# THE ROLE OF STROMAL CELLS IN HEPATITIS C VIRUS INFECTION

by

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**Abstract**

Hepatitis C virus (HCV) is a major cause of global morbidity, causing long-term pathologies, including cirrhosis and hepatocellular carcinoma. While hepatocytes are the major site of viral replication, the liver contains multiple non-parenchymal cells that regulate the hepatic microenvironment and may affect HCV infection *in vivo*. Current understanding of the role of non-parenchymal cells in HCV infection is limited. Therefore, this project aimed to establish co-culture systems that allowed investigations into interactions between hepatocytes and non-parenchymal cells, and how these interactions affected HCV infection.

The results showed that in co-culture, activated liver myofibroblasts (aLMFs) negatively regulate HCV entry, replication and spread of infection in a cell contact dependent manner. Soluble factors, including extracellular matrix proteins, and common antiviral pathways did not induce this effect. Instead, we found that aLMF-modulated cell-contact affected hepatocyte membrane receptor dynamics, reducing the mobility of the HCV receptor, CD81, impairing viral entry and replication. In addition, we found that aLMF surface expressed VAP-1 also significantly reduced virus infection independently of receptor modulation. These findings greatly improved our understanding of how the interactions between hepatic cells affect HCV, highlighting the importance of non-parenchymal cells in mediating infection in the liver microenvironment.

**“Curiouser and Curiouser!”**

*-Alice in Wonderland*

## Dedication

This thesis is dedicated to my family, friends and Nikki, for all the support, advice and for always believing in me – you are all truly amazing.

I would also like to dedicate this thesis in loving memory of my grandmother, Swarn Kaur Galsinh, your kind words of encouragement still give me strength today.

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**Frequently used abbreviations**

| | |
|---|---|
| aLMF | Activated liver myofibroblasts |
| FBS | Foetal bovine serum |
| CHO | Chinese hamster ovary cells |
| HCVcc | Cell culture hepatitis C virus |
| HCVpp | Hepatitis C virus pseudoparticles |
| HCV | Hepatitis C virus |
| HSC | Hepatic stellate cell |
| IFN | Interferon |
| ISG56 | Interferon stimulated gene-56 |
| LMF | Liver myofibroblast |
| LX-2 | Lieming Xu-2 stellate cell line |
| PHH | Primary Human Hepatocytes |
| SR-B1 | Scavenger Receptor Class B member I |
| TGFβ | Transforming Growth Factor-beta |

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# Chapter 1 Introduction

## 1.0 General introduction

The liver is a large, complex organ responsible for a number of important functions and contains of a variety of different cell types. It is involved in many functions such as: processing digested foods, controlling metabolic homeostasis, synthesising plasma proteins for example, albumin or clotting factors, detoxifying harmful products, drug metabolism, synthesising, and excreting bile, which is involved in lipid fat digestion, and removing other metabolised compounds from the body (Taub, 2004b).

The liver is a unique organ because it has the ability to regenerate, which is clinically very important as this allows the liver to repair itself especially following acute liver failure caused by factors such as drugs or toxins. This also has great implications for treating patients suffering from chronic liver failure. Patients can either undergo a liver transplant; where the donor liver is divided and used to treat several patients as it is able to regenerate over time, or they can undergo a partial hepatectomy to remove a section of their own liver, which may be damaged or contain a tumour, and the remaining liver is left to regenerate (Bismuth, 1982, Couinaud, 1957, Lefkowitch, 2011). Chronic liver failure is often caused by repetitive liver injury, which causes chronic inflammation. Factors such as high alcohol intake, having a fatty liver or viral infection can cause chronic liver disease. Prolonged inflammation of the liver initiates fibrosis, the liver wound healing response, which, although reversible, causes scarring of the liver and can lead to severe scarring of the liver, known as cirrhosis. Liver cirrhosis is often irreversible, causing liver failure and increasing the risk of

hepatocellular carcinoma or liver cancer. At this stage of liver disease, although patients can undergo a liver transplant or partial hepatectomy this may not always be possible due to the limited availability of donor organs. As a result, liver disease is a major cause of death. Furthermore, viral infection, a large proportion of which is due to Hepatitis C virus (HCV), causes a great burden on liver transplantation in the UK and western world (Brown, 2005, Gitto et al., 2009).

The infection caused by HCV can be acute and asymptomatic but approximately 80% of infected individuals develop persistent and chronic infection, which can progress to hepatocellular carcinoma when the patient will require a liver transplant. Treatments for patients with chronic HCV infection have recently advanced with newly licensed antivirals, which specifically target HCV. Before these new treatments, the standard treatment for HCV infected patients was a combination therapy of interferon-α and pegylated ribavirin, which had many adverse effects. The new antivirals are also being offered in combination therapy with the standard treatment of interferon-α and pegylated ribavirin, which not only increases the adverse effects but also increases the cost of treatment. There is still a need for better therapies that have fewer side effects and are more cost effective to ensure wider use. In order to develop these new therapies, new targets need to be identified, preferably at earlier stages of HCV disease (Blight et al., 2003b, Lindenbach et al., 2005a, Evans et al., 2007, Lemon et al., 2010, Sharma and Feld, 2014, Meredith et al., 2012a).

The key liver cell type involved in fibrosis is the hepatic stellate cell (HSC), which in response to liver injury such as viral disease, is activated to become a liver

myofibroblast (aLMF) to help repair the damage. This process of liver repair involves aLMFs secreting various extracellular matrix components, which would normally be degraded once the liver has repaired. However, when there is an accumulation of extracellular matrix (ECM) in the liver it leads to fibrosis and eventually cirrhosis. Thus, this project aimed to understand the role of aLMF in hepatocellular HCV infection, the impact these cells have on the hepatitis C viral lifecycle and their mechanism of action, potentially identifying new pathways that could be targets for novel therapies (Wynn, 2008, Kisseleva and Brenner, 2008, Kisseleva et al., 2012, Seldon et al., 1999).

## 1.1 The liver function and microenvironment

### *The liver anatomy*

The liver is a large organ weighing between 1.2-1.5 kilograms and is located in the upper right area of the abdomen cavity below the diaphragm and next to the stomach. The liver is protected by the rib cage as it is a key organ responsible for a number of vital functions such as the synthesis, storage, and secretion of factors involved in metabolic homeostasis (Taub, 2004b). Anatomically, the liver can be separated into two distinct lobes visible from the anterior. The right lobe is the larger of the two lobes, which are separated by the falciform ligament anteriorly and the ligamentum teres inferiorly. These ligaments provide a structural support to the liver and assist in holding the liver in position together with the abdomen walls (Bismuth, 1982, Diehl-Jones and Fraser Askin, 2002, van Leeuwen et al., 1994).

The location of the liver within the body assists with its function, as does its rich dual blood supply. The liver is supplied with blood directly from the gut via the hepatic vein accounting for 80% of the blood supply. The remaining 20% of the blood flow is supplied from the heart via the hepatic artery. The blood supplied from the gastrointestinal tract is deoxygenated blood rich in nutrients and the blood supplied from the heart is oxygenated blood. Upon entering the liver, they become mixed as the blood travels along a vast network of branched sinusoidal blood vessels. The mixed blood supply is collected in the central vein where it then travels to the inferior vena cava via the hepatic vein and here the blood is re-oxygenated (Lalor et al., 2002a). The liver can be further separated into 8 distinct functional segments based on the network of blood supply, identified by Couinaud and illustrated in **Figure 1-1** (Couinaud, 1957; Bismuth, 1982; Bismuth, 2014; Yoshida et al., 2012). Each segment has its own major vascular supply (a separate arterial blood supply and bile drainage system), which allows them to be surgically separated from the remaining liver during liver resection surgery (Bismuth, 1982).

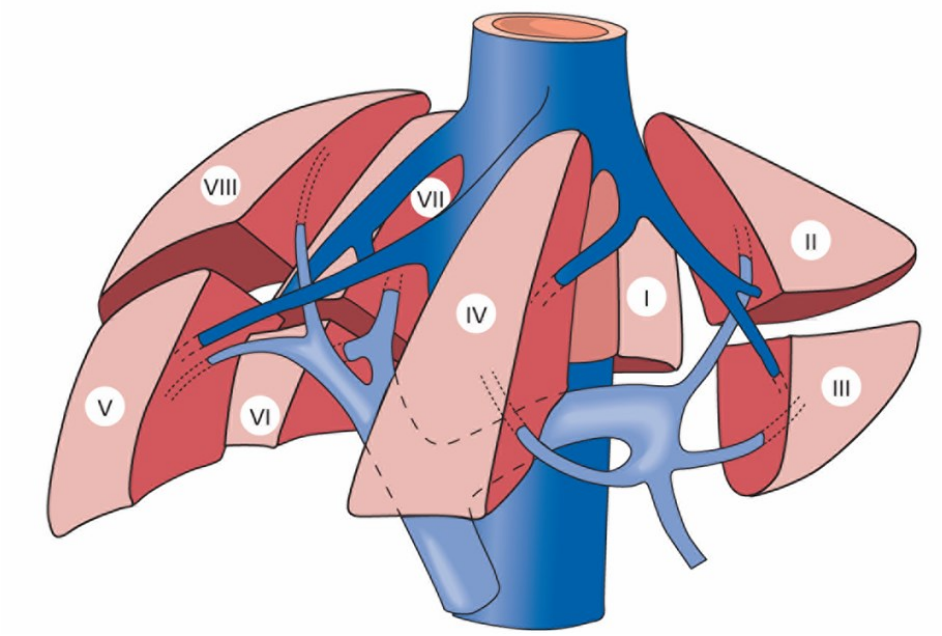



**Figure 1-1 Anatomy of the liver**

Cartoon illustrating the functional anatomy of the liver, with each of the 8 distinct functional segments identified by Couinaud labelled (I-VIII). The light blue vessels illustrate the blood supply from the portal vein and the dark blue illustrate drainage into the hepatic veins (Lefkowitch, 2011)

***The liver microenvironment***

The liver is a complex organ and contains of a variety of cells, but the main cell type, responsible for most of it functions, is the hepatocyte. Hepatocytes account for approximately 80% of the liver mass and are also referred to as liver parenchymal cells. These epithelial cells are large (20-30 μm across) and polygonal in shape, which allows hepatocytes to form multiple contacts with neighbouring hepatocytes and non-parenchymal cells (Pertoft and Smedsrod, 1987).

These multiple contacts are achieved as a result of the complex polarity hepatocytes have, where the membranes are linked by tight junction proteins. The basolateral surface of hepatocytes allows the cells to secrete various serum factors into the venous blood, conditioning the blood as it passes through the liver. Whereas the apical surface forms the canalicular structures and allow the hepatocytes to secrete bile into canaliculi, which then merge with bile ducts (Decaens et al., 2008; Musch, 2014; Perrault and Pecheaur, 2009; Selden et al., 1999). The correct physiological functioning of the liver is a result of complex polarity and the tight junction proteins which help maintain it, and separate the blood flow from the secreted bile (Decaens et al., 2008, Perrault and Pecheur, 2009, Adams and Eksteen, 2006, Selden et al., 1999, Cereijido et al., 1998).

The remaining 20% of the liver mass consists of non-parenchymal cells, also referred to as the stroma, which include: liver sinusoidal endothelial cells (LSEC), hepatic stellate cells (HSC), biliary epithelial cells (BEC) (also referred to as cholangiocytes), Kupffer cells, activated liver myofibroblasts (aLMF), Pit cells (NK cells), hepatic

dendritic cells, and NK T cells. Details of the proportions for each different cell type are listed in **Table 1-1** (Millward-Sadler et al., 1992, Lavon and Benvenisty, 2005b, Meredith et al., 2012a). These non-parenchymal cells have complex and multiple interactions with the hepatocytes. The hepatocytes form plates, which are one cell thick, and in between two plates is the sinusoid, a vascular channel supplying the hepatocytes with blood. The walls of sinusoids are lined by LSEC, a specialised type of endothelial cell with fenestrations that act as a sieve, allowing various nutrients to pass through into the Space of Disse (Braet et al., 2009, Lai et al., 2006, Lozach et al., 2004, Pohlmann et al., 2003).

**Table 1-1 Cellular composition of the liver[a]**

| Cell Type | Number % | Volume % | Cell diameter (μm) |
|---|---|---|---|
| Parenchymal | 65 | 92.5 | 10-34 |
| Non-parenchymal | 35 | 7.5 | 6-15 |
| Sinusoidal endothelial | 21 | 3.3 | 6-11 |
| Kupffer | 8.5 | 2.5 | 7-15 |
| Stellate | 5.5 | 1.7 | 10-13 |

[a]Table adapted from (Pertoft 1987) detailing the cellular composition of a rat liver and the proportions of different cell types. The proportion of cells is similar to that found in the human liver.

Within the Space of Disse there are various extracellular matrix components such as collagen, fibronectin and HSCs. The location of HSCs in relation to hepatocytes is illustrated in **Figure 1-2** (Millward-Sadler, 1992, Lavon and Benvenisty, 2005a, Meredith et al., 2012b). HSCs store vitamin A and become activated in response to liver injury into aLMF cells. These aLMF synthesize collagen and various other factors which ultimately lead to liver fibrosis (Taub, 2004a). Kupffer cells and pit cells are liver specific macrophages, which are responsible for phagocytising various pathogens and foreign particles that enter the liver and are found in the peripheral region of the liver (Millward-Sadler, 1992, Lavon and Benvenisty, 2005a). The liver also contains BEC that are located in the biliary tracts. As a result these cells often become the first cells a pathogen will encounter when it enters the liver via the bile duct, so they produce a range of cytokines in response to various pathogens (Millward-Sadler, 1992, Lavon and Benvenisty, 2005a, Kanno et al., 2000).

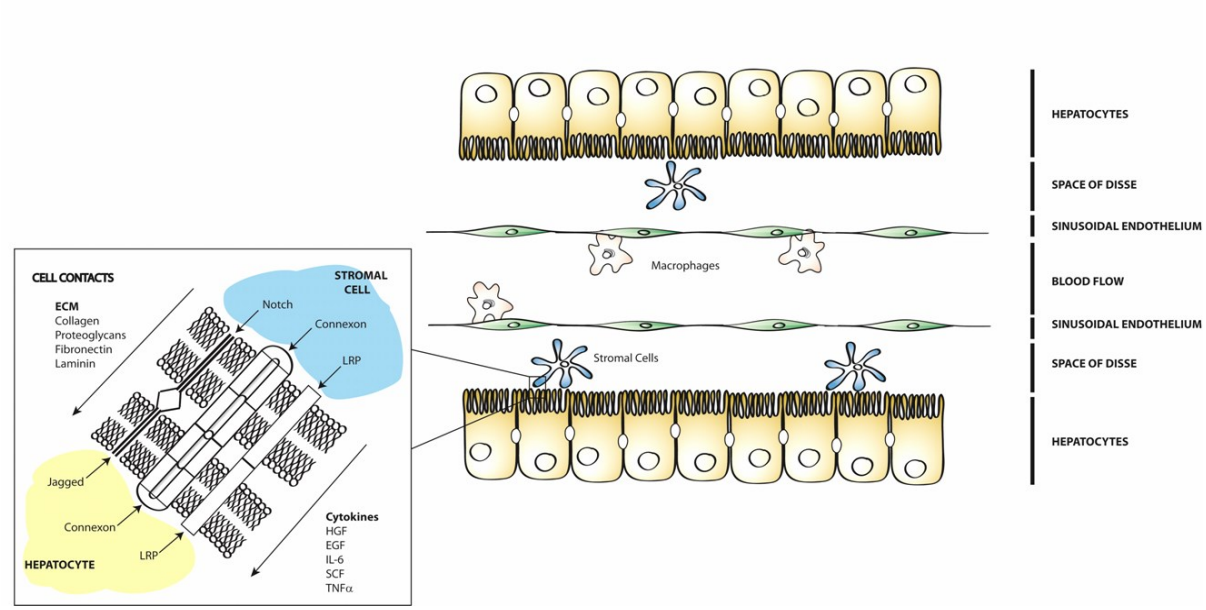



**Figure 1-2 The liver architecture**

An illustration showing the organisation of different cell types in the liver. Within the Space of Disse there are various extracellular matrix components such as collagen and fibronectin, and stromal cells such as hepatic stellate cells which are in direct contact with hepatocytes. Upon activation in response to liver injury such as viral infection, the stromal hepatic stellate cells become activated liver myofibroblasts (aLMF).

## 1.2 Fibrosis and aLMF

Fibrosis is a dynamic process between the deposition of extracellular matrix components (ECM) and the degradation of ECM. It is defined as the excessive healing response with scarring of the various tissues and excess ECM, particularly collagen, in response to continuous liver injury. Liver fibrosis is a result of chronic inflammation of the liver, which can be caused by a number of stimuli including: autoimmune diseases, metabolic diseases, alcohol abuse, viral hepatitis, cholestatic liver disease or toxins (Wynn 2008; Kisseleva et al., 2011; Kisseleva et al., 2008; Selden, Khalil et al., 1999). Myofibroblast cells are the key player in liver fibrosis as determined both clinically and experimentally. The origin of myofibroblasts is still in debate and it has been suggested that fibrosis induced by different types of injury may give rise to different fibrogenenic cells as illustrated in **Figure 1-3** (Kisseleva et al 2008; (Iredale, 2007). Several potential sources giving rise to myofibroblasts have been identified and it is thought that HSCs are the primary source as demonstrated by Mederacke and colleagues (Mederacke et al., 2013). They identified that HSCs can give rise to 82-96% of myofibroblasts using a novel Cre-transgenic mouse and fate tracing (Su et al 2014; Mederacke et al., 2013).

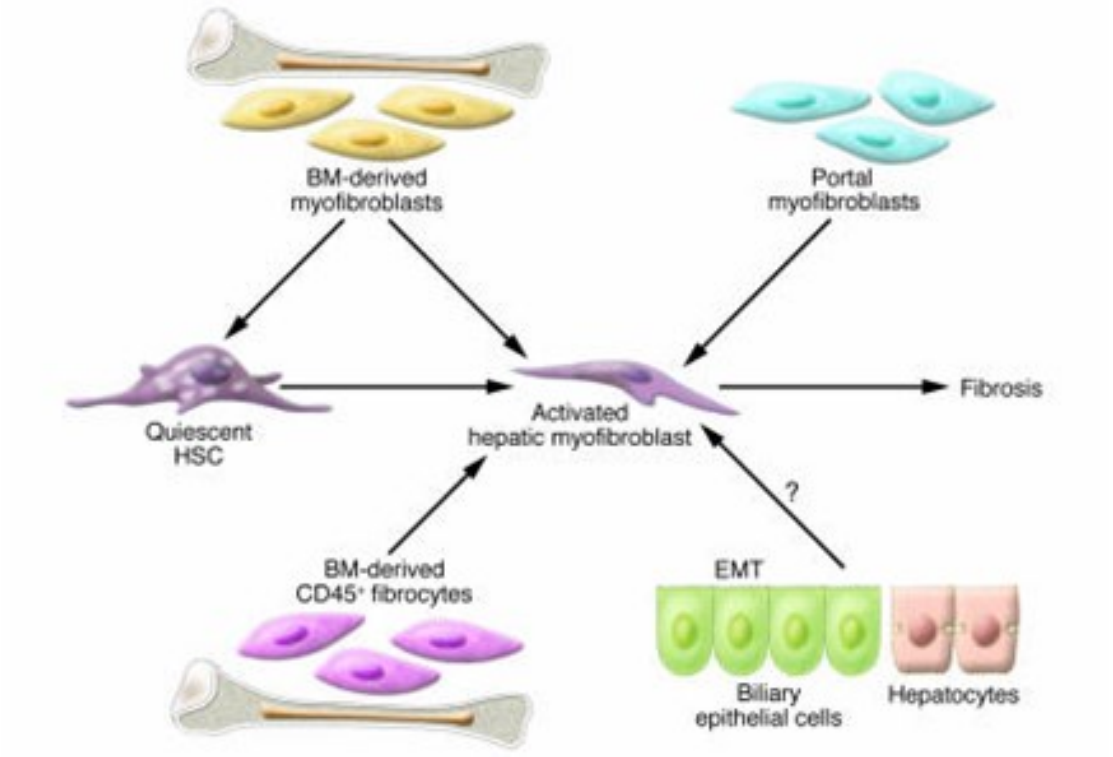



**Figure 1-3 Origins of liver myofibroblasts**

Diagram illustrating the possible sources of liver myofibroblasts. Current evidence suggests quiescent hepatic stellate cells (HSC) are the major source of myofibroblasts. The contribution of hepatocytes and biliary epithelial cells to myofibroblasts, via epithelial-mesenchymal transition (EMT) and endothelial to mesenchymal transition, was considered controversial but studies have shown these cells types are minor contributors of fibrogenic cells (Iredale, 2007).

Under normal conditions, aLMF are not found in the liver but HSC can be found in the space of Disse in a quiescent phenotype. HSC store vitamin A in lipid droplets which when activated in response to liver injury, lose the stored vitamin A and acquire contractility (Taub, 2004a). HSC express neural markers including synaptophysin, glial fibrilar acidic protein (GFAP) and synemin but once activated, these neural markers are down regulated and mesenchymal markers are up regulated such as α-SMA and fibronectin (Iredale, 2007; Geerts, 2001; Iredale et al., 2010). Myofibroblasts can also be derived from portal fibroblasts and bone marrow (BM)-derived mesenchymal cells. Portal fibroblasts do not store vitamin A and do not express α-SMA, unlike HSC and BM stroma do not express hematopoitic markers (Kisseleva et al., 2008). Hepatic epithelial cells can undergo epithelial-to-mesenchymal transition (EMT) into liver myofibroblasts. This process of differentiation is reversible and studies have shown transforming growth factor (TGF)-β is a key cytokine closely linked to driving EMT (Taura et al., 2010; Kisseleva et al., 2008). Interestingly, there have also been suggestions of endothelial cells differentiating into aLMF in a similar process called endothelial-mesenchymal transition (EndMT) (Kalluri et al., 2003; Wynn, 2008). Stromal cells create the microenvironment in the liver, supporting hepatocyte function via various cytokines, extracellular matrix (ECM) and cell contacts as shown in **Figure 1-4**. The myofibroblast synthesises various ECM components particularly collagen (types I, III, IV and V), elastin, laminin, fibronectin and proteoglycans, which can lead to scar tissue when excess is produced and not degraded. ECM tends to accumulate during fibrosis when ECM-removing matrix metalloproteinases (MMPs) are down-regulated, whilst tissue inhibitors of MMPs (TIMPs) are up-regulated, particularly TIMP-1

(Schulze-Krebs et al., 2005; Gomez et al., 2009). Although myofibroblasts create the microenvironment and are the key player in liver fibrosis, the cellular mechanisms of fibrogenesis actually involve a number of different liver cell types in a complex interplay illustrated in **Figure 1-5.** Fibrogenesis can be resolved if the underlying cause of liver injury is treated and cured, leading to fibrosis resolution which involves aLMF cells undergoing apoptosis and the regeneration of hepatocytes (Bataller and Brenner 2005).

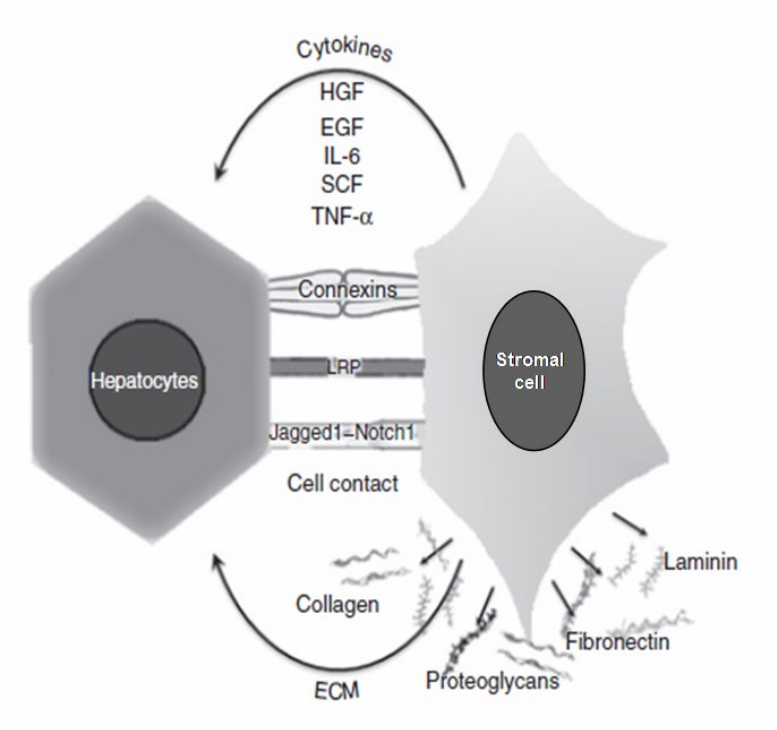



**Figure 1-4 Stromal cells create the microenvironment**

Mechanisms by which non-parenchymal stromal cells create a microenvironment for the hepatocytes to function, via the release of various cytokines, extracellular matrix and cell contacts. The cytokines secreted also mediate hepatocyte proliferation, differentiation, maturation and enhance hematopoiesis (Gomez-Aristizabel et al.,2009).

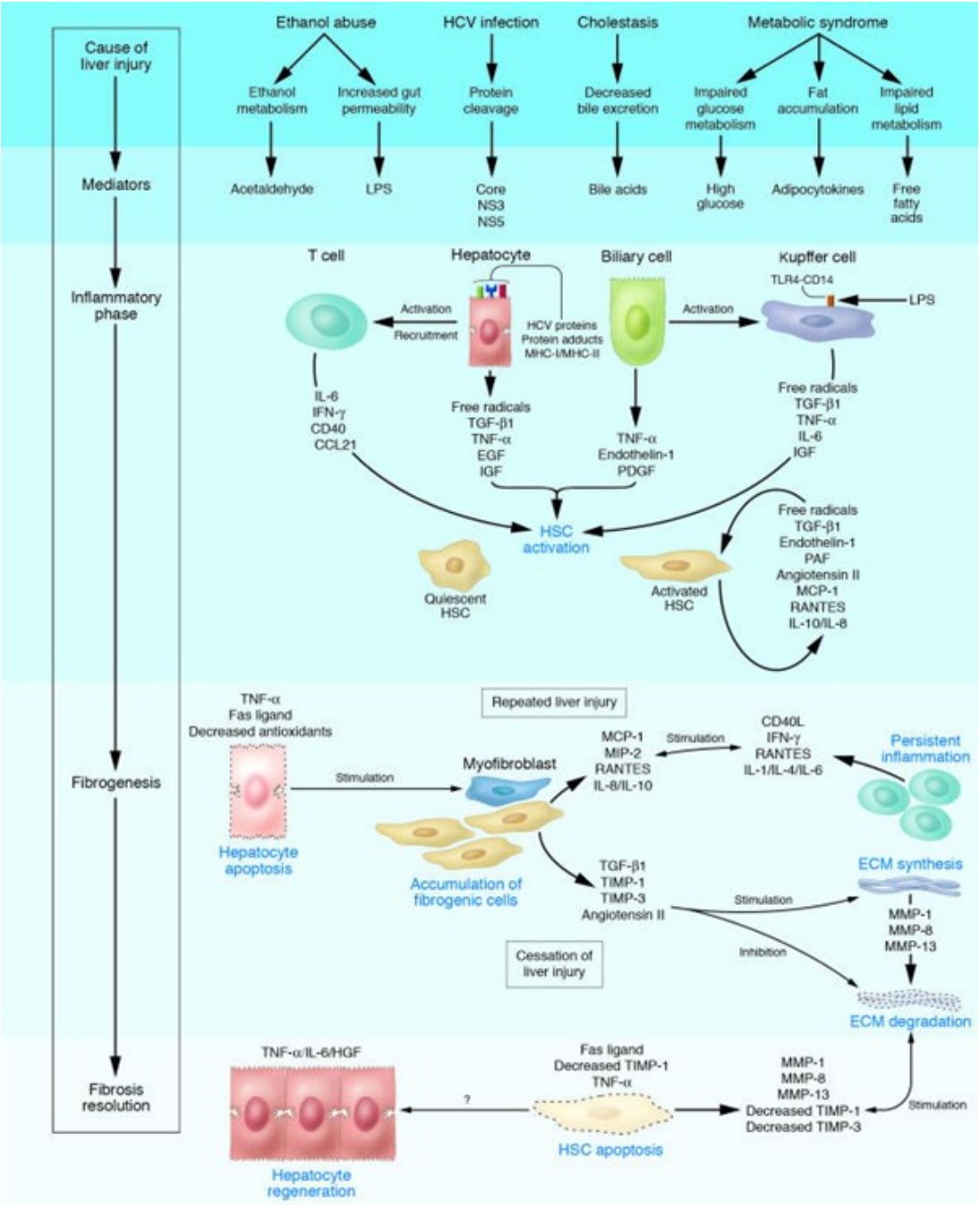



**Figure 1-5 Cellular mechanisms of liver fibrosis**

Schematic diagram of the complex interplay between different liver cell types during hepatic fibrogenesis. Following liver injury, damaged hepatocytes and biliary epithelial cells release inflammatory cytokines and various other soluble factors which activate and recruit T cells as well as activate Kupffer cells. This inflammation state leads to the activation of quiescent HSC to release various cytokines and extra cellular matrix components (ECM). There is an accumulation of aLMF, cytokines and ECM following repeated liver injury during fibrogenesis, which can be resolved once the underlying cause of liver injury has been removed. During fibrosis resolution,

activated HSC undergo apoptosis and there is a regeneration of hepatocytes (Bataller and Brenner 2005).

## 1.3 Hepatitis C virus

### *Discovery of HCV*

Hepatitis C virus (HCV) is one of five hepatitis viruses (named hepatitis virus A, B, C, D and E) that all cause acute or chronic hepatitis in humans. HCV is a blood borne virus that was first discovered during the 1970s, when it was noticed that post-blood transfusion, a viral agent that was a non-A and non-B , was causing hepatitis disease progression in patients. When the virus successfully crossed from humans to chimpanzees in the late 1970's Choo and colleagues worked on isolating and sequencing clones from chimpanzees which lead to the identification of a clone that was a single–stranded, positive sense RNA genome and was named hepatitis C virus (Choo et al., 1989, Lindenbach et al., 2005b, Wakita et al., 2005b, Zhong et al., 2005b).

### *HCV genotypes and epidemiology*

HCV is an enveloped virus belonging to the genus, *Hepacivirus,* of the *Flaviviridae* family. Other hepaciviruses include novel viruses identified across a range of animals including rodents, bats, dogs, horses, and primates (Kapoor et al., 2013, Drexler et al., 2013, Lauck et al., 2013). It can be divided into six major genotypes, which genetically vary in complete viral genome by approximately 30-35% (Simmonds, 1993; Simmonds, 2004). Currently, HCV infects approximately 170 million individuals worldwide and the distribution for each genotype varies geographically. Patients in Europe and North Americas are more commonly infected with genotypes 1a, 1b and 3a. However patients in Asia, the Middle East and North

Africa are more commonly infected with genotypes 2, 4, 5 and 6 (Simmonds, 1993 et al; Simmonds 1995; Simmonds, 2004;Ishrad et al 2010; Bruggmann 2014; Simmonds, 2005). There are reports of a seventh HCV genotype, genotype 7a, which has recently been found in blood samples from Belgium, Central Africa and Canada. The sequence diversity is illustrated by phylogenetic analysis in **Figure 1-6** (Simmonds et al., 1993, Gottwein et al., 2009). Within the genotype subtypes there appears to be further sequence differences which give rise to more than 70 subtypes (labelled a, b, c and so forth) (Simmonds et al., 1993, Shi et al., 2012). To add further to the genetic variation, HCV infected individuals are not positive for a single sequence of HCV but instead positive for a population of sequences which, although they vary with one another, are evolutionary closely related. This population of variants are known as quasispecies, which arise from the high mutation and replication rate of the HCV RNA polymerase. The rate of fixation of mutations in the HCV genome per year has been estimated as $1.44 – 1.92 \times 10^{-3}$ substitutions per genomic site. As a result, within the pool of variants there are mutants that are better adapted to escaping the immune response and antiviral therapies, for examples some mutations can alter the conformation of site where DAA compounds bind, leading to variants which are resistant to various antiviral therapies (Sarrazin et al., 2007, Simmonds et al., 2005, Gomez et al., 1999).

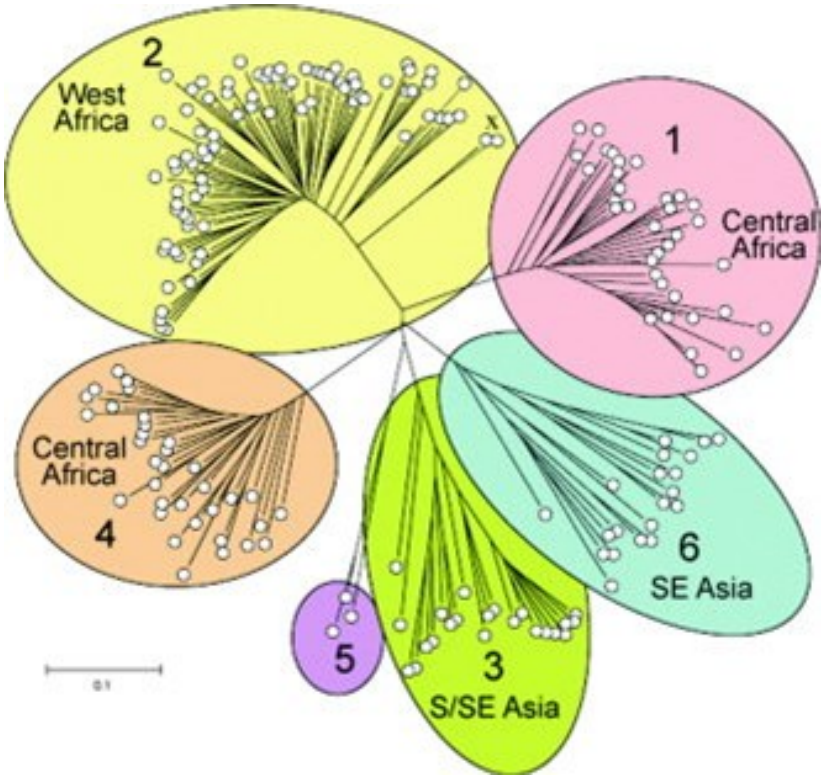



**Figure 1-6 HCV genotypes**

A phylogenetic analysis of HCV RNA polymerase (non-structural protein 5B) which illustrates the areas with high sequence diversity (Simmonds et al., 2005).

Hepatitis C viral particles have a diameter of approximately 50-60nm. The viral envelope is formed of a lipid bilayer containing E1 and E2 glycoproteins, which interact with the host cell. This envelope surrounds a capsid made up of core proteins, which contains the positive sense, single-stranded RNA genome. Despite variation between genotypes, subtypes and quasispecies, all HCV genomes are have a length of approximately 9.6 kilo bases, containing one open reading frame (ORF) with non-translated regions (NTR) at both the 5' and 3' terminus. This genomic material acts as messenger RNA (mRNA) from which the viral proteins can be translated. The ORF encodes for a polyprotein, which is cleaved by both viral and cellular proteinases, in a co- and post- translational manner, into several non-structural proteins (NS2, NS3, NS4A, NS4B, NS5A, NS5B) and structural proteins (core, envelope 1 (E1), envelope 2 (E2), p7). An additional protein of unknown function has also been identified and is a result of a ribosomal frameshift occurring during translation, and so it has been termed the F (frameshift) or ARF (alternative reading frame) protein (Lemon et al., 2010, Lemon, 2001, Brass et al., 2006, Appel et al., 2006, Blight et al., 2003a, Rehermann, 2009).

The structural genes are located at the 5' terminal and the non-structural genes are located at the 3' terminal, and the locations of various cleavage sites, cleaved by either host or viral enzymes, are demonstrated in **Figure 1-7A-C** (Lemon et al., 2010, Rehermann, 2009). As mentioned, the structural proteins core, E1 and E2 are essential components of the HCV virion as they form the viral capsid and envelope. The core protein has a molecular weight of 21kDa and mature core molecules are thought to form homo-dimers *in vivo*, at the endoplasmic reticulum (ER) membrane.

Not only does the core protein form the viral capsid, but it also regulates viral particle assembly, viral RNA binding and RNA translation. More recently, it has been suggested that the core protein may also play a role in various cell signalling pathways such as apoptosis, carcinogenesis and lipid metabolism (Matsumoto et al., 1996, Ait-Goughoulte et al., 2006, Santolini et al., 1994, Tellinghuisen et al., 2005). There are two envelope structural proteins, E1 and E2, which are both type I transmembrane proteins that have short transmembrane domains (TMD) and large hydrophilic ectodomains. The TMD is involved in anchoring E1 and E2 in the ER membrane and is involved in forming E1-E2 heterodimer complexes. These E1-E2 complexes are key in HCV adsorption and entry steps as they interact with CD81 and low density lipoprotein receptor (LDLR) leading to the viral envelope fusing with the host cell membrane, however other factors are also involved in the complicated entry mechanism and are discussed further in **section 1.5**. The E2 protein contains two hypervariable regions (HVR1 and HVR2) which can vary in amino acid sequence by 80% as a result of the immune response and exposure to HCV specific antibodies, therefore it is a key region for neutralising antibodies to target (Cocquerel et al., 1999, Duvet et al., 1998, Flint et al., 1999, Farci et al., 1996, Op De Beeck et al., 2000, Brass et al., 2006).

Between the E2 and N2 regions, there is a small protein called the p7 protein which forms an ion channel in the ER and it is thought to play a role in the assembly and formation of infectious virus particles but has been shown to be non-essential for the replication stage (Blight et al., 2000, Lohmann et al., 1999, Haqshenas et al., 2007, Griffin et al., Pavlović et al., 2003). It is the non-structural (NS) proteins which are

associated with HCV replication. NS2 forms the NS2/NS3 cysteine protease with the NS3 protein (N-terminal portion), which can cleave the polyprotein precursor by autocatalysis between the NS2 and NS3 region. The NS2 protein is thought to play a role in the virus assembly stage, possibly by interacting with E1-E2 complexes and the NS3/NS4A complexes. However, it has also been shown to interact with a number of cellular pathways including pro-apoptotic pathways, cell growth inhibition, arresting cell growth in the S phase and inhibiting IFNβ production (Grakoui et al., 1993, Santolini et al., 1995, Jones et al., 2007, Erdtmann et al., 2003, Kaukinen et al., 2013). The NS3 protein has fewer functions and is thought to interact with other viral proteins to assist its various functions however further work is required to fully understand these interactions and their role in HCV replication. Nevertheless, the C terminal of NS3 has been shown to have ATPase/helicase activity and the N terminal has been shown to utilise a portion of NS54 in order to have serine protease activity (Jennings et al., 2008, Failla et al., 1994, Bartenschlager et al., 1995, Lin et al., 1995, Tanji et al., 1995). The NS4A protein is actually a small polyprotein which acts as a cofactor for the NS3 serine protease and it also targets NS3 to the ER where it acts to stabilise NS3 further (Failla et al., 1994, Bartenschlager et al., 1995, Lin et al., 1995, Tanji et al., 1995, Wölk et al., 2000). The N terminal of the NS4B protein targets it the ER where it forms oligomers as an integral membrane protein. The localisation of NS4B at the ER is key for its functions as NS4B is essential in HCV replication because it can bind RNA, induces an ER derived membranous web structure and is thought to be responsible for forming the HCV RNA replication complex at the ER (Yu et al., 2006, Elazar et al., 2004, Gretton et al., 2005, Einav et al., 2004, Blight, 2011).

The membrane associated phophoprotein NS5A is involved in virus assembly, replication and virus release. The role of NS5A in HCV RNA replication is not fully understood however it is thought to have an essential but complicated role as it has been shown to interact with numerous cellular proteins, making it difficult to determine its main functions. Studies have shown NS5A to have an IFNα sensitivity-determining role (ISDR), interact with cytosolic cyclophilin A (CypA) (essential for HCV replication), and prevent oxidative stress mediated apoptosis to ensure the virus can continue to produce viral particles within a live host cell (Enomoto et al., 1995, Enomoto et al., 1996, Chatterji et al., 2009, Amako et al., 2013, Appel et al., 2008). The NS5B protein is the HCV RNA polymerase which is essential to the HCV replication complex located at the NS4B induced membranous web structure at the ER. NS5B has a high replication error rate as it lacks a proof-reading mechanism and as a result, this gives rise to high genetic variability between patients and within a patients own liver also (Behrens et al., 1996, Schmidt-Mende et al., 2001).

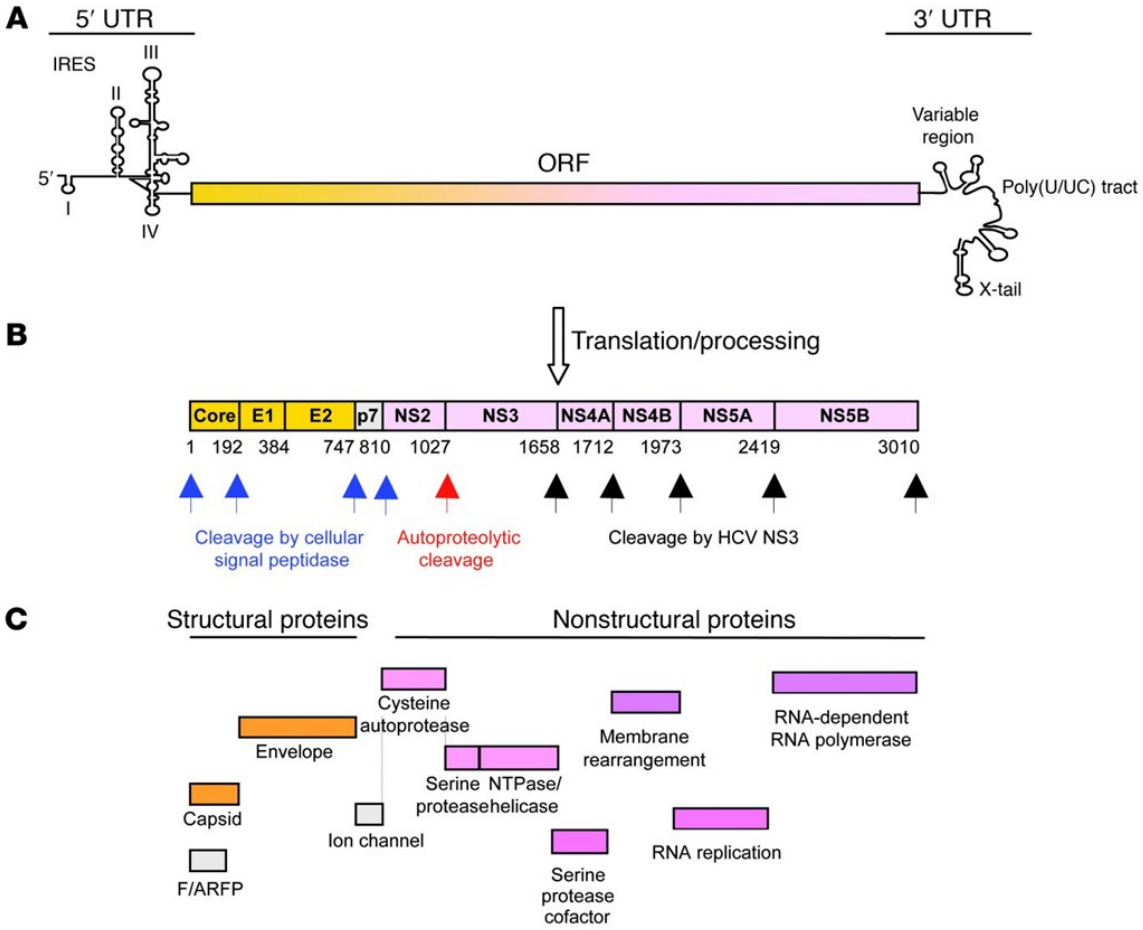



**Figure 1-7 HCV genome**

(**A**) The single-stranded HCV RNA genome encoded along one ORF, flanked by two untranslated regions (UTR). Translation is initiated at the 5' UTR, which contains the internal ribosomal entry site (IRES). (**B**) Following translation, the polyprotein is processed by host and viral proteases. The cleavage sites are labelled with the amino acid number below the polyprotein. (**C**) There are a total of 10 structural and non-structural proteins, each labelled the diagram. There is also a short protein of unknown function as a result a frameshift (F) leading to the translation of an alternative reading frame (ARF) which is labelled F/ARF (Rehermann, 2009)

***HCV disease progression***

HCV is transmitted through the sharing of contaminated needles among injection-drug users. It can also be transmitted via blood transfusions or organ transplants from infected donors, however this route of transmission has become less common since more robust screening tests were introduced. Other routes of transmission include sexual and perinatal transmission (Busch, 2001). Once HCV particles enter the blood stream, the virus reaches the liver via the sinusoidal vascular channels running between plates of hepatocytes. The sinusoidal endothelial fenestrations act like a sieve allowing the virus to pass through into the Space of Disse, although it is thought that they may also help the virus infect hepatocytes by binding infectious virus particles (Braet et al., 2009, Lai et al., 2006, Lozach et al., 2004, Pohlmann et al., 2003).

Hepatocytes are the primary target cells for HCV, as they support entry and full HCV replication, however recent studies suggest there may be extrahepatic sites of replication with the detection of HCV RNA in the brain, CNS tissue and in peripheral blood mononuclear cells (Murray et al., 2008, Fishman et al., 2008, Weissenborn et al., 2009, Weissenborn et al., 2004). In order to accurately determine HCV replication, both negative- and positive-stranded RNA must be detected and other studies have shown primary lymphocytes cannot support the entry and replication of HCV but associate with the virus and this association allows the lymphocytes to transfer infectious virus to hepatocytes *in vitro* (Marukian et al., 2008, Meredith et al., 2012b). A number of studies have also detected HCV RNA in the CSF and CNS tissue, with some identifying genetic variations between the sequences isolated from

the liver and brain from the same patient, but proof of HCV replication within these tissues is required before it can be stated that they support the full HCV lifecycle (Murray et al., 2008, Fishman et al., 2008, Forton et al., 2004, Wilkinson et al., 2010, Meredith et al., 2012b). A recent study by Fletcher and colleagues (2010) has shown several neuroepithelioma cell lines can support high levels of HCV entry and low levels of replication, indicating hepatocytes are not the only cells that can support HCV infection (Fletcher et al., 2010, Fletcher et al., 2012b, Fletcher et al., 2012a).

