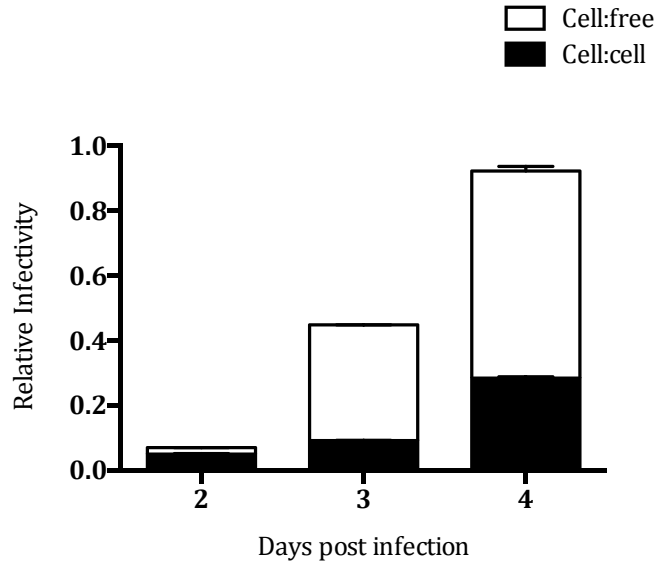


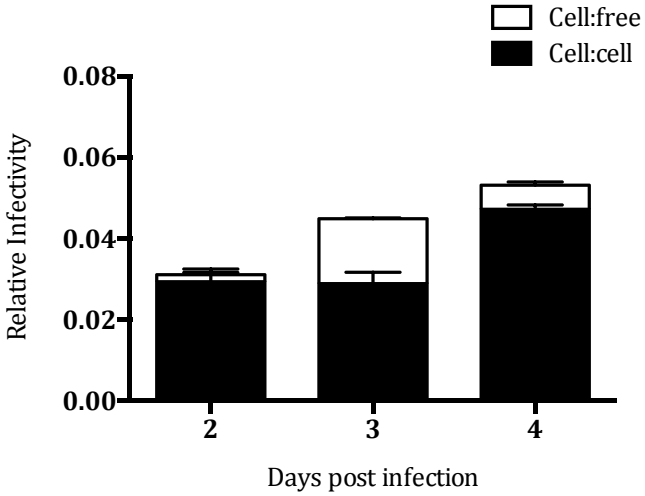
Figure 4.21 Differentiated cells show reduced permissivity to HCVcc infection

Huh-7 were differentiated as previously with DMSO for 10 days. Huh-7 in duplicate plates were differentiated using the Protzer method and differentiation status was confirmed by N Frampton. Cells were infected with HCVcc (SA13/JFH). 72 hours later cells were stained and NS5A positive foci counted. Results are NS5A positive cells per ml of inoculating virus and are triplicate wells and representative of two independent experiments. Results were analysed using students t-test and compared to Huh-7 untreated cells. ****$p \leq 0.0001$.

A


Cell:free
Cell:cell
Relative Infectivity
1.0
0.8
0.6
0.4
0.2
0.0
2
3
4
Days post infection

B


Cell:free
Cell:cell
Relative Infectivity
0.08
0.06
0.04
0.02
0.00
2
3
4
Days post infection

C

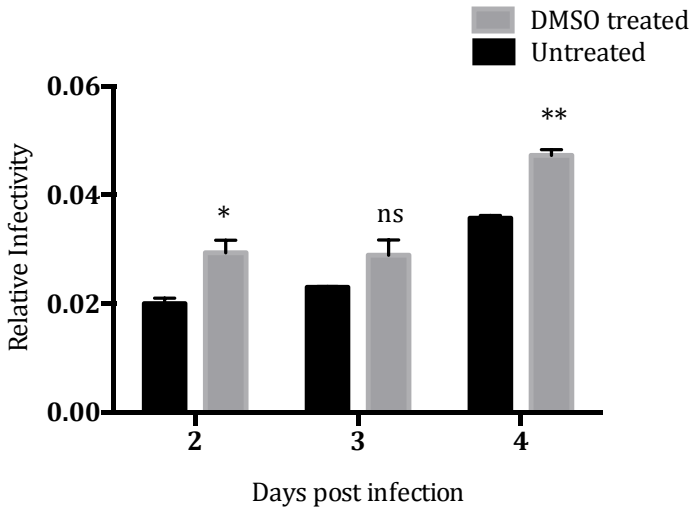

DMSO treated
Untreated
Relative Infectivity
0.06
0.04
0.02
0.00
*
ns
**
2
3
4
Days post infection

Figure 4.22 Huh-7^{diff} support minimal amounts of cell free viral spread

Huh-7(A) and Huh-7^{diff} (B) were infected with SA13/JFH at an equivalent MOI. 24h post infection, anti-HCV or control IgG was added to inhibit cell-free infection. Cells were fixed and stained for NS5A expression at indicated timepoints, and quantified by flow cytometry. Results are represented as relative infectivity with number of infected cells as a proportion of total cells in culture. (C) Spread was then corrected for cell division, allowing for doubling of cell number in the Huh-7 cells every 24 hours resulting in direct comparison of estimated cell-cell viral spread with Huh-7^{diff}. Results are analysed using students t-test ** $p \leq 0.01$ * $p \leq 0.05$ ns= not significant

4.9: The defect in permissivity can be overcome by cell delivered virus

Previous results showed that HCV has limited cell free viral spread in the differentiated system. However, cell-to-cell spread appeared comparable. Therefore we investigated the role of cell-cell viral delivery in Huh-7^{diff}.

We utilised a previously published co-culture assay where infected Huh-7 cells (HCV SA13/JFH and J6) were labelled with CMFDA and co-cultured with naïve Huh-7^{diff} or non-differentiated targets cells (**Figure 4.23**) (Meredith et al, 2013; Xiao et al, 2014). Co-cultures were incubated for 1 hour to allow cell contacts to form before adding anti-HCV IgG or control IgG to neutralize extracellular infectious particles. Co-cultures were incubated for a further 72 hours and the frequency of newly infected target cells normalized to the number of infected producer cells (**Figure 4.24**). Comparable frequencies of HCV infected Huh-7^{diff} and non-differentiated cells were observed for both viral strains. Given the upregulation of innate IL29 responses in differentiated cells, we investigated whether cell-to-cell delivery of virus altered IL29 mRNA. **Figure 4.25** shows that IL29 levels are higher when cells are infected using cell-to-cell transmission, consistent with the higher levels of infection in these cells, making it less likely that these virus delivered in this manner evades innate immune signalling.

In summary HCV delivered cell–to-cell can overcome the reduction in permissivity associated with cell free viral spread in differentiated cells, despite a robust innate response in these cells.

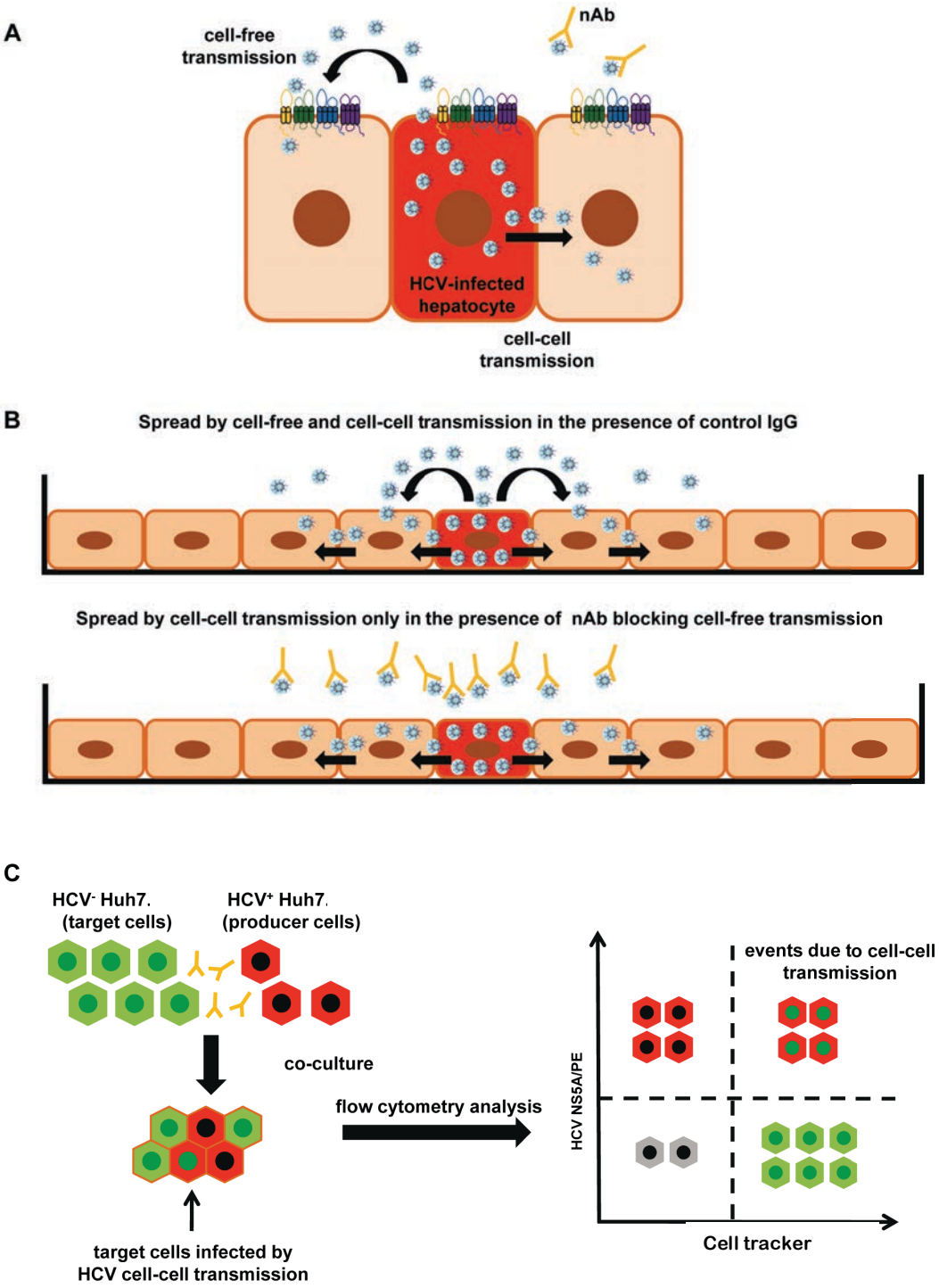


Figure 4.23 Assay for measuring cell-to-cell HCV dissemination.

Figure from Xiao et al (2014). (A) HCV can transmit by cell free or cell-to-cell routes of transmission. (B) In the absence of neutralising antibody (nAb) or control Ab virus can spread cell-to-cell and cell free. When nAb is present cell free spread is blocked allowing cell-to-cell transmission. (C) Huh-7 cells were electroporated with SA13/JFH or J6/JFH and are labeled with CMFDA Cell-tracker Green (Invitrogen) (labeled red) (Producer cells). These are then mixed with Huh-7^{diff} or Huh-7 (target cells, labeled green) in a 1:1 ratio and incubated for 72h in the presence of a neutralizing antibody. De novo transmission events were determined by staining for HCV NS5A and were quantified by flow cytometry.

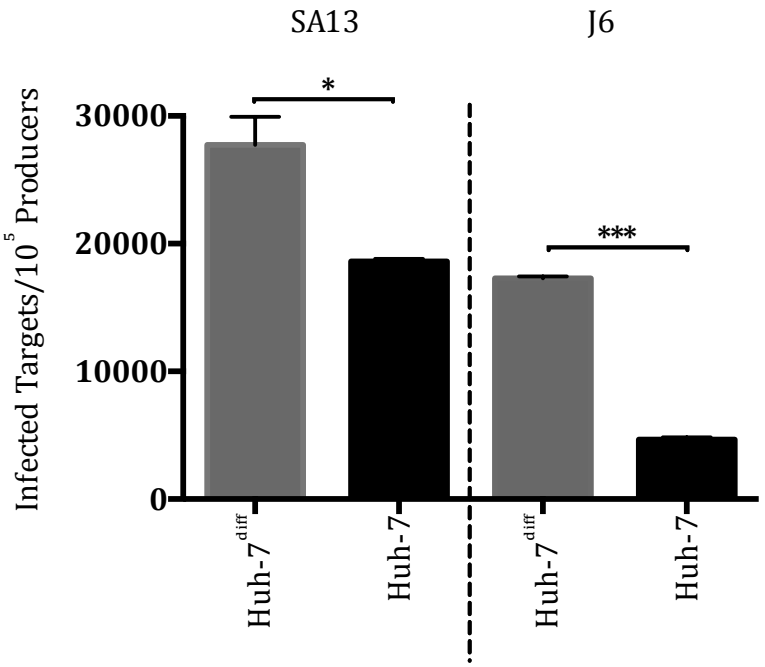


Figure 4.24 Cell-to-cell transmission can overcome the defect in permissivity of differentiated cells

Huh-7^{diff} cells were co-cultured with CMFDA-labelled SA13/JFH infected producer cells (Huh-7). Co-cultures were then incubated for 48 h in the presence of control or anti-HCV IgG, before being fixed, and infection quantified by flow cytometry, detecting NS5A. Results are the mean and standard deviation of three independent experiments. Results are analysed by students t-test ** $p \leq 0.01$ * $p \leq 0.05$ ns= not significant

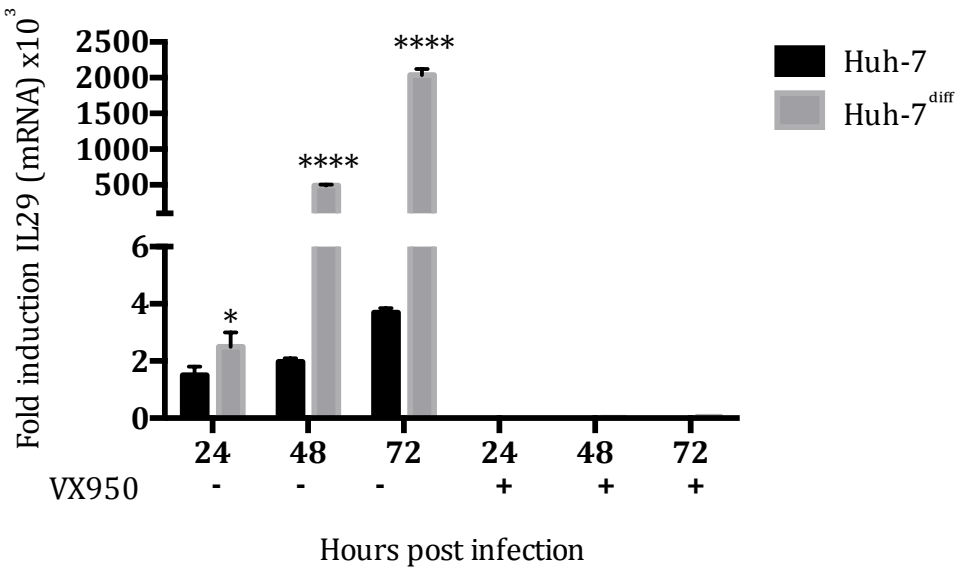


Figure 4.25 IL29 levels are increased by cell–to-cell delivery of HCV

Huh-7^{diff} or Huh-7 control cells were co-cultured with CMFDA-labelled, SA13/JFH infected producer cells (Huh-7). Co-cultures were then incubated for the indicated timepoints +/- VX950 in duplicate wells. Cellular IL29 mRNA was quantified by qPCR. Means from triplicate wells are plotted and representative of two independent experiments. Results were analysed using students t-test and are compared to Huh-7. ****$p \leq 0.0001$ * $p \leq 0.05$.

4.10: Discussion

Results showed that DMSO induced differentiation of Huh-7 cells limits HCV RNA translation, early replication and the infectivity of extracellular HCV particles, resulting overall in a 100-fold reduction in viral infectivity. Importantly, the reduction in cellular permissivity to support viral replication was only apparent following extracellular virus inoculation. When the virus was delivered via cell mediated contact the restrictions were overcome, suggesting cell-cell dependent signalling events between differentiated hepatoma cells that promote viral replication.

Assessing steps in the viral lifecycle to investigate reduced permissivity in differentiated cells, we have shown that there was no difference in viral entry. Using a pseudoparticle system, of varying genotypes, rates of viral entry were not altered (**Figure 4.5** and **Table 4.1**). This was particularly interesting, as receptor localisation changes over time. Cells appear to become polarised, although these changes are not consistent or convincing enough to be comparable to other polarised cells, such as HepG2 (Mee et al, 2009).

Hepatocytes in the liver are highly polarised with bile canaliculi, surrounded by tight junctions, running between adjacent cells at the apical membrane (Thorley et al, 2010). HCV enters through the sinusoidal blood and is likely to encounter the basolateral surface of hepatocytes, where CD81 and SRB1 localise (Reynolds et al, 2008). In our model system CD81 was expressed throughout the culture and there was no alteration with increasing differentiation (**Figure 4.2**). Similarly, there were small circular structures that

retained CMFDA (**Figure 4.3**), but these were not consistently expressed and we did not go on to confirm whether these were functionally important. There did appear to be a restriction in para-cellular permeability as a surrogate for polarisation (**Figure 4.4**), but is unlikely to impact on viral entry as disruption of tight junctions did not alter this (**Figure 4.6**).

Results showed that receptor localisation continued to change for up to 21 days post differentiation (**Figure 4.1**). However, all the data presented has been on cells differentiated for 7-10 days, based on maximal expression of liver markers, such as CYP3A4 and Albumin. Although we did not look at viral entry at 21 days post differentiation, it is unlikely this will make a difference to the results. Claudin-1 had already moved baso-laterally by 7 days (**Figure 4.1**) and there was a significant difference in para-cellular permeability. Therefore we were likely to see a decrease in viral entry by 7 days if this was an important mechanism in restricting permissivity.

Using a g-Luc virus we showed that viral translation and early replication were delayed in differentiated cells (**Figure 4.7**). The addition of VX950 to abrogate viral replication, allowed us to be more certain about the luciferase signal in the differentiated cells, as g-Luc delivery and subsequent levels of replication were low. HCV translation occurs at the rough ER and is facilitated by a number of viral and cellular proteins (Reviewed in Paul et al, 2014). Cellular factors include nuclear factor proteins (Isken et al, 2007), insulin-like growth factor II mRNA binding protein (Weinlich et al, 2009), eukaryotic initiation factors (eIF) (Jaafar et al, 2016), ErbB3 binding protein 1 (Ebp-1) (Mishra et al, 2017) and micro-RNAs particularly miR122 (Jopling et al, 2005; Roberts et al,

2011). miR122 is an important factor, and expression correlates with cellular permissivity to HCV (Wu et al, 2012; Coto-Llerena et al, 2017). Utilising a luciferase reporter construct with a miR122 inhibitor we were able to show that although overall translation was reduced in Huh-7^{diff} as compared to Huh-7, the proportion of miR122 driven translation was comparable (**Figure 4.8**). We did not go on to look at any specific cellular factors associated with HCV translation in differentiated cells, even though several candidate micro-RNAs were identified as being altered by differentiation. It is known that DMSO can alter a broad range of genes involved in cellular processing (N Frampton, personal communication). Therefore this would be an important area for future work and may be important in understanding how the virus initiates translation and replication in the liver.

Cells infected with HCV are known to have altered glucose metabolism. (Ripoli et al, 2010) and can affect glucose homeostasis within the liver. We attempted to switch cells into a glycolytic state, by limiting cellular glucose availability. This resulted in a step-wise decline in HCV replication in a replicon cell line (**Figure 4.11**). There is also significant interplay between glucose homeostasis and cellular oxygen levels. Hepatocytes within the liver are exposed to a significant oxygen gradient across the lobe, and peri-venous cells are exposed to low oxygen levels (pO2 3-5%). These cells respond to hypoxia by a transcriptional response mediated by hypoxia inducible factors (reviewed by Wilson et al, 2014), leading to upregulation of enzymes in the glycolytic pathway. Cellular hypoxia can promote HCV replication (Wilson et al, 2012) and HCV itself can also stabilize Hif and lead to a 'pseudohypoxic' state in the cell and is likely to perturb glucose metabolism. We have not shown convincingly that altering the

glucose environment can affect established HCV replication in these cells. Furthermore, we did not confirm with functional data, such as lactate production, that we were able to induce glycolysis in these cells, relying on mRNA of G-6-Pase, a key enzyme in the glycolytic pathway. It would be interesting to go on and look at metabolic functioning in these cells, including lipid metabolism, cellular response to hypoxia and Hif expression. Additionally, HCV infection in Huh-7 has been shown to activate the glucocorticoid receptor via Hif 1 alpha, which blunts the response to interferons (IFNα and IFNλ), allowing the virus to evade the innate immune response (Wilson et al, 2014). Given the significantly more robust innate response in Huh-7^{diff} there is much scope for looking at this in a differentiated system.

An important recent discovery in the field of HCV is SEC14L2 (SEC14 like lipid binding 2), a cytosolic protein that stimulates an enzyme in the cholesterol biosynthesis pathway. This protein enables hepatoma cell lines to support HCV replication with patient sera, which has previously proved impossible (Saeed, et al, 2015). SEC14L2 is increased in differentiated cells, however using a Huh-7.5 cell overexpressing SEC14L2, results showed that this phenotype was not sufficient to restore HCV permissivity to Huh-7.5^{diff} (**Figure 4.12**). We used Huh-7.5 in these experiments, in order to be able to compare results with parental cells (JW cells). Results confirmed that both Huh-7 and Huh-7.5 had comparable loss of permissivity when infected and therefore this change in hepatoma cell line is not likely to be a significant.

We have shown that differentiated cells upregulate the innate sensor TLR3 and can signal via TLR3 and RIGI/MDA5 to increase interferon lambda

levels (**Figure 4.13-4.17**). Although Bauhofer et al (2012) has shown that differentiated cells express a broad range of ISGs in response to exogenous type 1 interferon, the finding that hepatoma cells can initiate an innate response to replicating virus when differentiated, is novel and exciting (**Figure 4.19**). It was previously established that hepatoma cells do not express TLR3, however we have shown significant and robust upregulation of mRNA, although a limitation of this is that we did not confirm protein or localisation. We attempted to show that cell surface/endosomal TLR3 and the associated pathway was active by stimulation with PolyIC, a well-known synthetic viral mimic. Given that we did not simultaneously inhibit RIGI signalling we cannot say for certain that TLR3 protein is present and active. This requires further investigation.

Stimulation via RIGI/MDA5 resulted in a massive increase in IL29 expression in Huh-7^{diff}, although we failed to show any significant protein expression extracellularly (**Figure 4.18**). Supernatant failed to inhibit HCV replication, using a replicon cell line. Interestingly, we were able to show increase in IL29 mRNA levels using a full length HCVcc infection. It is known that during infection with HCV that hepatocytes are able to limit, but not completely eliminate infection. The virus is able to evade innate signalling due to NS3/4A protease cleaving the accessory proteins for both TLR3 (TRIF) (Li K et al, 2005) and RIG-I (MAVS) (Li XD et al, 2005). Therefore eliciting an innate response in these cells when infected with HCV has potential for helping our understanding of viral persistence despite a host innate response.

There are a number of limitations associate with these particular sets of experiments. We did not measure or localise TLR3 protein or RIG-I within these

cells and therefore conclusions drawn are based largely on mRNA levels. Furthermore we did not investigate the relative contributions of the RLRs, MDA5 and RIG-I to the downstream upregulation of IL29, although all are likely to be active. The liver itself is complex, and any innate immune response needs to be considered in the light of associated contributions from Kupffer cells, stellate cells, liver sinusoidal endothelial cells and infiltrating immune cells (reviewed in Protzer et al, 2012). For example, a recently discovered mouse model of HCV-related hepacivirus infection (Billerbeck et al, 2017) highlighted the importance of a predominantly T-cell response in controlling infection. Even the most physiological cell line cannot recapitulate the environment within the liver and this is a limitation of many experiments done with cell lines or primary hepatocytes alone.

Interestingly, the reduced permissivity of differentiated cells to support HCV replication is overcome when the virus is delivered cell-to-cell suggesting the defect is mediated by extracellular virus infection (**Figure 4.22** and **4.24**). Cell-to-cell transmission is utilised by a number of viruses, including HCV (Brimacombe et al, 2011; Timpe et al, 2008] and has a number of advantages including efficient viral spread (Meredith et al, 2013), direct infection of target cells by neighbouring cells and kinetic and stearic shielding of the virus from the host immune response. Mechanisms for cell-to-cell transmission have been described for a number of other viruses (reviewed in Sattentau, 2008) however this is not well understood for HCV. It is likely that the virus is transmitted across a synapse between two adjacent cells and although many of the HCV receptors are known to localise to tight junctions the abrogation of cell-cell transmission with anti-E2 antibodies

(Brimacombe et al, 2011] or nanoparticles [Tarr et al, 2013] suggests that the synapse is not completely shielded from neutralization. Other receptors such as CD81 do not appear essential for HCV cell-to-cell transmission (Timpe et al, 2008; Witteveld et al, 2009), although Claudin-1 and Niemann Pick cholesterol uptake receptor are important (Timpe et al, 2008; Barretto et al, 2014). HCV may be exploiting the virological synapse similar to that used by HIV. Furthermore, given the observation that cell-cell delivery can overcome the reduction in replication initiation in non-differentiated cells it is likely that there are additional effects on the target cell of the virus being delivered cell-to-cell. Nanbo et al (2012), for example, described altered cell signalling in B cells (producers) and epithelial cells (targets) in cell-cell delivery of EBV. This allowed the enhancement of the viral lytic cycle and increased viral transmission. The effects of cell contact mediated viral delivery on the target cell are not well studied, however the differentiated cell model system suggests this is an area that may be important in fully understanding cell-cell transmission.

Xiao et al (2014) also showed that cell-to-cell transmission was the predominant route of spread for direct acting antiviral-resistant HCV (in differentiated hepatoma cells). Given the recent advances in treatment of HCV, and concern about resistance, Huh-7^{diff} may provide an excellent model system for studying the HCV dissemination route, which is likely to be increasingly important.

A significant limitation with this study, and an important next step, is to investigate lipid metabolism with Huh-7^{diff}, particularly when considering the loss of specific infectivity of the viral particle. Lipid droplets are known to be

important for HCV production [Miyanari et al, 2007]. Furthermore, DMSO is known to affect cellular lipid and results in a reduction in lipid accumulation within hepatoma cell lines [Song at al, 2014]. Huh-7 are known to have VLDL defects and virus released from these cells is associated with apoE but not apoB (Jammart et al, 2013]. This in part could account for altered viral packaging within Huh-7^{diff}, since alteration of density of the viral particle by serial passage has direct implications for RNA replication and specific infectivity of released virus [Lindenbach et al, 2006], which is likely to impact on cell-free virus spread. The VLDL pathway, despite being involved in the secretion of infectious viral particles, is interestingly redundant in cell-cell viral spread in DMSO differentiated cells [Barretto et al, 2014]. It is therefore possible that alterations in the VLDL pathway in differentiated cells allow the virus to spread efficiently cell-cell but inhibit cell-free spread and this requires investigating.

