

Inhibition of HCV Replication by Host Cell Innate

Antiviral Defences

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Abstract

RIG-I and MDA5 are the major intracellular sensors for the induction of IFN- β , by dsRNA, in Huh7 cells. It was found that the expression of RIG-I, MDA5 and the signalling mediators TRIF, MAVS and MyD88 were upregulated in Huh7 HCV RNA replicon cells. Similar findings were obtained in Huh7 cells infected with JFH-1 HCV. When replicon and HCV infected cells were further stimulated by dsRNA the induction of these genes were reduced compare to control cells. The expression of NS3 and NS3/4A proteins, using adenovirus vectors, reduced RIG-I, MDA5, MAVS and MyD88 expression levels in response to dsRNA stimulation. Ablation of MDA5, MAVS, MyD88 and TRIF in HCV infected Huh7.5 cells, using siRNAs, increased virus replication demonstrating their importance in inhibiting HCV infection. These results show that HCV inhibits the induction of interferon in response to dsRNA and that NS3 plays an important role in this inhibition.

Analysis of gene expression during HCV infection showed significant changes in the expression of genes involved in the innate immune response, interferon, apoptosis and cell cycle regulation pathways. These include SOCS1, 2 and 3, OAS3, MXA1, IRF9, IRF1, IFNAR2 and STAT1. SOCS3 knockdown by siRNAs reduced HCV replication indicating an additional mechanism of IFN inhibition by HCV.

Infection with HCV also caused cell cycle arrest leading to DNA fragmentation and apoptosis. A decrease in cyclin B1 and an increase in GADD45- β expression during HCV infection indicates that they may play an important role in HCV induced alterations to the cell cycle.

Declaration:

This thesis is a presentation of my original research work. The microarray gene transcription analysis performed by Andrew J Baillie in collaboration with GlaxoSmithKline, USA. Results analysis including gene ontology, gene clustering and heatmaps construction were performed in Dr McGarvey's laboratory by Andrew J Baillie, Samantha Blackham and myself. This work is cited with reference to the literature and acknowledgement.

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ABBREVIATIONS LIST

β-gal	β-galactosidase
aa	amino acid(s)
ADP	adenosine diphosphate
ALT	alanine aminotransferase
AP	alkaline phosphatase
ApoE	Apolipoprotein E
ApR	ampicillin resistance
APS	ammonium persulphate
ATP	adenosine triphosphate
ATPase	adenosine triphosphatase
bp	base pairs
BSA	bovine serum albumin
BVDV	bovine viral diarrhoea virus
CCNB1	cyclin B1
CDC25C	cell division cycle 25 C
cDNA	Complementary DNA
CLDN 1, 6, 9	Claudin 1, 6, 9
CMV	Cytomegalovirus
CSFV	classical swine fever virus
CTL	cytotoxic T lymphocytes
CTP	cytidine triphosphate
Da	Daltons
dATP	deoxyadenosine triphosphate
DC	dendritic cells
dCTP	deoxycytidine triphosphate
DEPC	Diethyl pyrocarbonate
DF	dengue fever
dGTP	deoxyguanosine triphosphate
DMEM	Dulbecco's modified Eagle's medium
DMSO	Dimethylsulphoxide
DNA	deoxyribonucleic acid
DNase	Deoxyribonuclease
dNTP	deoxynucleotide 5' triphosphate
ds	double strand
dTTP	deoxythymidine triphosphate
<i>E. coli</i>	<i>Escherichia coli</i>
E1, 2	envelope proteins 1, 2
EDTA	ethylene diamine tetra acetic acid
eIF	elongation initiation factor
EIF	eukaryotic transcription initiation factor
ELISA	enzyme-linked immunosorbent assay
EMCV	encephalomyocarditis virus
ER	endoplasmic reticulum
EtOH	Ethanol
FCS	fetal calf serum
ffu	foci forming units
FITC	fluorescein isothiocyanate
GADD45-α	growth arrest and DNA-damage-inducible, alpha

GADD45- β	growth arrest and DNA-damage-inducible, beta
GAGs	Glycosaminoglycans
HAV	hepatitis A virus
HBsAg	hepatitis B surface antigen
HBV	hepatitis B virus
HCC	hepatocellular carcinoma
HCV	hepatitis C virus
HCV c	HCV core protein
HCVcc	HCV cell culture
HCV-LP	HCV-like particles
HCVpp	HCV pseudo particles
HDV	hepatitis delta virus
HGV	hepatitis G virus
HIV	human immunodeficiency virus;
HRP	horseradish peroxidase
HVR	hypervariable region
IFN	Interferon
IFNAR	Interferon alpha receptor
IFNGR	Interferon gamma receptor
IKK ϵ	inducible I κ B kinase epsilon
IL	Interleukin
IRES	Internal ribosome entry site
IRF1, 3, 7	Interferon regulatory factor1, 3, 7
ISDR	Interferon sensitivity determining region
ISGF3	IFN-stimulated gene factor-3
ISGs	IFN-stimulated genes.
ISRE	Interferon stimulated regulatory element
JAK	Janus kinase
JEV	Japanese encephalitis virus
JFH-1	Japanese fulminant hepatitis-1
kbp	kilobase pairs
kDa	KiloDaltons
LB	Luria broth
LBA	Luria broth agar
LDL	Low density lipoprotein
LiCl	lithium chloride
LPS	Lipopolysaccharide
MAPK	mitogen activated protein kinase
MAVS	mitochondrial antiviral signaling protein
MDA5	Melanoma differentiation-associated gene
MHC-I, II	Major histocompatibility class I, II
MOI	Multiplicity of infection
MOPS	3-(N-morpholino)propane sulphonic acid
mRNA	messenger RNA
MSM	Men sex men
MVEV	Murray valley encephalitis virus
MW	molecular weight
MX1	Myxovirus resistance 1,
MyD88	myeloid differentiation primary response protein 88
NANBH	non-A non-B hepatitis

NF-κB	nuclear factor κ B
ng	Nanograms
NK,	Natural killer
NLS	nuclear localization signal
NS	non-structural protein
Nt	Nucleotide
NTP	nucleoside triphosphate
NTPase	nucleoside triphosphatase
OCLN	Occludin
OD	optical density
ORF	open reading frame
PBMC	peripheral blood mononuclear cell
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PEG	polyethylene glycol
Pfu	plaque forming units
PI	Protease inhibitor
PI	Propidium iodide
PIAS1, 2	protein inhibitor of activated STAT,1, 2
PKA	protein kinase A
PKI	protein kinase A inhibitor
PKR	protein kinase R
Pmole	Picomole
Poly (I:C)	Polyinosinic-polycytidylic acid
RdRP	RNA dependant RNA polymerase
REF	rat embryo fibroblasts
RIG-I	retinoic-acid-inducible gene I
RNA	ribonucleic acid
RNase	Ribonuclease
rRNA	ribosomal RNA
RT	reverse transcriptase
RT-PCR	reverse transcriptase PCR
SDS	sodium dodecyl sulphate
SH	Src homology regions
siRNA	small interfering RNA
SKP2	S-phase kinase-associated protein 2 (p45)
SLEV	Saint Louis encephalitis
SOCS	Suppressor of cytokine signaling
SP	Signal peptidase
SPP	Signal peptide peptidase
Ss	single strand
STAT	signal transducer and activator of transcription
STAT-C	Specifically Targeted Antiviral Therapy for hepatitis C
STD	Sexually transmitted disease
STING	Stimulator of interferon genes
TAE	tris acetate buffer
TBEV	tick-borne encephalitis virus
TE	tris EDTA buffer
TEMED	N N N' N' Tetramethylethylenediamine
TGF	tumour growth factor

TLR-3	toll-like receptor 3
TM	transmembrane domain
TNF	tumour necrosis factor
TNFR	tumour necrosis factor receptor
TRAF6	Tumor necrosis factor (TNF) receptor-associated factor 6
TRIF	TIR domain-containing adaptor inducing IFN- β
Tris	tris[hydroxymethyl]aminoethane
UTR	untranslated region
UV	Ultraviolet
V	Volts
v/v	volume to volume
V _c	volume of cells
VLP	virus like particle
w/v	weight to volume
WNV	West Nile virus
X GAL	5 bromo 4 chloro 3 indoyl β D galactopyranoside
YFV	yellow fever virus

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CHAPTER ONE:

1. INTRODUCTION:

1.1. History of Hepatitis C Virus

Hepatitis C virus (HCV) is a major cause of liver disease and is also considered to be one of the leading causes of liver disease. It was identified as the causative agent for most post-transfusion and sporadic non-A, non-B hepatitis cases in 1989.

During the 1960`s blood screening measures were developed, to detect Post Transfusion Hepatitis (PTH) using serological tests to detect hepatitis B virus (HBV) and hepatitis A virus (HAV) antigens. In a study of 30 episodes of acute viral hepatitis, 53% of these were not attributable to HAV or HBV (1). During the 1970s, 7-10% of patients from blood transfusions developed viral hepatitis and serological tests failed to identify the agent as Hepatitis A or B and thus the condition was termed Non A non B Hepatitis (2). When plasma or serum from 4 patients with non-A, non-B (NANB) PTH and from a blood-donor implicated in two cases of PTH was inoculated into chimpanzees (3) it gave rise to hepatitis in the animals. This indicated that there may be an additional hepatitis agent (or agents) in addition to HAV and HBV

Attempts were made to identify the causative agent or agents of NANB hepatitis by using standard immunological techniques but these were not successful. In 1989, a random-primed cDNA library was constructed from plasma containing the uncharacterized NANBH agent and screened with serum from a patient diagnosed with NANBH. One cDNA clone was shown to encode an antigen associated specifically with NANBH infections (4). This cDNA clone was then shown to correspond to part of an agent that consisted of at least 10,000 nucleotides that was positive-stranded and was similar in structure to members of the Flaviviridae (4).

Later, an assay was developed to detect antibodies specific to the antigen encoded by this cDNA clone. It found that 88% of NANB hepatitis cases tested had antibodies against this antigen and the agent was then called Hepatitis C Virus (HCV) (5). By 1990, HCV was identified as the main cause of post transfusion associated NANB hepatitis and a universal donor screening programme was developed and the detection of anti-HCV antibodies using an ELISA assay became the standard test for blood transfusion and blood products. Introduction of this assay resulted in a 70% decrease in transfusion associated hepatitis in first generation of assay (6). A second generation (PCR based) assay developed in 1992 together with the ELISA assay has nearly eliminated HCV transmission (6) through blood transfusions and blood products.

1.2. The *Flaviviridae*

Flaviviruses comprise a family of greater than 70 enveloped, positive sense RNA viruses (7). The classification of the Flaviviridae, based on the study of virus genes, virus replication cycles and virus structure has divided then into three distinct evolutionary sub-groups namely, (1) the flavivirus, (2) the pestivirus and (3) the hepacivirus genera (7).

HCV is the main member of the hepacivirus genus. Based on comparison of genome organization and sequence homologies, GBV-B/HGV is the most closely related virus to HCV, and it is a second member of the hepacivirus genus. Other members of the Flavivirus family that can infect humans are usually transmitted by infected arthropod vectors such as mosquitoes or ticks. These include yellow fever virus (YFV), dengue virus (DENV), Japanese encephalitis virus (JEV), tick-borne encephalitis virus (TBEV) Murray valley encephalitis virus (MVEV), Saint Louis encephalitis virus

(SLEV) and West Nile virus (WNV). Animal viruses within the Pestivirus genus, including classical swine fever virus (CSFV) and bovine viral diarrhoea virus (BVDV), are more closely related to the GB viruses (which also included GBV-B and GBV-A) and HCV (7, 8). The phylogenetic relationships between these flaviviruses are shown in (figure 1.1).

The members of the *Flaviviridae* have diverse biological properties and they show little serological cross-reactivity, however they have common features in terms of genome organisation, RNA replication strategy and possibly virion morphology. For example they have similar genome sizes, they have a lipid envelope which contains virally encoded glycoproteins and their genomes are translated to produce a single polyprotein which is cleaved by both virus encoded and host cell proteases. They also express an RNA dependant RNA polymerase required for virus genome replication (7). Viruses within the flaviviruses genus have capped genome (5' type I cap, m7GpppAmpN2), which its translation is cap dependent. In contrast, the hepacivirus and pestivirus genomes are not capped and have an IRES in the 5' nontranslated region (7).

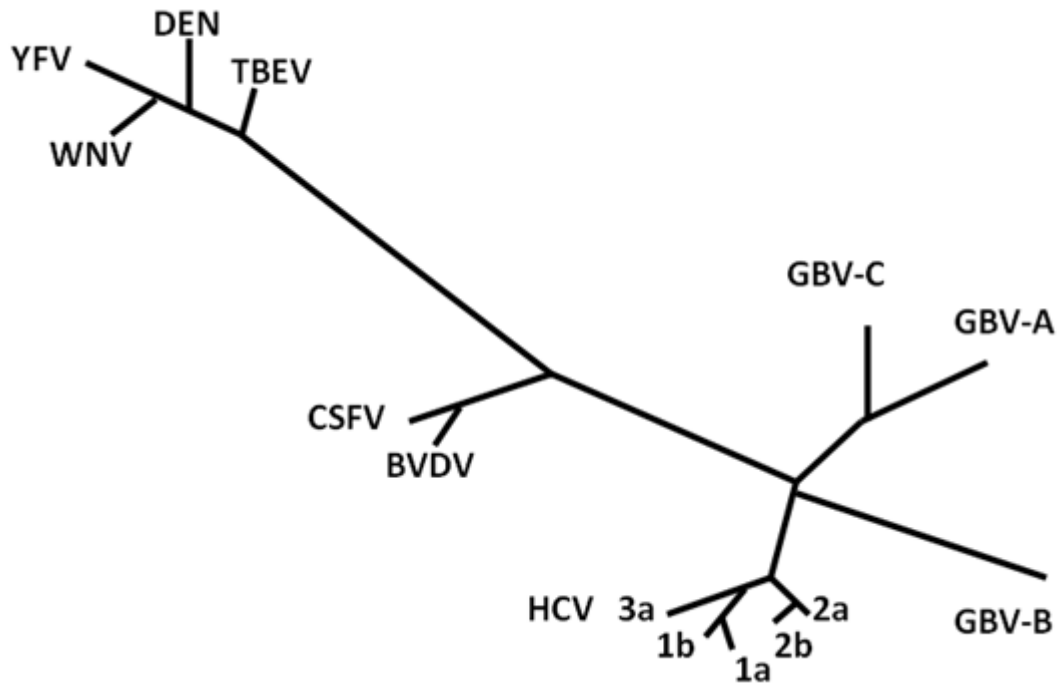


Figure 1.1: Phylogenetic tree based on analysis of amino acid sequences of flavivirus genomes. The flavivirus family are more distantly related to HCV and include tick-borne encephalitis virus (TBEV); yellow fever virus (YFV); West Nile virus (WNV); dengue virus (DEN), than the pestiviruses: bovine viral diarrhoea virus (BVDV). The hepaciviruses include, HCV (genotypes 1a, 1b, 2a, 2b, and 3a) and the most closely related viruses to HCV, GBV-A, GBV-B (HGV) and GBV-C. Figure adapted from Polgreen *et al*, 2003 (8).

1.3. Epidemiology

According to WHO, it is estimated that around 170 million people, representing about 3% of the world population, are infected with HCV and 3 to 4 million people are newly infected each year. HCV genotypes and subtypes have different geographical distributions around the world.

There is a large degree of geographic variability in the distribution of HCV, with rates of infection ranging from 1% to more than 10%. Countries with the highest reported prevalence rates are located in Africa and Asia. China has a reported seroprevalence

of 3.2% (9). In India rate of HCV infection is 0.9% (10). In a few countries such as Egypt, HCV prevalence is between 10-20 % of the population. In Egypt, a national campaign to treat schistosomiasis infection is suspected to be the major cause of the increase in HCV prevalence (11). Most developed countries have prevalence rates of less than 2.5%, although it varies among different countries. In USA 4.1 million (1.6%) Americans have been infected with HCV, of whom 80% are chronically infected (12). The prevalence in the United Kingdom has been estimated to be 0.5% and 1.3% in France (13).

1.4. HCV transmission:

In the majority of cases, infections are spread through exposure to the blood of chronically infected individuals. However, the serological tests for anti-HCV antibodies that were introduced for blood screening has reduced transfusion-related HCV infection and infection from blood products.

Many studies have been carried out to identify the prevalence rates of HCV within certain areas or age groups and also with race, economic and marital status, sex, and education. For instance, HCV infection through perinatal transmission is estimated to occur in 5% of infants born to HCV infected mothers, and a higher proportion of infants born to HIV/HCV coinfecting mothers (14). Transmission of HCV occurs only when serum HCV RNA is detectable (about 10^6 copies or more per ml) and co-infection with HIV increases the rate of transmission 4–5 fold (15).

Illegal drug use has been found to be the factor most strongly correlated with HCV infections e.g. in people who use cocaine, particularly with intranasal administration, using shared equipment. The Hepatitis C European Network for C-operative Research

(HENCORE) group also reported a prevalence of hepatitis C of 80% among intravenous drug users (IVDU) (16).

People with high-risk sexual behaviour, multiple partners, and sexually transmitted diseases are also at risk of HCV transmission (17). Sexual practices have not correlated significantly with HCV seroprevalence in IVDUs. However, those infected with both HCV and HIV are much more likely to transmit HCV to sexual partners (18). Transmission of HCV has been reported among HIV-infected men who have sex with men (MSM). However, this increase in prevalence is controversial; a study in the Netherlands has reported a high and increasing HCV prevalence in HIV-infected MSM. Two of 532 (0.4%) HIV-negative MSM and 28 of 157 (17.8%) HIV-positive MSM were infected with HCV. Over the study period, HCV prevalence among HIV-infected MSM increased from 14.6% to 20.9%. Seven of 28 (25.0%) HIV/HCV coinfecting MSM had acute HCV infection (18). By contrast, study in the UK showed that over 6 months, 3365 MSM attended for STI screening. Of 2309 MSM who agreed to be screened for HCV (69%) the prevalence of HCV was 0.65%. This is similar to the prevalence of HCV in the general population within the UK (19).

1.5. Hepatitis C Virus (HCV) Biology:

HCV is a small, enveloped virus containing a positive sense single-stranded RNA. The HCV genome is approximately 9.6 kilobases in length and contains a single open reading frame (ORF), which encodes a single polyprotein that is ≈ 3000 aa in length. This polyprotein is co and post-translationally processed by a combination of host and viral proteases to produce at least ten different proteins in the following order; core, envelope proteins E1, E2 and p7, and non-structural proteins NS2, NS3, NS4A, NS4B, NS5A and NS5B (figure 1.2). The ORF is flanked by 5' and 3' nontranslated

regions (NTRs). These NTRs contain *cis*-acting RNA elements (CREs), which regulate major steps of the viral life cycle.

HCV isolates show substantial divergence in their genomic sequences. This is because of the high rate of virus replication and its high mutation rate. HCV has an estimated virion half-life ($t_{1/2}$) of 2.7 hours and a clearance rate of around 10^{10} to 10^{12} virions per day in an infected patient (20, 21). The HCV RNA-dependent RNA-polymerase (NS5B) has poor fidelity and lacks proof reading activity. This leads to viral variants being produced continuously during infection. It is estimated that the error rate is approximately 10^{-3} to 10^{-5} mutations per nucleotide per genomic replication (22). These different variants, which are present in an infected patient, are called a *viral quasispecies*. On the basis of these variations, the phylogenetic evolution of HCV isolates has been classified into seven genotypes (numbered 1-7) with nucleotide sequence divergence of as much as 31% to 33%. Genotypes are further classified into subtypes, such as 1a and 1b with a difference of 20% to 25% between their genomes (23-25). Thus, the development of HCV therapies is challenged by the presence of different genotypes and subtypes of virus in different patients and also the variation in viral quasispecies in each patient. These heterogeneities in viral sequence mean that not all antiviral drugs will be equally effective for all HCV genotypes, subtypes, and isolates.

1.5.1. Non translated regions (NTRs)

1.5.1.1. 5' NTR

The highly conserved 5' NTR is 341-344 nucleotides long and contains a highly structured internal ribosome entry site (IRES) in the HCV 5' NTR. The 5' NTR has two independent functions that are necessary for the virus life cycle. The recognition

of a short 5' NTR sequence by microRNA122 (miR122), which is highly expressed in the liver, enhances replication and it allows protein synthesis to occur in a cap-independent manner (26).

Three dimensional structures of the 5' NTR have been elucidated by a combination of biochemical analysis and computer modelling (27). This revealed four distinct RNA domains in the HCV 5' NTR. The short stem-loop 1 is formed by residues 5 to 20 and it is not required for IRES activity. The HCV IRES is composed of three main structural domains comprising 340 nucleotides (27), stem loop II (SLII), 5' SLIII, and 5' SLIV (27, 28). SLIII is the core of the IRES and it has the highest degree of structural conservation and participates in the formation of an RNA pseudoknot that is essential for IRES activity. Domain IV contains a small stem loop which in turn contains the polyprotein start codon at nucleotide position 342, it also forms a pseudoknot by base pairing with a loop within domain III (29). The IRES also requires sequence elements downstream of the AUG in the core protein coding region to function correctly (see figure 1.2).

1.5.1.2. 3' NTR

The 3' NCR which follows the ORF stop codon, consists of a short variable (VR) region of about 40 nucleotides within which substantial sequence diversity is evident between different genotypes of HCV, this region is followed by a stretch of polyuridines, a polypyrimidine poly(U)-poly (UC) [poly(U/UC)] tract, and a highly conserved 98 nucleotide sequence termed the 3' X tail which forms three stem-loop structures (designated SL1, SL2, and SL3) (27, 28). The 3' RNA structures have important roles in RNA replication and polyprotein expression, in particular for minus RNA strand synthesis (30) (figure 1.2).

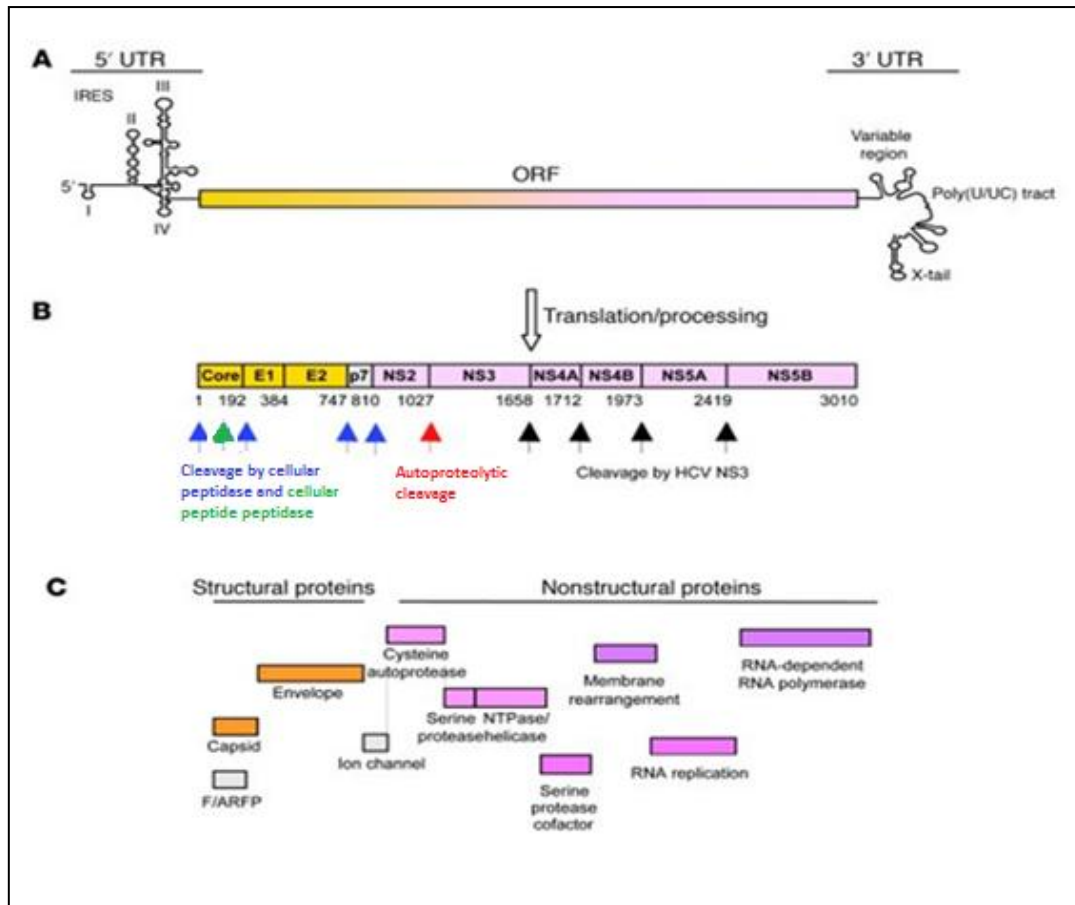


Figure 1.2: Structure and processing of the hepatitis C virus (HCV) genome: (a) The single-stranded RNA genome encodes a long open reading frame (ORF) flanked by 2 NTRs, which contain signals for viral protein and RNA synthesis and the coordination of both processes. Translation is initiated through an internal ribosomal entry site (IRES) in the 5' NTR. (b) The structural proteins are processed by cellular signal peptidases from the N-terminal region of the polyprotein: core, E1, E2, and p7. Non-structural proteins are processed by virally encoded proteases from the C-terminal region of the polyprotein: NS2, NS3, NS4A, NS4B, NS5A, and NS5B. (c) Functions of the resulting 10 structural and nonstructural proteins. A frameshift (F) protein is translated from a short alternate reading frame (ARF). Figure adapted from Rehmann B, 2009 (31).