The infection caused by HCV is often asymptomatic but once established in the liver, chronic infection can progress to scarring of the liver (fibrosis) and advanced scarring (cirrhosis), which in some cases can go on to develop into liver cancer (hepatocellular carcinoma) represented by the cartoon in **Figure 1-8A**. Of the approximately 170 million HCV infected people worldwide, about 70% of those acutely infected individuals go on to develop persistent and chronic infection with about 20-30% progressing to chronic active hepatitis with cirrhosis and about 7% developing hepatocellular carcinoma (HCC) which is fatal end stage liver disease (**Fig.1-8B**) (Lindenbach et al., 2005b, Blight et al., 2003a, Farquhar and McKeating, 2008, Lemon et al., 2010, Meredith et al., 2012b). Chronic HCV infection is defined as chronic if the infection has persisted for more than six months, however, it remains unclear why in many cases HCV develops into a chronic infection. Chronic HCV disease progression also varies in individuals and certain cohorts of patients. Although a number of factors which may contribute towards the variation in disease progression have been identified, we still do not fully understand why disease progression varies so much in individual patients (Vogel et al., 2009). Age and

gender appear to impact HCV disease progression as there are reports of faster disease progression in patients who became initially infected with HCV aged between 40 to 55 years compared to younger patients or children and faster disease progression has been observed in male patients (Svirtlih et al., 2007). Ethnic background also appears to impact disease progression with African-Americans showing slower disease progression and less severe changes in their liver histology (Sterling et al., 2004). Alcohol consumption has been linked with increased HCV replication, faster disease progression and liver injury, with even moderate amounts of alcohol having been shown to increase fibrosis, patients suffering from chronic HCV should avoid alcohol consumption (Gitto et al., 2009). In patients coinfected with other viruses such as HIV or acute HBV, there is generally an acceleration in HCV disease progression except for those patients infected with chronic HBV which may lead to lower levels of HCV replication compared to HCV mono-infected patients but even in this situation, HCV infection will predominate (Danta et al., 2008, Darby et al., 1997, Giordano et al., 2004, Chu and Lee, 2008, Jardi et al., 2001, Zarski et al., 1998). Other factors which may increase or accelerate HCV disease progression include the use of steroids, daily use of marijuana and host factors such as the HCV-specific immune response and the genetic polymorphisms of genes which may influence the progression rate of fibrosis for example adiponutrin (PNPLA3) or transforming growth factor B1 (TGF B1) (Jonsson et al., 2008, Zimmer and Lammert, 2011, Hraber et al., 2007)

Interestingly, 15-20% of infected individuals can spontaneously clear the acute infection following a strong immune response, the mechanism of which is not fully

understood (Rehermann, 2009). Although it is rare for patients with acute HCV infection to suffer from fulminant hepatic failure as a result of the infection, there is an increased risk of this occurring in patients who are coinfected with chronic HBV. In cohorts with HIV and HCV coinfection, the weakened immune response to HCV caused by the underlying HIV infection reduces the chances of spontaneously clearing the HCV infection even further (Chu et al., 1998, Vogel and Rockstroh, 2010, Thomson et al., 2011).

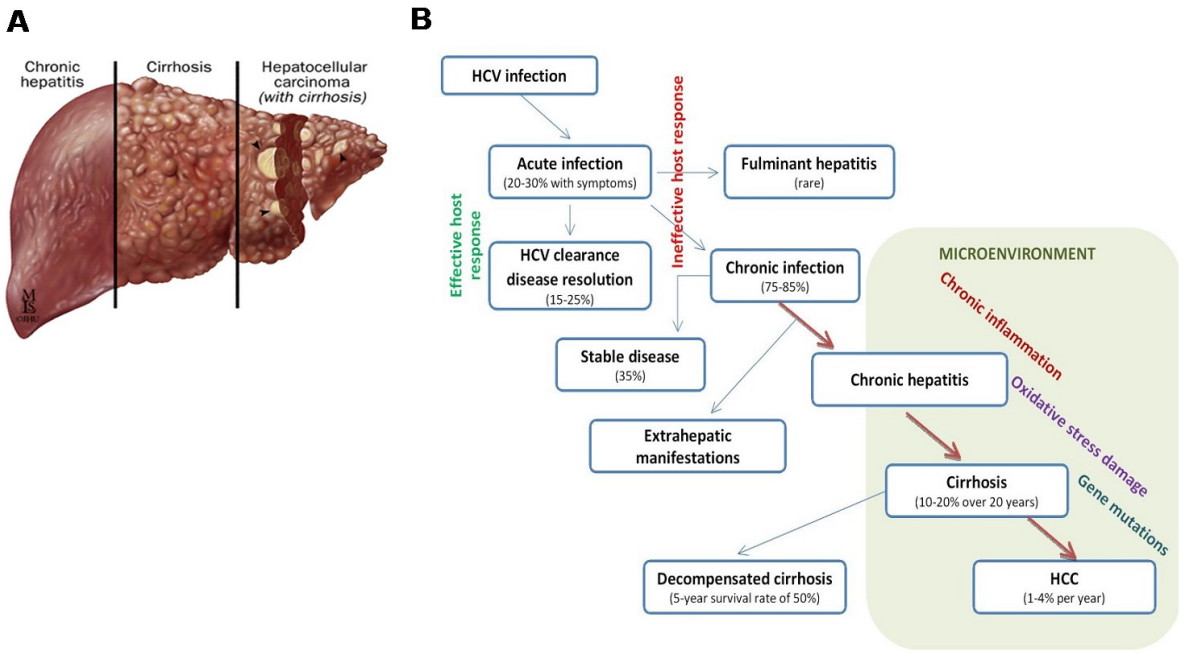



**Figure 1-8 HCV disease progression**

(**A**) Cartoon diagram of the liver illustrating liver disease progression from chronic hepatitis with scarring of the liver (fibrosis), to cirrhosis (advanced scarring) and then hepatocellular carcinoma (HCC) (Racino, 2013). (**B**) HCV disease progression detailing the percentages of patients who advance from acute infection to disease resolution or chronic infection advancing to cirrhosis and HCC.

### *HCV infection and immunity*

Most patients become aware that they are infected with HCV when the disease has progressed to end stage liver failure, typically over the course of 20-30 years, and symptoms of liver damage appear (Sharma and Feld, 2014; Ferenci et al, 2007). Patients suffering with either cirrhosis or hepatocellular carcinomas (HCC) are likely to undergo liver transplantation. Liver transplants in HCV infected people are fairly successful, however the new liver graft becomes reinfected in 100% of cases with circulating HCV particles. There is a possibility that some patients may require additional liver transplants over time, therefore HCV infection is the greatest burden on liver transplants (Brown, 2005).

Following initial HCV exposure there is often a variable incubation period when the viral RNA may be undetectable but 1-8 weeks after infection; HCV RNA can be detected in the blood plasma or the liver by PCR, reaching around $10^6$ genome copies per mL. The level of HCV RNA then increases to peak viral loads, often maintaining constant levels for 4-8 weeks before beginning to decrease as the adaptive immune system responds to the virus. HCV antibodies are produced typically 8 weeks following infection; however the antibodies are usually not detectable by ELISA until several months time (Vogel 2009; Neumann et al 1998; (Thimme et al., 2001, Major et al., 2004). Despite HCV antibodies being produced during the acute phase, the number of patients who can spontaneously clear the virus remains very low and infection persists into chronic stages for approximately 70% of those acutely infected patients. It is thought that $10^{12}$ viral particles are produced daily, and given the high mutation rate and genetic diversity, this may

assist HCV to escape immune recognition (Neumann et al 1998; Logvinoff et al 2004; Meunier et al 2005). High titres of neutralising HCV antibodies can be detected during chronic HCV infection which targets the HCV E1 and E2 proteins. However, exposure of the hypervariable regions (HVRs) within E1 and E2 to HCV specific antibodies leads to high variability with these regions. This selective pressure from the adaptive immune response, combined with the high viral mutation rate leads to the virus evolving to escape neutralising antibodies via epitope alterations (Weiner et al., 1991, Kato et al., 2001, Farci et al., 1996, Shimizu et al., 1996, Pantua et al., 2013). Some studies have also highlighted that the presence of these HCV neutralising antibodies may not be enough to clear the viral infection and that it also depends on the functional range of the antibodies, affinity for the highly variable binding site and ability to overcome the physical glycan 'shields' the virus uses to mask antibody binding sites thus escaping antibody neutralisation and viral clearance (Ball et al., 2014, Major et al., 2004, Prince et al., 1999, Pestka et al., 2007, Dowd et al., 2009, Osburn et al., 2014).

Successful clearance of HCV has been associated with host factors such a robust adaptive immune response with HCV-specific CD4+ and CD8+ T cell responses, high titres of broadly neutralising antibodies targeting HCV structural proteins, strong hepatocellular expression of IFNγ and IL28B gene polymorphisms (or IFNλ3 gene) (Lauer et al, 2001; Rauch et al, 2010; Thomas et al, 2009; (Billerbeck et al., 2013, Thimme et al., 2001). Patients with chronic HCV infection tend to have low or undetectable CD4+ and CD8+ T cell responses which may be a result of T cell exhaustion; however it is still unclear how infection with HCV leads to chronic

infection (McMahan et al., 2010). Chronic HCV disease progression varies in individual patients due a several factors previously discussed, which include: use of steroids, viral coinfection, age (Svirtlih et al., 2007), ethnic background (Sterling et al., 2004), and alcohol intake (Gitto et al., 2009). Polymorphisms of the IL28B gene have associated with spontaneous clearance of HCV infection and a better response to the standard HCV therapy of pegylated IFNα and ribavirin (Rauch et al., 2010, Tanaka et al., 2009).

HCV has other mechanisms that assist in evading the immune response to establish a successful chronic infection. There are several proteins HCV encodes able to disrupt various signalling pathways within the host cell, for example; interferon signalling can be blocked by either the core protein via modulating the JAK-STAT pathway or by NS3-4A via modulating IFNβ production (Foy et al., 2005; Li et al.,2005). HCV encoded proteins, NS5A, E2 and part of the internal ribosome entry site (IRES), can inhibit protein kinase receptor (PKR) signalling disrupting type 1 IFN signalling (Vyas et al 2003; Sklan et al., 2009). HCV can utilise cell-cell transmission instead of cell free transmission to evade immune responses. A number of other mechanisms HCV uses to evade the immune are summarised in **Table 1-2** (Brimacombe et al., 2011, Meredith et al., 2013, Grove et al., 2007, Barretto et al., 2014, Xiao et al., 2014).

**Table 1-2 Mechanisms by which HCV evades the host immune response[a]**

| Viral factor(s) | Evasion strategy |
|---|---|
| HCV genomic sequence | 2,5' OAS/RNase L pathway: RNase L digests viral RNAs. The genomic sequence of HCV has a paucity of RNase L cleavage |
| HCV proteins | Induces an ER stress response and increase PP2A expression, which inhibits the JAK-STAT pathway. Suppress ISG56, which normally antagonizes viral RNA translation |
| HCV IRES | Inhibits PKR, which normally antagonizes viral RNA translation |
| Core | Reduces the number of PDCs and decreases their ability to produce IFN-α, induces expression of SOCS, which down regulates the JAK-STAT pathway |
| E2 | Inhibits PKR, which normally antagonizes viral RNA translation |
| NS3-4A | Disrupts two independent viral recognition pathways, RIG-I and TLR3 |
| NS5A | Inhibits PKR, which normally antagonizes viral RNA translation, and activates the transcription factor IRF1, induces IL-8 production, which attenuates the activity of IFN-α |

[a]Table adapted from Sklan (2009). ER, endoplasmic reticulum; IFN, interferon; IL-8, interleukin 8; IRES, internal ribosome entry site; IRF1, interferon response factor 1; OAS, oligoadenylate synthetases; PDC, plasmactoid dendritic cell; PKR, double-stranded RNA-activated protein kinase; SOCS, suppressors of cytokine signalling.

***HCV therapies and treatments***

The standard HCV treatment is a combination therapy of ribavirin (a nucleoside inhibitor of viral replication) and pegylated interferon-α (Peg-IFN-α) which is toxic, costly and only effective in 20-50% of patients (~50% in genotype 1 infected patients) with some withdrawing from treatment due to the severe side effects (Lemon et al., 2010, Appel et al., 2006, Brass et al., 2006, Pezacki et al., 2010, Podevin et al., 2010, Lindenbach et al., 2005b, Pawlotsky, 2011). Responses to treatment can be genotype specific, with genotype 1 infected patients often requiring a longer treatment time which still results in a lower success rate compared to patients genotype 2 and genotype 3 infected patients (Ascione et al., 2010, Rumi et al., 2010; Feld and Hoofnagle 2005, Zeuzem, 2004).

There is currently still no vaccine against HCV; however HCV therapy was revolutionised when new direct-acting antiviral agents (DAAs) against HCV were developed. This included two new protease inhibitor drugs that target the viral NS3/NS4A serine protease, Boceprevir and Telaprevir, which have been licensed. These direct-acting anti-viral agents (DAA) can be used in combination with the current IFN-α and ribavirin treatment as monotherapy has been shown to result in the rapid emergence of drug resistance. They have shown promising results for genotype 1 infected patients (cure rates of up to 75%), however are associated with increased side effects (Poordad et al., 2011, Kwong et al., 2011, Lin et al., 2004, Sarrazin et al., 2007, Backus et al., 2014). More recently, a number of DAAs have been approved which show higher cure rates. The DAAs Simeprevir (Olysio®, Sovriad®) and Faldaprevir are two of the latest protease inhibitors designed to be

given once-daily to patients, however they will still be used in combination with the current IFN-α and ribavirin treatment. The cure rates for these newer DAAs are 72-80%, with the potential for a shorter treatment time if patients respond well 8-12 weeks after the treatment begins. In addition, there are fewer side effects with the newer DAAs (Welch and Jensen 2014, Manns et al 2014, Zeuzem 2014). Sofosbuvir (Solvaldi®) is the first DAA to be approved which inhibits HCV NS5B polymerase. Also a once-daily treatment for patients, Sofosbuvir, in combination with IFN-α and ribavirin has shown cure rates of 89% in treatment naïve genotype 1 infected patients (Lawitz et al., 2013). When in combination with ribavirin, Sofosbuvir has shown cure rates of 85-100% in treatments naïve genotype 2 and genotype 3 infected patients (Jacobson et al., 2013; (Lawitz et al., 2013, Zeuzem et al., 2014).

In general, IFN free HCV therapy for patients would be more ideal and DAA therapies would be cheaper and accessible to more patients. However, we have seen an improvement in response to HCV therapies over the last decade by tailoring the treatment doses and duration with IFN based treatments for individual patients taking into account key baseline factors including HCV genotype, viral load, coinfection, level of liver fibrosis, presence of liver steatosis, age, gender, ethnicity, body mass index and presence of insulin resistance. Some of these factors, for example insulin resistance, do not alter the treatment outcome when using triple therapy with PEG-IFN, ribavirin and DAAs (Backus et al., 2011, Veldt et al., 2007, van der Meer et al., 2012, McHutchison et al., 2009). A number of recent trials and studies have indicated differences in treating patients with different subtypes of the same HCV genotype, for example, HCV genotype 1a are at higher risk of developing antiviral

resistance than HCV genotype 1b patients (Sarrazin and Zeuzem, 2010). The development of these new DAAs does raise the potential issue of future drug resistance as a result of resistance associated amino acid variants of the virus. Ensuring patients follow their treatment regimes will be essential in trying to prevent antiviral drug resistance and additional measures such as routinely monitoring patient HCV sequences before and during therapy may also be helpful. Treating patients with different combinations of DAAs may also be another tactic to prevent drug resistance in the future (Schneider and Sarrazin, 2014, Sarrazin et al., 2007, Zeuzem et al., 2014, Manns et al., 2014).

Liver transplantation in HCV infected patients still remains a successful treatment, particularly for those with severe cirrhosis or hepatocellular carcinoma, even though donor livers are limited. In addition, the new liver can become reinfected with circulating HCV particles and in the long term, the prognosis appears to be worse with recurrent disease and potential loss of the graft. Currently, HCV infection is still a major burden on liver transplantation and major cause of global mortality. Taking in to account the new DAAs, there is still a need for novel HCV therapies that could protect the donor liver from reinfection during liver transplantation. There is also still no vaccine for HCV and novel HCV therapies which can treat more genotypes without the need for combination with IFN could be developed following a greater understanding of the mechanisms and host-virus interactions involved (Gao et al., 2010, Lemm et al., 2010, Meredith et al., 2012b, Pawlotsky, 2011).

## 1.4 Model systems to study HCV

### *HCV replicon systems*

The first *in vitro* model system to study HCV was a replicon system using cDNA clones, developed 10 years after the identification of HCV (Lohmann et al., 1999). This system first used RNA derived from HCV infected explants livers to clone the entire HCV ORF sequence; however the full length sequences failed to yield viral replication following transfection of Huh7 cells. The system was adapted by removing the region of the sequence encoding HCV structural proteins and inserting a neomycin resistance gene to create selectable subgenomic replicons (Lohmann et al., 1999). To improve the replication efficiency, cell culture adapted point mutations or deletions were introduced to the genome sequence (Blight et al., 2000; Lohmann et al., 2001; Bartenschlarger and Lohmann 2000; Krieger et al., 2001). Huh7 cells were used for the replicon system and following prolonged IFNα treatment of the transfected Huh7 cells, a sub-clone termed Huh7.5 was established by Blight and colleagues. This subclone appeared more permissive than Huh7 cells and later this was shown to be due to a defective retinoic acid inducible gene I (RIG-I), which is required for immune sensing of double-stranded RNA. A schematic diagram detailing the production of Huh7 cells containing subgenomic or genomic replicons and the generation of the Huh7.5 subclone is illustrated in **Figure 1-9A-B** (Blight et al 2002; Sumpter et al 2005; Regeard et al 2007). The replicon system was developed further and a selectable full-length HCV replicon is available, containing the structural genes (Kato et al., 2001; Kato et al., 2003). Furthermore subgenomic replicons derived from an HCV genotype 2a strain called JFH-1 have been generated with

even higher replication efficiency (Date et al., 2004; Kato et al., 2005). More recently the LucA2 subgenomic replicon-luciferase cell line was developed, using Huh7 cells expressing HLA-A2 and containing the replicating subgenomic JFH1 HCV strain. As this system contains a luciferase reporter, the level of replication can be easily determined by measuring the luciferase activity.

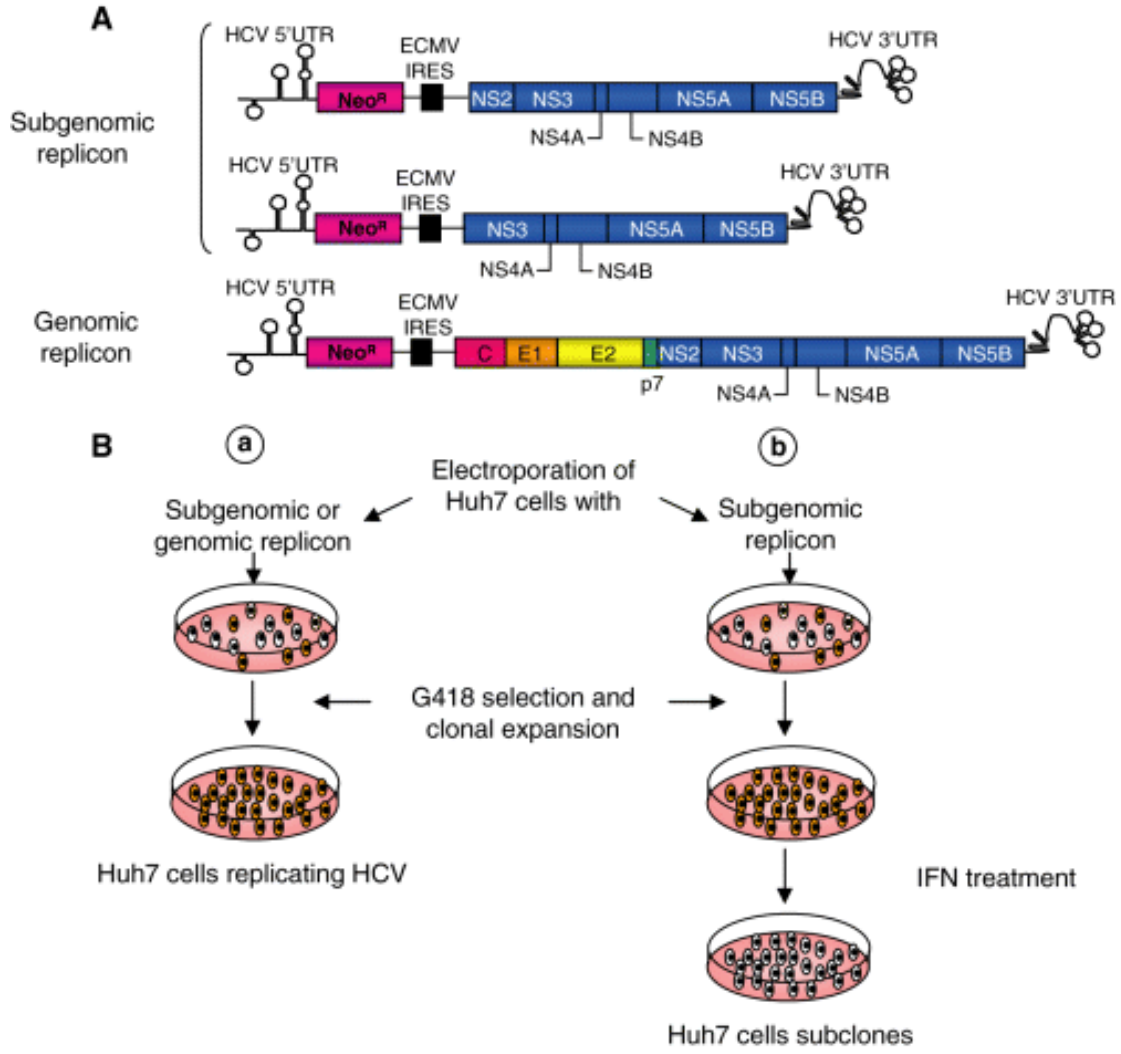



**Figure 1-9 HCV replicon system**

(**A**) Schematic diagram illustrating the replicon system from Regeard (2007). (**B**) Huh7 cells are electroporated with subgenomic or genomic replicon RNA and cells successfully replicating the HCV replicons are selected following treatment with G418 (Ba). In parallel, Huh7 subclones highly permissive to HCV replication can be selected after cells are transfected by first treating cells with G418 and then treating with interferon-α (IFN-α) to cure the HCV replicon (Bb)

***HCV pseudotype virus particles (HCVpp)***

Retroviral particles expressing the HCV glycoproteins E1 and E2 have been created and are called HCV pseudoparticles (HCVpp). This system allows the adsorption and entry steps of the HCV life cycle to be investigated, in contrast to the replicon system which bypassed entry steps by transfecting the HCV RNA into Huh7 or Huh7.5 cells. The pseudoparticles consist of HCV E1E2 glycoproteins, the gag-pol gene of HIV or murine leukaemia virus (MLV) and a reporter gene, as shown in **Figure 1-10**. The reporter gene allows easy detection of HCV entry and can be either a luciferase reporter or green fluorescent protein (GFP) (Bartosch et al 2003a, Hsu et al 2003, Zhang et al 2004; Drummer et al 2003).

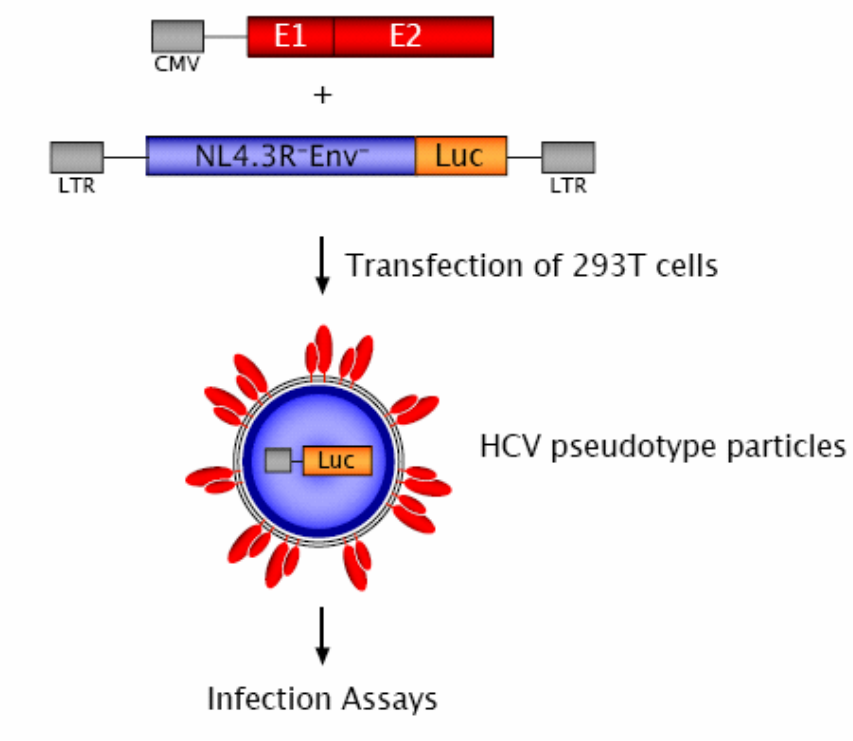



**Figure 1-10 HCV pseudotype virus particle (HCVpp) system**

A cartoon illustrating the generation of HCVpp, which can be used to quantify the level of HCVpp entry by luciferase activity within the target cells.

***HCV particles derived in cell culture (HCVcc)***

Several research groups have developed infectious HCV particles in cell culture (HCVcc), allowing the full HCV lifecycle to now be investigated. The development of HCVcc particles was first possible due to a unique clone isolated from a Japanese patient suffering severe acute HCV genotype 2a infection. The clone was termed Japanese Fulminant Hepatitis 1 (JFH-1) and it can infect cell lines such as Huh7 and Huh7.5 cells, however it is also infectious in chimeric mice with human liver cells and in chimpanzees (Wakita et al 2005; Zhong et al 2005; Lindenbach et al 2005). The system was developed further and chimeric viruses were produced expanding the range of genotypes. This system was a key breakthrough for HCV research allowing the full lifecycle of various HCV genotypes to be studied, including mechanisms of HCV pathogenesis and screening for HCV-specific antiviral compounds (Gottwein et al 2009; Lai et al 2010; Mancone et al 2011). HCVcc infection can also be monitored via luciferase activity by taking the complete HCV J6 viral strain containing a Gaussia luciferase reporter gene (HCVcc-gLuc) or the polymerase defective control HCV virus that is unable to replicate (HCVcc-gLuc) and transfecting both virus into hepatoma cells separately. The Gaussia luciferase signals can be detected by sampling the supernatants which contain *de novo* full length HCVcc particles and therefore allow the levels of replication to be quantified (Koutsoudakis et al 2012).

***Small animal models***

The only species naturally susceptible to HCV are humans and chimpanzees. Although chimpanzees have previously been used to research HCV and been a valuable model, studies using this model are hampered by increasingly limited

access to chimpanzees for research due to ethical concerns, high costs, small cohort sizes and genetic heterogeneity (Houghton 2009; Bukh 2004). The lack of small animal models that support HCV infection has limited research into virus-host interactions, immunity, and drug and vaccine candidates. Recently, two genetically humanized mouse models have been established which both show potential for future HCV research. However these model systems require improvement if they are going to be successfully used to study the complete HCV viral lifecycle, as they can not yet replicate HCV successfully. **Table 1-3** summarises the advantages and limitations of a number of animal models available to study HCV infection (Dorner 2011; Washburn 2011;Reeves and Manickam 2014). There is a need for small animal models to study HCV as the current animal models have a number of limitations, with the main issue being a lack of progressive HCV disease, which ultimately impacts the ability to study HCV immunity and therapeutics (Manickam and Reeves 2014).

**Table 1-3 Summary table of animal models used in HCV research[a]**

| Model | Advantages | Limitations |
|---|---|---|
| Chimpanzee | HCV discovery; *in vivo* virus replication | Expensive; limited availability; lack of liver fibrosis; low chronicity rate; ethical concerns |
| Tree shrew | Small animal susceptible to HCV infection | Lack of chronicity; only transient viremia |
| Humanized mouse model | Useful for immunisation and challenge studies | Low level of viral replication; lack of progressive liver pathology |
| GBV-B infection of New World Monkeys | GBV-B closely related to HCV; analogous disease course to HCV | Low frequency of chronic infection |
| Chimeric GBV-B infection of New World monkeys | Antiviral testing | Inability to cause chronic infection |

[a]Table summarizing the various animal models available for studying HCV infection, plus the advantages and disadvantages of each animal model. Adapted from Reeves and Manickam (2014).

### 1.5 HCV lifecycle

The exact mechanism(s) of the full hepatitis C viral lifecycle is yet to be fully understood due to several reasons including a limited number of physiologically relevant *in vitro* models which mimic the multi-cellular, complex liver microenvironment (Meredith et al., 2012b). In order for a virus to gain access to the host cell and establish infection, it must first bind to specific receptors or attachment factors expressed on the cell surface. Once the virus is bound to the cell membrane, it can be internalised via endocytosis or fusion of the viral envelope with the host plasma membrane. HCV entry is thought to be initiated by low affinity interactions with the attachment factors low density lipoprotein receptors (LDL-R), C-type lectins and glycosaminogylcans (GAGs) before the virus interacts with the four key HCV receptors essential for HCV entry; Scavenger Receptor Class B member I (SRBI), Tetraspanin CD81, Claudin-1 and Occludin (Meredith et al., 2012a). HCV is internalised via clathrin-dependant endocytosis and the viral genome is released following pH dependant fusion of the viral and endosomal membranes. The uncoated viral RNA can then undergo replication, which is a complicated process involving various viral and host proteins, the viral RNA and a membranous web structure which is the site of replication. Successful replication leads to the assembly and packaging of the replicated viral RNA and maturation of virions, which can be released from the cell to transmit infection to other host cells and new hosts (Blanchard et al 2006; Meredith, Wilson et al. 2012; Moradpour et al 2007; Bartenschlager et al 2011).

Despite the recent advances in HCV therapies with the latest DAAs, there are still issues with regards to successfully treating patients with different genotypes, potential for antiviral drug resistance, cost and availability of these therapies especially as many of the drugs are given as combination therapies. There is still a need to develop novel therapies and a vaccine, and so given the essential and conserved nature of the HCV entry step, there has been and currently still is a great deal of research in developing novel therapeutics which targets the HCV entry step. The HCV entry step in a complicated process, the mechanism of which we still do not fully understand, involving a number of entry attachment factors and receptors which are detailed below.

### 1.5.1 HCV attachment factors

HCV entry into hepatocytes is a complicated, multistep process for which the exact mechanism(s) is still unknown. Recently, a number of host factors involved in HCV entry have been identified in addition to the four key HCV receptors: tetraspanins CD81 (Hsu et al 2003) and scavenger receptor BI (SR-BI) (Grove et al 2007) and the two tight junction proteins claudin-1 (Kapadia et al 2007) and occludin (Ploss et al 2009), illustrated in **Figure 1-11**. When HCV first contacts hepatocytes, it is hypothesized that the virus is captured by low-density lipoprotein receptors (LDL-R), C-type lectins or heparin sulphate glycosaminoglycans. Although these interactions are low affinity and do not initiate the entry process, they do potentially concentrate the virus at the cell surface, tethering the virus before it interacts with the receptor molecules.

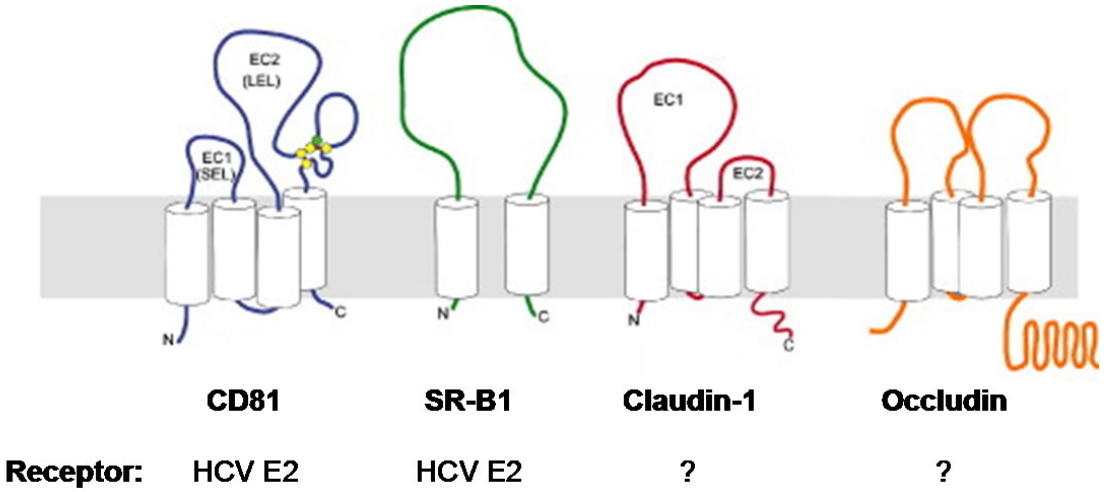



**Figure 1-11 Key HCV receptors**

The four key host HCV receptor molecules involved in HCV entry: tetraspanin CD81, scavenger receptor B I (SR-B1) and tight junction proteins Claudin-1 and Occludin. CD81, Claudin-1 and Occludin play a role in HCV internalisation and the extracellular loops 1 and 2 (EC1 and EC2), large extracellular loop (LEL) and small extracellular loop (SEL) are labelled. SR-B1 plays a role in HCV attachment. Adapted from Lemon and McKeating (2010).

***Low density lipoprotein receptors***

HCV particles *in vivo* in human sera are associated with LDL, very low density lipoproteins (VLDL) and high density lipoproteins (HDL). As a result of this association, several studies have suggested LDL-R found on hepatocytes are involved in HCV attachment and uptake (Agnello et al 1999; Monazahian 1999; Wunschmann 2000; Nahmais 2006; Molina 2007; Germi et al 2002)

***C-type lectins***

Although the C-type lectins, DC-SIGN and L-SIGN are not expressed on hepatocytes they are expressed on liver sinusoidal endothelial cells (LSEC) and dendritic cells and have been shown to bind many viruses including HCV. These molecules are thought to capture HCV particles and transfer them to the target hepatocytes (Lozach et al 2004; Cormier et al 2004; Pohlmann et al 2003).

***Glycosaminoglycans (GAGs)***

GAGs are polysaccharides present on the surface of many cell types and highly sulphated GAGs, such as heparin sulphate, have been shown to bind HCV via interactions with the HCV E2 glycoprotein. As heparin sulphate GAGs can bind many viruses this interaction is not specific for HCV and is thought to play more of a role in facilitating the interactions between HCV particles and the key receptors rather than act as a viral receptor itself (Germis 2002; Barth 2003; Basu 2004; Heo 2004; Jiang 2012).

### 1.5.2 HCV entry factors

Once HCV has interacted with the various attachment factors and become tethered to the hepatocyte cell membrane, the viral particle can then interact with entry factors that will facilitate uptake of the virus particle into hepatocytes. HCV is thought to interact with SR-BI first, then CD81-claudin-1 complexes followed by occludin, however the sequence of virus-receptor interactions is not yet fully understood (Meredith et al 2012).

#### *Scavenger Receptor Class B member I (SRBI)*

SR-BI is a multi-function lipoprotein receptor expressed on the cell surface of most mammalian cells, with high expression in hepatic and steroidogenic tissues. It is a 509 amino acid long glycoprotein with a large extra cellular loop between the N-terminal and C-terminal transmembrane domains, and also has a short cytoplasmic extension. It is expressed in areas of high blood flow where it is involved in mediating cholesterol ester uptake from HDL, LDL, and VLDLs, along with maintaining lipid homeostasis (von Hahn et al., 2006, Rhainds and Brissette, 2004, Rhainds et al., 2003, Dreux et al., 2006). Initially SR-B1 was identified as a HCV receptor with functional studies that showed SR-B1 binds soluble HCV E2 glycoprotein, later confirmed using the HCVpp and HCVcc systems. Later it was discovered that HDLs and oxidised LDLs can enhance or inhibit virus infection in an SR-BI dependent manner (Scarselli et al 2002; Meredith et al 2012). This interaction between HCV and SR-BI is complicated and it may lead to membranous or cytoplasmic rearrangements to help bring the HCV-SR-B1 complex closer to the

other factors required for virus entry. HCV binding SR-BI is required for it to interact with CD81 (Eyre et al., 2010, Meredith et al., 2012b, Brimacombe et al., 2011).

***Tetraspanin CD81***

CD81 is a tetraspanin, ubiquitously expressed in the body that is thought to play a role in various cell signalling pathways such as immune cell activation. However, the functions of CD81 are not fully defined. CD81 was identified as a potential HCV receptor following functional studies that showed CD81 binds soluble HCV E2 glycoprotein (Pileri et al 1998). It was later found that this interaction and subsequent HCV infection could be blocked using anti-CD81 antibodies and soluble CD81, using the HCVpp and HCVcc systems (Bartosch 2003a; Lindenbach 2005; Wakita 2005; Zhong 2005). Studies have also shown that non-permissive cell lines could become permissive to HCV infection following the expression of CD81 (Zhang 2004; Lavillette 2005; Akazawa 2007). However, CD81 alone is not sufficient for HCV entry as it requires co-factors such as SR-BI and claudin-1 to facilitate entry, and it appears CD81 is also involved in post entry steps and may promote endocytosis and particle internalisation (Bartosch 2003b; Hsu 2003 Cormier 2006; Bartaud 2006; Koutsoudakis 2006; Farquhar 2012).

***Claudin-1***

The role of claudin-1 is not as well defined as CD81, whose interaction with HCV induces a conformational change in the HCV E1 and E2 glycoproteins which go on to aid the pH-dependent fusion and endocytosis of HCV (Sharma et al., 2011, Meredith et al., 2012b). The tight junction protein, Claudin-1, is highly expressed in the liver

particularly around the bile canaliculi of hepatocytes and is thought play a role in a late step of virus entry (Fofana et al., 2010, Krieger et al., 2010). Recent data suggests that CD81 and claudin-1 may work together to assist HCV entry and that a direct interaction between the molecules is essential for HCV entry. It is thought CD81 and claudin-1 together have a role in virus internalisation and fusion, as they both co-endocytose and fuse with early endosomes (Meredith et al., 2012b, Farquhar et al., 2011, Farquhar and McKeating, 2008). Claudin-1 has also been shown to play a role in cell-cell transmission of HCV, independently of CD81 (Timpe 2007), furthermore, two other members of the claudin family, claudin-6 and claudin-9, may play a role in HCV infection (Zheng 2007; Meertens 2008).

***Occludin***

Occludin is a four-transmembrane domain tight junction protein, similar to claudin-1. It is essential for HCV infection, as demonstrated when cells expressing SR-BI, CD81 and claudin-1 were not susceptible to infection, compared to cells expressing all four key receptors; which were then susceptible to HCV (Evans 2007; Liu 2009; Ploss 2009). The role of occludin in entry and whether it interacts with HCV directly via E2 glycoproteins or indirectly via the CD81 and claudin-1 complex is yet to be determined (Thorley et al., 2010, Harris et al., 2010, von Hahn and Rice, 2008).

### 1.5.3 HCV replication, assembly and release

HCV RNA replication is a complex process which requires several cellular factors, in addition to viral factors, to form the HCV replication complex. The HCV NS5B protein has been identified as a RNA-dependant RNA polymerase and is responsible for viral RNA replication (Behrens et al., 1996). The HCV NS4B protein was later identified as the protein which induces the endoplasmic reticulum (ER) derived membranous web structure, the site of HCV replication, also containing other HCV non-structural proteins (Egger et al., 2002). The positive strand of HCV RNA released into the cytosol is used as a template by the NS5B RNA polymerase to synthesize the intermediate minus strand RNA. The HCV NS3 protein has helicase function and is thought to assist the RNA polymerase in synthesising minus strand RNA, by unwinding the template RNA. The NS3 helicase also unwinds the intermediate anti-sense RNA which then becomes the template for synthesis of numerous positive-stranded RNA to be used as either genomic RNA for HCV progeny or for polyprotein translation (Jin et al., 1995; Kim et al.,1995).

Research into HCV particle assembly and release has been limited as the *in vitro* models that could allow these processes to be studied have only been developed recently. The latest studies suggest that virus assembly occurs within the ER (Gastaminza et al., 2008) and that particle formation involves lipid droplets associating with various HCV proteins. The core protein, when associated with lipid droplets, has the ability to target viral structural proteins and HCV RNA from the ER to lipid droplets. NS5A associated with lipid droplets, together with apolipoprotein E (apoE), is thought to play a key role in forming infectious viral particles (Miyanari et

al., 2007; Appel et al., 2008; Benga et al., 2010). Based on current research highlighting the association of various HCV proteins with lipid droplets and spherical virus-like particles containing core protein and E2 found associated with membranes close to lipid droplets, it has been suggested that lipid droplet associated membranes may be the site of HCV particle assembly (Miyanari et al., 2007). The current model for the viral lifecycle is summarised in **Figure 1-12** (Feneant 2014).

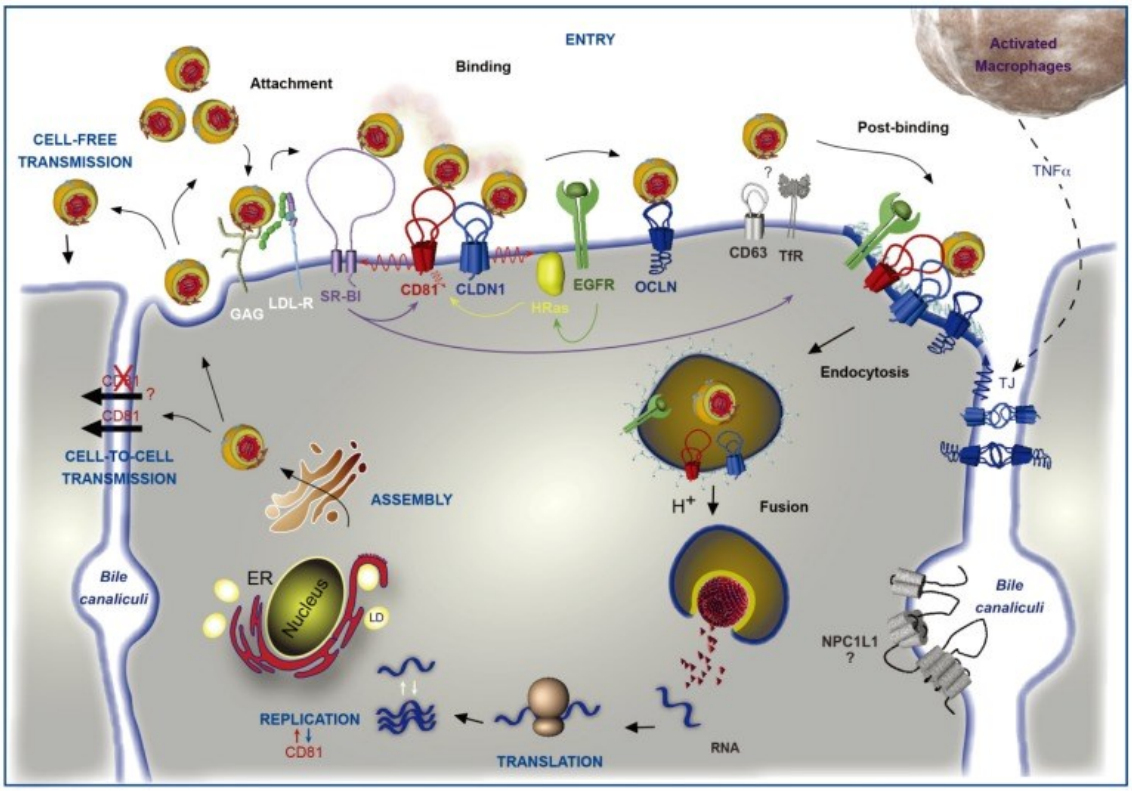



**Figure 1-12 HCV lifecycle**

HCV initiates infection following non-specific attachment to glycosaminoglycans (GAG) and Low Density Lipoprotein-Receptor (LDL-R). The HCV particle then interacts with SR-BI, CD81 and claudin-1 complexes and then occludin which leads to virus entry via clathrin-mediated endocytosis. HCV translation and replication occurs at the endoplasmic reticulum membrane, followed by assembly of viral particles associated with lipid droplets (Feneant 2014).

### 1.6 Role of aLMF in HCV pathogenesis

In developed countries alone, fibrotic diseases account for 45% of the total deaths but currently there are no anti-fibrotic therapies available for established cirrhosis. Therefore, there is a need to understand the molecular mechanisms of liver fibrogenesis in order to help develop new therapies for fibrotic diseases (Su et al 2014). Worldwide, chronic HCV infection is one of the leading causes of liver fibrosis progressing to cirrhosis. Several studies have shown the progression of fibrosis can be stopped and even reversed following successful antiviral therapies which treat the underlying cause of fibrosis. As there are no anti-fibrotic therapies available, we do not know whether combination therapy of antivirals and anti-fibrotic therapies would be beneficial for patients, and if this would lead to more successful therapies especially as the fibrosis is actually the liver's mechanism of repair in response to HCV infection and is a positive factor which becomes a negative issue if prolonged leading to irreversible damage (Su et al 2014; Wynn et al 2008; Friedman et al 2008; Friedman et al 2007).

The main driving force behind liver fibrosis is hepatic inflammation caused in response to liver injury such as HCV infection; however, host factors such as single nucleotide polymorphisms, in addition to viral factors such as viral load, mutations, and viral proteins are associated with fibrosis progression. The key characteristics of liver fibrosis include the activation of quiescent HSC into aLMF cells, proliferation of aLMFs, loss of hepatocytes, destruction of hepatic microarchitecture and excess ECM accumulation. In chronic viral hepatitis, HSCs are activated as a result of the host immunological response to initiate antiviral mechanisms, designed to clear

virally infected hepatocytes thus attempting to clear the viral infection (Friedman et al 2007; Su et al 2014; Wang et al 2013). A number of profibrogenic factors, including transforming growth factor beta (TGF-β) are released by HCV infected hepatocytes, which act on HSC modulating the expression of several genes associated with fibrosis (Schulze-Krebs et al 2005).

The role of HSCs in liver fibrosis is well known, however more recently, data is emerging that HSCs also play a role in liver immunity and could therefore impact HCV disease progression via this additional mechanism. During chronic HCV infection, infected hepatocytes can undergo apoptosis induced by the viral infection generating apoptotic bodies, which are cleared by phagocytosis as part of the innate immune response. HSCs have the ability to phagocytose these apoptotic bodies and this process leads to a profibrogenic response (Zhan et al 2006; Jiang et al 2008; Deng et al 2008; Wang et al 2013). HSCs have been reported to function as liver resident antigen-presenting cells (APC), responsible for displaying foreign antigens in order to stimulate T cells production, and are also thought to enhance the differentiation and accumulation of regulatory T cells. The innate immune response also recognises pathogens such as HCV via toll-like receptors (TLRs) and HSCs express TLRs, in particular TLR-3, TLR-4 and TLR-9. Pathogen sensing via TLR-3 leads to the activation of the IFN signalling pathway and production of Type I and Type III IFNs which act to limit HCV infection in hepatocytes (Wang et al., 2013a, Wang et al., 2013c, Winau et al., 2007, Ichikawa et al., 2011, Seki et al., 2007, Watanabe et al., 2007, Kumar et al., 2006, Kawai and Akira, 2006).

Recent studies have suggested HCV particles and some HCV proteins can also directly interact with HSC inducing liver fibrosis and possibly the innate immune response. For example, the HCV E2 protein has been shown to bind directly to CD81 expressed on HSCs which induces fibrosis pathways such as the up regulation of MMP-2. MMP-2 is responsible for degrading ECM, including the degradation and remodelling of normal ECM in areas infected with HCV to allow the penetration of inflammatory cells for repair. However, HCV has been shown to utilise this inflammatory repair mechanism to its advantage, creating an environment which actually favours HCV virus replication and spread (Mazzocca et al 2002; (Mee et al., 2009). Other recombinant HCV proteins thought to interact directly with HSCs and induce fibrosis and inflammation include HCV core and non-structural proteins NS3-N5 (Bataller et al 2004; Wang et al 2013). The role HCV infection in inducing profibrogenic HSCs and fibrosis is summarized in **Table 1-4.**

These studies have highlighted an additional role for HSCs and aLMFs other than causing fibrosis in response to HCV infected hepatocytes and suggest that these cells may also be acting as a key regulatory bystander cell involved in the livers innate immune response against HCV infection and disease progression. A greater understanding of viral-host interactions is required here in order to determine the role(s) of HSC/aLMF in HCV disease progression and implications for treating HCV which may potentially help identify new targets for novel therapeutics.