We have used this physiological system to understand how viral replication is altered in a differentiated cell, which allows insight into viral/host interaction *in vivo.* The system has significant advantages over other differentiated model systems, as cells are readily available, easy and relatively fast to differentiate and can be maintained in culture for a long period of time. The parental cells, Huh-7, are readily available. Therefore they are an ideal model system for studying the HCV lifecycle, particularly cell-to-cell viral transmission.

In addition to the limitations discussed above, a further limitation was using a laboratory-adapted strain for most of the experiments. This was the only virus available that allowed us to get high levels of infection in Huh-7^{diff}. This virus

has been serially passaged and is a highly infectious SA13 (genotype 5a) that has developed mutations that increase specific infectivity of virus (Mathieson et al, 2015). The virus has comparable rates of cell-to-cell and cell-free spread (Meredith et al, 2013), although cell culture adaptations may make the virus more efficient at cell-to-cell spread than wild type virus (Mathieson et al, 2015). Ideally many of the experiments should be confirmed with an early passage virus, other viral genotypes and virus derived from patient sera. However this was practically difficult.

Summary

In summary, we have shown that Huh-7^{diff} limit HCV translation/early replication and have reduced specific infectivity of the released viral particle. Furthermore we have shown that Huh-7^{diff} produce a robust innate response to HCV infection. Virus delivered directly between cells does not show altered permissivity.

The observation that direct cell-to-cell delivery of HCV is efficient in this physiological system raises further questions and hypotheses regarding HCV dissemination *in vivo*. HCV can infect other cell lines, such as neuroepithelioma cells (Fletcher et al, 2010), and can cause clinical multi-system involvement. We were particularly interested in cell-to-cell delivery of HCV by cells other than hepatocytes, which may allow distant viral spread. The following chapter outlines the cell-to-cell delivery of HCV by T cells.

Chapter 5: T cells can deliver HCV to permissive cells

5.1 : Introduction

HCV can transmit rapidly between hepatocytes by direct cell-to-cell contact (Brimacombe et al, 2011; Timpe et al, 2008; Witteveldt et al, 2009) which can offer many advantages including evasion of the adaptive immune response (reviewed in Sattentau, 2008). In the previous chapter we have shown that direct cell-to-cell delivery of HCV (SA13:JFH) can efficiently infect differentiated hepatoma cells, whereas infection using cell-free virus was significantly reduced. Previous work has shown that cells other than hepatocytes, such as B cells, can internalize and are able to deliver infectious HCV to hepatocytes (Stamataki et al, 2009). The infectious JFH-1 HCV strain can bind primary B cells and B cell lines but is not able to establish a productive infection within these cells. Other blood cells (PBMCs) have been reported to have HCV cellular RNA detected, however HCVcc clones do not infect lymphocytes themselves (Marukian, 2008). B cells are able to bind HCV on their surface using viral receptors CD81, SRB1, DC-SIGN and L-SIGN (Stamataki et al, 2009) and transfer infection to Huh7.5 showing enhanced levels of infection compared to cell-free virus delivery, which is resistant to neutralizing antibody infection. HCV cellular associations with other cell types, such as B cells, therefore, provides further mechanisms for immune evasion and transfer of HCV to the liver.

Other cell types are known to trans-infect viruses; dendritic cells expressing the C-type lectin DC-SIGN, can capture and internalize HIV-1 at mucosal surfaces and efficiently transfer to CD4+ T cells in lymph nodes, where

viral replication then occurs (Arrighi et al, 2004; van Montfort, 2007). Interestingly, C-type lectin domain family 4 (CLEC4M) is a trans-membrane protein expressed on liver sinusoidal endothelial cells, and has been shown to capture HCV and transinfect a permissive hepatoma cell line (Ishibashi et al, 2014). This may allow virus to be captured from the circulation and transferred to hepatocytes. These models provide biological plausibility to T cell trans-infection of HCV.

Further recent work by Stamataki et al (unpublished data) has shown that activated CD4+ T cells are capable of transferring HCV on their surface and infecting differentiated polarized hepatoma cells (HepG2) and neuroepithelioma cells (hCEMC/D3) which are difficult to infect with cell free virus. Stimulated CD4+ T cells were not amenable to support HCV replication however provide a mechanism for cell delivery to differentiated cells and distant sites such as the blood-brain barrier or the placenta. We therefore hypothesized that stimulated CD4+ T cells (both primary and immortalized lines) could be used to transfer HCV (SA13) to Huh-7 and Huh-7^{diff} and could provide an alternative mechanism for differentiated cell infection.

Given that CD4+ T cells are the primary host cell for replicating HIV we were interested to see whether HIV infection of CD4+ T cells altered HCV trans-infection. Co-infection with HIV-1 and HCV is common, and is reaching epidemic proportions in certain parts of the world, particularly Eastern Europe and Central Asia where around 40% of people with HIV are HCV co-infected, accounting for over one quarter of HIV/HCV co-infections worldwide (Platt et al,

2016). Clinically, co-infected individuals are at increased risk of rapid and progressive liver cirrhosis than HCV mono-infected individuals (de Lédinghen et al, 2008), with over 49% of co-infected patients having cirrhosis within 30 years (Thein et al, 2008) with an increased risk of hepatitis/liver related deaths despite anti-retroviral therapy in co-infected patients (Smit et al, 2008). The landscape of liver disease in co-infected patients is changing due to DAA's, however, these patients are still complex to manage due to drug interactions and the management of rampant liver disease (Schlabe and Rockstroh, 2018).

Co-infection is also relevant in the context of pregnancy where co-infection with HIV/HCV doubles the transmission of HCV from mother to child (5.8% to 10.8%) and maternal HIV co-infection is the single most important determinant of HCV vertical transmission risk (Benova et al, 2012). This is often thought to result from higher HCV viral load in women who are also co-infected with HIV (Thomas et al, 1998), however the mechanism behind vertical transmission is not well defined. Azzari et al (2000) reported that virus associated with PBMCs is related to vertical transmission of HCV independent of viral genotype or maternal viral load. We therefore hypothesized that HIV infection of CD4+ve T cells altered HCV carriage and delivery, thereby providing a novel mechanism for HIV-1 promotion of HCV infectivity and transmission.

Chapter Aims:

- To determine whether T cells bind HCV and trans-infect permissive hepatoma cells.
- To investigate the effect of activated T cells on HCV hepatocellular replication.
- To investigate the effect of HIV co-infection of T cell HCV trans-infection.

5.2 : Hepatitis C virus can be transferred on the surface of a CD4+ T cell

Stamataki et al (unpublished) showed that HCV could be trans-infected by a T cell. In order to confirm these results, SupT1 cells were incubated with HCVcc (SA13:JFH) prior to washing to remove unbound virus. Cells were then incubated with Huh-7 cells for 48 hours. HCV infection was quantified by staining for NS5A. **Figure 5.1** outlines the method of T cell trans-infection. SupT1 cells were used as they are easy to replicate between experiments and are a robust model for HIV infection. Results confirm that T cells are able to trans-infect permissive Huh-7 with HCV (**Figure 5.2**).

Results show that inoculating T cell number is important and can mediate cytotoxicity of the hepatoma cell monolayer. SupT1 added as high number per well ($2x10^7$/ml) resulted in T cells being tightly adhered to Huh-7 and loss of viability of the monolayer (**Figure 5.2**). SupT1 added at a lower number of cells per well ($1 x10^7$/ml) resulted in a healthy monolayer with spaces between the T cells where the Huh-7 can be visualised. NS5A staining in Huh-7 reveals clusters of infected cells, confirming that T cells can trans-infect HCV, although they have a cytopathic effect on healthy hepatoma cells at high numbers.

Previous results have shown that Huh-7^{diff} are permissive to HCVcc delivered cell-to-cell. Therefore we investigated whether cell delivered HCV by a T cell could overcome the inherent replication restriction of HCV seen when virus is delivered cell free in Huh-7^{diff}. **Figure 5.3** shows that Huh-7^{diff} can be infected with HCV delivered by a T cell, however have a 100-fold reduced

permissivity, confirming that T cell delivered virus is not able to infect differentiated cells as efficiently as hepatoma cell-to-cell infection. These results suggest that cell associated viral delivery to a differentiated cell differs depending on type of cell used.

In summary, T cells are able to trans-infect HCV to permissive cells, despite having a cytopathic effect on target cells. T cell delivered virus was unable to overcome restrictions to HCV permissivity seen in differentiated cells.

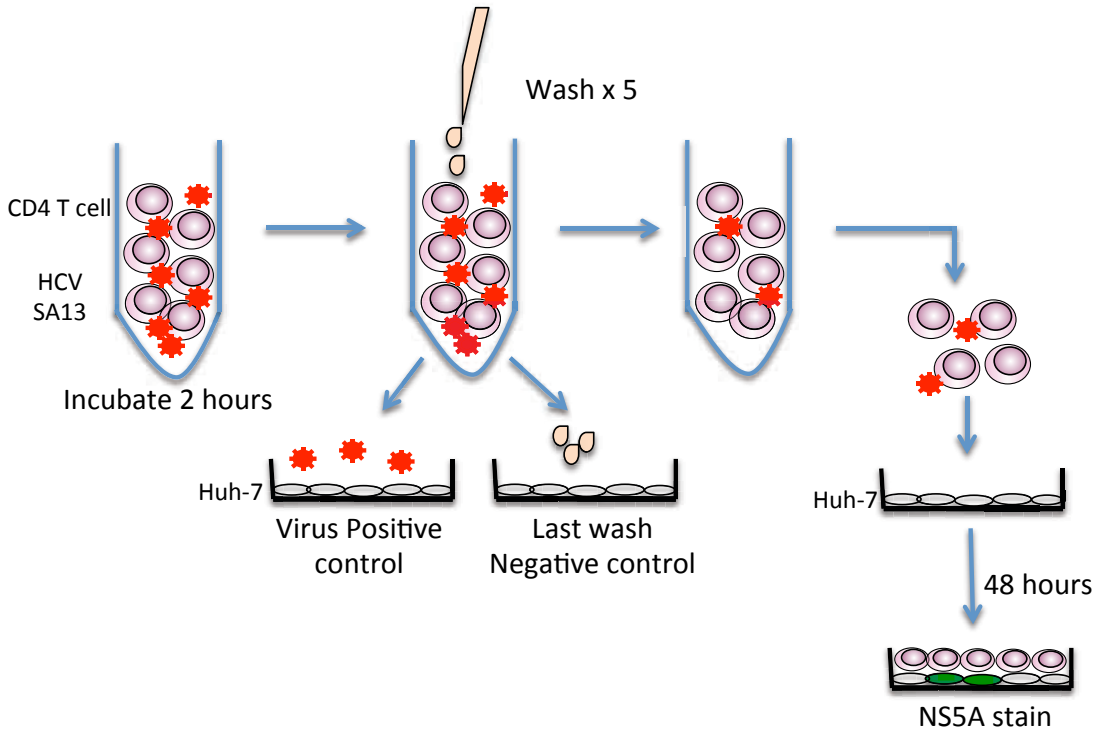


Figure 5.1 T cell trans-infection assay

T cells are incubated with HCVcc (neat virus) for 2 hours (200µl SA13 per 1 million T cells). Following incubation cells were washed with RPMI 5 times. Media from the last washing step is used in a second round infection as a negative control to ensure no cell-free virus is present at the time of trans-infection. Cells are then co-cultured with target cells (Huh-7 or LucA2 replicon cells) plated 24 hours prior to infection in 48 well plates ($5x10^4/cm^2$). T cells are added at varying numbers per ml to a final volume of 500µl per well. 48 hours post co-culture cells are methanol fixed and stained for NS5A. Positive cells are enumerated.

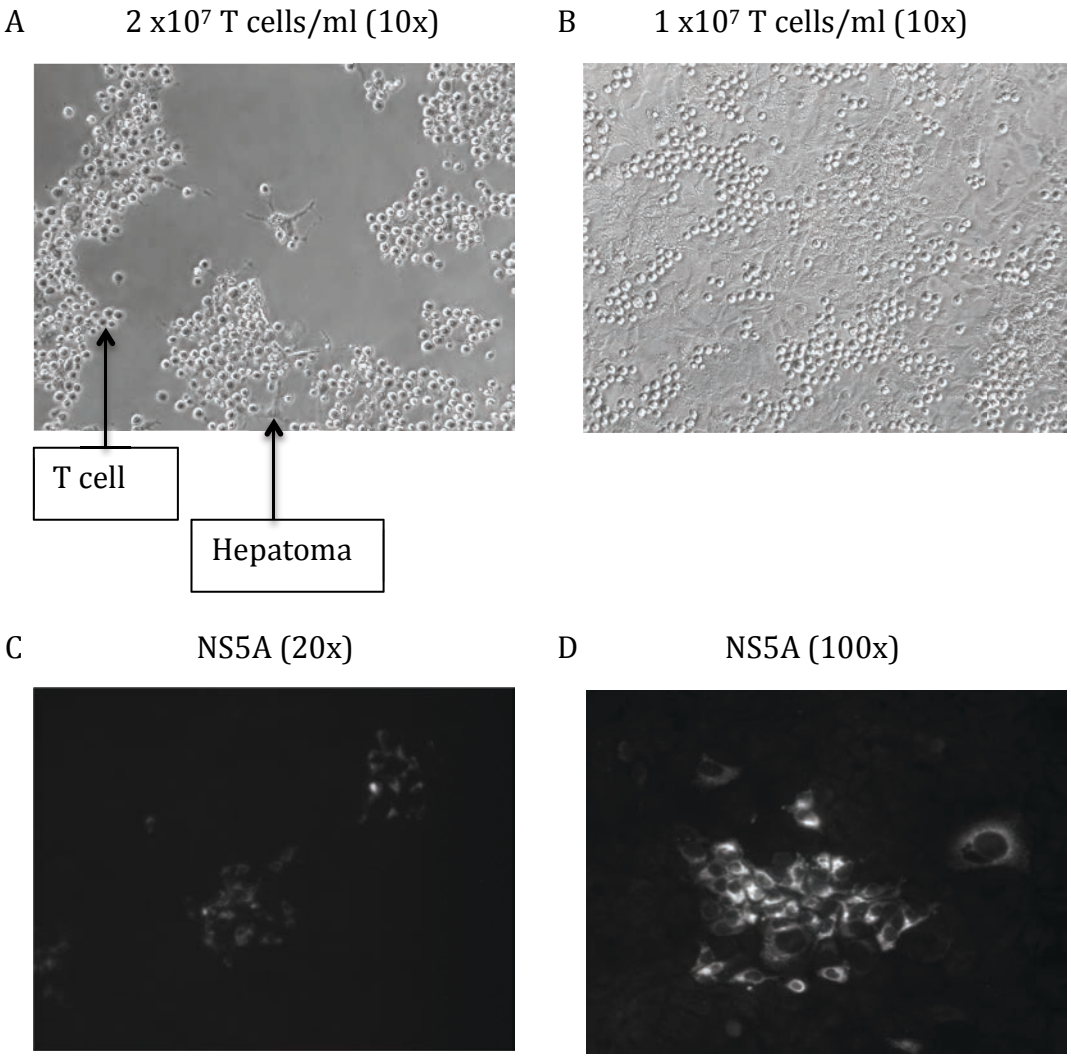


Figure 5.2: SupT1 cells can mediate HCV trans-infection of Huh-7

Huh-7 permissive cells were trans-infected with SA13 as described. (A) Light microscopy of the co-culture at 48 hours post trans-infection shows SupT1 cells adhered to the hepatoma monolayer causing cell death at 2 x10^7 T cells/ml. (B) The monolayer was intact when 1 x10^7 T cells/ml were added to the hepatoma cells. T cells are shown adherent to the healthy Huh-7. (C and D) NS5A staining showing T cells/Huh-7 with clusters of HCV infected cells.

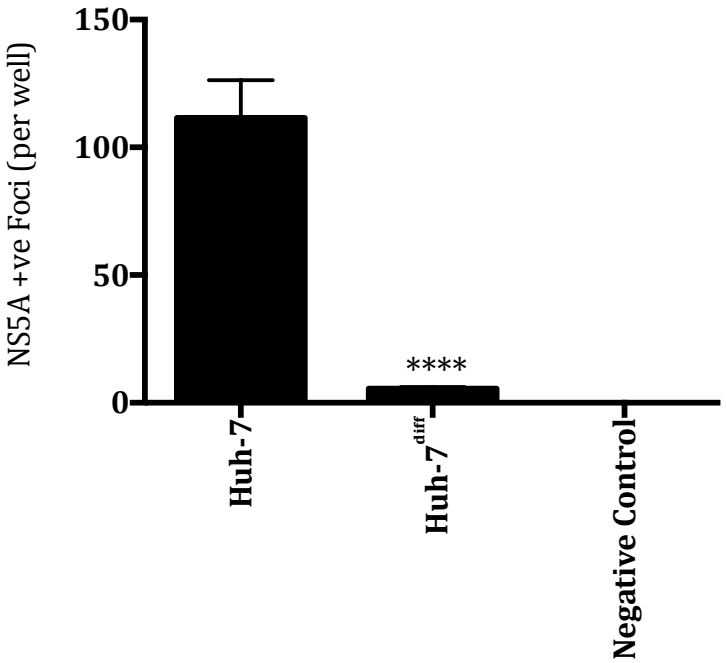


Figure 5.3 Huh-7^{diff} support reduced HCV infection when virus is delivered using SupT1

HCV was trans-infected by SupT1 cells into Huh-7 and Huh-7^{diff} as previously described. 48 hours post co-culture NS5A positive foci were enumerated. There was an intact monolayer (pictures not shown) and no foci seen in the negative control. Results are means of triplicate wells and representative of 3 independent experiments. Results were analysed with students t-test and compared to Huh-7 ****$p \leq 0.0001$

5.3: Primary CD4+ T cells and immortalized T cell lines can support HCV trans-infection

Results have showed that the immortalized T cell line, SuptT1, is able to mediate trans-infection of a permissive cell line with HCV (SA13). It is important to confirm that these results are reproducible with other T cell lines and primary CD4+ T cells.

The T cell lines SupT1, Jurkats and MOLT-4 cells were identified as candidate cell lines, due to their availability and ability to support HIV-1 infection (Dejucq, 2000; Hesselgesser et al, 2000). **Figure 5.4A** shows that all cell lines tested are equally able to trans-infect HCV. The number of T cells used in each trans-infection were titrated, as high T cell numbers appeared to disrupt the monolayer. These results suggest that T cell number has a direct effect on HCV delivery.

Stamataki et al showed that resting PBMC derived T cells were unable to support HCV trans-infection, and that T cells needed to be activated. Therefore the addition of T cell stimulatory molecules was investigated to assess impact on trans-infection by a T cell line. T cell lines were grown in the presence of anti-CD3/anti-CD28 antibody (Raulf-Heimsoth, 2008) for 7 days prior to use. This provides antigen-independent signaling of the TCR. IL-2 was added to the media as a T cell proliferating agent (Hedfors and Brinchman, 2003; Stamataki unpublished). **Figure 5.4B** shows that the addition of anti-CD3/anti-CD28 in the media to T cell lines does not alter HCVcc trans-infection. These are already a

highly activated immortalised T cell line, and further stimulation via CD3/CD28 does not potentiate the ability of these cells to trans-infect HCV.

In order to make results translatable, we confirmed that activated primary CD4+ T cells isolated from PBMCs could also trans-infect HCV and compared their ability to do this with T cell lines. CD4+ T cells were isolated, as described, from PBMC from healthy anonymous donors. Primary cells were activated with anti-CD3/anti-CD28 and maintained in media containing recombinant IL-2. Cells were then used at 5-7 days post isolation (as described by Stamataki et al, unpublished). **Figure 5.5** confirms that CD4+ T cells are able to trans-infect HCV to hepatoma cells. Although there is some variation in ability between donors, they generally transfer lower levels of HCV than equivalent numbers of immortalised T cells. Interestingly, they were able to support some HCV transfer even at very high numbers of T cells with the maintenance of the hepatoma monolayer, unlike that seen with T cell lines. However, higher primary CD4+ T cell numbers seem to support less HCV transfer, even without a cytotoxic effect, suggesting that T cell number has a direct effect on delivery or HCV replication.

In summary, we confirm that T cell lines and activated primary CD4+ T cells are able to support HCV trans-infection. Final HCV infectivity is affected by both the number and type of T cell used.

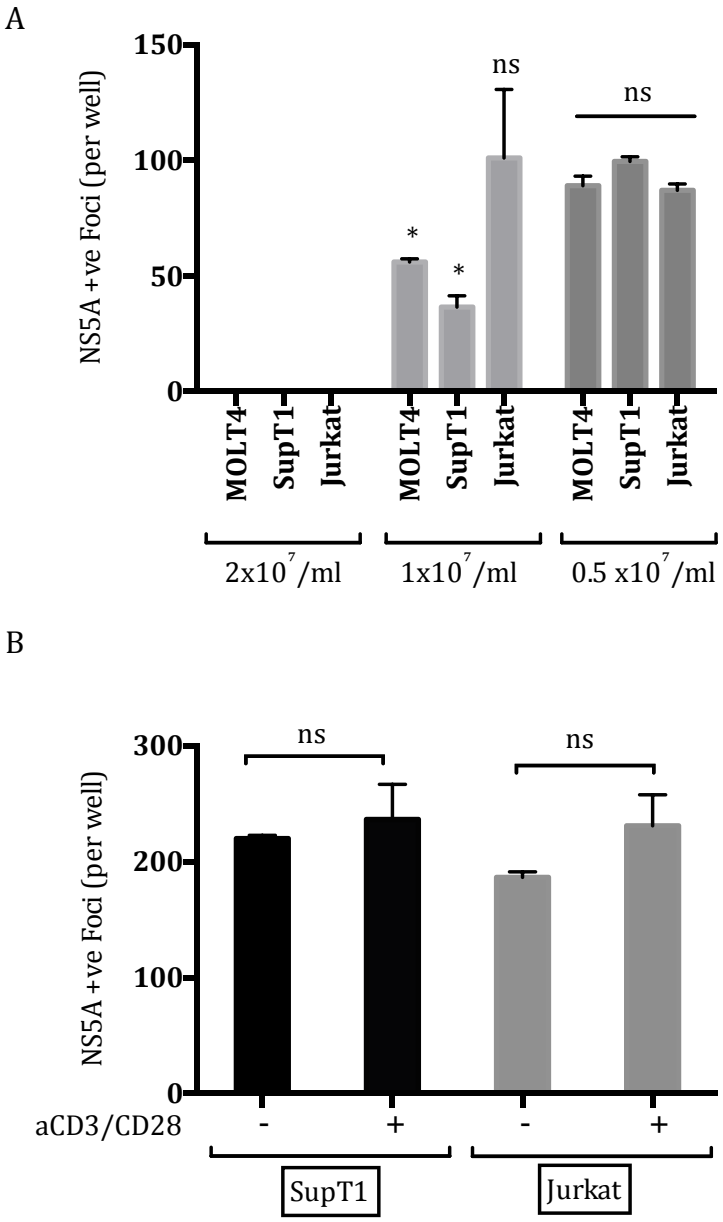


Figure 5.4 T cell lines support comparable HCV trans-infection and this is not altered by T cell stimulatory antibodies.

(A) MOLT4, Jurkat and SupT1 cells were used to trans-infect HCV at indicated T cell numbers in 48 well plates. 48 hours post co-culture plates were fixed and Huh-7 were stained for NS5A. There was no viable monolayer in any of the wells where high T cell numbers were used. (B) Trans-infection was repeated using T cells that had been activated with anti-CD3/anti-CD28 (1mcg/ml) 7 days prior to use. Data shown is for T cells added at $0.5x10^7$/ml in 48 well plates. Results are means of triplicate wells and representative of two independent experiments. Results are analysed using Students t-test *p≤0.05, ns=non significant.

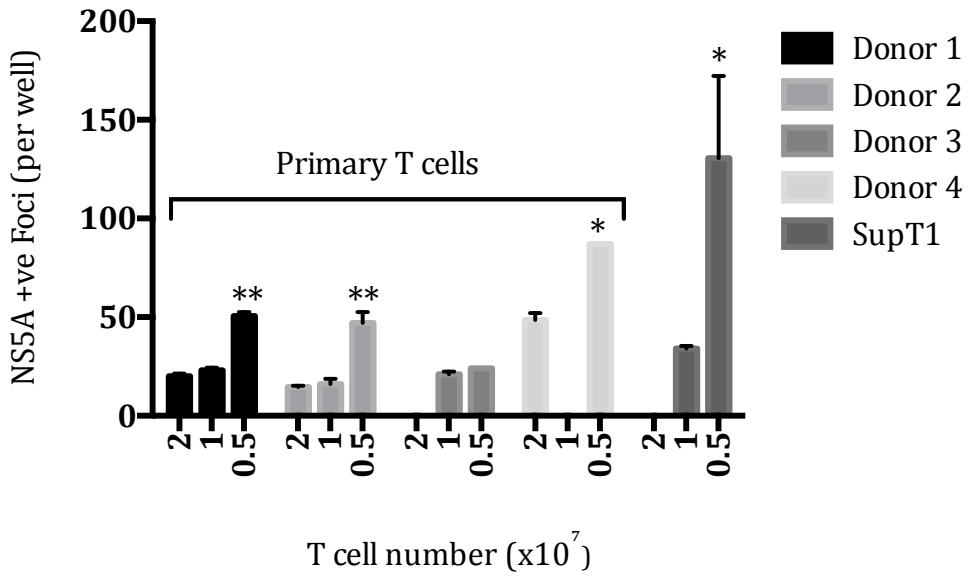


Figure 5.5 Primary CD4+ T cells are able to support trans-infection of HCVcc

Primary CD4+ T cells were isolated from healthy anonymous donors as described. Cells were activated with anti-CD3/anti-CD8 (1mcg/ml). 5-7 days post isolation cells were used to trans-infect HCV as previously described alongside SupT1 cells. 48 hours post infection Huh-7 were fixed and stained for NS5A. Results are means of triplicate wells and representative of three independent experiments. Analysis was done using a students t-test and results compared to number foci at $2x10^7$ cells/ml. Non-significant results are not marked. **p≤0.01 *p≤0.05

5.4 : T cell number affects HCV foci and size via a cell contact mediated effect

Results have consistently shown that T cell number affects HCV infectivity in a trans-infection. In order to optimise the assay we therefore investigated the role of T cell number on HCV trans-infection. T cells were titrated from $2x10^7$ to $1.5x10^6$ cells/ml and used in a trans-infection. **Figure 5.6A** shows NS5A positive cells by T cell number. Both high and low T cell numbers are associated with a reduction in the amount of HCV transferred. The optimum number of T cells per well is $6x10^6$ T cells/ml cultured in a 48 well plate and therefore will be used at this number in future experiments for SupT1 cells. This represents a 2:1 T cell:Huh-7 ratio (at 24 hours post plating). **Figure 5.6B** shows NS5A staining; at high T cell numbers there are fewer foci and these are small or single cell. At lower T cell numbers much larger foci are seen. These results indicate that T cells are mediating an anti-viral effect at high numbers inhibiting initial HCV infection and spread within Huh-7.

We hypothesize that there is a direct anti-viral effect associated with high T cell numbers. In order to test this an HCV trans-infection was repeated using SupT1 cells and primary cell lines with the target cell harboring a subgenomic replicon (LucA2) to study the effect of T cells on HCV replication directly. 48 hours post infection cells were lysed and HCV replication measured by luciferase. **Figure 5.7A** shows that T cells inhibit established HCV replication in a dose dependent manner, whether they are loaded with HCV or not. Primary cells also appear to have a larger effect per cell than the cell lines.