1.5.2. The structural proteins:

The structural proteins include the core protein, which forms the viral nucleocapsid, and the envelope glycoproteins E1 and E2 and p7 (figure 1.2).

1.5.2.1. Core (C) protein:

The HCV core protein is an RNA-binding protein, 191 aa in length, highly basic and it associates with HCV genomic RNA to form the viral nucleocapsid. It is released from the viral polyprotein by nascent proteolytic cleavage at amino acid 191 by cellular proteases (32). It consists of three distinct domains: an N-terminal hydrophilic domain 1 (D1) formed by the first 117 aa; a hydrophobic D2 directly after the C-terminal of D1 with 169 aa; and a highly hydrophobic domain 3 (D3) spanning the C-terminal 20 aa, which serves as the signal peptide of the C-terminal E1 protein (33). This immature form of core protein (p23) generated by the primary cleavage at the C terminus by signal peptidase (SP) undergoes additional C-terminal processing leading to the removal of most or all of the D3 by the intramembrane-cleaving protease signal peptide peptidase (SPP). This cleavage produces mature p21 core, that contains D1 and D2, which forms a dimeric, α -helical protein exhibiting features that are consistent with those of a membrane protein (34). D2 associates with lipid droplets (LDs) and endoplasmic reticulum (ER) membranes. This interaction between HCV core and LDs is vital for viral replication and production of infectious virus particles. The binding strength of the D2 domain of core for LDs is crucial in determining the efficiency of virus assembly (35).

Core protein has several additional functions. Nucleolar localization of core may be caused by its ability to bind to ribosomes assembled in the nucleus (36). It has been shown that expression of the core protein affects mitochondrial function and lipid metabolism. The core protein increases the cellular production of reactive oxygen species with subsequent increases in lipid peroxidation (37). The core protein also colocalizes with human apolipoprotein AII leading to an increase in the capacity to influence metabolic events involving lipid storage. Analysis of the triglyceride

populations within the cell revealed that core protein expression stimulates a change in cellular metabolism of triglycerides (38). In addition, the core protein reduces microsomal triglyceride transfer, leading to defects in very low density lipoprotein assembly and secretion (39). Also liver steatosis, which is considered a characteristic of HCV infection, may be a result of the direct effect of the core protein on lipid metabolism.

In addition to its function as a nucleocapsid component, the core protein has been incorporated in several cellular processes which contribute to alterations of host cell functions upon HCV infection. For instance, core can specifically suppress programmed cell death and interact with the cytoplasmic tail of the lymphotoxin- α receptor, a member of the tumour necrosis factor family, suggesting a possible immunomodulatory function and a critical role in the establishment of persistence and disease pathogenesis (40).

1.5.2.2. Envelope (E) proteins:

The major viral structural envelope proteins are the glycoproteins, E1 and E2, with molecular weight 31 and 70 kDa, respectively. They interact with each other to form a heterodimer (41). These proteins are released from the viral polyprotein by the action of host-cell signal peptidases. Both proteins contain hydrophobic domains in their C-terminal portions. These domains presumably act as membrane anchors (41).

E2 contains hypervariable regions, called HVR 1 (amino acids 390-410) and HVR 2 (amino acids 474-480). It is believed that E2 may elicit production of virus-neutralizing antibodies (42, 43). These antibodies against E2 have been shown to have the ability to protect against HCV infection in cell culture (44) and in chimpanzees (45). Despite the protective capacity of these antibodies, the virus can escape from

neutralizing antibodies produced in the host. This is caused by random mutation and selection of mutations in HVR-1 which occur during the course of HCV infection in individual patients. The importance of the selective pressure of the host immune system on the HVR region is shown by the escape of HCV from neutralization because of its extreme variability and because this variability was not observed in a patient with agammaglobulinemia (46). Although the development of HCV neutralizing antibodies occurs, the extremely high degree of variability tolerated by the virus in the HVRs enables the selection and immune escape of viruses that may initially represent only a small fraction of the viral population. As a result the development of a prophylactic vaccine which targets the E2 protein remains doubtful. E2 also has a significant role in evading the innate immune response during HCV replication. The HCV E2 protein contains a sequence identical to phosphorylation sites of the interferon (IFN)-inducible protein kinase (PKR), and the translation initiation factor eIF2, a target of PKR, leads to inhibition of PKR activation (47). Additionally, E2 protein interacts with and inhibits PKR-like ER resident kinase (PERK) as well (48). These interactions of E2 help to promote the persistence of HCV infection by suppressing the antiviral effect of interferon.

HCV E2 has been shown to bind to CD81 (49), a cellular protein expressed on the surface of membranes of mammalian cells that is essential for the entry of HCV into the hepatocytes and B lymphocyte (50). CD81 is a member of the tetraspanin family and is required to mediate HCV cell entry in combination with other co-receptors. The low density lipoprotein receptor (LDLR) (51) and scavenger receptor class B type I (SR-BI) (52) have also been proposed as components of the HCV receptor complex. Lipoproteins have also shown to play an important role for cell entry, (53, 54) in studies on HCV pseudotypes. The association with high density lipoprotein (HDL)

seems to enhance SR-BI guided cell entry and protect viral particles from neutralizing antibodies (55).

1.5.2.3. P7 protein:

This 63-amino acid protein is located at the junction between the structural and nonstructural region. It is unknown whether p7 is packaged into viral particles. It is composed of two transmembrane domains and is mainly located in the ER, mitochondrial and plasma membranes (56, 57). Different reports have shown that p7 can form hexamers that have ion channel activity (58, 59). It is believed that p7 could be important for viral assembly because the corresponding protein of the related bovine viral diarrhoea virus (BVDV) is essential for the production of infectious progeny virus but not for RNA replication (60). Recent work using HCV cell culture showed that p7 could facilitate virus assembly and release (61, 62). Other work, showed that p7 functions mainly at the late stage of virus propagation and that, iminosugars coupled to long alkyl chains like N-nonyl deoxygalactonojirimycin (NN-DGJ) not only interfere with ion channel activity but also repress the release of infectious particles from transfected Huh7 cells (62). Beside ion channel activity, p7 harbours a signal-like sequence in its C-terminal domain that directs the insertion of the N terminus of NS2 into the lumen of the ER (56). Brohm et al, developed a trans-complementation assay permitting the analysis of p7 outside of the HCV polyprotein to analyse the role of the native signal sequence of p7 and p7-containing precursor. They found that p7 is absolutely essential for the production of infectious HCV particles and can operate independently of an upstream signal sequence and that the cleavage of an E2- p7- NS2 intermediate, and tyrosine residue close to the conserved dibasic motif of p7 are important for optimal virus production. Mutation of the

tyrosine residue led to defects in HCV morphogenesis. In contrast, neither influenza A virus M2 nor HIV-1 vpu compensated for defective p7 in HCV morphogenesis (63).

1.5.3. Non structural proteins (NS):

1.5.3.1. NS2:

NS2 is 23 kDa transmembrane protein which has cysteine protease activity. The N-terminus of this protein is separated from the E2/p7 polypeptide by cleavage by host peptidases, whereas cleavage at the NS2/NS3 junction is mediated by HCV NS2-3 proteinase which is comprised of NS2 and the serine proteinase domain of NS3 (64-66). The HCV NS2-NS3 proteinase was originally thought to be a metalloprotease based on the observation that its enzymatic activity was stimulated by zinc and inhibited by metal chelators. Site directed mutagenesis studies have shown that amino acids His-952 and Cys-993 are important for proteolytic activity (67). Using HCV JFH-1 to assess the NS2-3 protease, with selected NS2-3 point mutants, demonstrate that these active site mutations do not solely affect the cleavage activity of the HCV NS2-3 protease but significantly affect the integrity of the global protein fold (68).

Recent work has shown that NS2 plays an important role in the production of infectious HCV particles. Cell culture adaptation mutations in NS2 led to an increased titre of either inefficient HCV chimeras or the JFH-1 as major contributors to assembly (69). Moreover, analyzing a set of NS2 mutants provides evidence that NS2 appears to play an essential role in HCV particle production (61, 70). HCV assembly defects caused by mutations in NS2 can also be rescued by trans-complementation (70).

Although the precise role of NS2 during virus production is unclear, genetic evidence suggests it may interact with viral structural proteins or other NS protein. Schregel et

al, defines NS2 as a bona fide protease, NS3 act as its regulatory cofactor, and the functional subdomains in NS3 that cooperate in NS2 protease activation (66). Besides this, NS2 also interacts with cellular factors. It has been noted that the function of NS2 relies on cyclophilin A, which via its cis-trans prolyl isomerise activity, may modulate NS2 protein folding (71).

1.5.3.2. NS3:

NS3 protein is a 70-kDa non-structural protein. The N-terminal 180 aa of NS3 has serine protease activity, while the C-terminal portion encodes helicase and nucleoside triphosphatase activities (NTPase). NS3 is multifunctional protein involved in polyprotein processing and virus replication and is therefore, an attractive target for antiviral drug development. The N-terminal serine protease requires NS4A, a 54-residue viral protein, co-factor required for efficient proteolytic processing for cleavage at the NS3/NS4A, NS4A/4B and NS4B/NS5A sites and it accelerates the rate of cleavage at the NS5A/NS5B junction.

The NS3 Protease domain:

The N-terminal portion of the NS3 protein is a serine protease which is necessary for the cleavage of certain regions of the HCV polyprotein. This protease activity cleaves most of the polyprotein to form the mature non-structural proteins: NS3, NS4A, NS4B, NS5A and NS5B. It rapidly cleaves the NS3/NS4A junction in *cis* while the rest of the junctions are cleaved in *trans* (72). The catalytic domain of the NS3 protease has been mapped to the amino-terminal 181 amino acids (amino acids 1027 to 1207 of the polyprotein), with the minimal protease region being narrowed down to amino acids 1059 to 1204. The N-terminal amino acid region contains a characteristic

serine protease catalytic triad (His-1083; Asp-1107; Ser-1165) (figure 1.3) (72, 73). These residues have been shown to be critical for polyprotein processing, since substitution at these positions abolished cleavage at the NS3/4A, NS4A/4B, NS4B/5A and NS5A/B junctions but not at the other sites (74, 75).

NS4A has been shown to be a cofactor for the NS3 protease, which is required for efficient proteolytic processing. NS4A had been shown to bind to NS3 and enable the protease to cleave peptide substrates more efficiently in particular at the NS4B/NS5A (65, 76).



Figure 1.3: Three dimensional conformational structure of the HCV NS3 protease domain showing the catalytic triad of amino acids forming the active site, the portion of NS4A which associates with NS3 is shown in red. Side-chains of active-site residues His-1083, Asp-1107 and Ser-1165, as well as the zinc ligands Cys-1123, Cys-1125 and Cys-1171 (yellow spheres). The tetrahedrally co-ordinated zinc ion in the C-terminal -barrel is coloured blue. Figure from Bartenschlager R. 2002 (73).

The RNA Helicase domain:

The HCV RNA helicase is composed of the C-terminal two-thirds of NS3. X-ray crystallography has showed that the helicase region of NS3 forms a three-domain Y-shaped structure (figure 1.4) (77, 78). Domains 1 and 2 are similar to those seen in other helicases, and they contain several conserved motifs characteristic of superfamily 2 helicases while domain 3 seems to be novel with no motifs found in other helicases. Based on its sequence homology to other RNA helicase domains, HCV NS3 RNA helicase is classified as a member of the DEAD-box family of RNA helicases. This group of helicases consists of three subfamilies of proteins that contain either a DEAD, DEAH or DExH amino acid motif. NS3 contains an Asp-Glu-Cys-His (DECH) motif at position 1315-1318 of the polyprotein, and is therefore, a member of the DExH subfamily (79).

It is thought that the main role for HCV RNA helicase is to assist the NS5B RNA-dependent RNA polymerase with viral replication by resolving RNA secondary structures and/or double-stranded replication intermediates. The HCV helicase carries out this function by binding and hydrolyzing nucleoside triphosphatase, translocating in a 3'-to-5' direction along the template RNA, separating nucleic acid base pairs, and displacing nucleic acid binding proteins. One strand of nucleic acid binds in the cleft between domain 3 and the first two domains, and ATP probably binds within the cleft separating domains 1 and 2 (80, 81).

To initiate unwinding, the HCV helicase requires a single-stranded region of RNA with a 3' overhang on which to load, and the energy from ATP hydrolysis is believed to fuel both translocation and unwinding. ATPase activity is stimulated up to 100-fold by nucleic acids depending on the nucleic acid sequence and whether or not the protease region is present (82-84). HCV NS3 alone is a poor helicase on RNA, and

helicase activity on RNA is promoted by cofactor NS4A (84). In contrast, NS3 alone is a highly processive helicase on DNA substrates (84), although HCV replication does not involve any known DNA intermediate during its replication cycle.

Besides unwinding viral RNA, the motor action of HCV helicase could also perform other cellular functions such as assisting translation, disrupting RNA-protein interaction, packaging RNA in viral capsids, and even interacting with cellular DNA to alter host gene expression. Mutational analyses of the helicase domain have shown that residues of the conserved motifs of DEAD-box helicases are essential for NTPase and helicase activities and also that NTPase activity is essential but not sufficient for helicase activity (85-88).

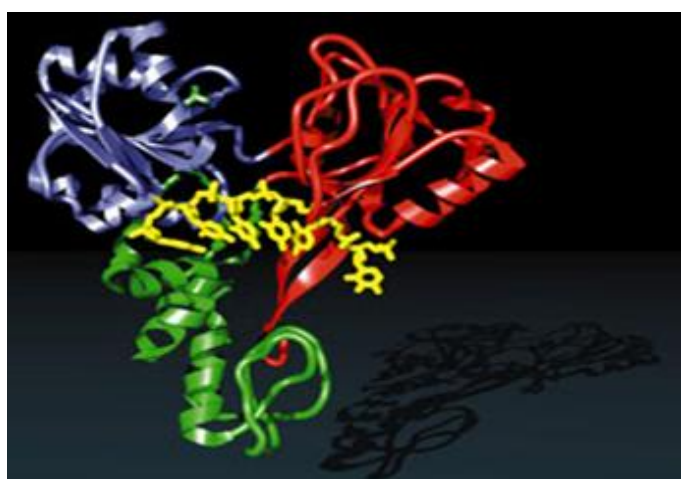


Figure 1.4: Ribbon diagram of the HCV NS3 helicase domain (3D image) with a bound oligonucleotide (yellow). The helicase domain has three distinct domains; Domain 1 (blue), Domain 2 (red) and Domain 3 (green). Figure from Kim *et al*, 1998 (89)

Recent research suggests that NS3 may have a role in viral assembly. Yi and colleagues have shown, that a chimeric HCV genome, which is comprised of genotype 1a H77c spanning the core to the NS2 region, placed within the corresponding region of the genotype 2a JFH-1 genome, did not produce infectious

virus despite evidence for robust RNA replication in the transfected cells until approximately after 9 days when large amounts of infectious virus began to be released from the cells (69). Sequencing revealed that a single amino acid change (NS3-Q221L) within the helicase domain of NS3 was responsible for this change in the virus production phenotype. Also work with other flavivirus NS3 proteins supports these findings. For instance, Kunjin virus (KUN), from genus *Flavivirus*, NS3 protein is involved in assembly in *cis* but its RNA sequence or secondary structure are not essential for efficient RNA packaging (90). Moreover, a defective mutation within the NS2A sequence of yellow fever virus (YFV) can be rescued by a second-site mutation within the NS3 helicase domain, suggesting that NS3 is involved in the production of infectious YFV (91). Additionally, a mutation in NS3, W349A, had no effect on viral replication, but caused defects on release of infectious virus particles which indicate the role of NS3 in virus assembly (92). However, the precise biological roles that the NS3 RNA helicase plays during the replication cycle of the virus are still not fully understood.

1.5.3.3. NS4B

In last few years more studies have revealed the importance of NS4B for virus replication. NS4B is a 261 amino acid, hydrophobic protein with a molecular weight of about 30 kDa. It is a highly hydrophobic, integral ER-membrane protein which contains four transmembrane domains (TM1-TM4) and is palmitoylated at two C-terminal cysteine residues (93). In addition, several critical domains within NS4B have been identified and genetically validated as essential for HCV genome replication. These include: an N-terminal amphipathic helix called 4BAH1 (94). Palmitoylation of 4B protein facilitates oligomerization which appears to be essential

for HCV replication (95). NS4B induces the formation of a structure termed the membranous web which is comprised of small vesicles (80-180 nm in diameter) embedded in a membranous matrix and is found closely associated (96) with the rough ER. HCV proteins and genomic RNA are associated with the membranous web indicating that it is a platform upon which HCV replication occurs (96, 97). This protein has also been implicated in interacting with NS5B together with NS3 to form a regulatory complex important in RNA replication (98).

Recently, NS4B has been shown to bind the 3' terminus of the negative RNA strand of the viral genome with a dissociation constant (Kd) of ~ 3.4 nM (99, 100). Additionally when pairs of highly conserved positively charged arginine rich amino acid motifs were mutated to alanine, either partially or completely, NS4B RNA binding activity was abrogated. Moreover, introduction of these same mutations into HCV replicons resulted in similar partial or complete abrogation of RNA genome replication (99, 100). These findings suggest NS4B could be a new potential target for antivirals. A clemizole hydrochloride compound was found to inhibit HCV RNA replication in cell culture and its activity has been shown to be mediated by its suppression of RNA binding by NS4B, with little toxicity for the host cell (99, 100).

1.5.3.4. NS5A:

Two phosphorylated variants of NS5A have been described and designated according to their molecular masses as 56 kd and 58kd, which correspond to the basal and hyperphosphorylated forms. NS5A is composed of three domains. Domain I is relatively conserved among HCV genotypes compared to domains II and III. Analysis of the crystal structure of the conserved domain I, which immediately follows the membrane-anchoring α -helix localized at the N terminus revealed a dimeric structure

(101). The interface between the dimer is characterized by a large, basic groove, which has been proposed as an RNA binding site. Domain II contains a region referred to as the interferon sensitivity determining region (ISDR), and this region and its C-terminal 26 residues have been shown to be essential for interaction with the interferon-induced, double-stranded RNA-dependent protein kinase (101).

NS5A is implicated in multiple interactions with the host cell to support persistent infection and is involved in RNA replication. NS5A has an important role in resistance against IFN- α induced antiviral response. The IFN sensitivity determining region (ISDR) can bind to PKR inhibiting dimerization, resulting in the repression of PKR function and inhibition of PKR-mediated eIF-2 α phosphorylation (102, 103). Moreover, interfering with the activity of PKR, which is responsible for translational arrest and the induction of apoptosis, leads to resistance to apoptosis by NS5A (104). Alanine substitution for serine residues in the central cluster 1 enhances RNA replication but reduces NS5A hyperphosphorylation. This indicates that NS5A might be a key regulatory molecule in the HCV replication cycle (105).

1.5.3.5. NS5B:

NS5B is a 68 kDa protein with RNA-dependent RNA polymerase (RdRp) which contains a GDD motif, at the residues 317-319, which is essential for RNA polymerase function and it can synthesize RNA by using various RNAs as templates in vitro. As is the case with other polymerases, the three dimensional structure of the HCV polymerase resembles a right hand, complete with finger, palm and thumb domains (106).

The full-length HCV NS5B protein contains 591 amino acids, and the catalytic core domain consists of the N-terminal 530 amino acids. The final 21 amino acids at the C-

terminus of NS5B are hydrophobic and responsible for the anchorage of NS5B to the ER membrane. Deletion of this fragment enhanced the RNA synthesis rate up to 50 fold (107). It has been shown that the C-terminal 21 amino acids not only have a membrane-anchoring function but also may perform additional functions for RNA synthesis in vivo (108).

1.6. HCV replication Cycle

HCV RNA has been detected in the livers, peripheral blood mononuclear cells (PBMCs) and bone marrow of infected patients, confirming that as well as occurring in the liver, virus replication may also occur at extra-hepatic sites including hematopoietic cells, dendritic cells and central nervous system (109-111). HCV particles have a density of about 1.15-1.17 g/ml and a spherical morphology with an average diameter of about 55 nm (112). HCV is surrounded by a host membrane-derived envelope containing the virus-encoded glycoproteins, E1 and E2. The E1 and E2 interact with specific molecules or receptors on the cell surface that promote the attachment and internalisation of particles into the cell (113, 114).

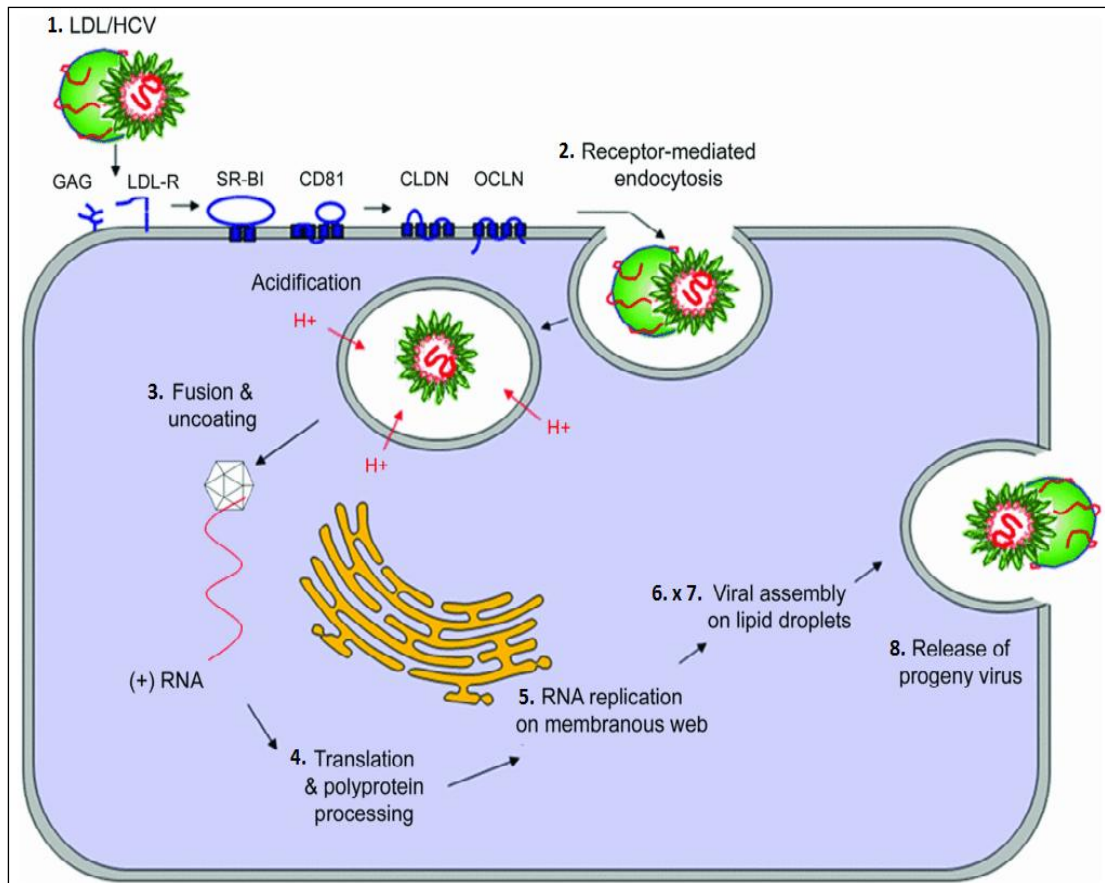
Until recently the investigation of the HCV life cycle in detail had been hampered by the lack of a cell culture system. The first studies for virus life cycles and the characterisation of HCV non-structural proteins was based on analogies with flavi- and pesti- viruses. The development of *in vitro* models for HCV RNA replication using replicon cell lines has helped to elucidate some of processes involved. Studies of HCV replication using replicon cell lines initially showed that the amount of replicon RNA in cells was dependent on the degree of cell confluence where the highest levels were found in exponentially growing cells, followed by a sharp decline in resting cells which indicates that HCV RNA replication or translation is tightly

linked to host cell metabolism (115). Point mutations within the NS5A and NS3 encoding sequences can lead to an increase in the rate of HCV RNA replication. Further analysis of replicon cell lines indicated that HCV RNA replication is not cytopathic to the cells (116).

However, in 2005, HCV isolated from fulminant Japanese hepatitis, demonstrated that genome-length RNA from a genotype 2a HCV strain JFH-1 could produce infectious virus in cell culture (112, 117, 118). This development opened a new era for investigating the mechanisms responsible for HCV particle assembly and release. Along with JFH-1, studies using chimeric derivatives encoding structural proteins from other HCV genotypes established roles for several non-structural proteins in the production of infectious virus. HCV from cell culture (HCVcc) has been found to be infectious in chimpanzees and mice transplanted with human hepatocytes (117). More importantly, isolation of JFH-1 has permitted the analysis of the essential contribution of host cell factors to virus production.