**Table 1-4 HCV mediated liver fibrosis[a]**

| HCV/HCV proteins | HSC |
|---|---|
| HCV | Induces liver injury, which activates HSC. |
| E2 | Engulfs apoptotic bodies through phagocyotosis triggers a profibrogenenic response in HSC. Up regulates matrix metalloproteinase-2 expression, increasing degradation of the normal hepatic extracellular matrix in HSC. |
| Core | Induces fibrogenic actions and stimulates intracellular signalling pathway in HSC. |
| NS3-N35 | Induces pro-inflammatory cytokines in HSC. |

[a]Table detailing how HCV and its proteins can interact with HSC and induce liver fibrosis, adapted from Wang et al (2013). HSC, hepatic stellate cells.

### 1.7 Project Aims

The overall aim of this study was to understand the role of aLMF cells in hepatitis C virus (HCV) infection of the liver. Firstly, we wanted to investigate whether aLMF could support HCV infection and the impact of aLMF cells on the various steps of the HCV lifecycle in hepatocytes. Secondly, we wanted to investigate the complex cell-cell interactions between aLMF and hepatocytes to increase our understanding of the mechanisms of host-viral interactions, and to potentially determine new pathways that can be targets for novel therapies.

# Chapter 2 Materials and methods

## 2.0 Tissue culture, Cell lines and primary cells

### 2.0.1 Tissue culture

Dulbecco's modified Eagle's medium (DMEM) (Gibco, CA, USA) was used to maintain all cells, unless otherwise stated, and was supplemented with varying quantities of foetal bovine serum (FBS), 1% non-essential amino acids, 1% L-Glutamine and 50 units/mL penicillin/streptomycin (Gibco, CA, USA) at 37ºC, 20% $O_2$ and 5% $CO_2$. Cells under hypoxic conditions were maintained at 37ºC, 1% $O_2$ and 5% $CO_2$. The different FBS quantities used and other supplements required for cells used in this study are detailed in **Table 2-1**.

Cells were stored in liquid nitrogen, briefly; cells were dissociated using trypsin, centrifuged at 1500 rpm for 5 minutes and resuspended in freezing media (95% FBS and 5% DMSO (Sigma-Aldrich)). The cells were stored in cryovials and placed into a -80ºC freezer overnight and then transferred to liquid nitrogen the following day. To thaw cells, cryovials were incubated at 37ºC, suspended in media, centrifuged at 1500 rpm for 5 minutes and resuspended in fresh media before transferring to tissue culture flasks for propagation.

**Table 2-1 Cell lines and primary cells with details of cell growth medium**

| Cell Type | Source | Medium | % FBS | Supplied by |
|---|---|---|---|---|
| Huh 7.5 | Human hepatoma | DMEM | 10 | Charles Rice, Rockerfellar University, NY, USA |
| Huh 7 | Human hepatoma | DMEM | 10 | American Type Culture Collection, VA, USA |
| HepG2 | Human hepatoblastoma | DMEM | 10 | American Type Culture Collection, VA, USA |
| HepG2-CD81 | Human hepatoblastoma | DMEM | 10 | In house |
| HepG2-CD81-GFP | Human hepatoblastoma | DMEM | 10 | In house |
| HepG2-EGFR-GFP | Human hepatoblastoma | DMEM | 10 | In house |
| HepG2-DPP IV | Human hepatoblastoma | DMEM | 10 | In house, UMCG |
| A2-HCV replicon-luciferase Huh 7 cells | Human hepatoma | DMEM | 10 | Robert Thimme, University of Freiburg, Germany |
| 293-T (HEK 293T/17) | Human Embryonic Kidney | DMEM | 3 | American Type Culture Collection, VA, USA |
| Chinese Hamster Ovary (CHO) cells | Chinese Hamster Ovary cell line | DMEM | 10 | American Type Culture Collection, VA, USA |
| Lieming Xu-2 (LX-2) stellate cell line | Human cell line | DMEM | 2.5 | Centre for Liver Research, UoB, UK |
| Activated liver myofibroblasts (aLMF) | Donor liver tissue | DMEM | 16 | Centre for Liver Research, UoB, UK |
| Primary Human Hepatocytes (PHH) | Human liver tissue | Williams E | 10 HS | Ragai Mitry, Kings College London, UK |
| Dermal fibroblasts (DM) | Human tissue | DMEM | 16 | Rheumatology Research Group, UoB, UK |
| Synovial fibroblasts (SY) | Human tissue | DMEM | 16 | Rheumatology Research Group, UoB, UK |
| Bone marrow fibroblasts (BM) | Human tissue | DMEM | 16 | Rheumatology Research Group, UoB, UK |

UoB, University of Birmingham; HS, human serum.

### 2.0.2 Liver tissue samples

All human liver tissue samples used to isolate various liver cell types were obtained from patients attending the Queen Elizabeth Hospital, Birmingham. The samples were collected with the consent from the patients or their relatives, and according to local research ethics committee approval. These samples were at various end stage chronic liver disease aetiologies, including patients undergoing liver transplantation for cryptogenic cirrhosis, primary biliary cirrhosis, alcoholic liver disease or hemochromatosis. Tissues were processed promptly after collection to ensure a high yield of viable cells, with each sample used to isolate activated liver myofibroblasts (aLMF), liver sinusoidal endothelial cells (LSEC) and biliary epithelial cells (BEC).

### 2.0.3 Isolation of primary aLMF

The liver tissue samples were processed by enzymatic digestion, differential density centrifugation and immunomagnetic separation in order to recover a variety of liver cell types. Firstly, the liver tissue was cut into smaller pieces and digested using type-1A collagenase (Sigma Aldrich, UK) at 0.4µg/mL. After enzymatic digestion for 25-45 minutes at 37ºC, the tissue mixture was sieved using a sterile fine mesh to remove any undigested tissue, washed with PBS several times and centrifuged at 2000rpm for 5 minutes. The pellet was resuspended in PBS and separated by centrifugation using a 33/77 wt/vol Percoll gradient (Amersham, Biosciences, GE) at 2300rpm for 20 minutes. Biliary epithelial cells (BEC) were isolated first, using an antibody targeting a glycoprotein specifically expressed on the surface of epithelial cells, HEA-125 (10µg/mL, Progen Biotechnik), followed by a sheep anti-mouse

secondary antibody conjugated to magnetic beads (Dynal, Invitrogen), allowing separation using a magnet. Subsequently LSEC were then isolated using an antibody targeting CD31 (10µg/mL, Progen Biotechnik) and magnetic bead separation as described above. aLMF were remaining in the cell suspension and thus purified by negative immunomagentic selection, following the removal of BEC and LSEC. The viability of each cell type was confirmed by trypan blue exclusion (Holt et al., 2009, Joplin et al., 1990).

Isolated cells were routinely assessed for cell morphology and purity to ensure cultures used were >95% pure. To assess purity, cells were stained and imaged by immunofluorescence microscopy using a number of lineage specific markers: for aLMF (CD90, desmin, vimentin or αSMA), hepatocytes (albumin, α-feto-protein, CK7, CK18), biliary epithelial cells (CK17, CK18, CD19, and EpCAM) and liver sinusoidal endothelial cell (CD31). The morphology was assessed by phase contrast microscopy and aLMF cells were kept in culture until at least passage 6. Primary cells were routinely isolated and supplied by Gill Murihead, Janine Youster and Elizabeth Humpreys (Centre of Liver Research, University of Birmingham).

### 2.0.4 Isolation and cryopreservation of primary human hepatocytes

Primary human hepatocytes (PHH) used in this study were a kind gift from Dr Ragai Mitry (King's College, London). These were isolated according to published protocols from donor liver tissue (Mitry, 2009; Hughes et al 2010). Once isolated, PHH were cryopreserved, stored in a liquid nitrogen tank and transported on dry ice. In order to thaw the PHH, briefly; the primary human hepatocytes were incubated at 37ºC,

centrifuged at 50xg, 4ºC for 5 minutes and resuspended in Minimal Essential Medium containing 20% human serum albumin (Baxter AG, UK), 25mM HEPES without phenol red and calcium (1:100 dilution) (Lonza, UK) plus 25% Percoll (GE Healthcare, UK). This cell suspension was then centrifuged at 50xg, 4ºC for 20 minutes to separate non-viable and viable cells. The viable cell pellet was resuspended in Minimal Essential Medium containing 20% human serum albumin (Baxter AG, UK), 25mM HEPES without phenol red and calcium (1:100 dilution) (Lonza, UK) and viability further assessed using trypan blue exclusion, then seeded accordingly in mono- and co- culture with aLMF, LX-2 and CHO cells (at a 1:1 ratio) in Williams Essential Eagles Medium (Sigma, UK) containing 10% human serum, 1% non-essential amino acids, 1% L-Glutamine and 50units/mL penicillin/streptomycin (Gibco, CA, USA), 1M HEPES, 1% Insulin (Sigma, UK). Cells were maintained at 37ºC, 20% $O_2$ and 5% $CO_2$, and the media replaced 24 hours post seeding to DMEM containing 10% FBS, 1% non-essential amino acids, 1% L-Glutamine and 50units/mL penicillin/streptomycin (Gibco, CA, USA), then infected with high titre HCVcc as detailed in **section 2.1.3**.

### 2.0.5 Isolation of primary fibroblasts from different sites

The fibroblasts from different sites used in this study were kind gifts from Professor Chris Buckley (University of Birmingham, UK). Dermal fibroblasts (DM), synovial fibroblasts (SY) and bone marrow fibroblasts (BM) were all isolated from clinical samples as previously described (Salmon et al., 1997), from patients with rheumatoid arthritis or osteoarthritis who gave consent according to local research ethics committee approval. Briefly; the tissue samples were cut into smaller pieces, washed

in RPMI containing 20mM HEPES and centrifuged at 300g for 6 minutes. The cells were resuspended in digestion buffer (RPMI containing 20mM HEPES and 0.2% collagenase type 1A) then incubated for 4-5 hours at 37ºC with vigorous shaking. The cell mixture was centrifuged at 300g for 6 minutes, and the pellet containing the adherent fibroblast cells was resuspended in DMEM containing 10% FBS, 1% non-essential amino acids, 1% L-Glutamine and 50units/mL penicillin/streptomycin (Gibco, CA, USA), then incubated at 37ºC, 20% $O_2$ and 5% $CO_2$. The cells were grown to confluency and fresh media replaced weekly. Fibroblast morphology was assessed by phase microscopy over time and the cultures were assessed for contamination by staining for markers of the excluded cell populations. Cultures were routinely stained and assessed using endothelial cell markers such as CD31 and von Willebrand factor, epithelial cell markers such as cytokeratin and macrophage markers such as CD14 and CD68 (Zimmermann et al., 2001, Salmon et al., 1997).

## 2.1 Routine techniques

### 2.1.1 Antibodies and application

Details of all antibodies used in this study, the application, and working concentrations used are detailed in **Table 2-2** and **Table 2-3**.

**Table 2-2 Primary Antibodies**

| Antibody name and clone | Target antigen | Type | Specificity | Species | Application | Working conc. (µg/mL) | Source |
|---|---|---|---|---|---|---|---|
| Anti- claudin-1 (polysera) | Human Claudin-1 | Purified IgG | Poly | Rabbit | IF, WB | 1 | Zymed, CA, USA |
| Anti-occludin (OC-3F10) | Human Occludin | Purified IgG | Poly | Rabbit | IF, WB | 1 | Zymed, CA, USA |
| Anti-CD81 (2s131) | Human CD81 | Purified IgG | Mono | Mouse | IF, WB | 1 | In-house |
| Anti-SRB1 (R25) | Human SRB1 | Purified IgG | Mono | Mouse | WB | 1 | BD Biosciences, UK |
| Anti-β-actin (AC-74) | Human β-actin | Purified IgG | Mono | Mouse | WB | 0.5 | Sigma Aldrich |
| Anti-NS5A (9E10) | HCV NS5A | Hybridoma supernatant | Mono | Mouse | IF | 2 | Rockefellar University, NY, USA |
| Anti-CD90 (5E10) | Human CD90 | Purified IgG1 | Mono | Mouse | IF | 0.25 | eBiosciences |
| Anti-Desmin (DE-R-11) | Human Desmin | Purified IgG1 | Mono | Mouse | IF | 0.5 | Vector Labs, UK |
| Anti-Vimentin (V9) | Human Vimentin | Purified IgG1 | Mono | Mouse | IF | 0.5 | Vector Labs, UK |
| Anti-VEGF-A (293) | Human VEGF-A | Hybridoma supernatant Purified IgG2B | Mono | Mouse | Neutralising | 100 | R&D systems |
| Anti-CD81 (2s131) | Human CD81 | Purified IgG | Mono | Mouse | Neutralising | 10 | In-house |
| Anti-VAP-1 (BTT1023) | Human VAP-1 | Purified IgG | Mono | Mouse | Neutralising | 20 | Biotie Therapies |

Poly, polyclonal; Mono, monoclonal; IF, Indirect immunofluorescence; WB, Western Blotting.

**Table 2-3 Secondary Antibodies**

| Antibody name | Antigen | Type | Specificity | Species | Application | Working dilution | Source |
|---|---|---|---|---|---|---|---|
| Rabbit Alexa Fluor 488 | Rabbit IgG | Purified IgG (H+L) | Poly | Goat | IF | 1/500 | Molecular Probes, Invitrogen, CA |
| Mouse Alexa Fluor 488 | Mouse IgG | Purified IgG (H+L) | Poly | Goat | IF | 1/500 | Molecular Probes, Invitrogen, CA |
| Rabbit Alexa Fluor 594 | Rabbit IgG | Purified IgG (H+L) | Poly | Goat | IF | 1/500 | Molecular Probes, Invitrogen, CA |
| Rabbit Alexa Fluor 633 | Mouse IgG | Purified IgG (H+L) | Poly | Goat | IF | 1/500 | Molecular Probes, Invitrogen, CA |
| Anti-Rabbit HRP | Rabbit IgG | Purified IgG | Poly | Donkey | WB | 1/1000 | GE Healthcare, PA |
| Anti-Mouse HRP | Mouse IgG | Purified IgG | Poly | Sheep | WB | 1/1000 | GE Healthcare, PA |

IF, Indirect immunofluorescence; WB, Western Blotting; Poly, polyclonal.

### 2.1.2 List of plasmids

Details of all plasmids used in this study are in **Table 2-4**

**Table 2-4 List of plasmids**

| Plasmid details | Supplied by |
|---|---|
| HCVcc JFH-1 | Tajika Wakita, National Institute of Infectious Disease, Tokyo, Japan (Wakita et al., 2005a, Zhong et al., 2005a) |
| HCVcc J6/JFH-1 | Charles Rice, Rockefellar University, NY, USA (Lindenbach et al., 2005a) |
| HCVcc SA13 | Jens Bukh, Copenhagen Hospital, Denmark (Jensen et al., 2008) |
| ISG-56 luciferase reporter | Michael Gale, University of Texas Southwestern Medical centre, TX, USA |
| H77 E1E2 | Zie Zhang, Rockefellar University, NY, USA (Bartosch et al., 2003, Zhang et al., 2004) |
| JFH-1 E1E2 | Zie Zhang, Rockefellar University, NY, USA (Bartosch et al., 2003, Zhang et al., 2004) |
| VSV-G | Aaron Diamond, AIDS Research Centre |
| HIV gag-pol | Aaron Diamond, AIDS Research Centre |
| CD81-GFP | In house |
| EGFR-GFP | In house |
| TRIP gag-pol | In house |

### 2.1.3 HCV cell culture (HCVcc) generation and infection

The HCVcc system is based on a unique clone, termed Japanese Fulminant Hepatitis 1 (JFH-1) which can infect hepatoma cell lines and has since been used to generate all other HCVcc strains (Wakita et al 2005; Zhong et al 2005; Lindenbach et al 2005(Jensen et al., 2008). SA13 (genotype 5a) HCVcc infection was achieved through electroporation of early passage Huh7.5 cells with SA13 RNA transcripts, allowing the entry of the RNA transcripts into the Huh7.5 cells. The RNA transcripts encoding the HCV genome SA13 were generated using the T7 RNA polymerase kit (Promega, UK) according to the manufacturer's instructions. The electroporation protocol consists washing and resuspending Huh7.5 cells in ice cold PBS, before gently mixing with SA13 RNA and transferring into a 0.2cm gap electroporation cuvette (Sigma, UK). Using the BTX Electro Square Porator (Harvard Appartus, USA), the cells were electroporated at 815 volts and then allowed to rest in the cuvette for 5 minutes at room temperature before gently transferring into IMDM containing 10% human serum, 1% L-Glutamine and 50units/mL penicillin/streptomycin . Cells were seeded in a 24 well tissue culture plate and taken to the category 3 containment laboratory for culturing and harvest of HCVcc particles. The level of HCV expression after 48 hours was determined by staining for NS5A positive cells indicating HCV infected cells, as shown in **Figure 2-1**. After confirming the level of HCV infected cells 48 hours post electroporation, HCVcc particles were harvested by collecting and replacing the media every 4 hours throughout the day, for 4 to 7 days post electroporation. Each virus harvest was immediately stored at -80ºC, and then pooled and spin clarified by centrifugation at 3000rpm for 5 minutes, and

stored at -80ºC. The virus stock was titrated by infecting naive Huh7.5 cells with varying dilutions to calculate foci forming units per mL (FFU/mL), according to NS5A positive staining at 48 hours post infection.

This harvested virus was used in all HCVcc infection assays as follows (unless otherwise stated). Mono- and co- cultures of cells, at a density of $2.5x10^4$ cells per well in a 48 well tissue culture plate, were infected 24 hours post seeding in the category 3 containment laboratory. The media was carefully removed and replaced with 200µL of the HCVcc particles diluted in complete DMEM media containing 10% FBS. The cells were incubated with the virus inoculum for 6 hours at 37ºC, washed with PBS carefully to remove any unbound virus particles. Complete DMEM media, containing 10% FBS, was added to the cells and the infection allowed to proceed for 48 hours at 37ºC (unless otherwise stated). Cells were fixed with ice cold methanol then blocked using PBS containing 1% BSA with 0.1% saponin or Triton X-100 added to permeabilise the cells. HCV positive cells were determined by staining for NS5A positive cells using the primary mouse anti-NS5A mono-clonal antibody (clone 9E10), as described in **section 2.1.6.** Staining was visualised using a fluorescent UV microscope (Nikon eclipse TE2000-5 inverted) and infection quantified by counting either the number of foci or total number of infected cells.

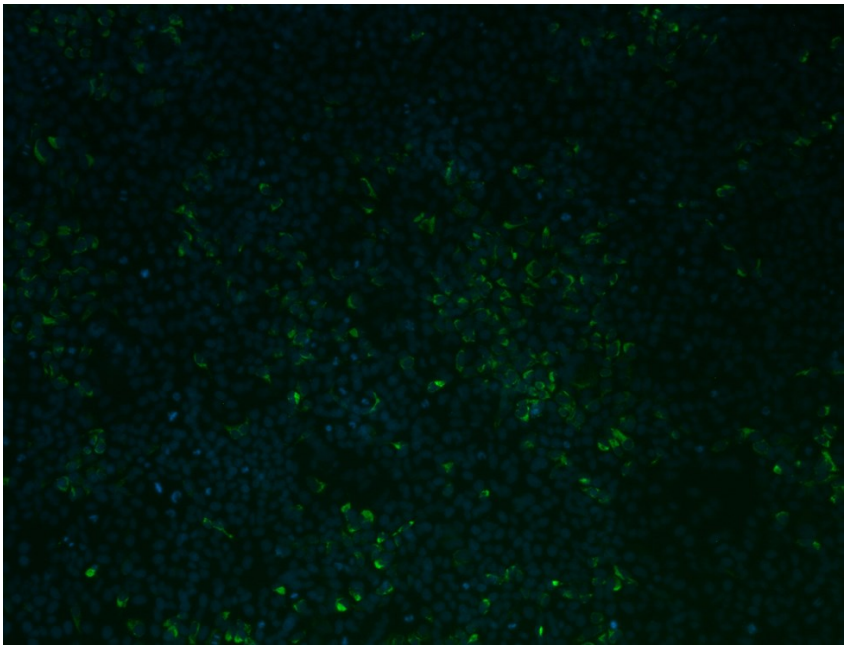


**Figure 2-1 Electroporated Huh7.5 cells stained for HCV NS5A**

Immunofluorescence image of Huh7.5 cells fixed and stained for HCV NS5A (green), with DAPI nuclear stain (blue), 48 hours post electroporation with SA13 RNA, at magnification x10.

### 2.1.4 Pseudoparticle virus generation and infection

Pseudoparticle viruses were generated using 293-T cells and a Fugene transfection protocol, according to the method published by Hsu et al (2003). Briefly, 293-T cells were seeded at $7x10^5$ cells per well in a 6 well tissue culture plate coated with poly-L-lysine at 0.1 mg/mL (Sigma, UK) using DMEM containing 10% FBS, 1% non-essential amino acids, 1% L-Glutamine (Gibco, CA, USA). After 24 hours media was replaced with DMEM containing 3% FBS, 1% non-essential amino acids and 1% L-Glutamine (Gibco, CA, USA). Plasmids were delivered to the cells using 6 µg/well Fugene (Roche) and 100 µL/well optimum (Gibco). 1000 ng/well of the pNL4.3luc plasmid, which encodes a HIV virus competent for a single round of replication containing a firefly luciferase gene, was co-transfected with 10 ng/well of the viral envelope construct of interest e.g. HCV E1E2, VSV, or the control plasmid encoding no envelope (NE). After incubating the cells with the transfection mixture for 6 hours at 37ºC, the media was removed and fresh DMEM containing 3% FBS, 1% non-essential amino acids, 1% L-Glutamine and 50units/mL penicillin/streptomycin (Gibco, CA, USA) added to the cells. The supernatants from the transfected cells containing pseudoparticle virus were collected at 48 and 72 hours post transfection, pooled, spin clarified by centrifugation at 3000rpm for 5 minutes and stored at -80ºC. The virus stocks were titrated by infecting naive Huh7.5 cells with varying dilutions, the cells were lysed 48 hours post infection using 1x Cell Culture Lysis Buffer (Promega), prepared according to the manufacturer's instructions, for 2 hours at room temperature. The luciferase activity was detected using the Luciferase Assay System

(Promega) and a Centro LB960 Luminometer (Berthold Technologies, UK) calculate relative light units (RLU) per mL.

The harvested pseudoparticle viruses were used in all pseudoparticle infection assays as follows; mono- and co- cultures, seeded at a density of $1.2x10^4$ per well in a 96 well plate, were infected 24 hours post seeding with the relevant pseudoparticle virus, or NE control, for 8 hours at a dilution defined for each batch based on a titration. Typically HCVpp were diluted 1:2, NEpp diluted 1:2 and VSVpp diluted 1:100 in DMEM containing 3% FBS, 1% non-essential amino acids, 1% L-Glutamine and 50units/mL penicillin/streptomycin (Gibco, CA, USA) and then added to the cells. After the virus inoculums were removed, the cells were lysed and luciferase activity measured after 48 hours as described above.

### 2.1.5 HCVcc-gLuc generation

The HCVcc-gLuc system is based on a HCV J6 viral strain containing a Gaussia luciferase reporter gene (HCVcc-gLuc), or the polymerase defective control HCV virus that is unable to replicate and thus unable produce Gaussia luciferase (HCVcc-GNN) as it encodes two stop codons in the NS5B region resulting in a catalytically inactive RNA dependent RNA polymerase. Both HCV viruses were transfected into hepatoma cells to determine the level of replication by measuring the Gaussia luciferase activity of Huh7.5 cells containing either the HCVcc-gLuc RNA or HCVcc-GNN RNA and then comparing the Gaussia luciferase signals. Gaussia luciferase signals can be detected by sampling the supernatants which contain *de novo* full length

HCVcc particles if replication is successful (Koutsoudakis et al 2012(Phan et al., 2011). Huh7.5 cells were transfected with either the HCVcc-gLuc virus, or the control HCV-GNN virus and then used in co-culture with stromal cells. First, Huh7.5 cells were seeded at $10x10^4$ cells per well in a 6 well tissue culture plate using DMEM containing 10% FBS, 1% non-essential amino acids, 1% L-Glutamine (Gibco, CA, USA). Cells settled for 24 hours and 1 hour prior to the transfection, the media was replaced with DMEM containing 3% FBS, 1% non-essential amino acids, 1% L-Glutamine (Gibco, CA, USA). The two different viral RNAs were delivered to the cells using Mirus Delivery TransIT®-mRNA Transfection kit (Mirus Bio LLC, UK). For each well, 4.5µL Mirus Transit mRNA was added to 300µL optimem and mixed well before 3µL Mirus Boost reagent was added and incubated at room temperature for 3 minutes. Next 1.5µg of RNA (HCVcc-gLuc or the negative control HCVcc-GNN) was diluted in 300µL optimem and added to the transfection mixture, then incubated at room temperature for 5 minutes before adding to the cells. After incubating for 4 hours at 37ºC, 50µL of supernatant was transferred to a white polystyrene 96-well fluorescent assay plate (Corning, USA), mixed with 50µL of Gaussia luciferase substrate reagent (Luciferase Assay System, Promega) and the Gaussia luciferase activity detected using a Centro LB960 Luminometer (Berthold Technologies, UK) for 1 second per well, measured as RLU, to determine whether the transfection was successful. Following successful transfections for both the HCVcc-gLuc virus and the control HCV-GNN virus, the transfected Huh7.5 cells were co-cultured with aLMF, LX-2 or CHO cells at a 1:1 ratio, at a density of $2.5x10^4$ cell per well in a 24 well plate, leading to co-cultures with either the HCVcc-gLuc infected Huh7.5 cells or the

replication deficient HCVcc-GNN infected Huh7.5 cells. The supernatants were sampled 24, 48, 72 and 96 hours post co-culturing, mixed with Gaussia luciferase substrate and the Gaussia luciferase readings measured using a Centro LB960 Luminometer (Berthold Technologies, UK), as previously described. By monitoring the HCVcc-gLuc Gaussia luciferase activity, thus monitoring *de novo* viral replication, and comparing it to the negative HCVcc-GNN control which is replication deficient, the level of replication was monitored in real time from the same cultures without the need to harvest cells at each time point. To determine the level of cell-cell transmission when in a co-culture, cells were treated with 10 µg/mL anti-CD81 antibody in order to block cell free infection and then the luciferase activity measured as the various time points described above.

### 2.1.6 Indirect immunofluorescence

Cells were seeded for the detection of various cellular proteins or HCV infection by immunofluorescence at a density of 0.05 cells per $mm^2$ then fixed using either ice cold methanol (Fisher Scientific, UK) for 5 minutes or 3.6% para-formaldehyde (PFA) (TAAB, UK) for 20 minutes, at room temperature. Cells were blocked using a wash buffer consisting of PBS containing 1% BSA and 0.1% saponin or Triton X-100, to permeabilise the cells, for 30 minutes at room temperature. Cells were washed twice with PBS before adding primary antibody diluted in wash buffer for 60 minutes at room temperature. Cells were then washed twice with PBS before the addition of the secondary fluorescent conjugated antibody diluted in wash buffer and incubated in the dark for 60 minutes at room temperature. Cells were washed twice with PBS,

then the nuclei were counterstained with 4', 6-diamidino-2-phenylindole (DAPI) at 10µg/mL (SIGMA, UK) for 5 minutes in the dark and finally PBS was added to the wells. Staining was visualised using fluorescent UV microscope (Nikon eclipse TE2000-5 inverted) and imaged with a digital camera (Hammatsu, Japan) at magnification x10, unless otherwise stated.

### 2.1.7 Western blotting

Cells were seeded in a 6 well plate at $4x10^4$ cells per well and 24 hours post seeding, lysed for western blotting. To prepare the cell lysates, media was removed and the cells washed carefully with PBS twice. Then the cells were then treated with ice-cold lysis buffer (PBS, 1% Brij97, 20mM/L Tris [pH 7.5], 300 mM/L $CaCl_2$ and 2nM/L $MgCL_2$) also containing protease and phosphate inhibitors (Roche, UK) for 30 minutes and kept on ice for the duration of the lysis process. Cell lysates were centrifuged at 20,000xg for 20 minutes at 4ºC and the supernatant was collected and stored at -20ºC. The protein concentration of each cell lysate was determined using a BCA Protein Assay Kit (Thermo Scientific, USA) according to the manufacturer's instructions.

Proteins were separated using 8% sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE). Cell lysates were diluted in 4x Laemmli loading dye ($H_2O$, 6% w/v SDS, 30% Glycerol, 0.02% v/v Bromophenol Blue and 0.2M Tris, pH 6.8) under reducing conditions (except for CD81 detection) to a final volume of 20µL and equal protein concentrations (20µg) based on the BCA Protein Assay Kit results. The samples were heat denatured at 95ºC for 5 minutes and allowed to cool prior to loading. Protein samples were

loaded, with a prestained standard (Novex Sharp, Invitrogen) and electrophoresis carried out at 200V for 35 minutes using the Mini Protein 3 system (Bio-Rad Laboratories, USA) according to the manufacturer's instructions. Proteins were transferred on to polyvinylidene membranes (Milipore, USA) washed in methanol, rinsed with water and in transfer buffer (25nM Tris, 0.2M Glycine, 200mL methanol and 10% SDS dissolved in $H_2O$, pH 8.3) for approximately 15 minutes. The transfer occurred at 350A for 60 minutes using a Mini Trans-Blot Electrophoresis system (Bio-Rad Laboratories, USA) according to the manufacturer's instructions. The membranes were blocked in a solution containing 5% Marvel dry milk powder dissolved in antibody buffer (10mM Tris, 100nM Sodium Chloride and 10% v/v Tween-20 dissolved in $H_2O$, pH 7.5) for 60 minutes with agitation. The blocking buffer was removed and the membrane incubated with the primary antibody diluted in antibody buffer (see **Table 2-2**) overnight with agitation at 4ºC. Following overnight incubation, the membranes were washed 5 times using the antibody buffer for 5 minutes with agitation, before the membranes were incubated with HRP-conjugated antibodies for 60 minutes with agitation. The membranes were washed 5 times and the HRP-conjugated antibodies detected by chemoluminescence using an ECL Western Detection System (Amersham, UK) according to the manufacturer's instructions.

### 2.1.8 Real time quantification PCR (qRT-PCR)

For all cell culture samples used in polymerase chain reactions (PCR), the RNA was lysed and extracted using the RNeasy mini kit (Qiagen) according to the manufacturer's instructions, and eluted with 35µL of RNase/DNase free

water at the end step. A Cells Direct One Step qRT-PCR kit (Life Technologies, UK) was used to quantify the sample RNA according to the manufacturer's instructions. Specific primers were then added to this PCR mix to detect on the FAM channel the copies of HCV (primer-unlimited; Applied Biosystems), or the relative level of the differentiation markers albumin, α-fetoprotein (α-FP), hepatocyte nuclear factor 4α (HnF4α) or cytochrome P450 family 3 - subfamily A, polypeptide 4 (cyp3a4) (Applied Biosystems). To quantify the copies of HCV RNA in the samples, a HCV positive control standard (Primer design) was used to produce a standard curve from a dilution series (ranging from $10^0$ to $10^7$ copies per µL) which was then used to calculate the copies of HCV RNA in the unknown samples. In all qRT-PCR reactions the house-keeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) with VIC was included as an internal endogenous control for amplification efficiency and RNA quantification (primer-limited endogenous control; Applied Biosystems).

Samples were run in triplicate using a MicroAmp 96 well optical reaction plate (ABPrism, Applied Biosciences, USA) and fluorescence was monitored in a Stratagene RT-PCR machine (MX3000P, Stratagene, Agilent, UK). The PCR reaction was set up with the following thermal settings:

30 minutes at 50º

5 minutes at 95ºC

15 seconds at 95ºC*

60 seconds at 60ºC* *repeated for 50 cycles

The PCR data was analysed using the MXpro software with the threshold values for the FAM and VIC signals set manually at the exponential phase of the amplification plots.

## 2.2 Specific techniques

### 2.2.1 Co-culture of stromal cells with hepatoma cells

Hepatoma cells were co-cultured with stromal cells: aLMF, LX-2, fibroblasts from different sites (dermal, synovial or bone marrow fibroblasts) or the control CHO cells. Mono- and co- cultures were seeded at a density of $2.5x10^4$ cells per well in a 48 well tissue culture plate or $10x10^4$ cells per well in a 12 well tissue culture plate. Both cell types were seeded at the same time, allowed to settle for 24 hours before being either transferred into the category 3 containment laboratory for HCVcc infection assays (detailed in **section 2.1.3**) or infected with pseudoparticle viruses (detailed in **section 2.1.4**). All co-cultures were maintained in DMEM containing 10% FBS, 1% non-essential amino acids, 1% L-Glutamine and 50units/mL penicillin/streptomycin (Gibco, CA, USA), throughout the duration of the co-culture assays and infection. Co-cultures were seeded at a 1:1 ratio, unless otherwise stated and infections allowed to proceed for 48 hours, unless otherwise stated. A schematic diagram of a co-culture set up is shown in **Figure 2-2.**

Co-cultures seeded using the LucA2 subgenomic replicon-luciferase hepatoma cell line (**section 3.3.3**) were seeded at a density of $10x10^4$ cells per well in a 48 well plate, at a 1:1 ratio and also maintained in complete DMEM containing 10% FBS for the duration of the co-culture assay. Under normal propagation conditions, the LucA2 subgenomic replicon-luciferase cells are maintained using DMEM containing 10% FBS supplemented with high glucose at 4.5 g/L, 1% non-essential amino acids, 1% L-Glutamine and

50units/mL penicillin/streptomycin (Gibco, CA, USA), plus G418 at 1mg/mL (PAA Laboratories, GmbH) and blasticidin S hydrochloride at 3µg/mL (Carl Roth GmbH+Co, Germany). Cultures were lysed 24 and 48 hours post seeding, using 100µL lysis buffer, to determine changes in replication over time. The luciferase activity was measured and data is expressed as relative light units (RLU) of the subgenomic-luciferase reporter activity.

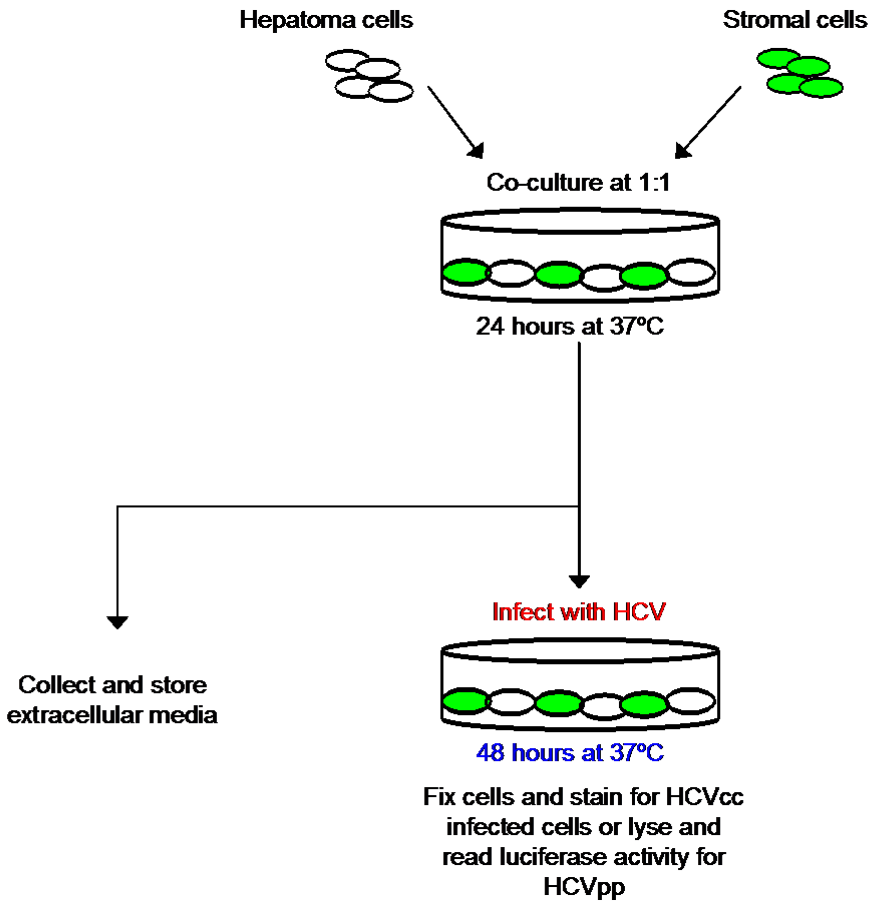



**Figure 2-2 Schematic diagram illustrating co-culture set up**

Huh7.5 cells were seeded in co-culture with stromal cells (LX-2 or aLMF) or the control CHO cells at a 1:1 ratio, and 24 hours post seeding, infected with HCVpp or HCVcc. Infection levels were determined 48 hours post infection by immunofluorescence staining for HCVcc infection assays, or luciferase readings for HCVpp assays. Conditioned media samples were collected prior to infection, spin clarified and stored at -80ºC for further testing.

### 2.2.2 Transwell assay

Transwell inserts with a pore size of 0.4μm (BD Falcon, USA) were used to separate cells but still allow the exchange of soluble secreted factors between the two types. The transwell inserts were placed in a 24 well plate with each well containing 800μL of fresh media, then aLMF, LX-2, dermal fibroblasts (DM), synovial fibroblasts (SY), bone marrow fibroblasts (BM) or CHO cells were seeded inside the transwell inserts at $2.5x10^4$ cells per insert in a volume of 300μL media. Next Huh7.5 cells were seeded in separate 24 well plates at $2.5x10^4$ cells per well in a volume of 800μL media, thus the number of cells in the inserts and tissue culture plates were in a 1:1 ratio. The cells were allowed to settle separately for a few hours before the inserts were carefully added to the wells containing Huh7.5 cells. Then 24 hours post adding the transwell inserts to the wells, Huh7.5 cells were infected with either HCVcc (detailed in **section 2.1.3**) or the pseudoparticle viruses (detailed in **section 2.1.4**), by carefully lifting the inserts, removing the media, adding the virus inoculums diluted in fresh media to give an end volume of 800μL and then adding back the transwell inserts carefully. The infection proceeded for 48 hours at which point the inserts were removed and discarded, and the Huh7.5 cells either fixed (if infected with HCVcc) or lysed (if infected with pseudoparticle viruses) to determine the level of infection.

### 2.2.3 Conditioned media

Conditioned media was collected from various mono- and co –culture conditions 24 hours post seeding but prior to infection, to ensure all samples

were virus free. Conditioned media samples were spin clarified by centrifugation at 3000rpm for 5 minutes and stored at -80ºC. Conditioned media samples were routinely collected from various co-culture assays and the volume of conditioned media necessary to achieve an equal ratio of co-culture cells to target cells was calculated. Naïve Huh7.5 mono-cultures were treated with a 1:1 mix of conditioned media and fresh media on the day of seeding and 24 hours later infected with either HCVcc (detailed in **section 2.1.3**), or pseudoparticle viruses (detailed in **section 2.1.4**), for 48 hours in the presence of the conditioned media, with naïve Huh7.5 cells cultured in normal media as a control.

### 2.2.4 ISG56 detection

Huh7.5 cells were transfected with an *ISG56* luciferase reporter plasmid and co-cultured with stromal cells. Briefly, Huh7.5 cells were seeded at $10x10^4$ cells per well in a 6 well tissue culture plate using DMEM containing 10% FBS, 1% non-essential amino acids and 1% L-Glutamine (Gibco, CA, USA). Cells settled for 24 hours and 1 hour prior to the transfection, the media was replaced with DMEM containing 3% FBS, 1% non-essential amino acids and 1% L-Glutamine (Gibco, CA, USA). The ISG56 plasmid was delivered to the cells using Lipofectamine Delivery (Life technologies, UK); per well, 4µL of lipofectamine was mixed with 250µL optimem and incubated at room temperature for 5 minutes. Then 4µg of the *ISG56* plasmid was mixed with 250µl optimem, added to the transfection mixture and incubated at room temperature for 20 minutes before adding to the Huh7.5 cells. After incubating the cells with the transfection mixture for 6 hours at 37ºC, the media was

removed and fresh DMEM containing 3% FBS, 1% non-essential amino acids, 1% L-Glutamine and 50 units/mL penicillin/streptomycin (Gibco, CA, USA) was added to the cells. After 24 hours the cells were re-seeded into co-culture with the stromal cells at a 1:1 ratio or seeded in mono-culture in order to screen the conditioned media for potential ISGs.

To screen the conditioned media, samples were added to reporter plasmid-transfected cells at a 1:1 ratio with fresh media. Exogenous IFNα (1, 10, 100 IU), IFNβ (1, 10, 100 units), IFNλ1 (0.3, 1, 3 ng/ml) and IFNλ2 (1, 10, 30 ng/ml) (Peprotech) were also added alongside conditioned media as positive controls. Type I IFN (IFNα and IFNβ) doses were based on previous concentrations used by our group and others, which have shown maximal inhibition of HCV replication at > or =50 U/ml (Meredith et al., 2014, Marcello et al., Marcello et al., 2006, Macejak et al., 2001). Type III IFN (IFNλ1 and IFNλ2) doses were based on concentrations shown by other research groups to have maximal inhibition on HCV genotype 2a replication at 10 ng/ml (Marcello et al., 2006) and ranges between 0.2-0.5 ng/ml showing 50% inhibition of HCV replication (Park et al., 2012, Pagliaccetti et al., 2008). The cells were lysed 24 hours post the addition of the samples with 50µL of 1x Cell Culture Lysis Buffer (Promega), prepared according to the manufacturer's instructions, for 2 hours at room temperature. Then 45µL of each cell lysate was transferred to a white polystyrene 96-well fluorescent assay plate (Corning, USA), mixed with 45µL of luciferase substrate reagent (Luciferase Assay System, Promega) and then the luciferase activity detected using a Centro LB960 Luminometer (Berthold

Technologies, UK) as relative light units (RLU) of the plasmid signal relative to the untransfected control Huh7.5 cells containing no plasmid (Luc:no plasmid).

In co-culture HCVcc infection assays, the transfected Huh7.5 cells were re-seeded with the stromal cells at a 1:1 ratio in a 96 or 48 well plate, as previously described in **section 2.2.1**, along with mono-cultures for positive controls. Once the cells had settled, mono-cultures of transfected Huh7.5 cells were either left untreated or treated with the positive control of 100 IU of IFNα. In parallel, the Huh7.5 co-cultures with aLMF, LX-2 and CHO cells were infected with a high titre HCVcc virus (detailed in **section 2.1.3**). The cells were lysed 24 hours post the addition of HCVcc virus and the positive IFNα control with 50µL (96 well plate) or 100µL (48 well plate) of 1x Cell Culture Lysis Buffer (Promega), prepared according to the manufacturer's instructions, for 2 hours at room temperature. Then 45µL of each cell lysate was transferred to a white polystyrene 96-well fluorescent assay plate (Corning, USA), mixed with 45µL of luciferase substrate reagent (Luciferase Assay System, Promega) and then the luciferase activity detected using a Centro LB960 Luminometer (Berthold Technologies, UK) as relative light units (RLU).

### 2.2.5 Human anti-viral Response PCR array

Huh7.5 cells were seeded in mono- and co- culture with aLMF as described in **section 2.2.1** then left uninfected or infected with a high MOI of HCVcc (MOI 10) to be lysed for RNA extraction, resulting in the following samples: Huh7.5 mono-culture uninfected, Huh7.5 mono-culture infected, Huh7.5+aLMF co-culture uninfected and Huh7.5+aLMF co-culture infected. The level of HCV

infection was detected using qRT-PCR (as described in **section 2.1.8**). To run the samples in the Human anti-viral Response PCR array, cDNA was first synthesized with the RNA as template, using the $RT_2$ First Strand Kit (SABiosciences, USA), according to the manufacturer's instructions. The PCR array constituted a 384-well plate (SABiosciences, USA) divided into 4 x 96 well formats, 1 for each sample. The gene expression was then measured according to the manufacturer's instructions using the Absolute Quantity RT-qPCR program monitored in a Stratagene RT-PCR machine (MX3000P, Stratagene, Agilent, UK). The array contains 84 key anti-viral genes and the control housekeeping genes: GAPDH, actin, B2M, RPLP0 and HPRT. Data were analysed using the online $RT_2$ Profiler PCR Array Data Analysis Program (www.sabiosciences.com/pcrarraydataanalysis.php, SABiosciences), by normalising the target genes to the housekeeping control genes, then comparing all four samples in pair wise comparisons, calculating the 2^ΔΔ Ct levels for each target gene. The data are presented in the form of heat-maps that display the fold-differences.

### 2.2.6 Inhibition of nitric oxide (NO) pathway and detection of NO in extracellular media

To inhibit the NO pathway, Huh7.5 cells were seeded in co-culture with aLMF, LX-2 and CHO cells (as detailed in **section 2.2.1**) at a 1:1 ratio for HCVcc infection and immediately treated with the anti-nitric oxide compound L-monoethlyl arginine (L-NMMA) (Sigma, UK) at 1 mM, and then seeded into co-culture. The L-NMMA compound can inhibit the NO pathway for

approximately 72 hours, and so cultures were infected with HCVcc 24 hours post seeding, in the presence or absence of L-NMMA. The cells were methanol fixed 48 hours post infection (detailed in **section 2.1.3**), to enumerate the foci. The supernatants removed from these cultures were collected and screened using the Griess assay (Promega) to determine the level of NO, following manufacturer's instructions. In order to test these supernatants using the Griess assay, they were removed from the category 3 containment laboratory; this involved treating them with 1% empigen (Sigma Aldrich Ltd, UK) at room temperature for 30 minutes to inactivate any HCVcc virus present. Then samples were spin clarified by centrifugation at 3000rpm for 5 minutes. This treatment did not interfere with results from the Griess assay.

### 2.2.7 Matrigel 3D cultures

To establish a 3D liver organoid Matrigel sandwich culture, I undertook a research visit to UMCG, The Netherlands, where under the guidance and help of Prof. Sven van Ijzendoorn and his research group; I learnt how to establish 3D liver organoid Matrigel sandwich cultures. Upon return to The University of Birmingham, the 3D Matrigel™ culture technique was adapted to grow Huh7.5 cells and HepG2 cells in co-culture with aLMF, LX-2 and CHO cells for HCV infection assays. BD Matrigel™ Basement Membrane Matrix (BD Biosciences) was stored in aliquots at -80ºC to avoid repeated freeze-thawing. Required aliquots were thawed on ice for approximately 30 minutes and then used to coat a Nunc™ Lab-Tex™ II 8 well Chambered Coverglass (Thermo Scientific, USA). Each chamber was coated with 5µL of BD Matrigel™ and

incubated at 37ºC for 30 minutes to set. Cells were then seeded to a total of 5000 cells per chamber in 300µL media. Huh7.5 and HepG2 cells were seeded in mono- and co- culture with aLMF, LX-2 and CHO cells at a 4:1 ratio of hepatoma to stromal cells. As a control, cultures were also seeded in Labtex Chambers without the BD Matrigel™ to give 2D cultures with the same number of cells. The 3D cultures were assessed 72 hours post seeding for the formation of organoid cultures using phase microscopy and if required, the cultures were given an additional day to form organoids before the cultures were infected. Once the organoids had formed, they were infected alongside the 2D control cultures with either HCVcc (detailed in **section 2.1.3**) or pseudoparticle viruses (detailed in **section 2.1.4**) for 48 hours.