In order to establish whether this is mediated by a cell contacts or a soluble mediator we removed the supernatant from wells at 48 hours post infection and placed directly onto LucA2 replicons. **Figure 5.7B** indicates that supernatants taken from wells with high and low T cell numbers (Jurkat and SupT1 cells) do not appear to have an affect on HCV replication. There was a modest inhibitory effect of supernatant from primary cells. This may explain the enhanced inhibition of replication seen with primary CD4+ T cells compared to T cell lines. We confirmed that HCVcc itself did not have an independent effect on ongoing HCV replication within the LucA2 replicon (data not shown).

In summary, T cells are able to transfer HCV to permissive hepatoma cell lines, despite a direct anti-viral effect of T cells on HCV replication, mediated by cell contacts. This effect results in inhibition of HCV transfer and spread at high T cell numbers.

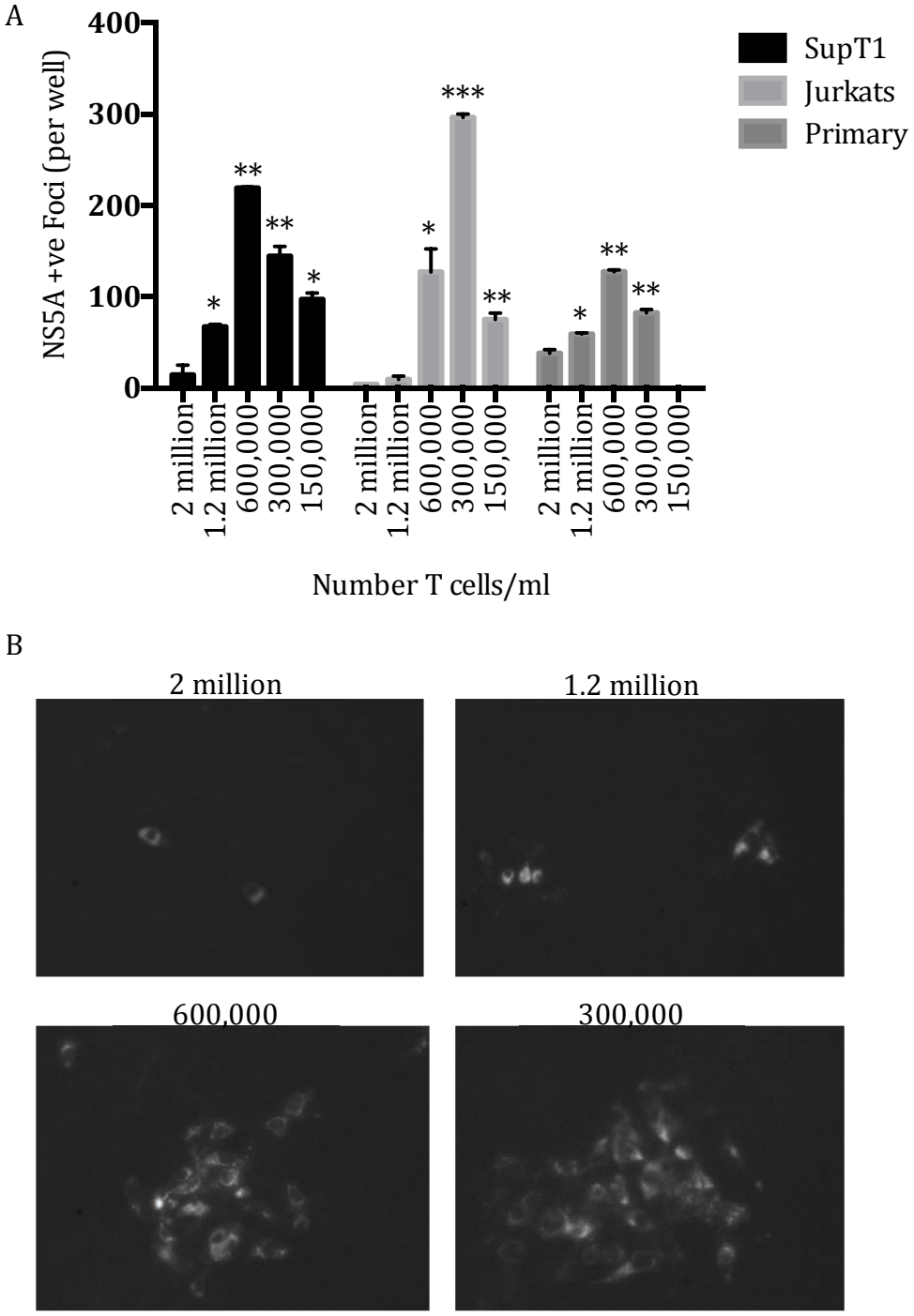


Figure 5.6: HCV transfer and spread is directly related to T cell number

T cell trans-infection was carried out as previously described using HCVcc (SA13) delivered to Huh-7. SupT1, Jurkat and primary T cells were titrated from $2x10^7$/ml to $1.5x10^6$/ml in 48 well plates. 48 hours post infection cells were fixed and NS5A stained. NS5A positive foci were counted in duplicate wells (A) and results are representative of 3 independent experiments. Results were analysed using students t-test and compared to 2 million cells/ml. (B) Representative images were taken (x20) showing foci size with the indicated number of cells/ml. *** $p \leq 0.001$ ** $p \leq 0.01$ * $p \leq 0.05$

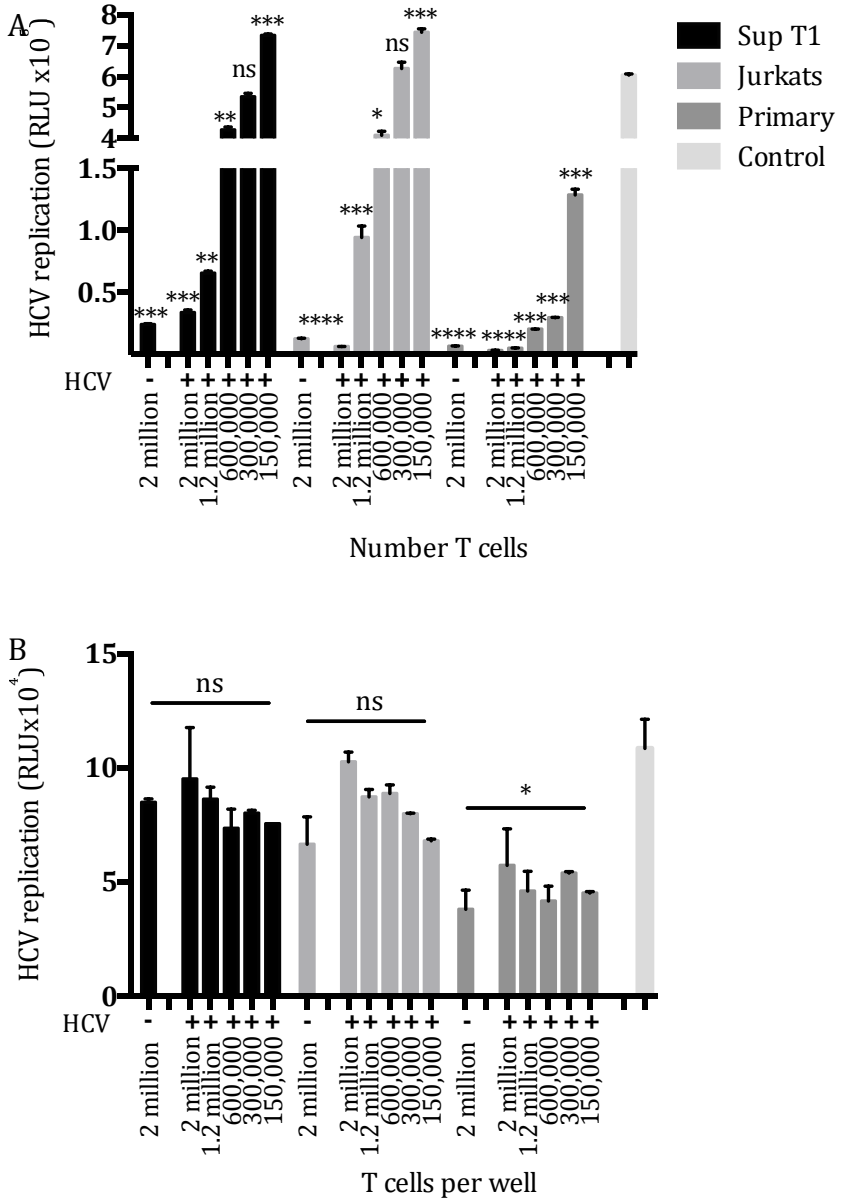


Figure 5.7 T cells inhibit HCV replication in a cell contact dependent manner

(A) SupT1, Jurkats and primary CD4+ T cells were used to trans-infect LucA2 replicon cells with HCV. 48 hours post infection cells were lysed and luciferase was read as a marker of HCV replication. (B) Supernatant from a matched trans-infection was taken at 48 hours post co-culture and added to LucA2 replicons. 48 hours later replicons were lysed and luciferase read as a marker of HCV replication. Results are means of triplicate wells and representative of three independent experiments. Analysis is with a students t-test. Results were compared to control LucA2 with no trans-infection or supernatant treatment. **** p≤0.0001 *** p≤0.001 **p≤0.01 *p≤0.05 ns=not significant.

5.5 : Infecting T cells with HIV

Results have shown that T cells are able to trans-infect HCV to a permissive cell line. Given that T cells are the target cell for HIV infection *in vivo* we were interested to see whether HIV infection altered HCV trans-infection. The first step was to confirm strains of HIV that efficiently infect T cell lines and primary cells.

HIV strains HXB2, NL4.3 and NL4.3 GFP (CXCR-4) and R5 (CCL-5) were made as previously described. Infectivity of virus was tested using an ELISA for HIV-1-p24 antigen. NL4.3 consistently had higher measured extracellular p24 (**Figure 5.8A**) and was able to infect GHOST cells (**Figure 5.8B**). NL4.3 was therefore used for all subsequent experiments with HIV, unless stated otherwise.

HIV strain NL4.3 was then used to infect T cell lines and primary CD4+ T cells. T cell lines shown to support HCV transfer (SupT1, MOLT4 and Jurkats) and primary activated CD4+ T cells from 4 healthy anonymous donors were infected with HIV (pnL4.3 20ng/ml). Supernatant was harvested 48 hours post infection and extracellular p24 as a marker of HIV infection was measured. **Figure 5.9A** shows that SupT1 cells supported the most productive HIV infection as indicated. Primary CD4+ T cells did support infection however at a much lower level. Extracellular p24 is a recognized sensitive test of HIV infectivity (Klein et al, 2003), however for this cell-based assay it was important to identify the number of cells infected with HIV. Therefore, SupT1 cells infected with HIV-1 (NL4.3) were additionally stained for intracellular p24. **Figure 5.9B** shows that only

1.5% of the cell culture was p24 positive. Despite low numbers of cells being infected with HIV this appears consistent with the number of T cells infected both in culture and *in vivo* (Pace et al, 2012; Eckstein et al, 2001; Gadol et al, 1994).

In order to utilise T cells when they had most replicating HIV present, the optimum time post infection was investigated. Primary CD4+ T cells were isolated from healthy anonymous donors as described, and infected with NL4.3 (20ng/ml) for 8-12 hours, alongside SupT1 cells. Cells were then washed and incubated in RPMI (+/- IL2). Supernatant was taken daily post infection and extracellular p24 measured by ELISA (**Figure 5.10**). Although there is significant variability between donors and SupT1 cells, results indicate that generally SupT1 cells should be used for trans-infection at 3 days post infection and primary cells between 3-5 days post infection, in order to maximize HIV infection and minimise cell death. Therefore all cells were infected with HIV for 72 hours prior to use in trans-infections.

In summary, T cell lines and primary CD4 cells support HIV infection with NL4.3 at 3 days post infection. Despite measurable extracellular p24, there are only 1.5% of cells infected with HIV within a T cell population.

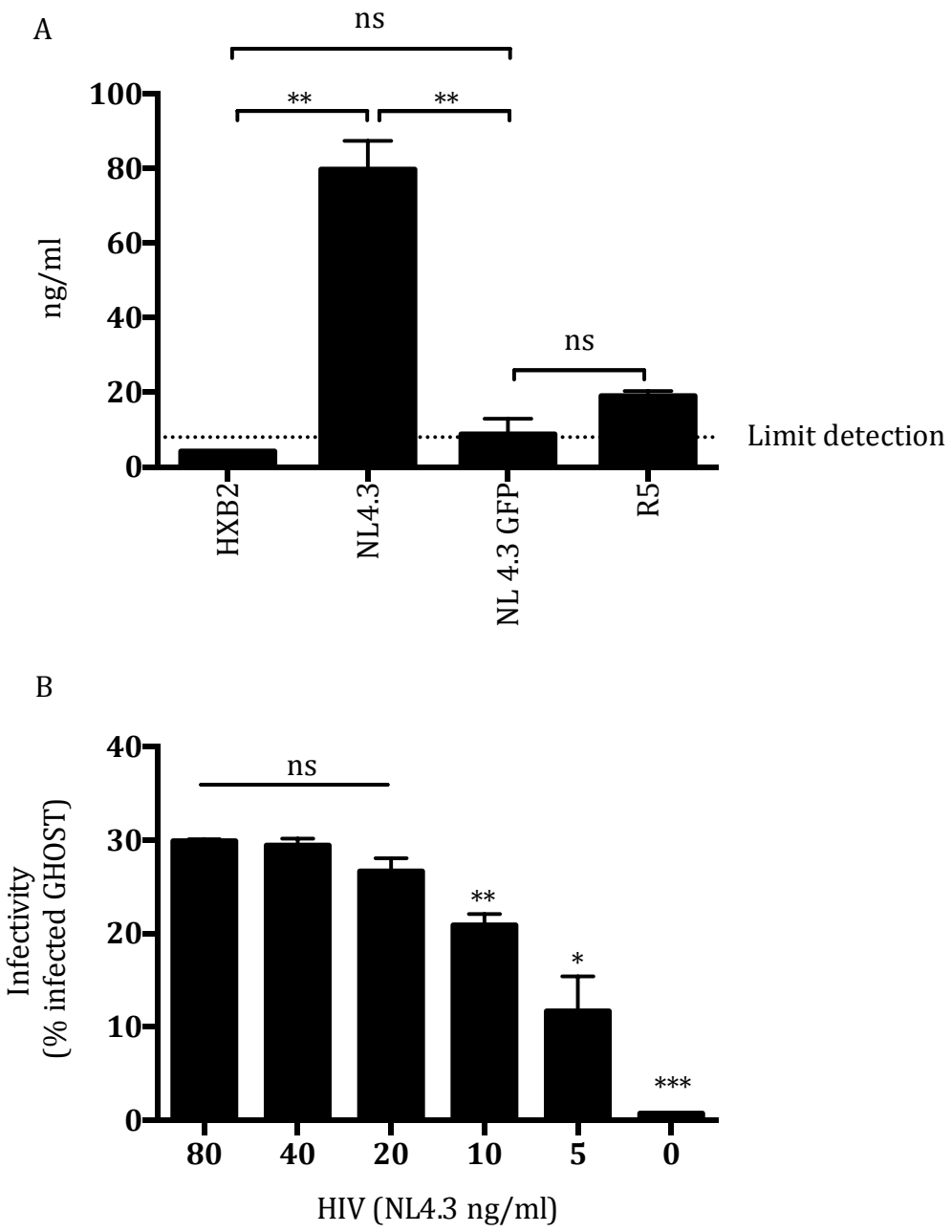


Figure 5.8 HIV strain NL4.3 has the highest infectivity

(A) Extracellular p24 was measured by ELISA in supernatant collected from 293T cells 72 hours post-transfection with indicated HIV strains. (B) GHOST indicator cell line was infected with NL4.3 at viral concentrations as indicated for 48 hours. Cells were fixed and the number of GFP positive cells (as a percentage of total cell population) was calculated using Flow cytometry. Results are means of duplicate pooled wells, representative of two independent experiments and are analysed using students t-test *** $p \leq 0.001$ ** $p \leq 0.01$ * $p \leq 0.05$ ns=not significant.

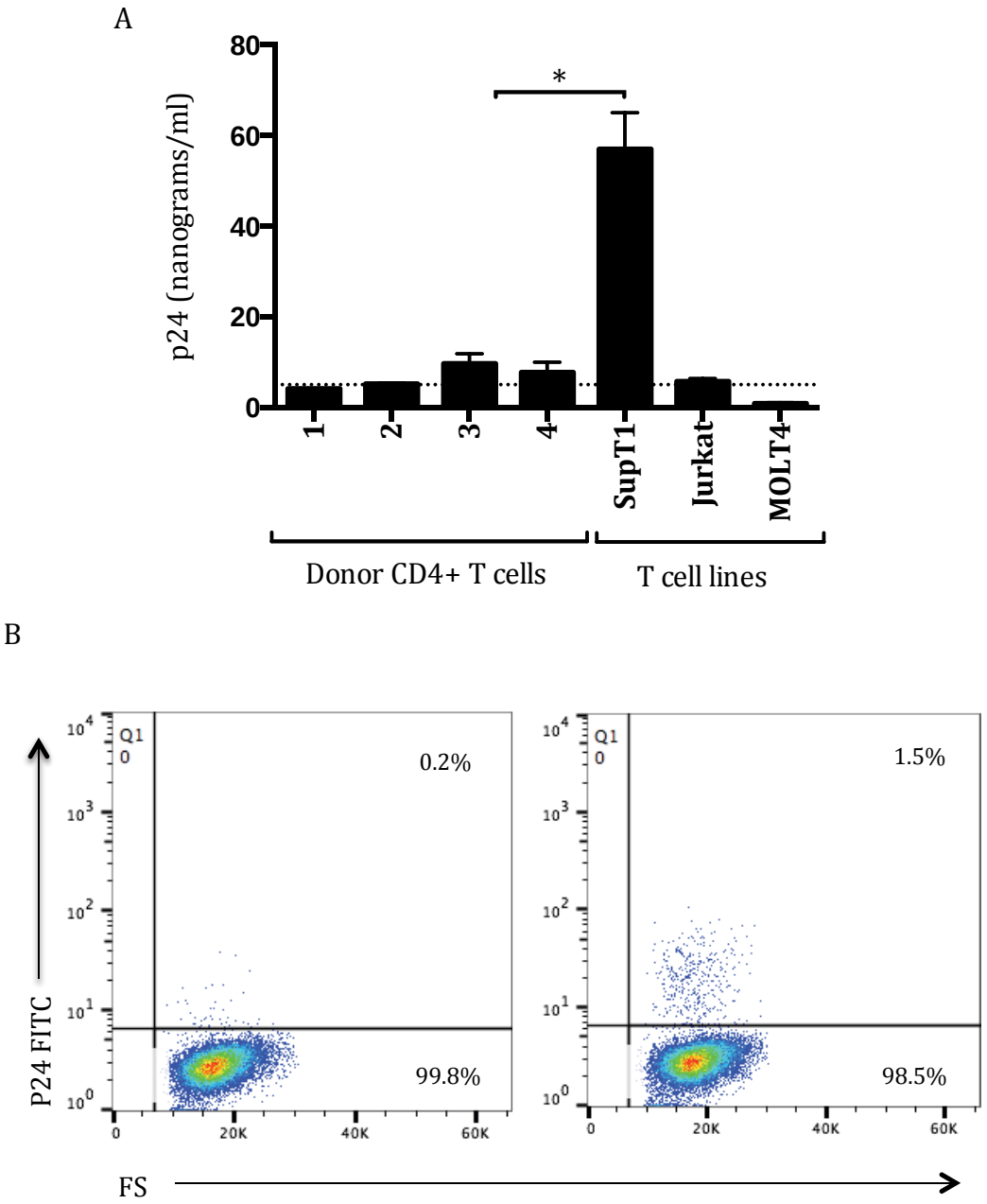


Figure 5.9 SupT1 cells support productive NL4.3 infection

(A) T cell lines and primary CD4+ T cells isolated from 4 independent donors were infected with NL4.3 (20ng/ml) for 12 hours. Cells were washed and incubated for a further 48 hours in RPMI (+/- IL2). Extracellular p24 was quantified in the supernatant by ELISA and corrected for final T cell number. Results are means of duplicate wells and of two independent experiments. Data was analysed using a students t-test and results show SupT1 compared to Donor 3 (two highest values) *p≤0.05. Limit of detection of assay is marked by a dotted line. (B) SupT1 cells were infected with HIV NL4.3 for 12 hours prior to washing. 72 hours post infection cells were stained with intracellular p24 as described and acquired by flow cytometry (FACSCalibur). Results show percentage cells positive for p24 gated on live cells only. Results are pooled triplicate wells and representative of two independent experiments.

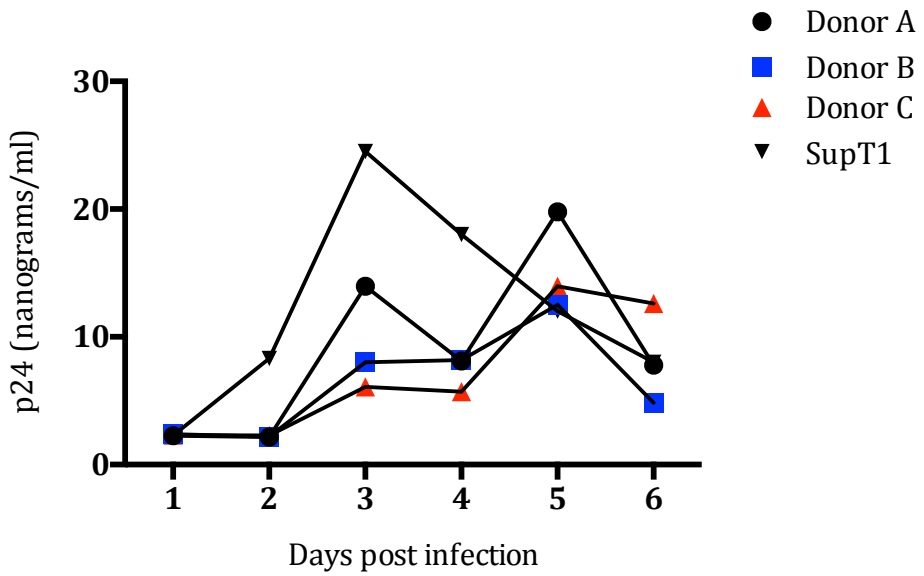


Figure 5.10 T cells show maximally HIV infectivity 3 days post infection.

SupT1 and primary CD4+ T cells isolated from three anonymous donors (A, B and C) were infected with NL4.3 for 8 hours. Cells were then washed and incubated for up to 6 days in RPMI (+/- IL2). Supernatant was taken daily post infection (and replenished in the wells to keep a constant volume) and extracellular p24 quantified by ELISA as described.

5.6 : HIV Infection boosts HCV transfer by T cells

Results have previously shown that T cells are able to support HIV infection and replication, and can transfer HCV to permissive cells. There is a known correlation between HIV infection and HCV viral load (Thomas et al, 2000; Matthews-Greer et al, 2001) and we hypothesize that HIV infected T cells may be able to deliver more HCV to target cells via trans-infection. Therefore we investigated whether the presence of HIV infection altered HCV trans-infection.

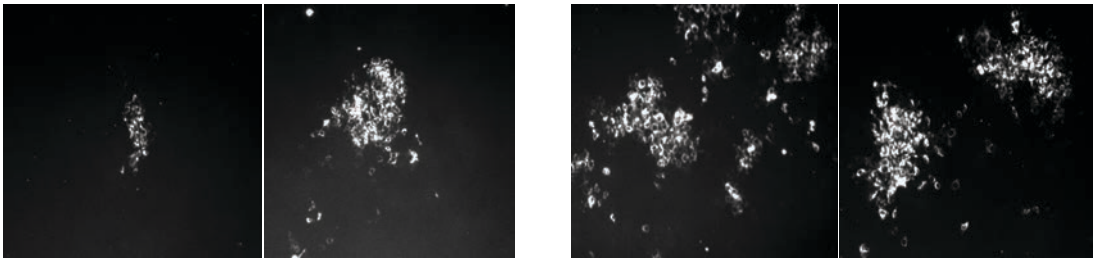
SupT1 cells were infected with HIV (NL4.3 X4 tropic strain) for 72 hours. HIV infection was confirmed by intracellular p24 staining and p24 ELISA, as described. 1.5% of cells were intracellular p24 positive, and mean extracellular p24 levels were 25ng/ml at 72 hours post infection. Cells were washed to remove inoculating virus and incubated with HCV (SA13) or media for 2 hours. Cells were washed again to remove cell free virus, and T cells co-cultured with human hepatoma cells for 48 hours to allow trans-infection. Infected target cells were detected by NS5A staining as described. **Figure 5.11A** shows representative images of wells with NS5A positive cells. **Figure 5.11B** shows mean foci per well. T cells that had been incubated with HIV were associated with higher levels of HCV trans-infection than control cells. To ensure that HIV itself was not altering the antiviral activity of T cells as shown in **Figure 5.7**, T cells were infected with HIV and used to trans-infect HCV onto LucA2 replicon cells. Uninfected T cells and HIV infected T cells that were not incubated with HCV were used as controls. **Figure 5.11C** shows that HIV infected and uninfected

T cells have an equal antiviral effect on HCV replication. These results confirm that HIV infection can boost HCV trans-infection mediated by T cells.

Results were confirmed with primary CD4+ T cells. CD4+ T cells were isolated from 4 anonymous donors. 72 hours post infection with HIV, T cells were used to trans-infect HCV to permissive Huh-7. HIV infection was confirmed by p24 ELISA. All donors showed detectable p24 in the supernatant (**Figure 5.12A**). Results show that primary CD4+ T cells incubated with HIV also promoted HCV transfer to hepatoma cells, although the magnitude of the effect was donor dependent (**Figure 5.12B-E**) but did not correlate with p24.

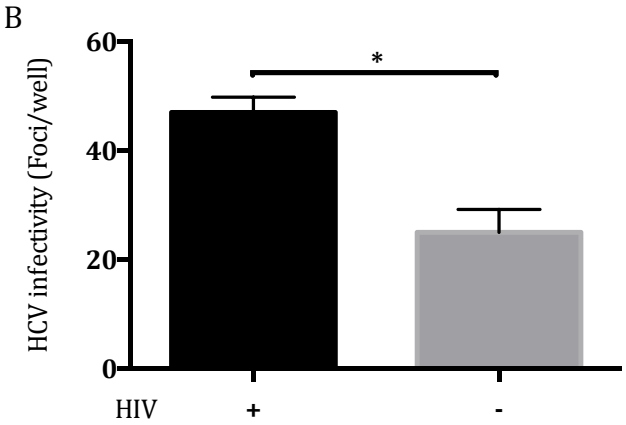
In summary, SupT1 and primary CD4+ T cells infected with HIV-1 can increase HCV trans-infection of hepatoma cell lines.