The life cycle of HCV may be summarised as follows (figure 1.5):

- Attachment to receptors on the cell surface, penetration of host cell and liberation of genomic RNA into cytoplasm of cell.
- Translation of the RNA genome; processing of the polyprotein generated.
- Synthesis of a minus strand intermediate from the plus strand genomic RNA.
- Generation of new positive strand molecules which may then be used for production of more negative strand molecules or packaging into progeny virions.
- Virion maturation and release of virus from the infected cell.



1.5: Model of the HCV replication cycle: Host cell (large rounded rectangle shape-blue). (1) Binding of the virus to the cell receptors by interactions with glycosylaminoglycans (GAG) and/or the LDLR and then the virions interact with entry receptors CD81 and SR-BI, (2) receptor-mediated endocytosis facilitated, by translocation of the virus–receptor complex to the tight junctions, where claudin (CLDN) and occludin (OCLN) act as cofactors and induce receptor- endocytosis (3) virion-membrane fusion and uncoating, (4) translation and polyprotein processing, (5) RNA replication at ER membrane where synthesis of (-) strand RNA takes place and serves as template for the production of plus strand (genomic RNA), (6) and (7) assembly of (+) strand with the interaction of RNA with the structural proteins to form virus particles, transport of virus particle via vesicles, and (8) vesicle fusion and release of the virus. Figure adapted from Tang & Grise, 2009 (119).

1.6.1. Attachment and Entry

HCV initiates its lifecycle by the attachment of virus particles to a specific host cell. Therefore, the interaction between receptors on the cell surface and the attachment

proteins on the virus surface is necessary. As has been described earlier, CD81, scavenger receptor class B member I (SR-BI) and the tight junction proteins Claudin-1 (CLDN1) (120) and Occludin (OCLN) (121, 122) are critical factors defining HCV entry (figure 1.6). Two additional Claudin family members, CLDN6 and CLDN9, have also been reported to confer HCV entry (123). In addition, it is thought that HCV may utilize glycosaminoglycans (GAGs) and low density receptors, low-density and very-low-density lipoproteins receptors (LDLR and VLDLR) as initial attachment factors (124).

The major envelope glycoprotein E1 and E2, both are believed to be responsible for initiating virus attachment to the host cell. HCV pseudoparticles (HCV pp) have been used to study virus entry mechanisms. HCVpp are produced in 293T cells by expressing the HCV glycoproteins, retroviral core proteins and a packaging-competent retrovirus-derived genome containing a reporter gene. During budding, HCV envelope glycoproteins are incorporated into the pseudoparticle envelope. As no retroviral glycoproteins are present in this system, the cell entry properties of HCVpp are determined by HCV glycoproteins. HCVpp entry results in the delivery of the retroviral nucleocapsid into the cytosol followed by reverse transcription and integration of the retroviral-derived genome into the cellular genome. Retroviral pseudoparticles (HCVpp) bearing HCV E1E2 gps infect hepatocytes and hepatoma derived cell lines suggest that virus-receptor interactions may in part define HCV tropism for the liver (125). Also E2-specific antisera prevent attachment of HCV to mammalian cells (126). HCV-like particles (HCV-LPs) have also been isolated from insect cells, infected with recombinant baculovirus expressing HCV structural proteins (Core, E1 and E2). Isolated HCV-LPs are composed of all the structural proteins (E1, E2 and C). Further analysis of HCV-LPs by cryoelectron microscopy

(CryoEM) revealed that they are spherical particles with smooth surfaces. They were found to be consistent with the native HCV virions isolated from HCV infected patients. HCV-LPs for the first time allowed 3D structural analysis of HCV particles, which further allows analysis of the complex mechanisms of HCV assembly and maturation (127).

After attachment, the nucleocapsid of enveloped viruses is released into the cell cytoplasm as a result of a fusion process between viral and cellular membranes. Fusion is mediated by specialized viral proteins and takes place either directly at the plasma membrane or following internalization of the particle into endosomes. The entry process is controlled by viral surface glycoproteins that trigger the conformational changes required for mediating fusion. At least two different classes of fusion proteins (I and II) found in enveloped viruses have been determined and shown to be activated by two general mechanisms based upon their requirement for low pH. Fusion of pH-independent viruses, such as HIV, is activated by receptor binding at the plasma membrane. In contrast, the fusion of pH-dependent viruses, such as influenza or alphaviruses/flaviviruses, is triggered by low pH in the endosome (128). By analogy, HCV envelope glycoproteins belong to class II fusion proteins (129) and enter target cells by receptor-mediated endocytosis and use class II fusion proteins (130, 131).

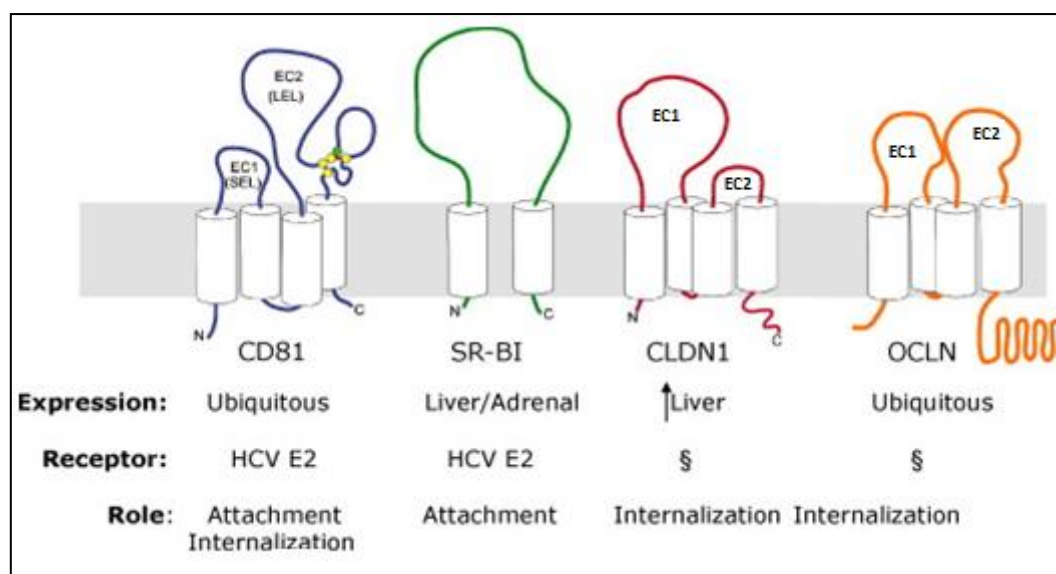


Figure 1.6: Host cell molecules involved in HCV entry. Expression and role of host tetraspanin CD81, scavenger receptor B I (SR-BI) and tight junction proteins Claudin-1 (CLDN1) and Occludin (OCLN). Extracellular loops 1 and 2 (EC1 and EC2). Figure adapted from Lemon *et al*, 2010 (94).

1.6.2. HCV RNA Translation and Proteolytic Processing

Once the virus is in the cytoplasm, the viral RNA is directly translated in a cap-independent way mediated by the viral IRES element, which interacts with the host cell ribosomes (29, 132). Ribosomes appear to bind in close proximity to the polyprotein initiator codon AUG with little or no scanning (133, 134).

Recent work has identified other factors involved in HCV RNA translation. For instance non-coding RNA molecules or microRNAs (miR) are important in the control of gene expression and regulation. Transcription of miR-122 stimulates HCV RNA translation and virus production via the direct interaction of miR-122 with two sites S1 and S2 in the 5'NTR, whereas its sequestration in liver cell lines strongly reduced HCV translation (135). This important role of miR-122 in HCV replication has led to it being considered as a potential therapeutic target. Base substitutions in both S1 and S2 failed to produce infectious virus in transfected cells, while virus production was rescued to near wild-type levels in cells supplemented with a

complementary miR-122 mutant and translation of HCV RNA was reduced over 50% by mutations in either S1 or S2 (136).

Another factor involved in RNA translational control is proteasome alpha-subunit PSMA7 which has been shown to have important role in regulating HCV IRES mediated translation (137). Unlike capped eukaryotic cellular mRNAs, The HCV IRES has been shown to bind directly to the 40S ribosomal subunit without the requirement for other translation factors, in a way which resembles the function of the Shine-Delgarno sequence in prokaryotes (138).

The polyprotein mediated by IRES translation is targeted to the rough endoplasmic reticulum, where the polyprotein is then cleaved co- and post-translationally by host cell signal peptidase and the two viral proteases. Polyprotein processing cleavage junctions have been deduced by the presence of hydrophobic sequences preceding the cleavage sites and the dependence on microsomal membranes. Cleavage at the C-NS2 region is processed by host cell encoded signal peptidases, with cleavage occurring at the following junctions: C/E1; E1/E2; E2/p7; p7/NS2. Cleavage of the signal sequence by the host signal peptidase yields the immature form of the core protein p23 (139, 140). The signal peptide is further processed by a host signal peptide peptidase to yield the mature form of the core protein p21 (139, 140). The host signal peptidase also ensures cleavage at the E1-E2 junction in the ER lumen. Additional signal peptidase cleavage at the C-terminal end of E2 and between p7 and NS2 gives rise to p7. E1 and E2 subsequently undergo several maturation steps, including N-glycosylation, conformational changes, and assembly of E1E2 heterodimers (140, 141).

The zinc-dependent NS2-3 autoprotease ensures *cis*-cleavage of NS3 from NS2. NS3 interacts with its cofactor NS4A to catalyze *cis*-cleavage at the NS3-NS4A junction

and *trans*-cleavage at all downstream junctions including NS4A-NS4B, NS4B-NS5A, and NS5A-NS5B (74, 75). The cleavage sites recognized by the NS3/NS4A proteinase have a common sequence: Asp/GluXXXXCys/Thr-Ser/Ala, with *trans*-cleavages occurring downstream of a cysteine residue and the *cis*-cleavage occurring downstream of a threonine residue (141).

1.6.3. HCV replication complex formation

Positive-strand RNA viruses, including HCV replicate their genome by inducing alterations in cellular membranes and interacting with them to form replication complexes. For HCV, the membrane structure ‘membranous web’ has been observed in cells expressing mature NS4B protein (96) or the precursor NS4A-B (142) which were seen in subgenomic replicon (143), or in HCV JFH-1 infected cells (144). The NS4B induced membranous web contains the HCV replication complex (RC), which also contains NS proteins (NS3, NS4A, NS4B, NS5A and NS5B), host factors and viral RNA.

It is not known whether NS4B recruits cellular proteins responsible for vesicle formation or induces vesicle formation by itself. Recent work has identified different host cellular factors that contribute to the formation of NS4B foci. For example, it is reported that the early endosome (EE) protein Rab5 is associated with NS4B foci, binds to NS4B and is required for HCV genome replication (145). Additionally, a recent report indicates that Rab5 is required for cell culture JFH-1 genome replication (146, 147).

1.6.4. RNA Replication

The 5' and 3' nontranslated regions of the HCV genome are presumed to contain the signals required for initiation and termination of viral replication, and transcriptional initiation occurs in both the 3' and 5' NTRs. The RdRp enzyme (NS5B) initiates synthesis of complementary negative-strand RNA using the HCV genome (positive polarity) as a template. The negative-strand RNA serves as a template to produce numerous strands of positive polarity. Subsequently, these are used for polyprotein translation and the synthesis of new intermediates of RNA replication, or packaging into new virus particles. Studies on subgenomic replicons cells showed that HCV RNA replicons efficiently replicating in cell lines accumulate 50-5,000 copies per cell and that negative strands are 5 to 10 fold less abundant than plus strands (148).

It is believed that a primer is required for the initiation of RNA replication, either by elongation of a primer hybridised to an RNA homopolymer or via a copy back mechanism when heteropolymeric templates are used. Sequences at the 3' end would fold back intramolecularly and hybridise producing a 3' end which may then be used for elongation (141). Evidence for this fold back mechanism in HCV has been shown by interaction of the 3' end of the plus and minus strand to form a G/C-rich stem loop. This involves domain I of the 5'NTR, the 3'NTR, and a *cis*-acting replication element (5BSL3.2) consisting of 50 bases located in a large predicted cruciform structure at the 3' end of the HCV NS5B-coding region (149). HCV RNA replication is initiated by interactions between proteins of the replication complex, the 3'X region of the 3'NTR, and 5BSL3.2 that forms a pseudoknot structure with a stem loop in the 3'NTR (149, 150).

Cellular and other viral factors, in addition to NS5B, are also required for RNA replication. For example, the NS3 helicase unwinds a stable double-stranded RNA

structure which facilitates replication. NS5A bind to NS5B and modulate its polymerase (poly A template/poly U primer) activity (151). NS5A stimulates replication on templates derived from the 3' end of the positive strand during elongation and this, could either be due to the stimulation of its catalytic activity or due to a conformational change induced by NS5A binding (152). However the mechanism of action is not fully clear.

In addition, cellular factors which might be involved in HCV RNA synthesis have also been described. For instance, PTB was found to interact with sequences at the 3' NTR (153, 154). Also Glyceraldehyde-3-phosphate dehydrogenase (GAPDH), bound efficiently to the full-length HCV RNA and revealed critical dependence upon the presence of the poly (U)-sequence in the 3' NTR (155). Recent work has shown that cyclosporin A (CsA) inhibits HCV RNA replication *in vitro*, by targeting by cyclophilin B (156). Cyclophilin B interacts with NS5B thereby stimulating its RNA binding activity, and associates with cyclophilin A (CypA) via its enzymatic pocket and exploits the isomerase/chaperone activity of CypA to help HCV replication (157).

1.6.5. Virion Assembly and Release

The development of cell culture systems which allow the production of significant quantities of virus particles, has led to further understanding of the mechanisms of HCV assembly and release. The production of virus like particles (VLP), by expression of the structural proteins of the virus, demonstrated that viral nucleocapsids acquire their envelope by budding through the ER membranes which retain E1 and E2 in HCV infected cells and the virus may then be exported through the secretory pathway (22).

It has been established that core protein is associated with lipid droplets (LDs) (35, 158). LDs are intracellular organelles that store neutral lipid. They are composed of a core of triacylglycerol and cholesterol ester, surrounded by a unique phospholipid leaflet, which in turn is surrounded by a protein layer (35, 158). Core protein recruits viral nonstructural proteins and replication complexes to lipid droplet-associated membranes, this recruitment is critical for producing infectious viruses (159). Plasma-derived HCV has been reported to be in complex with low-density and very-low-density lipoproteins (LDL and VLDL) (124). Furthermore, HCV production in hepatoma cells is dependent on assembly and secretion of VLDL lipoproteins (160). Thus, it appears that HCV particles interact with VLDL during the assembly of the lipoprotein particles and are secreted together with VLDL.

Many studies have demonstrated that lipoproteins are required for HCV virion infectivity and virus production. Human apolipoproteins have been detected in infectious HCV virions purified from low-density fractions of cell culture supernatants. These are assembled as ApoE-enriched lipoprotein particles and can be specifically precipitated by anti-ApoE and anti-E2 monoclonal antibodies (161). Moreover, inhibition of ApoE by using ApoE-specific monoclonal antibodies efficiently neutralized HCV infectivity, resulting in a reduction of infectious HCV by nearly 4 orders of magnitudes. Knockdown of ApoE expression by specific siRNAs also reduced the levels of intracellular as well as secreted HCV virions (161).

The infectious HCV cell culture model system has been used to demonstrate the role of NS proteins in virus production. Both intra- and inter-genotypic chimaeras require the JFH-1 derived NS proteins to produce infectious virus (162, 163). A detailed mapping study identified NS3, NS5B and the 3'-X tail of the JFH-1 isolate as important determinants of its unique ability to generate virions in cell culture (164).

Recent work has shown that the NS proteins are associated with or incorporated into the viral particles, suggesting a role of these NS proteins in the assembly and/or release of viral particles. NS2 and P7 (61), NS3 (165) and NS5A (166) have been shown to be required for the production of infectious viruses. The introduction of specific mutations into the NS5A showed that mutations in NS5A C-terminal domain III abolished core-NS5A colocalization in the HCV-replicating cells and hampered virion production (105). NS5A specifically interacted with ApoE but not with other viral proteins. The relevance of ApoE-NS5A interaction for viral infection was confirmed by co-immunoprecipitation and co-localization studies of ApoE and NS5A (167).

1.7. Pathogenesis and Disease Progression

The liver is the primary site of HCV infection and contains the highest levels of HCV RNA though relatively low levels of HCV antigens can be detected in the liver (1-10% of cells). Using RT-PCR, HCV RNA can be detected in PBMCs (168), lymph nodes, pancreas, and less frequently within the adrenal glands, bone marrow, thyroid tissue and the spleen (169).

Hepatitis C infection follows a slow progressive course. Initial infection leads to the establishment of an acute phase that is often asymptomatic making it difficult to detect at an early stage. Around 10–20% of HCV infected individuals spontaneously clear the virus during the acute phase, within 6 months after infection. The majority of HCV-infected individuals are unable to clear the virus during the acute phase and become chronically infected. Chronic hepatitis C is characterized by liver inflammation, fibrosis/cirrhosis and, in some patients, HCC (hepatocellular carcinoma) (170, 171). A broad clinical spectrum of extra-hepatic complications and

diseases are associated with chronic HCV infection including mixed cryoglobulinemia, non-Hodgkin's lymphoma, cutaneous vasculitis, glomerulonephritis, neuropathy, and lymphoproliferative disorders (172, 173). Approximately one-fifth of chronically infected individuals go on to develop cirrhosis over a 5–30 year period. Among those with cirrhotic livers, 1%–4% progress to hepatocellular carcinoma (figure 1.7).

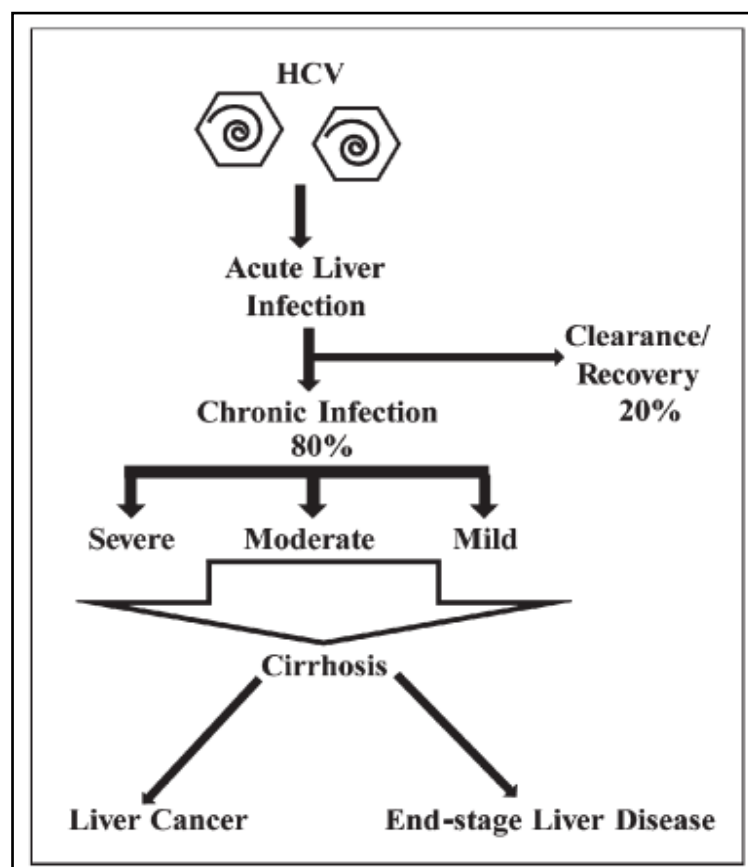


Figure 1.7: Natural course of HCV infection: progression of disease to the next level (arrows), percentage of patients progressing to the next disease state per year (%). In acute infection 20% of infected can clear and recover from infection. 80% develop chronic infection, among them 20% develop cirrhosis in over a 5–30 year period and 1%–4% progress to hepatocellular carcinoma (HCC). Figure from Sharma, 2010 (174).

The incubation period for HCV is approximately 7 weeks but may range from between 2 to 26 weeks. Acute hepatitis caused by HCV resembles other forms of acute viral hepatitis. Symptoms appear in about one third of patients and include malaise, nausea, right upper quadrant pain, dark urine and jaundice. HCV infection leads to an elevation level of serum ALT, which is observed in approximately 80% of cases (175).

Chronic hepatitis has been shown to develop within the majority of patients infected with HCV, therefore one of the most important factors regarding HCV infection is its ability to cause persistent infection. Chronic infection is, in most patients, asymptomatic however, some may experience increased fatigue as their only symptom or alternatively others may suffer overt symptomatic liver disease. Patients may develop severe chronic liver disease over only a few years but more commonly this occurs over several decades (174, 176).

In cell culture systems, HCV expression, replication and infection can induce oxidative stress (177, 178). Individual viral proteins, such as core, NS3 and NS5A, have all been reported to increase ROS production and induce oxidative stress (179). A role of ROS in liver fibrosis has been suggested by the ability of ROS to up-regulate the profibrogenic cytokine TGF- β (transforming growth factor- β) (180). A recent microarray study, which the current study is associated with, of Huh7 cells infected with JFH-1- HCV revealed an increase in intracellular reactive oxygen species and the inhibition of antioxidant proteins (e.g., TXNIP gene) and the production of proteins which induce reactive oxygen species (e.g., DDAH1 and AOX1 genes). By contrast, the same study showed a decrease in the expression of genes involved in cellular antioxidant responses, including, genes transcribed following NRF2 activation (e.g., CAT and EPHX1 genes), members of the

metallothionein gene family, genes for the glutathione S-transferase enzymes (e.g., GSTM1 to -4 genes), and genes for other key antioxidants (e.g., ATOX1 and GLRX genes) (177). Another proteomics study from patient biopsies identified 210 proteins exhibiting statistically significant differences associated with the fibrosis stage. Amongst the down-regulated proteins were mitochondrial proteins of the oxidative phosphorylation system and proteins responding to oxidative stress and reactive ROS. Similarly more proteins were also upregulated in this study including mitochondrial and antioxidant proteins, for example, glucose-regulated protein, calreticulin, ER protein 29, heat shock protein 1B, protein disulfide-isomerase A3 precursor, and ubiquitin-activating enzyme E1) (181). The study suggests a correlation between the down-regulation of antioxidant proteins and the later stages of liver fibrosis, consistent with a role of oxidative stress in the progression of liver fibrosis and cirrhosis (181).

Hepatic steatosis, an excessive lipid accumulation in the cytoplasm of hepatocytes, is a frequent histological feature in patients with chronic hepatitis C infection. It occurs in between 40% and 80% of chronic HCV patients and is also associated with the development of HCC (182, 183). The prevalence of steatosis varies depending on the features of the population studied in terms of, alcohol consumption, prevalence of obesity, diabetes and other risk factors of fatty liver (184). An association of HCV core protein in insulin resistance and hepatic steatosis has been proposed. Core induces lipid droplet accumulation and redistribution in cell culture and significant correlation between the steatosis score and titre of intra-hepatic HCV RNA was found in patients infected with genotype 3 (183).

It has been proposed that liver steatosis in hepatitis C could be a storage disease induced by the effects of the virus and of its proteins on the intracellular lipid

machinery and on microsomal triglyceride transfer protein (MTP) (185). MTP gene expression and protein activity in liver biopsy specimens from patients with CHC were significantly reduced in the presence of severe steatosis, particularly in patients infected with HCV-3 and that impairment in MTP activity showed a direct relation to levels of HCV replication (185, 186). This reduction is correlated with reduced serum cholesterol, ApoB, and low-density lipoproteins (185). Moreover, MTP 493 G/T polymorphism showed significant association between MTP polymorphism and liver fat accumulation. The MTP T allele was shown to be an independent risk factor for high grades of steatosis in the total cohort of HCV patients (187). Recently, an inverse relationship was also found between HCV related steatosis and liver expression of SREBP-1c and GPAT, two genes involved in fatty acid and triglyceride synthesis (186). These findings support the hypothesis that HCV may cause accumulation of lipids in infected hepatocytes by perturbing hepatic lipid metabolism through a direct interference with MTP, leading to VLDL/triglyceride retention (185).

1.8. Humoral and cellular Immune Responses to HCV infection:

The humoral immune response determines the outcome of infection. HCV neutralizing antibodies were first described after the experimental infection of chimpanzees. These antibodies were directed against epitopes in the hypervariable region-1 (HVR-1) of HCV envelope glycoprotein E2 (45) and antibodies directed against HVR-1 has also been associated with viral clearance in HCV-infected humans (42). However, despite the high titres of these antibodies they fail to clear the virus as they continue to exert selection pressure on HCV quasispecies and thereby contribute to the evolution of the HCV envelope sequences throughout the course of infection (188). Using a single-source outbreak of HCV it was shown that viral clearance was

associated with a rapid induction of neutralizing antibodies in the early phase of infection (189). However, HCV specific antibodies do not prevent reinfection and are not maintained in many individuals who have recovered from HCV infection (190).

There is evidence for an important role of both virus-specific CD4⁺ and CD8⁺ T-cells in controlling the levels of virus present as well as in causing liver injury. CD4⁺ T-cells recognize antigens presented by major histocompatibility complex (MHC) class II molecules on the surface of professional antigen presenting cells (APCs). Subsequently, CD4⁺ T-cells perform multiple effector functions, including direct activation of macrophages and antigen-specific B-cells as well as activation of CD8⁺ T-cells in a cytokine-dependent manner. CD8⁺ T-cells, in contrast, recognize antigens presented by MHC class I molecules on the surface of infected cells. The effector functions of CD8⁺ T-cells comprise two different mechanisms. The killing of infected target cells (cytotoxicity), which has been thought to be the primary function of CD8⁺ T-cells and secretion of cytokines such as IFN- γ and tumour necrosis factor that can inhibit viral replication without killing the infected cell (31, 191).