### 2.2.8 Cell IQ live cell imaging

Huh7.5 cells were seeded in mono- and co- culture at a 1:1 ratio with aLMF, synovial, bone marrow and dermal fibroblasts as previously described in **section 2.2.1**, on glass bottomed tissue culture plates. The fibroblasts were labelled using CMFDA green cell tracker dye (Invitrogen, USA) prior to co-culturing. Briefly; cells were incubated with 5µM CMFDA in complete DMEM containing 3% FBS at 37ºC for 30 minutes. Cells were then carefully washed with PBS twice, fresh complete DMEM containing 3% FBS added and incubated at 37ºC for 30 minutes before seeding in co-culture with unlabelled Huh7.5 cells. After 24 hours the cultures were placed in a Cell-IQ® SLF (single-label fluorescence) machine. This maintained normal tissue culture conditions and imaged multiple points in each well every 30 minutes over night, on both the phase channel and green fluorescence channel, obtaining images

of unlabelled Huh7.5 cells and CMFDA labelled fibroblasts over time. These images were used to create time lapse movies showing the cells migrating from which representative images were taken at approximately 1, 4, 7 and 10 hours. The time lapse movies were also used to track individual CMFDA labelled fibroblasts over time using ImageJ software frame by frame (tracking data performed by Dave Mason).

### 2.2.9 Virus binding to cell membrane and ECM assay

Huh7.5, Huh7, HepG2, aLMF, LX-2 and the non-permissive control CHO cells were seeded in mono-culture in duplicate wells to assess the level of virus binding to the cells and also virus binding to the ECM produced by each cell type. Cells were seeded at $4x10^4$ cells per well in a 24 well plate. To remove the cells and leave behind the ECM produced on the tissue culture plate, 24 hours post seeding, the cells were treated with a lysis buffer consisting of PBS containing 0.5% Triton X (v/v) and 20mM ammonium hydroxide ($NH_4OH$) until they detached, which was observed by phase microscopy (Butler 2005). The remaining ECM was washed carefully with PBS three times. HCVcc inoculum was added to wells containing either cells or ECM alone for one hour, the remaining virus inoculum was removed, spin clarified and transferred to naïve Huh7.5 mono-cultures seeded at $2.5x10^4$ cells per well in a 48 well plate to determine the level of infection compared to a control virus inoculum incubated in an empty well to control for potential static or non-specific binding caused by the tissue culture plate, as detailed in **section 2.1.3**.

### 2.2.10 Generation of TRIP virus

The TRIP virus system is used to generate retrovirus gene expression vectors that allow us to transduce cells to express a protein of interest. The retrovirus gene expression vector is formed using a replication deficient gag-pol core bearing vesicular stomatitis virus (VSV-G) envelope glycoproteins. These TRIP virus particles can then be used to enclose the RNA transcript of the gene of interest to be delivered to the target cells by transduction (Gottwein et al 2009, Zennou et al). In this study, transduced cells were maintained under normal tissue culture conditions without selection and typically maintained exogenous gene expression for up to 4 weeks, at which point cells were discarded. The TRIP virus system was used to transduce Huh7.5 cells with plasmids encoding CD81-GFP or EGFR-GFP. Briefly, TRIP virus particles were generated in 293-T cells using a Fugene transfection protocol. First, 293-T cells were seeded at $7x10^5$ cells per well in a 6 well tissue culture plate coated with poly-L-lysine at 0.1 mg/mL (Sigma, UK) using DMEM containing 10% FBS, 1% non-essential amino acids and 1% L-Glutamine (Gibco, CA, USA). After 24 hours the media was replaced with DMEM containing 3% FBS, 1% non-essential amino acids and 1% L-Glutamine (Gibco, CA, USA). The TRIP gag-pol plasmid encoding a HIV virus deficient of replication (600ng/well) was transfected with VSV-G envelope plasmid (600ng/well) and the target gene of interest (600ng/well), using 6µL Fugene in 100 µL optimem on a per well basis, as described previously in **section 2.2.4**. As the target genes express GFP, the transfection efficiency was assessed using UV microscopy. The supernatants from the transfected cells, containing TRIP virus particles, were collected at 48 and 72 hours post transfection, pooled, spin clarified by

centrifugation (at 3000rpm for 5 minutes) and used to transduce the target cells immediately. The target Huh7.5 cells were seeded 24 hours prior to transduction at $4x10^4$ cells per well in a 6 well plate. To transduce the Huh7.5 cells, the supernatants containing TRIP virus were diluted 1:2 in DMEM containing 3% FBS, 1% non-essential amino acids, 1% L-Glutamine, 50units/mL penicillin/streptomycin (Gibco, CA, USA) and 1.6µg/mL polybrene (Sigma, UK), then added to the cells and incubated overnight. The following day the media was replaced with fresh DMEM containing 3% FBS, 1% non-essential amino acids, 1% L-Glutamine and 50units/mL penicillin/streptomycin (Gibco, CA, USA).

### 2.2.11 Fluorescence recovery after photobleaching (FRAP)

Real-time fluorescence recovery after photobleaching (FRAP) was used to investigate the effects of aLMF cells in co-culture with Huh7.5 cells transduced with either CD81-GFP or EGFR-GFP (transduction protocol detailed in **section 2.2.10**). The transduced Huh7.5 cells were seeded, in mono- and co-culture with aLMF cells at a 1:1 ratio, onto glass bottomed 24 well tissue culture plate at $4x10^4$ cells per well in DMEM containing 10% FBS, 1% non-essential amino acids, 1% L-Glutamine and 50units/mL penicillin/streptomycin (Gibco, CA, USA). After 24 hours the cells were imaged using a Zeiss LSM 780 Confocal microscope at a 100x Plan Apochromat 1.4NA oil immersion objective. The media was replaced 1 hour prior to imaging with phenol red free DMEM/IMDM containing 10% FBS, 1% non-essential amino acids, 1% L-Glutamine and 50units/mL penicillin/streptomycin and 1M HEPES (Sigma, UK). In order to determine the impact of heterotypic cell-cell contact on membrane

dynamics of Huh7.5 cell expressed CD81 and EGFR, Huh7.5 cells in co-culture and direct contact with aLMF cells were selected for FRAP and compared to Huh7.5 cells in contact with other Huh7.5 cells in mono-culture. Also, 16-bit images were obtained with optimal pixel resolution. The GFP-tagged CD81 and EGFR proteins were excited using an argon 488 laser, photobleached using full laser power on circular regions of interest (ROI) selected in the planar membrane. FRAP measurements were taken prior to and after photobleaching at 0.08 s per frame, for approximately 2 minutes until the ROI recovery reached a plateau. The mean fluorescent intensity over time for the photobleached ROIs and a background ROI (containing no cells) were acquired, and then analysed using the Zeiss Zen analysis software. Data were normalised for potential fluctuations in laser strength, image capture and overall loss of fluorescence in the cells by subtracting the background ROI from the photobleached ROIs. To calculate the relative fractional recovery for each photobleached ROI, the values acquired before the bleaching were set as 100% and then post-bleaching calculated as relative to the mean fluorescence intensity prior to photobleaching for that ROI. Graphpad Prism software was used to fit the data to a single exponential decay algorithm Y = span (1-exp (-K*X)) + plateau. The mobile fraction (MF) was calculated from the span and plateau. The diffusion coefficient (D) was calculated using the equation D = 0.224 x (radius2/t½) for each circular bleached ROI in a two-dimensional diffusion model. In each independent experiment, a minimum of 10 cells and 100 ROIs were selected for each condition.

### 2.3 Statistical analysis

Statistical analysis was conducted using PRISM software (Graphpad). Non-parametric statistical tests were used when assumptions of a normal or Gaussia distribution could not be tested, allowing statistical significance to be determined without assuming normal distribution of data. This included all HCVcc based assays which were tested using the Mann-U-Whitney test followed by Wilcoxson test with multiple corrections (small sample sizes) or the Kruskal-Wallis test with Dunn's corrections (larger sample sizes). These non-parametric tests were used to compare the median infection levels across the different co-culture conditions and data are shown as the median ± standard deviation unless otherwise stated. Parametric tests were used for data which passed the normality test (D'Agostino-Pearson omnibus test in Graphpad) indicating a Gaussia distribution. This included all luciferase based assays such as pseudoparticle infections or luciferase reporter plasmids, which were tested using a One way ANOVA test with Bonferroni's corrections. Data are shown as the mean ± standard deviation unless otherwise stated.

# Chapter 3 aLMF are non-permissive to HCV but have the ability to inhibit HCV infection in co-culture

## 3.0 Introduction

The liver contains multiple types of cells with the parenchymal cells, also referred to as hepatocytes, occupying the majority of the total volume. Additional types of cells include the non-parenchymal cells, which provide many important functions. One such cell type is hepatic stellate cells (HSC) which store vitamin A and become activated in response to liver injury, such as viral infection, into activated liver myofibroblast cells (aLMF) (Yoshida et al 2012; Ishibashi et al 2009; Pertoft 1987). Several potential sources are thought to give rise to aLMF cells however HSCs have been identified as the main source. aLMF cells are the key players in liver fibrosis, a dynamic process designed to heal the liver however in response to chronic inflammation, which can cause liver scarring and progress further to cirrhosis or advanced scarring (Su et al 2014; Mederacke et al 2013). Fibrosis, or scarring of the liver, is a result of the excess ECM produced by the aLMF which is not degraded, particularly collagen (types I, III, IV and V), elastin, laminin, fibronectin and proteoglycans (Schulze-Krebs et al 2005). HCV infection of hepatocytes induces a profibrogenic response, stimulating the HSCs to become activated and driving fibrosis via a number of mechanisms (Su et al 2014; Wang et al 2013).

Although hepatocytes are the primary target for HCV in the liver, the role of HSC or aLMF in the viral lifecycle is poorly understood. HSCs have been

reported to express the HCV receptor CD81 and the attachment factor, low density lipoprotein receptor (LDL-r) (Bataller et al 2004). We wanted to determine if HSCs and primary aLMF are permissive to HCV infection and then given their close proximity to hepatocytes in the liver, establish a co-culture system to study the role these cells plays in HCV infection of the liver.

### 3.1 Isolation and phenotyping of primary activated liver myofibroblasts

aLMF cells were isolated from human liver samples obtained from patients attending the Queen Elizabeth Hospital Birmingham for a range of liver diseases as listed in **Table 3-1**. These human tissues were at various end stage chronic liver disease aetiologies providing the opportunity to examine and interpret the function *in vivo* of the aLMF. Many of the human tissues were obtained from patients undergoing liver transplantation for various diseases or occasionally obtained from tissue rejected for use as a donor liver due to damage from illness or disease. These tissues all have underlying disease aetiologies and normal healthy tissue is only obtained if there is surplus tissue to requirements at transplant which is often very limited amounts. The tissue was processed by enzymatic digestion, differential density centrifugation and immunomagnetic separation in order to recover cells from the liver tissue and generate a single cell suspension, see materials and methods (**section 2.0.3**) (Joplin et al 1990; Holt et al 2009). The aLMF were only used when they were 95% pure as assessed by staining for lineage specific markers for aLMF, hepatocytes, biliary epithelial cells (BEC) and liver sinusoidal endothelial cell (LSEC), which is also detailed in the materials and methods (**section 2.0.3**). After isolation, these cells maintained classical aLMF elongated morphology in culture until at least passage 6 (**Fig.3-1A**) and the expression of the characteristic aLMF phenotype markers cluster of differentiation 90 (CD90), desmin, and vimentin were monitored over 6 passages in culture by immunofluorescence microscopy (**Fig.3-1B**).

**Table 3-1 Disease aetiologies of patient derived aLMF[a]**

| Abbreviation | Disease |
|---|---|
| ALD | Alcoholic Liver Disease |
| Donor | Tissue from rejected donor liver |
| HCC | Hepatocellular Carcinoma |
| NASH | Non-alcoholic Steatohepatitis |
| NAFLD | Non-alcoholic fatty liver disease |
| PBC | Primary Biliary Cirrhosis |
| PSC | Primary Sclerosing Cholangitis |
| Seronegative | Negative reaction to serological tests for viral infections including HIV and hepatitis viruses |

[a]Table summarising the different disease aetiologies of the human tissues obtained and routinely used to isolate aLMF cells by the Centre for Liver Research, University of Birmingham.

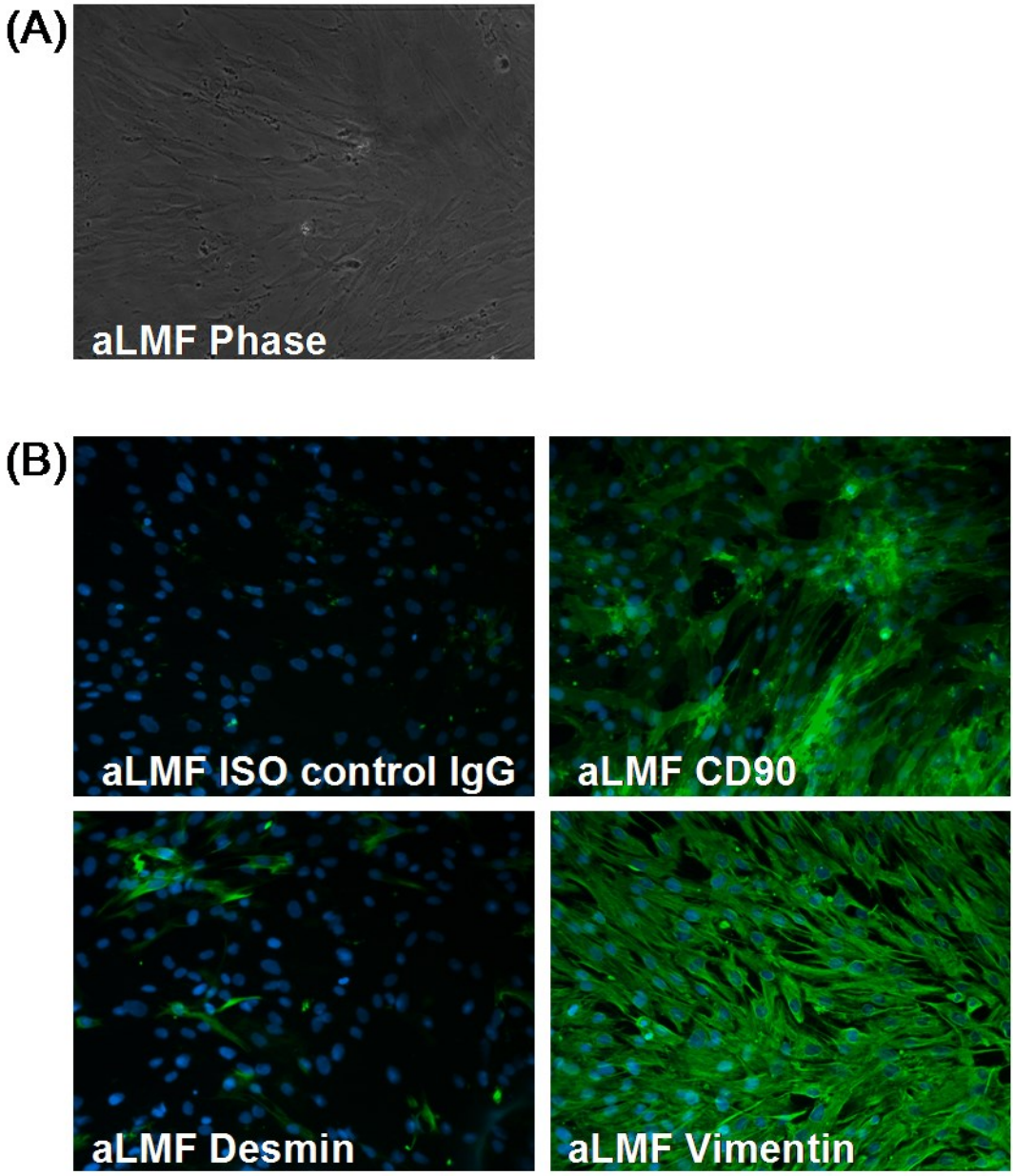



**Figure 3-1 Phenotype of primary isolated aLMF**

(**A**) Representative phase contrast image of aLMF morphology under tissue culture, taken at magnification x10. (**B**) aLMF were fixed and stained for fibroblast specific markers including CD90, vimentin and desmin (green) with the mouse anti-human IgG isotype control. Cell nuclei were stained using DAPI (blue).

### 3.2 aLMF do not support HCV infection

HCV primarily targets the liver and due to the location of aLMF within the liver and given their role in fibrosis progression, we first wanted to determine whether aLMF could support HCV infection. To ascertain whether aLMF primary cells and the Lieming Xu-2 (LX-2) stellate cell line can support HCV infection, mono-cultures were infected with high titre high titre hepatitis C virus derived from cell culture (HCVcc) inoculum with mono-cultures of Huh7.5 included as a positive control and the Chinese Hamster Ovary (CHO) cell line included as a non-permissive control. Mono-cultures of the various cell types were incubated with a high Multiplicity of Infection (MOI) of HCVcc (MOI of 1 on Huh7.5) for 6 hours before the cells were washed, fresh media added and cells incubated for a further 48 hours. The supernatant was then removed, clarified of cell debris by centrifugation (20,000 g, 5 minutes) and incubated with naïve Huh7.5 “target” cells for 48 hours in order to determine whether or not infectious Hepatitis C viral particles were being produced by the mono-cultures. The mono-cultures incubated with the high titre virus inoculum and the naïve Huh7.5 “target” cells incubated with the supernatants were then assessed for HCV infection using immunofluorescence and staining for non-structural 5A (NS5A) protein of HCV.

As expected, high levels of HCVcc infection were detected in the control Huh7.5 cells incubated with the high virus infection and in the naïve Huh7.5 target cells treated with the supernatant collected from these cells (**Fig.3-2**). This confirmed that the Huh7.5 cells are highly permissive to the HCVcc inoculum used and highly supportive of HCVcc infection as the supernatant

transferred to the naïve Huh7.5 target cells also produced high levels of HCVcc infection. In the negative control CHO cells, there was no detectable HCVcc infection by immunofluorescence, similarly there was no detectable virus in their supernatant when incubated on the naïve Huh7.5 target cells. Notably, there was no detectable HCVcc infection in the aLMF and LX-2 mono-cultures or in the target cells treated with supernatants collected from these cells. Taken together, these results indicate that aLMF and LX-2 cells are not permissive to HCVcc infection.

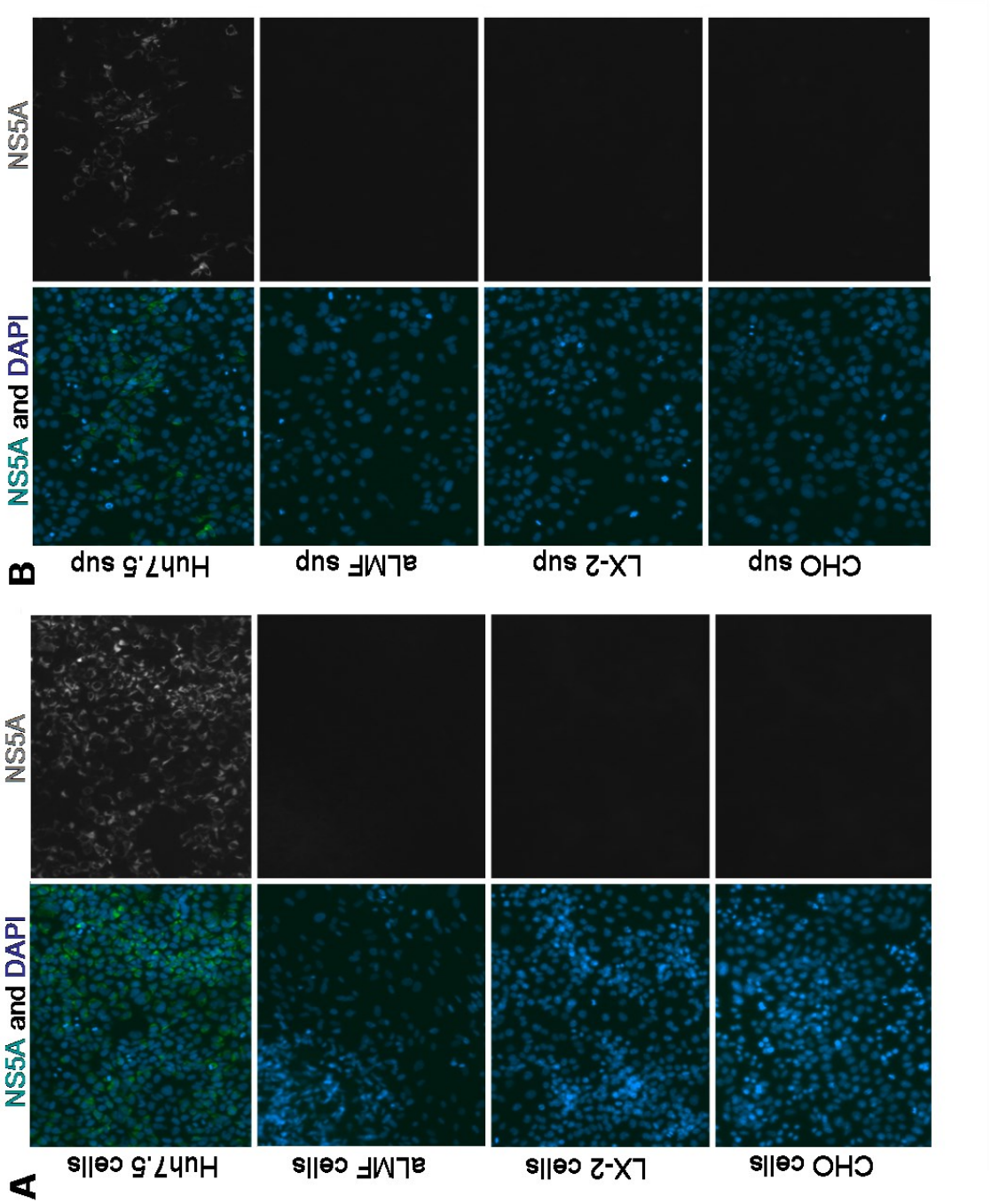



**Figure 3-2 aLMF do not support HCVcc infection**

**(A)** Representative immunofluorescence images of Huh7.5, aLMF, LX-2 and CHO mono-cultures infected with a high MOI of HCVcc followed by incubation with fresh media for a further 48 hours. Cells were fixed and stained for NS5A (green) and also stained with DAPI to show nuclei (blue). NS5A alone staining is shown alongside (grey). **(B)** Representative immunofluorescence images of naive Huh7.5 target cells treated with supernatants (sup) transferred from the Huh7.5, aLMF, LX-2 and CHO mono-cultures infected with a high MOI of HCVcc. Cells were fixed 48 hours post treatment with the supernatants and stained for NS5A (green) and with DAPI nuclei (blue). NS5A alone staining is shown alongside (grey) (magnification x10). Data representative of n=2 independent repeats (n=2 aLMF donors).

### 3.3 Key receptors mediating HCV entry are not present on aLMF

HCV entry into the cell is mediated by four well-established HCV receptors: CD81, Claudin-1, Occludin, and the human scavenger receptor class B type I (SR-BI) (Hsu et al 2003; Kapadia et al 2007; Ploss et al 2009; Grove et al 2007). Several studies have identified other possible entry factors which HCV may also utilise to enter cells, including epidermal growth factor receptor (EGFR), low-density lipoprotein receptor (LDLR), Neimann-Pick C1-like cholesterol absorption receptor (NPC1L1), and ephrin receptor A2 (EphA2) (Meredith et al 2012; Lupberger et al 2011). As aLMF and LX-2 cells were unable to support HCV infection, we therefore investigated if these cell types expressed the four well-established HCV receptors: CD81, Claudin-1, Occludin, and SR-BI which mediate HCV entry into the cell (Meredith et al 2012). Receptor expression was investigated using Western blot analysis of the aLMF and LX-2 cells. As a positive control the Huh7.5 cell line was included because they have previously been shown to express all four receptors. As a negative control the Chinese Hamster Ovary (CHO) cell line was included because they do not express any of the human receptors. As expected, the controls stained for their respective receptors (**Fig.3-3A**). Analyses revealed that aLMF express CD81, but showed no expression of Claudin-1, SR-BI, or Occludin. In contrast, the LX-2 cell line expresses all receptors except Claudin-1.

We then used immunofluorescence to characterize the expression levels and localisation of the receptors on aLMF. Using Confocal microscopy, cells were imaged at 24 hours post adherence to a glass-bottomed tissue culture plate.

A representative image for CD81, Occludin, and Claudin-1 staining of aLMF is shown in **Fig.3-3B**. For both Claudin-1 and Occludin, a staining pattern was constant over time but very faint, indicating very low levels of detectable expression. The staining which was seen appeared to be localised mainly in the cytoplasm, with some nuclear staining. These faint staining patterns could also be due to some autofluorescence or background noise. aLMF often appear autofluorescent under microscopy due to the vitamin A stored within the cells and exacerbated by the high FBS content in the culture medium. At the time, we were unable to visualise SR-BI by immunofluorescence as there was no access to SR-B1 antibodies for this method.

Together these two independent experimental methods, western blot analysis and immunofluorescence, show that aLMF do not express all four key HCV receptors required for viral entry. This lack of receptor expression explains why aLMF cells failed to support HCV infection.

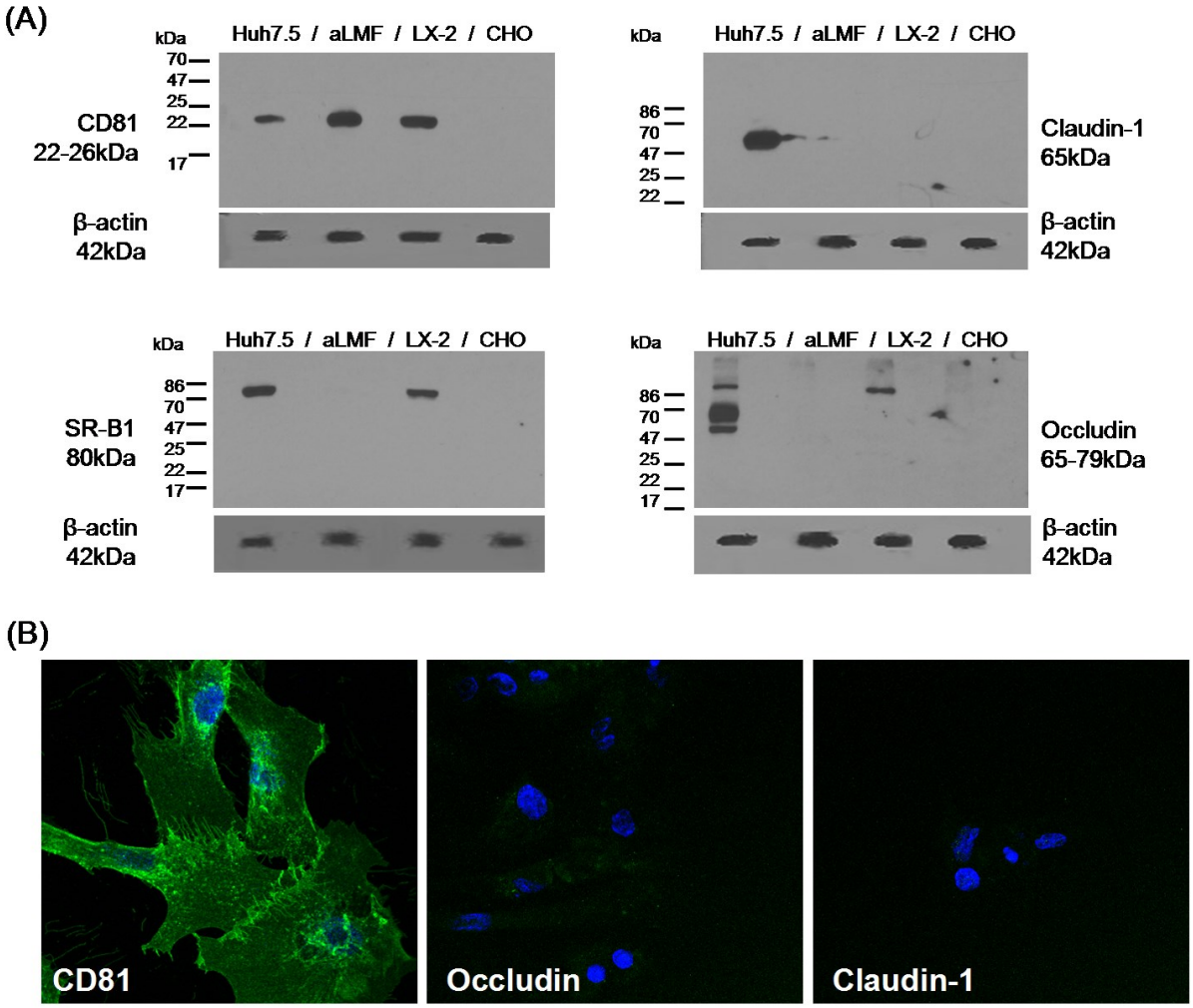



**Figure 3-3 aLMF lack expression of key HCV receptors**

**(A)** Cell lysates from Huh7.5, aLMF, LX-2 and CHO cell mono-cultures were prepared for Western blot detection of the four key HCV receptors; CD81, Claudin-1, SRB1 and Occludin. β-Actin is used as a loading control. Data is representative of n=3 independent repeats (n=3 aLMF donors). **(B)** aLMF were seeded on glass cover slips in a 48 well tissue culture plate at a seeding density of $2.5x10^4$ cells per well and stained for CD81, Occludin and Claudin-1 staining (green). Imaging was done by Confocal microscopy (Zeiss 510 inverted using a 100x Plan Apochromat 1.4NA oil immersion objective). The cells were left for 48 hours to adhere before being fixed at 24, 48 and 72 hours post adherence. A representative image from 24 hours post adherence is shown.

### 3.4 The role of aLMF on HCV infection in a co-culture system

The interactions of different cells in the liver are of crucial importance for tissue formation and homeostasis (Taub, 2004a). The majority of work done in laboratories involves studying cells of one origin and therefore lacks the dimension introduced by heterotypic cell-cell interactions found *in vivo*. Within the liver, aLMF are found in close proximity to hepatocytes which support HCV infection (Taub, 2004a). We have demonstrated aLMF do not support HCV infection. Therefore, to further investigate if aLMF have a role in modulating hepatocellular HCV infection and lifecycle we developed a co-culture model.

#### 3.4.1 Establishing a co-culture system to investigate the effects of aLMF on hepatocellular HCV infection

In order to establish a co-culture system, initially Huh7.5 cells were seeded 45-60 minutes before the addition of the secondary cells: aLMF, LX-2 or CHO cells. In addition both cell types were seeded together at the same time in co-culture. Both methods of seeding resulted in the same pattern with the two cells types separating to form islands of matched cell types which resemble “rivers” and “islands” (**Fig.3-4A**). This separation was clearly visible by phase microscopy due to the differences in morphology and is possibly mimicking the cell arrangements in the liver environment. This separation is seen when other secondary cell types are co-cultured with Huh7.5 cells, including liver sinusoidal endothelial cells (LSEC) (Rowe et al 2014). Given that the various cell types are propagated in DMEM containing different levels of bovine serum

(FBS), see materials and methods (**section 2.0.1**), it was decided to maintain all co-cultures in DMEM containing 10% FBS. Typically 3% FBS is used during HCV infection assays, however the higher level of FBS helps maintain the aLMF at optimal density and aids aLMF adherence in co-culture.

Next, the effect of varying the number of target cells was investigated by varying the number of target cells on HCV infection. Using Huh7.5 cells as the target cells because they have previously been shown to be highly permissive to HCV infection, we seeded a full well of target cells, $2.5x10^4$ cells per well in a 48 well tissue culture plate giving a density of 0.05 cells per $mm^2$. Huh7.5 cells were then seeded a half, a fourth and an eighth of the number of target cells in the full well, infected with the same virus inoculum and cells fixed 48 hours post infection. Cells were stained for NS5A positive cells and the level of infection determined by counting the number of foci per well. Altering the number of target Huh7.5 cells and infecting the mono-cultures with the same virus inoculum led to a decreasing level of HCV infection in proportion with the number of target cells present (**Fig.3-4B**). By altering the number of Huh7.5 target cells in the same sized tissue culture wells, we were of course also altering the density and previous studies have previously shown hepatoma density impacts HCV infection via HCV receptor expression levels (Schwarz et al., 2009, Grove et al., 2007, Koutsoudakis et al., 2007). HCV entry is dependent on the target cell density due to the increased level of claudin-1 and SRB1 expression at the points of cell-cell contact. Therefore, as the target cell density increases, the level of HCV entry for both HCVpp and HCVcc particles also increases (Schwarz et al 2009; Grove et al 2007;

Koutsoudakis et al 2007). Based on these observations, the non-permissive CHO cell line was included in co-culture assays as a control in order to take up space controlling for cell density by cell contact inhibition of cell proliferation. Thus, comparing aLMF or LX-2 containing co-cultures to the CHO containing co-cultures is more accurate than comparing to the half density well of Huh7.5 cells alone.

We next co-cultured target Huh7.5 cells:stromal cells (aLMF, LX-2 or the control CHO cells) in both a 1:1 and 2:1 ratio condition. We wanted to model the ratio of hepatocytes to liver myofibroblasts at a more physiologically relevant level and therefore wanted to test aLMF seeding at a range of ratios. However there are always a small number of aLMF cells which do not adhere due to the nature of primary cells being more difficult to maintain in culture compared to cell lines. The lower the number of aLMF present in the culture the less likely they were to adhere and therefore the increased cell death in the aLMF would distort the final ratio. Based on this observation we decided not to seed the ratio of Huh7.5 cells to aLMF at any less than a 2:1 ratio. aLMF, LX-2 and the control CHO cells were then co-cultured with Huh7.5 cells at a 1:1 and 2:1 hepatoma:stromal cell ratio, then infected with HCVcc. The level of HCV infection observed in the co-cultures seeded at a 1:1 and 2:1 ratio were comparable but there was an overall modest increase in HCV infection in the 2:1 ratio co-culture. This modest increase in HCV infection supports our prediction that the level of HCV infection is proportional to the number of target hepatoma cells present and the cell density. Interestingly, in co-culture the aLMF from three separate donors and LX-2 cells reduced the

level of HCV infection to a greater extent than the control CHO cells. Furthermore, the ability of aLMF cells to significantly reduce the levels of HCV infection in co-culture was not restricted to any specific disease aetiology or passage number, as demonstrated over n=4 independent experiments and n=7 aLMF donors at different passages as detailed in **Table 3-2** for co-cultures seeded at a 1:1 hepatoma:stromal cell ratio. The data indicates the aLMF can reduce the level of HCVcc infection in neighbouring Huh7.5 cells by 93.5%±5.7 when compared to Huh7.5 mono-cultures or by 65.3%±43.4 when compared to the control CHO co-cultures. LX-2 cells can reduce HCVcc infection in neighbouring Huh7.5 to lesser extent of 59.4%±29.1 when compared to Huh7.5 mono-cultures or by 71.9%±15.2 when compared to the control CHO co-cultures.

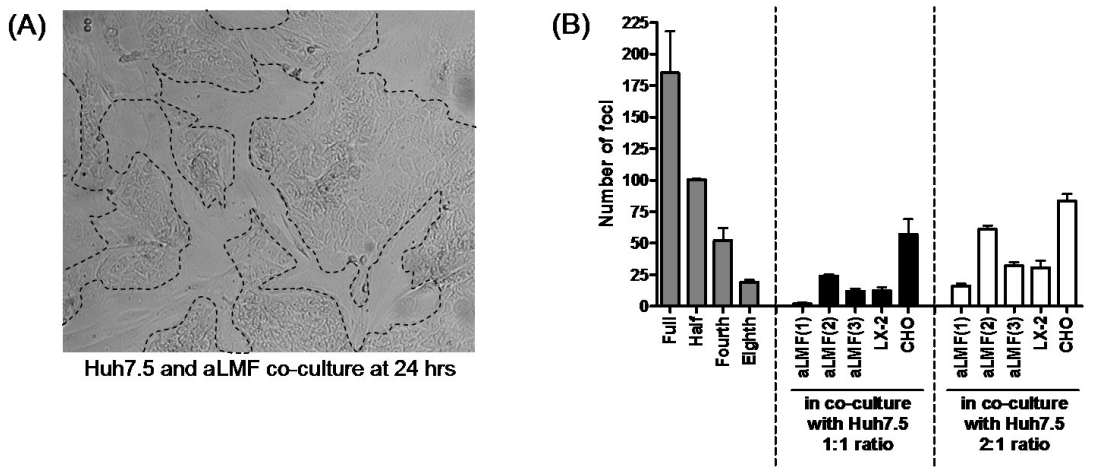



**Figure 3-4 The effects of aLMF on hepatocellular HCV infection in co-culture**

**(A)** Phase image of Huh7.5 cells and aLMF cells in co-culture 24 hours post seeding (magnification x10). **(B)** In a 48 well tissue culture plate, Huh7.5 target cells were seeded in mono-culture at $2.5x10^4$ cells per well in the full well, then a half, a fourth and an eighth of the number of target cells in the full well. Huh7.5 cells were also seeded in co-culture with either three separate aLMF donors (numbered 1-3), LX-2 or CHO cells at both a 1:1 and 2:1 hepatoma:stromal cell ratio. 24 hours post seeding, the cultures were infected with HCVcc for 48 hours after which the cells were fixed and stained for NS5A and the number of NS5A positive cells counted as foci to determine the level of infection. Data representative of n=4 independent repeats (n=7 aLMF donors). Statistical comparison was made using the Kruskal-Wallis test and Dunn's corrections were applied.

**Table 3-2 Hepatitis C viral inhibition by aLMF in co-culture with Huh7.5 cells[a]**

| Disease | Passage | HCVcc viral inhibition (%) relative to | |
|---|---|---|---|
| | | Huh7.5 mono-culture | CHO co-culture |
| Donor | 6 | 98.7 | 95.0 |
| Donor | 5 | 93.7 | 77.0 |
| PBC | 3 | 97.5 | 90.8 |
| ALD | 6 | 90.6 | 0.0 |
| PBC | 6 | 100.0 | 100.0 |
| NASH | 3 | 89.7 | 89.2 |
| ALD | 6 | 84.1 | 5.4 |

[a]Table summarising data from n=4 independent co-culture experiments seeded at a 1:1 hepatoma:stromal cell ratio, infected with HCVcc 24 hours post seeding and infection levels counted as either foci or total number of infected cells per well, 48 hours post infection. Data is expressed as percentage viral inhibition relative to either the full Huh7.5 mono-culture (stromal cell co-culture infection calculated as a percentage relative to Huh7.5 mono-culture expressed as 100% infection) or the CHO control co-culture (stromal cell co-culture infection calculated as a percentage relative to Huh7.5+CHO co-culture expressed as 100% infection) from a total of n=7 aLMF donors at different passages and of different disease aetiologies.

### 3.4.2 aLMF can limit HCV entry in co-culture

After observing that the aLMF can reduce the level of HCV infection in neighbouring hepatoma cells, we wanted to determine at which stage in the virus lifecycle the aLMF are acting to reduce the level of viral infection. We first started by investigating whether or not this reduction in infection was due to an effect on HCV virus entry. For this approach, we used the HCV pseudoparticles (HCVpp) system to determine whether or not the aLMF can inhibit HCVpp entry into the Huh7.5 cells when in co-culture (Bartosch et al 2003a, Hsu et al 2003, Zhang et al 2004; Drummer et al 2003).

As before, Huh7.5 cells were first seeded in mono-culture at target cell number varying from a full well of target cells ($1.2x10^4$ cells per well in a 96 well tissue culture plate), then a half, a fourth and an eighth of the density of the full well. Cultures were allowed to adhere for 24 hours before infecting with the same virus inoculum for the indicated pseudoparticles; HCVpp strain H77, Vesicular Stomatitis virus pseudoparticles (VSVpp), and the negative control of no envelope pseudoparticles (NEpp). VSVpp were included as a control for HCVpp specificity. The infections proceeded for 48 hours before the cells were lysed for the detection of the luciferase reporter gene activity, which indicates the level of pseudoparticle entry. A 48 hour infection period was used instead of the standard 72 hours as this matches the infection time used in previous experiments with HCVcc (**detailed in section 3.4.1**). By matching the infection time, we sought to ensure that both experimental set ups are matched for infection duration and cell density throughout the assays. The luciferase signals from the negative NEpp control give the background

level of relative light units (RLU), which is then used to normalise the data for the pseudoparticle infections. The level of pseudoparticle virus entry for both the HCVpp and VSVpp, showed a decreasing entry in proportion with the number of target cells present (**Fig.3-5A**). This indicates that the target cell number of Huh7.5 cells present determines the level of HCV entry and is comparable to the results seen with the full length HCVcc virus in **section 3.4.1**.

Before co-culturing the hepatoma and stromal cells and infecting with HCVpp, monocultures were first infected with HCVpp and VSVpp (**Fig.3-5B.i**). Cultures were allowed to adhere for 24 hours before infecting for 48 hours with the same virus inoculum for the indicated pseudoparticles: HCVpp, VSVpp, and negative control NEpp. Huh7.5 cells served as a positive control and CHO cells as a negative control. The results for the mono-cultures indicate aLMF, LX-2, and CHO cells alone were non-permissive to HCV and VSV pseudoparticle viruses reaffirming our observations in **section 3.2** (**Fig.3-5B.i**).

Afterwards, Huh7.5 cells were seeded in co-culture with aLMF, LX-2 and CHO cells at a 1:1 ratio, allowed to adhere for 24 hours, and then infected for 48 hours (**Fig.3-5B.ii**). In co-culture with Huh7.5 cells, aLMF, LX-2 and CHO cells significantly reduced the level of HCVpp entry in the Huh7.5 cells (**Fig.3-5B.ii**). These results indicate that stromal cells can impact HCV infection at the entry stage. Interestingly, stromal cells also significantly reduced VSV (**Fig.3-5B.ii**) and the murine leukaemia virus (MLV) (data not shown)

pseudoparticle entry in Huh7.5 cells, which suggests stromal cells can inhibit multiple viral entry pathways, and not just HCV in hepatoma cells. The percentage inhibition of HCVpp and VSVpp entry and the details of the various aLMF donors tested are summarised in **Table 3-3**.

Based on the previous results indicating that stromal cells have the ability to modulate viral entry, we wanted to determine whether they are modulating the pseudoparticle's ability to enter the cell or the transcription of the luciferase reporter plasmid after it has entered the cell. To test this, the luciferase reporter plasmid, pNL4.3luc, was transfected into Huh7.5 cells alone. The transfected cells were trypsinised and reseeded in co-culture with the stromal cells at a 1:1 ratio as done previously; 24 hours post transfection. Then 24 hours after co-culture, the cells were then lysed to allow for the detection of the luciferase reporter gene activity. The luciferase readings for the transfected Huh7.5 cells in co-culture with three different aLMF donors, LX-2 and the control CHO cells were compared to transfected Huh7.5 cells alone to assess the effect of the stromal cells on pNL4.3luc transcription (**Fig.3-5C**). Important to realize, is that when the Huh7.5 cells are co-cultured with stromal cells there are 50% less Huh7.5 cells compared to the mono-culture. Therefore, the approximate 50% decrease in luciferase signals in all the co-culture compared to mono-culture condition is relative to the number of Huh7.5 cells. As a result, this indicates that there is no modulation of the luciferase reporter plasmid in co-culture and supports the hypothesis that co-culture with stromal cells modulate the pseudoparticle entry.

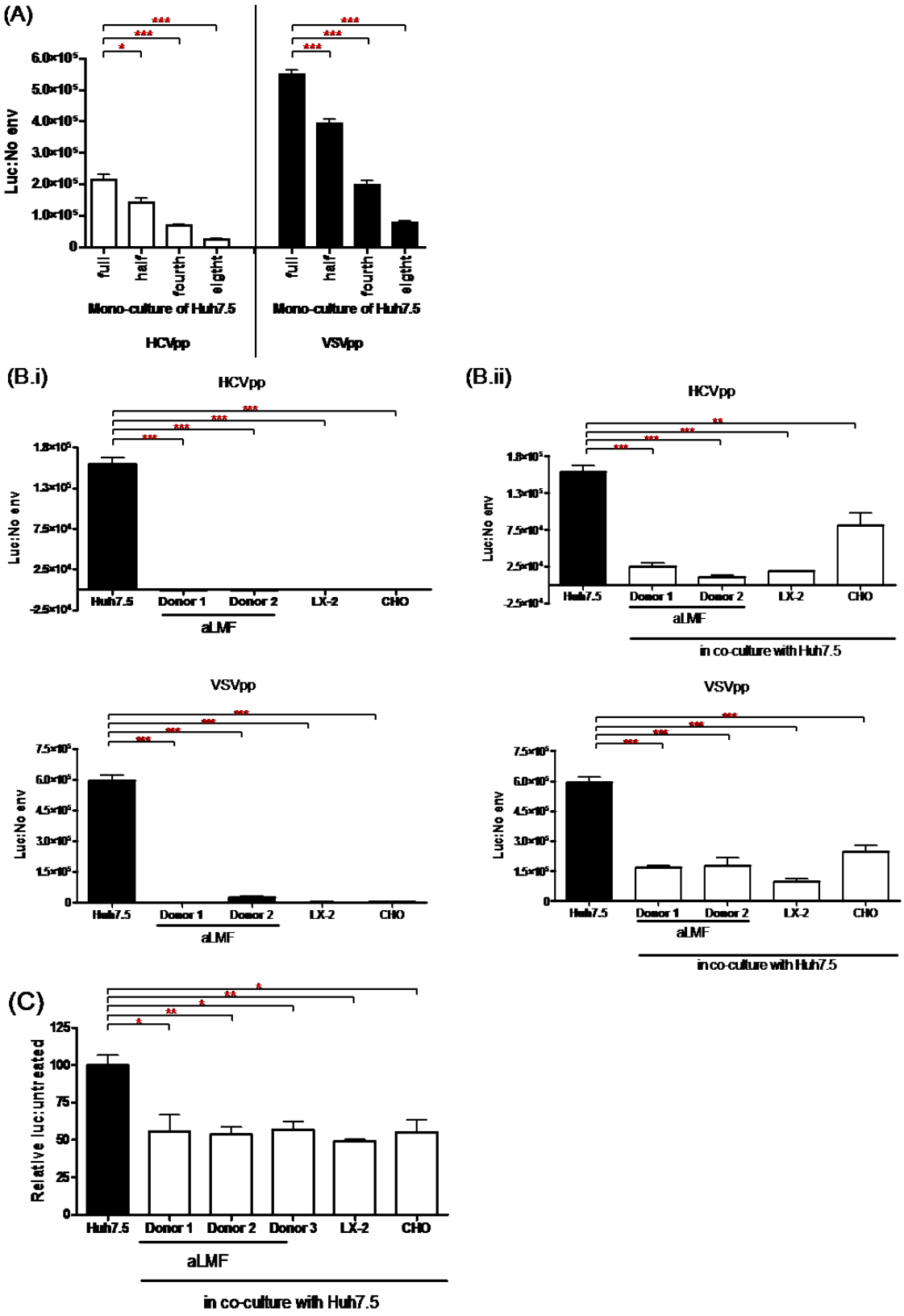



**Figure 3-5 aLMF can limit HCVpp entry in co-culture**

(**A-B**) Cells were seeded in a 48 well tissue culture plate, allowed to adhere for 24 hours, infected with HCVpp, VSVpp, and NEpp for 48 hours, then lysed,

and the luciferase activity measured. For each sample, data is expressed as RLU of luciferase activity normalised to the negative NEpp control (Luc:No env). (**A**) Huh7.5 cells were seeded at $2.5x10^4$ cells per well in a full well, then at a half, a fourth and an eighth of the number of cells in the full well. Data is representative of n=4 independent repeats. (**B.i**) aLMF, LX-2, and CHO cell were seeded in mono-culture and (**B.ii**) in co-culture with Huh7.5 cells at a 1:1 ratio. Data is representative of n=4 independent repeats (n=7 aLMF donors). (**C**) Huh7.5 cells were transfected with the luciferase reporter gene, pNL4.3luc, for 24 hours then seeded either in mono- or co-culture with aLMF, LX-2, and CHO cells at a 1:1 ratio. Cells were lysed and luciferase activity measured 24 hours after seeding. Data is expressed as RLU of pNL4.3luc luciferase activity normalised to the untransfected control Huh7.5 cells (Luc:untreated). Data representative of n=3 independent repeats (n=7 aLMF donors). Statistical comparison was made using one way ANOVA tests to compare groups of data and Bonferroni's corrections (parametric) were applied for pair wise comparisons of all data where * $P<0.05$, ** $P<0.01$ and *** $P<0.001$.