A



-HIV +HIV

B


HCV infectivity (Foci/well)
60
40
20
0
*
HIV
+
-

C

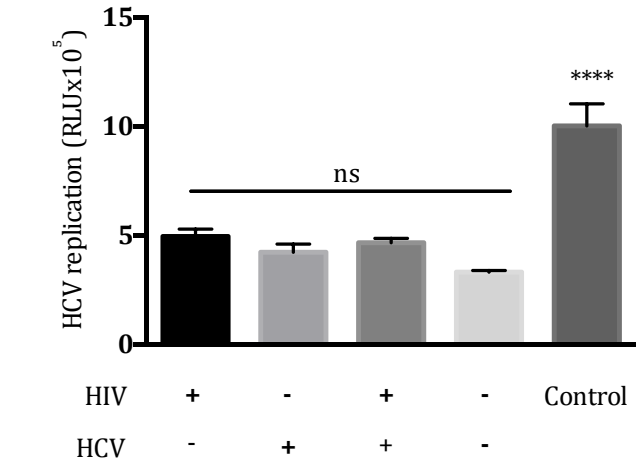

HCV replication (RLUx10^5)
15
10
5
0
ns
****
HIV + - + - Control
HCV - + + -

Figure 5.11 HIV infected T cells boost HCV trans-infection

SupT1 cells were infected with NL4.3 (p24 20ng/ml) for 12 hours, prior to washing and then incubated for a further 72 hours. Cells were then washed and used in T cell trans-infection as previously described. SupT1 cells not exposed to HIV were used as a control. 48 hours post trans-infection wells were washed and stained for NS5A. (A) Fluorescent microscopy showing duplicate representative wells staining for NS5A at 600,000 T cells per/ml in a 48 well plate (x100). (B) NS5A positive foci per well (600,000 T cells/ml). Results are means of duplicate wells and representative of three independent experiments. (C) SupT1 cells +/- HIV were used in a trans-infection onto LucA2 replicon cells. Control cells not incubated with HCV were included. 48 hours post co-culture cells were lysed and replication assessed by luciferase activity . Results are means of duplicate wells and representative of three independent experiments. All results were analysed using a students t-test. Results for the replicon were compared against untreated LucA2 (control) **** $p \leq 0.0001$ * $p \leq 0.05$ ns=not significant.

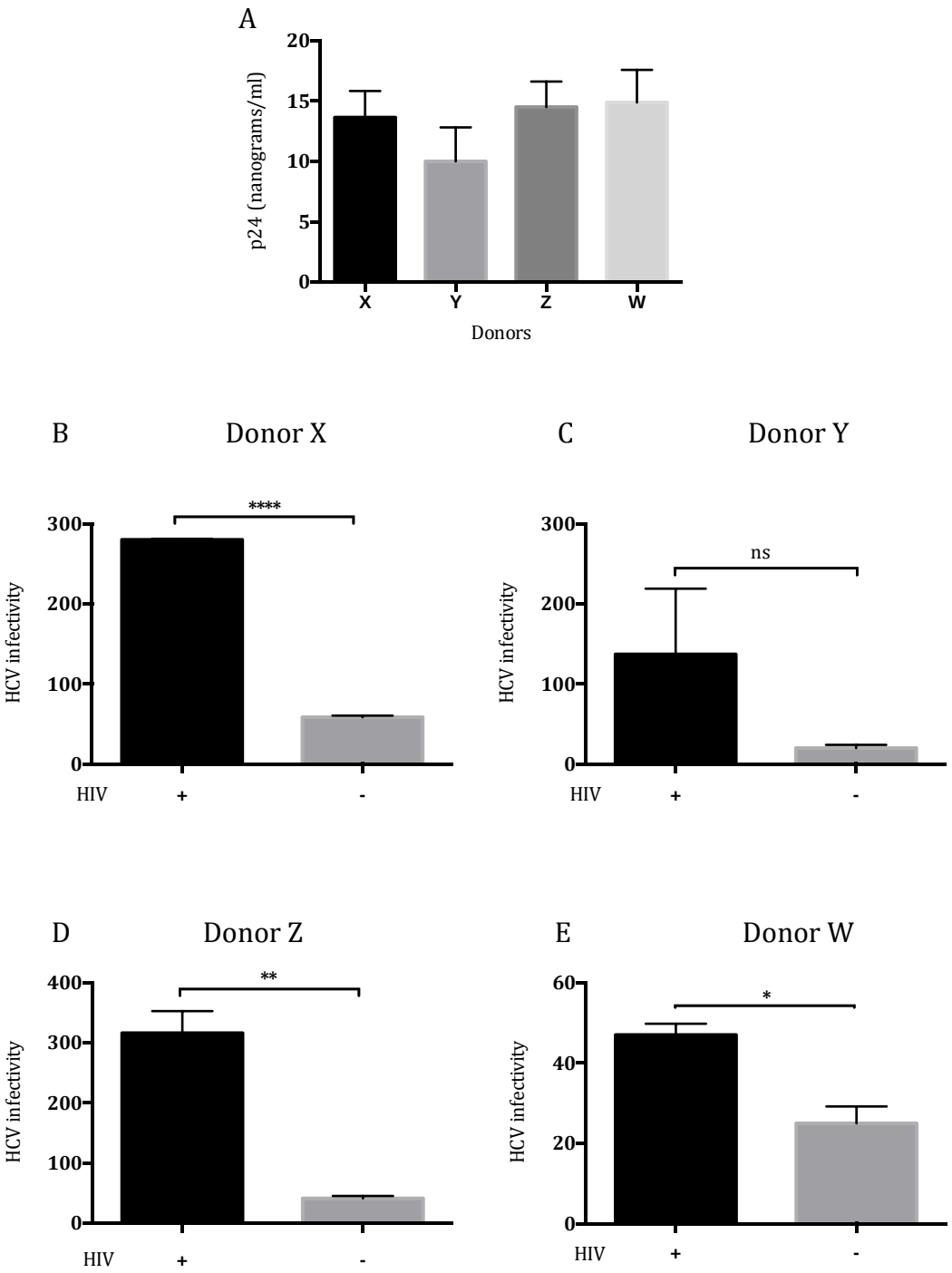


Figure 5.12 HIV infected CD4+ T cells boost HCV trans-infection

CD4+ T cells were extracted from whole blood from 4 anonymous donors (X,Y,Z,W) as previously described, activated with CD3/CD28 and maintained in media containing IL2. 5 days post isolation cells were infected with NL4.3 (20ng/ml p24) for 12 hours, then washed and used in a trans-infection 72 hours later. (A) Supernatant was taken 72 hours post infection and extracellular p24 measured by ELISA. (B-E) 48 hours post co-culture Huh-7 cells were stained for NS5A. Results are NS5A positive foci per well and means of triplicate wells in duplicate experiments. Statistical analysis was using students t-test **** p≤0.0001 **p=≤0.01 *p≤0.05 ns=not significant

5.7 : HIV co-receptor engagement is not required for HCV transfer

Results have shown that HCV trans-infection is boosted by the presence of an X4 tropic HIV-1 strain. We wanted to investigate the mechanism behind HIV boosted HCV transfer by T cells.

Results so far have all been with an X4-tropic virus. HIV-1 uses both X4 and R5 as major co-receptors for viral entry and viral tropism is associated with clinical deterioration in patients (Clapham et al, 2001). We therefore investigated whether HCV delivery by a T cell was altered by co-receptor use. T cells (SupT1) were infected with pNL4.3 (X4) and BAL (R5) for 48 hours and then used for trans-infection as described. **Figure 5.13A** shows NS5A positive foci per well. HIV infectivity was confirmed with supernatant p24 ELISA 48 hours after infection (**Figure 5.13B**). Results indicate that both X4 and R5 tropic virus are associated with boosted HCV delivery and the effect is not co-receptor dependent.

To confirm this the HIV pseudoparticle system was used. Pseudoparticles harbouring the pnL4.3 genome with a luciferase reporter and with HIV envelopes ADA (R5) or LAI (X4), VSV envelope or no envelope (NE) control were used to infect SupT1 cells for 48 hours. Cells were then used in a trans-infection. Infection of T cells was confirmed by quantifying luciferase. Interestingly VSV, LAI and BAL all mediated a boost in HCV transfer (over NE control) (**Figure 5.14**). The amount of HCVcc transferred was comparable, even by VSVpp, and significantly more than uninfected SupT1 cells. Overall these results indicate that

the presence of a HIV genome, with a single round of replication can mediate the effect, independent of route of viral entry.

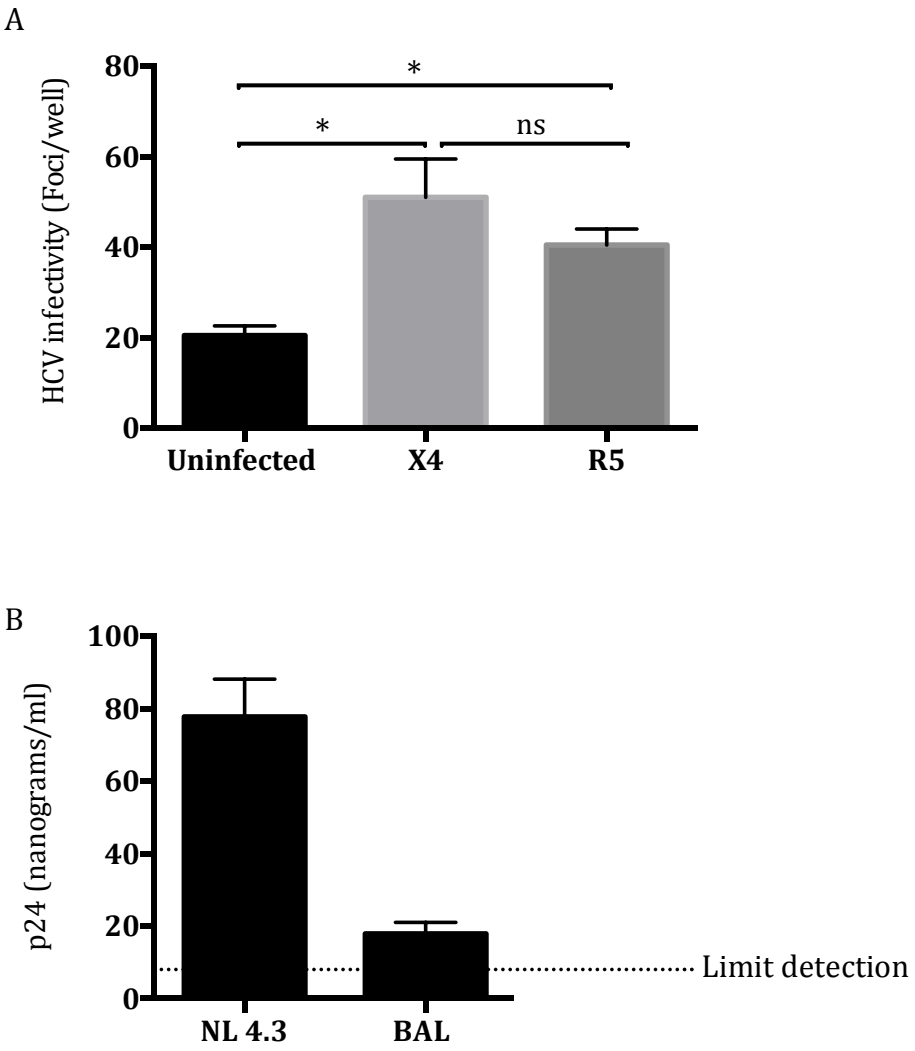


Figure 5.13 X4 and R5 tropic HIV can boost T cell trans-infection of HCV

(A) SupT1 cells were infected with pNL4.3 (X4 tropic HIV-1) and BAL (R5 tropic HIV-1) as described. 72 hours post infection, cells were used to transfer HCVcc to Huh-7 hepatoma cells as described. Huh-7 were stained for NS5A after 48 hours and foci per well plotted. Results are means of triplicate well and representative of two independent experiments. Results were compared to uninfected cells and analysed by students t-test *p=≤0.05 ns=not significant (B) p24 was measured by ELISA from supernatant taken at 48 hours post infection.

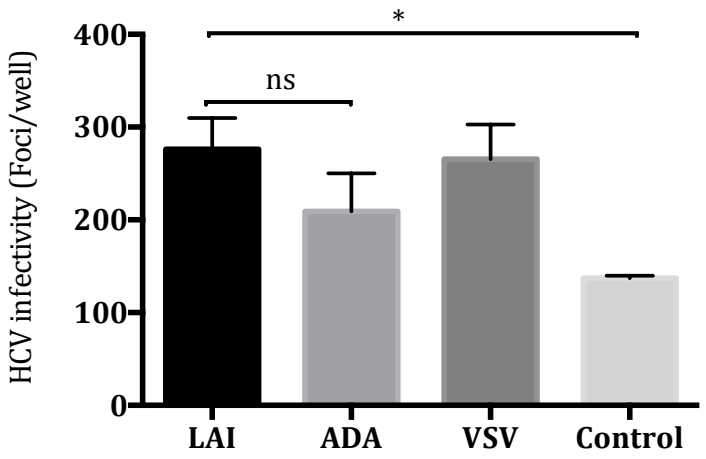


Figure 5.14: HIV pseudoparticle infected T cells can boost HCV transfer to hepatoma cells

SupT1 cells were infected with LAIpp, ADApp (1:2 dilution), VSVpp and No Envelope (NE) control (1:100 viral dilution) for 48 hours. Cells infected with pseudoparticles or control (NE) were used in a T cell trans-infection as described. HCV infection was quantified using NS5A staining and number of positive foci per well was plotted. Results are means of duplicate wells and representative of two independent experiments. Pseudoparticle infection was confirmed by luciferase and was comparable for HIV-1 and VSV envelopes. Analysis is by students t-test and compared to control (NE) *p≤0.05 ns=not significant.

5.8 : Replicating HIV is required for boosted HCV transfer

Results so far have shown that HIV infection, in a small number of T cells, can mediate increased HCV delivery via T cell trans-infection. This is independent of HIV-1 co-receptor engagement. We therefore wanted to investigate whether HIV replication is required.

The ACH2 cell line, a model of HIV latency (Clouse et al, 1989; Folks et al, 1989), was used to investigate whether latently infected T cells can transfer HCVcc. ACH2 cells can become active with the addition of TNFα or PMA (10nM) and rapidly start replicating HIV-1. This model was used to assess whether the amount of replicating intracellular HIV has an effect on HCV transfer. **Figure 5.15A** and **B** show the percentage of cells positive for intracellular p24 within a culture of activated ACH2 cells at 24 hours post activation. There was baseline p24 positivity in ACH2 cells of around 15% and this increased markedly with both PMA and TNFα treatment.

ACH2 cells were then used to trans-infect HCVcc following activation. ACH2 cells were activated with TNFα and used to trans-infect Huh-7 with HCV at 24 hours post activation. 48 hours post co-culture Huh-7 were fixed and stained for NS5A positive foci (**Figure 5.16A**). There was significantly more HCV trans-infected in activated compared to latently infected ACH2. Control cells (SupT1) were used alongside, showing that the addition of TNFα to T cells alone did not alter HCV transfer or infectivity (**Figure 5.16B**). Results indicate that HCV

transfer may be related to the amount of replicating HIV present within the transferring T cell.

In order to confirm this observation SupT1 cells were infected with HIV and then cured using the anti-viral Zidovudine (AZT). SupT1 were infected with HIV (NL4.3) then treated with AZT (4 nM) to inhibit HIV replication (Mitsuya et al, 1985. Broder, 2009). Cells were washed and incubated with HCV as previously described prior to co-culture with hepatoma cells for 48 hours. Cells were fixed and HCV infectivity quantified by enumerating NS5A positive cells (**Figure 5.17A**). SupT1 infected with HIV have boosted HCVcc transfer, which is reduced to control levels when treated with AZT. Controls confirm that AZT itself is not having an effect on the HCVcc transfer. Supernatant from T cells were taken at the end of the experiment to confirm that p24 was significantly reduced in cells treated with AZT (**Figure 5.17B**).

In order to show that HIV itself is not having an effect on HCVcc replication in permissive cells, HIV (pnL4.3) was added (20ng/ml) to an end-point dilution of SA13 titrated onto permissive Huh-7. **Figure 5.17C** shows that the presence of HIV alone does not have an effect on HCVcc infectivity. To confirm that HIV infection of T cells is not altered by the presence of HCV in the media, HIV infected T cells were incubated with HCVcc for 48 hours. Supernatant taken at the end of 48 hours shows no difference in p24 production and therefore the presence of HCVcc does not alter HIV infectivity of SupT1 cells (**Figure 5.17D**).

Overall these results show that increased HCV transfer to a permissive cell line requires replicating HIV to be present in the T cell. Replicating HIV in a small number of T cells is required for boosted HCV delivery to permissive cells.

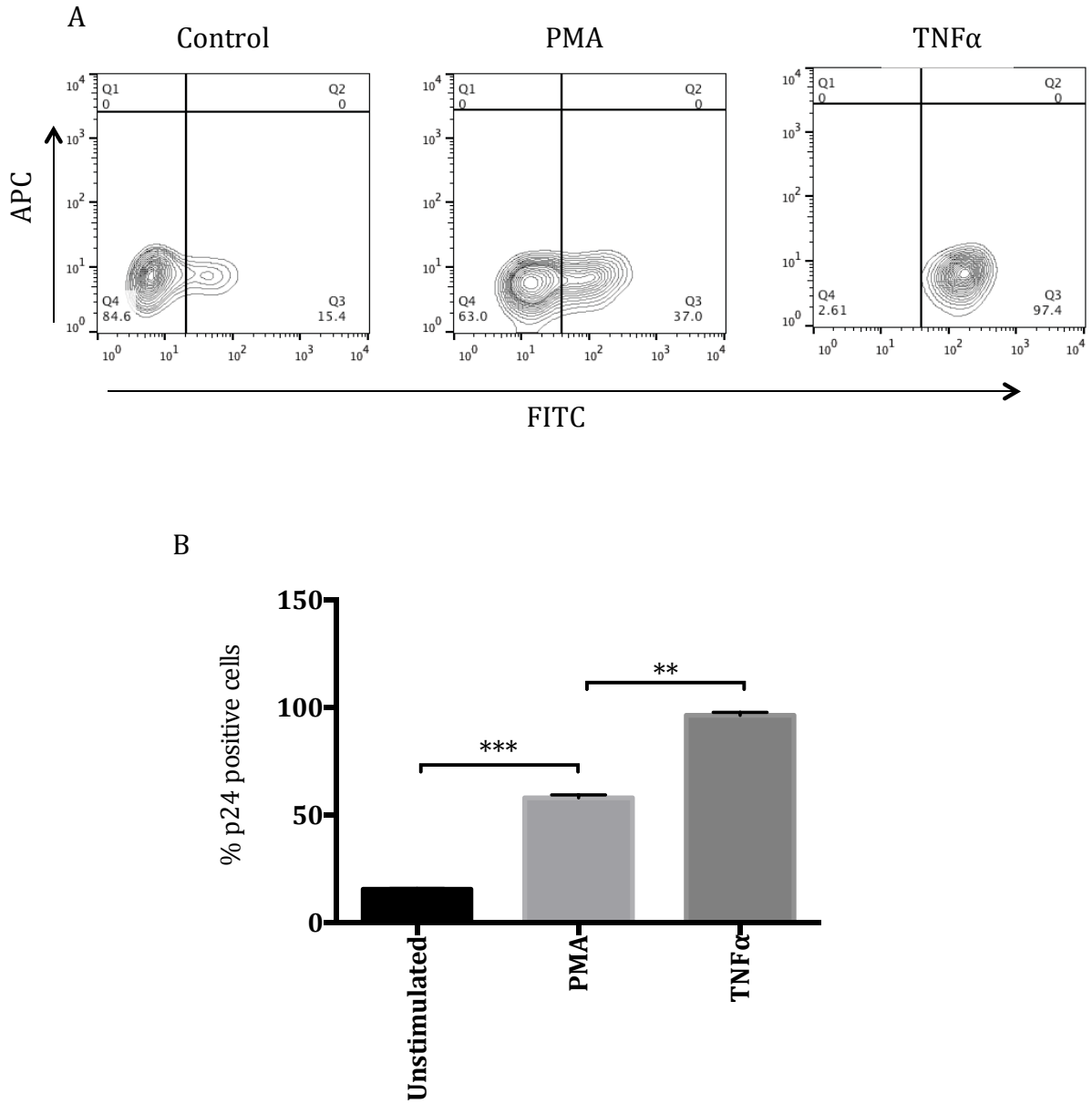


Figure 5.15 ACH2 cells can be activated and replicate HIV

(A) ACH2 cells were maintained in RPMI and activated with PMA (10nM) or TNFα (10ng/ml). 24 hours post activation cells were washed and stained for intracellular p24 as described. Cells were acquired by Flow Cytometry and gated on live, CD4 positive cells. (B) Graphical representation of the cells in (A) shows the percentage of p24 positive cells by activation status. Results are representative of three independent repeats and means of triplicate wells with a minimum of 20,000 cells per well. Results were analysed by Students t-test ***p≤0.001 ** p≤0.01

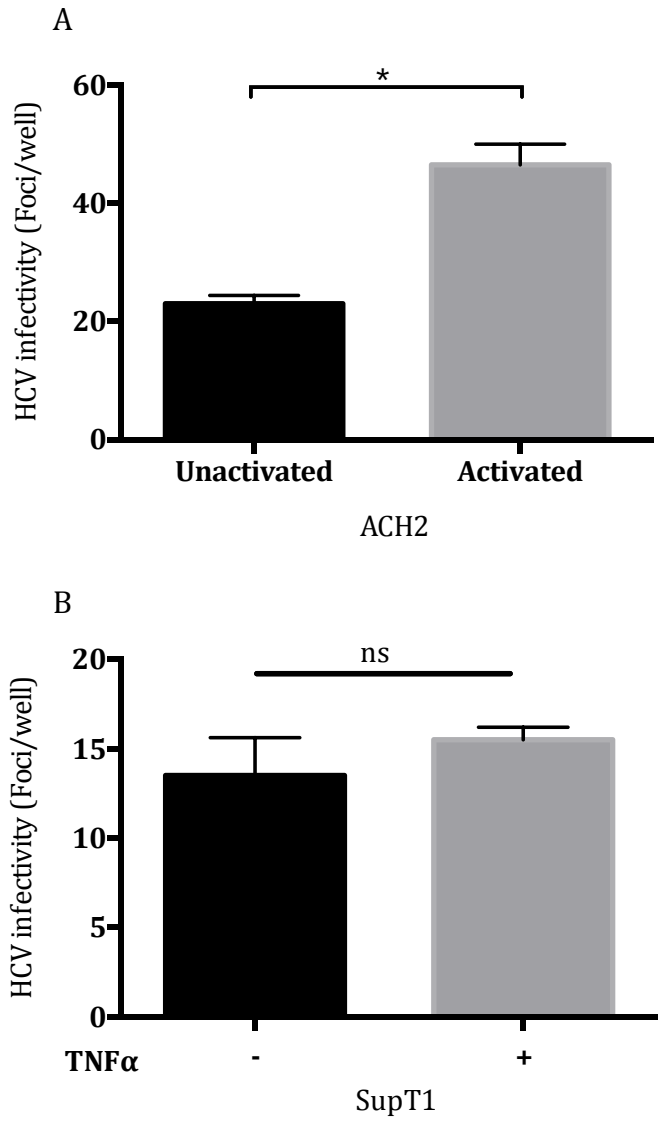


Figure 5.16: Activated ACH2 cells transfer more HCV to Huh-7

(A) ACH2 cells or (B) SupT1 control cells were activated with TNFα (10ng/ml). 24 hours post activation cells were washed and used in an HCV trans-infection as described, using 600,000 T cells/ml in a 48 well plate onto Huh-7. 48 hours post co-culture Huh-7 were washed fixed and stained for NS5A. Results are means of duplicate wells and two independent experiments. Results are analysed using the students t-test *p≤0.05, ns=not significant.

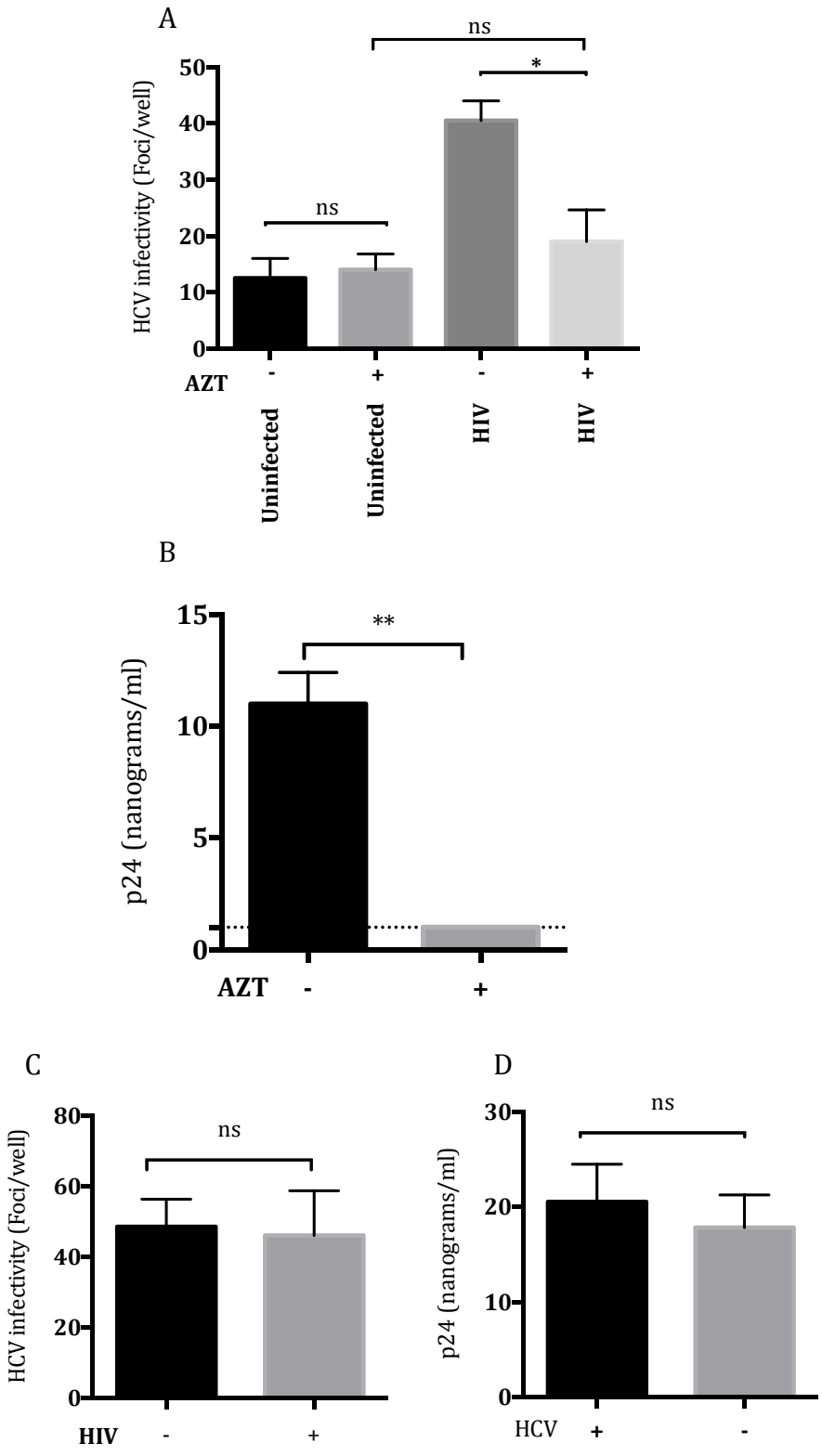

A
ns
*
ns
HCV infectivity (Foci/well)
50
40
30
20
10
0
AZT
-
+
-
+
Uninfected
Uninfected
HIV
HIV
B
**
p24 (nanograms/ml)
15
10
5
0
AZT
-
+
C
ns
HCV infectivity (Foci/well)
80
60
40
20
0
HIV
-
+
D
ns
p24 (nanograms/ml)
30
20
10
0
HCV
+
-

Figure 5.17: Ablation of HIV replication results in HCV transfer at the level of control

A) SupT1 cells were infected with HIV for 48 hours as described. Cells were then washed and incubated with AZT (at 4nM) for 48 hours. Cells were then used in a HCV trans-infection as previously described onto Huh-7. 48 hours post trans-infection cells were fixed and stained for NS5A as described. Results are foci per well (mean of 3 wells 48 well plate) and representative of two independent experiments. (B) Supernatant from AZT treated SupT1 was pooled and p24 quantified using ELISA as previously described. Results are means of duplicate wells and representative of two independent experiments. (C) HCVcc was added to Huh-7 (MOI 0.1)) and HIV (20 ng/ml) was added to duplicate wells. 48 hours post infection cells were fixed and stained for NS5A. Results are means of foci per well. (D) SupT1 infected with HIV for 48 hours were then washed and incubated for 48 hours with SA13 HVCcc (MOI 0.1). Supernatant was taken and p24 was quantified by ELISA. Results are analysed by Students t-test. *$p \leq 0.05$, ns=not significant

5.9: HIV increases HCV capture by T cells

In order to understand the mechanism behind HCV trans infection by T cells containing replicating HIV, HCV capture was investigated.