HCV-specific CD4⁺ T cells are essential in the generation of a successful HCV-specific immune response (31). Rapid clearance of circulating HCV is associated with a vigorous and sustained response of IFN- γ production and proliferating CD4⁺ T cells in the blood. In contrast, those who showed a weak IFN- γ (+) T-cell response maintained small quantities of circulating HCV (192, 193).

Several studies have shown that HCV-infected patients who develop chronic infection typically have only weak, oligo-/mono-specific or no virus-specific CD4⁺ and CD8⁺ T cell responses in the acute (194) and chronic phases of infection (195, 196). Several studies have shown that cytotoxic T-cell dysfunction may occur either due to primary T-cell failure or due to T-cell exhaustion and that this plays a role in virus persistence.

The expression of high levels of PD-1 (a mediator of T-cell exhaustion and viral persistence) on HCV specific CD8⁺ and CD4⁺ has been shown early in acute infections irrespective of clinical outcome (197). It has also been suggested that antigen presentation by dendritic cells (DCs) and macrophages might be impaired in HCV infection (31).

1.9. Treatment and Prevention

The limitations and the side effects of the current treatments for chronic HCV infection have led to the search for new antivirals. The primary goal of treatment of chronic HCV infection is to eliminate the virus from the body so that the level of HCV RNA is undetectable. When achieved clinically it is called a sustained virological response (SVR) (198).

In 1991, IFN- α became the first treatment for chronic HCV infection. This treatment was improved by binding IFN- α to polyethylene glycol (Pegylated-IFN- α) which increases the half-life of circulating IFN- α without significantly reducing its effectiveness and also reduces the dose needed to once a week (199). Ribavirin was then combined with IFN- α , as an additional antiviral agent, used in the treatment of HCV. Ribavirin is a nucleoside analog of guanosine which has been shown to have antiviral activity against many RNA and DNA viruses (200). However the mode of action for Ribavirin is not completely understood. It has been suggested that it acts directly by becoming incorporated into RNA during replication causing chain termination, or indirectly by inhibiting inosine monophosphate dehydrogenase (IMPDH). Inhibition of IMPDH leads to reduced intracellular levels of GTP which is required for HCV RNA synthesis. Another suggested mode of action is that ribavirin causes the RNA-dependent RNA polymerase to become more error-prone causing it

to introduce higher numbers of mutations which dramatically reduce the infectivity of the new virions (201, 202).

Combination therapy of ribavirin with peg-IFN- α , which is the present standard treatment regimen, results in a sustained response rate close to 90% for patients carrying the less common genotypes 2, 66-80% for genotype 3, but is less effective for genotype 1, with around 50% SVR (198).

However in addition to these limitations in its efficiency the therapy is expensive, produces unpleasant side effects that include depression, haemolytic anaemia, fever and chills. This means that new and effective therapeutic agents are needed to increase the SVR.

In last decade, different promising new therapeutic approaches based on antiviral agents that target specific steps in the virus life cycle have been developed and are collectively called Specifically Targeted Antiviral Therapy for hepatitis C (STAT-C). The first small molecule to be used in clinical trials was BILN 2061, a macrocyclic peptidomimetic inhibitor designed to inhibit NS3/4A activity. Oral administration of BILN 2061 in patients infected with genotype-1 was found to dramatically reduce HCV RNA levels within 1–2 days (203). This was considered a landmark study which demonstrated for the first time that potent small molecule inhibitors were effective against HCV in vivo. However in spite of these impressive results, further clinical development of BILN 2061 had to be terminated as it was found to be toxic. It was also found that replicons containing single mutations in the NS3 protease gene (A156V or D168V), resulted in high levels of resistance to BILN-2061 (204). Similar findings were confirmed with single amino acid substitution A156T, R155Q, or D168V which resulted in 357-fold, 24-fold, and 144-fold reductions, respectively, compared to the wild-type replicon (205).

These results opened a new era for the development of new protease inhibitors (PI) which, aimed to produce high levels of inhibition of viral replications with less toxicity and resistance. Currently, multiple HCV PI are in various stages of clinical development, including telaprevir/VX-950 (Phase 3), VX-500 (Phase 1), VX-813 (Phase 1), boceprevir/SCH503034 (Phase 3), ITMN-191/R7227 (Phase 1b), TMC435 (Phase 2), MK-7009 (Phase 2) and PHX1766 (Phase 1) (198).

VX-950 for example VX-950 is a peptidomimetic compound that has been structurally optimized to inhibit NS3/4A activity and can be taken orally, shows potent inhibition of HCV replication (206), and like BILN 2061, VX-950 also generates resistance mutations within NS3 (207). Lin et al, have identified mutants that contain cross-resistance mutations, conferred by substitution of Ala (156) with either Val or Thr that resist inhibition by BILN 2061 are sensitive to VX-950, or vice versa (207).

The RNA-dependent RNA polymerase (NS5B) enzyme is essential for HCV genome replication indicating that it would be a good target for anti-viral intervention. To date, several nucleoside and non-nucleoside NS5B inhibitors have been developed. NM-283 (Valopicitabine), is a nucleoside analog, prodrug of 2'-C-methyl-cytidine and, serves as a substrate for viral RNA polymerase where its incorporation leads to premature chain termination (208). In phase-II clinical trials NM-283 treatment in patients infected with HCV genotype-1 either alone or in combination with interferon-alpha results in synergistic inhibition of HCV genome replication in cell culture (209).

1.10. Vaccination:

Development of an effective vaccine against the hepatitis C virus (HCV) remains a challenge. This is due to several factors including; (1) The large number of mutations

in the HCV genome, for example, the hypervariable regions HVR1 and HVR2 within the E2 gene; (2) the observation that convalescent humans and chimpanzees could be re-infected after re-exposure. HCV envelope proteins can induce broadly neutralizing antibodies against conserved domains for HCV binding to the cellular receptors (45) (42). Consequently the HCV envelope proteins have been considered as the major HCV vaccine candidates. A vaccine based on the E1 and E2 glycoproteins has been tested in chimpanzees (210, 211). However, the neutralizing activity of HCV specific antibodies is: low in infected patients, short lived and needs to be boosted frequently and it only gave partial protection against challenge with HCV of the same genotype as the E1 and E2 proteins used in the vaccine (212, 213). Other virus proteins have also been used e.g. TG4040 is a candidate vaccine based on the attenuated modified vaccinia Ankara (MVA) expressing NS3, NS4 and NS5B proteins (214).

Since naturally produced HCV antibodies do not seem to completely eradicate the virus, there is interest in stimulating the production of antibodies and promoting T-cell activity. Many current vaccine candidates aim to stimulate both types of immune response and mainly prime broad HCV-specific Th1 and CTL responses (215).

1.11. Interferons (IFNs):

Interferons are critical signalling proteins which are secreted by cells in response to viral infections and which activate host cell antiviral responses. Three classes of IFN have been identified, designated types I, II and III, and are classified according to the receptor complex they signal through. Class II contains one member, IFN- γ produced by T cells and NK cells in response to immune and inflammatory stimuli. IFN- γ binds the IFN- γ receptor (IFNGR) complex, and mediates broad immune responses (figure 1.8) (216, 217).

More recently a third type of IFN designated type III IFN- λ (represented by IL-28 and IL-29), shown to be structurally and functionally related to type I IFNs. They are regarded as a distinct family and signal through a separate receptor complex, which signal through receptors containing IFNLR1 (IFN- λ receptor 1; also known as IL-28Ra) and IL-10R2 (also known as IL-10R β). There are several type I IFNs, subtyped IFN- α (13 subtypes) and one subtype of IFN- β one gene, IFN- κ , IFN- ϵ , IFN- ω , IFN- τ and IFN- δ of which bind to IFNAR (IFN- α receptor) (216, 217). Type I IFNs are the most important for the innate host response viral infections.

1.11.1. IFN signalling:

Interferons (IFN) effect their responses by binding to specific receptors on the cell surface. The binding of IFN- α and β to the extracellular domains of their receptor initiates a signal transduction cascade. Type I IFNs binds to a common heterodimeric receptor interferon alpha receptor 1 and 2 (IFNAR1 and IFNAR2), inducing oligomerization. Prior to activation, the cytoplasmic tail of IFNAR1 is associated with tyrosine kinase 2 (Tyk2) and the IFNAR2 subunit is associated with the tyrosine kinase JAK1. This cascade involves activation of Jak1 and Tyk2 and the subsequent tyrosine-phosphorylation of STAT1 and STAT2 results in their translocation to the nucleus. The STAT1–STAT2 complex associate with a monomer of IRF9 to form the ISGF3 heterotrimer that binds to the IFN-stimulated response element (ISRE), located in the promoters of most IFN-responsive genes, and enhances transcription.

IFN- γ binds to IFNGR1 and JAK2 binds to the intracellular domain of IFNGR2. JAK1 and JAK2 are then, activated by autophosphorylation. Once activated, two latent STAT1 proteins bind to these JAKs. STAT1 proteins are phosphorylated by the

JAKs, and dissociate from the receptor and translocate to the nucleus. The activated STAT1 binds to specific gamma activated sequence (GAS) and stimulates the expression of the genes downstream (figure 1.8) (216, 217).

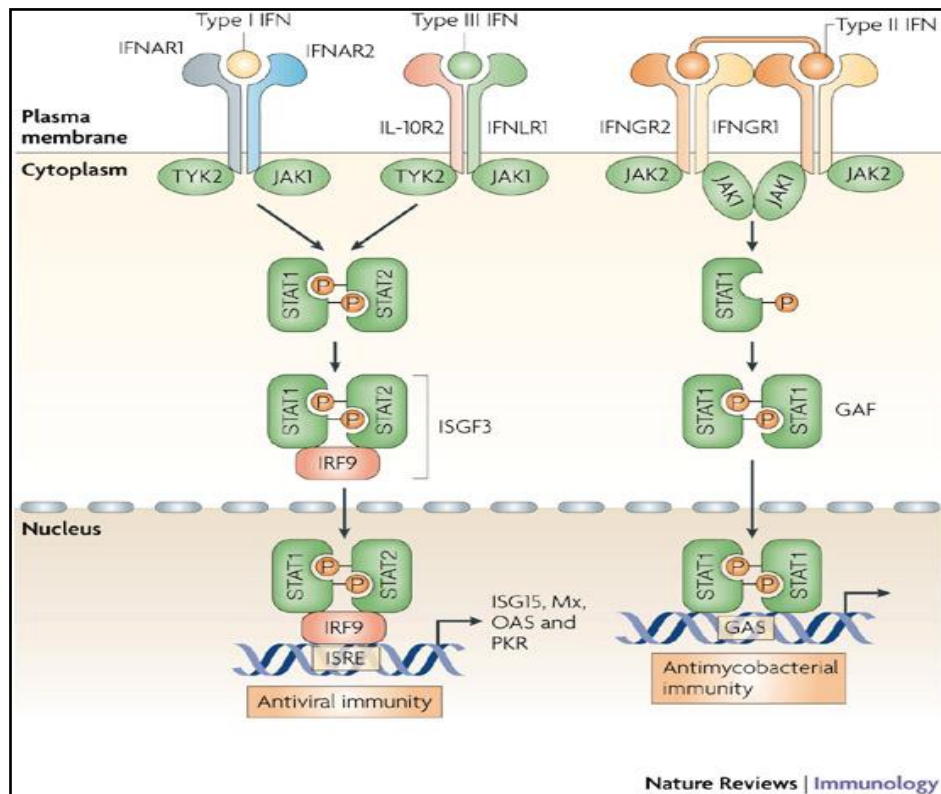


Figure 1.8: IFN signaling: IFNs bind to three receptor complexes: a heterodimer of IFN- α receptor 1 (IFNAR1) and IFNAR2 binds type I IFNs; the interleukin-10 receptor 2 (IL-10R2) associates with IFNLR1 (IFN- λ receptor 1) to bind the three IFN- λ subtypes; and a tetramer consisting of two IFNGR2 (IFN- γ receptor 2) chains and two IFNGR1 chains binds dimers of the type II IFN- γ . Following IFN binding translocated to the nucleus to induce IFN-stimulated genes from IFN-stimulated response elements (ISREs) or GAS promoter elements, for type I and type III, or type II IFN responses, respectively. Figure from Sadler & Williams, 2008 (217).

1.11.2. IFN biological effects:

IFN α/β induce an antiviral effect in cells on the onset of viral infection through activation of expression of more than 300 IFN-stimulated genes (ISGs) which have antiviral, antiproliferative, and immunomodulatory functions. Some ISGs are mediated through a cascade of antiviral pathways leading to several antiviral responses including proteins that catalyse cytoskeletal remodeling, that induce apoptosis, that regulate post-transcriptional events (splicing, mRNA editing, RNA degradation and the multiple steps of protein translation) and proteins that are involved in subsequent post-translational modification. Several IFN-induced proteins have documented anti-HCV activity ISG15 (IFN-stimulated protein of 15 kDa), the GTPase Mx1 (myxovirus resistance 1), ribonuclease L (RNaseL) and protein kinase R (PKR).

IFN induces resistance to viral replication in uninfected cells by activating genes that cause destruction of mRNA and inhibit the translation of viral and host proteins. A good example is protein kinase R (PKR) which is normally present in an inactive form in uninfected cells, but on binding to double stranded RNA it undergoes autophosphorylation and a conformational change unmasking its C terminal catalytic domain (218). Activated PKR phosphorylates the alpha subunit of the initiation factor eIF2, which results in the formation of an inactive complex resulting in the rapid inhibition of translation (219). Another inhibitory pathway is activated through oligoadenylate synthetase, which converts ATP into a series of 2' – 5' linked oligomers which activate RNase L that then degrades cellular and viral RNA (208, 220). ADAR 1 catalyzes the deamination of adenosine on target dsRNAs to yield inosine which destabilizes the secondary structure due to a change from an AU base

pair to a less stable IU base pair and it also causes mutations which accumulate within the viral genome (221).

Several microarray studies performed with HCV infected liver samples, obtained from transiently infected chimpanzees and chronically infected humans, have shown that more genes are involved in the induction of interferon (IFN)-stimulated genes (ISGs) in HCV infection compared to uninfected controls (222). Helbig et al, revealed that viperin has antiviral effects on HCV infection (223). Viperin is identified as an antiviral pathway that works together with ISG20 and PKR, to mediate the IFN response against HCV infection (224). This work revealed the molecular mechanism of viperin, which is a putative radical S-adenosyl-L-methionine (SAM) enzyme (224), induces the formation of crystalline ER and interferes with protein secretion (225). Therefore, it is hypothesized that viperin inhibits HCV replication by interfering with the formation activity of HCV RC on ER (226). Murao K et al, demonstrated that IFN- α inhibits the expression of hSR-BI/CLA-1, a cell surface receptor that probably serves a dual role; which might facilitate the entry of HDL particles into liver cells and function as a receptor for HCV (227). IFN- α / β help to activate the immune response by induction of MHC class I expression on cells newly infected by the virus to enhance the degradation by CTL. HCV infection also increases production of class II MHC in macrophages.

1.11.3. Inhibition of IFN pathway by HCV:

Several HCV structural and non-structural proteins have been shown to have a significant influence in suppressing the antiviral effects of interferon which helps to promote the persistence of HCV infection. The HCV E2 protein contains a sequence identical to phosphorylation sites of the interferon (IFN)-inducible protein kinase (PKR), and the translation initiation factor, eIF2, a target of PKR. E2 therefore acts as

a pseudosubstrate for PKR and consequently it can confer resistance to PKR in HCV infected cells by increasing the level of protein translation (47, 228).

HCV replicons were found to replicate more efficiently in PKR deficient mouse embryonic fibroblasts (MEFs) than wild type MEF, and PKR expression knockdown by specific siRNAs significantly enhanced the level of HCV RNA replication (229). NS3/4A serine protease blocks the phosphorylation and effector actions of IFN regulatory factor 3 (IRF-3), the key molecule in the antiviral signalling pathway (230). NS5A has also been shown to block the activation of interferon regulatory factor 1 (IRF1) through the PKR pathway which is important for IFN-induced transcriptional programs (231).

1.11.4. Innate immunity and HCV:

The innate immune response is the first line of defence against an invading pathogen. Interferon type I (IFN- α and IFN- β) responses are key to innate antiviral immune defences. To trigger this antiviral state it is necessary to 1. Detect the invading pathogen and 2. Initiate a cascade of signalling proteins that regulate the synthesis of type I IFN. To achieve this, the cells of the innate immune system express sensing proteins called Pattern Recognition Receptors (PRR) which recognises various molecules from pathogens collectively known as pathogen associated molecular patterns (PAMP) (232, 233).

1.11.4.1. Antiviral innate immunity induction by Toll Like Receptors (TLRs):

TLRs are transmembrane receptors that recognize PAMPs through leucine-rich repeats (LRRs) in their extracellular domains and transmit this information by the Toll/IL-1R (TIR) intracellular signaling domain. TIR interacts with several TIR-

containing adaptor molecules including, myeloid differentiation primary response protein88 (MyD88), MyD88 adaptor-like (also known as TIR domain-containing adaptor protein, TIRAP), TIR domain-containing adaptor inducing IFN- β (TRIF) and TRIF-related adaptor molecule (TRAM). These interaction results in the activation of different intracellular signalling cascades, generally resulting in the activation of NF- κ B and activating protein-1 (AP-1) in MyD88-dependent pathways and the activation of type I interferons (IFNs) in TRIF-dependent anti-viral pathways (234).

Among the eleven members of the human TLR family, TLR2, TLR 3, TLR 4, TLR 7, TLR8 and TLR9 are involved in the initial sensing of viral components. TLR3 recognizes dsRNA and may also be involved in sensing dsRNA released from dying cells. Detection of dsRNA by TLR3 in Dendritic cell requires phagocytosis of infected material (235). TLR7 and TLR8 are receptors for ssRNA associated with viruses that enter cells through endocytosis. TLR9 recognizes unmethylated CpG DNA present in DNA viruses such as herpesvirus. TLR4 may also be involved in the recognition of viral glycoproteins. These receptors are localized in the endosomal membranes, with the ligand binding domain facing the lumen of the endosomes, and the TIR signaling domain positioned on the cytoplasmic side (234, 236).

1.11.4.2. Antiviral innate immunity induction by RIG-I and MDA5 pathways:

TLR3 was first identified as the main dsRNA receptor capable of inducing IFN expression. However, TLR3 was found not to be required in four different infectious viral models (lymphocytic choriomeningitis virus (LCMV), vesicular stomatitis virus (VSV), murine cytomegalovirus (MCMV), and reovirus) when TLR3 genetically deficient (-/-) mice were infected. This showed that TLR3 absence does alter either

viral pathogenesis or impair the host's generation of adaptive antiviral responses to these viruses (237). This led to the subsequent identification of other crucial dsRNA sensors. These include cytoplasmic RNA helicases Retinoic acid – inducible gene I (RIG-I) and melanoma differentiation-associated gene (MDA5), which ensure that viruses that avoid recognition by TLRs in endosomal locations will still be detected by the innate immune system (238). RIG-I and MDA5 recognize a variety of RNA viruses and trigger innate antiviral responses independently from TLR dependent pathways. RIG-I is a member of the DExD/H box-containing RNA helicase family of proteins that unwind dsRNA in an ATPase dependent manner (238). The helicase domain of RIG-I can bind both synthetic dsRNA poly (I:C) and viral dsRNA. Besides the C-terminal helicase domain, RIG-I also contains two tandem caspase recruitment domains (CARDs) at its N-terminus. MDA5 has also been identified as a cytoplasmic DExD/H box RNA helicase sensor that activates innate antiviral immune responses. Like RIG-I, MDA5 also contains two N-terminal CARD domains which can activate the IFN- β promoter (239, 240). Through the CARD domain, both RIG-I and MDA5 recruit an adaptor protein containing an N-terminal CARD, designated MAVS (also known as IPS-1, VISA, or Cardif) (238).

Various studies have demonstrated that the I κ B kinase (IKK) family members are critically involved in virus-triggered and MAVS mediated induction of type I IFNs. IKK- α and IKK- β are essential for virus-triggered NF- κ B activation, the noncanonical IKK family members TBK1 and IKK3 are responsible for phosphorylating and activating IRF3 and IRF7 (241-243). The core complex of IKKs also contains the regulatory unit IKK- γ (called NEMO) protein (244). The NEMO protein is essential for ensuring the oligomerization state of the complex since without NEMO, the complex is inactive (245). MAVS can interact with tumour necrosis factor (TNF)

receptor-associated factor 6 (TRAF6) through its conserved TRAF6-binding motifs. MAVS mediated NF- κ B but not IRF3 activation is diminished in TRAF6-deficient fibroblasts, suggesting that TRAF6 acts downstream of MAVS in the NF- κ B but not IRF3-activation pathway (246). Finally, MAVS mediates the sequential recruitment and activation of TANK-binding kinase-1, inducible I κ B kinase (IKK- ϵ), IFN regulatory factor 3 (IRF3) and NF- κ B transcription factors leading to type I IFN synthesis and secretion (figure 1.9) (244, 245).

Although the links between MAVS and these transactivators is not completely elucidated, recently many novel proteins have been discovered to be involved in the downstream signalling of IFN. In two independent studies, a protein called stimulator of interferon genes (STING) (247) also called MITA (248) was identified as a significant component to this downstream pathway able to activate both NF- κ B and IRF3 transcription pathways to induce expression of IFN- α and IFN- β and exert a potent anti-viral state following expression (247, 248). Experiments by both groups were able to show that STING localize to the outer membrane of mitochondria and interacts with both MAVS and RIG-I and is needed for transmission of virus-induced signals through these molecules. Both groups found that overexpression of STING activated IRF3, whereas knockdown of MITA inhibited virus-triggered activation of IRF3, expression of type I IFNs, and cellular antiviral response (247, 248).

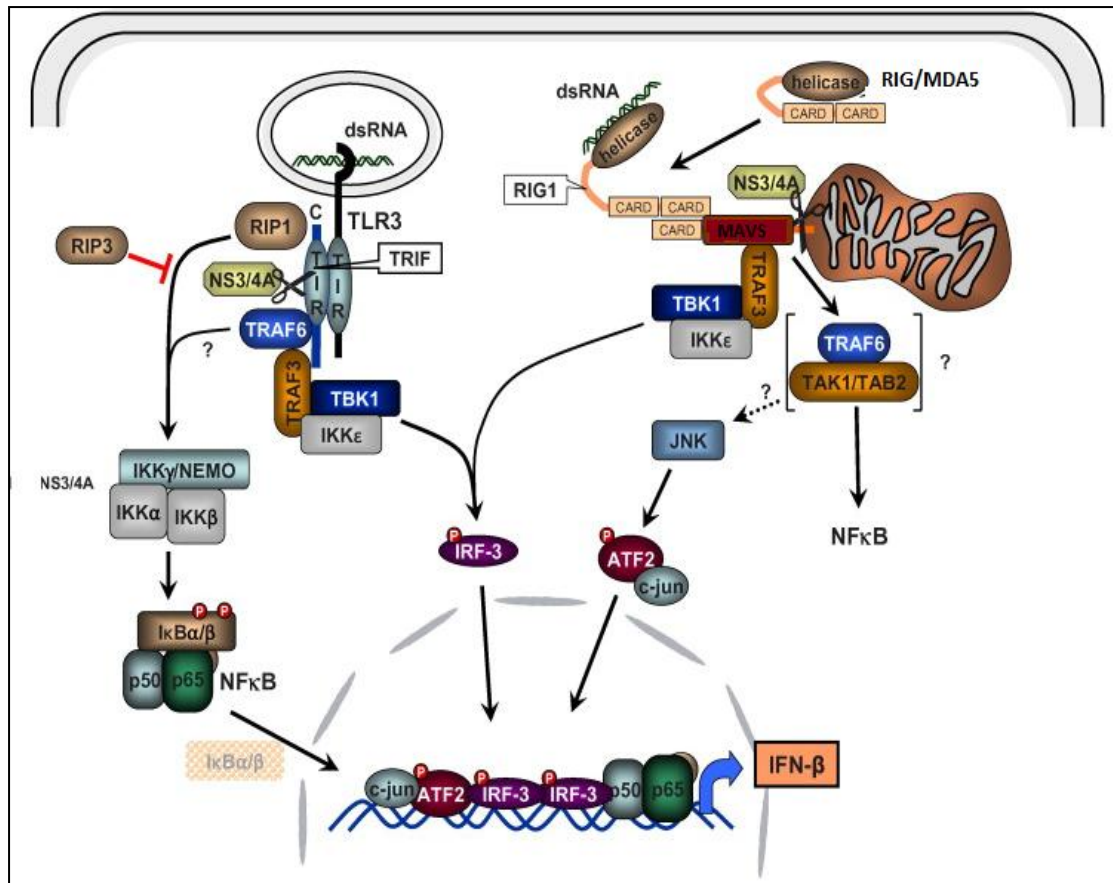


Figure 1.9: Induction of IFN gene expression by dsRNA. On dsRNA binding within endosomes, TLR3 recruits the adaptor TRIF through a TIR–TIR interaction. TRIF, in turn, recruits RIP1 to activate NF- κ B, via TRAFs and the IKK (I κ B kinase) complex. TRIF also recruits TBK1/IKK ϵ and TRAF3 to activate IRF3/7. RIG-I and MDA5 recruits mitochondrially anchored MAVS, which, in turn, recruits appropriate IKKs to activate NF- κ B and IRF, resulting in the induction of type I IFN. During an HCV infection, both TRIF and MAVS are cleaved and inactivated by the HCV NS3/4A protease. NS3/4A of HCV interrupts these pathways by cleavage of TRIF or MAVS. Figure adapted from Bode *et al*, 2007 (249).