**Table 3-3 Pseudoparticle entry inhibition by aLMF in co-culture with Huh7.5 cells[a]**

| Disease | Passage | HCVpp viral inhibition (%) relative to | |
|---|---|---|---|
| | | Huh7.5 mono-culture | CHO co-culture |
| NASH | 4 | 82.1 | 69.8 |
| ALD | 3 | 84.0 | 74.0 |
| | 4 | 91.6 | 39.0 |
| Donor | 3 | 67.9 | 64.0 |
| | 4 | 89.3 | 42.0 |
| Seronegative | 3 | 92.0 | 48.7 |
| NAFLD | 5 | 87.6 | 73.8 |

[a]Table summarising data from n=3 independent co-culture experiments seeded at a 1:1 hepatoma:stromal cell ratio, infected with either HCVpp, VSVpp or the negative NEpp control 24 hours post seeding, cells were lysed and luciferase activity measured 48 hours post infection to determine pseudoparticle entry. Data is expressed as percentage viral inhibition relative to either the full Huh7.5 mono-culture (stromal cell co-culture infection calculated as a percentage relative to Huh7.5 mono-culture expressed as 100% infection) or the CHO control co-culture (stromal cell co-culture infection calculated as a percentage relative to Huh7.5+CHO co-culture expressed as 100% infection) from a total of n=7 aLMF donors at different passages and of different disease aetiologies.

### 3.4.3 aLMF can reduce HCV replication over time in co-culture

After observing that the aLMF reduce HCV entry in neighbouring hepatoma cells, we next sought to determine if they also modulate HCV replication. For this approach, we first decided to use a subgenomic replicon-luciferase cell line, Huh7A2HCV, in co-culture with aLMF. This cell line is a human hepatoma Huh7 cell line containing the replicating subgenomic JFH1 HCV strain encoding luciferase. The cell line is under selection and allows for easy and accurate quantification of HCV RNA replication over time (Jo et al 2009).

Huh7A2HCV replicon cells were seeded in mono- and co- culture at a 1:1 ratio with aLMF, LX-2 and CHO cells as previously done so. Huh7.5 cells were also included in co-culture to control for density because density is critical in maintaining optimal luciferase plasmid activity within the Huh7A2HCV replicon cells. It has previously been observed that at higher densities there is a loss of luciferase activity. Cells were lysed 24 and 48 hours post seeding to determine changes in HCV RNA replication over time. The luciferase activity was measured and data expressed as relative light units (RLU) of the subgenomic-luciferase reporter activity (**Fig.3-6**).

At 24 hours post seeding, the cell density control, Huh7.5 cells, in co-culture had slightly lower replication levels (39.5%+/-2.5) than expected (50%) when compared to the Huh7A2HCV mono-culture. Given that there is 50% less Huh7A2HCV in the co-culture compared to the mono-culture control, we would have expected approximately 50% of the replication. This suggests there is a low level reduction in replication when the Huh7A2HCV cells are in

contact with Huh7.5 cells and possibly other cell types. Nevertheless, the co-cultures with Huh7A2HCV cells appeared to reduce the level of replication in the Huh7A2HCV cells further than the co-culture of stromal cells with the Huh7.5 cells, with levels at 24.1%+/-2.9 for the aLMF and 23.7%+/-4.8 for the LX-2 in co-culture with Huh7A2HCV cells. Both of these reductions are lower than the level of replication in the Huh7.5 or CHO containing control co-cultures (35.7+/-13.3) (24 hour time point **Fig.3-6**). The percentages of replication in co-culture conditions for each time point were calculated relative to the Huh7A2HCV cell mono-cultures for the appropriate time point. In some conditions, high standard deviations were observed which may be as a result of the high sensitivity in detection for the luciferase based system.

At 48 hours post seeding, the level of HCV RNA replication in the Huh7A2HCV mono-culture and Huh7.5 co-culture increased compared to 24 hours, which indicates successful HCV replication over time. The control CHO cells slightly reduce the level of replication to 66.9%+/-21.9 of the Huh7A2HCV mono-culture. The stromal cell containing co-cultures significantly reduce the levels of replication in the Huh7A2HCV cells, when compared to the Huh7.5 containing co-culture with aLMF containing co-cultures at 20.5%+/-5.7 and LX-2 at 26.1%+/-14.0 (48 hour time point **Fig.3-6**). Interestingly, the levels of replication detected in both the aLMF and LX-2 containing co-cultures at 48 hours are comparable to the levels at 24 hours. This suggests that little replication has occurred in the Huh7A2HCV cells over time as a result of co-culturing with stromal cells which leads us to hypothesize that the stromal cells are also inhibiting HCV replication over time.

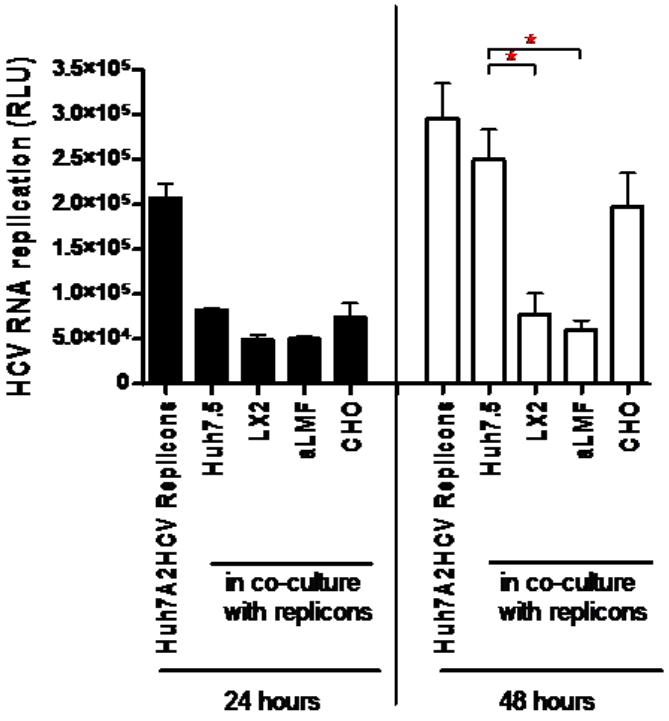



**Figure 3-6 aLMF can reduce HCV replication over time in co-culture**

Huh7A2HCV cells were seeded in either mono- or co-culture with Huh7.5, aLMF, LX-2, or CHO cells at a 1:1 ratio. Cultures were lysed and luciferase activity measured at 24 and 48 hours post-seeding. Data is expressed as HCV RNA replication (RLU) and is representative of n=2 independent repeats (n=2 aLMF donors). Statistical comparison was made using one way ANOVA tests and Bonferroni's corrections (parametric) were applied where * $P<0.05$.

### 3.4.4 Co-culture of aLMF with Huh7.5 cells inhibits HCVcc spread at 48 hours

While counting the level of HCVcc infection as foci per well in the previous co-culture assays, it was observed that the foci size could vary from foci containing as little as 1-6 infected cells to foci greater than 25 infected cells. These observations lead us to conclude that counting HCVcc foci was a subjective way to count the level of HCVcc infection and that there were clearly differences in HCV spread. These differences in HCVcc foci size are visible in the representative immunofluorescence images of HCV NS5A positive stained Huh7.5 cells in mono- and co-culture conditions, with very small foci detected in the co-culture conditions compared to the Huh7.5 mono-cultures (**Fig.3-7A**). In co-culture conditions, we observed very small foci compared to the Huh7.5 mono-culture. The number of individually infected cells per foci was counted to determine the changes in HCV spread over time in Huh7.5 mono-culture (**Fig.3-7B**). There was a significant increase in the number of infected cells per foci for the Huh7.5 mono-culture from 24 to 48 hours. This indicates that the virus has established in the initial infected cells by 24 hours, and then by 48 hours it has successfully replicated and spread to neighbouring Huh7.5 cells, thus increasing the number of HCVcc infected cells per foci. However, part of this increased infection is also due to cell division occurring between the 24 to 48 hour time points.

When the aLMF, LX-2 and CHO cells were co-cultured with Huh7.5 cells at a 1:1 ratio, the number of HCVcc infected cells per foci after 48 hours was moderately decreased in the aLMF co-culture, but significantly decreased in

the LX-2 co-culture compared to the Huh7.5 mono-culture (**Fig.3-7C**). The control CHO cells also appear to inhibit HCV spread however to a lesser extent than the stromal cell containing conditions. These data suggest the presence of stromal cells in co-culture inhibit HCVcc spread in the Huh7.5 cells.

In order to bypass the entry step and only measure replication post viral entry, Huh7.5 cells were transfected with either the RNA of the complete HCV virus (J6 strain) containing a Gaussia luciferase reporter gene (HCVcc-gLuc) or the RNA of the polymerase defective control HCV virus (HCVcc-GNN). The HCVcc-GNN control is unable to replicate and thus unable to produce Gaussia luciferase as it encodes two stop codons in the HCV RNA dependent RNA polymerase (NS5B) region which results in a catalytically inactive RNA polymerase (Koutsoudakis et al., 2012, Phan et al., 2011). This system allows *de novo* full length HCVcc virus particles to be detected by sampling the supernatants periodically over time to detect the levels of Gaussia luciferase following successful HCV replication and thus measure replication in real time without having to set up cultures at each time point. Supernatants were sampled 4 hours post transfection in order to first determine whether or not the transfection was successful by reading the luciferase activity of *de novo* virus released into the supernatant following replication (**Fig.3-7D.i**). The transfected cells were then trypsinised and re-seeded into co-culture with aLMF, LX-2 or CHO cells from which point on, supernatants were sampled every 24 hours in order to measure HCV replication in real time, and expressed as HCVcc-gLuc luciferase activity over HCVcc-GNN luciferase

activity. The results indicate that the aLMF and LX-2 cells can strongly inhibit HCVcc-gLuc replication when in co-culture over the course of 96 hours.

In addition, the mono- and co- culture conditions were treated with mouse IgG control (**Fig.3-7D.i**), and parallel cultures were treated with anti-CD81 antibody (**Fig.3-7D.ii**) in order to inhibit cell free infection and cell-cell transmission. These results indicate the level of HCVcc-gLuc virus in Huh7.5 mono-culture conditions can be significantly inhibited in the presence of the anti-CD81 antibody; however there is very little difference in the level of infection in co-culture conditions with or without the anti-CD81 antibody. This data suggests the aLMF and LX-2 cells may be able to inhibit HCV spread in a similar manner to the anti-CD81 antibody via cell free and cell-cell transmission however further work would be required to confirm this and establish which mode of transmission is impacted when Huh7.5 cells are in co-culture with stromal cells.

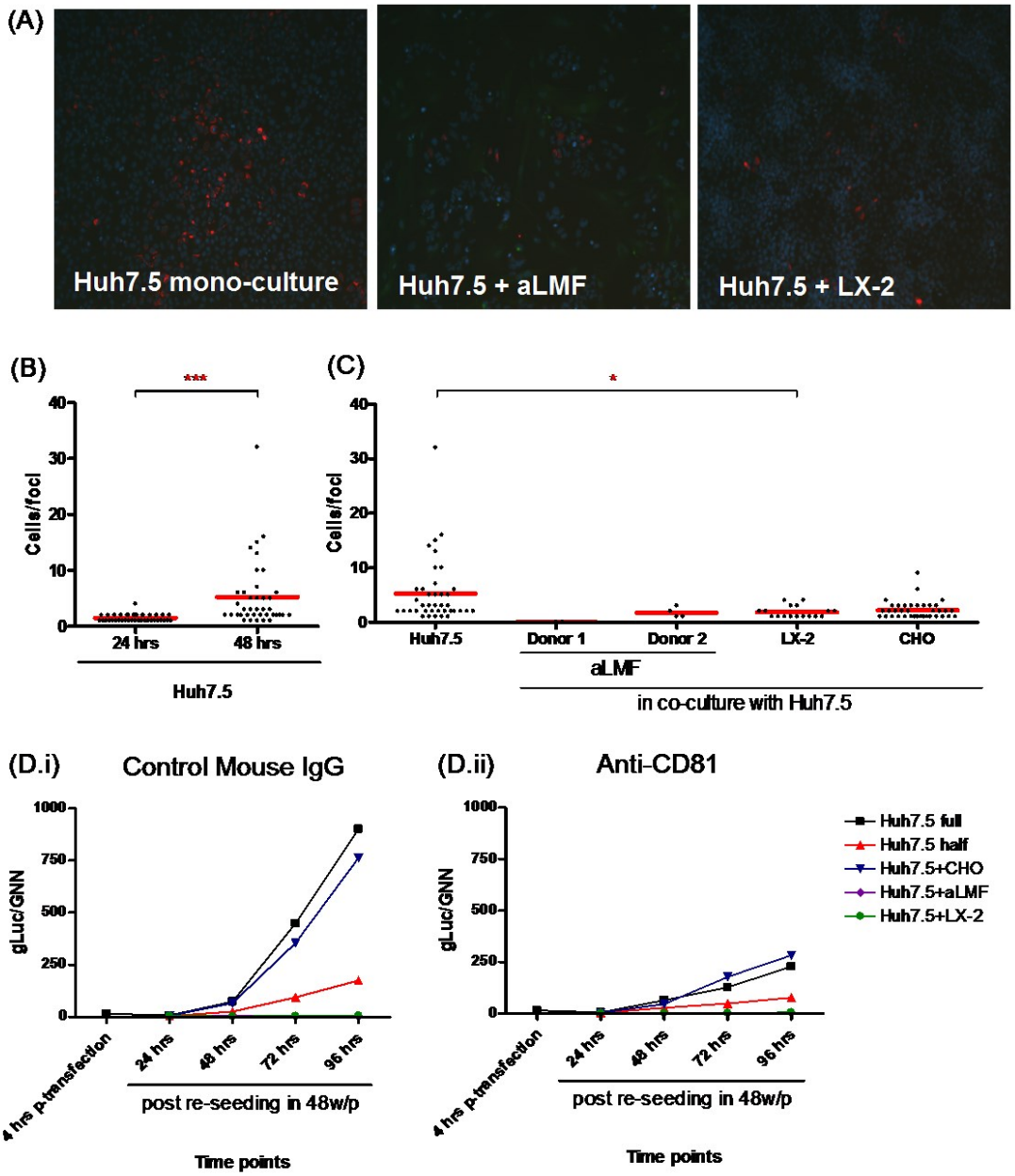



**Figure 3-7 Co-culture of aLMF with Huh7.5 cells inhibits HCVcc spread at 48 hours**

(**A**) Microscopy images 48 hours post infection (magnification x10). HCVcc foci in Huh7.5 cells were visualised using anti-NS5A antibody detected by a secondary antibody conjugated to Alexa594 (red) and nucleus detected by DAPI (blue). (**B-C**) Cells were fixed, stained for NS5A positive cells and the number of NS5A positive cells per foci of infection counted. (**B**) Huh7.5 cells were seeded in mono-culture. 24 hours post seeding, they were infected with HCVcc and the infection proceeded for 24 and 48 hours. (**C**) Huh7.5 cells were seeded in either mono- or co-culture with aLMF, LX-2, or CHO cells at a 1:1 ratio. Then 24 hours post seeding, they were infected with HCVcc and the

infection proceeded for 48 hours. Data is representative of n=4 independent repeats (n=6 aLMF donors). Statistical comparison was made using the Mann-U-Whitney test where * P<0.05 and *** P<0.001. (**D**) Huh7.5 cells were transfected with either HCVcc-gLuc or HCVcc-GNN. After measuring transfection success at 4 hours post transfection, cells were reseeded into mono- and co-culture conditions with aLMF, LX-2, or CHO cells at a 1:1 ratio. Cultures were treated with control mouse IgG antibody (**D.i**) or treated with an anti-CD81 antibody at 5µg/mL (**D.ii**) for the duration of the infection. Data is expressed as the luciferase activity measured from the HCVcc-gLuc transfected cells over the HCVcc-GNN transfected cells. Data representative of n=3 independent repeats (n=3 aLMF donors).

### 3.8 Discussion

In this chapter, we first characterized primary aLMF and established they are non-permissive to HCV infection, using both the HCVcc and pseudoparticle systems to confirm that the stromal cells tested do not support HCV infection or entry. Primary human HSCs have been reported to express the HCV receptor CD81 and the attachment factor, low density lipoprotein receptor (LDL-r) by mRNA expression (Bataller et al 2014). So we demonstrated that aLMFs isolated from patients express CD81 however they do not express the other key HCV receptors, Claudin-1, SRB1, and Occludin as confirmed by Western blot analysis and immunofluorescence. Interestingly, the LX-2 cell line expresses all receptors except Claudin-1, which illustrates there are differences between the two stromal cell types tested in this thesis. Nonetheless, aLMF and LX-2 cells are non-permissive to HCV infection as they do not express the full set of HCV receptors required for successful entry of the viral particles.

We then wanted to design a co-culture system to study if stromal cells, in particular fibroblasts could impact the HCV lifecycle in hepatocytes, the cells that can support HCV infection. When establishing this co-culture model to study the role of fibroblasts on hepatocellular HCV infectivity, there were many factors to take into consideration and so the co-culture model under went a series of optimisation steps. The primary aLMF cells gave us a great opportunity to study how these primary human liver derived cells may impact HCV infection in the liver *in vivo* however working with primary cells poses a number of challenges in itself with care needed to be taken during the

isolation process to avoid contamination of the aLMF cell cultures with other liver cell types, as well as monitoring the phenotype of the cells over time to ensure they still maintained aLMF function and differentiation. Some of the major issues faced whilst working with aLMF cells were the limited availability and the slow proliferation rate, often requiring time to expand the aLMF cells to ensure there were enough cells to perform the required assays. The delicate nature of the aLMF cells also lead to a very small proportion of the cell death occurring upon each trypsination of these cells, with an increased level of aLMF cell death observed as the number of aLMF in co-culture was reduced lower than a 2:1 ratio (hepatoma:fibroblast) in order to more accurately represent the proportion of aLMF *in vivo*. This increased level of cell death could be due to a number of factors such as the cultured aLMF cells may require a certain level of homotypic cell-cell contact in order for successful adherence to occur. Under normal propagation of aLMF, there are soluble secreted factors produced by the aLMF cells which help these cells to proliferate and maintain phenotype in culture. So another possible explanation could be that the lower levels of soluble secreted factors as a result of fewer aLMF cells present could be leading to increased aLMF cell death. Despite the 1:1 ratio not accurately representing the hepatocyte:stellate cell ratio found in a normal healthy liver which is approximately 11:1, this ratio could be used to mimic the level of fibroblasts present in a highly fibrotic liver (Pertoft and Smedsrod, 1987, Taub, 2004b, Ishibashi et al., 2009). However the major advantage of a 1:1 ratio is that not only does it help ensure sufficient cell-cell contacts between the two cells

types are established, but it also helps maintain the aLMF cells in culture as well as making the interpretation of the data easier.

One of the other issues with using aLMF cells is that they represent the activated liver myofibroblasts found in the liver during fibrosis, and not the quiescent stellate cells typically found in a healthy liver prior to liver injury, for example by viral infection. Unfortunately this is an artefact of culturing these primary cells. When primary HSCs are isolated from the liver and cultured on normal plastic tissue culture surfaces, molecular signals are triggered, similar to those during activation in response to liver injury, leading to their gradual activation into liver myofibroblasts or aLMF. Once the cultured HSCs are activated, they undergo a number of phenotypic and functional differences associated with their role in fibrosis including an increased cellular contractility, increased cell mobility, elongated cell morphology, increased expression and deposition of ECM including collagens. Once activated, HSCs also lose the stored vitamin A, down-regulate a number of neural markers and up-regulate mesenchymal markers such as aSMA and fibronectin, therefore it is not surprisingly that the LX-2 cells and aLMF cells demonstrated differential expression of HCV receptors in **section 3.3** (Herrmann et al 2007; Taub 2004; Iredale et al 2007; Geerts et al 2001; Kisseleva et al 2008).

In terms of developing a system which mimics the role of fibroblasts in the liver microenvironment and HCV infection, the use of primary fibroblasts would be physiologically more relevant compared to immortalized cell lines, which as mentioned often display altered phenotypes and differentiation gene

expression compared to primary cells (Herrmann et al 2007). However, if we wanted to understand virus-host mechanisms occurring in the earlier stages of infection in healthy livers which could trigger liver disease progression, then aLMF may not be suitable as they already display a fairly activated phenotype. This is of course assuming that when patients are initially infected with HCV, their livers are healthy with no signs of mild or acute fibrosis from other underlying health problems such as alcoholic liver disease or unhealthy diets leading to fatty liver related diseases. This is very difficult to determine as patients often do not know when they acquired the virus as the infection caused by HCV is often asymptomatic with symptoms of liver end stage liver disease being the only indication after the virus has established infection for a considerable number of years (Lindenbach et al., 2005b, Blight et al., 2003a, Farquhar and McKeating, 2008, Lemon et al., 2010, Meredith et al., 2012b). However, understanding the role of aLMF on HCV infection would prove very valuable when trying to treat patients with HCV as therapies are given to patients with varying levels of fibrosis during which time there is an increased number of aLMF in the liver. Therefore, to address these issues and the aims of this study, both the LX-2 cell line and primary aLMF were used in parallel representing the quiescent HSC and liver myofibroblasts, respectively. The LX-2 cell line was characterised by Xu and colleagues in 2005 and has since been described as the stellate cell line which is the most similar to primary HSC, hence is a commonly used in studies investigating liver fibrosis (Xu et al 2005; Herrmann et al 2007). Despite the potential issues surrounding the pre-activated stated of the aLMF prior to infection in our system, we were keen to still investigate these cells as they represent what we are faced with clinically,

which is HCV patients with chronic fibrosis. By focusing slightly more on the role of aLMF in HCV infection and disease we hoped to increase over understanding of the mechanism(s) of host-viral interactions, potentially leading to new pathways which can be targeted for novel and improved therapies.

Generally aLMF cells grow significantly slower than the Huh7.5 hepatoma cell line used in these co-culture assays and so there is a certain degree of competition between the two cells type for space and nutrients when cultured together, with the more rapidly dividing Huh7.5 having more space when there are fewer aLMF present. For consistency across the entry, replication and spread assays, cultures were generally infected for 48 hours and maintained in DMEM containing 10% FBS for the duration of the assay. Normally for the duration of HCV infections cells are grown the DMEM containing 3% FBS in order to control the growth rate of the cells which avoid over confluent mono-layers, but I found that reducing the FBS content to such a low level compared to the DMEM containing 16% FBS plus 33% conditioned media used to propagate fibroblasts affected their adherence and growth which overall impacted the experiment negatively. These factors may contribute to fewer aLMF settling when lower numbers are seeded. This difference in proliferation rates was taken into consideration when optimising this co-culture system and attempts were made to arrest cell growth of all the cell types in order to help maintain the initial ratio throughout the assay and help with interpreting the impact of infection under arrested cell growth. Two methods were used to arrest cell growth, gamma irradiation of cells in suspension

before seeding and mitomycin C treatment post seeding however both methods were unsuccessful for a number of reasons including cell growth was not completely arrested for some of the cell types including the aLMF and LX-2 cells, but also there was a high level of aLMF cell death and so given the limited availability of these cells and wanting to maintain both their function and phenotype it was decided to continue co-culturing without arresting cell growth. HCV entry is dependant on the target cell density due to the increased level of claudin-1 and SRB1 expression at the points of cell-cell contact. Thus cultures were seeded at a slightly higher seeding density than normal (at $2.5x10^4$ cells per well in a 48 well tissue culture plate) adjusted according to the surface area of different sized tissue culture plates as this higher density would lead to contact inhibition cell growth thus assisting in controlling the cell growth of these different cell types in culture.

Having established the co-culture system, we assessed the impact of co-culturing aLMF cells with the highly permissive Huh7.5 cells, on various stages of the HCV lifecycle. The data demonstrated that in co-culture, the aLMF can limit HCV infection at multiple stages of infection; entry, replication and spread. Interestingly, we saw no difference in the level of inhibition at any stage of infection when testing aLMF isolated from various different end stage liver disease aetiologies or the passage at which the fibroblasts were used. Also, the level of aLMF mediated inhibition was comparable to the LX-2 cells, which probably became activated in response to the virus infection leading to similar wound healing mechanisms as the already activated primary aLMF. When enumerating the level of HCVcc inhibition using immunofluorescence

microscopy, I noticed the variability in the size of the focus forming units (FFUs) typically counted when assessing HCVcc infection. Further microscopy analysis highlighted the subjectiveness of counting FFUs to assess the overall level of HCV infectivity in co-culture. I noticed the Huh7.5 mono-cultures produced a greater number of large foci compared to the co-cultures which produced a greater number of smaller foci. As a result, the number of foci does not accurately reflect the infectivity as a foci made up of 10-15 cells can not be compared to a foci of 2-3 cells. Consequently, I counted the total number of individually infected cells when assessing overall levels of HCV infection, and counted the number of cells per foci when assessing the level of HCVcc spread.

Having demonstrated that stromal cells can impact HCV infection of hepatocytes at various stages of the viral lifecycle, we wanted to investigate the anti-viral mechanism but wanted to first investigate the role of cytokines and other soluble factors in our system. During HCV infection, a number of profibrogenic factors, including transforming growth factor (TGF-β) are released by HCV infected hepatocytes. These secreted factors act on fibroblasts modulating the expression of several genes associated with fibrosis thus initiating the wound healing and innate immune response which could be limiting HCV infection (Schulze-Krebs et al 2005; Wang et al 2013; Friedman et al 2007; Su et al 2014). The fibroblasts could also be responding directly to interaction with the virus. Some recent studies have artificially stimulated LX-2 cells and shown anti-viral properties of the conditioned media collected post stimulation on Huh7 cells infected with HCV (Wang et al.,

2013b, Wang et al., 2013c). Whilst other studies have shown HCV or recombinant HCV proteins can act via a range of mechanisms which could potentially contribute towards the anti-viral activity observed in our co-culture system. Some of these mechanisms include the induction of pro-inflammatory cytokines in HSC, stimulation of intracellular signalling pathways in HSC, or the activation of HSC leading to fibrogenic action (Wang et al 2013; Friedman 2008; Mazzocca 2005; Coenen et al 2011).

Overall, there could be multiple pathways or mechanism by which the stromal cells can inhibit HCV infection in neighbouring hepatocytes. In summary, I have shown aLMF have the ability to significantly limit the level of HCV entry, replication and spreading infection in neighbouring hepatoma cells when in seeded together in co-culture.

# Chapter 4 Pathways and mechanisms not contributing to the anti-viral activity of aLMF in co-culture

## 4.0 Introduction

We have determined that aLMF limit hepatocellular HCV infection at multiple stages in the virus lifecycle: entry, replication, and spread (Chapter 3). In order to better characterize HCV and host cell interactions, we sought to define the mechanism leading to aLMF facilitated anti-viral activity. A complex crosstalk of cell membrane interactions and signalling pathways exist between hepatocytes and stromal cells, which are responsible for creating the liver microenvironment (Taub, 2004b, Gomez-Aristizabal et al., 2009). Therefore, we hypothesized that there would be crosstalk between the hepatoma and aLMF upon co-culturing and further crosstalk in response to viral infection. For this approach, we first established if the anti-viral mechanism required cell-cell contact or soluble secreted factors. Next, we wanted to investigate which signalling pathways were activated during the hepatoma innate immune response when aLMF were present. To determine if the aLMF could be modulating the immune response in neighbouring hepatoma cells, HCV infected Huh7.5 cell mono-cultures were compared to aLMF containing co-cultures.

Given the lack of small animal models to study HCV infection in the liver, our co-culture system could provide a physiologically relevant cell culture model for studying HCV lifecycle. Thus, it was important to determine the physiological relevance of our model by testing the aLMF limitation of

hepatocellular HCV infection in different hepatoma cells, particularly primary human hepatocytes (PHH), and under low oxygen levels that mimic the liver environment *in vivo*. By using this co-culture system to understand the mechanism by which aLMF limits HCV infection, the knowledge we obtain could provide new therapeutic avenues for treating viral infection and the underlying inflammatory response in chronic hepatitis.

### 4.1 Stromal cell anti-viral activity is mediated primarily via a cell contact dependant mechanism

To begin characterizing HCV and host cell interactions that lead to aLMF facilitated anti-viral activity, we first established if the anti-viral mechanism required cell-cell contact or soluble secreted factors. For this approach, transwell inserts (0.4μm pore size) were used to physically separate the stromal and Huh7.5 cells but still allow the cells to exchange soluble secreted factors. The different cell types were seeded at a 1:1 ratio and allowed to settle separately for 2 hours before the inserts were added to the wells. Then 24 hours post adding the inserts to the wells, the co-culture conditions were infected with HCVcc (**Fig.4-1A**). Infecting with HCVcc allowed us to investigate the effect of cell separation on the complete viral lifecycle. CHO cells were included as a control for cell numbers and to act as a non-stromal cell control. When aLMF, LX-2, and CHO cells were in co-culture but separated from hepatoma cells using transwells there was minimal reduction in HCVcc infection compared to the Huh7.5 mono-culture control which suggests stromal cells mediate their effects mainly via cell contacts.

In order to investigate the effects of cell separation on viral entry alone, pseudoparticles HCVpp (**Fig.4-1B.i**), VSVpp (**Fig.4-1B.ii**), and the negative control NEpp were used to infect the same transwell system and were then compared to co-culture conditions where the cells were in contact and infected with that same pseudoparticle viral inoculums. The HCVpp and VSVpp data both show a decrease of approximately 50% infectivity in all transwell co-culture conditions compared to a decrease of approximately 80%

in all contact co-culture conditions. This indicates that although some inhibition of HCV and VSV entry can be detected in transwell, cell contact is required in order to see a significant reduction in viral entry.

To confirm that aLMF facilitated anti-viral activity required cell-cell contact, virus free conditioned media clarified (**section 2.3.3**) and frozen from previous Huh7.5 co-culture assays was used to further investigate the role of soluble secreted factors on HCV infectivity. Mono- and co- culture conditioned media was collected to establish whether or not cell contact in co-culture versus mono-culture produces soluble secreted factors that have anti-viral activity. Huh7.5 mono-cultures were treated with a 1:1 mix of conditioned and fresh media on the day of seeding. Cultures were infected 24 hours after seeding with HCVcc, and the infection proceeded for 48 hours in the presence of the 1:1 conditioned:fresh media mix. The level of HCV infection was quantified by counting NS5A positive foci per well in order to determine the effects of mono- or co- culture conditioned media on full length HCV infection (**Fig.4-1C**). A Huh7.5 mono-culture treated with fresh media was used as a control. The difference in the level of HCVcc infection when Huh7.5 cell were treated with conditioned media was not significant when compared to the normal media control. This indicates that the conditioned media from both mono- and co-culture conditions does not impact overall HCV infectivity.

To compare to the observations seen with HCVcc, we next investigated the effects of conditioned media on viral entry alone by using pseudoparticles. Huh7.5 cells treated with 1:1 conditioned:fresh media mix for 24 hours were

then infected with HCVpp, VSVpp, or the negative control NEpp for 48 hours (**Fig.4-1D**). Again the difference in the level of HCVpp or VSVpp infection when Huh7.5 cells were treated with conditioned media compared to the fresh media control was not significant. However, there was one exception where aLMF donor 1 mono-culture conditioned media showed a significant decrease in the level of VSVpp entry (**Fig.4-1D**, right panel; *$p<0.05$). Together with the transwell data, the modest decreases in the number of HCVcc foci and HCVpp entry levels when treated with conditioned media indicates a minimal role for soluble secreted factors on HCV infectivity. Therefore, we hypothesize that the mechanism by which the aLMF reduce HCV infectivity is a cell contact dependant mechanism.

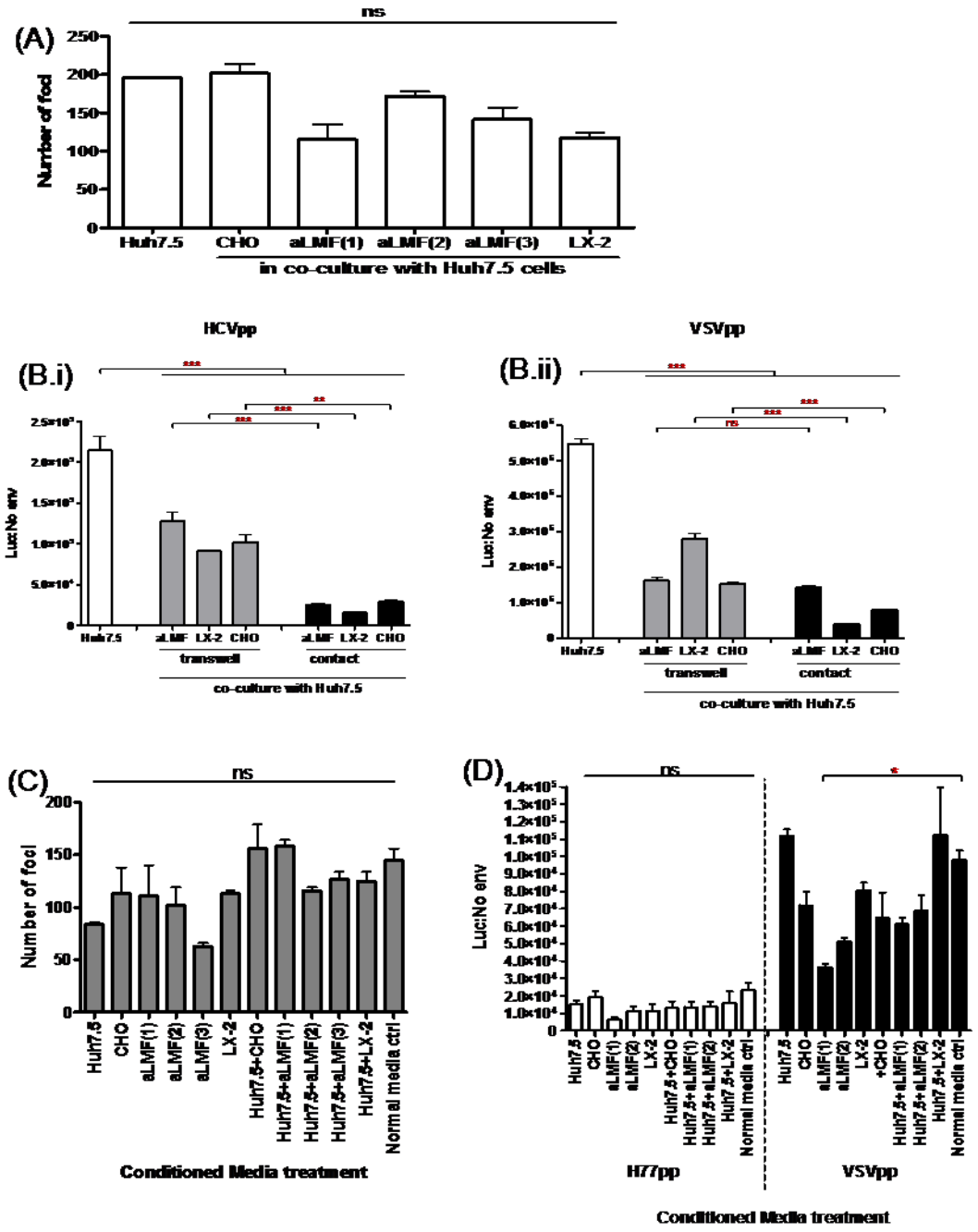



**Figure 4-1 Stromal cells mediate their anti-viral effect primarily via cell contact**

(**A-B**) Stromal cells were seeded in the transwell inserts and Huh7.5 cells seeded in the tissue culture plates. After adhering, the inserts were added to the tissue culture plate. After 24 hours, the cells were infected with (**A**) HCVcc virus or (**B.i**) HCVpp or (**B.ii**) VSVpp for 48 hours. (**A**) HCVcc infected Huh7.5 cells were fixed, stained, and counted for NS5A positive foci. Data representative of n=2 independent repeats (n=4 aLMF donors). Statistical comparison was made using the Kruskal-Wallis test and Dunn's corrections were applied. (**B**) Cells infected with pseudoparticles were lysed and luciferase activity measured. Data is expressed as RLU of HCVpp (left panel)

or VSVpp (right panel) luciferase activity normalised to the no envelope control by subtracting the no envelope signals from the pseudoparticle virus signals (Luc:No env). Data representative of n=2 independent repeats (n=4 aLMF donors). Statistical comparison was made using one-way ANOVA tests and Bonferroni's corrections (parametric) were applied for pair wise comparisons of all data. (**C-D**) Huh7.5 mono-cultures were treated with 1:1 conditioned:fresh media. The source of the conditioned media is as indicated. After 24 hours, they were infected with (**C**) HCVcc or (**D**) HCVpp, VSVpp, or NEpp for 48 hours in the presence of the 1:1 conditioned:fresh media. (**C**) HCVcc infected Huh7.5 cells were fixed, stained, and counted for NS5A positive foci. Data representative of n=4 independent repeats and aLMF conditioned media n=8 in both mono- and co-culture. Statistical comparison was made using the Kruskal-Wallis test and Dunn's corrections were applied. (**D**) Cells infected with pseudoparticles were lysed and luciferase activity measured. Data is expressed as RLU of HCVpp (left panel) or VSVpp (right panel) luciferase activity normalised to the no envelope control by subtracting the no envelope signals from the pseudoparticle virus signals (Luc:No env). Data representative of n=3 independent repeats and aLMF conditioned media n=6 in both mono- and co-culture. Statistical comparison (B-D) was made using One-way ANOVA tests with Bonferroni's corrections to compare the treatment groups of data and Bonferroni's corrections (parametric) were applied for pair wise comparisons of all data. * $P<0.05$, **$P<0.01$ and *** $P<0.001$. All error bars show SD.

## 4.2 Stromal cell anti-viral activity is also mediated via a cell contact dependant mechanism in cytokine sensitive hepatomas

Huh7.5 cells are highly permissive to HCV infection when compared to other hepatoma cell lines commonly used in the study of HCV infection. This high permissivity is due to a defective RIG-1 pathway in Huh7.5 cells. Huh7.5 cells have also been shown by our group to be less responsive to cytokines compared to other hepatoma cells lines, such as Huh7 and HepG2 cells (unpublished data; Wang et al., 2013). Therefore, we decided to also test the role of soluble secreted factors in aLMF facilitated anti-viral activity on a more sensitive hepatoma cell line. We decided to use Huh7 as this cell line still provides a level of permissivity that will allow a countable level of HCVcc infection instead of HepG2 cells which would potentially produce too low and uncountable levels of HCVcc infection.

As previously detailed in **section 4.1**, Huh7 mono-cultures were treated with a 1:1 mix of conditioned and fresh media on the day of seeding. The conditioned media was collected from previous Huh7.5 containing co-culture assays, from both the mono- and co- culture conditioned, spin clarified and stored as described in **section 2.3.3**. Then 24 hours after seeding and adding the conditioned media, the cultures were infected with HCVcc for a 48 hour infection in the presence of the 1:1 conditioned:fresh media mix. The level of HCVcc infection was quantified by counting NS5A positive foci per well in order to determine the effects of mono- and co- culture conditioned media on full length HCV infection (**Fig.4-2A**). As a control, Huh7 mono-cultures were also treated with fresh media only. The difference in the level of

HCVcc infection when Huh7 cells were treated with conditioned media was not significant when compared to the normal media control. This indicates that the conditioned media collected from the previous Huh7.5 assays, mono- and co- culture conditions, does not impact overall HCV infectivity even in cytokine sensitive hepatoma cells.

We next investigated the effects of the conditioned media collected from previous Huh7.5 assays on the entry of viruses into the cytokine sensitive Huh7 hepatoma cell line using pseudoparticles. Huh7 cells were treated with 1:1 mix of conditioned:fresh media mix on the day of seeding and 24 hours after, were infected with HCVpp, VSVpp, or the negative control NEpp for a 48 hour infection in the presence of the 1:1 mix of conditioned:fresh media mix (**Fig.4-2B**). Again the difference in the level of HCVpp or VSVpp infection when Huh7 cells were treated with conditioned media compared to the fresh media control was not significant and consistent with the HCVcc infection data (**Fig.4-2A**). Overall these data indicate that any soluble secreted factors found in conditioned media do not impact HCV infection. Therefore, we conclude that the mechanism controlling aLMF facilitated anti-viral activity requires cell-cell contact.

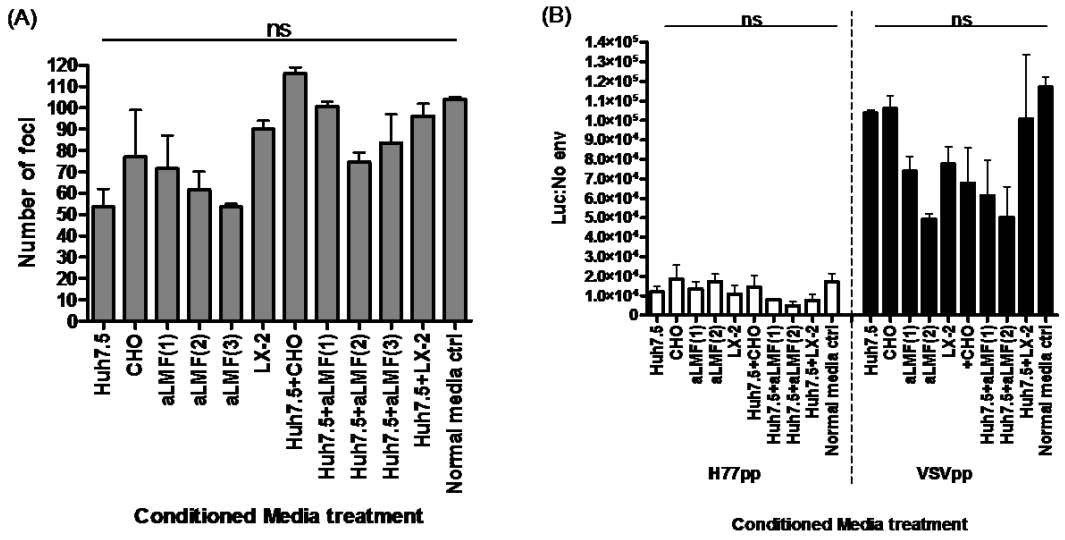



**Figure 4-2 Testing conditioned media using cytokine sensitive hepatoma cells**

(**A-B**) Huh7 mono-cultures were treated with 1:1 conditioned:fresh media. The source of the conditioned media is as indicated. After 24 hours, they were infected with (**A**) HCVcc or (**B**) HCVpp, VSVpp, or the negative control NEpp for 48 hours in the presence of the 1:1 conditioned:fresh media. (**A**) HCVcc infected Huh7 cells were fixed, stained, and counted for NS5A positive foci. Data representative of n=3 independent repeats and aLMF conditioned media n=6 in both mono- and co-culture. Statistical comparison was made using the Kruskal-Wallis test and Dunn's corrections were applied. (**B**) Cells infected with pseudoparticles were lysed and luciferase activity measured. Data is expressed as RLU of HCVpp (left panel) or VSVpp (right panel) luciferase activity normalised to the no envelope control by subtracting the no envelope signals from the pseudoparticle virus signals (Luc:No env). Data representative of n=3 and aLMF conditioned media n=6 in mono- and co-culture. Statistical comparison was made using one-way ANOVA tests to compare the treatment groups of data and Bonferroni's corrections (parametric) were applied for pair wise comparisons of all data. All error bars show SD.

### 4.3 Stromal cell anti-viral activity is not mediated through an interferon dependent pathway

Production of interferons is a key defence against viral infection as part of the innate immune response. After the detection of viruses, the signalling pathway leads to the phosphorylation of IRF3 and subsequent translocation into the nucleus where it initiates the production of interferons (IFNs) and numerous interferon stimulated genes (ISGs), including *ISG56* (!!! INVALID CITATION !!!, Horner and Gale, 2009, Marukian et al., 2011). We wanted to establish whether or not the interferon signalling pathway played a role in stromal cell anti-viral activity. For this approach, we decided to use the *ISG56* luciferase reporter to measure the level of IFN activation in hepatocytes when in co-culture with stromal cells compared to mono-culture.

The *ISG56* luciferase reporter allows the detection of interferon pathway activation by a luciferase assay. We have confirmed that the *ISG56* luciferase reporter is sensitive enough to detect IFNα, IFNβ, IFNλ1, and IFNλ2 as shown by the dose response graphs (**Fig.4-3A**). Exogenous Type I IFN (IFNα and IFNβ) doses were based on previous concentrations used by our group and others, which have shown maximal inhibition of HCV replication at concentrations greater then or equal to 50 U/ml (Meredith et al., 2014, Marcello et al., 2006, Macejak et al., 2001). Type III IFN (IFNλ1 and IFNλ2) doses were based on concentrations shown by other research groups to have maximal inhibition on HCV genotype 2a replication at 10 ng/ml (Marcello et al., 2006) and ranges between 0.2-0.5 ng/ml showing 50% inhibition of HCV replication (Park et al., 2012, Pagliaccetti et al., 2008). Time course assays

were used to investigate the *ISG56* reporter response after the addition of exogenous IFNα and the data indicated that the luciferase activity increases over 8 to 24 hours, with the peak signals achieved 24 hours post addition of IFNα (100 IU) even when the time course was extended beyond 24 hours up to 48 hours (**Fig.4-3B**). Based on the results of the time course and high sensitivity of the assay, 24 hours post addition of interferon was chosen as the end time point for the *ISG56* reporter assays.

Alternative reporters which also detect the interferon pathway were tested alongside the *ISG56* reporter however the *ISG56* reporter showed the highest sensitivity and consistency in dose and time response assays, and is capable of detecting both Type I and III interferons. The other luciferase reporter plasmids tested included: *PRDII* (the nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) dependent region of the *IFNβ* promoter which induces a proinflammatory response following induction by pattern recognition receptors (PRRs) such as Toll-like receptors (TLRs) that recognise and respond to virus associated pathogen-associated molecular patterns or PAMPS), *pConA* (an artificial reporter construct with NF-κB responsive sequence taken from the concanavalin A promoter induced by virus infection), *pIFNβ* (a full length IFNβ promoter), *pISRE* (binds to the IRF3 transcription factor) and *pNIFTY* (artificial construct of the NF-κB region from the ELAM-1 promoter which is induced by PRRs such as TLRs that recognise and respond to PAMPS) (data not shown) (Macdonald 2003; Richards and Macdonald 2011; Wu et al 2012; Singhi et al 2004; Leblanc 1990; (Mogensen, 2009).

The *ISG56* luciferase reporter plasmid was transfected into Huh7.5 cells with an average transfection efficiency of approximately 12%. Then 24 hours after transfection, Huh7.5 cells were seeded in mono-culture and co-culture with the stromal cells at a 1:1 ratio as done previously (**section 3.4**). After 4-6 hours, the co-cultures were infected with high titre HCVcc virus. Huh7.5 mono-cultures were treated with IFNα as a positive control. As a negative control, cells were untransfected or left untreated after transfection. After 24 hours, the cells were lysed and the *ISG56* expression determined by measuring luciferase activity (**Fig.4-3C**). In the aLMF and LX-2 containing co-cultures, the levels of *ISG56* activation were comparable to the no IFN control. This suggests there is no IFN being produced in co-culture and therefore it has no role in the inhibition of HCVcc when aLMF or LX-2 cells and hepatoma cells are in co-culture.

Taking into account the 12% transfection efficiency, we wanted to demonstrate that the levels of HCVcc infection were sufficient to induce the *ISG56* reporter. After transfection and infection as done above, we fixed co-cultures for immunofluorescence staining. Representative images of NS5A positive cells in mono- and co- culture conditions indicate high levels of HCVcc infection (**Fig.4-3D**). Therefore, despite high levels of HCVcc infection, IFN stimulated pathways leading to ISG56 activation were not activated in co-culture, which suggests no role for them in stromal cell anti-viral activity.