Stamataki et al (2009) showed that the amount of HCV captured by a B cell directly translates to the amount of HCV delivered to a permissive cell. Furthermore, robust work has shown that PBMC cannot be infected with HCV (Laskus et al, 2007; Blackard et al, 2007; Marukian et al, 2008). However many authors have shown HCV associated with PBMC (Qian et al, 1992; Zignego et al, 1992; Wang, et al, 1992; Muller et al, 1993; Azzari et al, 2000), which may represent HCV capture but not infection. Therefore SupT1 cells were infected with HIV (pNL4.3) as previously and then incubated with HCVcc (SA13:JFH) for 2 hours. Cells were then fixed and HCV RNA load quantified by qPCR. Interestingly, results show that cells infected with HIV were associated with significantly more HCV RNA than cells that were not infected with HIV (**Figure 5.18**). Duplicate plates in this experiment were used for T cell trans-infection (see **Figure 5.17A**) to confirm that increased RNA correlated with increased HCV transfer.

HIV infection of T cells associate with significantly increased levels of HCV RNA than uninfected cells. This is likely to represent HCV carriage and is likely to be a significant mechanism when considering the way in which HCV is delivered to naïve hepatoma cells in the presence of HIV.

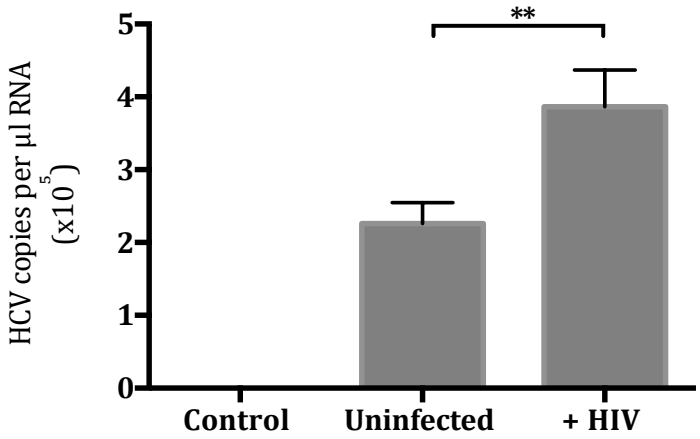


Figure 5.18 HIV infected T cells capture more HCV

SupT1 cells were infected with pNL4.3 (20ng/ml) for 48 hours. Cells were then washed and incubated with SA13 HCVcc as described for 2 hours (as per trans-infection). HIV infected SupT1 and control uninfected cells were washed x5 to remove any non-bound HCVcc. RNA was extracted (Qiagen) and HCV RNA quantified by qPCR and normalised to GAPDH. Results are mean of six wells and representative of three independent experiments. Duplicate plates were used in a T cell trans-infection. Results were analysed with Students t-test ** p=<0.01

5.10 : Discussion

Work throughout this chapter aimed to show that HCVcc could be transferred by a CD4+ T cell and used to infect a permissive cell (trans-infection). Results confirm that all T cell lines tested, in addition to activated primary CD4+ T cells, were able to transfer HCVcc (late adapted SA13 virus) to Huh-7 cells (**Figures 5.4 and 5.5**). T cells were repeatedly washed after incubation with HCVcc to remove the presence of any cell free virus and negative controls were included in all experiments (outlined in **Figure 5.1)** in order to confirm that the virus transferred was cell-associated and not cell-free. HCVcc infection of Huh-7^{diff} using T cell transinfection was significantly reduced compared to Huh-7. T cell delivered virus is not able to overcome the inherent restrictions when Huh-7 are differentiated, thereby suggesting that efficient cell-to-cell delivery shown in previous chapters is dependent on the type of cell used. Furthermore, T cell transfer is less likely to be an important mechanism within the liver of viral dissemination and spread. However, we have shown that T cells are able to associate virus and deliver to permissive cells, which may be important in setting up distant sites of infection.

Results also show that HCV transfer and infectivity occurred even in the presence of an anti-viral **(Figure 5.7)** and cytotoxic **(Figure 5.2)** effect of high T cell numbers. We observed a direct effect of the activated CD4+ T cells and T cell lines on the monolayer, with adherent cells leading to monolayer breakdown. CD4+ T cells can have a direct cytotoxic effect, through cell contact mediated apoptosis (Fas/Fas Ligand) (reviewed in Green and Ferguson, 2001) or through

release of toxic effector molecules such as perforin or granzyme resulting in target cell death (Trapani and Smyth, 2002). We did not investigate the mechanism behind this, however noted that T cell lines had much greater effect than primary cells on direct cytotoxicity. T cell from immortalised cell lines were much larger than primary cells and may have had more cell contact mediating direct cell toxicity.

In addition we noted that CD4+ primary T cells and T cell lines had a significant anti-viral effect, inhibiting HCV replication in a dose dependent manner **(Figure 5.7A)**. Direct cell contact was necessary as supernatant did not have the same effect (**Figure 5.7B**). However, primary cells do appear to secrete anti-viral mediators into the supernatant that have a modest effect on replication and may explain why primary cells have a much larger effect on replication than T cell lines. This difference may also be explained by innate immune gene expression between cell lines and primary cells. SupT1 cells, for example downregulate TLR7/8 and are unable to produce IL-6 in response to TLR stimulation, a pathway that is intact in primary cells (Rausell et al, 2016). Remarkably, HCVcc is able to trans-infect permissive cells and establish a spreading infection, despite the direct anti-replicative effect. Understanding this further may be important in identifying therapeutic targets for modulating T cell mediated trans-infection.

We then went on to show that HIV-1 infection, using primarily an X4 tropic virus boosts T cell trans-infection using a T cell line and primary CD4+ T cells (**Figure 5.11**). There was inter-experimental variability and donor

variation, particularly in the amount of HCV transferred; however the presence of HIV boosted this in all experiments. This occurred even with very low numbers of T cells within the culture being infected with HIV (**Figure 5.9**), although numbers of infected cells are comparable to that seen *in vivo*, even in the presence of high viraemia (Walker and McMichael, 2012; Gadol et al, 1994). This makes the results applicable to an *in vivo* setting.

Although we have consistently seen that HCV transfer is boosted in the presence of HIV, it is not possible to tell exactly which cells are mediating this effect. Given that such small numbers of cells are infected with HIV, it is likely that uninfected T cells are also able to transfer HCV. Pathogenic infections of human CD4+ T cells are characterised by generalised immune activation, including TNFα, IFNγ and IL-6 release (Rausell et al, 2012) leading to progressive CD4 T cell depletion (Sodora and Silvestri, 2008). Therefore, mediators released into the supernatant from HIV infected cells are likely to act more widely. Studies of HIV-exposed but not infected individuals have shown that HIV-1 specific T cells exist (Ritchie et al, 2011β). Therefore it is likely that the presence of replicating HIV infection has effects on many cells within the culture. This requires further investigation.

Interestingly, results show that co-receptor engagement is not essential, but presence of replicating HIV is important for HIV boosted T cell transfer. A pseudoparticle system with pNL4.3 genome and differing HIV envelopes (HIV R5, HIV X4) and non-HIV (VSV) all boosted HCV transfer (**Figure 5.14**), suggesting that replicating HIV is important, as VSVpp was associated with

increased HCV trans-infection. Further results show that ablating replication, within cells that had been infected with HIV and then cured, also reduced HCV transfer to a level of control (**Figure 5.17**). Therefore HCV transfer requires replicating HIV.

Lastly, we were able to show that T cells that have been exposed to HIV are associated with increased amounts of HCV RNA (**Figure 5.18**). We did not investigate whether these cells had been infected with HCV, however previous studies have shown that lymphocytes are not amenable to infection (Marukian et al, 2008). Therefore it is likely that HCV can associate with a T cell and is bound to the surface. Natarajan et al (2010) showed that HCV is carried on the surface of T cells, but also on NK cells, monocytes and B cells in co-infected patients. Elucidating the mechanism would be the next step. Stamataki has shown that cell surface LDL receptor and CD81 are important in HCV capture (unpublished data) and CD81 expression on T cells is altered by HIV infection (Meroni et al, 2007). Future work should include investigating the role of the tetraspanin CD81 and LDL R in HCV capture in HIV infected T cells.

In summary this chapter has described HCV trans-infection mediated by CD4+ T cells to a permissive hepatoma cell line. HCV transfer is boosted by HIV exposed T cells, and the presence of replicating HIV is important. However, there are significant limitations associated with this work so far. Much of the data is descriptive and requires more investigation to identify the mechanisms involved. The current inability to identify the exact cells that are HIV infected and then go on to transfer HCV on their surface makes investigating this particularly

challenging. We have not characterized the T cells at all or looked at the signaling pathways set up within the cells on exposure to HIV/HCVcc. We have also not used patient derived virus or T cells isolated from HIV positive individuals to see whether the effect can still be demonstrated. It would also be interesting to show that HCV transfer can occur in the presence of anti-HCV neutralising antibody. We would also like to further investigate the alterations in cellular signaling both within the T cell and the target cell when HCV is delivered in this way. Despite the shortcomings, we have robustly shown that T cells can transfer HCVcc and the presence of HIV impacts this significantly.

These novel results provide a possible insight into HCV access to distant sites *in vivo*, a model that is used by other viruses, such as HIV (van Montfort, 2007). Similarly, other cells bearing CD4 on their cell surface, such as human placenta, may also be able to transfer HCV, offering insight into maternal to child transmission which is poorly understood, particularly in the context of co-infection.

Chapter 6: Discussion and Future Work

6.1 : General Discussion

The data presented in this thesis describes the development of cell models for the study of HCV, viral host interactions and HCV/HIV co-infection.

We initially describe how the addition of DMSO to a human hepatoma cell line is able to drive the cell into a differentiated state. These cells are readily available and are easy to differentiate and maintain. The addition of DMSO to drive differentiation appears to effect multiple and broad ranging changes within the cell, many of which are known to interact with the HCV lifecycle. Huh-7^{diff} upregulate key hepatic enzymes in vivo, including enzymes involved in drug metabolism, and therefore provide a functional xenobiotic model. Despite slowed cell cycle progression they are not metabolically quiescent, showing evidence of altered glucose metabolism. Cells also show significant upregulation of candidate micro-RNAs, which are likely to have complex interactions with virus and host.

We went on to show that Huh-7^{diff} are able to support the entire HCV lifecycle, although are significantly less permissive than undifferentiated hepatoma cell lines. Unlike other available differentiated models for studying HCV replication, human hepatoma cells can support HCV entry, replication and egress, without the exogenous addition of key cellular factors. Differentiated cells, therefore, can give insight into the likely viral lifecycle within the human liver. Interestingly, the reduction in permissivity seen was a compound effect of

reduced early viral replication and reduction of specific infectivity of the released viral particle.

Despite identifying key steps in the viral lifecycle that were altered by differentiation we were not able to define the mechanism behind this. Candidate cellular factors that are known to alter viral infectivity, including miR122 (Jopling et al, 2005; Wu et al, 2012) and SEC14L2 expression (Saeed et al, 2015) do not alter permissivity in differentiated hepatoma cells. Altered innate signaling pathways in hepatoma cells are key to the ability of these cells to support HCVcc (Sumpter et al, 2005). We were able to show that differentiation induced the expression of TLR3 and IL29 in response to agonists and HCVcc. However, there was no detectable IL29 protein and we could not robustly confirm that the altered innate pathways impacted viral replication. However, interestingly, these cells appear to respond to HCVcc and can provide a model for studying the host immune response.

Cellular differentiation also reduced specific infectivity of the released viral particle. We confirmed these results functionally, however did not investigate the further mechanisms behind this. The HCV particle is closely associated with apolipoproteins, particularly apoE, apoB, apoA1 and apoC (Catanese et al, 2013; Chang et al, 2007; Gastaminza et al, 2008; Meunier et al, 2008) and are particularly rich in ApoE (Merz et al, 2011). ApoE is important for infectivity of the viral particle (Chang et al, 2007). Hepatoma cells are known to have defective lipoprotein biogenesis (Jammart et al, 2013) and this may be altered by cellular differentiation, since we have shown that other metabolic

pathways can affected. Furthermore, the density of HCVcc *in vitro* is lower than that found *in vivo*, likely due to associations with lipoproteins (Lindenbach et al, 2006). Viral assembly and egress is closely related to the lipoprotein metabolic pathways (Popescu et al, 2014) and DMSO is known to affect cellular lipid and results in a reduction in lipid accumulation within hepatoma cells (Song et al, 2014). Therefore investigating lipid metabolism and the lipid content of the released virion from differentiated cells would be important to consider. The VLDL pathway, despite being involved in the secretion of infectious viral particles, is interestingly redundant in cell-cell spread in Huh-7^{diff} (Barretto et al, 2014). It is therefore possible that alterations in the VLDL pathway in differentiated cells allow the virus to spread efficiently cell-cell but inhibit cell-free spread.

The reduced permissivity of differentiated cells to support HCV replication is overcome when the virus is delivered cell-to-cell. Many viruses use cell-to-cell spread as it can be efficient way of infecting neighbouring cells, whilst protecting the virus from the host immune response (Sattentau, 2105). Differentiated hepatoma cells would provide an excellent model for studying viral dissemination route and host responses. We went on to explore the possibility that HCV could be delivered to a human hepatoma cell via cell-to-cell transmission, mediated by another cell type such as a T cell. We showed that T cells could deliver HCV to permissive cells, although they were not able to overcome the restriction in Huh-7^{diff} suggesting that cell type is important in the mechanism of cell-to-cell delivery. CD4+ T cells or T cell lines infected with HIV can transfer more HCV to a permissive cell line. This is independent of receptor

engagement or soluble mediators, but requires replicating HIV. T cells appear to associate with more HCV on their surface when exposed to HIV, although it is not clear whether cells themselves need to be infected with HIV for this effect to occur. The mechanism behind this requires further investigation. Figure 6.1 provides a model of known and proposed modes of HCV transmission based on findings presented in this thesis.

The observation that virus can associate with immune cells is biologically relevant as it raises the possibility that HCV can use immune cells to spread to distal sites. One particular area of interest is in mother to child transmission. The mechanism behind HCV maternal to child transmission has not been clearly defined (Le Campion et al, 2012) but HIV co-infection is associated with significantly increased risk of HCV transmission (Gibb et al, 2000; Marine-Barjoan et al, 2007). HCV itself is unable to infect trophoblasts (Le Campion et al, 2012; Rajgor, personal communication), which are foetal derived cells that form the feto-maternal interface. However, trafficking of maternal immune cells, including T cells, across the placenta has been well described (reviewed in Jeanty et al, 2014). Association of HCV with cells that are directly transferred across the placenta from mother to child is a novel concept in vertical transmission. The finding that HIV co-infection increases the quantity of HCV that is transferred is a biologically plausible mechanism and provides targets for therapeutic intervention.

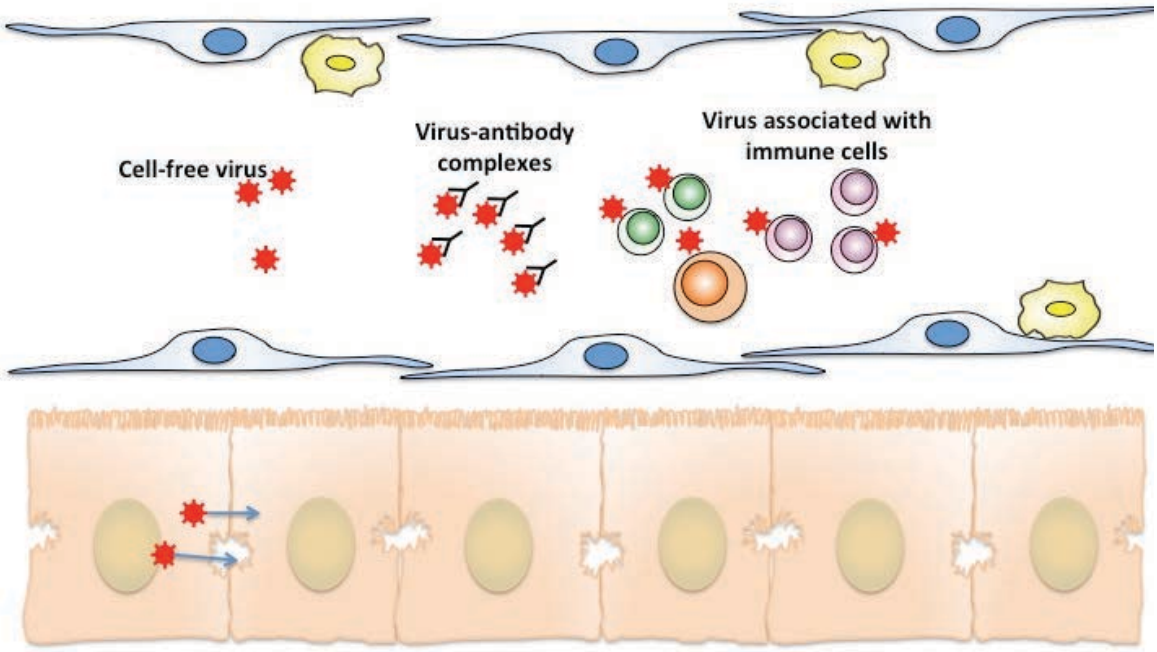


Figure 6.1 Model of modes of viral transmission

Model of current proposed mechanisms of HCV dissemination and spread, including cell-free and cell-to-cell disseminations. Virus may use immune complexes or cells to disseminate to distal sites causing damage, infection or viral transmission.

6.2: Future work

This thesis has described the development of a differentiated cell system, that supports HCV infection but at lower levels than in standard hepatoma cells. There are restrictions in early viral replication and specific infectivity of the released viral particle, which can be overcome by cell-to-cell delivery. Despite changes in many key candidate cellular factors we have not been able to elucidate the exact mechanism behind this and therefore requires further investigation.

Much of the work described is transcript data, and it would be pertinent to look at protein expression and location, particularly in relation to innate signaling. Much recent work has highlighted the importance of IL29 in cellular response to HCV (reviewed in Breuning et al, 2017). The expression of TLR3 mRNA at detectable levels in differentiated cells, which are absent in hepatoma cells (Li et al, 2005) is a potentially important finding. TLR3 expression is central in viral recognition and downstream innate signaling. Therefore investigating protein levels and location in differentiated cells would be a useful additional step. Additionally there are candidate ISG's, particularly ISG15 (Kim et al, 2000; Schneider et al, 2014) that are known to directly inhibit HCV replication. Results so far would suggest that further understanding of innate signaling in Huh-7^{diff} would be important, not only in investigating HCV replication, but also in utilizing these cells as a model with intact innate signaling pathways.

The effect of differentiation of the production of HCV lipoviral particle is also important, and has not yet been studied. Future work should include assessing the buoyant density of the released viral particle and the ability of the isolated released particle to then enter and replicate in differentiated cells. We have looked at the viral lifecycle of cell adapted HCVcc but not the virus once it has been released from differentiated cells. Given the importance of lipoproteins in viral entry (Popescu et al, 2012; Jiang, et al 2012) this would be important to consider.

Much of the viral work so far has been using laboratory adapted SA13/JFH and should be expanded to include other viral strains, and patient derived virus. Differentiated cells express SEC14L2, which was recently identified as the missing cellular factor that allowed hepatoma cells to support HCV from patient sera (Saeed et al, 2015). It would be interesting to look at whether differentiated cells were able to support patient derived virus.

We have shown that Huh-7^{diff} are a good model for cell-to-cell viral transmission, which is an efficient mode of viral dissemination (Meredith et al, 2013) and may represent the predominant route of viral spread *in vivo* (Carloni et al, 2012). We used newly infected Huh-7 as 'producer cells'; in order to better model the *in vivo* environment, chronically infected Huh-7^{diff} could be utilized as producer as well as target cells. We were able to show that virus can also be transmitted in association with other cell types, although the mechanism behind this is likely to be different; virus is delivered from the surface of a T cell rather than directly cell-to-cell. It would be interesting to investigate whether T cells

are able to deliver virus in the presence of anti-HCV neutralizing antibody, as cell-to-cell transmission *in vivo* is thought to evade this response.

The mechanism by which HIV is able to increase HCV trans-infection by CD4+ T cells requires further investigation. Initial steps would be to look at LDLR and CD81 expression on the surface of cells exposed to HIV. These have been shown to be important for HCV capture on lymphocytes (Stamataki, unpublished data). There is evidence that HIV can alter CD81 expression on a T cell (Meroni et al, 2007) and this is a plausible mechanism for HCV capture. We have showed that HIV only infects a small number of cells within the culture, which is likely representative of the number of cells infected *in vivo*(Pace et al, 2012). However, we have not been able to confirm whether it is the cells that are infected that are able to mediate the effect. It is possible that HIV infection of a small number of cells alters the cellular micro-environment allowing a larger number of cells to associate with HCV. We have shown that cell contacts are important, suggesting the cellular changes, rather than released soluble mediators are important for HCV capture. Clearly this is a huge area for future work, and focus initially would be on candidates for cell surface HCV capture. Understanding the T cell changes that are effected by HIV infection/exposure that interact with HCV capture and delivery would be important in going forward.

Furthermore most of the work is with CXCR4 tropic virus. It would be important to confirm the findings with a CCR5 tropic virus. The shift from a CCR5 to a CXCR4 virus is associated with clinical decline and T cell depletion (Naif, 2013), and therefore the finding of increases HCV capture may correlate with

HIV viral tropism. Similarly, it would be interesting to see if T cells isolated from an HIV positive patient were also able to mediate the same effect.

It is not clear whether T cells are the only cells able to transfer HCV to permissive cells. Dendritic cells are particularly important at mucosal surfaces for HIV capture and infection (Wu and KewalRemani, 2006; Lambotin et al, 2010). No work has been done with dendritic cells, or indeed other cells that support HIV infection and that could be used as a model for HCV trans-infection.

Exciting future work in this area would also include modelling T cell trans-infection at sites distant to the liver, such as the human placenta, or blood/brain barrier. Placental models, such as BeWo cells or primary trophoblast cultures could be utilised for studies of HCV transmission. Initial steps would be to look at cell-free and cell-associated viral delivery to placental cells. We would then go on to look at whether T cells were able to cross the placental barrier and trans-infect to permissive cells.

In summary, we have described models that allow the study of viral host interactions and co-infection. Future work based on these findings will allow progress in understanding HCV pathogenesis, dissemination and transmission, particularly in HIV co-infected.

Chapter 7: References

Abdel-Hady, M. et al., 2011. Chronic hepatitis C in children--review of natural history at a National Centre. *Journal of Viral Hepatitis*, 18(10), pp.e535–40.

Agnello, V. et al., 1999. Hepatitis C virus and other flaviviridae viruses enter cells via low density lipoprotein receptor. *Proceedings of the National Academy of Sciences of the United States of America*, 96(22), pp.12766–12771.

Allen-Baume, V., Ségui, B. & Cockcroft, S., 2002. Current thoughts on the phosphatidylinositol transfer protein family. *FEBS letters*, 531(1), pp.74–80.

Amadei, B. et al., 2010. Activation of natural killer cells during acute infection with hepatitis C virus. *Gastroenterology*, 138(4), pp.1536–1545.

André, P. et al., 2002. Characterization of low- and very-low-density hepatitis C virus RNA-containing particles. *Journal of Virology*, 76(14), pp.6919–6928.

Andrus, L. et al., 2011. Expression of paramyxovirus V proteins promotes replication and spread of hepatitis C virus in cultures of primary human fetal liver cells. *Hepatology*, 54(6), pp.1901–1912.

Anggakusuma et al., 2015. Control of hepatitis C virus replication in mouse liver-derived cells by MAVS-dependent production of type I and type III interferons. *Journal of Virology*, 89(7), pp.3833–3845.

Appel, N., Pietschmann, T. & Bartenschlager, R., 2005. Mutational analysis of hepatitis C virus nonstructural protein 5A: potential role of differential phosphorylation in RNA replication and identification of a genetically flexible domain. *Journal of Virology*, 79(5), pp.3187–3194.

Armitage, A.E. et al., 2014. Distinct patterns of hepcidin and iron regulation during HIV-1, HBV, and HCV infections. *Proceedings of the National Academy of Sciences*, 111(33), pp.12187–12192.

Arrighi, J.-F. et al., 2004. DC-SIGN-mediated infectious synapse formation enhances X4 HIV-1 transmission from dendritic cells to T cells. *The Journal of experimental medicine*, 200(10), pp.1279–1288.

Arshad, M., El-Kamary, S.S. & Jhaveri, R., 2011. Hepatitis C virus infection during pregnancy and the newborn period--are they opportunities for treatment? *Journal of Viral Hepatitis*, 18(4), pp.229–236.

Asselah, T. et al., 2008. Liver gene expression signature to predict response to pegylated interferon plus ribavirin combination therapy in patients with chronic hepatitis C. *Gut*, 57(4), pp.516–524.

Azzari, C. et al., 2000. Vertical transmission of HCV is related to maternal peripheral blood mononuclear cell infection. *Blood*, 96(6), pp.2045–2048.

Balagopal, A., Thomas, D.L. & Thio, C.L., 2010. IL28B and the control of hepatitis C virus infection. *Gastroenterology*, 139(6), pp.1865–1876.

Bandyopadhyay, S. et al., 2011. Hepatitis C virus infection and hepatic stellate cell activation downregulate miR-29: miR-29 overexpression reduces hepatitis C viral abundance in culture. *Journal of Infectious Diseases*, 203(12), pp.1753–1762.

Barba, G. et al., 1997. Hepatitis C virus core protein shows a cytoplasmic localization and associates to cellular lipid storage droplets. *Proceedings of the*

National Academy of Sciences of the United States of America, 94(4), pp.1200–1205.