Recent work with different viruses has led to the suggestion that RIG-I and MDA5 detect different subtypes of RNA ligands and this reflects virus-specific recognition by these helicases. For instance, when poly (I:C) is added to cells it activates TLR3, but when directly introduced into the cytoplasm, it preferentially activates MDA5, whereas RIG-I is activated by in vitro transcribed dsRNAs (250). Recently, it was

discovered that ssRNA with 5' triphosphate (5'ppp ssRNA) is a ligand for RIG-I (251) (252). Thus, it could be defined that dsRNA and 5'ppp ssRNA are recognized by innate immune sensors as “nonself” RNA, whereas “self” RNAs escape from detection because of capping of the 5'-triphosphate end or by nucleoside modification of RNA, both occurring during posttranscriptional RNA processing in eukaryotes (252). However, work conducted by Gale et al, has shown that 5'ppp is necessary but not sufficient for RNA binding by RIG-I and they identified a 100-nucleotide poly-U/UC region of the HCV genome and replication intermediate RNA as a ligand of RIG-I signalling which is independent of MDA5, MyD88 or TRIF dependent PRR pathways (253).

It has also become clear that RIG-I and MDA5 function differently in the recognition of RNA viruses (250, 254). RIG-I is essential for the production of interferons in response to RNA viruses including, influenza virus and Japanese encephalitis virus, whereas MDA5 is critical for picornavirus detection. The importance of MDA5 as an anti-viral protein had been suggested based on the finding that paramyxovirus V protein binds MDA5 and inhibits its function (250). In addition a recently discovered paramyxovirus Human metapneumovirus (HMPV), that causes a significant proportion of respiratory infections, activates the RIG-I/MAVS signalling pathway in airway epithelial cells, but MDA5 does not play a significant role (255). HMPV G protein was identified as virulence factor which inhibits the production of antiviral mediators by targeting RIG-I (255).

1.11.4.3. Negative regulation of RIG-I/MDA5:

The RIG-I/MDA5 signalling pathway is regulated by several specialized proteins which act as negative regulators and inhibitors of the JAK-STAT pathway. The

suppressor of cytokine signalling (SOCS) proteins specifically prevent STAT activation by binding to activated cytokine receptors, inhibiting the activity of JAKs, and targeting bound signalling proteins for proteasomal degradation (256). The SOCS family proteins is comprised of eight members (SOCS-1 to SOCS-7 and CIS) all sharing a central SH2 domain and a C-terminal SOCS box. SOCS-1 and SOCS-3 are the most effective members of this family and act as negative regulators of several intracellular pathways and particularly the Jak–STAT pathway. There is evidence that SOCS-1 and SOCS-3 inhibit the IFN-induced signal cascade and in hemopoietic tumour cells, overexpression of SOCS-1 or SOCS-3 inhibits the antiproliferative and antiviral activity of IFN- α/β (257, 258) and also IFN- γ (259). Silencing either SOCS1 or SOCS3 by small interfering RNA dramatically augments the production of IFN- γ in T cells. In chronically infected HCV patients, it has been shown that the HCV core/gC1qR-induced T-cell dysfunction involves the induction of SOCS (260). Recent studies showed a relationship between treatment failure and expression of SOCS3 in HCV patients who did not respond to type I IFN treatment, causing lack of response to IFN therapy (261, 262).

Recent findings have identified tumour suppressor CYLD (cylindromatosis) as a negative regulator of RIG-I-mediated innate antiviral response. These results showed that expression of CYLD inhibits the IRF3 signalling pathway and IFN production triggered by RIG-I; in contrast CYLD knockdown enhanced the response. CYLD removes polyubiquitin chains from RIG-I as well as from TANK binding kinase 1 (TBK1), the kinase that phosphorylates IRF3, coincident with an inhibition of the IRF3 signalling pathway (263).

Recently several regulatory proteins of the RIG-I pathway have been identified. It has been demonstrated that RIG-I-mediated signalling is positively and negatively

controlled by ubiquitination of RIG-I and this ubiquitination is necessary for efficient activation of the RIG-I signalling pathway (264, 265). CARD domains of RIG-I undergo Lys 63-linked ubiquitination by tripartite motif (TRIM) 25, a ubiquitin E3 ligase composed of a RING finger domain, a B box/coiled-coil domain and a SPRY domain (265). TRIM25^{-/-} cells showed impaired production of type I IFNs against viral infection. RIG-I also undergoes ubiquitination by the ubiquitin ligase RNF125, which leads to its proteasomal degradation (264).

As described before LGP2, which lacks a CARD, also function as a negative regulator of RIG-I/MDA5 signalling. LGP2 contains repressor domain (RD), which interact with the RD of RIG-I and suppress its self-association (266). Overexpression of Lgp2 inhibits Sendai virus and Newcastle disease virus signalling to IFN-stimulated regulatory element- and NF-κB-dependent pathways (239, 267). LGP2 is a negative regulator of RIG-I, but not of MDA5 (268). Lgp2^{-/-} mice exhibited resistance to lethal VSV infection, by an enhanced RIG-I response to viral replicative intermediates, which triggers an increase in the production of type I IFN. However, mice lacking LGP2 were observed to exhibit a defect in type I IFN production in response to infection by the encephalomyocarditis virus (EMCV), the replication of which activates MDA5-dependent innate immune responses (268). These results showed that both poly (I:C) and EMCV are recognized by MDA5.

1.11.4.4. HCV innate immune response evasion:

Persistence of HCV infection reflects its ability to disrupt virus-induced signalling pathways activating cellular antiviral defenses. As mentioned above, NS3/4A can inhibit IRF3 phosphorylation and activation (269). Different studies demonstrated that NS3/4A has the ability to inhibit both TLRs and RIG-I mediating signalling pathways

(230). NS3/4A cleaves Toll-IL-1 receptor domain which contains an adaptor-inducing interferon- β (TRIF or TICAM-1) by specific proteolysis (270, 271). Another study demonstrated that HCV NS3/4A interacts directly with (TANK-binding kinase-1) TBK1, and that binding inhibits the association between TBK1 and IRF-3, leading to the inhibition of IRF-3 activation (272).

In a similar way, NS3/4A disrupts the RIG-I pathway through proteolysis of essential signalling components of IRF-3 activation (269). Two independent studies have shown that HCV NS3/4A protease cleaves MAVS to block RIG-I signalling (273, 274). This cleavage by NS3/4A results in subcellular redistribution of MAVS and loss of interaction with RIG-I which preventing downstream activation of IRF-3 and IFN- β induction (273).

Interferon (IFN) therapy is the current therapy for chronic HCV infection, either IFN alone or in combination with ribavirin. Approximately 70% of patients infected with type 1b HCV are non-responders in terms of viral elimination (275).

Studies to elucidate the mechanism influencing IFN sensitivity in HCV infected cells may be crucial for improving the strategy of IFN therapy. Consequently, the study of the HCV genotype specific IFN responses by comparing the effects of the genotype 2a full-length JFH-1 genome and genotype 1b proteins on IFN signalling pathways should lead to a greater understanding of the mechanisms of IFN signalling suppression. By elucidating the innate immune response induction pathways, it is possible that new strategies that modify these pathways could lead to the development of novel antiviral agents against HCV.

1.12. Aims of Project:

- Examine the expression and activity of key innate immune response proteins including TLRs, RIG-I, MDA5, TRIF, MAVS and MyD88 in the presence and absence of HCV NS proteins in RNA replicons and HCV infected cells.
- Identify possible novel innate response proteins that are up or down regulated by IFN- α , in RNA replicons and HCV infected cells, by microarray analysis.
- Identification of the IFN components in the regulation of innate immune responses such as apoptosis, oxidative stress and cell cycle regulation.
- Examine the roles of interferon response pathway proteins to HCV expression by using RNAi to knock out the expression of individual host genes, in particular TLRs, RIG-I, MDA5, TRIF, MAVS and MyD88 in RNA replicons and HCV infected cells.
- Compare the effects of expressing NS3, and other HCV NS proteins from different genotypes on the interferon response pathway, in liver cell lines.

CHAPTER TWO:

2. MATERIALS AND METHODS

2.1. Materials

2.1.1. Molecular biological reagents

2.1.1.1. Chemicals and Molecular biological reagents

Agarose (Molecular biological grade, 4% agarose stock was prepared by Invitrogen) dissolving 4µg of agarose and in 100 ml distilled sterile water. The solution was autoclaved at 121°C for 20 minutes to sterilize. Before use the solution was equilibrated at 65°C in a water bath.

DEPC water Milli Q water treated with 0.1% v/v diethylpyrocarbonate (DEPC) (Sigma) for at least 1 hour at 37°C and then autoclaved (at least 15 min) to inactivate traces of DEPC

EDTA solution 0.5M EDTA in Milli Q water pH 8.0.

Ethidium bromide stock 10mg/ml ethidium bromide in sterile Milli Q water

MTT (3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide; Thiazolyl blue (Sigma) 5mg/ml MTT stock solution was prepared in PBS. The solution filtered by syringe and stored at +4°C

Paraformaldehyde	4% paraformaldehyde solution: 4g of paraformaldehyde was dissolved in 100 ml PBS.
Phosphate Buffered Saline (PBS)	137mM NaCl, 2.7mM KCl, 4.3mM Na ₂ HPO ₄ •7H ₂ O, 1.4mM KH ₂ PO ₄ . (pH 7.1). Or prepared by dissolving 1 PBS tablet (Sigma) per 200 ml Milli Q water. Autoclave to sterilize
PCR dNTPs (50x stock)	10mM each of dATP, dTTP, dCTP, dGTP (Promega) in water and stored at -20°C.
Phenol: Chloroform	Phenol : Chloroform : Isoamyl alcohol 125:24:1, pH 4.7
Sodium Acetate	3M Sodium Acetate in distilled water. Adjust pH to 5.2 with glacial acetic acid. Autoclave to sterilize.
TAE Buffer (50x)	2M Tris base, 50mM EDTA, 5.71% (v/v) acetic acid.
TBE Buffer (5x)	0.9M Tris base, 0.9M boric acid, 25mM EDTA.

TE buffer	10mM Tris-HCl pH 7.5, 1mM EDTA
Triton X-100 (Sigma)	0.1% (v/v) Triton X-100 in PBS

2.1.1.2. Tissue culture materials and reagents

Dulbecco's Modification of Eagle's Minimum essential medium (DMEM) (GIBCO, Invitrogen)	DMEM growth media supplemented with 2mM L-glutamine, 10% Fetal Calf Serum (FCS), 100 IU/ml penicillin and 100µg/ml streptomycin
Fetal Calf Serum (FCS) (Invitrogen)	Inactivation of complement in FCS was achieved by incubation at 56°C for 60 minutes
Trypsin-EDTA (TE) (GIBCO, Invitrogen)	10x (TE-EDTA)
Penicillin-Streptomycin (GIBCO, Invitrogen)	10,000 units penicillin; 10,000µg streptomycin

2.1.1.3. Reagents used for transfection purposes and assays preformed prior to transfection:

Lipofectamine 2000	Invitrogen
OptiMEM	Invitrogen
Trizol	Invitrogen
Geneticin (50mg/ml)	Invitrogen
Lipopolysaccharide (LPS) (E. coli O111:B4)	Sigma

poly (I:C) 10µg/ml. (polyinosinic-polycytidylic acid dsRNA) Sigma

2.1.1.4. Bacterial growth media

Luria Broth (LB)	1% (w/v) tryptone, 0.5% (w/v) yeast extract, 1% (w/v) NaCl
Luria Broth agar (LBA)	Bacto-agar added to 1.5% (w/v) to LB
SOC medium	2% Tryptone, 0.5% Yeast Extract, 10mM NaCl 2.5mM KCl, 10 mM MgCl ₂ , 10mM MgSO ₄ 20 mM glucose

2.1.1.5. FACS analysis solutions

Propidium iodide (PI) (Sigma)	200 mg of PI was dissolved in 10 ml of PBS, aliquoted into 1.5 Eppendorf tubes and kept at -20 °C in the dark.
Ribonuclease A (RNase A) (Sigma).	1:100 RNase A (50µg/ml)
Staining buffer	PBS, pH 7.4-7.6 2% (v/v) heat inactivated FCS 0.2% (w/v) sodium azide
4% paraformaldehyde (fixation buffer)	14 ml 10x PBS 10.8 ml 37.5% formaldehyde

	75.2 ml PBS
Permeabilization (PERM) buffer	FACS staining buffer + 0.5% saponin (Sigma S-2149)
SUPERPERM buffer	75% (v/v) PERM buffer, + 25% (v/v) FCS (filtered)

2.1.1.6. SDS-PAGE

Protein lysis buffer	50mM Tris, 150mM NaCl pH 7.4. 1% (v/v) NP40, 2.4M Sodium deoxy cholate, 1% (v/v) Triton X-1-00, 100mM Ethylene glycol-bis (β - Aminoethylether)-N-N-N'-N'- Tetra acetic acid (EDTA). Made up to 100ml and stored at 4oC.
Protease Inhibitor Cocktails	Protein inhibitor: 0.1% of inhibitors cocktail (20mM Phenylmethysulfonyl fluoride (PMSF), 20mM Sodium orthovanadate (Na ₃ VO ₄), 20mM NAF, 0.5mM Aprotinin, 0.01mM Leupeptin, 0.5mM Pepstatin)
SDS-PAGE separation gel	30% w/v acrylamide, 0.8 % w/v bis- acrylamide (National Diagnostics) 1.5M Tris-HCl, pH8.8, 0.1% (w/v) Sodium

	dodecyl sulphate (SDS) 0.1% (w/v)
	ammonium persulphate (APS) 0.5% (v/v)
	N,N,N',N'-tetramethyl-ethylenediamine (TEMED).
SDS-PAGE stacking gel	30% w/v acrylamide, 0.8 % w/v bis-acrylamide (National Diagnostics), 1M Tris-HCl, pH6.5 0.1% (w/v) SDS 0.1% (w/v) ammonium persulphate (APS) 0.5% (v/v) N,N,N',N'-tetramethyl-ethylenediamine (TEMED).
10x SDS-PAGE Gel Running Buffer	25mM Tris, 192mM Glycine, 3.5mM SDS (National Diagnostics).

2.1.1.7. Western blot reagents

APS 10% (w/v) (ammonium persulphate)	0.5g in 5ml of water, aliquoted and stored at -20°C
10x Tris –EDTA reducing buffer	Prepared by adding: 10% (v/v) Beta-mercaptoethanol, 0.5M Tris HCl, 8% (w/v) SDS. Aliquots and stored -20°C.
Bromophenol- blue/glycerol	0.02% Bromophenol blue in EDTA = 5ml. 5ml glycerol + 5ml bromophenol blue
Transfer Buffer	25mM Tris, 192mM Glycine, 20% (v/v) Methanol

PBS/Tween buffer solution	1x PBS/0.1% (v/v) Tween
PBS/Tween/skimmed	1x PBS/0.1% (v/v) Tween/2% (w/v)
	skimmed milk

2.1.2. Kits used in experiments

MEGAscript kit (Ambion)

DNA-free Kit (Ambion)

First-Strand cDNA Synthesis (Invitrogen)

ECL Western blotting detection reagents and analysis system (Amersham Biosciences)

QuantiTect™ SYBR® Green PCR (QIAGEN)

QIAquick PCR Purification Kit (QIAGEN)

QIAquick Gel Extraction Kit (QIAGEN)

HiSpeed Plasmid Midi Kit (QIAGEN)

ViraPower Adenovirus Expression System (Invitrogen)

pAd/CMV/V5-DEST vector (Invitrogen)

LR Clonase enzyme (Invitrogen)

2.1.3. Molecular weight markers

100bp DNA ladder (Promega): contains DNA molecules of 100, 200, 300, 400, 500, 600, 700, 800, 900, 1000, and 1500 base pairs. (Appendix 1)

1000bp DNA ladder (Promega): contains DNA molecules of 250, 500, 750, 1000, 1500, 2000, 2500, 3000, 4000, 5000, 6000, 8000 and 10000 base pairs. (Appendix 1)

RNA marker (Ambion): contains RNA molecules of 0.5, 1, 1.5, 2, 2.5, 3, 4, 5, 6, and 9 kilobases. (Appendix 1)

SDS-PAGE prestained molecular weight marker (Invitrogen). (Appendix 1)

2.1.4. Oligonucleotides

Oligonucleotides were synthesized by Invitrogen. The primers were dissolved in distilled water at a concentration of 100 μ M and stored at –20°C.

2.1.4.1. DNA primers for qRT-PCR of innate immune response genes:

Table 2.1 primers used in qRT-PCR for innate immune response genes. Sequences, gene accession numbers and PCR product size are detailed.

Target gene	Primers sequences	Accession number	PCR product size
TLR3-F	5- CCGCCAACCTTCACAAGGTAT	NM_003265	130 bp
TLR3-R	5-AGCTCATTGTGCTGGAGGTT		
TLR7-F	5- GCTCTGTGGGAGTTCTGTCC	NM_016562	129 bp
TLR7-R	5- ACCGTTTCCTTGAACACCTG		
TLR8-F	5-TGAAGCACATCCCAAATGAA	NM_138636	126 bp
TLR8-R	5-GCAACTCGAGACGAGGAAAC		
MDA5-F	5-GGGGCATGGAGAATAACTCA	AF095844	132 bp
MDA5-R	5- TGCCCATGTTGCTGTTATGT		
RIG-I-F	5- GTTGTCCCATGCTGTTCTT	NM_014314.3	124 bp
RIG-I-R	5- GCAAGTCTTACATGGCAGCA		

MAVS-F	5-GGGTAAACAGGGTGCAGAGA	NM_020746	129bp
MAVS-R	5-ACAGGCATCAAGGTGGTAGG		
TRIF-F	5- TCCTCCTCCTCCTCCTTCAT	AB093555	120 bp
TRIF-R	5- GCGTGGAGGATCACAAAGTT		
MyD88-F	5- GCACATGGGCACATACAGAC	NM_002468	132bp
MyD88-R	5- TAGCTGTTCTGAGGAGCTGT		
IFN- β -F	5-GACATCCCTGAGGAGATTAAGCA	NM_002176	130bp
IFN- β -R	5-CAACAATAGTCTCATTCCAGCCA		
JFH-1 F	5-TCTGCGGAACCGGTGAGTA	AB047639	131bp
JFH-1 R	5-TCAGGCAGTACCACAAGGC		
GAPDH	5-TCTCTGCTCCTCCTGTTTCGAC	NM_002046	118
GAPDH	5-CGGATTGTCGTATTGGG		

2.1.4.2. Primers sequences for AdNS3 and AdNS3/4A construction:

Table 2.2: primers used in cloning NS3 and NS3/4A genes which were amplified with PCR using high fidelity Taq polymerase for cloning into TOPO vector.

Primer Name	Sequence
NS3 1a F	<u>CACCAT</u> GGCGCCCATCACGGCGTA
NS3 1b F	<u>CACCAT</u> GGCGCCTATTACGGCCTA
NS3 2a F	<u>CACCAT</u> GGCTCCCATCACTGCT
NS3 1a R	CGTGACGACCTCCAGCA
NS3 1b R	CGTGACGACCTCCAGGTCAG
NS3 2a R	GGTCATGACCTCAAGGTCAGC
NS3/4A 1 R	GCACTCTTCCATCACATCG

NS3/4A 1b R	GCACTCTTCCATCTCATCG
NS3/4A 2a R	GCATTCCTCCATCTCATC

Bold italic: start codon

Underline: “CACC” topoisomerase recognition directional cloning sequence.

2.1.4.3. Primers used for plasmids sequencing:

Table 2.3 primers used for sequencing insert in vectors that were used for, NS3 and NS3/4A constructs.

Vector	Primer	Sequence
pENTR TOPO	M13 forward	5' GTAAAACGACGGCCAGT3
pENTR TOPO	M13 reverse	5' GGAAACAGCTATGACCATG
pAd/CMV/V5-DEST	T7 promoter	5' TAATACGACTCACTATAGGG

2.1.4.4. Primers used for adenovirus quantitation:

Table 2.4 primers used in quantifying NS3 and NS3/4A genes which cloned into adenovirus vectors.

Primer	Sequence
NS3-2a qRT-PCR forward	GGCGCCATAGTGGTGAGTAT
NS3-2a qRT-PCR reverse	TCCGTGGTAAACAGTCCACA
NS3-1a qRT-PCR forward	AGGGCTACAAGGTGTTGGTG
NS3-1a qRT-PCR reverse	GGCTGCCAGTGGTAATTGTT
NS3-1b F qRT-PCR forward	5' ATGGCGCCTATTACGGCCTA
NS3-1b R qRT-PCR reverse	5' ACACACGCCATTGACGCAGG

2.1.4.5. Synthetic siRNAs sequences duplexes used for RNAi work:

Table 2.5 siRNAs used in RNAi for various genes knockdown and their sequences.

Target gene	Sequence	Position
RIG-I sense	5- GGAAGAGGTGCAGUAUAUUuu	263-281
RIG-I antisense	5- AAUAUACUGCACCUCUCCuu	
MDA5 sense	5- GGUGAAGGAGCAGAUUCAGuu	345-362
MDA5 antisens	5- CUGAAUCUGCUCCUUCUCCuu	
MAVS sense	5'-UAGUUGAUCUCGCGGACGAuu	411-429
MAVS antisense	5- UCGUCCGCGAGAUCAACUAuu	
TRIF sense	5- GACCAGACGCCACTCCAACuu	1238-1259
TRIF antisense	5-GUUGGAGUGGCGUCUGGUCuu	
MyD88 sense	5'AAGGCAAUGAAGAAAGAGUUCuu	995-1015
MyD88 antisense	5-AACUCUUUCUUCAUUGCCUUuu	
SOCS3 sense	5-UGAUUUGGUUUAACCUGAtt	1484-1502
SOCS3 anti sense	5-UCAGGUUUAACCAAUAUCAaa	
Non-Targeting 1	5-GAUCAUACGUGCGAUCAGAtt	
	5-UCUGAUCGCACGUAUGAUCtt	

2.1.4.6. Primers used for microarray validation work:

Table 2.6 primers used in qPCR for microarray validation work and their sequences and PCR products size.

Target gene	Primers sequences	Accession number	PCR products size
SOCS1 F	GACCCCTTCTCACCTCTTGA	NM_003745.1	116
SOCS1 R	TAGGAGGTGCGAGTTCAGGT		
SOCS2 F	AGGGACTCGTTTTGGGATTC	NM_003877	111
SOCS2 R	GGGTCATGAGAGAAGGGTCA		
SOCS3 F	AGTTGTGTGTTGGGGTGGTT	NM_003955.3	178
SOCS3 R	TAGCCTCAAGGGCCTGAGTA		
IRF1 F	ACCCTGGCTAGAGATGCAGA	NM_002198	131
IRF1 R	AGGCATCCTTGTTGATGTCC		
IRF9 F (ISGF3)	ATGGATGTTGCTGAGCCCTA	NM_006084	140
IRF9 R (ISGF3)	ATGGCATCCTCTTCCTCCTT		
CCNB1 F	ATAAAGCTTGTGGCCCCTTT	NM_031966	149
CCNB1 R	TCAATTGGATCCCCAGGTAA		
CDC25C F	ATGCATCATCAGGACCACAA	NM_001790	139
CDC25C R	GCCAGTGGCTGGAATGTTAT		
GADD45- α F	GGCCCGGAGATAGATGACTT	NM_001924	120
GADD45- α R	TTTTCCTTCCTGCATGGTTC		
GADD45- β F	ACAGTGGGGGTGTACGAGTC	NM_015675	127
GADD45- β R	GGATGAGCGTGAAGTGGATT		
SKP2 F	GCTGAAGAGCAAAGGGAGTG	NM_005983	137
SKP2 R	GGAGGCACAGACAGGAAAAG		
MxA1 F	CCACATCCCTTTGATCATCC	NM_002462	111

MxA1 R	TTCAGGAGCCAGCTGTAGGT		
OAS3 F	TGCTGTGTACCCAAGAGCAG	NM_006187	117
OAS3 R	GGGATCTGAGACAGGTCCAA		
IFNAR2 F	GTGAGCAGTGGAGAGAAGGG	NM_207585.1	121
IFNAR2 R	GGAATTCCAATTGCAGGAGA		
STAT1 F	CAGCTGAACATGTTGGGAGA	NM_007315	120
STAT1 R	CCAAAGCCAGAAGGGAAAAT		
STAT2 F	AAGCACTGCTAGGCCGATTA	NM_005419	132
STAT2 R	ACCATGTCTCCAGCTGTTCC		
IFNAR1 F	AGGCAGTTGCATTCCACTCT	NM_000629	124
IFNAR1 R	TACCCCCAAGTGCATGGTAT		
PIAS1 F	TCCACATCAAGCATCTCCAG	NM_016166	125
PIAS1 R	AGTCGTAAGGCATGGGTGTC		
PIAS2 F	AGTTTGGATGGTGGCTCATC	NM_173206	122
PIAS1 R	TGAAGCAGCACAGAACCAAC		

2.1.5. Antibiotics:

Antibiotics used for bacterial cell culture were purchased from Sigma. Antibiotics were made as stock solutions 1000 times the working concentration, filter sterilized (0.2µm) and were stored as aliquots at -20°C. The autoclaved media were cooled to 45°C prior to addition of antibiotic.