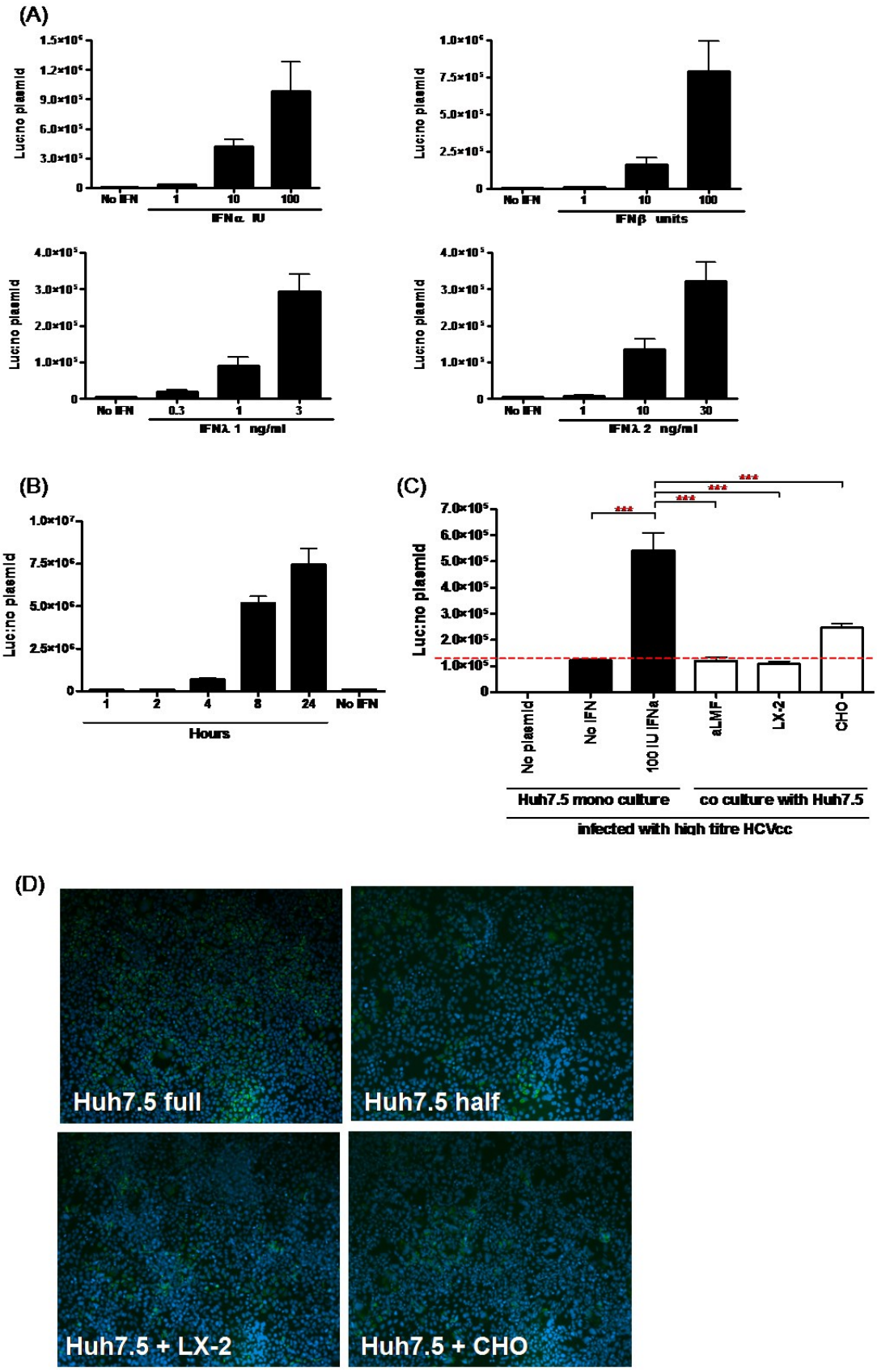



**Figure 4-3 Measuring *ISG56* activation in co-culture**

(**A-D**) 24 hours after Huh7.5 cells were transfected with the *ISG56* luciferase reporter plasmid or untransfected as a negative control, they were re-seeded

in mono-culture or co-culture and allowed to adhere for 4-6 hours before treatment. (**A-C**) Cells were lysed 24 hours after the indicated treatment and luciferase activity (RLU) measured. *ISG56* activity is expressed as RLU normalised to the untransfected Huh7.5 cells by subtracting the no plasmid signals (Luc:no plasmid). (**A**) Huh7.5 mono-cultures were treated with IFNα (1, 10, 100 IU), IFNβ (1, 10, 100 units), IFNλ1 (0.3, 1, 3 ng/ml), or IFNλ2 (1, 10, 30 ng/ml). Data representative of n=7 independent repeats. (**B**) Huh7.5 mono-cultures treated with IFNα (100 IU) were collected at 1, 2, 4, 8, and 24 hours post treatment. (**C**) Mono-cultures of Huh7.5 cells were either untreated or treated with IFNα (100 IU). Huh7.5 co-cultures with aLMF, LX-2, or CHO cells were infected with a high titre HCV virus. Data representative of n=3 independent repeats (n=4 aLMF donors). Statistical comparison was made using one-way ANOVA tests to compare and Bonferroni's corrections (parametric) were applied for pair wise comparisons of all data where *** $P<0.001$. All error bars show SD. (**D**) Microscopy images, 24 hours after infection with HCVcc, foci in mono- and co-cultures were visualised using anti-NS5A antibody detected by a secondary antibody conjugated to Alexa488 (green) and nucleus detected by DAPI (blue) (magnification x10). Data representative of n=3 independent repeats (n=4 aLMF donors).

### 4.4 Anti-viral response PCR array shows no significant contribution to stromal cell anti-viral activity

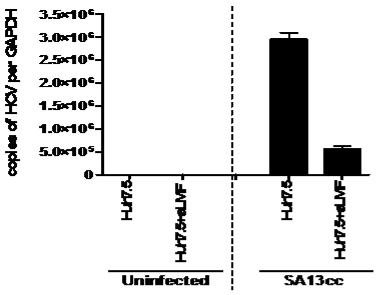
In order to investigate further the reduction in HCV infectivity when hepatoma cells are co-cultured with stromal cells, we used a Human Antiviral Response PCR array from SABiosciences. The human antiviral response PCR array allowed us to determine the expression of 84 key genes involved in the innate antiviral immune response. The genes are a mixture of receptors, chaperones, downstream signalling effectors, genes responsive to these pathways and signalling pathways belonging to the following functional groups; Toll-Like receptor (TLR) signalling, Nod-Like receptor (NLR) signalling, RIG-I-Like receptor signalling and Type-I-Interferon signalling and response. TLRs, NLRs and RIG-I-like receptors are different groups or pattern recognition receptors (PRRs) which can initiate the innate immune response following the detection of common pathogens including viruses, leading to a cascade of immune signalling pathways. Type-I interferon signalling leads to the activation of natural killer cells and dendritic cells but can also activate the adaptive immune response (Foy et al., 2005; Wang et al., 2013a; Wynn 2008).

Huh7.5 cells were seeded in mono-culture or in co-culture with aLMF. After 24 hours, the cells were infected with a high level of HCVcc (MOI 10) or left uninfected. First, the infection was confirmed by reverse transcription polymerase chain reaction (RT-qPCR) detecting copies of HCV over GAPDH (**Fig.4-4A**). Once the infection was confirmed, the cDNA was isolated and used in the antiviral PCR array. The antiviral PCR array includes a number of controls in addition to the 84 key genes of interest involved in the innate

antiviral immune response, as shown in the plate map (**Fig.4-4B**). The controls included: housekeeping genes (wells H1-H5), human genomic DNA contamination control (HGDC) (well H6), reverse transcription controls (RTC) for testing the efficiency of the $RT^2$ First strand kit (wells H7-H9) and positive PCR controls (PPC) for testing the efficiency of the polymerase chain reaction itself using an artificial DNA sequence and primer set pre-dispensed (wells H10-12). The controls for each sample were checked and passed. The data was analysed and heat maps used to display the fold-difference in gene expression when making pair wise comparisons of the samples for example the fold-difference in expression levels between the uninfected co-culture and infected co-culture (**Fig. 4-4C**). To investigate the reduction in HCV infectivity when Huh7.5 cells are co-cultured with aLMF cells, the relative level of genes in mono-culture and co-culture samples were compared. Gene differences above two fold were considered significant and genes with a cycle threshold value (Ct) above 30 classed as undetectable for all pair wise comparisons made, summarised according to functional family in **Table 4-1**. A large number of genes for all four samples were classified as undetectable, and many more genes classified as non-significant and so were not included in the summary table. The genes classified as undetectable were not due to issues with RNA or cDNA as the controls to test for the $RT^2$ First strand kit (wells H7-9) and positive PCR controls (PPC) (wells H10-12) passed confirming there were no issues with RNA or cDNA and that there was enough genomic material added to the array. When comparing the effect of HCVcc infection on Huh7.5 cells by comparing the Huh7.5 mono-culture uninfected vs the Huh7.5 mono-culture infected, there were no significant gene changes

suggesting the viral infection did not initiate any of the anti-viral pathways included on the array. The same was true when comparing the effect of HCVcc infection on the Huh7.5 and aLMF co-cultures. Significant gene changes of 2-fold, either up-regulated or down-regulated genes were only detected when a comparisons were made between either; Huh7.5 mono-culture uninfected vs Huh7.5 and aLMF co-culture uninfected or Huh7.5 mono-culture infected vs Huh7.5 and aLMF co-culture infected. There were some similarities in the genes change across these two comparisons, however, it is difficult to interpret some gene changes are a result of the aLMF being present in co-culture or the viral infection of mono-culture compared to the co-culture conditions. In order to interpret this data better and understand the effect of HCVcc infection on the aLMF, additional antiviral response PCR array kits should have been used to run the following samples: aLMF mono-culture uninfected and aLMF mono-culture infected. As a result, it was difficult to conclude whether the mechanism of stromal cell anti-viral activity is likely to be associated with an anti-viral mechanism used in this PCR array, especially given the large number of genes which were undetermined due to a high Ct value (>30). However, there were also a number of genes which remained unchanged when comparing the uninfected vs infected co-culture conditions, and taken together with the ISG56 data in this chapter, it is likely that the mechanism of anti-viral activity does not involve the IFN pathway, TLR, NLR or RIG-1 signalling pathways but further work may be required to confirm this data.

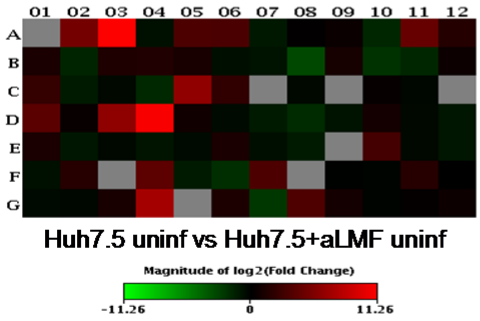
(A)



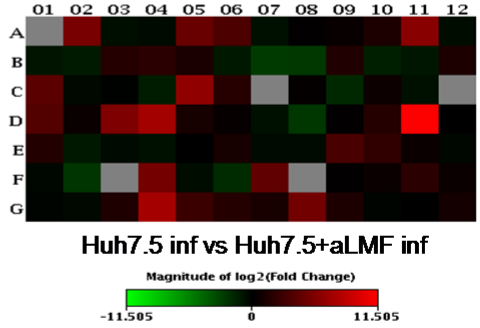

(B)

| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| A | AIM2 | APOBEC3G | ATG5 | AZI2 | CARD9 | CASP1 | CASP10 | CASP8 | CCL3 | CCL3 | CD40 | CD80 |
| B | CD86 | CHUK | CTSB | CTSL1 | CTSS | CXCL10 | CXCL11 | CXCL9 | CYLD | DAK | DDX3X | DDX58 |
| C | DHX58 | FADD | FOS | HSP90AA1 | IFIH1 | IFNA1 | IFNA2 | IFNAR1 | IFNB1 | IFBKB | IL12A | IL12B |
| D | IL15 | IL18 | IL1B | IL6 | IL8 | IRAK1 | IRF3 | IRF5 | IRF7 | ISG15 | JUN | MAP2K1 |
| E | MAP2K3 | MAP3K1 | MAP3K7 | MAPK1 | MAPK14 | MAPK3 | MAPK8 | MAVS | MEFV | MX1 | MYD88 | NFKB1 |
| F | NFKB1A | NLRP3 | NOD2 | OAS2 | PIN1 | PSTPIP1 | PYCARD | PYDC1 | RELA | RIPK1 | SPP1 | STAT1 |
| G | SUGT1 | TBK1 | TICAM1 | TLR3 | TLR7 | TLR8 | TLR9 | TNF | TRADD | TRAF3 | TRAF6 | TRIM25 |
| H | ACTB | BSM | GAPDH | HPRT1 | RPLP0 | HGDC | RTC | RTC | RTC | PPC | PPC | PPC |

(B.ii)




(B.iii)




(B.iv)

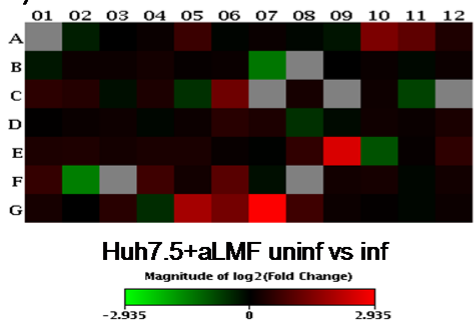



**Figure 4-4 Human anti-viral response PCR array**

Huh7.5 cells were seeded in mono-culture and in co-culture with aLMF donor at a 1:1 hepatoma:stromal cell ratio. The cultures were infected 24 hours post seeding with a high titre HCVcc (MOI 10) or left uninfected. After 48 hours post infection, the cells were lysed for RNA extraction. (**A**) RT-qPCR detecting

copies of HCV over GAPDH. (**B**) Plate map with gene names and locations of the genes included in the 2-step $RT^2$ profiler SABiosciences human anti-viral mirco array. There were 84 key genes involved in the innate immune response and the controls: housekeeping genes (wells H1-H5), human genomic DNA contamination control (HGDC) (well H6), reverse transcription controls (RTC) (wells H7-H9) and positive PCR controls (PPC) (wells H10-12). (**C**) Data analysed using the $RT^2$ profiler SABiosciences software and displayed as heat maps showing the fold-difference in gene expression when making pair wise comparisons of the samples labelled beneath the heat map. The colour represent the magnitude of log2 (fold changes) with green representing decreased expression and red representing increased expression.

| Sample comparison | Up-regulated genes (>2-fold) | Down-regulated genes (>2-fold) | Undetectable (>30Ct) | Pathways |
|---|---|---|---|---|
| Huh7.5 mono-culture *uninfected* vs. Huh7.5 mono-culture *infected* | - | - | CCL3, CCL5, CD40, CD80, CD86, CXCL11, CXCL9, IL15, IL1B, IL6, IRF5, IRF7, JUN, TLR3, TLR7, TLR8, TLR9, TNF, IFNA1, IFNA2, IFNB | Toll-Like Receptor signalling |
| | - | - | AIM2, CARD9, CASP1, IL1B, MEFV, NLRP3, NOD2, OAS2, PSTPIP1, PYCARD, PYDC1 | Nod-Like Receptor Signalling |
| | - | - | ATG5, DHX58, IFIH1, IFNA1, IFNA2, IFNB1, IL12B, TNF | RIG-I-Like Receptor Signalling |
| | - | - | APOBEC3G, IL15, MX1, TLR3, IFNA1, IFNA2, IFNB1 | Type-1-Interferon Signalling |
| Huh7.5 mono-culture *uninfected* vs. Huh7.5+aLMF co-culture *uninfected* | CTSB, CTSL1, CTSS, MAP2K3, MAPK3, SPP1, TICAM1 | CHUK, IRF3 | CCL3, CCL5, CD40, CD80, CD86, CXCL11, CXCL9, IL15, IL1B, IL6, IRF5, IRF7, TLR3, TLR7, TLR8, TLR9, TNF, IFNA1, IFNA2, IFNB1 | Toll-Like Receptor signalling |
| | PYCARD | HSP90AA | AIM2, CARD9, CASP1, MEFV, NLRP3, NOD2, OAS2, PSTPIP1, PYDC1 | Nod-Like Receptor Signalling |
| | CYLD | CASP10, CHUK, DAK, DDX3X, FADD, IRF3, MAVS, PIN1 | ATG5, DHX58, IFIH1, IFNA1, IFNA2, IFNB1, IL12B, TNF | RIG-I-Like Receptor Signalling |
| | - | - | APOBEC3, MX1, IFNA1, IFNA2, IFNB1 | Type-1-Interferon Signalling |
| Huh7.5 mono-culture *infected* vs. Huh7.5+aLMF co-culture *infected* | CTSB, CTSL1, CTSS, IL15, MAP2K3, MAPK3, SPP1, TICAM1 | CXCL10 | CCL3, CCL5, CD40, CD80, CD86, CXCL11, CXCL9, IFNA1, IFNA2, IFNB1, IL12B, IL1B, IL6, IRF5, IRF7, JUN, TLR3, TLR7, TLR8, TLR9, TNF | Toll-Like Receptor signalling |
| | - | HSP90AA1, | AIM2, CARD9, CASP1, IL1B, MEFV, NLRP3, NOD2, OAS2, PSTPIP1, PYCARD, PYDC1 | Nod-Like Receptor Signalling |
| | CYLD, DDX58, ISG15, TRADD | CHUK, CXCL10, DAK, DDX3X, MAP3K1 | DHX58, IFIH1, IFNA1, IFNA2, IFNB1, IL12B, IRF7, TNF | RIG-I-Like Receptor Signalling |
| | IL15, ISG15 | - | APOBEC3, IFNA1, IFNA2, IFNB1, MX1, TLR3 | Type-1-Interferon Signalling |
| Huh7.5 mono-culture *uninfected* vs. Huh7.5+aLMF co-culture *infected* | - | - | CCL3, CCL5, CD40, CD80, CD86, CXCL11, CXCL9, IFNA1, IFNA2, IFNB1, IL12B, IRF5, IRF7, TLR7, TLR8, TLR9, TNF | Toll-Like Receptor signalling |
| | - | - | AIM2, CARD9, MEFV, NLRP3, NOD2, OAS2, PSTPIP1, PYCD1 | Nod-Like Receptor Signalling |
| | - | - | IFNA1, IFNA2, IFNB1, IL12B, IRF7, TNF | RIG-I-Like Receptor Signalling |
| | - | - | APOBEC3, IFNA1, IFNA2, IFNB1, MX1 | Type-1-Interferon Signalling |

**Table 4-1 Summary of anti-viral array data**.

Table summarising anti-viral array data obtained as the gene differences above two fold that were considered significant and genes with a cycle threshold value (Ct) above 30 classed as undetectable for all pair wise comparisons made, and grouped according to functional family. A large number of genes for all four samples were classified as undetectable, and many more genes classified as non-significant and so were not included in the summary table

### 4.5 Blocking the VEGF pathway does not suppress stromal cell anti-viral activity

Previous work in our group, identified bone morphogenetic protein 4 (BMP4) as a pro-viral molecule expressed by liver sinusoidal endothelium cells (LSEC) in chronic liver diseases including HCV (Rowe et al 2014). BMP4 was negatively regulated by vascular endothelial growth factor A (VEGF-A). Based on these observations and having ruled out other anti-viral pathways, we wanted to investigate whether or not VEGF-A contributes to the reduced HCV infectivity observed in aLMF and hepatoma cell co-cultures.

For this approach, Huh7.5, aLMF, LX-2, and CHO cells were treated with anti-VEGF-A antibody to block VEGF-A activity or left untreated while in suspension. Then Huh7.5 cells were seeded in co-culture with aLMF, LX-2, or CHO cells at a 1:1 ratio. 24 hours after seeding, the cultures were infected with HCVcc and cultured in the absence or presence of the anti-VEGF-A antibody for 48 hours. The cultures were then fixed, stained for NS5A, and the level of HCV infection quantified by counting the number of NS5A positive cells per foci, which indicates the spread of the infection (**Fig.4-5A**) and total number of infected cells (**Fig.4-5B**). There was no difference in the level of HCV spread or overall number of HCV infected cells between mono-cultures and co-cultures in the absence or presence of anti-VEGF-A antibody. This anti-VEGF-A antibody has been extensively used by our group and we have previously shown it can inhibit the pro-viral action of BMP4, however, additional positive controls which could have been included here are: BMP4 alone at doses of 1, 10, 100 ng/ml (which would demonstrate a dose

dependant increase in HCV infection of Huh7.5 cells) and BMP4 doses with anti-VEGF-A antibody (which would show the anti-VEFG-A antibody can inhibit the increased HCV infection caused by BMP4 increasing hepatocyte permissivity to support HCV infection) (Rowe et al., 2014). Under these circumstances, this data indicates that VEGF-A does not contribute towards stromal cell anti-viral activity as blocking VEGF-A did not restore HCV infection in co-culture.

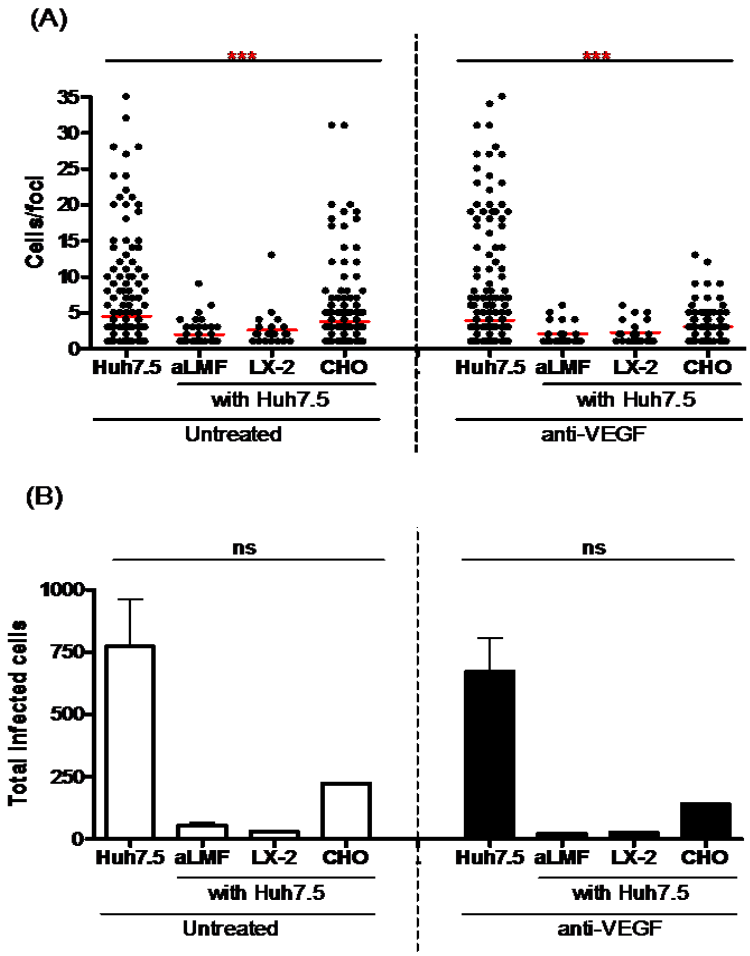



**Figure 4-5 Blocking the VEGF pathway does not restore HCV infection in co-culture**

After cells were either treated with anti-VEGF-A antibody (1000µg/mL) or left untreated, Huh7.5 mono-cultures were seeded alone and in co-cultures with aLMF, LX-2, or CHO cells at a 1:1 ratio. Cells were infected 24 hours post seeding and left for 48 hours in the absence or presence of anti-VEGF-A antibody. The level of HCVcc infection was determined by counting the number of NS5A positive cells per foci (**A)** and the total number of infected cells (**B**). Data representative of n=1 (n=1 aLMF donor). Statistical comparison was made using the Kruskal-Wallis test and Dunn's corrections were applied *** P<0.001. All error bars show SD.

### 4.6 Stromal cell anti-viral activity is independent of the nitric oxide pathway

After investigating various anti-viral pathways, we were left to explore other pathways not traditionally classified as anti-viral, but may in this model contribute to the anti-viral mechanism of aLMF. One potential pathway that could account for the inhibition of HCVcc infection is the nitric oxide (NO) pathway, which is activated following induction by interferons and thought to play a role in controlling viral infection but can also act independent to the IFN pathway (Ibrahim M, et al 2010; Mehta D R et al 2012; (Mehta et al., 2012). An increase in NO production as a result of HCV infection has been reported as well as its links to oxidative stress, carcinogenesis, and vascular thickening (Bruckdorfer, 2005a, Ren et al., 2008, Lukacs-Kornek et al., 2011). To investigate if this pathway could explain the mechanism of stromal cell anti-viral activity, the NO pathway was blocked using the chemical inhibitor L-monomethyl arginine (L-NMMA) (Bruckdorfer, 2005b). The NO inhibitor L-NMMA was added to the cells in suspension (1mM working concentration) and then the cells were seeded in mono- and co- culture. Then 24 hours after seeding, the cells were infected with HCVcc and cultured in the absence or presence of L-NMMA for 48 hours. The cultures were fixed, stained for NS5A, and the level of HCV infection quantified by counting the number of NS5A positive cells per foci, indicating spread (**Fig.4-6A**), and total number of infected cells (**Fig.4-6B**).

When comparing the level of HCVcc infection in mono-cultures of Huh7.5 cells to co-cultures as cells per foci, there appeared to be a significant

difference (*** P<0.001) in each co-culture condition when compared to the mono-culture conditions (**Fig.4-6A**). This is expected as we have previously shown that stromal cells can limit HCVcc infection in Huh7.5 cells when in co-culture. When the infection level of HCVcc was compared as total number of infected cells, there was statistically minimal difference in the co-culture conditions compared to the Huh7.5 mono-cultures (untreated or treated with anti-NO inhibitor) however there is clearly a reduction in infection levels in the co-culture conditions (**Fig.4-6B**). Moreover, there was no difference in HCVcc infection between the untreated cultures compared to the cultures infected in the presence of the NO pathway inhibitor L-NMMA (**Fig.4-6A-B**). If the NO pathway was required for stromal cell anti-viral activity, we would have expected to see the levels of infection in the L-NMMA treated co-cultures to be higher than the untreated, which we did not.

In parallel to this assay, as a control, supernatants from cells in mono- and co-culture were collected after 48 hours of HCVcc infection. Using the Griess assay, the supernatants were screened for the presence of any NO produced by the cells in response to infection. The Griess assay indicated the supernatants were negative for NO (data not shown). By measuring the production of NO in the supernatants and by blocking the production of NO using L-NMMA, it is clear NO production plays no role in control of HCV infection. Additional controls which could be added to future NO assays include using poly I:C to stimulate NO production which should lead to an reduction in HCV infection, or adding exogenous NO to the cultures and then

using exogenous NO and the L-NMMA inhibitor in combination to show the L-NMMA inhibitor does block the NO pathway (Mehta et al., 2012).

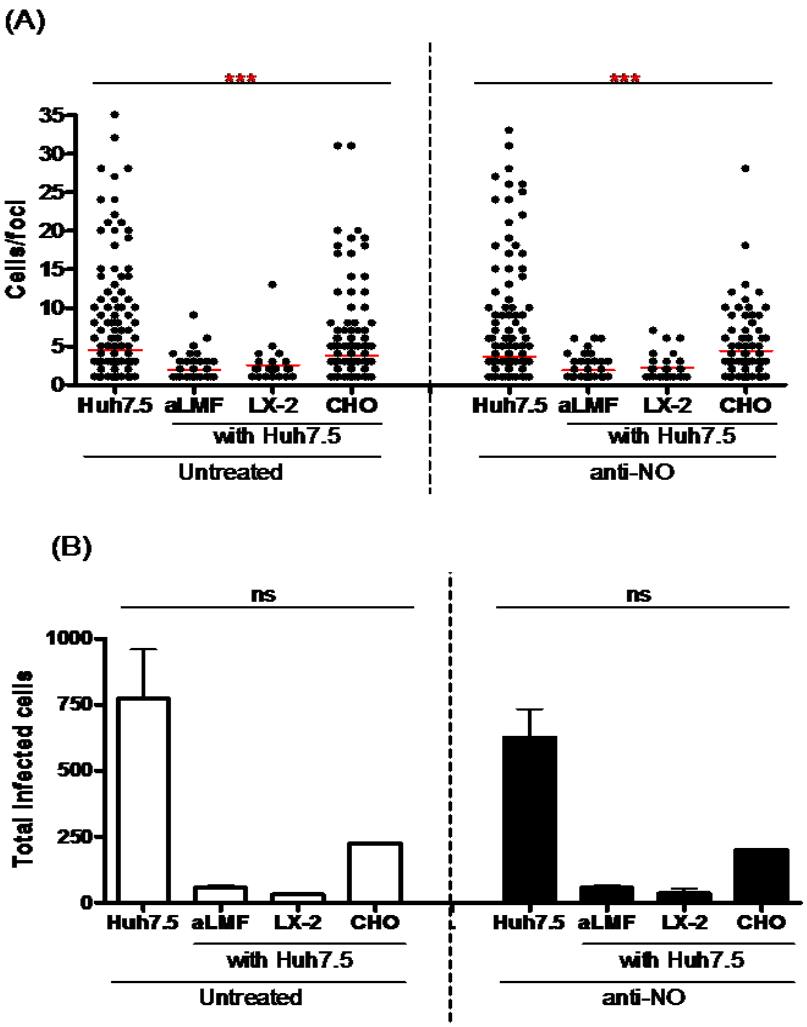



**Figure 4-6 Blocking the NO pathway does not restore HCV infection in co-culture**

After cells were either treated with the NO inhibitor, L-NMMA (1mM), or left untreated, Huh7.5 mono-cultures were seeded alone and in co-cultures with aLMF, LX-2, or CHO cells at a 1:1 ratio. 24 hours post seeding, cells were infected and left for 48 hours in the absence or presence of L-NMMA. The level of HCVcc infection was determined by counting the number of NS5A positive cells per foci (**A**) and the total number of cell infected (**B**). Data representative of n=1 (n=1 aLMF donor). Statistical comparison was made using the Kruskal-Wallis test and Dunn's corrections were applied *** P<0.001. All error bars show SD.

### 4.7 aLMF limit HCVcc infection in PHH

The ideal *in vitro* physiological model to study the human liver uses primary human hepatocytes (PHH), which are the closest system we have available to study hepatocytes *in vivo.* PHH are metabolically and phenotypically more similar to hepatocytes in the liver than the Huh7 cell lines. PHH express various hepatocyte differentiation markers and do not divide in culture like hepatocytes *in vivo*. The highly differentiated phenotype of PHH means these cells have an anti-viral response that is more intact compared to immortalised hepatoma cell lines. Therefore to investigate the physiological relevance of our Huh7.5 co-culture model, we used the PHHs in the co-culture system established in **section 3.4** and looked for aLMF anti-viral activity.

The caveat of this model is that the differentiation status of PHH in culture declines over time along with metabolic activity. Therefore, we first monitored the differentiation status of the PHH by using qRT-PCR to measure the expression levels of hepatocyte differentiation markers: alpha-fetoprotein (αFP), hepatocyte nuclear factor 4α (HnF4α), albumin, and a cytochrome P450 enzyme called CYP3a4. The PHHs maintained all four differentiation markers at a consistent level of expression over a period of 96 hours, observed using three different PHH donors (**Fig.4-7A**).

Our research group first characterised the PHH donors provided by Dr Ragai Mitry (King's College, London), comparing the PHHs to hepatoma cells, examining HCV receptor expression and location, and testing the ability of PHHs to support HCV entry and replication over time, in assays performed by

Garrick Wilson. We determined the time it takes for PHHs to de-differentiate in culture, which were monitored over a period of 12 days. The hepatocyte morphology was monitored by phase microscopy and samples collected every 24 hours to investigate albumin secretion and differentiation status in assays performed by myself. We found that the PHHs maintained their differentiation status for 10 days, at which point the morphology had changed from a hepatocyte like shape (a unique polygonal shape) to a more elongated fibroblast like shape. The level of albumin secretion decreased slowly over time, starting at approximately 350ng/mL on the day of seeding down to approximately 20ng/mL after 10 days in culture, thus indicating the PHH were de-differentiating (unpublished data). This data correlates with reports from other groups, highlighting the difficulties in culturing PHH and maintaining the phenotype and morphology in culture (Bhogal 2011; Yang 2011; Podevin 2010; Ploss 2010).

Next, we investigated the effect aLMF would have on HCV infection of PHH in co-culture. As established in **section 3.4**, PHHs were seeded in mono- and co- culture with aLMF, LX-2 and CHO cells at a 1:1 ratio. In mono-culture, the PHHs were seeded varying the number of target cells by starting with a full well of target cells ($2.5x10^4$ cells per well in a 48 well tissue) then a half the number of target cells in the full well. Cultures were allowed to adhere for 24 hours before infecting HCVcc. 48 hours post infection, cells were lysed for RNA extraction and RT-PCR used to detect the levels of HCV RNA. qRT-PCR was used to quantify infection because of the difficulties we found with staining and detecting HCV positive cells due to the autofluorescent nature of

PHH in immunofluorescence. In the PHH mono-cultures seeded at a full and half well of target cells, the level of HCV copies per GAPDH was higher when half the number of target cells are available (**Fig.4-7B**). This is in contrast to the Huh7.5 mono-culture where the level of infection is proportional to the number of target hepatoma cells available (**section 3.4.1**). However, this discrepancy could be due to PHH donor variability, as previous work by Garrick Wilson indicated that donor variation in PHHs did affect HCVcc replication in PHHs. Moreover, this initial data shows that HCVcc infection of PHHs is inhibited when they are in co-culture with aLMF, LX-2, and CHO cells.

Given the difficulties in isolating and culturing PHH, plus the limited availability, repeat assays were not feasible at this time and there weren't enough PHHs from a single donor to split the culture in order to set up two experiments in parallel. Under these circumstances, the data indicates that aLMF reduce HCV infection in both Huh7.5 cells and the physiologically relevant PHH, thus supporting the above conclusions made with our co-culture model.

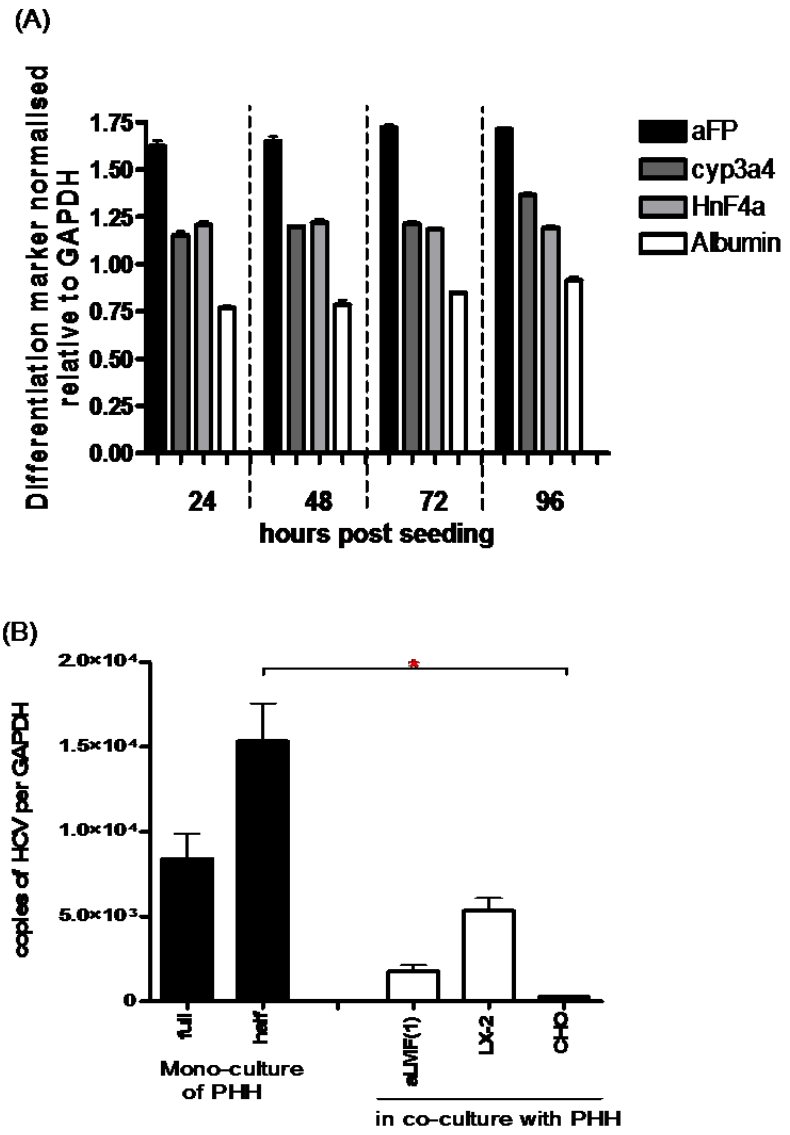



**Figure 4-7 aLMF limit HCVcc infection in PHH**

(**A**) Mono-cultures of PHHs were lysed at 24, 48, 72 and 96 hours post seeding for RNA extraction and qRT-PCR detection of the differentiation genes αFP, HnF4α, albumin, and CYP3a4 and the control house keeping gene GAPDH. The expression levels were quantified and normalised relative to the level of GAPDH detected at each time point. Data representative of n=3 independent repeats (n=3 PHH donors). (**B**) PHHs were thawed and seeded in mono-culture or co-culture with aLMF, LX-2, or CHO cells at a 1:1 ratio. 24 hours post seeding, cells were infected with high titre HCVcc. Then 48 hours post infection, cells were lysed for RNA extraction and gene expression of HCV RNA and GAPDH detected by qRT-PCR with data expressed as copies of HCV per GAPDH. Data representative of n=1 independent repeats (n=1 PHH donor and n=1 aLMF donor). Statistical comparison was made using the Kruskal-Wallis test and Dunn's corrections were applied * $P<0.05$. All error bars show SD.

### 4.8 The differentiation status of hepatoma cells in co-culture with aLMF remain unchanged

The role of aLMF within the liver is to support the hepatocytes in cell growth and survival. Therefore, we hypothesized that the aLMF may push the Huh7.5 cells to become more differentiated when in co-culture. More differentiated hepatomas would ultimately lead to a decrease in infection, similar to the lower levels of infection seen when using highly differentiation PHH.

To investigate the level of differentiation of the Huh7.5 cells in mono- and co-culture, the expression levels of hepatocyte differentiation genes α-FP (**Fig.4-8A**), albumin (**Fig.4-8B**), HnF4α (**Fig.4-8C**), and CYP3a4 (**Fig.4-8D**) was assessed using qRT-PCR. The results indicate that the expression level of the hepatocyte differentiation genes was not significantly different when comparing Huh7.5 cells in mono-culture to Huh7.5 cells in co-culture with aLMF. Therefore, the differentiation status of the Huh7.5 cells is not a potential mechanism for the stromal cell anti-viral activity.

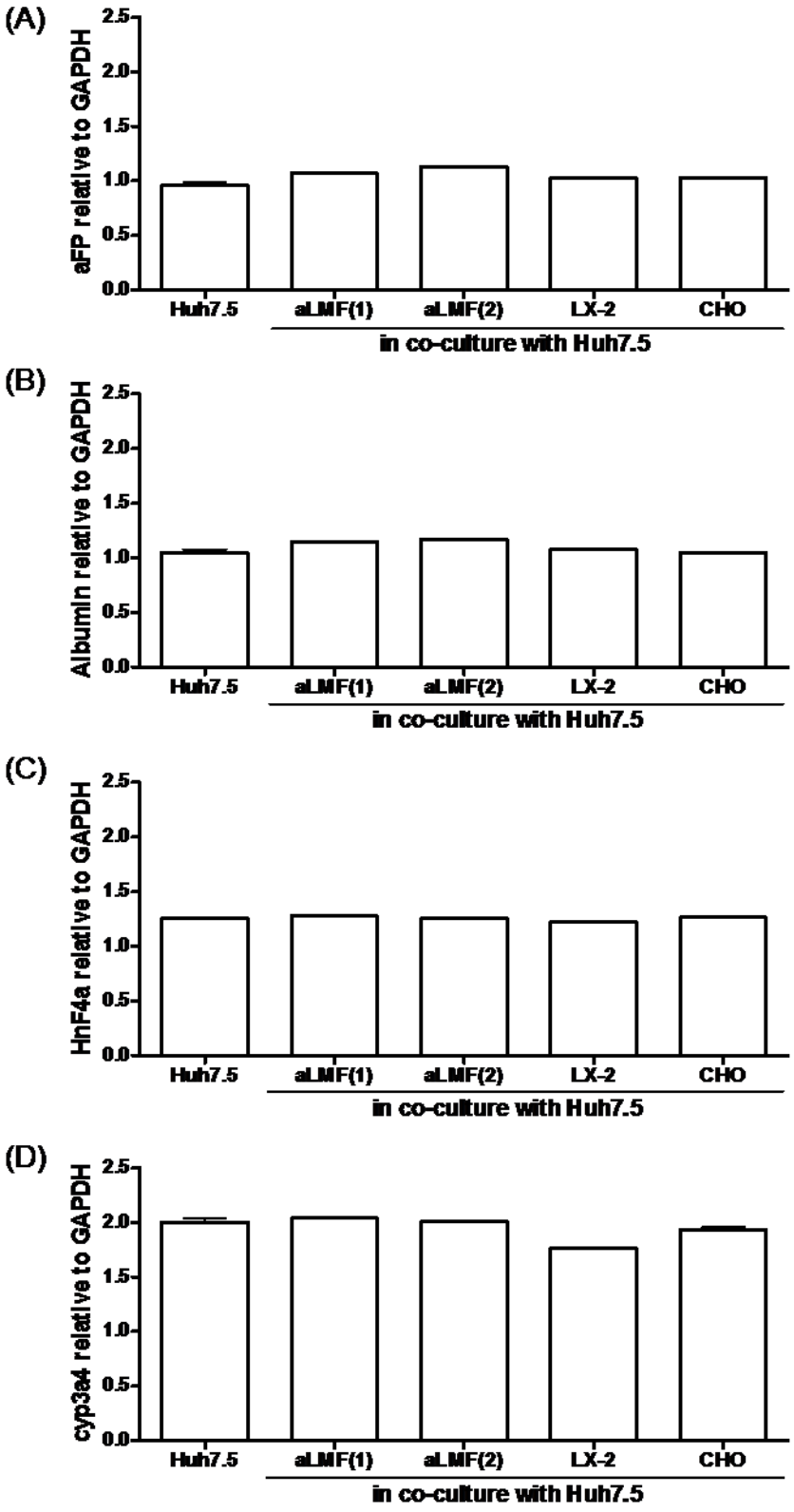



**Figure 4-8 The differentiation status of hepatoma cells when in co-culture with aLMF remains unchanged**

Huh7.5 cells were seeded in mono- or co- culture with aLMF (two donors, denoted 1 and 2), LX-2, or CHO cells at a 1:1 ratio. 72 hours post seeding,

cells were lysed for RNA extraction and qRT-PCR used to detect the expression level of the differentiation markers and the control house keeping gene GAPDH. The expression levels of α-FP (**A**), albumin (**B**), HnF4α (**C**), and CYP3a4 (**D**) were normalised relative to the level of GAPDH detected at each time point. Data representative of n=3 independent repeats. Statistical comparison was made using the Kruskal-Wallis test and Dunn's corrections were applied. All error bars show SD.

### 4.9 HepG2 polarity is altered when in co-culture with aLMF

The differentiation status and polarity of hepatocytes is key to maintaining their phenotype and function. After showing that aLMFs do not alter the differentiation status of Huh7.5 cells in co-culture, we next investigated if they could alter the polarity.

For this approach, we used HepG2 cells because this hepatoma cell line has the ability to polarize whereas Huh7.5 cells do not polarize in culture. Hepatocyte polarity is key in the ability of hepatocytes to function and is described as the distinct distribution of lipids and proteins in the sinusoidal and bile canaliculi membranes (Chiu et al 1990; Wang and Boyer 2004). HepG2 expressing DPP IV (tagged with red fluorescent protein) were seeded in mono- or co- culture with aLMF, LX-2 and CHO cells. DPP IV is a membrane expressed protein which is trafficked to the bile canaliculi in hepatocytes and so can be used to visualise the formation of bile canaliculi in HepG2 cells when polarised (Wojtal et al 2006; Ait Slimane et al 2003). The cells were allowed to settle for 3 days after seeding to give them time to polarize, after which the cultures were assessed for polarity using immunofluorescence (**Fig. 4-9**). We did not observe significant differences in polarity when the LX-2 or CHO cells were in co-culture with the HepG2-DPP IV cells. However we did observe a noticeable difference in polarity when aLMF (green) were co-cultured with HepG2-DPP IV cells (red) compared to the HepG2-DPP IV cells alone by immunofluorescence (**Fig. 4-9**). The aLMFs appeared to be causing the HepG2-DPP IV cells to form larger lumens that were more cis like in structure, compared to when the HepG2-DPP IV

cells are alone or in co-culture with LX-2 or CHO cells, thus appearing to have altered the polarity. The cis like lumens observed in the aLMF containing co-cultures can be described as larger lumens than normal, appearing bright which indicates there is a high level of DPP IV located around the lumen labelled with red fluorescent protein. There were also some lumens in the aLMF containing co-culture, which had not formed very well as a result of the HepG2-DPP IV cells being in direct contact with the aLMF, and sometimes, the long spindly extensions of the aLMF appeared to be running across where the lumen should have formed, disrupting the formation.

This data suggest that the aLMF are altering the polarity of the hepatocytes when in co-culture. The role of the altered hepatocyte polarity requires further functional assays in order to investigate the potential impact on viral infection and hepatocyte function.

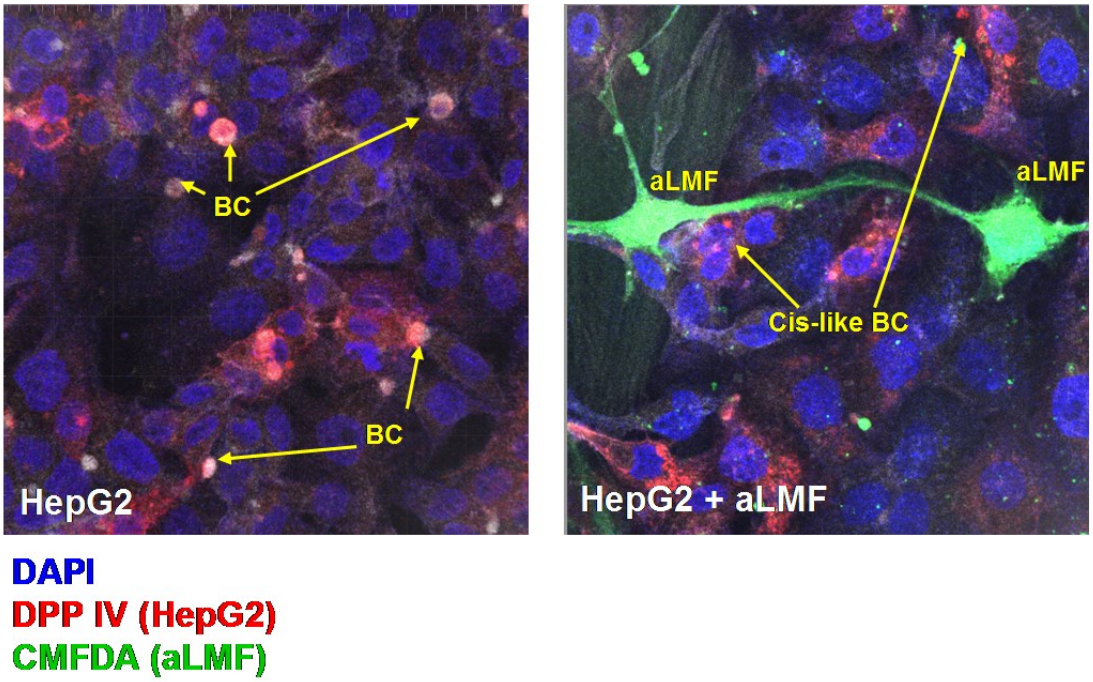



**Figure 4-9 HepG2 polarity is altered when in co-culture with aLMF**

Representative immunofluorescence images of HepG2-DPP IV cells (red) seeded in mono- or co- culture with aLMF. Cells were fixed 3 days post seeding, the nuclei stained using DAPI (blue) and the immunofluorescence visualised by immunofluorescence microscopy (magnification x50). Data representative of n=2 independent repeats (n=2 aLMF donors). BC - bile canaliculi structures.