Barretto, N. et al., 2014. Determining the involvement and therapeutic implications of host cellular factors in hepatitis C virus cell-to-cell spread. *Journal of Virology*, 88(9), pp.5050–5061.

Barré-Sinoussi, F., Ross, A.L. & Delfraissy, J.-F., 2013. Past, present and future: 30 years of HIV research. *Nature Reviews Microbiology*, 11(12), pp.877–883.

Bartenschlager, R., Frese, M. & Pietschmann, T., 2004. Novel insights into hepatitis C virus replication and persistence. *Advances in virus research*, 63, pp.71–180.

Barth, H. et al., 2003. Cellular binding of hepatitis C virus envelope glycoprotein E2 requires cell surface heparan sulfate. *Journal of Biological Chemistry*, 278(42), pp.41003–41012.

Bartosch, B. & Cosset, F.-L., 2006. Cell entry of hepatitis C virus. *Virology*, 348(1), pp.1–12.

Bartosch, B. et al., 2003. Cell entry of hepatitis C virus requires a set of co-receptors that include the CD81 tetraspanin and the SR-B1 scavenger receptor. *Journal of Biological Chemistry*, 278(43), pp.41624–41630.

Basu, A. et al., 2002. Hepatitis C virus core protein is necessary for the maintenance of immortalized human hepatocytes. *Virology*, 298(1), pp.53–62.

Bauhofer, O. et al., 2012. Persistence of HCV in Quiescent Hepatic Cells Under Conditions of an Interferon-Induced Antiviral Response. *Gastroenterology*, 143(2), pp.429–438.e8.

Bellecave, P. et al., 2010. Cleavage of mitochondrial antiviral signaling protein in the liver of patients with chronic hepatitis C correlates with a reduced activation of the endogenous interferon system. *Hepatology*, 51(4), pp.1127–1136.

Benga, W.J.A. et al., 2010. Apolipoprotein E interacts with hepatitis C virus nonstructural protein 5A and determines assembly of infectious particles. *Hepatology*, 51(1), pp.43–53.

Bengsch, B. et al., 2010. Coexpression of PD-1, 2B4, CD160 and KLRG1 on exhausted HCV-specific CD8+ T cells is linked to antigen recognition and T cell differentiation. *PLoS pathogens*, 6(6), p.e1000947.

Benhamou, Y. et al., 1999. Liver fibrosis progression in human immunodeficiency virus and hepatitis C virus coinfected patients. The Multivirc Group. *Hepatology*, 30(4), pp.1054–1058.

Benova, L. et al., 2014. Vertical transmission of hepatitis C virus: systematic review and meta-analysis. *Clinical infectious diseases : an official publication of the Infectious Diseases Society of America*, 59(6), pp.765–773.

Billerbeck, E. et al., 2017. Mouse models of acute and chronic hepacivirus infection. *Science*, 357(6347), pp.204–208.

Blackard, J.T. et al., 2007. Compartmentalization of hepatitis C virus (HCV) during HCV/HIV coinfection. *The Journal of infectious diseases*, 195(12), pp.1765–1773.

Blight, K.J., McKeating, J.A. & Rice, C.M., 2002. Highly permissive cell lines for subgenomic and genomic hepatitis C virus RNA replication. *Journal of Virology*, 76(24), pp.13001–13014.

Boisvert, J. et al., 2001. Quantitative analysis of hepatitis C virus in peripheral blood and liver: replication detected only in liver. *The Journal of infectious diseases*, 184(7), pp.827–835.

Braeuning, A. et al., 2006. Differential gene expression in periportal and perivenous mouse hepatocytes. *The FEBS journal*, 273(22), pp.5051–5061.

Briat, A. et al., 2005. Hepatitis C virus in the semen of men coinfected with HIV-1: prevalence and origin. *AIDS (London, England)*, 19(16), pp.1827–1835.

Broder, S., 2010. The development of antiretroviral therapy and its impact on the HIV-1/AIDS pandemic. *Antiviral research*, 85(1), pp.1–18.

Bruening, J., Weigel, B. & Gerold, G., 2017. The Role of Type III Interferons in Hepatitis C Virus Infection and Therapy. *Journal of immunology research*, 2017, p.7232361.

Carloni, G. et al., 2012. HCV infection by cell-to-cell transmission: choice or necessity? *Current molecular medicine*, 12(1), pp.83–95.

Castoldi, M. & Muckenthaler, M.U., 2012. Regulation of iron homeostasis by microRNAs. *Cellular and molecular life sciences : CMLS*, 69(23), pp.3945–3952.

Catanese, M.T. et al., 2010. Role of scavenger receptor class B type I in hepatitis C virus entry: kinetics and molecular determinants. *Journal of Virology*, 84(1), pp.34–43.

Catanese, M.T. et al., 2013. Ultrastructural analysis of hepatitis C virus particles. *Proceedings of the National Academy of Sciences*, 110(23), pp.9505–9510.

Chang, J. et al., 2008. Liver-specific microRNA miR-122 enhances the replication of hepatitis C virus in nonhepatic cells. *Journal of Virology*, 82(16), pp.8215–8223.

Chang, K.-S. et al., 2007. Human apolipoprotein e is required for infectivity and production of hepatitis C virus in cell culture. *Journal of Virology*, 81(24), pp.13783–13793.

Chattergoon, M.A. et al., 2011. High plasma interleukin-18 levels mark the acute phase of hepatitis C virus infection. *Journal of Infectious Diseases*, 204(11), pp.1730–1740.

Chen, Limin et al., 2005. Hepatic gene expression discriminates responders and nonresponders in treatment of chronic hepatitis C viral infection. *Gastroenterology*, 128(5), pp.1437–1444.

Chen, M et al., 1999. Limited humoral immunity in hepatitis C virus infection. *Gastroenterology*, 116(1), pp.135–143.

Chen, Yemiao & Verfaillie, C.M., 2014. MicroRNAs: the fine modulators of liver development and function. *Liver international : official journal of the International Association for the Study of the Liver*, 34(7), pp.976–990.

Chen, Yemiao et al., 2016. MicroRNAs as key mediators of hepatic detoxification. *Toxicology*, 368-369, pp.80–90.

Chen, Yue et al., 2016. Identification of the transcripts associated with spontaneous HCV clearance in individuals co-infected with HIV and HCV. *BMC infectious diseases*, 16(1), p.693.

Cheng, J.-C. et al., 2012. Let-7b is a novel regulator of hepatitis C virus replication. *Cellular and molecular life sciences : CMLS*, 69(15), pp.2621–2633.

Chew, K.W. & Bhattacharya, D., 2016. Virologic and immunologic aspects of HIV-hepatitis C virus coinfection. *AIDS (London, England)*, 30(16), pp.2395–2404.

Chodosh, J. et al., 2000. Patterned entry and egress by Epstein-Barr virus in polarized CR2-positive epithelial cells. *Virology*, 266(2), pp.387–396.

Choi, J.E. et al., 2014. MicroRNA-27a modulates HCV infection in differentiated hepatocyte-like cells from adipose tissue-derived mesenchymal stem cells. *PLoS ONE*, 9(5), p.e91958.

Clouse, K.A. et al., 1989. Monokine regulation of human immunodeficiency virus-1 expression in a chronically infected human T cell clone. *Journal of immunology (Baltimore, Md. : 1950)*, 142(2), pp.431–438.

Collins, S.J. et al., 1978. Terminal differentiation of human promyelocytic leukemia cells induced by dimethyl sulfoxide and other polar compounds. *Proceedings of the National Academy of Sciences of the United States of America*, 75(5), pp.2458–2462.

Connolly-Andersen, A.-M., Magnusson, K.-E. & Mirazimi, A., 2007. Basolateral entry and release of Crimean-Congo hemorrhagic fever virus in polarized MDCK-1 cells. *Journal of Virology*, 81(5), pp.2158–2164.

Conrad, K.D. & Niepmann, M., 2014. The role of microRNAs in hepatitis C virus RNA replication. *Archives of virology*, 159(5), pp.849–862.

Conrad, K.D. et al., 2013. MicroRNA-122 dependent binding of Ago2 protein to hepatitis C virus RNA is associated with enhanced RNA stability and translation stimulation. *PLoS ONE*, 8(2), p.e56272.

Conte, D. et al., 2000. Prevalence and clinical course of chronic hepatitis C virus (HCV) infection and rate of HCV vertical transmission in a cohort of 15,250 pregnant women. *Hepatology*, 31(3), pp.751–755.

Cormier, E.G. et al., 2004. L-SIGN (CD209L) and DC-SIGN (CD209) mediate transinfection of liver cells by hepatitis C virus. *Proceedings of the National Academy of Sciences of the United States of America*, 101(39), pp.14067–14072.

Costiniuk, C.T. & Jenabian, M.-A., 2014. Cell-to-cell transfer of HIV infection: implications for HIV viral persistence. *Journal of General Virology*, 95(Pt 11), pp.2346–2355.

Coto-Llerena, M. et al., 2017. Permissiveness of human hepatocellular carcinoma cell lines for hepatitis C virus entry and replication. *Virus Research*, 240, pp.35–46.

Cox, A.L. et al., 2005. Comprehensive analyses of CD8+ T cell responses during longitudinal study of acute human hepatitis C. *Hepatology*, 42(1), pp.104–112.

Craigie, R. & Bushman, F.D., 2012. HIV DNA integration. *Cold Spring Harbor perspectives in medicine*, 2(7), p.a006890.

Cuninghame, S., Jackson, R. & Zehbe, I., 2014. Hypoxia-inducible factor 1 and its role in viral carcinogenesis. *Virology*, 456-457, pp.370–383.

Czysz, K., Minger, S. & Thomas, N., 2015. DMSO efficiently down regulates pluripotency genes in human embryonic stem cells during definitive endoderm derivation and increases the proficiency of hepatic differentiation. *PLoS ONE*, 10(2), p.e0117689.

Da Costa, D. et al., 2012. Reconstitution of the entire hepatitis C virus life cycle in nonhepatic cells. *Journal of Virology*, 86(21), pp.11919–11925.

Dalgleish, A.G. et al., 1984. The CD4 (T4) antigen is an essential component of the receptor for the AIDS retrovirus. *Nature*, 312(5996), pp.763–767.

Dansako, H. et al., 2013. Class A scavenger receptor 1 (MSR1) restricts hepatitis C virus replication by mediating toll-like receptor 3 recognition of viral RNAs produced in neighboring cells. *PLoS pathogens*, 9(5), p.e1003345.

de Lédinghen, V. et al., 2008. Liver fibrosis on account of chronic hepatitis C is more severe in HIV-positive than HIV-negative patients despite antiretroviral therapy. *Journal of Viral Hepatitis*, 15(6), pp.427–433.

Decaens, C. et al., 2008. Which in vitro models could be best used to study hepatocyte polarity? *Biology of the cell*, 100(7), pp.387–398.

Dehouck, M.P. et al., 1992. Drug transfer across the blood-brain barrier: correlation between in vitro and in vivo models. *Journal of neurochemistry*, 58(5), pp.1790–1797.

Dejucq, N., 2000. HIV-1 replication in CD4+ T cell lines: the effects of adaptation on co-receptor use, tropism, and accessory gene function. *Journal of leukocyte biology*, 68(3), pp.331–337.

Delorme-Axford, E. et al., 2013. Human placental trophoblasts confer viral resistance to recipient cells. *Proceedings of the National Academy of Sciences*, 110(29), pp.12048–12053.

Deng, X.-G. et al., 2014. Overexpression of miR-122 promotes the hepatic differentiation and maturation of mouse ESCs through a miR-122/FoxA1/HNF4a-positive feedback loop. *Liver international : official journal of the International Association for the Study of the Liver*, 34(2), pp.281–295.

Di Martino, V. et al., 2001. The influence of human immunodeficiency virus coinfection on chronic hepatitis C in injection drug users: a long-term retrospective cohort study. *Hepatology*, 34(6), pp.1193–1199.

Diamond, D.L. et al., 2010. Temporal proteome and lipidome profiles reveal hepatitis C virus-associated reprogramming of hepatocellular metabolism and bioenergetics. *PLoS pathogens*, 6(1), p.e1000719.

Dill, M.T. et al., 2011. Interferon-induced gene expression is a stronger predictor of treatment response than IL28B genotype in patients with hepatitis C. *Gastroenterology*, 140(3), pp.1021–1031.

Dixit, E. et al., 2010. Peroxisomes are signaling platforms for antiviral innate immunity. *Cell*, 141(4), pp.668–681.

Dolganiuc, A. et al., 2012. Type III interferons, IL-28 and IL-29, are increased in chronic HCV infection and induce myeloid dendritic cell-mediated FoxP3+ regulatory T cells. *PLoS ONE*, 7(10), p.e44915.

Drakesmith, H. & Prentice, A., 2008. Viral infection and iron metabolism. *Nature Reviews Microbiology*, 6(7), pp.541–552.

Drummer, H.E., Maerz, A. & Poumbourios, P., 2003. Cell surface expression of functional hepatitis C virus E1 and E2 glycoproteins. *FEBS letters*, 546(2-3), pp.385–390.

Du, X. et al., 2016. Hepatitis C virus replicative double-stranded RNA is a potent interferon inducer that triggers interferon production through MDA5. *Journal of General Virology*, 97(11), pp.2868–2882.

Dubuisson, J., Helle, F. & Cocquerel, L., 2008. Early steps of the hepatitis C virus life cycle. *Cellular Microbiology*, 10(4), pp.821–827.

Duggal, P. et al., 2013. Genome-wide association study of spontaneous resolution of hepatitis C virus infection: data from multiple cohorts. *Annals of internal medicine*, 158(4), pp.235–245.

Duh, E.J. et al., 1989. Tumor necrosis factor alpha activates human immunodeficiency virus type 1 through induction of nuclear factor binding to the NF-kappa B sites in the long terminal repeat. *Proceedings of the National Academy of Sciences of the United States of America*, 86(15), pp.5974–5978.

Duong, F.H.T. et al., 2014. IFN-λ receptor 1 expression is induced in chronic hepatitis C and correlates with the IFN-λ3 genotype and with nonresponsiveness to IFN-α therapies. *The Journal of experimental medicine*, 211(5), pp.857–868.

Eckstein, D.A. et al., 2001. HIV-1 actively replicates in naive CD4(+) T cells residing within human lymphoid tissues. *Immunity*, 15(4), pp.671–682.

European Association for the Study of the Liver. Electronic address: easloffice@easloffice.eu, 2017. EASL Recommendations on Treatment of Hepatitis C 2016. *Journal of hepatology*, 66(1), pp.153–194.

European Paediatric Hepatitis C Virus Network., 2001. Effects of mode of delivery and infant feeding on the risk of mother-to-child transmission of hepatitis C virus. European Paediatric Hepatitis C Virus Network. *BJOG : an international journal of obstetrics and gynaecology*, 108(4), pp.371–377.

Evans, M.J. et al., 2007. Claudin-1 is a hepatitis C virus co-receptor required for a late step in entry. *Nature*, 446(7137), pp.801–805.

Farquhar, M.J. et al., 2012. Hepatitis C virus induces CD81 and claudin-1 endocytosis. *Journal of Virology*, 86(8), pp.4305–4316.

Feldman, G.J., Mullin, J.M. & Ryan, M.P., 2005. Occludin: structure, function and regulation. *Advanced drug delivery reviews*, 57(6), pp.883–917.

Filipowicz, W., Bhattacharyya, S.N. & Sonenberg, N., 2008. Mechanisms of post-transcriptional regulation by microRNAs: are the answers in sight? *Nature reviews. Genetics*, 9(2), pp.102–114.

Fillebeen, C. et al., 2007. Expression of the subgenomic hepatitis C virus replicon alters iron homeostasis in Huh7 cells. *Journal of hepatology*, 47(1), pp.12–22.

Fillebeen, C. et al., 2005. Iron inactivates the RNA polymerase NS5B and suppresses subgenomic replication of hepatitis C Virus. *Journal of Biological Chemistry*, 280(10), pp.9049–9057.

Fiore, M. & Degrassi, F., 1999. Dimethyl sulfoxide restores contact inhibition-induced growth arrest and inhibits cell density-dependent apoptosis in hamster cells. *Experimental cell research*, 251(1), pp.102–110.

Fields Virology 13th Edition. Lipcott Williams and Wilkins

Fletcher, N.F. et al., 2014. Activated macrophages promote hepatitis C virus entry in a tumor necrosis factor-dependent manner. *Hepatology*, 59(4), pp.1320–1330.

Fletcher, N.F. et al., 2010. Hepatitis C virus infection of neuroepithelioma cell lines. *Gastroenterology*, 139(4), pp.1365–1374.

Fletcher, N.F. et al., 2017. TNF superfamily members promote hepatitis C virus entry via an NF-κB and myosin light chain kinase dependent pathway. *Journal of General Virology*, 98(3), pp.405–412.

Flint, M. et al., 2006. Diverse CD81 proteins support hepatitis C virus infection. *Journal of Virology*, 80(22), pp.11331–11342.

Folks, T.M. et al., 1989. Tumor necrosis factor alpha induces expression of human immunodeficiency virus in a chronically infected T-cell clone. *Proceedings of the National Academy of Sciences of the United States of America*, 86(7), pp.2365–2368.

Fraser, C.S. et al., 2007. eIF3j is located in the decoding center of the human 40S ribosomal subunit. *Molecular cell*, 26(6), pp.811–819.

Freed, E.O., 2015. HIV-1 assembly, release and maturation. *Nature Reviews Microbiology*, 13(8), pp.484–496.

Friedman, R.C. et al., 2009. Most mammalian mRNAs are conserved targets of microRNAs. *Genome research*, 19(1), pp.92–105.

Fromentin, R., Tardif, M.R. & Tremblay, M.J., 2010. Human hepatoma cells transmit surface bound HIV-1 to CD4+ T cells through an ICAM-1/LFA-1-dependent mechanism. *Virology*, 398(2), pp.168–175.

Fukuhara, T. et al., 2012. Expression of microRNA miR-122 facilitates an efficient replication in nonhepatic cells upon infection with hepatitis C virus. *Journal of Virology*, 86(15), pp.7918–7933.

Gadol, N., Crutcher, G.J. & Busch, M.P., 1994. Detection of intracellular HIV in lymphocytes by flow cytometry. *Cytometry*, 15(4), pp.359–370.

Galbraith, R.A., Sassa, S. & Fujita, H., 1988a. Regulation of heme synthesis in HepG2 human hepatoma cells by dimethyl sulfoxide. *Biochemical and Biophysical Research Communications*, 153(2), pp.869–874.

Galbraith, R.A., Sassa, S. & Fujita, H., 1988b. Regulation of heme synthesis in HepG2 human hepatoma cells by dimethyl sulfoxide. *Biochemical and Biophysical Research Communications*, 153(2), pp.869–874.

Gale, M. et al., 1998. Control of PKR protein kinase by hepatitis C virus nonstructural 5A protein: molecular mechanisms of kinase regulation. *Molecular and cellular biology*, 18(9), pp.5208–5218.

Gallastegui, E. et al., 2012. Combination of biological screening in a cellular model of viral latency and virtual screening identifies novel compounds that reactivate HIV-1. *Journal of Virology*, 86(7), pp.3795–3808.

Gamage, D.G. et al., 2011. Incidence of hepatitis-C among HIV infected men who have sex with men (MSM) attending a sexual health service: a cohort study. *BMC infectious diseases*, 11, p.39.

Gao, L. et al., 2004. Interactions between viral nonstructural proteins and host protein hVAP-33 mediate the formation of hepatitis C virus RNA replication complex on lipid raft. *Journal of Virology*, 78(7), pp.3480–3488.

Gastaminza, P. et al., 2008. Cellular determinants of hepatitis C virus assembly, maturation, degradation, and secretion. *Journal of Virology*, 82(5), pp.2120–2129.

Ge, D. et al., 2009. Genetic variation in IL28B predicts hepatitis C treatment-induced viral clearance. *Nature*, 461(7262), pp.399–401.

German Advisory Committee Blood (Arbeitskreis Blut), Subgroup 'Assessment of Pathogens Transmissible by Blood', 2016. Human Immunodeficiency Virus (HIV). *Transfusion medicine and hemotherapy : offizielles Organ der Deutschen Gesellschaft fur Transfusionsmedizin und Immunhamatologie*, 43(3), pp.203–222.

Ghany, M.G. et al., 1996. Effect of human immunodeficiency virus infection on hepatitis C virus infection in hemophiliacs. *Digestive diseases and sciences*, 41(6), pp.1265–1272.

Gibb, D.M. et al., 2000. Mother-to-child transmission of hepatitis C virus: evidence for preventable peripartum transmission. *Lancet (London, England)*, 356(9233), pp.904–907.

Gilbert, P.B. et al., 2003. Comparison of HIV-1 and HIV-2 infectivity from a prospective cohort study in Senegal. *Statistics in medicine*, 22(4), pp.573–593.

Girard, M. et al., 2008. miR-122, a paradigm for the role of microRNAs in the liver. *Journal of hepatology*, 48(4), pp.648–656.

Giraudon, I. et al., 2008. Increase in diagnosed newly acquired hepatitis C in HIV-positive men who have sex with men across London and Brighton, 2002-2006: is this an outbreak? *Sexually transmitted infections*, 84(2), pp.111–115.

Goergen, D. & Niepmann, M., 2012. Stimulation of Hepatitis C Virus RNA translation by microRNA-122 occurs under different conditions in vivo and in vitro. *Virus Research*, 167(2), pp.343–352.

Gondar, V. et al., 2015. Apolipoprotein E, but Not Apolipoprotein B, Is Essential for Efficient Cell-to-Cell Transmission of Hepatitis C Virus. *Journal of Virology*, 89(19), pp.9962–9973.

Gondeau, C. et al., 2014. In vitro infection of primary human hepatocytes by HCV-positive sera: insights on a highly relevant model. *Gut*, 63(9), pp.1490–1500.

Götz, H.M. et al., 2005. A cluster of acute hepatitis C virus infection among men who have sex with men--results from contact tracing and public health implications. *AIDS (London, England)*, 19(9), pp.969–974.

Graham, C.S. et al., 2001. Influence of human immunodeficiency virus infection on the course of hepatitis C virus infection: a meta-analysis. *Clinical infectious diseases : an official publication of the Infectious Diseases Society of America*, 33(4), pp.562–569.

Grassi, G. et al., 2016. Hepatitis C virus relies on lipoproteins for its life cycle. *World journal of gastroenterology*, 22(6), pp.1953–1965.

Green, D.R. & Ferguson, T.A., 2001. The role of Fas ligand in immune privilege. *Nature reviews. Molecular cell biology*, 2(12), pp.917–924.

Greenberg, C.C. et al., 2006. Glycogen branches out: new perspectives on the role of glycogen metabolism in the integration of metabolic pathways. *American journal of physiology. Endocrinology and metabolism*, 291(1), pp.E1–8.

Gretton, S.N., Taylor, A.I. & McLauchlan, J., 2005. Mobility of the hepatitis C virus NS4B protein on the endoplasmic reticulum membrane and membrane-associated foci. *The Journal of general virology*, 86(Pt 5), pp.1415–1421.

Greub, G. et al., 2000. Clinical progression, survival, and immune recovery during antiretroviral therapy in patients with HIV-1 and hepatitis C virus coinfection: the Swiss HIV Cohort Study. *Lancet (London, England)*, 356(9244), pp.1800–1805.

Gripon P. et al., 2002. Infection of a human hepatoma cell line by hepatitis B virus. *PNAS*, 99(24), 15655-60.

Grove, J. et al., 2008. Identification of a residue in hepatitis C virus E2 glycoprotein that determines scavenger receptor BI and CD81 receptor dependency and sensitivity to neutralizing antibodies. *Journal of Virology*, 82(24), pp.12020–12029.

Grove, J. et al., 2007. Scavenger receptor BI and BII expression levels modulate hepatitis C virus infectivity. *Journal of Virology*, 81(7), pp.3162–3169.

Guedj, J. et al., 2010. A perspective on modelling hepatitis C virus infection. *Journal of Viral Hepatitis*, 17(12), pp.825–833.

Guillouzo, A. et al., 2007. The human hepatoma HepaRG cells: a highly differentiated model for studies of liver metabolism and toxicity of xenobiotics. *Chemico-biological interactions*, 168(1), pp.66–73.

Guo, X. et al., 2017. Efficient replication of blood-borne hepatitis C virus in human fetal liver stem cells. *Hepatology*, 66(4), pp.1045–1057.

Hantz, O. et al., 2009. Persistence of the hepatitis B virus covalently closed circular DNA in HepaRG human hepatocyte-like cells. *The Journal of general virology*, 90(Pt 1), pp.127–135.

Harris, H.J. et al., 2008. CD81 and claudin 1 coreceptor association: role in hepatitis C virus entry. *Journal of Virology*, 82(10), pp.5007–5020.

Harris, H.J. et al., 2010. Claudin association with CD81 defines hepatitis C virus entry. *The Journal of biological chemistry*, 285(27), pp.21092–21102.

Harris, H.J. et al., 2013. Hepatoma polarization limits CD81 and hepatitis C virus dynamics. *Cellular Microbiology*, 15(3), pp.430–445.

Hartsock, A. & Nelson, W.J., 2008. Adherens and tight junctions: structure, function and connections to the actin cytoskeleton. *Biochimica et biophysica acta*, 1778(3), pp.660–669.

Hedfors, I.A. & Brinchmann, J.E., 2003. Long-term proliferation and survival of in vitro-activated T cells is dependent on Interleukin-2 receptor signalling but not on the high-affinity IL-2R. *Scandinavian journal of immunology*, 58(5), pp.522–532.

Heim, M.H. & Thimme, R., 2014. Innate and adaptive immune responses in HCV infections. *Journal of hepatology*, 61(1 Suppl), pp.S14–25.

Hellen, C.U.T., 2009. IRES-induced conformational changes in the ribosome and the mechanism of translation initiation by internal ribosomal entry. *Biochimica et biophysica acta*, 1789(9-10), pp.558–570.

Henke, J.I. et al., 2008. microRNA-122 stimulates translation of hepatitis C virus RNA. *The EMBO journal*, 27(24), pp.3300–3310.

Hesselgesser, J. et al., 1998. Identification and characterization of the CXCR4 chemokine receptor in human T cell lines: ligand binding, biological activity, and HIV-1 infectivity. *Journal of immunology (Baltimore, Md. : 1950)*, 160(2), pp.877–883.

Hiscott, J., Kwon, H. & Génin, P., 2001. Hostile takeovers: viral appropriation of the NF-kappaB pathway. *The Journal of clinical investigation*, 107(2), pp.143–151.

Hoffman, B. & Liu, Q., 2011. Hepatitis C viral protein translation: mechanisms and implications in developing antivirals. *Liver international : official journal of the International Association for the Study of the Liver*, 31(10), pp.1449–1467.

Horner, S.M. & Gale, M., 2009. Intracellular innate immune cascades and interferon defenses that control hepatitis C virus. *Journal of interferon & cytokine research : the official journal of the International Society for Interferon and Cytokine Research*, 29(9), pp.489–498.

Hou, W. et al., 2009. Lambda interferon inhibits human immunodeficiency virus type 1 infection of macrophages. *Journal of Virology*, 83(8), pp.3834–3842.