1000x antibiotic stock solutions:

50mg/ml in Milli Q water.	Ampicillin:
25mg/ml in Milli Q water.	Kanamycin

2.1.6. Antibodies for immunofluorescence staining and Western blotting:

Table 2.7 Antibodies used in immunofluorescence staining and Western blotting and their concentration used during the experiments.

Antibody	Isotype	Supplier	Working concentration
Polyclonal sera NS3-7	Rabbit IgG	Raised against the NS3 peptide FIPVENLETTMRS (amino acids 1195 – 1207). Wardell et al, 1999 (88)	1:40
Anti V5	Mouse IgG	Invitrogen	1:1000
Anti GADD45- β	Mouse IgG	Guido Fransozo (Department of Medicine, Imperial College).	200ng/mL
Anti cyclin B1	Mouse IgG1	Santa Cruz Biotechnology.	200ng/mL
Anti tubulin	Mouse IgG1	Sigma	1:10000
Anti core	Mouse IgG	Abcam	IF: 1:400 or WB: 2 μ g/ml
Anti NS3	Mouse IgG	Novagene	IF: 1:100 or WB: 2 μ g/ml

Anti NS5A	Sheep IgG	Mark Harris, University of Leeds	1:100
Anti rabbit	Sheep IgG-HRP	Sigma	1:2000
Anti mouse	Goat IgG-HRP	Sigma	1:2000
anti-rabbit	AlexaFluor 488- conjugated IgG	Invitrogen	1:1000
anti-mouse	AlexaFluor 594- conjugated IgG	Invitrogen	1:1000

2.1.7. Restriction enzymes:

Table 2.8 restriction digestion enzymes used in plasmids cloning and their incubation time, temperature and concentration. All enzymes were from commercial suppliers.

Enzyme	Source	Incubation conditions	Concentration /dilution
EcoRI	Promega	Buffer H. 37°C. 4 h	1µl (10u/µl)
BamHI	Promega	Buffer E. 37°C. 4 h	1µl (10u/µl)
HindIII	Promega	Buffer E. 37°C. 4 h	1µl (10u/µl)
PacI	New England Biolab	1X NEB buffer 1. 37°C. 18 h	2.5µl (10u/µl)

2.1.8. X-Ray film:

X-OMAT AR film was used for Western blots and BioMax MR was used for all radiography, both were from VWR.

2.1.9. Cell lines:

2.1.9.1. Bacterial Cells:

Table 2.9: The following strains of chemically component *E.coli* cells were purchased from Invitrogen:

Cells	Genotype Characteristics
Subcloning Efficiency DH5 α	F- ϕ 80lacZ Δ M15 Δ (lacZYA-argF)U169 recA1 endA1 hsdR17(rk -, mk +) phoA ^{sup} E44 thi-1 gyrA96 relA1 λ -
MAX Efficiency DH5 α	F- ϕ 80lacZ Δ M15 Δ (lacZYA-argF) U169 recA1 endA1 hsdR17 (rk-, mk+) phoA supE44 λ - thi-1 gyrA96 relA1
Competent TOP10	F- mcrA Δ (mrr-hsdRMS-mcrBC) 80lacZ Δ M15 Δ lacX74 recA1 araD139 Δ (araleu) 7697 galU galK rpsL (StrR) endA1 nupG
INV α F'	F' endA1 recA1 hsdR17 (rk-, mk+) supE44 thi-1 gyrA96 relA1 ϕ 80lacZ Δ M15 Δ (lacZYA-argF)U169 λ -

2.1.9.2. Mammalian cell lines:

Table 2.10 cells lines used during the experiments.

Cells	Characteristics
Huh7	A differentiated human hepatoma cell line established from a hepatocellular carcinoma (276). Huh7 cells were a gift from John Monjardino (277)
Huh7.5	Derivative of Huh7 contained defective RIG-I mutant pathway. Highly permissive to replicate HCV than in the parental Huh7 cells (278). Huh7.5 cells were a gift from Charles Rice (Rockefeller University, USA).
HEK293A	A subclone of the 293 Cell Line. The 293 cell line is a permanent line established from primary embryonal human kidney transformed with sheared human adenovirus type 5 DNA (279) (280).
Rep4	Huh7 cell lines carrying S1179I HCV subgenomic replicons. Produced in Dr McGarvey's laboratory (Department of Medicine, Imperial College).

2.1.10. Plasmids

Table 2.11 plasmids used during the experiments.

Plasmid	Source
pHCV JFH-1 isolate	Dr Takaji Wakita (Tokyo Metropolitan Institute for Neuroscience, Tokyo, Japan.).
pHCV 1a, isolate H77	Dr. Charles Rice (Rockefeller University, New York USA).
pHCVrep1bBB7 (S1179I)	Dr. Charles Rice (Rockefeller University, New York USA).
pCR®2.1	Invitrogen
pENTR TOPO	Invitrogen
pAd/CMV/V5-DEST	Invitrogen

2.2. Methods

2.2.1. Cells Culture methods

2.2.1.1. Growth and maintenance:

Huh-7, Huh7.5 and 293A cell lines were routinely grown in Dulbecco's Modification of Eagle's Minimum essential medium supplemented with 1X nonessential amino acids, 100 unit/ml penicillin, 100µg/ml streptomycin and 10% Fetal Calf Serum (FCS). Inactivation of complement in FCS was achieved by incubation at 56°C for 60 minutes. 10x Trypsin-EDTA (TE-EDTA) was also purchased from Invitrogen. Cells were grown as a monolayer in 25, 75 or 175 cm² sterile tissue culture flasks in humid incubators at 37°C and 5% CO₂. Once the cells had reached confluence, growth medium was removed and the cells were washed once with sterile PBS pre-warmed at 37°C. 1X Trypsin-EDTA solution was added and incubated at 37°C until cells began to detach from the flask. Trypsin activity was neutralized by the addition of 5 volumes of DMEM contains 10% FCS and the cells were transferred to 15 ml sterile falcon tubes and spun for 5 minutes at 400 x g. The supernatant as discarded to remove dead cells and the pellet resuspended in DMEM and pipetted up and down until a homogenous cell suspension was achieved. An aliquot was transferred to a new flasks containing fresh DMEM with 10% FCS and then incubated at 37°C. Cells were passaged two or three times per week by adding them at a ratio of 1:4-1:8 and transferring them to new flasks depending on the growth characteristics of each individual cell line.

Huh7 cell lines carrying S1179I HCV subgenomic replicons (Rep4) were grown in DMEM with the supplements indicated above plus 500µg /ml G418 (geneticin, GIBCO/ Invitrogen).

2.2.1.2. Stimulation of cells with LPS and poly I:C

Huh7 and replicon cells were seeded into 6 wells or 24 well plates and allowed to grow until 60 - 70% confluent. Cells were then stimulated either by the addition of 100ng/ml of LPS (*E. coli* O111:B4, Sigma) or poly (I:C) 10ng/μl. At specified times post-stimulation, cells were lysed and RNA extracted using TRIZOL /chloroform method as described below and genomic DNA digested.

2.2.2. Preparation of real-time PCR samples:

2.2.2.1. RNA extraction:

The cells were plated in six well plates and treated with the appropriate stimulus. The medium was removed and 0.5ml Trizol (Invitrogen) was added and left for 5 minutes to allow for complete dissolution of the cells. The Trizol system uses a formulation of guanidinium thiocyanate and urea together with a phenol solution. The Trizol cell extract solution was then mixed by pipetting up-and-down and then placed in a 1.5ml Eppendorf tube. 250μl of chloroform were added and the mixture was shaken vigorously for 15 seconds and incubated at room temperature for 5 minutes. The organic and aqueous phases were separated by centrifugation at 12,000g for 15 minutes at 4°C. The aqueous phase (top layer) was then transferred to a new tube and the RNA was precipitated by the addition of an equal volume of isopropanol. The mixture was incubated at room temperature for 15 minutes and then centrifuged at 12,000g for 30 minutes at 4°C. The RNA pellet was washed with 70% ethanol and resuspended in 50μl of DEPC-treated water and stored at -80°C until further use.

2.2.2.2. DNase treatment:

This was carried out by using a DNA-free Kit (Ambion). 3µl of 10x volume DNase I buffer and 1µl of DNase I (2units) were added 1-5µg RNA in 30µl reaction, mixed gently, and incubated at 37°C for 20-30 minutes. 0.1 volume of DNase Inactivation Reagent was added to the mixture, which was then vortexed, and incubated for 2 minutes at room temperature. The tube was centrifuged at 10,000 x g for 1 minute to pellet the DNase Inactivation Reagent and the RNA was then transferred to a new tube.

2.2.2.3. Agarose gel electrophoresis of RNA

The horizontal gel electrophoresis apparatus used for DNA gels was also used for RNA gels but the apparatus was pre-treated to remove RNases which could degrade the RNA. Pre-treatment involved washing the apparatus with detergent and Milli Q water, then soaking it with a 3% (v/v) hydrogen peroxide solution (using a 30% (v/v) stock H₂O₂ solution and diluted in DEPC treated water). Lastly the hydrogen peroxide was washed off with DEPC water and the apparatus was left to air dry before use. RNA gels were prepared in a similar way to DNA gels, 1% (w/v) agarose in 1x TAE buffer (made and diluted with DEPC treated water) was melted in a microwave oven, 5µg/ml ethidium bromide was added, it was allowed to cool to 65°C and then poured into the assembled apparatus taking care not to introduce air bubbles. Once the gel had set it was covered to a depth of 5mm of appropriate 1x buffer (diluted in DEPC treated water) and the combs removed to reveal the wells. 5µl of RNA samples were added to 5µl of glycerol based loading buffer (glycerol: DEPC water (50% v/v) and the mixture was carefully pipetted into the wells. 1x agarose gel sample buffer (which contained blue tracking dye) was added to the last well of the gel and the gel was run at constant voltage of 80 volts (or at a constant current of 100mA until the sample, as

indicated by the position of the blue tracking dye present in the agarose sample buffer) had migrated to the end of the gel. The bands were visualized on a UV transilluminator and photographed.

2.2.2.4. Primer design:

Primers were designed using Primer3 software (available online at: http://www.broad.mit.edu/cgi-bin/primer/primer3_www.cgi/) and had a low probability of forming duplexes and hairpin loops using Oligo6 software (available online at: <http://www.oligo.net>). All primers were tested for specificity using standard PCR with total cDNA obtained from Huh7 or HCV RNA replicon cell RNA. These PCR products were characterized by agarose gel (1%) electrophoresis.

2.2.2.5. First-strand cDNA synthesis using M-MLV RT

RNA was extracted from mammalian cells and cDNA was synthesized by MMLV reverse transcriptase (Promega). In a nuclease-free microcentrifuge 1.5 ml tube: 1µl of 50µM oligo (dT) and 2µg total RNA were combined. The mixture was heated to 65°C for 5 minutes and quickly chilled on ice. The following were added to the contents of the tube: 5µl 5x MMLV Buffer, 2µl 0.1 M DTT, and 1µl RNasin (40 units/µl) (Promega) and 1µl 10 mM dNTP Mix. The mixture was then mixed gently and incubated at 37°C for 2 minutes. 1µl (200 units) of MMLV RT was added to the mixture and mixed by gently pipetting up and down. The tubes were then incubated at 37°C for 50 minutes. The reactions were inactivated by heating at 70°C for 15 minutes. Subsequently, 900ng of the first-strand cDNA was used for PCR.

2.2.2.6. Polymerase Chain Reaction (PCR):

PCR was performed by using specific sets of primers (table 2.1) separately in individual reactions for each sample. In a sterile Eppendorf tube, a mastermix

prepared by adding 5µl 10X Red Taq (NH₄) polymerase buffer (Biolone), 1µl of 2mM MgCl₂, 1µl (100µM each dNTPs, 10pmole of each primer) and 1 units of Red Taq polymerase (Bioline) and the volume adjusted to 50µl with HPLC water. Red Taq polymerase has a nontemplate-dependent terminal transferase activity that adds a single deoxyadenosine (A) to the 3' ends of PCR products. This allows PCR inserts to be ligated into the vector which has single overhanging 3' deoxythymidine (T) residues, as described below in section 2.2.2.7.

Reaction tubes were then placed in a thermal cycler (The GeneAmp® PCR System 9700, Applied Biosystem) and the DNA template was melted at 94°C for five minutes and then amplified by 30 rounds of 1 minute at the annealing temperature between 55-60°C for 1 minute at 72°C (elongation step), and 1 minute at 94°C (denaturation step). Following the final annealing step, the polymerization was allowed to proceed for 7 minutes to minimize the formation of single strands at the ends of the amplified DNA molecules. The PCR products were stored at -20°C and 10µl analysed by agarose gel electrophoresis. To avoid PCR contamination negative PCR controls were always included. If these gave positive results, then the experiment and reagent aliquots were discarded.

2.2.2.7. Cloning into pCR2.1:

PCR products of each target gene plus β-actin that showed single, clear and distinct bands of the correct predicted size bands were then ligated separately into pCR2.1 by TA cloning (TA cloning kit, Invitrogen) and transformed into INVα competent cells overnight. TA cloning provides a highly efficient cloning strategy were used for the direct insertion of Taq polymerase-amplified PCR products into a plasmid vector. The plasmid vector pCR2.1 was linearized and contains overhanging 3' deoxythymidine

(T) that allows PCR inserts produced previously Red Taq polymerase to be ligated efficiently using T4 DNA ligase. In a 0.2ml sterile PCR tube 10µl ligation reactions were performed by adding 1µl fresh PCR product, 1µl 10x ligation buffer, 2µl pCR2.1 vector (25ng) and 1µl T4 DNA ligase enzyme then the reaction was completed to 10µl final total volume with sterile water. The tubes were incubated at 14°C overnight. Next day, 2µl of each ligation reaction was directly pipetted into a vial of 50µl INVαF' *E.coli* competent cells for transformation. The cells were incubated on ice for 30 minutes and then heat shocked the cells for 30 seconds at 42°C without shaking. Immediately, the vials were transferred to ice. 250µl of SOC medium were added to each vial and incubated in shaking horizontally at 37°C for 1 hour at 225 rpm in an orbital incubator. Finally, 50µl to 200µl from each transformation vial were spread on LB agar plates containing X-Gal and 50µg/ml of kanamycin.

The next day, white colonies on X-Gal plates were selected and into 50ml tubes containing 5ml of LB plus 50µg/ml kanamycin were inoculated with individual bacterial colonies. The tubes were incubated at 37°C with shaking for a minimum of six hours and DNA miniprep were carried out for plasmid extraction as described on 2.2.3.1 section. The ligation of fragments within the pCR2.1 was confirmed by sequencing samples with an M13 forward primer.

2.2.2.8. Real-time PCR:

QuantiTech SYBR RT-PCR (Qiagen) kit was used to carry out Real-Time PCR analysis. The *QuantiTech™ SYBR Green Master Mix* contains an optimized concentration of the fluorescent dye SYBR Green. SYBR Green binds to all double-stranded DNA molecules during PCR, emitting a fluorescent signal on binding. The reactions are characterized at the point, during cycling, when amplification of the

PCR product is first detected, rather than by the amount of PCR product accumulated after a fixed number of cycles.

A master mix for PCR was prepared as follows: 10µl of SYBR Green, 0.2µl of RT enzyme, 1µl of 3.2µM of each primer, and 6.8µl of water per reaction. The master mix was then distributed into the tubes, mixed, and centrifuged for 3 seconds in PCR capillaries. 300-500ng of each RNA sample was added to the individual capillaries and the LightCycler was programmed as follows: initial activation step at 95°C for 15 min, denaturing at 94°C for 15 s, annealing at between 55 - 60°C for 30 s, and extension at 72°C for 30 s. PCR capillaries were placed in the LightCycler machine and the cycling program was started. Finally, PRISM software was used to draw the peaks of the different samples.

2.2.2.9. Absolute and fold change calculation in host gene expression

RNA standards required for absolute quantification were generated by *in vitro* RNA transcription. RNA transcript levels were quantified by using a spectrophotometer and then diluted to generate a standard curve ranging from 4ng/µl – 400fg/µl. Fold change in host gene expression was calculated from the qRT-PCR data using both absolute quantification, using standards curves and relative quantification by fold change calculation.

2.2.2.10. mRNA quantification using standards curves:

Absolute quantification was used to quantify the amount of target gene mRNA transcript present within the Huh7 and Rep4 cells RNA samples. An RNA standard curve was included in each qRT-PCR experiment to quantify the abundance of target

RNA. The levels of the β -actin mRNA transcript were also quantified for each cellular RNA sample using an RNA standard curve. Fold change in host gene expression was calculated from the absolute quantification of the target gene, by dividing the quantity e.g. (in μg) of the target gene by quantity (in μg) of β -actin to give a normalised measure of gene expression. Then, the normalised value of treated target gene divided by normalized control gene (non- treated). The following equation:

$$1) \text{ Normalized target gene expression} = \frac{\text{Quantity of target RNA}}{\text{Quantity of } \beta\text{-actin RNA}}$$

$$2) \text{ Fold change in target gene expression} = \frac{\text{Normalized expression treated sample}}{\text{Normalised expression untreated sample}}$$

Absolute quantification was also used to calculate the abundance of JFH-1 RNA present in infected Huh7.5 cells using an RNA standard curve (appendix 8.3).

2.2.2.11. Relative quantification

The higher the starting copy number of the DNA target, the earlier the increase in fluorescence is observed. The parameter C_T (threshold cycle) is defined as the first detectable increase in fluorescence (281, 282). The target gene copy number in unknown samples is quantified by measuring the C_T to calculate fold change in gene expression using the comparative C_T method. C_T or ‘cycle threshold’ values obtained from the qRT-PCR reaction were used to calculate fold change in gene expression using the $\Delta\Delta C_T$ equation.

The $\Delta\Delta\text{CT}$ equation:

CT target gene – CT reference gene = ΔCT

ΔCT treated sample – ΔCT untreated sample = $\Delta\Delta\text{CT}$

Fold change in gene expression = $2^{-\Delta\Delta\text{CT}}$

Reference genes used to normalise the host gene expression data included; GAPDH, and β -actin (281, 282). Finally, Results graphs representing fold change in host gene expression were generated using software and error bars represent mean values $\pm$ standard deviation (appendix 8.3).

2.2.3. Plasmids preparation:

2.2.3.1. Extraction of plasmid from bacteria: Mini-prep:

Plasmid Mini Prep kit (Qiagen) was used in this method according manufacturer's protocol. 5mls of an overnight culture of *E. coli* containing a plasmid was used to inoculate 50ml of LB and 100 $\mu\text{g/ml}$ ampicillin selective antibiotics. This was incubated at 37°C overnight. The bacterial cells were harvested by centrifugation at 3000g for 20 minutes at 4°C. All traces of supernatant were removed and the bacterial pellet was resuspended in 250 μl buffer P1. Next 250 μl Buffer P2 was added to the resuspended pellet and was mixed by inverting 4–6 times. This was incubated at room temperature for 5 minutes. 250 μl buffer N3 was added to the lysate after the incubation period and mixed immediately but gently by inverting 4–6 times. After addition of Buffer P3, a fluffy white precipitate containing genomic DNA, protein, cell debris, and SDS became visible. The lysate was centrifuged for 10 minutes. Supernatant was applied to the DNA binding column and centrifuged for one minute.

The column was then washed with 750µl Buffer QC. The DNA attached to the resin was eluted into a sterile Eppendorf tube with 1ml of Buffer TE.

2.2.3.2. Determination of DNA concentration

DNA samples were diluted 1:100 so that an A_{260} absorbance of between 0.1 and 1.0 was obtained. The absorbance of the sample was determined at 260nm and 280nm in a quartz cuvette using an Ultraspec 2000 spectrophotometer and the DNA concentration calculated from the standard estimation that 1.0 A_{260} absorbance unit is equal to 50µg/ml double stranded DNA.

2.2.3.3. Agarose gel electrophoresis of DNA

1.0% agarose in 1x TBE buffer was melted in a microwave oven, allowed to cool and then poured into the assembled gel electrophoresis apparatus. Once the gel had set it was covered with 1x TBE buffer and the comb removed to reveal the wells. The gel was run at a constant current of 80mA for one hour. The gel was stained with ethidium bromide for 20 minutes and destained for 20 minutes with water. The DNA bands were then visualized on a UV transilluminator and photographed (GelDoc-It Imaging System, VPU).

2.2.3.4. Plasmid DNA Digestion:

1µg of each plasmid was linearized by digestion with *EcoR*I restriction enzyme for 3 hours at 37°C. To each reaction, 2µl of restriction enzyme buffer (10x buffer H), 1µl of restriction enzyme, 2µl of BSA (1mg/ml) and 14µl of sterile nuclease free water were added. After 3 hours incubation, each plasmid DNA was checked to ensure that complete linearization had been achieved by agarose gel electrophoresis.

2.2.4. In vitro RNA transcription using T7 RNA polymerase.

The RNA standards were *in vitro* transcribed using pCR 2.1 plasmid DNA templates and the T7 RNA polymerase. The plasmid DNAs were linearized with Bam H1 restriction enzyme downstream of the insert to be transcribed using 5µl buffer H, 2µl of BSA, 1µl of Bam H1 enzyme and 2µg of DNA template.

The reaction was incubated at 37°C for a minimum of three hours and the linearized DNA templates were examined on a gel to confirm that cleavage was complete. After linearization the restriction digestion was terminated by adding the following:

- 1/20th volume 0.5 M EDTA
- 1/10th volume of 3 M sodium acetate
- 2 volumes of ethanol

The reactions were mixed well and chilled at –20°C for at least 15 min and then centrifuged for 15 min at 13000 x g. The supernatant was removed and re-centrifuged for a few seconds to remove the residual fluid. The pellet was suspended in dH₂O at 0.5–1µg/µl.

2.2.5. Proteinase K treatment and phenol/chloroform extraction:

This was performed to remove any residual RNases, restriction enzymes that could introduce RNase or other inhibitors of transcription from DNA preparation. The linearized plasmid DNAs were treated with 1µl proteinase K (100–200µg/ml) and 0.5% SDS for 30 min at 50°C. This was followed with phenol/chloroform extraction (using an equal volume) and ethanol precipitation. 115µl of nuclease-free water and 15µl 5M ammonium acetate stop solution were added and mixed thoroughly, and then

an equal volume of chloroform was added to recover aqueous phase and transferred to a new tube.

2.2.6. RNA transcription:

To perform RNA transcription, the following components were added are for a single 20 μ l reaction; 2 μ l of each ATP, CTP, GTP, and UTP solution, 2 μ l enzyme mix, 2 μ l 10X reaction buffer and 1 μ g linear DNA template. The reaction was mixed gently and incubated at 37°C for 16 hours. RNA transcripts were precipitated by adding 30 μ l nuclease-free water and 30 μ l lithium chloride (LiCl 7.5M) precipitation solution. These were mixed thoroughly and chilled for 30 min at –20°C. RNA was pelleted by centrifugation at 4°C for 15 min in an Eppendorf mini-centrifuge at maximum speed. The supernatant was removed, and the pellet washed with 1 ml 70% ethanol, and re-centrifuged to maximize removal of unincorporated nucleotides. Finally, the 70% ethanol was removed, and the RNA was resuspended in 10 μ l RNase free water. The concentration of the RNA transcripts within the sample was estimated by measuring absorbance at 260 nm and the RNA was stored frozen at –80°C until further use.

2.2.7. Construction of recombinant NS3 and NS/4A adenoviruses

Adenoviruses containing individual NS3 and NS3/NS4A inserts from genotypes 1a, 1b and 2a DNA were prepared using a ViraPower Adenovirus Expression System (Invitrogen) according to the manufacturer's instructions.

2.2.7.1. Plasmids:

DNA fragments encoding each of the HCV NS3 and NS3/4A nonstructural proteins were generated from full-length cDNAs containing the whole genome of HCV clones of three different genotypes namely: HCV genotype 2a, JFH-1 isolate (Accession no. AB047639), HCV genotype 1b Con1 isolate (Accession no. AJ238799) and HCV genotype 1a, isolate H77 (Accession no. NC_004102).

2.2.7.2. Primers design and PCR:

Specific oligonucleotide PCR primers were generated to amplify the gene of interest. The sequence “CACC” was added to the 5’ end of forward primers to enable directional cloning. ATG initiation codons were also added for proper initiation of translation within the primer sequences.