## 4.10 aLMF may reduce HCVcc infection at physiologically relevant oxygen levels

Metabolic, vascular, and secretory functions of the liver are regulated by oxygen concentration, with a 3-9% oxygen gradient across the liver parenchyma. Therefore, hepatocytes are exposed to different levels of oxygen depending on their location within the liver (Wilson et al 2014; Wilson et al 2012). The oxygen level found in normal tissue incubators is 20% so the role of stromal cell anti-viral activity at physiologically relevant oxygen concentrations is unknown. Previous studies have shown that hypoxic conditions significantly increase HCV infection of hepatocytes and that under normal tissue culture conditions; HCV infection can induce a pseudohypoxic state by stabilizing HIF-1a expression (Nasimuzzaman et al 2007; Hassan 2009; Vassilaki 2013). Therefore it is hypothesized that HIF-dependent changes in hepatocyte metabolism and permeability can actually promote HCV replication and transmission (Wilson et al 2014; Vassilaki 2013; Mee et al 2009; Wilson 2012).

To investigate the role of oxygen concentration, Huh7.5 cells were seeded in mono-culture and co-culture with aLMF, LX-2, or CHO cells at a 1:1 ratio. Then 24 hours after seeding and incubating in the normal 20% oxygen incubator, the cultures were infected with HCVcc and immediately transferred to the 1% oxygen incubator (hypoxia) or left at 20% (normoxia conditions). The cells were fixed 48 hours post infection and the level of infection determined by staining for NS5A positive cells and counting the number of foci per well. The level of HCV infection under normoxia (**Fig.4-10; left**

**panel**) compared to hypoxia (**Fig.4-10; right panel**) was comparable in this preliminary data. The level of infection in the Huh7.5 mono-culture condition was unexpected as under hypoxia, the level of HCVcc infection in Huh7.5 is supposed to be significantly higher than under normoxia, however in this preliminary data, there appeared to be a slight decrease in the level of HCVcc infection of Huh7.5 mono-cultures which can not be explained but could be due to possible technical issues with the hypoxic incubator. Given that in this data the level of infection in both conditions is comparable it is difficult to establish whether or not hypoxia does play a role in stromal cell anti-viral activity but this potential mechanism warrants further investigation.

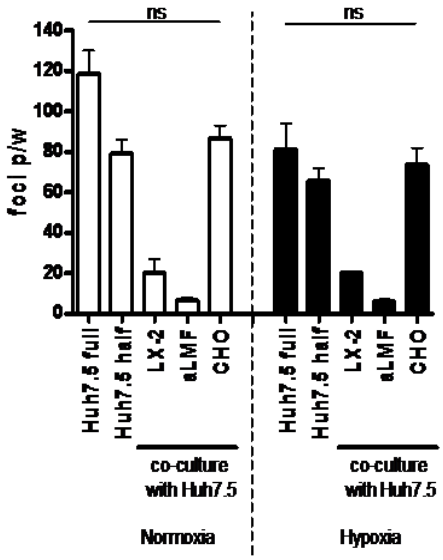



**Figure 4-10 aLMF can still reduce HCVcc infection at physiologically relevant oxygen levels**

Huh7.5 cells were seeded alone or in co-culture with aLMF, LX-2, or CHO cells at a 1:1 ratio in the normal 20% oxygen incubator. Then 24 hours post seeding, cells were infected with HCVcc and immediately transferred to the 1% oxygen incubator (hypoxia) or left at 20% (normoxia). Cells were fixed 48 hours after infection, stained for NS5A positive cells, and the number of foci per well counted. Statistical comparison was made using the Mann-U-Whitney test. All error bars show SD.

### 4.11 Discussion

There are limited studies investigating HCV infection of hepatoma cells that also take into account the complex nature and diverse cell types found within the liver. Previous work in our group from Rowe et al (2014) investigated the role of liver sinusoidal endothelium cells (LSEC) in regulating HCV replication and identified a secreted factor that promoted HCV infection within other cells. LSEC do not support HCV infection but do secrete bone morphogenetic protein 4 (BMP4), a soluble secreted factor that promotes HCV infection of hepatoma cells and increases HCV replication. However, when both cell types are in contact co-culture, there is actually an overall reduction in HCV infectivity of hepatoma cells when compared to mono-culture despite the LSEC producing the pro-viral BMP4 factor. Further research identified that although recombinant BMP4 increased hepatoma permissivity to HCV, hepatoma cells can actually negatively regulate BMP4 at the transcriptional level via vascular endothelial growth factor A (VEGF-A) activation, a factor known to protect the liver from injury (Rowe at el, 2014).

In chapter 3, we demonstrated that when aLMF, LX-2 and CHO cells were co-cultured with Huh7.5 cells at a 1:1 ratio, there were clearly less infected cells per foci after 48 hours in co-culture indicating that stromal cells can inhibit HCVcc spread. Despite the clear decrease in the level of spread in co-culture, there was only a moderate decrease in the aLMF co-culture, but a reasonable decrease in the LX-2 containing conditions ($P<0.05$) (**Fig.3-7C**). By using the various systems available to study HCV lifecycle, we were able to

demonstrate that stromal cells can limit HCV infection at various stages of the viral lifecycle including entry, replication and spread.

The data in this chapter demonstrates that the mechanism by which aLMF can limit HCV infection in neighbouring hepatoma cells is mediated via cell contacts determined by separating the cells in co-culture and testing the conditioned media from both mono- and co- culture conditions for potential soluble secreted factors with anti-viral properties. Huh7.5 cells are known to have a defective RIG-1 pathway due to a mutation in the *RIG-1* gene, which is involved in regulating the interferon pathway (Yao et al 2011; Feigelstock et al 2010; Blight et al 2002; Sumpter et al 2005; Regeard et al 2007). This defective pathway means the Huh7.5 cells are more permissive to viral infection, which works well in our co-culture system as it allows us to achieve higher infection levels and a better dynamic range in co-culture conditions. However, when trying to understand if the mechanism by which aLMF are inhibiting viral infection involves the innate immune response, this defective pathway means the system does not accurately mimic hepatocytes within the liver, which do have intact innate immune responses. On the other hand, even with this defective pathway in Huh7.5 cells we still observed a decrease in HCV infectivity, which could be suggesting that the mechanism is independent of this pathway. Interestingly, previous studies have shown that poly I:C stimulation of LX-2 cells in mono-culture leads to TLR-3 activation in the LX-2 which induces IFN-λ production Huh7 cells (which have an intact RIG-I pathway) when treated with conditioned media collected from the LX-2 cells. As a result, Huh7 cells pre-infected with HCV were treated with the

conditioned media collected from the stimulated LX-2 cells, which was shown to be anti-viral. In a similar manner, conditioned media collected from LX-2 cells stimulated with a RIG-I ligand were also shown to suppress HCV replication of pre-infected Huh7 cells, indicated RIG-I signalling of LX-2 cells can inhibit HCV infection via the production of IFN-β and IFN-λ (Wang et al., 2013b, Wang et al., 2013c). With such questions surrounding the physiological relevance of the Huh7.5 co-culture system, we wanted to validate the system and in this chapter we have shown data using PHH in co-culture demonstrating a similar level of viral inhibition in PHH co-culture to the Huh7.5 co-culture system.

A human anti-viral array was then used to screen the contribution of a vast number of anti-viral pathways and this data, combined with the *ISG56* data, was key in concluding that anti-viral mechanisms do not contribute to the reduction in HCV infectivity. When comparing the effect of HCVcc infection on the Huh7.5 and aLMF co-cultures, there were no significant gene changes and a large number of genes which remained unchanged suggesting the viral infection did not initiate any of the anti-viral pathways included on the array. However there were also a large number of genes which were undetermined due to a high Ct value (>30), and these genes could have possibly been altered significantly altered and so a few additional repeats would help make the data more robust (**Fig. 4-4B**). Nevertheless, the *ISG56* reporter, demonstrated to detect a range of IFNs, confirmed these various IFN pathways do not contribute to the stromal cell anti-viral mechanism. Interestingly, there was a low level of *ISG56* activation in the cell density

control co-cultures containing CHO cells (**Fig. 4-3C**). This could be possibly be due to CHO cells being recognised as a non-human cell type therefore potentially stimulating an anti-viral response in the Huh7.5 cells and so could possibly explain why the CHO cells can also inhibit HCV infection in neighbouring hepatoma cells, although to a lesser extent than the stromal cells (**Fig 3-4B and Table 3-2**). Another factor to bear in mind with using CHO cells or any other cell type as a control for such experiments is that no cell is an inert cell and there will inevitably be a certain degree of cross-talk between two different cell types when they are co-cultured together.

Our research group have previously characterised the phenotype of PHH in culture and investigated HCV infection of PHH using the various HCV infection systems present (unpublished data). PHH infection was compared to several hepatoma cell lines, the optimal day post seeding for HCV infection established and the expression of the four key HCV receptors required for entry monitored over time in culture. HCVpp infection of PHH indicated there was no donor variability at the entry step of HCV infection, however there was donor variability in HCV infection when the PHH were infected with HCVcc. This indicated that donor variability impacted the replication steps of HCV infection which has been previously reported by Marukian et al (2008). Marukian and colleagues went on to demonstrate the variation in replication was due to the variation in the interferon response and ISGs after HCVcc infection of PHH. The overall level of HCV infection in PHH is lower when compared to various hepatoma cells due to factors such as the more intact anti-viral response and the PHH being more differentiated (Marukian et al

2008; Bhogal 2011; Yang 2011; Podevin 2010; Ploss 2010). One of the key challenges with PHH culture in addition to the limited availability is that they de-differentiate over time in culture. The role of stromal cells in the liver is to support hepatocyte function and so a number of studies have used stromal cells including non-human fibroblast cell lines, in co-culture systems to assist in maintaining hepatocyte function (Ploss 2010; Goulet 1988; Khetani and Bhatia 2008; Hui and Bhatia 2007). So we investigated the role of aLMF in maintaining hepatoma cell function, focusing on differentiation status and polarity, two key characteristics of hepatocytes which are vital for their function contributing to the lower permissivity in PHH when compared to hepatoma cell lines. Huh7.5 cells are not known as a differentiated cell line and unlike HepG2 cells, they can not polarize in culture. The differentiation status of Huh7.5 cells remained unchanged in our co-culture system suggesting this does not account for the reduction in HCV infectivity. aLMF did however appear to alter HepG2 polarity observed by microscopy however given that the majority of the data in this study used Huh7.5 cells, this was not investigated further. Nevertheless, investigating the impact of stromal cells on hepatocyte polarity and how this impacts HCV infectivity would be interesting for further work and increase our understanding of the role stromal cells play on HCV disease progression in the liver.

This chapter demonstrates the physiological relevance of our co-culture system, validating our data using this system and highlighting the importance of understanding the mechanism for future therapeutic avenues. Interestingly, we were able to rule a number of potential pathways that would have

indicated a typical anti-viral response such as the innate immune response, IFNs and ISGs, cytokines and other soluble factors. We were also able demonstrate that some mechanisms which could alter hepatocyte function and thus HCV infection such as albumin production and differentiation status, do not contribute to the ability of aLMF to reduce HCV infection. Yet, HepG2 polarity when in co-culture was altered with the preliminary data showing altered lumens in co-culture with aLMF compared to mono-cultures of HepG2 cells (**Fig.4-9**) and occasionally observed what appeared to be membrane ruffling on the HepG2 cells in contact with the aLMF however this was preliminary data (data not shown). Overall, these data lead us back to the inhibition being a cell contact based; possibly impacting the membrane dynamics or structure and so we wanted to investigate the cell-cell contacts occurring between these two cell types in more detail to elucidate a potential mechanism.

# Chapter 5 aLMF inhibit HCV infection via two independent mechanisms: limiting CD81 lateral diffusion and VAP-1 expression

## 5.0 Introduction

Having investigated and ruled out antiviral immune defence mechanisms playing a role in aLMF limitation of HCV infection in co-culture, we decided to focus on potential non-immune related mechanisms particularly cell mobility and membrane dynamics. In Chapter 4, we established that the mechanism by which aLMF limit HCV infection is cell contact dependent. When in contact with HepG2 cells, aLMF also appeared to alter the polarity of the hepatocytes. Based on these observations, we decided to focus on potential cell-cell contact mechanisms.

The number of heterotypic cell-cell contacts occurring in our conventional 2D co-culture system could be influenced by the arrangement of the cells compared to the 3D arrangement found in the liver. Therefore, we decided to model the co-culture system in 3D in order to mimic the spatial arrangement of these cell types *in vivo*. We would then use this 3D model to establish if the cellular arrangement has an impact on the ability of aLMF to inhibit HCV infection in hepatoma cells.

aLMFs are mobile cells (Brandao et al 2006; Iredale 2007; Friedman 2008) which led us to hypothesize that in co-culture they may be in contact with multiple hepatoma cells at different points in time as they migrate through the

culture responding to cell signals. As the aLMF are migrating there is also the potential that they are laying down extracellular matrix (ECM) that may or may not be degraded. This ECM could potentially act as a trap binding virus inoculum. Therefore, we wanted to study virus binding to the aLMF cell membrane and ECM in order to establish a mechanism by which aLMF deplete virus and consequently reduce HCV infection.

### 5.1 aLMF in 3D co-culture have the ability to limit HCVpp entry

The co-culture system established in Chapter 3 used conventional 2D co-cultures that may not accurately mimic the complex multi-cellular environment found in the liver. Thus, we wanted to develop a 3D organoid culture that would contain multiple liver cell types in order to create a more realistic model of the liver microenvironment.

With the guidance of Professor van Ijzendoorn at UMCG, we adapted the established 3D liver organoid Matrigel sandwich culture system to grow Huh7.5 cells (**Fig.5-1A**) and HepG2-CD81 cells (**Fig.5-1B**) (Elamin et al 2012; Molina et al 2012). Unlike Huh7.5 cells, HepG2 cells do not express CD81 and so they are non-permissive to HCV infection. However, by endogenously expressing CD81 in HepG2 cells, they can be rendered permissive to HCV infection. These cells were seeded in co-culture with aLMF, LX-2, or CHO cells at a 4:1 ratio of hepatoma:stromal cells. This ratio was selected after optimisation steps with different ratios were tested which indicated that the larger size of the aLMF in relation to the HepG2-CD81 cells often disrupted the organoid cultures from forming over time. The 4:1 ratio of hepatoma:stromal cells allowed the successful formation of organoid cultures in a 3D arrangement containing both cell types within the standard time frame of this system. The 3D organoid cultures look like round 3D spheres made up of multiple cells, often with an empty space in the centre of the sphere shape which can be visualised by immunofluorescence imaging and taking z-stack image through the sphere like 3D organoid cultures. As a control, cultures were also seeded in 2D without the Matrigel as established in Chapter 3 but at

a matching 4:1 ratio of hepatoma:stromal cells. Cultures were infected 72 hours post seeding with either HCVpp (**Fig.5-1A-B**) or HCVcc (**Fig.5-1C**). The cultures infected with HCVpp were lysed 48 hours post infection and the level of HCVpp infection determined by measuring luciferase activity. Based on the comparable infection levels for the Huh7.5 mono-cultures in 2D without Matrigel and Huh7.5 mono-culture in 3D with Matrigel, we confirmed that the Matrigel sandwich culture system did not interfere with the luciferase readings (**Fig.5-1A-B**). In the 2D cultures, the aLMF appeared to not inhibit HCV infection in co-culture which is inconsistent with the previous co-culture data, yet in 3D with Matrigel, the aLMF are showing an inhibition of infection as expected. This could potentially be explained by the low number of aLMFs present in the 4:1 Huh7.5:aLMF co-culture ratio here, leading to fewer aLMF adhering, increased aLMF cell death and a even fewer aLMF cells present, as described in **section 3.4.1**. The aLMF cells may have settled better at the 4:1 ratio in the 3D Matrigel conditions as the Matrigel itself may have provided a scaffold for the cells to adhere to and is routinely used to enhance cell growth of primary cells or cells which are difficult to culture using normal tissue culture plates. Overall, the results indicate that aLMF have the ability to reduce HCV entry in 3D cultures to levels comparable to the 2D cultures as indicated by the raw RLU values. This suggests that even when the cells are cultured 2D co-cultures, the level of cell-cell contact achieved in 2D could potentially be mimicking the level of cell-cell contacts in 3D as found in the liver (**Fig.5-1A-B**). We had hypothesised that a 3D arrangement of cells could potentially allow one aLMF cell to contact multiple Huh7.5 cells and form more cell-cell contacts with Huh7.5 cells than in the conventional 2D cultures which

we hypothesised would then lead to an enhanced reduction in HCV infection of Huh7.5 cells.

Representative immunofluorescence images were taken of HCVcc infected Huh7.5 cells or HepG2-CD81 cells alone in 2D and 3D Matrigel cultures (**Fig.5-1C**). The images show how the cells grow in 3D organoids making it difficult to focus on HCV positive cells and thus making it difficult to count the level of infection. We concluded that HCVcc infections in 3D are difficult to enumerate at this time and so this system would be best suited for pseudoparticle infections. Given the limited availability of mouse models to study HCV, after further optimization the 3D 'liver' cultures could provide a significant advance in studying the interaction(s) between liver cells and their role in the HCV lifecycle.

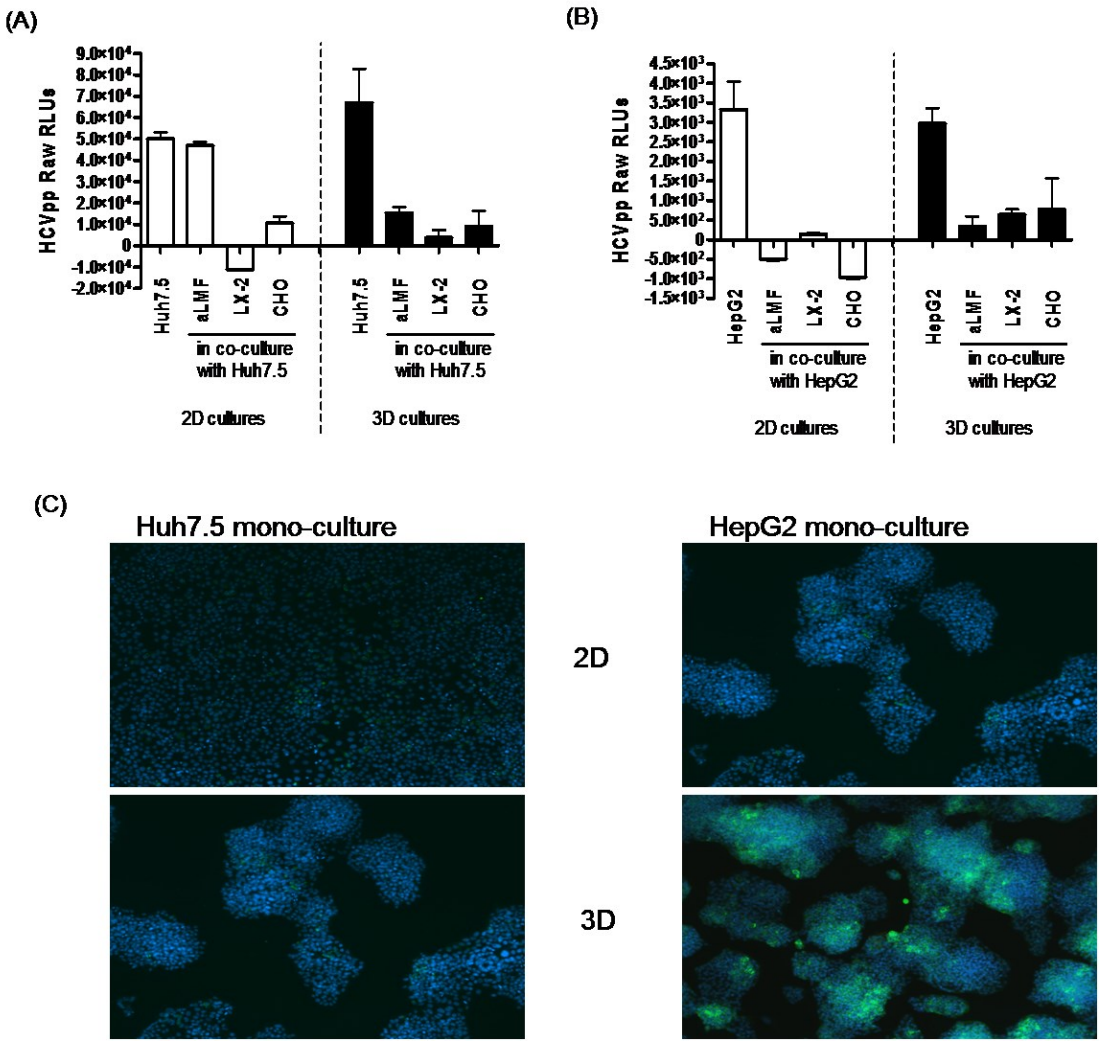



**Figure 5-1 aLMF cultured in 3D limit HCVpp entry**

Ibidi chamber slides (8 well slides) were either uncoated or coated with Matrigel 30 minutes prior to seeding. Huh7.5 cells (**A**) and HepG2-CD81 cells (**B**) were seeded in mono- or co- culture with aLMF, LX-2, and CHO cells at a 4:1 ratio of hepatoma:stromal cells. Cultures were infected 72 hours post seeding with either HCVpp (**A-B**) or HCVcc (**C**) for 48 hours. (**A-B**) Cells were lysed and luciferase activity measured. Data is expressed as relative light units (RLU) of HCVpp. Data representative of n=2 independent repeats (n=2 aLMF donors). Statistical comparison was made using the one way ANOVA and Bonferroni's corrections were applied. All error bars show SD. (**C**) Cells were fixed and stained with anti-NS5A antibody detected by a secondary antibody conjugated with the Alexa488 (green) and DAPI to show nuclei (blue) (magnification x10). Data representative of n=2 independent repeats (n=2 aLMF donors).

**5.2 aLMF are more mobile than hepatoma cells**

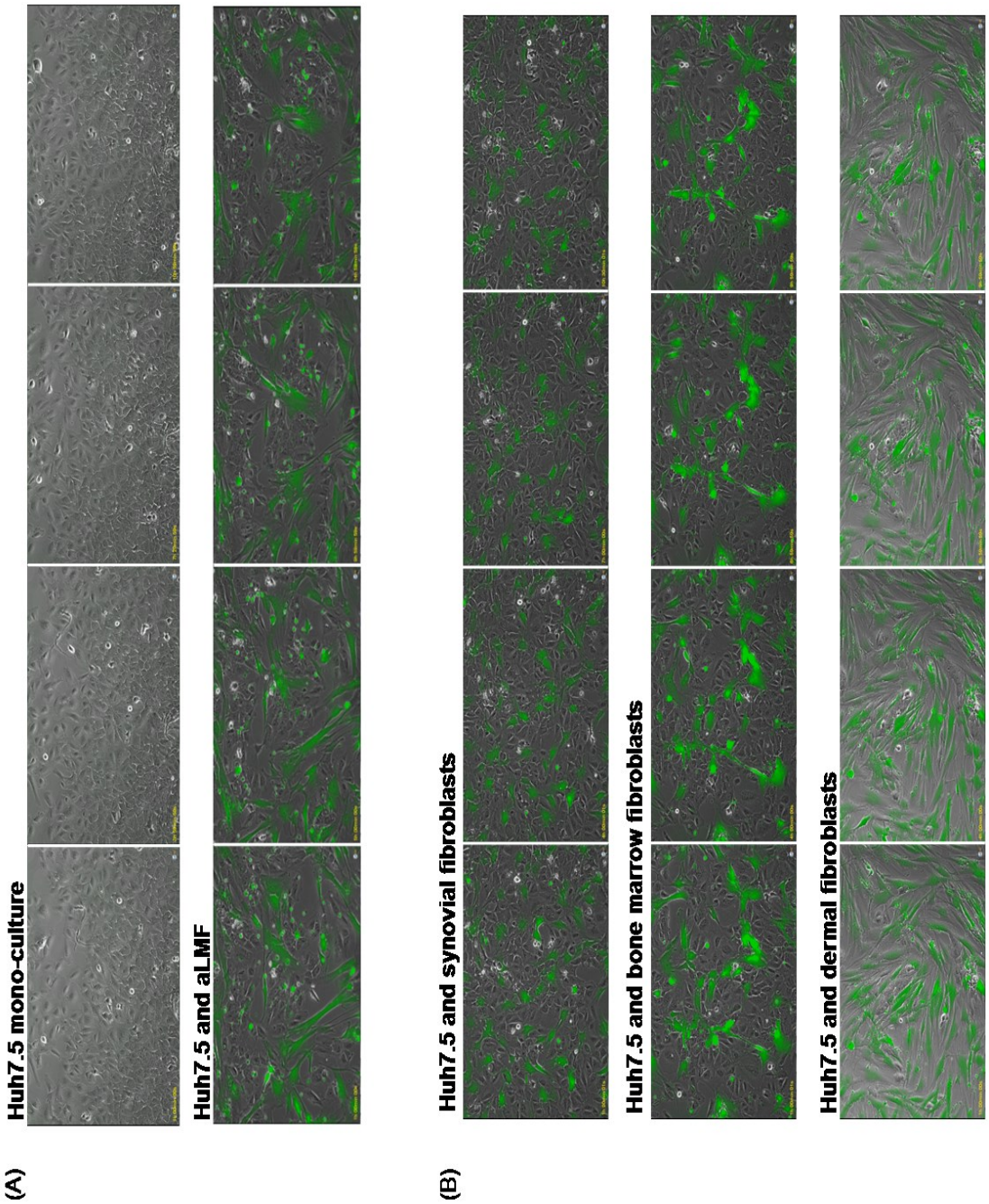
When HSCs become activated to aLMF in response to injury such as viral infection, the aLMF proliferate, synthesise various ECM including collagen, and become more mobile (Iredale 2007; Friedman 2008; Brandao et al 2006). As the mechanism of stromal cell anti-viral activity is dependent on cell contact, we wanted to explore the mobility of aLMF in co-culture. We hypothesized that there would be a difference in aLMF mobility compared to hepatoma cells given the contractile nature of aLMF cells and that this difference could possibly have implications on HCV infection in co-culture.

For this approach, we decided to use the Cell IQ for live cell imaging and cell tracking over time. The Cell IQ maintains normal tissue culture conditions for the duration of imaging and the Cell-IQ SLF (single-label fluorescence) allows detection and quantification of green fluorescence. Huh7.5 cells were co-cultured at a 1:1 ratio with aLMF labelled with CMFDA green cell tracker dye. The cultures were allowed to settle overnight under normal cell culture incubation conditions before the tissue culture plates were inserted into the Cell IQ for live cell imaging. The machine was programmed to obtain images at multiple positions in each well, every 30 minutes on both the phase and green fluorescence channel, which would allow us to distinguish between the unlabelled Huh7.5 cells and the CMFDA (green) labelled aLMF.

The visual analysis of the Cell IQ tracking data and the representative images collected at 1, 4, 7.5, and 11 hours clearly indicate the aLMF (green) are more mobile than the hepatoma cells (unlabelled, grey) when in co-culture, with the

Huh7.5 cells showing minimal mobility in both mono- and co- culture conditions (**Fig.5-2A**). We decided to also track the movement of synovial, bone marrow, and dermal fibroblasts in co-culture with Huh7.5 cells because we wanted to compare the mobility of aLMF to different types of fibroblasts in order to determine if liver derived fibroblasts are more mobile than other types of fibroblasts. As above, Huh7.5 cells (unlabelled, grey) were co-cultured at a 1:1 ratio with the different types of fibroblasts (green) and allowed to settle overnight before the cultures were imaged every 30 minutes on the Cell IQ. When comparing the mobility of these different fibroblasts in the representative images collected at 1, 4, 7, and 10.5 hours, we observed subtle differences (**Fig.5-2B**). Compared to the other fibroblasts, the aLMFs appeared to be more mobile, more flexible and more contractile, changing shape and size more than the other fibroblasts and covering more surface area faster when moving around too. The synovial fibroblasts and bone marrow fibroblasts behaved in a similar manner, with both fibroblast types appearing morphologically smaller and less elongated or spindle like. Both fibroblast types also moved slower than the aLMFs, not contracting as much with the movements appearing to be more localised. The dermal fibroblasts morphologically are more similar to the aLMF displaying a similar thin, stretched and elongated shape and size. The dermal fibroblasts covered more surface area than the aLMF but appeared to move slightly slower than the aLMF. Also the dermal fibroblasts appeared to have occupied majority of the space in co-culture with Huh7.5 cells, more space than any of the other fibroblast types had covered in co-culture.

When we tried to calculate the change in cell migration for the different fibroblasts, a number of key issues were identified. The CMFDA green cell tracker dye used to label the aLMF was visible for 24-36 hours post imaging in the Cell IQ but beyond 36 hours, the green fluorescent signal bleached rapidly over time. This rapid bleaching restricted the time available for imaging and made it increasing difficult to distinguish between the two cell types. The CMFDA bleaching is illustrated over 72 hours and shows a single aLMF moving across the field of view within 153 frames (**Fig.5-2C**). However, the visual analysis of the images provided by the Cell IQ indicate that fibroblasts are more mobile that hepatoma cells.


(A)
Huh7.5 mono-culture
Huh7.5 and aLMF
(B)
Huh7.5 and synovial fibroblasts
Huh7.5 and bone marrow fibroblasts
Huh7.5 and dermal fibroblasts

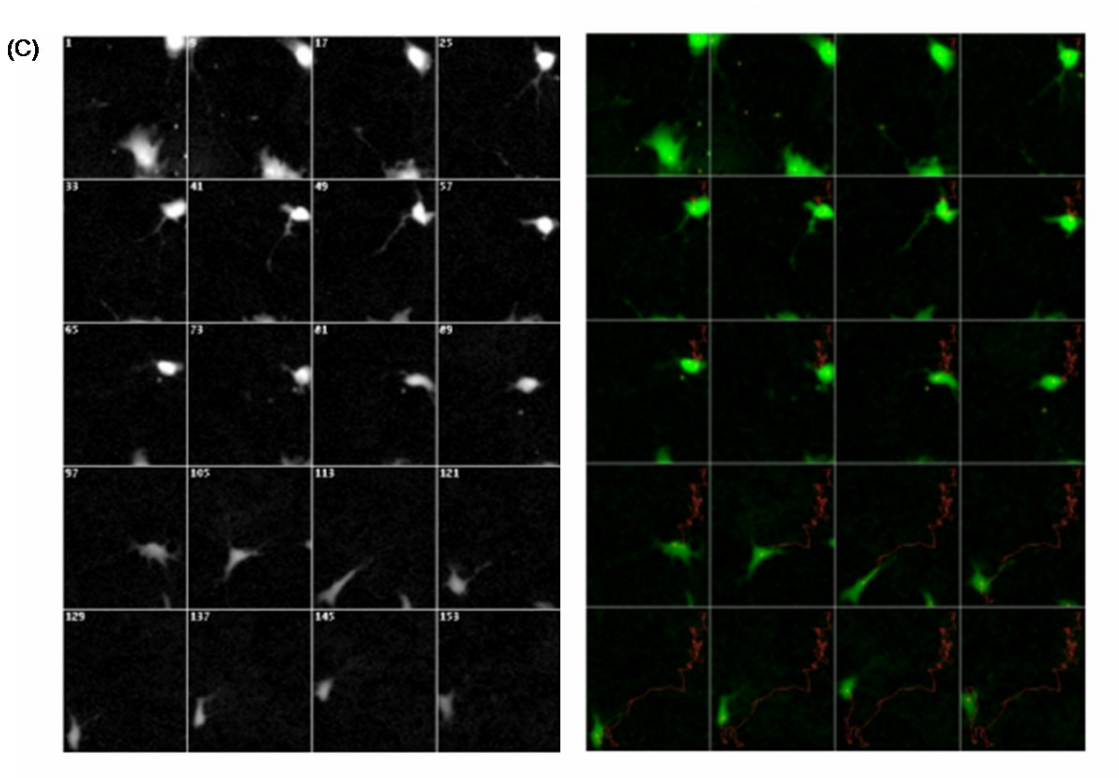



**Figure 5-2 aLMF are more mobile than hepatoma cells**

(**A-C**) Fibroblasts (green) were labelled with CMFDA green cell tracker dye then unlabelled Huh7.5 cells (grey) were seeded in mono-culture and in co-culture at a 1:1 ratio with the different types of fibroblasts: aLMF (**A**), synovial, bone marrow, or dermal fibroblasts (**B**). Cultures were allowed to settle for 24 hours before being placed in the Cell IQ, which was set to image multiple points in each well every 30 minutes over night. Representative images from the live cell time-lapse movies are shown at the indicated time points post incubating in the Cell IQ. (**C**) Using the live cell time-lapse movie on the Cell IQ, a single CMFDA labelled aLMF was tracked over 72 hours using ImageJ software frame by frame, the red line represents the continuous movement of the aLMF tracked frame by frame over 72 hours (tracking analysis performed by Dave Mason).

### 5.3 Binding of HCVcc by aLMF cells or ECM is minimal

Previous research indicates that aLMF play a role in response to liver injury such as viral infection using mechanisms such as synthesis of various ECM including collagen (Kisseleva 2008; Wang 2012; Schulze-Krebs et al 2005; Gomez et al 2009). Therefore, we wanted to investigate whether there is a role for ECM produced by aLMF in the stromal cell mediated anti-viral activity, using mechanisms such as binding HCV particles and consequently depletion of available virus for infection (Jiang et al 2012; Barth 2003; Harmaia 2001; Jiang 2013).

aLMF, LX-2, and the non-permissive control CHO cells were seeded in mono-culture. As a control for the stromal cells, three different hepatoma cell lines, Huh7.5, Huh7, and HepG2, were tested in parallel for their virus binding capacity. Duplicate wells were seeded for testing virus binding to the cells (**Fig.5-3,** left panel) compared to the ECM (**Fig.5-3,** right panel). Naïve Huh7.5 mono-cultures were seeded in order to test the infectivity of virus inoculum collected from the cells or ECM. Select wells were treated with a lysis buffer consisting of PBS containing 0.5% Triton X (v/v) and 20mM ammonium hydroxide ($NH_4OH$), 24 hours post seeding. The lysis buffer was removed once the cells detached, which was observed by phase microscopy, in order to remove the cells and leave the ECM on the tissue culture plate (Butler 2005). The remaining ECM was washed carefully with PBS three times. HCVcc was then added to the wells containing either the cells or ECM alone for one hour, after which the total virus inoculum was removed, spin clarified, and undiluted to the naïve Huh7.5 mono-culture wells. As a control

for virus binding to the tissue culture plate, HCVcc was incubated in an empty well and transferred to the naïve Huh7.5 mono-culture cells. The naïve Huh7.5 mono-cultures with transferred virus inoculum were fixed 48 hours post infection and stained for NS5A in order to enumerate the number of HCV foci. The foci count from the transferred virus inoculum is expressed relative to the empty well control.

The results indicate that the hepatoma (Huh7 and HepG2) and stromal cells (aLMF, LX-2, and CHO cells) bind approximately 10% of the total virus inoculum (**Fig.5-3,** left panel). Similarly, the ECM of the Huh7 and HepG2 bind approximately 10% of the virus inoculum (**Fig.5-3,** right panel). However, the ECM from the aLMF depleted approximately 21%±12.4 and the ECM from the LX-2 depleted approximately 35.6%±18.7 of the virus inoculum, ECM from the non-permissive control CHO cells bound approximately 21.9%±10.0 of the virus inoculum and Huh7.5 cells and ECM bound minimal virus inoculum.

All together, these results suggest that the cells bind small amounts viral particles (approximately 10%) whereas the ECM from the stromal cells can bind slightly more viral particles, depending on the cell type (ranging from approximately 10-30%). However, the level of virus inoculum bound is not equal to the level of viral inhibition seen in contact co-cultures in Chapter 3. Therefore, this suggests the role for stromal cell or ECM binding virus and consequently reducing HCV infectivity is minimal.

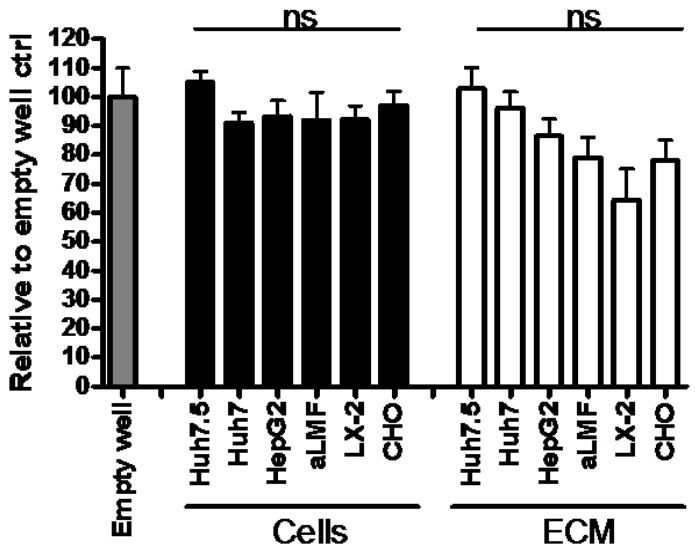



**Figure 5-3 aLMF do not bind virus whereas aLMF ECM depletes virus minimally**

Huh7.5, Huh7, HepG2, aLMF, LX-2 and CHO cells were seeded in mono-culture. Duplicate wells were seeded for testing virus binding to the cells and ECM. The wells for ECM testing were treated with a lysis buffer consisting of PBS containing 0.5% Triton X (v/v) and 20mM ammonium hydroxide ($NH_4OH$), 24 hours post seeding. The lysis buffer was removed once the cells detached, which was observed by phase microscopy, in order to remove the cells and leave the ECM on the tissue culture plate (Butler 2005). High titre HCVcc was added to the wells of cells or ECM and an empty well as a control. The virus inoculum was removed after 1 hour, spin clarified, and the total inoculum added to naïve Huh7.5 'target' cells for 48 hours before the cells were fixed, stained for NS5A, and the number of foci per well counted. Date is graphed relative to the empty well control. Data representative of n=1, aLMF=1. Statistical comparison to the relative empty well control was made using the Kruskal-Wallis test and Dunn's corrections were applied. All error bars show SD.

## 5.4 aLMF in co-culture limit the lateral diffusion speed of CD81 but not EGFR on membrane of hepatoma cells

Previous work in our group showed that polarised HepG2 cells limited lipid, CD81, and HCVpp mobility compared to non-polarised hepatoma cells which in turn is thought to limit HCVpp entry (Harris 2013; Farquhar 2012; Helen 2008). In our co-culture model, we have seen aLMF limit HCVpp entry in various hepatoma cells described in this thesis including non-polarised HepG2 cells (data not shown). We have also seen aLMF alter the polarity of HepG2 cells when in co-culture (**section 4.9**). With this information and given that aLMF reduce HCV infectivity in neighbouring cells using a cell contact dependant mechanism, we wanted to investigate HCV receptor dynamics on hepatoma cells in co-culture with aLMF using live cell imaging. For these studies, we decided to focus on CD81 and EGFR, which is a recently identified entry factor for HCV (Pileri et al 1998;Lindenbach 2005; Wakita 2005; Zhong 2005; Lupberger et al 2011; Diao 2012).

For this approach, we used real-time fluorescence recovery after photobleaching (FRAP) to investigate the effects aLMF have on membrane dynamics when in contact with Huh7.5 cells. We were able to calculate the speed at which the protein can diffuse (diffusion coefficient) and the level of protein present and mobile at the cell surface (mobile fraction) using Huh7.5 cells transduced with either green fluorescent protein (GFP)-tagged CD81 or GFP-tagged EGFR (Harris 2013; Mee 2009; Carter and Sorkin,1998). Huh7.5 transduced cells were seeded in mono- and co- culture with aLMF at a 1:1 ratio on glass bottomed tissue culture dishes. 24 hours post seeding, cells

were imaged on a Ziess Confocal microscope. Huh7.5 cells (green) in direct contact with aLMF (unlabelled) were selected for the FRAP studies and compared to Huh7.5 cells alone in mono-culture (**Fig.5-4A**). The GFP-tagged CD81 or EGFR proteins were photobleached and FRAP measurements taken prior to and after photobleaching. The FRAP data was normalised for background fluctuations and from these measurements we could determine mobile fraction and diffusion coefficient.

We found that there was no significant difference in the mobile fraction (data not shown for mobile fraction) or in the diffusion coefficient of EGFR (**Fig.5-4B**), indicating that there was no difference in the levels of receptor on the surface or in the speed of the receptor diffusion. There was also no difference in the mobile fraction of CD81; however, there was a significant decrease in the diffusion speed of CD81 when we compared the Huh7.5 mono-culture to the co-culture (**Fig.5-4C**).

This reduction in the CD81 diffusion coefficient on Huh7.5 cells when in co-culture offers a potential mechanism by which the aLMF may be limiting HCV entry in neighboring hepatoma cells. HCV requires Claudin-1 to associate with CD81 in order to enter the cell (Meredith et al., 2012b, Farquhar et al., 2011, Farquhar and McKeating, 2008). The complex interactions occurring between the aLMF and hepatoma cells cause a decrease in the speed of CD81 diffusion. This decrease in diffusion speed could in turn result in fewer CD81-Claudin-1 transient associations and therefore fewer viral particles entering the cells, which would result in a decrease in HCV entry.

This mechanism could also explain why we observed a decrease in HCV spreading in co-culture in Chapter 3. Any *de novo* virus, either cell free or from cell-cell transmission, would be limited by the decrease in the speed of CD81 thus limiting overall spread.

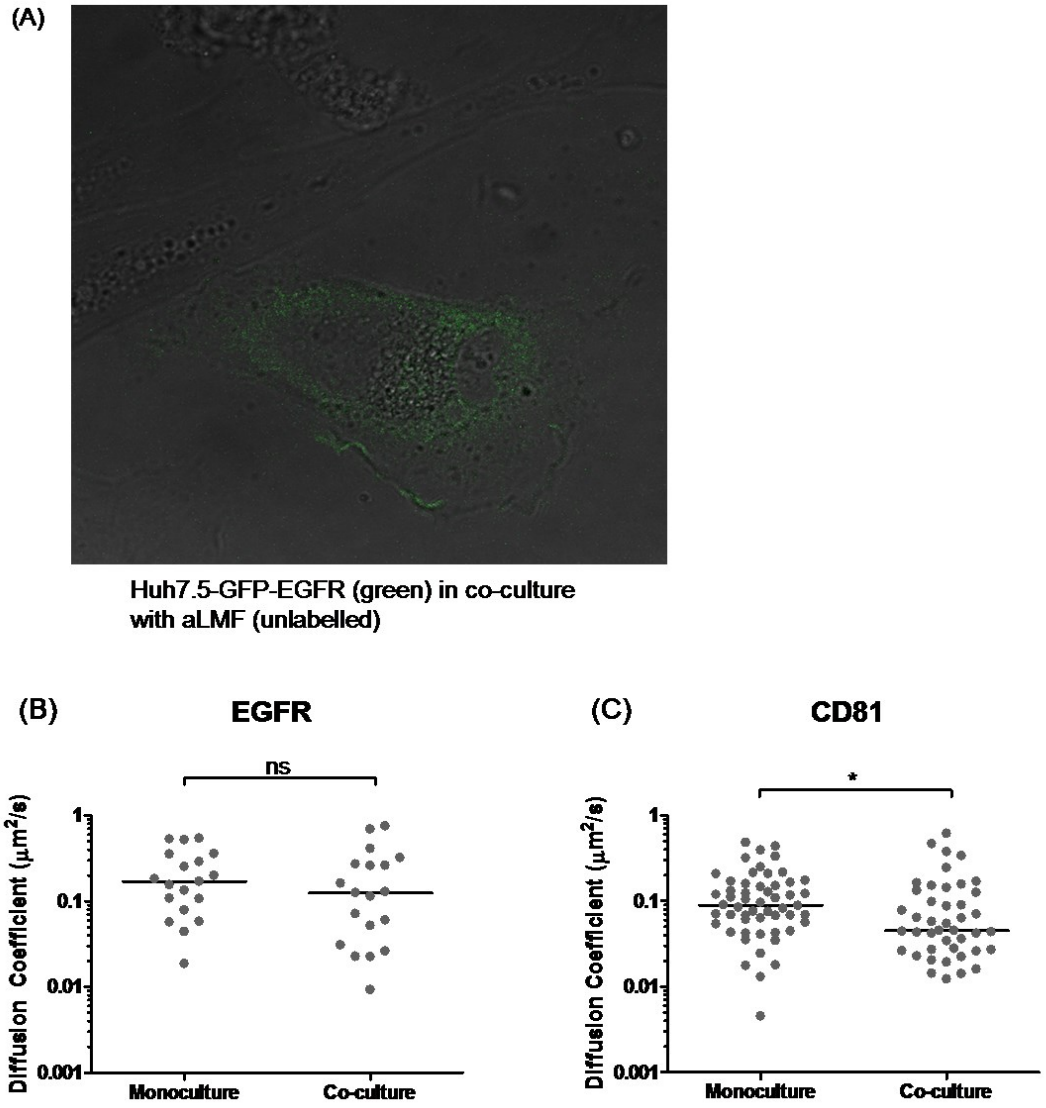



**Figure 5-4 aLMF limit CD81 lateral diffusion on the hepatoma cell membrane but have no effect on EGFR**

(**A**) Representative immunofluorescence image of Huh7.5 cells expressing GFP-EGFR (green) in co-culture with aLMF (unlabelled) 24 hours post seeding. Taken using the Ziess Confocal microscope (100x Plan Apochromat 1.4NA oil immersion objective). (**B-C**) Huh7.5 cells expressing GFP-EGFR (**B**) or GFP-CD81 (**C**) were seeded in mono- or co-culture with aLMF at a 1:1 ratio. 24 hours post seeding, cultures were imaged on a Ziess Confocal microscope. Huh7.5 cells in direct contact with aLMF were selected for FRAP analysis and compared to Huh7.5 cells in mono-culture. The Huh7.5 cells were photobleached and FRAP measurements taken prior to and after photobleaching at 0.08 s per frame. The FRAP data was normalised for background fluctuations. Data representative of n=2, aLMF=2. Statistical comparison was made using non-parametric Mann-Whitney t-tests * $P<0.05$.

### 5.5 Exploring the role of VAP-1 in HCV infection

After identifying the reduction in cell surface CD81 diffusion speed on hepatoma cells as a potential contributing mechanism for aLMF anti-viral activity in co-culture, we wanted to investigate other cell surface proteins that may potentially play a role. Emerging research in the Centre of Liver Research (CLR) identified VAP-1 as a potential target protein, therefore we collaborated with CLR researchers in order to investigate the role of fibroblast expressed VAP-1 on HCV infection in our co-culture system. VAP-1 is a primary amine oxidase found in both bound and soluble form. It has been implicated in the recruitment of leukocyte subtype to the liver where it is highly expressed by aLMF and liver sinusoidal endothelial cells (LSEC). It can also be expressed on endothelium in inflamed skin and normal endothelial cells in the gut, lymphatic endothelium, and follicular dendritic cells (Weston, C.J. and Adams, D.H. 2011; Weston C.J 2014; Lalor 2002).