Hsieh, M.-H. et al., 2014. Hepatitis C virus infection among injection drug users with and without human immunodeficiency virus co-infection. *PLoS ONE*, 9(4), p.e94791.

Hu, W.-S. & Hughes, S.H., 2012. HIV-1 reverse transcription. *Cold Spring Harbor perspectives in medicine*, 2(10).

Hullegie, S.J. et al., 2017. Decreased pro-inflammatory immune responses during recurrent acute HCV infections in HIV co-infected patients. *Journal of General Virology*, 98(6), pp.1294–1298.

Iannello, A. et al., 2008. Antiviral NK cell responses in HIV infection: II. viral strategies for evasion and lessons for immunotherapy and vaccination. *Journal of leukocyte biology*, 84(1), pp.27–49.

Ikeda, M. et al., 2002. Selectable subgenomic and genome-length dicistronic RNAs derived from an infectious molecular clone of the HCV-N strain of hepatitis C virus replicate efficiently in cultured Huh7 cells. *Journal of Virology*, 76(6), pp.2997–3006.

Ishibashi, M. et al., 2014. CLEC4M-positive and CD81-negative Huh7 cells are not susceptible to JFH-1 HCVcc infection but mediate transinfection. *Archives of virology*, 159(11), pp.2949–2955.

Ishida, H. et al., 2011. Alterations in microRNA expression profile in HCV-infected hepatoma cells: involvement of miR-491 in regulation of HCV replication via the PI3 kinase/Akt pathway. *Biochemical and Biophysical Research Communications*, 412(1), pp.92–97.

Isken, O. et al., 2007. Nuclear factors are involved in hepatitis C virus RNA replication. *RNA (New York, N.Y.)*, 13(10), pp.1675–1692.

Isom, H.C. et al., 1985. Maintenance of differentiated rat hepatocytes in primary culture. *Proceedings of the National Academy of Sciences of the United States of America*, 82(10), pp.3252–3256.

Israelow, B. et al., 2014. HepG2 cells mount an effective antiviral interferon-lambda based innate immune response to hepatitis C virus infection. *Hepatology*, 60(4), pp.1170–1179.

Iwamoto, M. et al., 2014. Evaluation and identification of hepatitis B virus entry inhibitors using HepG2 cells overexpressing a membrane transporter NTCP. *Biochemical and Biophysical Research Communications*, 443(3), pp.808–813.

Jaafar, Z.A. et al., 2016. Translation initiation by the hepatitis C virus IRES requires eIF1A and ribosomal complex remodeling. *eLife*, 5.

Jammart, B. et al., 2013. Very-low-density lipoprotein (VLDL)-producing and hepatitis C virus-replicating HepG2 cells secrete no more lipoviroparticles than VLDL-deficient Huh7.5 cells. *Journal of Virology*, 87(9), pp.5065–5080.

Jangra, R.K., Yi, M. & Lemon, S.M., 2010. Regulation of hepatitis C virus translation and infectious virus production by the microRNA miR-122. *Journal of Virology*, 84(13), pp.6615–6625.

Jeanty, C., Derderian, S.C. & Mackenzie, T.C., 2014. Maternal-fetal cellular trafficking: clinical implications and consequences. *Current opinion in pediatrics*, 26(3), pp.377–382.

Jiang, J. et al., 2012. Hepatitis C virus attachment mediated by apolipoprotein E binding to cell surface heparan sulfate. *Journal of Virology*, 86(13), pp.7256–7267.

Jo, J. et al., 2009. Analysis of CD8+ T-cell-mediated inhibition of hepatitis C virus replication using a novel immunological model. *Gastroenterology*, 136(4), pp.1391–1401.

Johnson, D.C. & Huber, M.T., 2002. Directed egress of animal viruses promotes cell-to-cell spread. *Journal of Virology*, 76(1), pp.1–8.

Jones, C.T. et al., 2010. Real-time imaging of hepatitis C virus infection using a fluorescent cell-based reporter system. *Nature biotechnology*, 28(2), pp.167–171.

Jopling, C.L. et al., 2005. Modulation of Hepatitis C Virus RNA Abundance by a Liver-Specific MicroRNA. *Science*.

Jopling, C.L., Schütz, S. & Sarnow, P., 2008. Position-dependent function for a tandem microRNA miR-122-binding site located in the hepatitis C virus RNA genome. *Cell Host and Microbe*, 4(1), pp.77–85.

Joshi, A. et al., 2017. CCR5 promoter activity correlates with HIV disease progression by regulating CCR5 cell surface expression and CD4 T cell apoptosis. *Scientific reports*, 7(1), p.232.

Jungermann, K. & Kietzmann, T., 2000. Oxygen: modulator of metabolic zonation and disease of the liver. *Hepatology*, 31(2), pp.255–260.

Kakizaki, S. et al., 2000. Iron enhances hepatitis C virus replication in cultured human hepatocytes. *Liver*, 20(2), pp.125–128.

Kanda, T. et al., 2006. Generation of infectious hepatitis C virus in immortalized human hepatocytes. *Journal of Virology*, 80(9), pp.4633–4639.

Kandathil, A.J. et al., 2013. Use of Laser Capture Microdissection to Map Hepatitis C Virus-Positive Hepatocytes in Human Liver. *Gastroenterology*, 145(6), pp.1404–1413.e10.

Kasai, D. et al., 2009. HCV replication suppresses cellular glucose uptake through down-regulation of cell surface expression of glucose transporters. *Journal of hepatology*, 50(5), pp.883–894.

Kataki, K. et al., 2017. Association of mRNA expression of toll-like receptor 2 and 3 with hepatitis B viral load in chronic hepatitis, cirrhosis, and hepatocellular carcinoma. *Journal of medical virology*, 89(6), pp.1008–1014.

Kell, A.M. & Gale, M., 2015. RIG-I in RNA virus recognition. *Virology*, 479-480, pp.110–121.

Kieffer, T.L. et al., 2007. Telaprevir and pegylated interferon-alpha-2a inhibit wild-type and resistant genotype 1 hepatitis C virus replication in patients. *Hepatology*, 46(3), pp.631–639.

Kim, M.-J. & Yoo, J.-Y., 2010. Inhibition of hepatitis C virus replication by IFN-mediated ISGylation of HCV-NS5A. *Journal of immunology (Baltimore, Md. : 1950)*, 185(7), pp.4311–4318.

Klatzmann, D. et al., 1984. T-lymphocyte T4 molecule behaves as the receptor for human retrovirus LAV. *Nature*, 312(5996), pp.767–768.

Klaunig, J.E. et al., 1981. Mouse liver cell culture. II. Primary culture. *In vitro*, 17(10), pp.926–934.

Klein, S.A. et al., 2003. Comparison of TaqMan real-time PCR and p24 Elisa for quantification of in vitro HIV-1 replication. *Journal of virological methods*, 107(2), pp.169–175.

Klenerman, P. & Thimme, R., 2012. T cell responses in hepatitis C: the good, the bad and the unconventional. *Gut*, 61(8), pp.1226–1234.

Koike, K., 2007. Hepatitis C virus contributes to hepatocarcinogenesis by modulating metabolic and intracellular signaling pathways. *Journal of gastroenterology and hepatology*, 22 Suppl 1, pp.S108–11.

Kong, L. et al., 2014. HIV infection of hepatocytes results in a modest increase in hepatitis C virus expression in vitro. *PLoS ONE*, 9(2), p.e83728.

Krishna, M., 2013. Microscopic anatomy of the liver R. E. Nakhleh, ed. *Clinical Liver Disease*, 2(S1), pp.S4–S7.

Kwiek, J.J. et al., 2006. Maternal-fetal microtransfusions and HIV-1 mother-to-child transmission in Malawi. *PLoS medicine*, 3(1), p.e10.

Lagos-Quintana, M. et al., 2002. Identification of tissue-specific microRNAs from mouse. *Current biology : CB*, 12(9), pp.735–739.

Laguette, N. et al., 2011. SAMHD1 is the dendritic- and myeloid-cell-specific HIV-1 restriction factor counteracted by Vpx. *Nature*, 474(7353), pp.654–657.

Laidlaw, S.M. et al., 2017. Tumor Necrosis Factor Inhibits Spread of Hepatitis C Virus Among Liver Cells, Independent From Interferons. *Gastroenterology*, 153(2), pp.566–578.e5.

Lambert, D., O'Neill, C.A. & Padfield, P.J., 2005. Depletion of Caco-2 cell cholesterol disrupts barrier function by altering the detergent solubility and distribution of specific tight-junction proteins. *The Biochemical journal*, 387(Pt 2), pp.553–560.

Lambotin, M. et al., 2010. A look behind closed doors: interaction of persistent viruses with dendritic cells. *Nature Reviews Microbiology*, 8(5), pp.350–360.

Lanford, R.E. et al., 1995. Lack of detection of negative-strand hepatitis C virus RNA in peripheral blood mononuclear cells and other extrahepatic tissues by the highly strand-specific rTth reverse transcriptase PCR. *Journal of Virology*, 69(12), pp.8079–8083.

Laskus, T. et al., 2007. Negative-strand hepatitis C virus (HCV) RNA in peripheral blood mononuclear cells from anti-HCV-positive/HIV-infected women. *The Journal of infectious diseases*, 195(1), pp.124–133.

Law, W.P. et al., 2004. Impact of viral hepatitis co-infection on response to antiretroviral therapy and HIV disease progression in the HIV-NAT cohort. *AIDS (London, England)*, 18(8), pp.1169–1177.

Lázaro, C.A. et al., 2007. Hepatitis C virus replication in transfected and serum-infected cultured human fetal hepatocytes. *The American journal of pathology*, 170(2), pp.478–489.

Le Campion, A. et al., 2012. Pathogenesis of hepatitis C during pregnancy and childhood. *Viruses*, 4(12), pp.3531–3550.

Levy, J.A., 1993. HIV pathogenesis and long-term survival. *AIDS (London, England)*, 7(11), pp.1401–1410.

Li, A. et al., 2013. Modulation of miR-122 expression affects the interferon response in human hepatoma cells. *Molecular medicine reports*, 7(2), pp.585–590.

Li, G. & De Clercq, E., 2016. HIV Genome-Wide Protein Associations: a Review of 30 Years of Research. *Microbiology and molecular biology reviews : MMBR*, 80(3), pp.679–731.

Li, K., Chen, Z., et al., 2005. Distinct poly(I-C) and virus-activated signaling pathways leading to interferon-beta production in hepatocytes. *Journal of Biological Chemistry*, 280(17), pp.16739–16747.

Li, K., Foy, E., et al., 2005. Immune evasion by hepatitis C virus NS3/4A protease-mediated cleavage of the Toll-like receptor 3 adaptor protein TRIF. *Proceedings of the National Academy of Sciences of the United States of America*, 102(8), pp.2992–2997.

Li, Q. et al., 2017. Cellular microRNA networks regulate host dependency of hepatitis C virus infection. *Nature communications*, 8(1), p.1789.

Li, T. et al., 2014. Infection of common marmosets with hepatitis C virus/GB virus-B chimeras. *Hepatology*, 59(3), pp.789–802.

Li, X.-D. et al., 2005. Hepatitis C virus protease NS3/4A cleaves mitochondrial antiviral signaling protein off the mitochondria to evade innate immunity. *Proceedings of the National Academy of Sciences of the United States of America*, 102(49), pp.17717–17722.

Li, Y., Masaki, T. & Lemon, S.M., 2013. miR-122 and the Hepatitis C RNA genome: more than just stability. *RNA biology*, 10(6), pp.919–923.

Li, Y., Masaki, T., Yamane, D., et al., 2013. Competing and noncompeting activities of miR-122 and the 5' exonuclease Xrn1 in regulation of hepatitis C virus replication. *Proceedings of the National Academy of Sciences*, 110(5), pp.1881–1886.

Liang, Y. et al., 2009. Visualizing hepatitis C virus infections in human liver by two-photon microscopy. *Gastroenterology*, 137(4), pp.1448–1458.

Lin, L., Fevery, J. & Hiem Yap, S., 2002. A novel strand-specific RT-PCR for detection of hepatitis C virus negative-strand RNA (replicative intermediate): evidence of absence or very low level of HCV replication in peripheral blood mononuclear cells. *Journal of virological methods*, 100(1-2), pp.97–105.

Lindenbach, B.D., 2013. Virion assembly and release. *Current topics in microbiology and immunology*, 369, pp.199–218.

Lindenbach, B.D. & Rice, C.M., 2013. The ins and outs of hepatitis C virus entry and assembly. *Nature Reviews Microbiology*, 11(10), pp.688–700.

Lindenbach, B.D. & Rice, C.M., 2005. Unravelling hepatitis C virus replication from genome to function. *Nature*, 436(7053), pp.933–938.

Liu, B., Xiang, Y. & Zhang, H.-S., 2015. Circulating microRNA-196a as a candidate diagnostic biomarker for chronic hepatitis C. *Molecular medicine reports*, 12(1), pp.105–110.

Liu, S. et al., 2009. Tight junction proteins claudin-1 and occludin control hepatitis C virus entry and are downregulated during infection to prevent superinfection. *Journal of Virology*, 83(4), pp.2011–2014.

Logvinoff, C. et al., 2004. Neutralizing antibody response during acute and chronic hepatitis C virus infection. *Proceedings of the National Academy of Sciences of the United States of America*, 101(27), pp.10149–10154.

Lupberger, J. et al., 2011. EGFR and EphA2 are host factors for hepatitis C virus entry and possible targets for antiviral therapy. *Nature medicine*, 17(5), pp.589–595.

Lusic, M. & Siliciano, R.F., 2017. Nuclear landscape of HIV-1 infection and integration. *Nature Reviews Microbiology*, 15(2), pp.69–82.

Machlin, E.S., Sarnow, P. & Sagan, S.M., 2011. Masking the 5' terminal nucleotides of the hepatitis C virus genome by an unconventional microRNA-target RNA complex. *Proceedings of the National Academy of Sciences*, 108(8), pp.3193–3198.

Maddon, P.J. et al., 1986. The T4 gene encodes the AIDS virus receptor and is expressed in the immune system and the brain. *Cell*, 47(3), pp.333–348.

Mailly, L. et al., 2015. Clearance of persistent hepatitis C virus infection in humanized mice using a claudin-1-targeting monoclonal antibody. *Nature biotechnology*, 33(5), pp.549–554.

Malim, M.H. & Emerman, M., 2008. HIV-1 accessory proteins--ensuring viral survival in a hostile environment. *Cell Host and Microbe*, 3(6), pp.388–398.

Malim, M.H. et al., 1989. Functional comparison of the Rev trans-activators encoded by different primate immunodeficiency virus species. *Proceedings of the National Academy of Sciences of the United States of America*, 86(21), pp.8222–8226.

Mandell, K.J. & Parkos, C.A., 2005. The JAM family of proteins. *Advanced drug delivery reviews*, 57(6), pp.857–867.

Mandorfer, M. et al., 2016. Advances in the management of HIV/HCV coinfection. *Hepatology international*, 10(3), pp.424–435.

Manns, M.P., Wedemeyer, H. & Cornberg, M., 2006. Treating viral hepatitis C: efficacy, side effects, and complications. *Gut*, 55(9), pp.1350–1359.

March, S. et al., 2015. Micropatterned coculture of primary human hepatocytes and supportive cells for the study of hepatotropic pathogens. *Nature protocols*, 10(12), pp.2027–2053.

Mariné-Barjoan, E. et al., 2007. HCV/HIV co-infection, HCV viral load and mode of delivery: risk factors for mother-to-child transmission of hepatitis C virus? *AIDS (London, England)*, 21(13), pp.1811–1815.

Marnata, C. et al., 2015. Determinants Involved in Hepatitis C Virus and GB Virus B Primate Host Restriction. *Journal of Virology*, 89(23), pp.12131–12144.

Martin, D.N. & Uprichard, S.L., 2013. Identification of transferrin receptor 1 as a hepatitis C virus entry factor. *Proceedings of the National Academy of Sciences*, 110(26), pp.10777–10782.

Martinez-Sierra, C. et al., 2003. Progression of chronic hepatitis C to liver fibrosis and cirrhosis in patients coinfected with hepatitis C virus and human immunodeficiency virus. *Clinical infectious diseases : an official publication of the Infectious Diseases Society of America*, 36(4), pp.491–498.

Marukian, S. et al., 2008. Cell culture-produced hepatitis C virus does not infect peripheral blood mononuclear cells. *Hepatology*, 48(6), pp.1843–1850.

Marukian, S. et al., 2011. Hepatitis C virus induces interferon-λ and interferon-stimulated genes in primary liver cultures. *Hepatology*, 54(6), pp.1913–1923.

Mason, A.L. et al., 1999. Association of diabetes mellitus and chronic hepatitis C virus infection. *Hepatology*, 29(2), pp.328–333.

Mathiesen, C.K. et al., 2015. Adaptive Mutations Enhance Assembly and Cell-to-Cell Transmission of a High-Titer Hepatitis C Virus Genotype 5a Core-NS2 JFH1-Based Recombinant. *Journal of Virology*, 89(15), pp.7758–7775.

Matthews-Greer, J.M. et al., 2001. Comparison of hepatitis C viral loads in patients with or without human immunodeficiency virus. *Clinical and diagnostic laboratory immunology*, 8(4), pp.690–694.

Mayer, K.H. & Krakower, D.S., 2016. Antiretrovirals for HIV Treatment and Prevention: The Challenges of Success. *JAMA*, 316(2), pp.151–153.

McGuinness, P.H. et al., 1994. False detection of negative-strand hepatitis C virus RNA. *Lancet (London, England)*, 343(8896), pp.551–552.

McLauchlan, J. et al., 2002. Intramembrane proteolysis promotes trafficking of hepatitis C virus core protein to lipid droplets. *The EMBO journal*, 21(15), pp.3980–3988.

Mee, C.J. et al., 2008. Effect of cell polarization on hepatitis C virus entry. *Journal of Virology*, 82(1), pp.461–470.

Mee, C.J. et al., 2010. Hepatitis C virus infection reduces hepatocellular polarity in a vascular endothelial growth factor-dependent manner. *Gastroenterology*, 138(3), pp.1134–1142.

Meertens, L. et al., 2008. The tight junction proteins claudin-1, -6, and -9 are entry cofactors for hepatitis C virus. *Journal of Virology*, 82(7), pp.3555–3560.

Mengshol, J.A. et al., 2010. A crucial role for Kupffer cell-derived galectin-9 in regulation of T cell immunity in hepatitis C infection. *PLoS ONE*, 5(3), p.e9504.

Meredith, L.W. et al., 2013. Early infection events highlight the limited transmissibility of hepatitis C virus in vitro. *Journal of hepatology*, 58(6), pp.1074–1080.

Meroni, L. et al., 2007. Altered expression of the tetraspanin CD81 on B and T lymphocytes during HIV-1 infection. *Clinical and experimental immunology*, 147(1), pp.53–59.

Merz, A. et al., 2011. Biochemical and morphological properties of hepatitis C virus particles and determination of their lipidome. *The Journal of biological chemistry*, 286(4), pp.3018–3032.

Meuleman, P. et al., 2008. Anti-CD81 antibodies can prevent a hepatitis C virus infection in vivo. *Hepatology*, 48(6), pp.1761–1768.

Meunier, J.-C. et al., 2008. Apolipoprotein c1 association with hepatitis C virus. *Journal of Virology*, 82(19), pp.9647–9656.

Mishra, P. et al., 2017. Modulation of HCV replication and translation by ErbB3 binding protein1 isoforms. *Virology*, 500, pp.35–49.

Mitry, R.R., 2009. Isolation of Human Hepatocytes. In *Hepatocyte Transplantation*. Methods in Molecular Biology. Totowa, NJ: Humana Press, pp. 17–23.

Mitsuya, H. et al., 1985. 3"-Azido-3-"deoxythymidine (BW A509U): an antiviral agent that inhibits the infectivity and cytopathic effect of human T-lymphotropic virus type III/lymphadenopathy-associated virus in vitro. *Proceedings of the National Academy of Sciences of the United States of America*, 82(20), pp.7096–7100.

Miyamoto, H. et al., 2007. Involvement of the PA28gamma-dependent pathway in insulin resistance induced by hepatitis C virus core protein. *Journal of Virology*, 81(4), pp.1727–1735.

Miyanari, Y. et al., 2007. The lipid droplet is an important organelle for hepatitis C virus production. *Nature Cell Biology*, 9(9), pp.1089–1097.

Mohan, N. et al., 2010. Chronic hepatitis C virus infection in children. *Journal of pediatric gastroenterology and nutrition*, 50(2), pp.123–131.

Molina-Jimenez, F. et al., 2012. Matrigel-embedded 3D culture of Huh-7 cells as a hepatocyte-like polarized system to study hepatitis C virus cycle. *Virology*, 425(1), pp.31–39.

Moradpour, D., Penin, F. & Rice, C.M., 2007. Replication of hepatitis C virus. *Nature Reviews Microbiology*, 5(6), pp.453–463.

Murphy, D.G. et al., 2015. Hepatitis C virus genotype 7, a new genotype originating from central Africa. *Journal of clinical microbiology*, 53(3), pp.967–972.

Müller, H.M. et al., 1993. Peripheral blood leukocytes serve as a possible extrahepatic site for hepatitis C virus replication. *The Journal of general virology*, 74 (Pt 4), pp.669–676.

Myers, R.F. & Siegel, M.I., 1984. The appearance of phospholipase activity in the human macrophage-like cell line U937 during dimethyl sulfoxide induced differentiation. *Biochemical and Biophysical Research Communications*, 118(1), pp.217–224.

Naif, H.M., 2013. Pathogenesis of HIV Infection. *Infectious disease reports*, 5(Suppl 1), p.e6.

Narbus, C.M. et al., 2011. HepG2 cells expressing microRNA miR-122 support the entire hepatitis C virus life cycle. *Journal of Virology*, 85(22), pp.12087–12092.

Nasimuzzaman, M. et al., 2007. Hepatitis C virus stabilizes hypoxia-inducible factor 1alpha and stimulates the synthesis of vascular endothelial growth factor. *Journal of Virology*, 81(19), pp.10249–10257.

Natarajan, V. et al., 2010. HCV in peripheral blood mononuclear cells are predominantly carried on the surface of cells in HIV/HCV co-infected individuals. *Journal of medical virology*, 82(12), pp.2032–2037.

Ndongo-Thiam, N. et al., 2011. Long-term propagation of serum hepatitis C virus (HCV) with production of enveloped HCV particles in human HepaRG hepatocytes. *Hepatology*, 54(2), pp.406–417.

Neddermann, P. et al., 2004. Reduction of hepatitis C virus NS5A hyperphosphorylation by selective inhibition of cellular kinases activates viral RNA replication in cell culture. *Journal of Virology*, 78(23), pp.13306–13314.

Negro, F., 2011. Mechanisms of hepatitis C virus-related insulin resistance. *Clinics and research in hepatology and gastroenterology*, 35(5), pp.358–363.

Negro, F. & Alaei, M., 2009. Hepatitis C virus and type 2 diabetes. *World journal of gastroenterology*, 15(13), pp.1537–1547.

Neil, S.J.D., Zang, T. & Bieniasz, P.D., 2008. Tetherin inhibits retrovirus release and is antagonized by HIV-1 Vpu. *Nature*, 451(7177), pp.425–430.

Nelson, H.B. & Tang, H., 2006. Effect of Cell Growth on Hepatitis C Virus (HCV) Replication and a Mechanism of Cell Confluence-Based Inhibition of HCV RNA and Protein Expression. *Journal of Virology*, 80(3), pp.1181–1190.

Netski, D.M. et al., 2007. CD4+ T cell-dependent reduction in hepatitis C virus-specific humoral immune responses after HIV infection. *The Journal of infectious diseases*, 195(6), pp.857–863.

Neuberger, J., 2016. Liver transplantation in the United Kingdom. *Liver Transplantation*, 22(8), pp.1129–1135.

Neumann, A.U. et al., 1998. Hepatitis C viral dynamics in vivo and the antiviral efficacy of interferon-alpha therapy. *Science*, 282(5386), pp.103–107.

Neumann-Haefelin, C. & Thimme, R., 2011. Success and failure of virus-specific T cell responses in hepatitis C virus infection. *Digestive diseases (Basel, Switzerland)*, 29(4), pp.416–422.

Ni, Y.H. et al., 1994. Temporal profile of hepatitis C virus antibody and genome in infants born to mothers infected with hepatitis C virus but without human immunodeficiency virus coinfection. *Journal of hepatology*, 20(5), pp.641–645.

Ni Y., Urban S. 2017. Stem cell-derived hepatocytes: A promising novel tool to study hepatitis B virus infection. *Journal of Hepatology,* 1540, pp.15-25.

Nikolaou, N. et al., 2016. Optimizing human hepatocyte models for metabolic phenotype and function: effects of treatment with dimethyl sulfoxide (DMSO). *Physiological reports*, 4(21).

Nishijima, T. et al., 2014. Incidence and risk factors for incident Hepatitis C infection among men who have sex with men with HIV-1 infection in a large Urban HIV clinic in Tokyo. *Journal of acquired immune deficiency syndromes (1999)*, 65(2), pp.213–217.

Odendall, C. et al., 2014. Diverse intracellular pathogens activate type III interferon expression from peroxisomes. *Nature immunology*, 15(8), pp.717–726.

Onabajo, O.O. et al., 2015. Expression of Interferon Lambda 4 Is Associated with Reduced Proliferation and Increased Cell Death in Human Hepatic Cells. *Journal*

of interferon & cytokine research : the official journal of the International Society for Interferon and Cytokine Research, 35(11), pp.888–900.

Ottosen, S. et al., 2015. In vitro antiviral activity and preclinical and clinical resistance profile of miravirsen, a novel anti-hepatitis C virus therapeutic targeting the human factor miR-122. *Antimicrobial agents and chemotherapy*, 59(1), pp.599–608.

Pace, M.J. et al., 2012. Directly infected resting CD4+T cells can produce HIV Gag without spreading infection in a model of HIV latency. *PLoS pathogens*, 8(7), p.e1002818.

Parczewski, M. et al., 2012. IL28B CC genotype is associated with higher all-cause mortality in antiretroviral-treated HIV-infected patients. *AIDS research and human retroviruses*, 28(12), pp.1640–1646.

Park, H. et al., 2012. IL-29 is the dominant type III interferon produced by hepatocytes during acute hepatitis C virus infection. *Hepatology*, 56(6), pp.2060–2070.

Park, S.-H. & Rehermann, B., 2014. Immune responses to HCV and other hepatitis viruses. *Immunity*, 40(1), pp.13–24.

Paul, D., Madan, V. & Bartenschlager, R., 2014. Hepatitis C virus RNA replication and assembly: living on the fat of the land. *Cell Host and Microbe*, 16(5), pp.569–579.

Pazienza, V. et al., 2007. The hepatitis C virus core protein of genotypes 3a and 1b downregulates insulin receptor substrate 1 through genotype-specific mechanisms. *Hepatology*, 45(5), pp.1164–1171.

Pedersen, I.M. et al., 2007. Interferon modulation of cellular microRNAs as an antiviral mechanism. *Nature*, 449(7164), pp.919–922.

Peng, M. et al., 2015. HIV Vpr protein upregulates microRNA-122 expression and stimulates hepatitis C virus replication. *Journal of General Virology*, 96(8), pp.2453–2463.

Penin, F. et al., 2004. Structural biology of hepatitis C virus. *Hepatology*, 39(1), pp.5–19.