Full-length NS3 and NS3/4A sequences were amplified by PCR with ACCUAZYME proofreading polymerase (Accuzyme polymerase; Bioline, Germany). Reaction tubes were then placed in a thermal cycler (The GeneAmp® PCR System 9700, Applied Biosystem). Thirty cycles of PCR were performed at 94°C for 60 s, at 55°C for 30 s, at 72°C for 120 s, and at 72°C for 10 min in 50µl reaction mixture containing 0.3µl (1 U/µl) Taq polymerase, 5µl 10x PCR buffer, 1µl dNTPs (20 mmol/l), 1µl of each primer (10µmol/l), 1µl DNA as template, and 41.45µl of distilled water. The PCR products were subjected to electrophoresis on a 1% agarose gel for 60 min (voltage

80V) and visualized using a UV transilluminator and photographed (GelDoc-It Imaging System, VPU).

2.2.7.3. Subcloning into the entry vector:

The PCR products of NS3 and NS3/4A were subcloned into the pENTR/D-TOPO vector with a pENTR Directional TOPO Cloning kit (Invitrogen). The pENTR Directional TOPO cloning Kits utilize the blunt-end PCR products produced by adding CACC sequence from 2.2.7.2 into one of the pENTR TOPO vectors to generate an entry clone. These clones generate an expression construct by performing an LR recombination reaction between the entry clone and a Gateway destination vector of choice as described below. In a 0.2ml PCR tube 2µl of fresh PCR product was combined with 1µl salt solution (1.2 M NaCl, 0.06 M MgCl₂, Invitrogen) and sterile water to a final volume of 5µl. Finally 1µl of pENTR TOPO vector was added, mixed gently and incubated for 30 minutes at room temperature. At the end of incubation period, the reaction tubes were placed on ice until proceed to transforming into TOP 10 *E. coli* competent cells. Transformation was performed by mixing 2µl of cold TOPO cloning reaction mixture, and 50µl of competent *E. coli* cells (TOP 10). The mixture was incubated on ice for 30 minutes, and then heat shocked for 30 seconds at 42 °C and placed back on ice. SOC medium (250µl) was added to cells which were then incubated at 37°C for 1 hour in an orbital shaker at 200 rpm. After incubation 50 and 100µl aliquots of the cell suspension were spread onto two LB agar plates which contained 50µg/ml kanamycin, and incubated at 37°C overnight. Next day, colonies were selected and grown in 50ml universal tubes which contained 5 ml of LB broth with 50µg/ml kanamycin at 37°C for 16 hours. A QIA Spin Miniprep Kit

was used to prepare the plasmid DNAs. The inserts were then sequenced with an M13 forward primer to confirm that they were correct.

2.2.7.4. Cloning into the pAd/CMV/V5-DEST adenovirus vectors plasmids:

The cDNA inserts were then transferred to the pAd/CMV/V5-DEST vector (Invitrogen) by means of the Gateway system using LR Clonase (Invitrogen). pAd/CMV/V5-DEST vector *E1/E3* is a replication-deficient adenovirus vector based on the adenovirus serotype 5 (Ad5) *E1/E3* deletion mutant. Recombination of the pENTR entry clones with the pAd/CMV/V5-DEST vector was performed using the LR Clonase enzyme mix. Briefly; 2µl 5x LR reaction buffer, 50-150ng entry clones and 300ng pAd/CMV/V5-DEST vector were mixed and made up to final volume 8µl with TE. Then 2µl of LR Clonase enzyme mix was added and mixed by briefly vortexing. The reaction was incubated at 25°C for one hour. To stop the reaction 1µl of Proteinase K was added and the reaction was incubated at 37°C for 10 minutes. The recombined plasmids were transformed into *E. coli* (TOP10) cells and expression resistant transformants were selected on LB plates containing 50µg/ml ampicillin.

2.2.7.5. Sequence analysis

To confirm that the HCV sequences had been correctly inserted, all samples with successful ligation were sent for DNA sequencing. For pENTR plasmids which contain M13 binding sequencing sites, 500ng of from each plasmid was mixed with 3.2µM of forward or reverse M13 primers. While for pAd/CMV/V5-DEST, the plasmid contains T7 primer binding sequences was mixed with 3.2µM of forward or reverse T7 primers. All sequencing materials were sent to the DNA sequencing service at the MRC Clinical Science Centre, Hammersmith Campus, Imperial College.

2.2.7.6. Production adenoviruses in 293A cells

Before transfection of the expression clones into 293A cells, the plasmids must expose the left and right viral ITRs on the vector to allow proper viral replication and packaging. pAd/CMV/V5-DEST vectors contain Pac I restriction sites. Digest at least 4µg of purified plasmid DNA of pAd/CMV/V5-DEST expression constructs were linearized with Pac I (New England Biolabs) by adding, 2µl of restriction enzyme buffer (NEB buffer 1), 2.5µl of Pac I, 2µl of BSA (100µg/ml BSA) sterile nuclease free water to a volume of 50µl. After overnight incubation, each plasmid DNA was checked, to ensure that complete linearization had been achieved, by agarose gel electrophoresis.

The linearized plasmids were then transfected into subconfluent 293A cells (Invitrogen) with lipofectamine 2000 in Opti-MEM medium. The day before transfection, 293A cells were seeded into 6-well plates at 5×10^5 cells per well with 2 ml containing 10% FCS DMEM. Next day the medium was removed and replaced with 1.5 ml of Opti-MEM I medium. Plasmid transformation solutions were prepared as follow, 4µg of Pac I-digested pAd-CMV-DEST expression plasmid DNA was mixed gently with 250µl of Opti-MEM I medium in a 1.5 Eppendorf tube. In a separate Eppendorf tube, 5µl of lipofectamine 2000 as added to 250µl of Opti-MEM I medium, mixed gently and incubated for 5 minutes at room temperature. After the incubation, the DNA with the Lipofectamine 2000 preparations were combined, mixed gently and incubated for 20 minutes at room temperature to allow the DNA-lipofectamine 2000 complexes to form. Following the incubation the DNA-lipofectamine 2000 complexes were added drop-wise to 293A cells in each well and the plates were rocked gently and incubated overnight at 37°C in 5% CO₂. The next day, the transfection mix was remove and replaced with 10% FCS DMEM culture

medium. 48 hours post-transfection, the cells were trypsinized and transferred to a sterile T25 tissue culture flask containing 10 ml of complete culture medium. The culture medium was replaced with fresh 10% FCS DMEM culture medium every 2-3 days until visible regions of cytopathic effect (CPE) were observed about 4-5 days post-transfection. The cells were kept until infections proceed until approximately 80% CPE was observed. At 10-13 days post-transfection, the adenoviruses were harvested by detaching cells from the flask by vigorous pipettes and transferred into a sterile 15 ml tube. The tubes were placed at -80°C for 30 minutes, and then placed in a 37°C water bath for 15 minutes to thaw. The freezing and thawing steps were repeated twice, followed by centrifugation of the cell lysate in a bench-top centrifuge (HERAEUS, MEGAFUGE 1.0R) at 3000 rpm for 15 minutes at room temperature to pellet the cell debris and the adenovirus-enriched supernatants were obtained. Six plasmids were transfected to produce six adenoviruses designated as AdNS3-1a, AdNS3-1b, AdNS3-2a, AdNS3/4A-1a, AdNS3/4A-1b and AdNS3/4A-2a. As a control, the pAd/CMV/V5-GW/*lacZ* vector (Invitrogen) was transfected into 293A cells to produce *LacZ*-bearing adenovirus.

2.2.7.7. Adenovirus stock amplification:

293A cells were trypsinized, counted and seeded in a T25 flask at 3×10^6 cells with normal growth medium 10% FCS DMEM at 80 - 90% confluency. Next day, 100µl of a suspension of AdNS3 and AdNS3/4A adenoviruses stocks added to the cells, which were then gently swirled and incubated at 37°C in a CO₂. The infection was allowed to proceed for 3 – 5 days until 80 - 90% of the cells had rounded up and either detached from or lightly attached to the tissue culture flask. Adenoviruses were harvested by vigorously pipetting with a 10 ml tissue culture pipette and transferred to

a sterile 15 ml tube. The tubes were placed at -80°C for 30 minutes to freeze the cells, and then placed in 37°C water bath for 15 minutes to thaw. The freezing and thawing steps were repeated twice, followed by centrifugation of the cell lysate in a bench-top centrifuge (HERAEUS, MEGAFUGE 1.0R) at 3000 rpm for 15 minutes at room temperature to pellet the cell debris. Finally, the supernatant containing viral particles was transferred to cryovials as 1.7 ml aliquots and stored at -80°C until further use.

2.2.7.8. Titration of adenoviruses stocks:

A day before infection, of 293A cells were trypsinized, counted and plated in 6 well plates with 1×10^6 cells per well and grown to 80 - 90% confluence. Next day, a vial of each six AdNS3 and AdNS3/4A adenoviruses stock in addition to AdLacZ viruses were thawed for 10 min at 37°C water bath. 10 fold serial dilutions ranging from 10^{-4} to 10^{-9} were prepared, by dilution in 10% FCS DMEM, to a final volume of 1 ml. The culture medium from the cells was removed and each 1 ml dilution of adenoviruses was added to one well of cells. The plate was then swirled gently and incubated at 37°C overnight. The following day, the medium containing virus was removed and the cells were gently overlaid with 2 ml of 4% agarose overlay solution per well. The overlay solution was prepared by mixing 12 ml of prewarmed 2% FCS DMEM (at 37°C) and 1.2 ml of pre-warmed (at 65°C) 4% agarose in PBS. The overlay solution was applied to the cells by gently pipetting the overlay down the side of each well. The plate was placed in a level tissue-culture hood at room temperature for 15 minutes or until the agarose overlay solidified and incubated at 37°C in a humidified atmosphere of 5% CO₂. Two days following the initial overlay, the cells were overlaid with an additional 1 ml of agarose overlay solution per well (prepared as described in material section) and allowed to solidify before returning the plate to a

37°C humidified 5% CO₂ incubator. The plates were monitored until plaques became visible, in about 8-12 days. After 8-12 days post-infection each well was gently overlaid with 300µl of 5 mg/ml MTT solution (1/10 the volume of the agarose overlay) on top of the solidified agar and incubated for 3 hours at 37°C. Finally the plaques were counted and the titer of adenoviral stocks was calculated and expressed as plaque forming units (pfu) per ml. The titer was calculated as follow: the number of plaques each well was counted, then the following formula was used to determine the titer (pfu/ml) of viral stock.

$$\text{The viral titer} = \frac{\text{Number of plaques per well}}{\text{Dilution factor X volume of diluted virus added to the well}} = \text{pfu/ml}$$

2.2.7.9. Adenoviruses genome copy number calculation using qPCR:

To assess the number of adenoviruses genome copies per cell, DNA was extracted from virus stocks using the Trizol method. Copy numbers were assessed using real-time PCR-based quantification. PCR was performed using a Light Cycler Real-Time PCR System using the SYBR Green PCR Mastermix as described for qRT-PCR. A set of primers was developed specifically for each genotype (table 2.4). Standard curves were run in duplicate and consisted of six ten-fold dilutions using total genomic DNA. Melting curve analysis confirmed the quality and specificity of the PCR products. The viral titer was expressed as number of viral particles per ml of supernatant (VP/ml). The virus concentration was calculated using the following equations.

The average genome size of an adenovirus is approximately 40 kbp, therefore,

Number of VP per ng adenoviral DNA =

$$\frac{\text{Mass of sample (g)} \times \text{Avogadro's Number}}{\text{Genome length (pb)} \times \text{mass of bp}}$$

$$\text{Virus Titer (VP/ml)} = \frac{\text{Amount of VP}}{\text{Viral sample volume (ml)}}$$

$$1 \text{ ng adenoviral DNA} = \frac{(1 \times 10^{-9}) \text{ g}}{40,000 \text{ bp} \times 660 \text{ g/bp} \times 6 \times 10^{23}} = 2.3 \times 10^7 \text{ VP}$$

$$\text{Virus Titer (VP/ml)} = \text{Amount of adenoviral ds DNA (ng)} \times 9.2 \times 10^7 \text{ VP/ng}$$

2.2.7.10. Adenovirus transduction:

One day before transduction 1×10^6 of Huh7 cells were seeded in 6 well plates with 10% FCS DMEM. Next day, the medium was removed and cells in each well were washed with PBS. The PBS was removed and adenovirus was added to the cells at an MOI of 100 pfu/cell. The cells were incubated with adenovirus overnight at 37°C after which the virus inocula were removed and replaced with complete tissue culture medium and the cells were then incubated at 37°C overnight.

2.2.8. Immunofluorescence confocal microscopy of HCV NS3 proteins in

Huh7 cells:

Glass cover slips (22 x 22 mm) were sterilized (dipped in alcohol and flamed) and placed in the bottom of the wells of 6 well plates. Huh7 cells were seeded at 2×10^5 per well with 10% FCS DMEM to grown to 60-70 confluence. Next day, the cells were transduced with the adenovirus constructs, AdNS3-1a, 1b, 2a, AdNS3/4A-1a, 1b, 2a and AdLacZ recombinant adenoviruses at MOI as detailed in section 2.5.11.

After 72 hours post infection the cells were prepared for immunofluorescence staining to assess NS3 recombinant protein expression. First, the mix was removed from the wells and the cells were washed with 1ml PBS followed by adding 2ml of 4% paraformaldehyde in PBS fixation solution and incubated for 20 minutes at room temperature in the hood. The mix was then removed from the wells and the cells were washed twice with 1ml PBS. The cells were permeabilised for 20 minutes with 0.1% Triton X-100 in PBS and then washed twice wash with PBS. Non-specific binding was blocked with 1ml 10% FCS for 30 minutes and then the cells were washed twice with PBS. Each of the cover slips was removed from the corresponding well and placed, cell coated face down, onto 50µl of rabbit polyclonal anti-NS3 antibody (NS3-7) diluted 1 in 50 in sterile PBS or 50µl mouse anti tag V5 antibody (anti-V5) diluted 1:5000 in sterile PBS on a clean glass microscope slide for one hour at room temperature. Unbound antibody was washed off by washing three times in PBS and the coverslips were then placed face down onto 50µl of FITC conjugated AlexaFluor 488-conjugated anti-rabbit IgG or AlexaFluor 594-conjugated anti-mouse IgG (Invitrogen), diluted 1:1000 in sterile PBS on a clean glass microscope and incubated for one hour at room temperature. The slide was washed again three times in sterile PBS, and placed face down on mounting medium (Vectashield with DAPI) on a clean

microscope slide. The cells were viewed at 400x magnification using an inverted microscope.

2.2.9. RNA interference (RNAi): Synthetic siRNA transfection:

A day before transfection, Huh7 or Huh7.5 cells were seeded at 1×10^5 cells per well in a 24 well plate in 500 μ l 10% FCS DMEM and grown until 40-50% confluent at the time of transfection.

For each well to be transfected, 1- 2 μ l of 50 μ M of synthetic siRNA duplex (siRNAs sequences table 2.5) was mixed with 50 μ l OptiMEM I reduced serum medium to a total volume of 50 μ l in a sterile Eppendorf tube. In a separate tube 1 μ l of lipofectamine 2000 was mixed with 50 μ l OptiMEM I reduced serum medium. Both of the preparations were incubated for 5 minutes at room temperature. The lipofectamine/ OptiMEM was then combined with the siRNA/ OptiMEM and incubated for 20 minutes at room temperature. Then 100 μ l of the transfection mixes were added into the wells and the cell were then incubated at 37°C in 5% CO₂ for 8 hours. Finally, the transfection mix was removed and replaced with 0.5 ml of 10% FCS DMEM and incubated again for 48 hours at 37°C in 5% CO₂. In all experiments GAPDH and scrambled non-targeting siRNAs were used as a control to examine the efficiency of transfection

2.2.10. HCV culture:

2.2.10.1. HCV Constructs and Transcription:

The HCV JFH-1 culture system was previously established in Dr. McGarvey's Lab. The JFH-1 clone was derived from a Japanese patient with fulminant hepatitis. This full genome cloned cDNA was inserted into a plasmid behind a T7 promoter to create

the plasmid pJFH-1, as well as a replication-defective NS5B negative control construct pJFH-1 GND (118) . These two plasmids, the pJFH-1 and pJFH-1- GND, were linearized at the 3' end of the HCV cDNA by XbaI digestion. The linearized DNA was then purified and used as a template for in vitro transcription (MEGAscript; Ambion, Austin, TX). 4µg of transcribed JFH-1 RNA was transfected into Huh7.5 cells using lipofectamine 2000 for 24 hours and then the cells were passaged every 2–3 days before the cells became confluent. The generated virus was checked by immunofluorescent staining for core protein and the titer of HCV RNA by quantitative RT-PCR after extracting RNA from medium, as described before.

2.2.10.2. Titration of Infectious HCV:

Cell culture supernatants which contained HCV were serially diluted 10-fold in 0.5 ml of 10% FCS DMEM and used to infect 10^5 naïve Huh7.5 cells per well in eight well chamber slides (Labtek). The inocula were incubated with cells for 4 h at 37°C and then supplemented with fresh 0.5 ml of 10% FCS DMEM. The level of HCV infection was determined 3 days post infection by immunofluorescence staining, the cells were washed with PBS, fixed with 0.5 ml 4% paraformaldehyde for 30 minutes at room temperature and then washed twice with PBS. The cells were permeabilised for 20 minutes with 0.5 ml 0.1% Triton X-100 (in PBS) at room temperature and incubated with 50µl HCV anti-core antibodies 1:400 dilution (C7-50, Abcam) for one hour at room temperature. The cells were washed with PBS three times, then 50µl of secondary antibody (Alexa-594 DAM dilution 1:1000 in 1% BSA in PBS) for 1 hour at room temperature. The cells were washed again for 5 minutes in PBS three times with a final wash in sterile distilled water. Excess water was removed and slides mounted with Mowiol plus DAPI. A fluorescence microscope (Nikon EclipseT2000S)

was used to visualize and count the number of positive staining foci. The viral titres were expressed as focus forming units per ml of supernatant (ffu/ml).

2.2.10.3. Production of HCV virus stock:

To generate viral stocks, infectious HCV cell culture media were diluted in 10% FCS DMEM and used to inoculate naive 10–15% confluent Huh7.5 cells at an MOI of 0.01 in a 175 cm² tissue culture flask. Infected cells were trypsinized and replated before confluence at day 4–5 postinfection. The infected cell culture media (35 ml per flask) were collected in sterile 50 ml tubes and cell debris was removed by low-speed centrifugation (5 minutes at 400 x g). The culture medium was then passed through a 0.45-µm syringe-top filter unit to remove dead cells debris. To concentrate the virus, the filtered culture medium was pipetted into an Amicon Ultra-15 filtration unit, (maximum volume of 15 ml per unit) and centrifuged for 30 minutes at 3,600 x g and 4°C (MSE Mistral 3000i centrifuge). The centrifugation supernatant was then collected and aliquoted into 1.7 ml cryogenic tubes for storage at -80°C. The titer of viral stock was determined as described above.

2.2.11. Flow cytometry analysis:

2.2.11.1. Cell FACS analysis of DNA fragmentation and Cell cycle using propidium iodide (PI) staining

PI is a fluorogenic compound that binds stoichiometrically to nucleic acids so that fluorescence emission is proportional to the DNA (and RNA, which has to be removed if DNA is to be measured) content of a cell. When apoptotic cells are stained with PI and analyzed with a flow cytometry, they display a broad hypodiploid (sub-

G1) peak, which can be easily discriminated from the narrow peak of cells with normal (diploid) DNA content in the red fluorescence channels.

Huh7 and Huh7.5 cells were seeded in 6 well plates at 2×10^5 per well and the next day they were infected or mock infected with HCV JFH-1 for an indicated time. At different time points, the cells were harvested by trypsinization at 37°C for 2-3 minutes, 2 ml of PBS was added and the cell suspensions were transferred to 12 x 75 mm polypropylene tubes and centrifuged at 1200 rpm for 5 minutes at 4°C. Then the supernatant was discarded and 1 ml of PBS was added to the cell pellet, mixed gently and centrifuged again at 1200 rpm for 5 minutes at 4°C. The supernatant was discarded completely and clean tissue paper was used to remove any residual PBS. The cells were fixed by adding 1ml of 70% (v/v) cold ethanol to the cell suspension drop-wise while being vortexed and then the tubes were incubated overnight at -20°C. Next day, the tubes were centrifuged at 1200 rpm for 5 minutes at 4°C and the supernatant was discarded. The cells were then washed twice with 1 ml of PBS for each tube, mixed gently and centrifuged again at 1200 rpm for 5 minutes at 4°C. The supernatant was discarded and the cells resuspended in 1 ml of DNA staining solution which containing 20µg/ml propidium iodide and 1:100 RNase A (50µg/ml). The cells were incubated for at least 3 hours on ice in the dark, and then the cell cycle profiles were determined by flow cytometry using a FACScan (Becton-Dickinson, USA). Cells were analyzed by flow cytometry using 488-nm and measure red fluorescence 620 nm and side scatter, which allow the discrimination of true apoptotic nuclei from nuclear debris. The apoptotic cells displayed a broad hypodiploid (sub-G1) peak, which can be discriminated from the narrow peak of cells with normal (diploid) DNA content. Also, the distribution of cells in the various phases of the cell cycle indicates a proliferating cell population: G1 and S (DNA synthesis phase), G2 and M-phase

(mitosis). To set the acquisition parameters correctly, experiments included the following; 1. Non-infected, unstained (no PI) negative control cells, 2. Mock infected stained with PI negative control and 3. Positive infected and stained with PI positive controls cells.

For accurate FACS analysis, at least 20,000 events were collected for reading; the first gate was performed to gate out residual debris. A second gate was carried for doublet discrimination. Then, the cell cycle profiles were obtained by detecting the cells stained with PI. The cells were also stained with anti-core antibodies and the infection efficiency was evaluated by calculating the percentage of FITC core positive cells. (appendix figures 8.4 and 8.5 show an example of this analysis)

2.2.11.2. FACS analysis of HCV core intracellular staining:

A day before infection 2×10^5 of Huh7 and Huh7.5 cells were seeded in six well plates and infected with HCV JFH-1 at different MOI as indicated and times as described in 2.9.1. For FACS staining all steps prior to fixation were carried out on ice. The cells were harvested by trypsinization at 37°C for 2-3 minutes, 2 ml of PBS was added and the cell suspensions were transferred to Falcon 12x75 mm polypropylene tubes and centrifuged at 1200 rpm for 5 minutes at 4°C. The supernatant was then discarded and 1 ml of PBS was added to the cell pellet, mixed gently and centrifuged again at 1200 rpm for 5 minutes at 4°C. The supernatant was discarded and 300µl of fixative (4% paraformaldehyde and formaldehyde in PBS) was added to each cell pellet, mixed gently and incubated on ice for a minimum of 30 minutes. After incubation, 1 ml of PBS was added to each tube, mixed gently and centrifuged again at 1200 rpm for 5 minutes at 4°C. The supernatant was discarded and then resuspended in 200µl PBS and transferred to a V-shape 96 well plate

(Becton-Dickinson). For intracellular staining all of the subsequent steps were carried out using saponin containing buffers. First 200µl PERM buffer (staining buffer with 0.5% (w/v) saponin) was added to each well, mixed thoroughly and then centrifuged at 1200 rpm 5 minutes using a plate centrifuge (SCI Quip, DJB Labcare, Sigma). This washing step was repeated once with 200µl SUPERPERM buffer. The supernatant was discarded and 80µl (1:400 in PBS) of the mouse monoclonal (anti core C7-50 Abcam) was added to each sample except the negative control and left for at least 1 hour at room temperature. Then, the cells were washed twice with 200µl PERM buffer per sample and spun as before. After removing the supernatant, the cell pellets resuspended in 100µl of the rabbit anti mouse IgG-FITC secondary antibody (DAKO), diluted 1:50 in PBS and left for at least 1 hour at room temperature. The plate was spun as before and the supernatant was removed. The cell pellets were washed with 200µl of PERM buffer, spun as before, and the supernatant was discarded again. After the last step, the cells were washed with PBS once as described before and the cells were then resuspended in 200µl of staining buffer (2% FCS in PBS), mixed gently and transferred to FACS analysis tubes.

Cell fluorescence signals were determined by a FACS caliber flow cytometer (Becton Dickinson). FITC was identified at 530nm. The analysis was performed using CELLQUEST software, version 4.3, (Becton Dickinson). The primary gate was set to exclude dead cells or debris. The background level was then estimated by comparison with cells in which primary antibody had been omitted during the staining procedure.

2.2.12. Protein and Western blotting work

2.2.12.1. Extraction from Mammalian Cells

Cells were grown to 70% confluence. On the day of protein extraction, cells were washed twice with 2ml cold PBS. 1 ml of protein lysis buffer was added to each flask

and cells were scraped off with a sterile scraper. The cells were then transferred to pre-cooled (to 4°C) 1.5 ml Eppendorf tubes and centrifuged for 12 minutes at maximum speed at 4°C (SORVALL Biofuge Fresco). The supernatant from each tube was transferred to a new pre-cooled Eppendorf tube and stored at -20°C.

2.2.12.2. Bradford assay of protein estimation using a 96 well plate:

10µl of each soluble cell extract was diluted 1/20 with the addition of 190µl milli Q water. 0.4 ml of each known BSA stock concentration protein standards were used to make serial dilutions of the 1mg/ml. The following 0.4 ml dilutions of BSA were made: serial dilutions in water of 1µg/µl BSA in water were used as known protein concentration in the Bradford assay; 0.0µg/µl, 0.05µg/µl, 0.1µg/µl, 0.25µg/µl, and 0.5µg/µl.