#### 5.5.1 VAP-1 expression on various liver cell types

First, we wanted to demonstrate the level of VAP-1 expression on cells used in this thesis and in other liver cell populations. RNA was extracted from biliary epithelial cells (BEC), mesenchymal stem cells (MSC), hepatocytes, hepatic sinusoidal endothelial cells (HSEC), a cell line overexpressing human VAP-1 (AxVAP-1), HEK293 cells expressing wild type VAP-1 (HEK293-wt-VAP-1), HEK293 cells expressing enzyme dead VAP-1 (HEK293-Y471F-VAP-1), aLMF from 2 donors (numbered 1 and 2), and hepatic stellate cells (HSC). Some cells were also treated with TNF-α, IFN-γ, or LPS as indicated

in **Fig.5-5A** . Data collected using semi-quantitative PCR and kindly provided by Dr. Chris Weston in the CLR indicate the level of VAP-1 expression in the various different cells types (**Fig.5-5A**). As a positive control, AxVAP-1, HEK293-wt-VAP-1, and HEK293-Y471F-VAP-1 all show high VAP-1 expression. In comparison to these over expressed bands, there are faint bands indicating low level expression of VAP-1 detected in the aLMF donor 1 and HSC both untreated and treated with TNF-α and IFN-γ for 24 hours (Weston C.J 2014).

Specific cells of interest including HSEC, aLMF, HSC, and LX-2 were further analysed for VAP-1 copies using an absolute-quantitative qPCR (**Fig.5-5B**). Data kindly provided by Dr. Chris Weston, show that VAP-1 can be detected at the levels of 1000 or more copies of VAP-1 in HSEC, aLMF, primary HSC, and the LX-2 stellate cell line compared to a negative control cell line (dotted line) (Weston C.J 2014). Taken together, this data reaffirms VAP-1 is expressed on primary aLMF and LX-2 cells, of which both have been shown in this thesis to limit HCV infection in hepatoma cells.

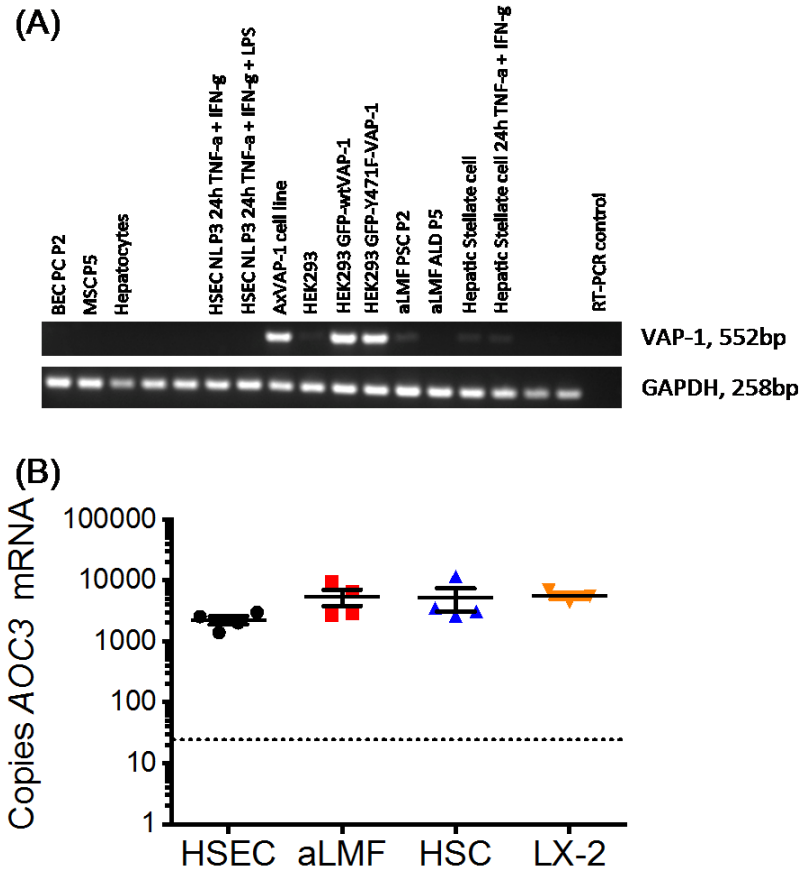



**Figure 5-5 VAP-1 expression on various liver cell types**

Semi-quantitative PCR (**A**) and absolute-quantitative qPCR (**B**) was used to detect the level of VAP-1 expression in various liver derived primary cells and cells lines. (**A**) VAP-1 is expressed at 552 bp and the control GAPDH at 258 bp. (**B**) Data is expressed as VAP-1 copies as determined by comparing results to a calibration curve of diluted VAP-1 expressing plasmid. The dotted line indicates the level of VAP-1 detected in a negative control cell line. Data kindly provided by Dr. Chris Weston in the CLR. BEC, biliary epithelial cells; MSC, mesenchymal stem cells; HSEC, hepatic sinusoidal endothelial cells; HSC, hepatic stellate cells.

### 5.5.2 Inhibiting VAP-1 restores HCV infection in co-culture

After establishing that the aLMF express VAP-1, we next wanted to investigate the role of aLMF expressed VAP-1 in the reduction of hepatocellular HCV infection when in co-culture. For this approach, we used the mono-clonal antibody BTT1023 to inhibit VAP-1 activity. Huh7.5 cells were seeded in mono-culture or co-culture with aLMF or LX-2. Then 24 hours after seeding, cultures were either left untreated or treated with 20μg/mL BTT1023. Cultures were infected 1 hour after BTT1023 treatment with HCVcc in the absence or presence of the antibody. Cells were fixed and stained for NS5A 48 hours after infection and the level of infection enumerated by counting NS5A positive cells (**Fig. 5-6**).

Dramatically, the co-cultures treated with the antibody BTT1023 showed levels of infection similar to that of the Huh7.5 mono-culture. This restoration in the level of HCV infection suggests that VAP-1 expression on stromal cells plays a key role in anti-viral activity.

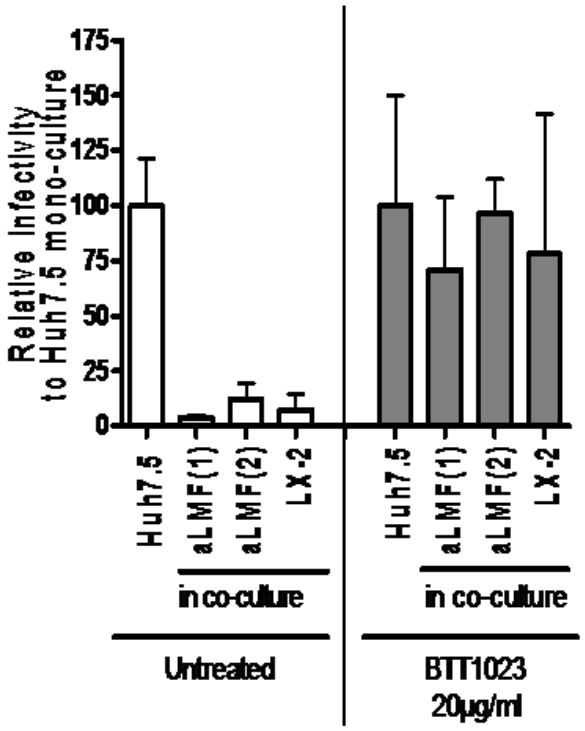



**Figure 5-6 Inhibiting VAP-1 restores HCV infection in co-culture**

Huh7.5 cells were seeded in mono-culture or co-culture with aLMF or LX-2 cells at a 1:1 ratio. 24 hours post seeding, cultures were treated with 20µg/mL BTT1023 mono-clonal antibody targeting VAP-1 for 1 hour prior to infection with HCVcc. After 48 hours in the absence or presence of BTT1023, cells were fixed, stained for NS5A, and the level of infection counted as the total number of infected cells per well. Data expressed relative to the Huh7.5 mono-culture condition. Data representative of n=3 independent repeats (n=4 aLMF donors). Statistical comparison was made using the Kruskal-Wallis test (non-parametric) and Dunn's corrections were applied. All error bars show SD.

### 5.5.3 Anti-viral activity of rVAP-1 is dose dependant

Having identified VAP-1 as a key player in stromal cell anti-viral activity, we next wanted to establish the level at which VAP-1 needs to be expressed in order to have an anti-viral effect. For this approach, we used a recombinant form of VAP-1 (rVAP-1) and Huh7.5 cells, which we confirmed, did not express VAP-1 (**Fig 5-5A**). Huh7.5 cells were seeded in mono-culture and 24 hours post seeding treated with rVAP-1 at 100, 300, and 1000 ng/mL or left untreated as a control. Cultures were infected with HCVcc 1 hour after being treated with rVAP-1 and the infection proceeded in the presence of rVAP-1.

Analysis of the data indicates that rVAP-1 inhibits HCVcc infection in a dose dependant manner with HCV infection decreasing as the doses of rVAP-1 are increased (**Fig 5-7**). Preliminary data demonstrated rVAP-1 inhibits HCVpp entry, VSVpp entry and inhibits HCV replication in Huh7A2HCV Replicon co-cultures in a similar manner (data not shown as it was preliminary data which required more biological repeats especially as there had been some experiments in which the VAP-1 batches varied slightly in activity).

This data combined with the data in **section 5.5.2** indicates that both the surface expressed and soluble forms of VAP-1 have the ability to reduce HCV infection of hepatoma cells. The mechanism by which VAP-1 decreases HCV infection needs to be further explored. One hypothesis is that enzymatic activity is responsible for the reduction of HCV infection *in vitro*. *In vivo* high expression of VAP-1 in the diseased liver could also be interacting with various immune cells that would play a role in controlling HCV infection and

could provide an additional mechanism by which VAP-1 may exert anti-viral activity *in vivo* but it could also be interacting with stromal cells as shown here (Bonder 2005; Lalor 2002 recruitment; Lalor 2002 VAP; Lalor 2007; Lee 2013).

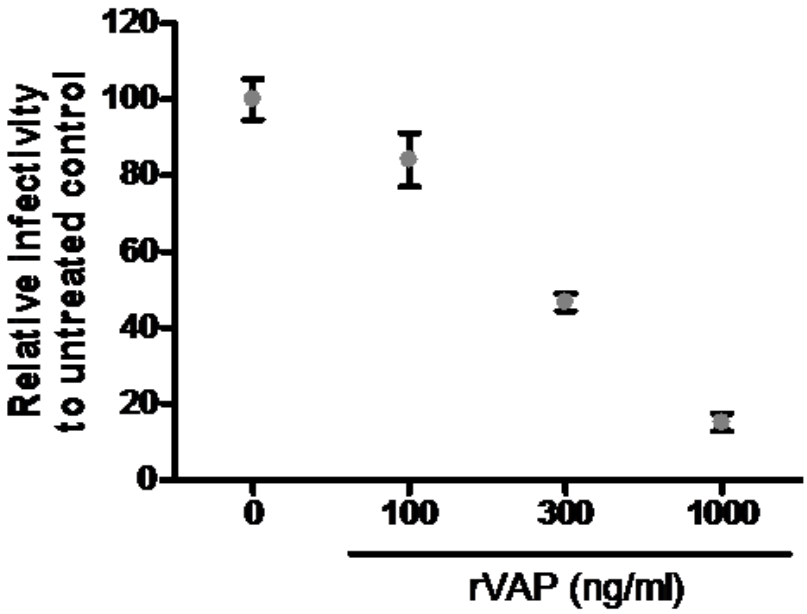



**Figure 5-7 Anti-viral activity of rVAP-1 is dose dependant**

Huh7.5 cells in mono-culture were treated 24 hours after seeding with 100, 300 and 1000 ng/mL rVAP-1 or left untreated as a control. Then 1 hour after treatment, cultures were infected with HCVcc and the infection proceeded in the absence or presence of rVAP-1. After 48 hours, the cells were fixed, stained for NS5A, and the level of infection enumerated as the total number of infected cells per well. Data is expressed relative to the untreated Huh7.5 mono-culture condition. Data representative of n=5 independent repeats. Statistical comparison was made using the Kruskal-Wallis test (non-parametric) and Dunn's corrections were applied. All error bars show SD.

### 5.5.4 rVAP-1 does not affect the speed CD81 diffuses

In **section 5.4**, we found that aLMF in co-culture limits the speed CD81 diffuses on the hepatoma cell membrane, which could explain their ability to reduce HCV infection in neighbouring hepatoma cells. We next wanted to determine if the decrease in CD81 diffusion speed is linked to aLMF expressed VAP-1.

For this approach, mono-cultures of Huh7.5 cells transduced with GFP-tagged CD81 were treated with either 1000ng/mL rVAP-1, control buffer, or left untreated one hour prior to imaging on a Ziess Confocal microscope. The GFP-tagged CD81 proteins were photobleached and FRAP measurements taken prior to and after photobleaching. The FRAP data was normalised for background fluctuations and from these measurements we could determine the mobile fraction and diffusion coefficient of CD81.

The data shows that after treating the Huh7.5 cells with a high dose of rVAP-1, there was no significant difference in the level of CD81 present and mobile at the cell surface (**Fig.5-8A**) and no change in the diffusion coefficient of CD81 (**Fig.5-8B**) when compared to the controls. With this data, we can conclude that the anti-viral action of VAP-1 is independent to the ability of aLMF to limit CD81 dynamics. Thus, the stromal cell anti-viral activity is mediated by two independent mechanisms.

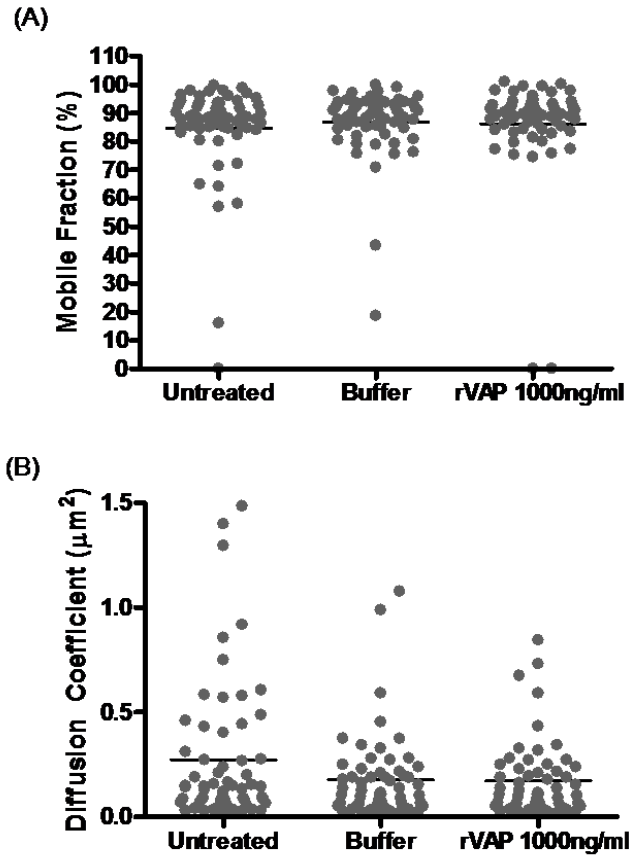



**Figure 5-8 rVAP-1 does not affect CD81 mobile fraction or diffusion coefficient**

Huh7.5 cells expressing GFP-CD81 were treated with 1000 ng/mL rVAP-1, control buffer, or left untreated as a control 24 hours post seeding in mono-culture. One hour post treatment, the cells were imaged on a Ziess Confocal microscope and the GFP-tagged CD81 proteins were photobleached and FRAP measurements taken prior to and after photobleaching. The FRAP data was normalised for background fluctuations. Data representative of n=2 independent repeats. Statistical comparison was made using non-parametric Mann-Whitney t-tests.

## 5.6 The ability of aLMF to reduce HCV infection is not specific to liver myofibroblasts

To determine if the mechanism by which aLMF can reduce HCV infectivity in neighbouring cells was specific to liver derived fibroblasts or a global affect, we decided to use fibroblasts isolated from different sites of the body in co-culture with infected hepatoma cells. Dermal, synovial, and bone marrow fibroblasts were isolated and supplied by the Centre for Translational Inflammation Research. The main function of fibroblasts in general is to assist in maintaining the structure of connective tissue by secreting various ECM which vary depending on their location. Phenotypically there are subtle differences between the fibroblasts from different sites. Bone marrow and synovial fibroblasts display a smaller size and shape compared to dermal fibroblasts which have a more elongated shape similar to aLMF (**Fig.5-9A**). These fibroblasts from different sites are also non-permissive to HCV infection (data not shown) as we established with aLMF (**section 3.2**).

Dermal fibroblasts (DM), synovial fibroblasts (SY), bone marrow fibroblasts (BM), LX-2, and control CHO cells were co-cultured with Huh7.5 cells at a 1:1 ratio, either in contact or separated by transwell inserts as detailed in **section 4.1**. Cultures were infected with HCVcc to investigate the complete virus lifecycle (**Fig.5-9B**) or infected with pseudoparticles HCVpp, VSVpp, and NEpp to investigate the entry step of viral infection (**Fig.5-9C-D**). All infections were allowed to proceed for 48 hours before the cultures infected with HCVcc were fixed and stained for NS5A and cultures infected with

pseudoparticle viruses were lysed to allow the detection of the luciferase reporter gene activity.

The results from co-culturing the various fibroblasts with hepatoma cells mimic the data obtained using aLMF. All the fibroblasts tested in co-culture have the ability to reduce HCVcc infection in a cell contact dependent manner by approximately 70-80% (**Fig.5-9B**) and HCVpp entry by approximately 50-75% (**Fig.5-9C**) compared to hepatoma mono-cultures. There was no significant difference in the level of HCVcc infection when comparing across the fibroblasts in contact or in transwell. This preliminary data also suggests that fibroblasts reduce HCVpp entry more significantly then VSVpp entry, which may indicate that these fibroblasts are targeting an entry step specific to HCV entry (**Fig.5-9C**).

Taken together, these data indicate the reduction in HCV infection in co-culture is not specific to liver fibroblasts as primary fibroblasts from other sites are capable of reducing HCV infection in a similar manner to aLMF. This data could lead to future studies identifying a mechanism of action such as a membrane expressed protein or function common to all fibroblasts, which may be contributing to the anti-viral responses.

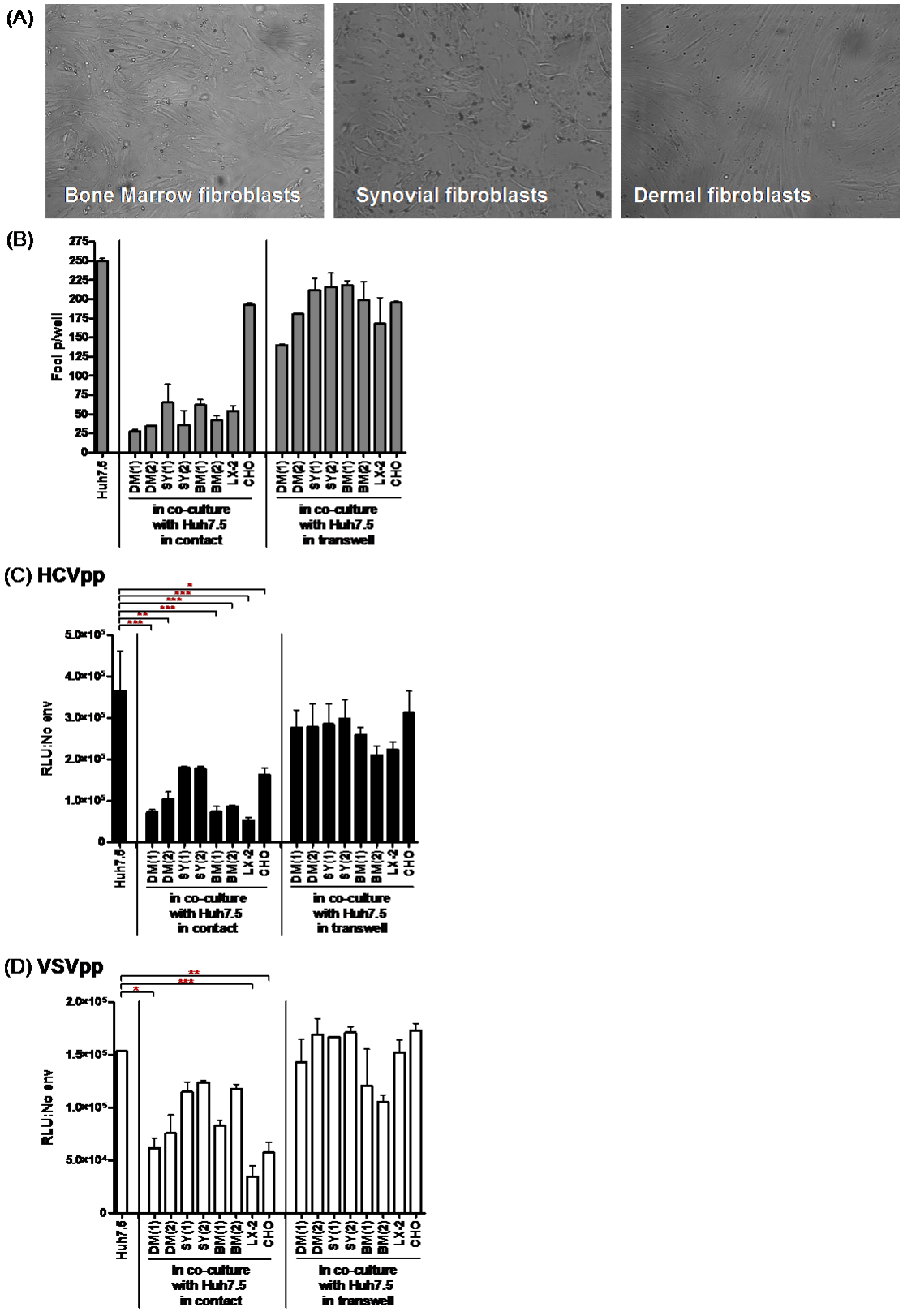



**Figure 5-9 The ability of aLMF to reduce HCV infection is not specific to liver myofibroblasts**

(**A**) Representative phase contrast images of aLMF (magnification x10). (**B-D**) Dermal (DM), synovial (SY), and bone marrow (BM) primary fibroblasts

along with LX-2 and the control non-permissive CHO cell were seeded in co-culture with Huh7.5 cells at a 1:1 ratio either in contact or separated using transwell inserts. The transwell inserts were added to the wells containing Huh7.5 cells 3 hours after seeding. 24 hours post adding the transwell inserts to the wells, the co-culture conditions were infected with either HCVcc (**B**) or the pseudoparticles HCVpp (**C**), VSVpp (**D**) and NEpp. 48 hours after infection, (**B**) HCVcc infected cultures were fixed, stained for NS5A, and the number of cells per foci were enumerated. Statistical comparison was made using the Kruskal-Wallis test and Dunn's corrections were applied. 48 hours after infection, (**C-D**) pseudoparticle infected cultures were lysed and the luciferase activity measured. Data is expressed as relative light units (RLU) of HCVpp (**C**) or VSVpp (**D**) normalised to the NEpp control (Luc:No env). Data representative of n=1 independent repeat (n=2 DM, n=2 SY and n=2 BM donors). Statistical comparison was made using one way ANOVA tests to compare groups of data and Bonferroni's corrections (parametric) were applied for pair wise comparisons of all data. * $P<0.05$, ** $P<0.01$ and *** $P<0.001$. All error bars show SD.

## 5.7 Discussion

The data in Chapter 3 demonstrates that stromal cells, both HSC and aLMF, can significantly limit HCV the level of HCV entry, replication and spread of infection in neighbouring hepatoma cells via a cell contact dependent mechanism. In the process of elucidating the mechanism of action, we were able to rule out a number of potential pathways that would have indicated a typical anti-viral response such as the innate immune response. We also demonstrated in Chapter 4 that some potential mechanisms which could alter hepatocyte function and thus HCV infection, such as albumin production and differentiation status, does not contribute to the ability of aLMF to reduce HCV infection. We then decided to focus how stromal cells could be acting on hepatoma cells in a cell contact dependant manner leading to inhibit HCV infection and factors which may influence the cell-cell contacts formed between these two cell types.

Stromal cells are located in the Space of Disse, where they are in direct contact with hepatocytes allowing these non-parenchymal cells to support and maintain hepatocyte function via multiple complex interactions. Also, as the HCV particle enters the liver it must pass through the sinusoids, across the sinusoidal endothelial into the Space of Disse in order to infect the hepatocytes, during which the viral particles could possibly, come into direct contact with HSCs ((Perrault 2009; Pohlmann, Zhang et al. 2003; Lozach, Amara et al. 2004; Lai, Sun et al. 2006; Lavon and Benvenisty 2005). Studies have indicated that direct contact of HCV particles or proteins with HSC can lead to their activation which then induces fibrosis (Mazzocca et al 2002;

Mazzocca 2005; Bataller et al 2004; Wang et al 2013). HSCs are the main source of aLMF, the key cells involved in fibrosis responsible for depositing ECM, described as elongated cells with highly proliferate, contractile and mobile qualities (Mederacke et al 2013; Taub 2004; Schulze-Krebs et al 2005; Gomez et al 2009; Friedman 2008; Brandao et al 2006)

The preliminary data investigating aLMF mobility using the Cell IQ machine highlighted some issues which would require optimisation in the future, but we were able to observe from this data the general differences in cell mobility. aLMFs are more mobile in co-culture compared to Huh7.5 cells, and in general, the fibroblasts from different sites were also more mobile than the Huh7.5 cells. The initial attempt at tracking the aLMF mobility raised an interesting point on which part of the cell should be tracked in our investigation, the cell nucleus or whole cell. A nuclear stain could be used to track aLMF mobility. However given the shape and size of the aLMF compared to the Huh7.5 cells and seeing as cell-cell interactions appear to be the key to aLMF limiting HCV infectivity, a more informative way of tracking the aLMF would be to track the whole cell. A possible technique would be to use a fluorescently tagged protein expressed on the aLMF membrane that doesn't bleach as fast over time. Providing we could optimise the tracking and quantification, we could measure the rate of mobility in order to determine if the different rates correlated to differing degrees of HCV inhibition when in co-culture. If so, we could then use various techniques such as agarose overlays, altering the temperature of live cell cultures to halt membrane dynamics or chemical inhibitors of cell migration or proliferation, to alter

fibroblast mobility and cell-cell contacts and then monitor how this also impacts HCV inhibition in co-culture.

Understanding the impact of aLMF mobility on neighbouring hepatoma cells and HCV infection could elucidate potential mechanisms to target during fibrosis stages of HCV infection to either promote the inhibition of HCV infection or minimize the damage of fibrosis. During fibrosis, aLMF lay down ECM, which under normal circumstances is degraded once the liver has repaired returning to a normal healthy state. However, when the ECM is not degraded and aLMFs continue to proliferate, the liver becomes fibrotic which can become chronic, leading to hepatocellular carcinoma as in many HCV infected patients (Wynn 2008; Kisseleva et al 2011; Kisseleva et al 2008; Selden, Khalil et al. 1999; (Blight, McKeating et al. 2003; Lindenbach, Evans et al. 2005; Farquhar and McKeating 2008;). Given the role of fibroblasts is to support the hepatocytes and one key mechanism by which this is achieved is the production of ECM, it was interesting to observe that neither the aLMF cells nor the ECM they produced can bind or deplete cell free HCV particles. Thus ruling out the role of aLMF and the ECM they produce in trapping hepatitis C viral particles. In this experimental design, when the cells were incubated with the virus inoculum, there is a high probability of various ECM components also present in combination with the cells. Interestingly, this potential combination of cells and ECM still bound less virus inoculum than the ECM itself, but one possibility could be the methodology used to remove the cells. There are a number of different methods in the literature which can be used to remove cells from tissue culture plastic leaving the ECM behind

but whether these methods destroy components of the ECM or leave membrane debris behind will need to be determined as they could impact the outcome of the assay. It could also be beneficial to measure the differences in amount of ECM produced and level of different ECM components produced when stromal cells are in mono- and co- culture, for example by using an ELISA. The data here used ECM produced by stromal cell mono-cultures which does not take into account that the reduction was seen in co-culture conditions where the mechanism is a cell contact dependent mechanism, and there are many complex cell-cell interactions and paracrine signalling pathways between both cells types which may impact ECM production (Crapo 2011).

Investigating the effect of aLMF contact on Huh7.5 membrane dynamics revealed two independent mechanisms which are contributing to aLMF limiting HCV infection. aLMF can limit CD81 lateral diffusion on Huh7.5 cells which in turn could lead to reduced HCV entry dynamics and an overall reduction in the level of HCV infection. aLMF also express VAP-1 on the surface and as a soluble secreted form. We have demonstrated both forms of VAP-1 appear to have anti-viral properties however as rVAP-1 failed to alter CD81 lateral diffusion it indicates the mechanism by which VAP-1 is anti-viral, is independent to CD81 dynamics. The mechanism by which VAP-1 has anti-viral properties is yet to be determined and there is still a lot to be understood about VAP-1. Some known functions of VAP-1 which could be of importance in determining its anti-viral mechanism include its primary amine oxidase enzymatic activity responsible for generating aldehydes, ammonia and $H_2O_2$,

which leads to the secretion of chemokine via NFκB dependent mechanism (Bonder 2005; Lalor 2002 recruitment; Lalor 2002 VAP; Lalor 2007; Lee 2013). Recently, the amine oxidase activity HSC and aLMF expressed VAP-1 has been shown to modulate leukocyte migration. Given that we know lymphocyte assisted HCV particles have enhanced hepatocyte infectivity, it would be interesting to expand this co-culture model and investigate if HSC or aLMF can differentially modulate leukocyte migration in the favour of HCV infection and if the anti-viral contact based mechanisms can counter the potential increased infection from infiltrating lymphocytes (Weston 2014; Stamataki 2009).

Testing fibroblasts from different sites revealed that these cells can also inhibit HCV infection which signifies that the mechanism is not specific to liver myofibroblasts. Different pseudoparticle viruses were also tested including measles, lassa and murine leukaemia virus (MLV) in our co-culture system (data not shown). These data showed fibroblasts can also inhibit the entry of other viruses and so this model could possibly be expanded to investigate the role of stromal cells on other hepatotrophic viruses such as HBV.

To determine the specificity of the mechanisms identified, future work would be needed to investigate whether the fibroblasts from different sites could also limit CD81 lateral diffusion and if they to express VAP-1. However, in the context of understanding the role of stromal cells on hepatocellular HCV infection, the data suggests aLMF and LX-2 cells may be inhibiting HCV infection of hepatoma cells with VAP-1 expressed at the membrane or the

soluble released form. This mechanism is independent to the ability of aLMF to reduce CD81 lateral diffusion speed thus leading to a reduction in HCV infection. These observations uncover a new role for VAP-1 to regulate HCV replication and provide new therapeutic avenues for treating both the underlying inflammatory response and viral replication in chronic hepatitis.

# Chapter 6 General Discussion

The HCV replicon system and successful development of infectious HCV particles in cell culture (HCVcc), were the first *in vitro* systems that studied HCV infection and since then, significant progress has been made in understanding the HCV lifecycle and developing *in vitro* models to study HCV (Wakita et al 2005; Zhong et al 2005; Lindenbach et al 2005). Recent advances in the field include the approval of a number of DAAs including the two protease inhibitors Simeprevir and Faldaprevir, and the NS5A polymerase inhibitor Sofosbuvir. These DAAs have shown higher cure rates than the previously developed DAAs, Boceprevir and Telaprevir. Despite the very promising cure rates, shorter treatment times, and fewer side effects with the latest DAAs, a patient's response to such DAA treatments is genotype specific, predominantly benefiting patients with either genotype 1, 2 or 3, and unfortunately the therapies are also very costly (Welch and Jensen 2014, Manns et al 2014, Zeuzem 2014; Lawitz 2013; Jacobson 2013).

In patients with chronic HCV, successfully treating the underlying viral infection has been shown to reverse fibrosis; however many patients often suffer severe cirrhosis, HCC or liver failure (Su et al 2014; Wynn et al 2008; Friedman et al 2008; Friedman et al 2007). Although liver transplantation in HCV infected patients is a successful treatment option, the new liver becomes reinfected with circulating viruses leading to recurrent disease and poorer prognosis (Mutimer et al 2006; Rowe et al 2008). Thus, HCV infection is still a major burden on liver transplantation. With no vaccines available there remains a need to develop a HCV vaccine and novel HCV therapies to treat

current HCV infected patients, which are more cost effective and beneficial to patients suffering from more advanced liver diseases and a wider range of HCV genotypes.

During chronic viral hepatitis, infected hepatocytes release various profibrogenic factors which activate HSCs into aLMF cells as part of the antiviral mechanisms designed to clear the infection. HSCs are the primary source of aLMF and it is these aLMF cells which are the key player in liver fibrosis. Liver fibrosis is defined as an excessive healing response with scarring and excessive ECM deposition, particularly collagen, in response to continuous liver injury (Kisseleva et al 2008; Su et al 2014; Mederacke et al 2013; Wynn 2008; Kisseleva et al 2011). Despite these cell types playing such a key role in fibrosis progression and HCV infection being a leading cause of liver fibrosis, there is little research into the understanding of the role of liver fibroblasts in hepatocellular HCV infection.

Some studies have shown that HCV proteins E2, core, and NS3-NS5 can mediate liver fibrosis via a few mechanisms such as inducing profibrogenic responses and pro-inflammatory cytokines in HSCs (Zhan et al 2006; Jiang et al 2008; Deng et al 2008; Wang et al 2013; Mazzocca et al 2002; Bataller et al 2004). However many of these finding are based on physiologically irrelevant systems which do not co-culture HSCs with hepatocytes to mimic the liver microenvironment but instead stimulate the HSCs in isolation using recombinant HCV proteins or artificial chemical stimulation such as poly I:C and transfer the conditioned media to hepatoma cells in isolation to examine

the effects on HCV infection. This technique allows the cytokines and soluble factors to be studied but ignores the role of cell-contact dependent mediators which may alter the profibrogenic mechanisms reported. Other studies have used fibroblasts such as 3T3 mouse fibroblasts or stroma in co-culture systems to maintain human hepatocyte function, which is another key function of stromal cells within the liver and physiologically, these studies are trying to model the liver microenvironment more accurately but unfortunately utilise irrelevant stroma or stromal cells instead of human derived primary stromal cells (Ploss 2010; Khetani and Bhatia 2008; Hui and Bhatia 2007; Bhatia 1999).

More research into the role of non-parenchymal cells in HCV infection is needed in order to identify host pathways involved in HCV disease progression which can be targeted for novel therapies. Studying the complex interplay between two cell types is not without its difficulties. However, such studies can identify novel findings such as the recent study investigating the role of LSEC on HCV infection. It was previously thought that LSEC would bind HCV particles in the sinusoids and transfer the virus to hepatocytes, but work by Rowe et al (2014) revealed that LSEC actually acted to limit HCV infection via cell contact-dependent mechanisms and soluble factors. The soluble factor VEGF-A secreted by hepatocytes suppresses LSEC expression of BMP4, a proviral factor which normally promotes HCV replication, thus highlighting a potential new therapeutic target (Protzer et al 2012; Goulet et al 1988; Rowe et al 2014).

This study investigated the role of stromal cells in HCV infection, focusing on the HSC and the activated form of HSCs, the activated liver myofibroblast (aLMF). The LX-2 stellate cell line was used to represent the HSC found in the liver alongside primary aLMFs in our study, neither of which supports HCV infection as they lack expression of all four key HCV receptors required for successful viral infection. Using co-culture systems, we were able to show that the non-permissive stromal cells could limit HCV infection of neighbouring hepatocytes at the entry step, replication stage, and limit spread of HCV infection in a cell contact dependent manner. Though hepatocytes are the major site of HCV replication and the virus appears to be efficient at replication with $10^{12}$ viral particles produced per day, the level of infected hepatocytes in the liver is low, at an estimated 10-25%. There are a number of known factors which contribute toward this, including the high mutation rate and genetic diversity which assist the virus in escaping immune recognition but may also result in defective HCV particles ((Murray et al., 2008, Fishman et al., 2008, Weissenborn et al., 2009, Weissenborn et al., 2004); Vogel 2009;(Gomez et al., 1999); Powers et al 2006; Liang et al 2009). Given the close proximity of stromal cells to hepatocytes in the liver, and the data presented here indicating that stromal cells can significantly inhibit the levels of hepatocellular HCV infection, we can suggest that stromal cells also contribute towards the low levels of HCV infected hepatocytes detected in the liver.

Given that the role of fibroblasts in the liver is to respond to chronic inflammation through various stimuli such as viral infection, as part of the host

immune response, one could argue that these observations would have been expected (Wynn 2008; Kisseleva et al 2011). Fibroblasts can detect viral infection via a number of mechanisms; for example, they express toll-like receptors (TLRs) which can detect viral genomic material and they respond to various profibrogenic cytokine stimuli released by infected hepatocytes (Novo et al 2014; Holt et al 2008; Zhan et al 2006; Jiang et al 2008; Deng et al 2008; Wang et al 2013). However, our investigations into the mechanism by which fibroblasts can reduce HCV infection, led us to quickly establish that the mechanism was a cell contact dependent mechanism, ruling out soluble factors such as cytokines. We also used inhibitors to rule out the involvement of both the VEGF and NO pathways, however, this data is preliminary and future assays should also include additional positive controls. Despite the anti-VEGF antibody having already been extensively used by our research group, the addition of exogenous BMP4 to increase HCV infection and BMP4 combined with the anti-VEGF antibody used in this study will provide positive controls to indicate the anti-VEGF antibody can inhibit HCV infection levels caused by BMP4 increasing hepatocyte permissivity to support HCV infection (Rowe et al., 2014). The Griess assay was used to assess supernatants for the presence of nitric oxide (NO) in untreated co-cultures compared to co-cultures treated with a NO inhibitor. Even though all the supernatants tested were negative for NO production, additional positive controls such as poly I:C stimulation of culture leading to the production of NO, could have been included on the Griess assay to prove the NO pathway can be stimulated in the cells tested. A human anti-viral PCR array containing 84 key genes involved in the innate immune response was one of the techniques used to

rule out the role of the innate immune response in the ability of aLMFs to limit hepatocellular HCV infection. The PCR array allowed us to compared 4 samples but the data could be expanded with additional anti-viral PCR array kits. The additional kits would allow us to run extra samples, in particular mono-cultures of fibroblasts uninfected and fibroblasts infected with HCV in order to help better interpret the array data is this study and better understand which signalling pathways are regulated as a result of the fibroblasts being stimulated from contact with HCV particles compared to signals from a neighbouring infected hepatocytes.

Stromal cells create the microenvironment in the liver which supports the hepatocytes to function via cell contacts and secreting various cytokines. In addition to releasing various cytokines, stromal cells can also produce various ECM components, particularly different types of collagen, which tends to accumulate during fibrosis; the liver's natural wound healing response. As fibrosis is triggered in HCV infected livers, we continued our investigation into the mechanism being related to the wound healing function of fibroblasts and their function as supportive cells to hepatocytes. We were able to demonstrate minimal virus binding to the fibroblast cells or the ECM produced by the fibroblasts in this study thus eliminating the binding and depletion of circulating virus in the liver by fibroblasts or their ECM as a potential mechanism. A number of previous studies have used stromal cells as supportive cells to maintain hepatocyte function in cell culture and so this lead us to investigate the differentiation status of hepatocytes in co-culture. We hypothesized the stromal cells could be differentiating the hepatoma cell line

used in this study, pushing it more towards a hepatocyte like differentiation status which could lead to a decrease in infection, similar to the low levels of infection observed with highly differentiated PHHs. However the differentiation status of the hepatoma cell line used in our co-culture system remained unchanged over time when in co-culture with stromal cells thus ruling out this mechanism also. The differentiation status of the hepatoma cell line used may have remained unchanged because this particular cell line is incapable of becoming differentiated and not because the stromal cells can not differentiate hepatocytes or maintain primary hepatocyte differentiation status in cell culture.

Two cell contact dependent mechanisms by which the fibroblasts can inhibit HCV infection in hepatocytes were identified in this study. The first mechanism is that aLMF can limit CD81 lateral diffusion on hepatocytes which would explain the ability for aLMF to limit HCV entry. Previous studies have shown that a decrease in CD81 lateral diffusion leads to fewer CD81-Claudin-1 complexes on the hepatocyte cell membrane, which in turn limits HCV entry leading to decreased HCV infection. The interaction between HCV particles and the receptors expressed by hepatocytes is key to facilitating the successful entry and infection of HCV. This mechanism could also potentially explain why we observed a decrease in the spread of HCV infection in co-culture. Any *de novo* cell free or cell-cell transmitted virus would have difficulty in overcoming the decreased CD81 diffusion coefficient thus also limiting the overall spread of infection (Harris 2013; Harris 2010; Meredith 2012).

The second mechanism identified in this study involved the fibroblast expressed VAP-1 molecule, which is expressed at the membrane surface but can also be secreted in a soluble form. VAP-1 in an amine oxidase that has previously been shown to recruit leukocyte subtypes to the liver and elevated levels of the soluble secreted form of VAP-1 have been detected in patients with chronic liver disease, correlating with the level of fibrosis (Weston 2014; Weston 2011). We were able to demonstrate that stromal cell expressed VAP-1 has the ability to inhibit HCV infection which can be restored following inhibition of VAP-1 using a mono-clonal antibody targeting VAP-1. Preliminary data indicates that stromal expressed VAP-1 can inhibit HCV infection at the entry step and replication stage. Recombinant VAP-1 (rVAP-1) was used to show that the anti-viral activity of rVAP-1 is dose dependent but does not contribute to the ability of aLMF to limit CD81 lateral diffusion, thus indicating the two mechanisms are independent.

Some studies have investigated inhibiting VAP-1 as a potential therapy for preventing liver disease progression; however in the context of the data in this study, patients with HCV may not benefit from this therapy as inhibiting VAP-1 increased the level of HCV infection in our study (Weston 2014; Lalor et al 2002). Therefore, further work investigating the role of stromal VAP-1 in HCV infection would provide valuable for HCV infected patients undergoing therapy, especially patients who may also have other underlying liver diseases which could impact the VAP-1 therapy. To determine whether or not the mechanism of VAP-1 anti-viral action is catalysed by its semicarbazide-sensitive amine

oxidase (SSAO) enzymatic activity, the GFP-(Y471)VAP-1 construct can be compared to the wild type GFP-wtVAP-1 construct in HCV infection co-cultures. In the GFP-(Y471)VAP-1 construct, the tyrosine at position 471 has been replaced with a phenylalanine rendering the enzyme incapable of catalysis, and so expressing both the enzyme dead and wild type VAP-1 constructs in cells which are negative for VAP-1 expression or over expressing the constructs in stromal cells and co-culturing with hepatoma cells could help determine the role of VAP-1 enzyme activity on HCV infection. As the constructs also express GFP, the cellular distribution of VAP-1 in stromal cells could also be investigated, to see if there are differences in localisation as a result of co-culturing or HCV infection (Jalkanen et al., 2007, Weston et al., 2014). Nevertheless, the ability for fibroblasts to limit CD81 dynamics and anti-viral properties of fibroblast expressed VAP-1 highlight the role stromal cells play in HCV infection of the liver and present two novel mechanisms by which the fibroblasts limit HCV infection.

By nature, aLMF cells are very contractile, long cells and highly mobile, as we observed when tracking the cell mobility in co-culture using the Cell IQ. Their large size allows them to be in contact with multiple hepatocytes in the liver which means even a low number of aLMFs could impact the level of HCV infection via these cell-contact based mechanisms. It would be interesting to investigate the mobility of stromal cells in co-culture with infected hepatoma cells using the Cell IQ to see if the stromal cells can detect neighbouring infected hepatoma cells and if the presence of these infected hepatoma cells can alter the migration speed or direction of stromal cells, and whether or not

the stromal cells migrate towards infected hepatoma cells to mediate their cell contact dependant anti-viral activity. While the immune response to HCV has been well studied, the role of non-parenchymal cell types and their effect on HCV infection has been studied to a lesser extent (Wynn 2008; Kisseleva et al 2011; Mazzocca et al 2002; Mazzocca 2005; Bataller et al 2004; Wang et al 2013). We intend to apply this knowledge to improve our understanding of the liver microenvironment and the role it plays in antiviral responses in working towards novel therapies. Despite the recent advances in DAA treatments for HCV infected patients, there are still issues surrounding high toxicity and costs associated with the therapies, some of which are also given in combination with the current standard of HCV treatment or ribavirin and pegylated interferon-α (Welch and Jensen 2014, Manns et al 2014, Zeuzem 2014). Another concern is that the patient's response to these new treatments is genotype specific with only 3 of the 7 genotypes appearing to show the most success in treatments (Lawitz 2013; Jacobson 2013). With approximately 170 million HCV infected people worldwide, about 70% of those acutely infected individuals go on to develop persistent and chronic infection, thus HCV infection is still a major burden on liver transplantation and a major cause of global mortality. There is also still no vaccine for HCV and so the need for novel HCV therapies still remains (Pawlotsky 2011; Meredith, Wilson et al. 2012 Mutimer et al 2006; Rowe et al 2008;).

We believed that novel therapies must be developed with a greater understanding of the liver microenvironment and host-virus interactions, potentially leading to possible pathways which can be targets for novel

therapies. We also believed that given the role of stromal cells in maintaining the liver microenvironment, their role in liver disease progression and limited understanding on the role of these cells in HCV infection, that we should develop a physiologically relevant system to study the role of stromal cells on hepatocellular HCV infection. This study established a co-culture system which allowed us to investigate the impact of stromal cells on HCV infection at various stages in the HCV lifecycle (entry, replication and spread), establish if the mechanism required cell contacts or was mediated via soluble factors, study the impact of stromal cells on hepatoma differentiation status and polarity, investigate HCV receptor membrane dynamics and also demonstrated a 3D co-culture technique which can move the conventional co-cultures into a more physiologically relevant organoid co-culture model. To develop the model further and to mimic the liver microenvironment even more closely, the 3D co-cultures could be set up using PHHs or polarised hepatoma cells which are growth arrested to mimic PHHs given the limited availability of PHHs for research. The 3D organoid system could also be cultured at physiologically relevant oxygen levels and other non-parenchymal cell types could be added to the co-cultures however this may complicate the ability to decipher mechanisms even further. The model could then be used to investigate further stromal cell mobility in 3D co-cultures and the impact of stromal cells on hepatoma membrane dynamics, polarity, tight junction formation and integrity, and how these factors impact HCV infection. The model could also be used to investigate and screen potentially novel therapies in a more physiologically relevant system, which could be used to also model a fibrotic patient liver by altering the ratio of hepatoma to fibroblast cells.

This study investigated the role of stromal cells in HCV infection of the liver. The study first established that these cells are not permissive to HCV infection and then investigated the impact of stromal cells on the various steps of the HCV lifecycle in hepatocytes, identifying that stromal cells can inhibit HCV entry, replication and spread in neighbouring hepatocytes in a cell contact dependent manner. Data in this thesis supports a cell contact mediated anti-viral mechanism where by fibroblasts can affect hepatocyte membrane receptor dynamics, reducing the mobility of the HCV receptor CD81, impairing both viral entry and replication. The data also indicates that VAP-1, which is expressed on the surface of fibroblasts, also significantly reduces virus infection independently of CD81 receptor modulation. These findings have greatly improved our understanding of how the interactions between hepatic cells affect HCV, and highlight the importance of non-parenchymal cells in mediating infection in the liver microenvironment.

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