Perrault, M. & Pécheur, E.-I., 2009. The hepatitis C virus and its hepatic environment: a toxic but finely tuned partnership. *The Biochemical journal*, 423(3), pp.303–314.

Pestova, T.V., Borukhov, S.I. & Hellen, C.U., 1998. Eukaryotic ribosomes require initiation factors 1 and 1A to locate initiation codons. *Nature*, 394(6696), pp.854–859.

Peters, L. et al., 2014. HIV and hepatitis C co-infection in Europe, Israel and Argentina: a EuroSIDA perspective. *BMC infectious diseases*, 14 Suppl 6, p.S13.

Petit, T. et al., 1995. A highly sensitive polymerase chain reaction method reveals the ubiquitous presence of maternal cells in human umbilical cord blood. *Experimental hematology*, 23(14), pp.1601–1605.

Pfeffer, S. & Baumert, T.F., 2010. Impact of microRNAs for pathogenesis and treatment of hepatitis C virus infection. *Gastroenterologie clinique et biologique*, 34(8-9), pp.431–435.

Pileri, P. et al., 1998. Binding of hepatitis C virus to CD81. *Science*, 282(5390), pp.938–941.

Pineda, J.A. et al., 2005. HIV coinfection shortens the survival of patients with hepatitis C virus-related decompensated cirrhosis. *Hepatology*, 41(4), pp.779–789.

Piroth, L. et al., 1998. Does hepatitis C virus co-infection accelerate clinical and immunological evolution of HIV-infected patients? *AIDS (London, England)*, 12(4), pp.381–388.

Platt, L. et al., 2016. Prevalence and burden of HCV co-infection in people living with HIV: a global systematic review and meta-analysis. *The Lancet infectious diseases*, 16(7), pp.797–808.

Ploss, A. et al., 2009. Human occludin is a hepatitis C virus entry factor required for infection of mouse cells. *Nature*, 457(7231), pp.882–886.

Ploss, A. et al., 2010. Persistent hepatitis C virus infection in microscale primary human hepatocyte cultures. *Proceedings of the National Academy of Sciences*, 107(7), pp.3141–3145.

Podevin, P. et al., 2010. Production of infectious hepatitis C virus in primary cultures of human adult hepatocytes. *Gastroenterology*, 139(4), pp.1355–1364.

Polis, C.B. et al., 2007. Impact of maternal HIV coinfection on the vertical transmission of hepatitis C virus: a meta-analysis. *Clinical infectious diseases : an official publication of the Infectious Diseases Society of America*, 44(8), pp.1123–1131.

Popescu, C.-I. et al., 2014. Hepatitis C virus life cycle and lipid metabolism. *Biology*, 3(4), pp.892–921.

Protzer, U., Maini, M.K. & Knolle, P.A., 2012. Living in the liver: hepatic infections. *Nature reviews. Immunology*, 12(3), pp.201–213.

Qian, C. et al., 1992. Replication of hepatitis C virus in peripheral blood mononuclear cells. Effect of alpha-interferon therapy. *Journal of hepatology*, 16(3), pp.380–383.

Raj, V.S. et al., 2017. Identification of HCV Resistant Variants against Direct Acting Antivirals in Plasma and Liver of Treatment Naïve Patients. *Scientific reports*, 7(1), p.4688.

Rauch, A., Rohrbach, J. & Bochud, P.-Y., 2010. The recent breakthroughs in the understanding of host genomics in hepatitis C. *European journal of clinical investigation*, 40(10), pp.950–959.

Raulf-Heimsoth, M., 2008. T cell - primary culture from peripheral blood. *Methods in molecular medicine*, 138, pp.17–30.

Rausell, A. et al., 2016. Innate immune defects in HIV permissive cell lines. *Retrovirology*, 13(1), p.43.

Ray, R.B., Meyer, K. & Ray, R., 2000. Hepatitis C virus core protein promotes immortalization of primary human hepatocytes. *Virology*, 271(1), pp.197–204.

Reeves, J.D. & Doms, R.W., 2002. Human immunodeficiency virus type 2. *The Journal of general virology*, 83(Pt 6), pp.1253–1265.

Reynolds, G.M. et al., 2007. Hepatitis C virus receptor expression in normal and diseased liver tissue. *Hepatology*, 47(2), pp.418–427.

Riemer, J. et al., 2004. Colorimetric ferrozine-based assay for the quantitation of iron in cultured cells. *Analytical biochemistry*, 331(2), pp.370–375.

Ringelhan, M., McKeating, J.A. & Protzer, U., 2017. Viral hepatitis and liver cancer. *Philosophical transactions of the Royal Society of London. Series B, Biological sciences*, 372(1732).

Ripoli, M. et al., 2010. Hepatitis C virus-linked mitochondrial dysfunction promotes hypoxia-inducible factor 1 alpha-mediated glycolytic adaptation. *Journal of Virology*, 84(1), pp.647–660.

Ritchie, A.J. et al., 2011. Differences in HIV-specific T cell responses between HIV-exposed and -unexposed HIV-seronegative individuals. *Journal of Virology*, 85(7), pp.3507–3516.

Roberts, A., Lewis, A.P. & Jopling, C.L., 2011. miR-122 activates hepatitis C virus translation by a specialized mechanism requiring particular RNA components. *Nucleic acids research*.

Roberts, P.C., Kipperman, T. & Compans, R.W., 1999. Vesicular stomatitis virus G protein acquires pH-independent fusion activity during transport in a polarized endometrial cell line. *Journal of Virology*, 73(12), pp.10447–10457.

Rodriguez, K.R., Bruns, A.M. & Horvath, C.M., 2014. MDA5 and LGP2: accomplices and antagonists of antiviral signal transduction. *Journal of Virology*, 88(15), pp.8194–8200.

Rutebemberwa, A. et al., 2008. High-programmed death-1 levels on hepatitis C virus-specific T cells during acute infection are associated with viral persistence and require preservation of cognate antigen during chronic infection. *Journal of immunology (Baltimore, Md. : 1950)*, 181(12), pp.8215–8225.

Saeed, M. et al., 2015. SEC14L2 enables pan-genotype HCV replication in cell culture. *Nature*, 524(7566), pp.471–475.

Sainz, B. & Chisari, F.V., 2006. Production of Infectious Hepatitis C Virus by Well-Differentiated, Growth-Arrested Human Hepatoma-Derived Cells. *Journal of Virology*, 80(20), pp.10253–10257.

Sainz, B. et al., 2012. Identification of the Niemann-Pick C1-like 1 cholesterol absorption receptor as a new hepatitis C virus entry factor. *Nature medicine*, 18(2), pp.281–285.

Sainz, B., TenCate, V. & Uprichard, S.L., 2009. Three-dimensional Huh7 cell culture system for the study of Hepatitis C virus infection. *Virology journal*, 6, p.103.

Sajadi, M.M. et al., 2012. Chronic immune activation and decreased CD4 cell counts associated with hepatitis C infection in HIV-1 natural viral suppressors. *AIDS (London, England)*, 26(15), pp.1879–1884.

Sarasin-Filipowicz, M. et al., 2009. Alpha interferon induces long-lasting refractoriness of JAK-STAT signaling in the mouse liver through induction of USP18/UBP43. *Molecular and cellular biology*, 29(17), pp.4841–4851.

Sarasin-Filipowicz, M. et al., 2008. Interferon signaling and treatment outcome in chronic hepatitis C. *Proceedings of the National Academy of Sciences*, 105(19), pp.7034–7039.

Sarrazin, C. et al., 2007. Dynamic hepatitis C virus genotypic and phenotypic changes in patients treated with the protease inhibitor telaprevir. *Gastroenterology*, 132(5), pp.1767–1777.

Sánchez, C. et al., 2013. Scaling up epidemics of acute hepatitis C and syphilis in HIV-infected men who have sex with men in Spain. *Liver international : official journal of the International Association for the Study of the Liver*, 33(9), pp.1357–1362.

Scarselli, E. et al., 2002. The human scavenger receptor class B type I is a novel candidate receptor for the hepatitis C virus. *The EMBO journal*, 21(19), pp.5017–5025.

Schaefer, A. et al., 2010. Suitable reference genes for relative quantification of miRNA expression in prostate cancer. *Experimental & molecular medicine*, 42(11), pp.749–758.

Scheel, T.K.H. & Rice, C.M., 2013. Understanding the hepatitis C virus life cycle paves the way for highly effective therapies. *Nature medicine*, 19(7), pp.837–849.

Schlabe, S. & Rockstroh, J.K., 2018. Advances in the treatment of HIV/HCV coinfection in adults. *Expert opinion on pharmacotherapy*, 19(1), pp.49–64.

Schmidt, B.L., 1991. A rapid chemiluminescence detection method for PCR-amplified HIV-1 DNA. *Journal of virological methods*, 32(2-3), pp.233–244.

Schneider, W.M., Chevillotte, M.D. & Rice, C.M., 2014. Interferon-stimulated genes: a complex web of host defenses. *Annual review of immunology*, 32, pp.513–545.

Schultze, B., Zimmer, G. & Herrler, G., 1996. Virus entry into a polarized epithelial cell line (MDCK): similarities and dissimilarities between influenza C virus and bovine coronavirus. *The Journal of general virology*, 77 (Pt 10), pp.2507–2514.

Schwartz, R.E. et al., 2012. Modeling hepatitis C virus infection using human induced pluripotent stem cells. *Proceedings of the National Academy of Sciences*, 109(7), pp.2544–2548.

Seal, K.H. et al., 2007. The association of human immunodeficiency virus infection with spontaneously resolved hepatitis C virus infection and level of viremia among injection drug users. *Digestive diseases and sciences*, 52(12), pp.3423–3430.

Sendenburg, Dufour, 2011. Liver Physiology in Gines and Kamath Chronic Liver Failure Clinical Gastroenterology. Humana Press

Sendi, H. et al., 2015. MiR-122 decreases HCV entry into hepatocytes through binding to the 3' UTR of OCLN mRNA. *Liver international : official journal of the International Association for the Study of the Liver*, 35(4), pp.1315–1323.

Seth, R.B. et al., 2005. Identification and characterization of MAVS, a mitochondrial antiviral signaling protein that activates NF-kappaB and IRF 3. *Cell*, 122(5), pp.669–682.

Shaw, R.J., 2006. Glucose metabolism and cancer. *Current opinion in cell biology*, 18(6), pp.598–608.

Sheahan, T.P. et al., 2014. Interferon Lambda Alleles Predict Innate Antiviral Immune Responses and Hepatitis C Virus Permissiveness. *Cell Host and Microbe*, (15), pp.190–202.

Sheehy, A.M. & Erthal, J., 2012. APOBEC3 versus Retroviruses, Immunity versus Invasion: Clash of the Titans. *Molecular biology international*, 2012, p.974924.

Sheehy, A.M. et al., 2002. Isolation of a human gene that inhibits HIV-1 infection and is suppressed by the viral Vif protein. *Nature*, 418(6898), pp.646–650.

Shin, K., Fogg, V.C. & Margolis, B., 2006. Tight Junctions and Cell Polarity. *Annual Review Cell and Developmental Biology*, 22, pp.207–235.

Shirasaki, T. et al., 2013. MicroRNA-27a regulates lipid metabolism and inhibits hepatitis C virus replication in human hepatoma cells. *Journal of Virology*, 87(9), pp.5270–5286.

Shoji, I., Deng, L. & Hotta, H., 2012. Molecular Mechanism of Hepatitis C Virus-Induced Glucose Metabolic Disorders. *Frontiers in Microbiology*, 2.

Shrivastava, S. et al., 2015. MicroRNAs: Role in Hepatitis C Virus pathogenesis. *Genes & diseases*, 2(1), pp.35–45.

Si-Tayeb, K. et al., 2010. Highly efficient generation of human hepatocyte-like cells from induced pluripotent stem cells. *Hepatology*, 51(1), pp.297–305.

Simmonds, P., 2004. Genetic diversity and evolution of hepatitis C virus--15 years on. *The Journal of general virology*, 85(Pt 11), pp.3173–3188.

Simmonds, P., Holmes, E.C., et al., 1993. Classification of hepatitis C virus into six major genotypes and a series of subtypes by phylogenetic analysis of the NS-5 region. *The Journal of general virology*, 74 (Pt 11), pp.2391–2399.

Simmonds, P., McOmish, F., et al., 1993. Sequence variability in the 5' non-coding region of hepatitis C virus: identification of a new virus type and restrictions on sequence diversity. *The Journal of general virology*, 74 (Pt 4), pp.661–668.

Singh, A.B. & Harris, R.C., 2004. Epidermal growth factor receptor activation differentially regulates claudin expression and enhances transepithelial resistance in Madin-Darby canine kidney cells. *Journal of Biological Chemistry*, 279(5), pp.3543–3552.

Smit, C. et al., 2008. Risk of hepatitis-related mortality increased among hepatitis C virus/HIV-coinfected drug users compared with drug users infected only with hepatitis C virus: a 20-year prospective study. *Journal of acquired immune deficiency syndromes (1999)*, 47(2), pp.221–225.

Sodora, D.L. & Silvestri, G., 2008. Immune activation and AIDS pathogenesis. *AIDS (London, England)*, 22(4), pp.439–446.

Song, Y.M. et al., 2014. Dimethyl sulfoxide reduces hepatocellular lipid accumulation through autophagy induction. *Autophagy*, 8(7), pp.1085–1097.

Stamataki, Z. et al., 2009. Hepatitis C virus association with peripheral blood B lymphocytes potentiates viral infection of liver-derived hepatoma cells. *Blood*, 113(3), pp.585–593.

Stanaway, J.D. et al., 2016. The global burden of viral hepatitis from 1990 to 2013: findings from the Global Burden of Disease Study 2013. *Lancet (London, England)*, 388(10049), pp.1081–1088.

Steenbergen, R.H.G. et al., 2013. Human serum leads to differentiation of human hepatoma cells, restoration of very-low-density lipoprotein secretion, and a 1000-fold increase in HCV Japanese fulminant hepatitis type 1 titers. *Hepatology*, 58(6), pp.1907–1917.

Stremlau, M. et al., 2004. The cytoplasmic body component TRIM5alpha restricts HIV-1 infection in Old World monkeys. *Nature*, 427(6977), pp.848–853.

Suhy, D.A. et al., 2012. Safe, long-term hepatic expression of anti-HCV shRNA in a nonhuman primate model. *Molecular therapy : the journal of the American Society of Gene Therapy*, 20(9), pp.1737–1749.

Sulkowski, M.S., 2008. Viral hepatitis and HIV coinfection. *Journal of hepatology*, 48(2), pp.353–367.

Sumpter, R. et al., 2005. Regulating intracellular antiviral defense and permissiveness to hepatitis C virus RNA replication through a cellular RNA helicase, RIG-I. *Journal of Virology*, 79(5), pp.2689–2699.

Syder, A.J. et al., 2011. Small molecule scavenger receptor BI antagonists are potent HCV entry inhibitors. *Journal of hepatology*, 54(1), pp.48–55.

Takahashi, K. et al., 2010. Plasmacytoid dendritic cells sense hepatitis C virus-infected cells, produce interferon, and inhibit infection. *Proceedings of the National Academy of Sciences*, 107(16), pp.7431–7436.

Tawar, R.G. et al., 2015. Claudins and pathogenesis of viral infection. *Seminars in cell & developmental biology*, 42, pp.39–46.

Terili, R.R. & Cox, A.L., 2013. Immunity and HCV a review. *Curr HIV/AIDS Rep*, 10(1), pp.51–58.

Thein, H.-H. et al., 2008. Natural history of hepatitis C virus infection in HIV-infected individuals and the impact of HIV in the era of highly active antiretroviral therapy: a meta-analysis. *AIDS (London, England)*, 22(15), pp.1979–1991.

Thenin-Houssier, S. & Valente, S.T., 2016. HIV-1 Capsid Inhibitors as Antiretroviral Agents. *Current HIV research*, 14(3), pp.270–282.

Theurl, I. et al., 2004. Iron regulates hepatitis C virus translation via stimulation of expression of translation initiation factor 3. *The Journal of infectious diseases*, 190(4), pp.819–825.

Thomas, D.L. et al., 2000. Determinants of the quantity of hepatitis C virus RNA. *The Journal of infectious diseases*, 181(3), pp.844–851.

Thomas, D.L. et al., 1996. Effect of human immunodeficiency virus on hepatitis C virus infection among injecting drug users. *The Journal of infectious diseases*, 174(4), pp.690–695.

Thomas, D.L. et al., 2009. Genetic variation in IL28B and spontaneous clearance of hepatitis C virus. *Nature*, 461(7265), pp.798–801.

Thomas, D.L. et al., 1998. Perinatal transmission of hepatitis C virus from human immunodeficiency virus type 1-infected mothers. Women and Infants Transmission Study. *The Journal of infectious diseases*, 177(6), pp.1480–1488.

Thomas, E. et al., 2012. HCV infection induces a unique hepatic innate immune response associated with robust production of type III interferons. *Gastroenterology*, 142(4), pp.978–988.

Timpe et al. 2008. Hepatitis C virus cell-cell transmission in hepatoma cells in the presence of neutralizing antibodies. *Hepatology,* 47(1), pp17-24.

Thorley, J.A., McKeating, J.A. & Rappoport, J.Z., 2010. Mechanisms of viral entry: sneaking in the front door. *Protoplasma*, 244(1-4), pp.15–24.

Tovo, P.A. et al., 1997. Increased risk of maternal-infant hepatitis C virus transmission for women coinfected with human immunodeficiency virus type 1. Italian Study Group for HCV Infection in Children. *Clinical infectious diseases : an official publication of the Infectious Diseases Society of America*, 25(5), pp.1121–1124.

Tran, N.M. et al., 2013. An appropriate selection of a 3D alginate culture model for hepatic Huh-7 cell line encapsulation intended for viral studies. *Tissue engineering. Part A*, 19(1-2), pp.103–113.

Trapani, J.A. & Smyth, M.J., 2002. Functional significance of the perforin/granzyme cell death pathway. *Nature reviews. Immunology*, 2(10), pp.735–747.

Treyer, A. & Müsch, A., 2013. Hepatocyte polarity. *Comprehensive Physiology*, 3(1), pp.243–287.

Tsang, J.S., Ebert, M.S. & van Oudenaarden, A., 2010. Genome-wide dissection of microRNA functions and cotargeting networks using gene set signatures. *Molecular cell*, 38(1), pp.140–153.

Tscherne, D.M. et al., 2006. Time- and temperature-dependent activation of hepatitis C virus for low-pH-triggered entry. *Journal of Virology*, 80(4), pp.1734–1741.

Tsukita, S., Furuse, M. & Itoh, M., 2001. Multifunctional strands in tight junctions. *Nature reviews. Molecular cell biology*, 2(4), pp.285–293.

Van Damme, N. & Guatelli, J., 2008. HIV-1 Vpu inhibits accumulation of the envelope glycoprotein within clathrin-coated, Gag-containing endosomes. *Cellular Microbiology*, 10(5), pp.1040–1057.

van de Laar, T. et al., 2009. Evidence of a large, international network of HCV transmission in HIV-positive men who have sex with men. *Gastroenterology*, 136(5), pp.1609–1617.

van de Laar, T.J.W. et al., 2007. Increase in HCV incidence among men who have sex with men in Amsterdam most likely caused by sexual transmission. *The Journal of infectious diseases*, 196(2), pp.230–238.

van Montfort, T. et al., 2007. Efficient capture of antibody neutralized HIV-1 by cells expressing DC-SIGN and transfer to CD4+ T lymphocytes. *Journal of immunology (Baltimore, Md. : 1950)*, 178(5), pp.3177–3185.

Vassilaki, N. et al., 2013. Low oxygen tension enhances hepatitis C virus replication. *Journal of Virology*, 87(5), pp.2935–2948.

Vidricaire, G., Gauthier, S. & Tremblay, M.J., 2007. HIV-1 infection of trophoblasts is independent of gp120/CD4 Interactions but relies on heparan sulfate proteoglycans. *The Journal of infectious diseases*, 195(10), pp.1461–1471.

Vienberg, S. et al., 2017. MicroRNAs in metabolism. *Acta physiologica (Oxford, England)*, 219(2), pp.346–361.

Wakita, T. et al., 2005. Production of infectious hepatitis C virus in tissue culture from a cloned viral genome. *Nature medicine*, 11(7), pp.791–796.

Walker, B. & McMichael, A., 2012. The T-cell response to HIV. *Cold Spring Harbor perspectives in medicine*, 2(11).

Wandeler, G. et al., 2012. Hepatitis C virus infections in the Swiss HIV Cohort Study: a rapidly evolving epidemic. *Clinical infectious diseases : an official publication of the Infectious Diseases Society of America*, 55(10), pp.1408–1416.

Wang, C. et al., 1995. An RNA pseudoknot is an essential structural element of the internal ribosome entry site located within the hepatitis C virus 5' noncoding region. *RNA (New York, N.Y.)*, 1(5), pp.526–537.

Wang, J.T. et al., 1992. Detection of replicative form of hepatitis C virus RNA in peripheral blood mononuclear cells. *The Journal of infectious diseases*, 166(5), pp.1167–1169.

Warburg, O., Wind, F. & Negelein, E., 1927. THE METABOLISM OF TUMORS IN THE BODY. *The Journal of general physiology*, 8(6), pp.519–530.

Waris, G. et al., 2007. Hepatitis C virus induces proteolytic cleavage of sterol regulatory element binding proteins and stimulates their phosphorylation via oxidative stress. *Journal of Virology*, 81(15), pp.8122–8130.

Wei, S. et al., 2016. HNF-4α regulated miR-122 contributes to development of gluconeogenesis and lipid metabolism disorders in Type 2 diabetic mice and in palmitate-treated HepG2 cells. *European journal of pharmacology*, 791, pp.254–263.

Weinlich, S. et al., 2009. IGF2BP1 enhances HCV IRES-mediated translation initiation via the 3'UTR. *RNA (New York, N.Y.)*, 15(8), pp.1528–1542.

Westhaus, S. et al., 2017. Scavenger receptor class B member 1 (SCARB1) variants modulate hepatitis C virus replication cycle and viral load. *Journal of hepatology*, 67(2), pp.237–245.

Wieland, S. et al., 2014. Simultaneous detection of hepatitis C virus and interferon stimulated gene expression in infected human liver. *Hepatology*, 59(6), pp.2121–2130.

Wilson, G.K. & Stamataki, Z., 2012. In vitro systems for the study of hepatitis C virus infection. *International journal of hepatology*, 2012, p.292591.

Wilson, G.K. et al., 2015. Permissivity of primary hepatocytes and hepatoma cell lines to support hepatitis C virus infection. *Journal of General Virology*, 96(Pt 6), pp.1369–1373.

Wilson, G.K., Tennant, D.A. & McKeating, J.A., 2014. Hypoxia inducible factors in liver disease and hepatocellular carcinoma: current understanding and future directions. *Journal of hepatology*, 61(6), pp.1397–1406.

Wilson, J.A. et al., 2011. Human Ago2 is required for efficient microRNA 122 regulation of hepatitis C virus RNA accumulation and translation. *Journal of Virology*, 85(5), pp.2342–2350.

Witteveldt, J. et al., 2009. CD81 is dispensable for hepatitis C virus cell-to-cell transmission in hepatoma cells. *The Journal of general virology*, 90(Pt 1), pp.48–58.

Witteveldt, J., Martin-Gans, M. & Simmonds, P., 2016. Enhancement of the Replication of Hepatitis C Virus Replicons of Genotypes 1 to 4 by Manipulation of CpG and UpA Dinucleotide Frequencies and Use of Cell Lines Expressing SECL14L2 for Antiviral Resistance Testing. *Antimicrobial agents and chemotherapy*, 60(5), pp.2981–2992.

World Health Organisation. Guidlelines for the screening, care and treatment of persons with chronic hepatitis C infection. April 2016, Geneva

World Health Organisation. Global Hepatitis Report, 2017, Geneva

Wu, L. & KewalRamani, V.N., 2006. Dendritic-cell interactions with HIV: infection and viral dissemination. *Nature reviews. Immunology*, 6(11), pp.859–868.

Wu, X. et al., 2012. Productive hepatitis C virus infection of stem cell-derived hepatocytes reveals a critical transition to viral permissiveness during differentiation. *PLoS pathogens*, 8(4), p.e1002617.

Xiao, F. et al., 2014. Synergy of entry inhibitors with direct-acting antivirals uncovers novel combinations for prevention and treatment of hepatitis C. *Gut,* 64(3), pp 483-94.

Xiao, P. et al., 2008. Characterization of a CD4-independent clinical HIV-1 that can efficiently infect human hepatocytes through chemokine (C-X-C motif) receptor 4. *AIDS (London, England)*, 22(14), pp.1749–1757.

Xiong, Y. et al., 2015. Hepatitis C virus represses the cellular antiviral response by upregulating the expression of signal transducer and activator of transcription 3 through sponging microRNA‐122. *Molecular medicine reports*, 11(3), pp.1733–1737.

Yang, F. et al., 2008. Cyclophilin A is an essential cofactor for hepatitis C virus infection and the principal mediator of cyclosporine resistance in vitro. *Journal of Virology*, 82(11), pp.5269–5278.

Yang, Z., Cappello, T. & Wang, L., 2015. Emerging role of microRNAs in lipid metabolism. *Acta pharmaceutica Sinica. B*, 5(2), pp.145–150.

Yeung, L.T.F. & Roberts, E.A., 2010. Current issues in the management of paediatric viral hepatitis. *Liver international : official journal of the International Association for the Study of the Liver*, 30(1), pp.5–18.

Yoshikawa, T. et al., 2012. Silencing of microRNA-122 enhances interferon-α signaling in the liver through regulating SOCS3 promoter methylation. *Scientific reports*, 2, p.637.

Zanetti, A.R. et al., 1998. A prospective study on mother-to-infant transmission of hepatitis C virus. *Intervirology*, 41(4-5), pp.208–212.

Zanetti, A.R. et al., 1995. Mother-to-infant transmission of hepatitis C virus. Lombardy Study Group on Vertical HCV Transmission. *Lancet (London, England)*, 345(8945), pp.289–291.

Zhang, S. et al., 2013. CD81/CD9 tetraspanins aid plasmacytoid dendritic cells in recognition of hepatitis C virus-infected cells and induction of interferon-alpha. *Hepatology*, 58(3), pp.940–949.

Zheng, A. et al., 2007. Claudin-6 and claudin-9 function as additional coreceptors for hepatitis C virus. *Journal of Virology*, 81(22), pp.12465–12471.

Zhong, W., Ingravallo, P., et al., 2000. RNA-dependent RNA polymerase activity encoded by GB virus-B non-structural protein 5B. *Journal of Viral Hepatitis*, 7(5), pp.335–342.

Zhong, W., Uss, A.S., et al., 2000. De novo initiation of RNA synthesis by hepatitis C virus nonstructural protein 5B polymerase. *Journal of Virology*, 74(4), pp.2017–2022.

Zhou, M. et al., 2014. Long-term maintenance of human fetal hepatocytes and prolonged susceptibility to HBV infection by co-culture with non-parenchymal cells. *Journal of virological methods*, 195, pp.185–193.

Zignego, A.L. et al., 1992. Infection of peripheral mononuclear blood cells by hepatitis C virus. *Journal of hepatology*, 15(3), pp.382–386.