200µl of 1x Bradford reagent was added to each well in addition to 10µl of either BSA standards or an unknown protein sample. All samples were mixed and incubated at room temperature for 30 minutes. The absorbance for each sample was measured at 595 nm and the test protein concentrations were calculated by generating a graph of the standards (the standard curve) with the dependent variable (µg/ml) on the X axis and the independent variable (595 nm) on the Y axis.

2.2.12.4. Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE):

Separation gel

A separation gel (10 or 15%) was prepared by the addition of 3.3 or 5 ml protogel (30% acrylamide, 0.8 % w/v bis-acrylamide, National Diagnostics), 2.5 ml resolving buffer (1.5M Tris, pH 8.8, 0.1 ml of 10% SDS, National Diagnostics), 100µl of 10%

ammonium persulphate, 10 μ l TEMED (Sigma) and made up to 10 mls with milli Q water. The separation gel mixture was pipetted into the pre-assembled glass plates in the mini gel apparatus. Once the mixture was added, propanol was pipetted above the separation gel to form a thin airtight layer, allowing polymerisation to proceed. The gel was left to stand at room temperature for 1 hour. Just before adding the stacking gel mix, the propanol was removed.

Stacking gel

A stacking gel mix consisted of 1.5 ml of protogel (30% acrylamide, 0.8 % w/v bis-acrylamide), 2.5ml staking buffer (contain 0.5M Tris, 0.1 ml 10% SDS, National Diagnostics), 0.1ml 10% ammonium persulphate, 10 μ l TEMED and 5.7ml double distilled water. The stacking gel pipetted above the separation gel and an 8 well comb was placed. The stacking gel was allowed to polymerise for 30 minutes at room temperature. Following polymerisation, the gel was transferred to the running gel apparatus and 1x running buffer (1.92 M glycine, 0.25 M Tris and 3.5mM SDS) was poured into the upper and lower compartments of the tank and the combs removed from the stacking gels.

2.2.12.3. Preparation of samples

Protein samples were prepared by adding 10 μ g of protein in sample buffer (2.5 μ l 10x Tris-EDTA reducing buffer and 10 μ l bromophenol blue/glycerol. The protein samples were then loaded into the gel in a total volume of 40 μ l. 15 μ l SDS-PAGE prestained molecular weight marker (Invitrogen) was also loaded onto each gel. Before loading, all protein samples were mixed with the sample buffer and boiled in a

heater block for 5-10 minutes, spun briefly transferred and loaded into pre-assembled apparatus (Bio-Rad). Once loaded, the gel was run at a constant voltage of 100V for 1 hour or when the dye front was 0.5cm from the bottom the power was switched off and the gels were prepared for either Western blotting or Coomassie staining.

2.2.12.4. Coomassie Blue staining

Staining was carried out using 0.1% (w/v) Coomassie Blue (CBB) dissolved in methanol: water: glacial acetic acid (ratios 45:45:10, by volume). This acid-methanol mixture acts as a denaturant to fix the protein in the gel, which prevents the protein from diffusing within the gel or being washed out while it is being stained. Gels were stained overnight, by gentle agitation, at room temperature. Following the staining, the gels were immersed in a destain solution (30% propan-2-ol, 10% acetic acid) for a minimum of 2 hours. Once they were sufficiently destained, the gels were placed in transparent plastic paper for scanning.

2.2.12.5. Western blotting

Nitrocellulose membrane and blotting paper were cut to the correct dimensions. The membrane was then immersed in Western transfer buffer (1.92 M glycine, 0.25 M Tris and 3.5mM SDS, 20% methanol). The scotch bright pads (from the Western blot transfer tank-BIO-RAD), blotting paper and the nitrocellulose membrane were then, placed in transfer buffer. SDS-PAGE gels were disassembled from the running apparatus, rinsed gently in transfer buffer, and set up in the transfer sandwich. This sandwich was prepared as follow; the plastic holder (black side) 1x sponge, 2 x filter paper, protein gel, membrane, 2x filter paper, 1x sponge, plastic holder (clear side).

The sandwich and cooling unit were placed in the transfer tank containing transfer buffer. The transfer unit was then run at 120V for 1 hour at 4°C to allow the transfer of the proteins from the gel to membrane.

2.2.12.6. Immunolabelling of the membrane

Following the transfer of proteins, the membrane was removed from the transfer tank and placed in water. The membrane was wiped gently to remove any acrylamide. To ensure there had been a successful transfer of proteins to membrane, membrane was immersed into Ponceau S solution (SIGMA) for 30-60 seconds. Ponceau S binds to proteins and allows their detection but does not interfere with the immuno-detection steps that follow. The membrane was then transferred to water and washed a further three times with water. The membrane was then transferred to a container and water added to ensure the membrane did not dry. The membrane was removed from the water and immersed in 10 ml of 10% blocking solution (PBS/Tween/4% skimmed milk). The membrane was left on a rocker (10-20 revolutions/min) at room temperature for 1 hour. Following incubation, the membrane was washed twice for 5 minutes with 10ml 0.1% PBS/Tween. The membrane was then placed on plastic film and 2µg of primary antibody diluted in 2ml of PBS/ Tween/BSA solution was added. A sheet of plastic film was placed on top and it was incubated on ice for overnight on (30 rev/min on rocker). Following the incubation, the membrane was washed three times for 5 minutes (30 rev/min on rocker) with 10ml 0.1% PBS/Tween/BSA solution and the secondary antibody (horseradish peroxidase (HRP) labelled anti-mouse or anti-rabbit) was added to the membrane at a dilution of 1:1000 in 2mls 0.1% PBS/Tween/BSA and incubated at room temperature for an hour, on a rocker (30

rev/min). Following the incubation, the membrane was washed three times for 5 minutes with PBS/Tween/BSA solution.

2.2.12.7. Western blotting detection

Following the final wash of the membrane (section 2.2.12.6) the PBS/Tween/BSA solution was removed. For blot detection SuperSignal West Pico Chemiluminescent Substrate was used (Thermo scientific). An equal volume of detection solution 1 (Lumino/ Enhancer solution) and 2 (stable peroxide solution) were mixed, ensuring there was a sufficient volume to cover the membrane. The excess wash buffer was drained away from the washed membrane which was placed protein side up on a sheet of cling film. The detection solution was then pipetted onto the membrane and incubated for 1 minute at room temperature. The detection solution was then drained away by holding the membrane gently with forceps and touching the edge of it against paper towel. The membrane was then placed protein side down onto fresh cling film, wrapped up and any air bubbles expelled by smoothing out. The wrapped membrane was then placed protein side up in an x-ray film cassette and a sheet of autoradiography film (Hyperfilm ECL, Amersham Biosciences) was placed on top of the wrapped membrane. The cassette was closed and the membrane was exposed for 15 seconds minutes. Following exposure, the film was developed immediately, and on the basis of its appearance, it was possible to estimate time required to produce an optimal signal with a second sheet of film. The second exposure time varied from 1 to 10 minutes.

2.2.13. Immunofluorescence double staining microscopy and subcellular

localization of HCV proteins and cyclin B1 in Huh7 cells:

Glass cover slips were sterilized (dipped in alcohol and flamed) and placed in the bottom of the wells of 24 well plates. 1×10^5 Huh7 cells were seeded in half of the

wells and infected with HCV JFH-1 at an MOI of 3. In other wells Huh7 (1×10^5) cells were mock infected. The infected and mock infected cells were incubated for 72 hours at 37°C at 5% CO₂.

The media were then removed from the wells and the cells were washed with 0.5 ml PBS followed by fixation with 0.5 ml of 4% paraformaldehyde in PBS for 20 minutes at room temperature. The cells were permeabilized for 20 minutes with 0.5 ml 0.1% Triton X-100 (in PBS) at room temperature. Non-specific binding of antibodies was blocked by adding 0.5 ml of 10% BSA in PBS for 30 minutes at room temperature and washing twice with 0.5 ml PBS. Each of the coverslips were removed from the corresponding well and placed, cell coated face down, onto 50µl of mouse monoclonal anti-cyclin B1 antibody (Santa Cruz), diluted 1 in 50 in sterile BSA, at room temperature on a clean parafilm sheet for one hour. Unbound antibody was washed off by washing three times in PBS and the coverslips were then placed face down onto 50µl AlexaFluor 594-conjugated anti-mouse IgG (Invitrogen), diluted 1:1000 in sterile 1% BSA/PBS and incubated for one hour. The coverslips were washed three times in sterile PBS and placed onto 50µl of sheep polyclonal anti-NS5A secondary antibody diluted 1 in 100 in sterile BSA at room temperature on a clean slide for one hour. Unbound antibody was washed off by washing three times in PBS and the coverslips were then placed face down onto 50µl FITC-conjugated anti-sheep IgG (DAKO), diluted 1:50 in sterile 1% BSA/PBS and incubated for one hour. Finally, the coverslips were washed again three times in sterile PBS, and placed face down on mounting medium (Vectashield with DAPI) on a clean microscope slide. The cells were viewed at 400x magnification with a fluorescent microscope (Nikon EclipseT2000S).

CHAPTER THREE

3.0. Effects of HCV replication on the host innate immune responses

3.1. Quantification of host gene expression:

Quantitative methods were used to measure the difference in the levels of host gene mRNAs in Huh7, Rep 4 and JFH-1 infected cells. RNA was extracted and cDNAs were generated and used for PCR using primers for individual host genes. Standard curves were generated using PCR standards of known concentration and the test PCR samples should then fall within the range of these standard curves. To create real-time PCR standards, PCR products were cloned in a plasmid vector. Small quantities of PCR products amplified by host gene specific primers, which also include β -actin and GAPDH primers that were used as a controls, were run on agarose gels. They were then eluted and ligated into pCR2.1 (Invitrogen), as described in the Materials and Methods. Plasmid DNAs corresponding to the standards were purified and samples were digested with EcoR1 restriction enzyme and separated on agarose gels to confirm that the ligations had been successful. Furthermore, the plasmids were sequenced to confirm the sequence orientation of the inserts. After confirmation of ligation, the plasmids were linearized using Pst1 and transcribed using a T7 transcription kit (Ambion). The transcripts were run on RNA gels to confirm that RNA transcripts of the correct size had been produced (figure 3.1). The transcripts were kept until needed for the real-time qRT-PCR reactions.

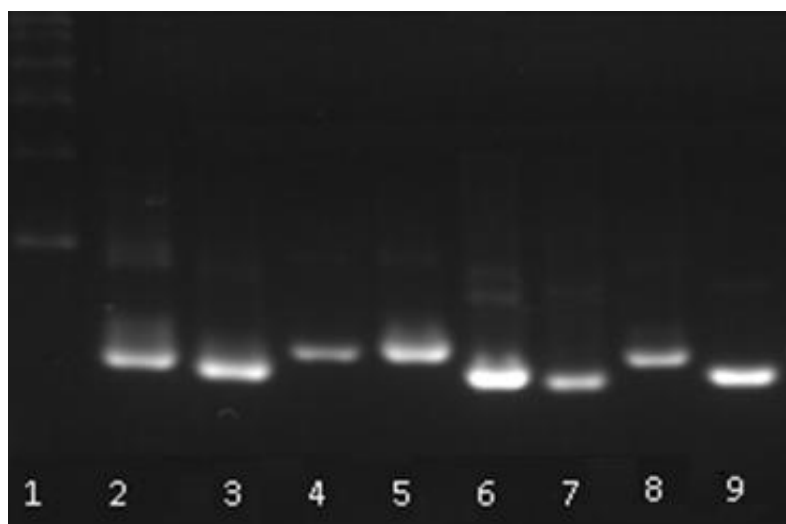


Figure 3.1: RNA transcripts for the host genes. 1µl of the RNA transcription reaction was run with 3x RNA loading buffer on a 2% agarose gel stained with SYBR safe. RNA ladders were included for RNA size estimation. Lane 1, RNA ladder, lane 2 RIG-I, lane 3 MDA5, lane 4 MAVS, lane 5 TRIF, lane 6 TLR7, lane 7 TLR3, lane 8 TLR8 and lane 9 MyD88.

3.2. RT-PCR Standard curves production:

Standard curves were produced to enable the determination of the copy numbers in all of the test samples. To generate a standard curve using a known template, the copy number had to be determined. Six sequential ten-fold dilutions were made for each standard. The unknown test samples from Huh7, Rep 4 cells, JFH-1 infected cells or siRNA transfected cells were compared to the 6 standards for the target standard curve of β -actin or GAPDH. The copy number of the target gene was normalized against β -actin or GAPDH by dividing it with the copy number of β -actin or GAPDH gene. This normalization is necessary to correct for any inaccuracies during the preparation of RNA e.g. RNA extraction from the cells or DNase treatment. In addition, each of the real-time RT-PCR runs included a water control to detect any contamination by PCR template.

Relative quantification is an alternative method for calculating fold change in the expression of host genes, in which RNA standard curves do not need to be included in the qRT-PCR reaction. Instead, fold changes in expression can be calculated directly from the qRT-PCR cycle threshold (CT) values using the $\Delta\Delta CT$ equation as described in materials and methods (section 2.2.2.11).

3.3. JFH-1 cell culture system

3.3.1. Production of JFH-1 infectious virus

A cell culture system for the production of full length JFH-1 infectious virus was previously established in Dr McGarvey's laboratory according the protocol proposed by Wakita et al ([118](#), [283](#)). Virus production was carried out using the highly permissive Huh7.5 cell line which expresses high levels of CD81 and has a defective interferon response due to an inactive RIG-I gene. HCV infection was determined by staining for HCV core protein (figure 3.2A).

3.3.2. Titration of Infectious HCV and determination of JFH-1 infectivity in Huh7.5 cells:

HCV culture cell supernatants were serially diluted 10-fold in 10% FCS DMEM and used to infect Huh7.5 cells using 8 well chamber slides. The original stock were diluted in 1:500, 1:1000 and 1:2000 dilution, and was incubated with cells for 4 h at 37°C. The level of HCV infection was determined 2 days post infection by immunofluorescence staining for HCV anti-core and foci were counted from the

different dilution to determine the stock concentrations. The viral titer is expressed as focus forming units per ml of supernatant (ffu/ml).

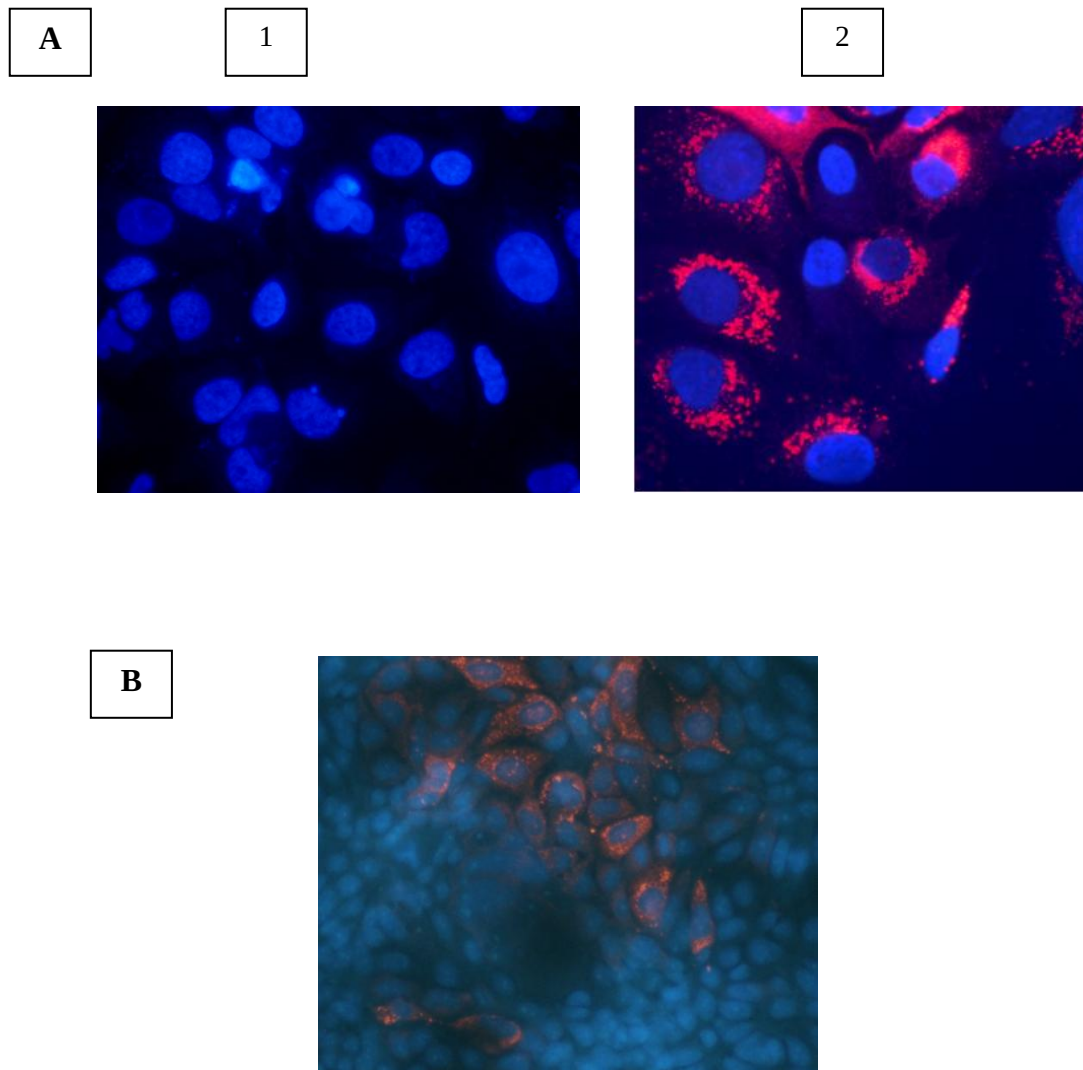


Figure 3.2 **(A)**: HCV core protein staining. Huh7.5 cells producing infectious JFH-1 virus particles were stained for HCV core protein using a mouse monoclonal antibody (red staining). Cell nuclei were stained with DAPI (blue staining). Images were captured at a magnification of 40x with a fluorescence microscope. (1). Mock infected Huh7.5 cells. (2). JFH-1 infected Huh7.5 cells. **(B)**: Titration of JFH-1 viral stocks. Huh7.5 cells were seeded into 8-well chamber slides. 1 μ l and 0.1 μ l of the concentrated JFH-1 virus stocks were used to infect the Huh7.5 cells in duplicate. After 48 hours, the chamber slides were stained for HCV core protein and foci were counted at a magnification of 20x with a fluorescent microscope.

3.4. Expression of extracellular and intracellular innate immune response receptors genes in Huh7 and replicon cells:

To investigate the molecular mechanisms of interferon induction during HCV infection first the basal expression levels of components of the dsRNA response pathway in naïve (untransfected) Huh7 cells and Huh7 cells transfected with the HCV subgenomic replicon (Rep4), were examined. The quantification of mRNA by qRT-PCR of the main intracellular and extracellular dsRNA (RIG-I, MDA5 and TLR3) and ssRNA recognition receptors (TLR7 and TLR8) in test samples was determined using standard curves. In Huh7 cells, RIG-I, MDA5 and TLR7 were expressed at higher levels than TLR3 and TLR8 was undetectable (Figure 3.3A). However, the presence of the HCV replicon causes the induction of gene expression of about 4-5 fold for RIG-I, MDA5 and TLR3, whereas TLR7 was induced by around two fold (figure 3.3B).

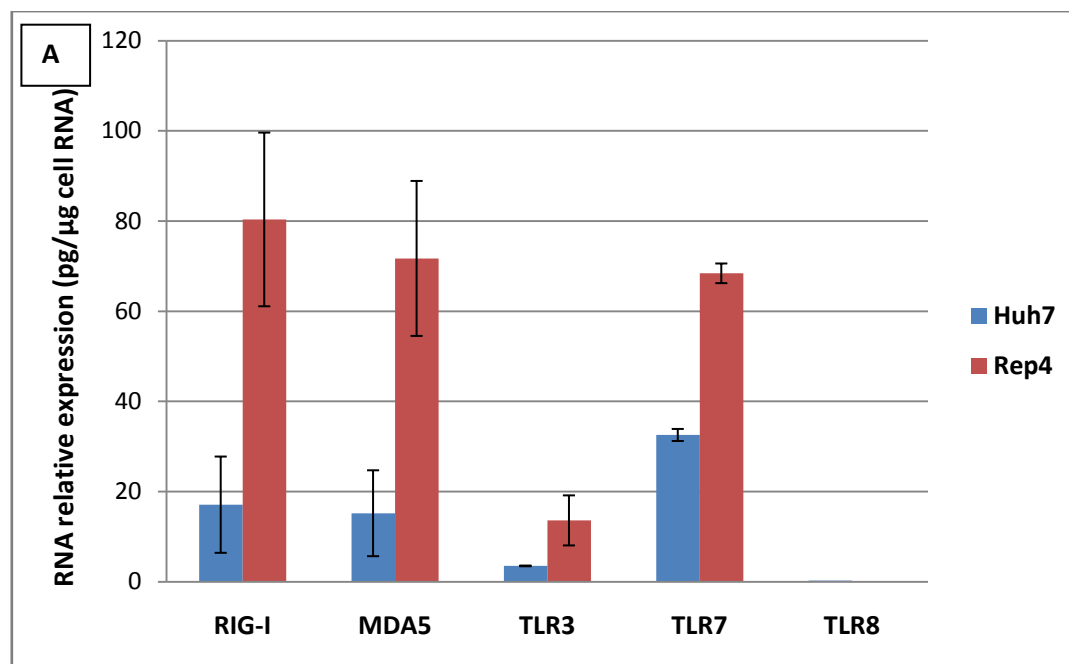
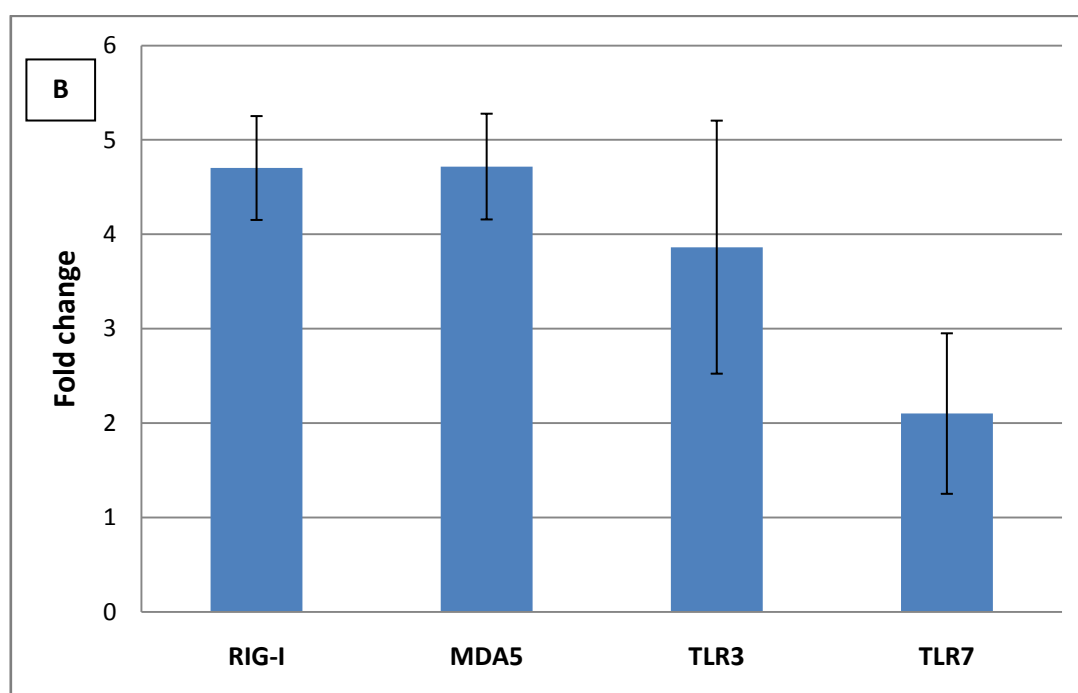


Figure 3.3: (A) Expression of RIG-I, MDA5, TLR3, TLR7 and TLR8 mRNA in Huh7 and Rep4 cells. Expression levels were determined from total RNA using real time qRT-PCR and normalized by comparison to β -actin. RNA standard curves for each target gene and the β -actin gene as control were used to quantify the mRNAs. (B) Fold change induction of RIG-I, MDA5, TLR3 and TLR7 mRNA in Rep4 cells. Fold change was calculated by comparing expression data for the Rep4 cells to the naïve control Huh7 cells. The expression data for relative quantification was normalized using β -actin.

To examine the effect of HCV non-structural proteins on the induction of IFN- β , cells were first treated with poly (I:C) a synthetic dsRNA which is a strong inducer of IFN- β . Cells were either transfected with 5ng/ μ l of poly (I:C) or poly (I:C) was added to the cell culture medium. Upon poly (I:C) treatment, Huh7 cells showed large increases in expression levels for MDA5 (50 fold) and RIG-I (12 fold). Additionally, TLR3 expression levels were also stimulated up to 17 fold (figure 3.4).

By contrast, although the expression of these genes was also induced in response to poly (I:C) treatment in Rep4 cells they were significantly less induced compare to Huh7 cells (figure 3.4). These data indicate that the HCV NS proteins have a role in reducing the induction of IFN- β and other components of the interferon induction pathway.

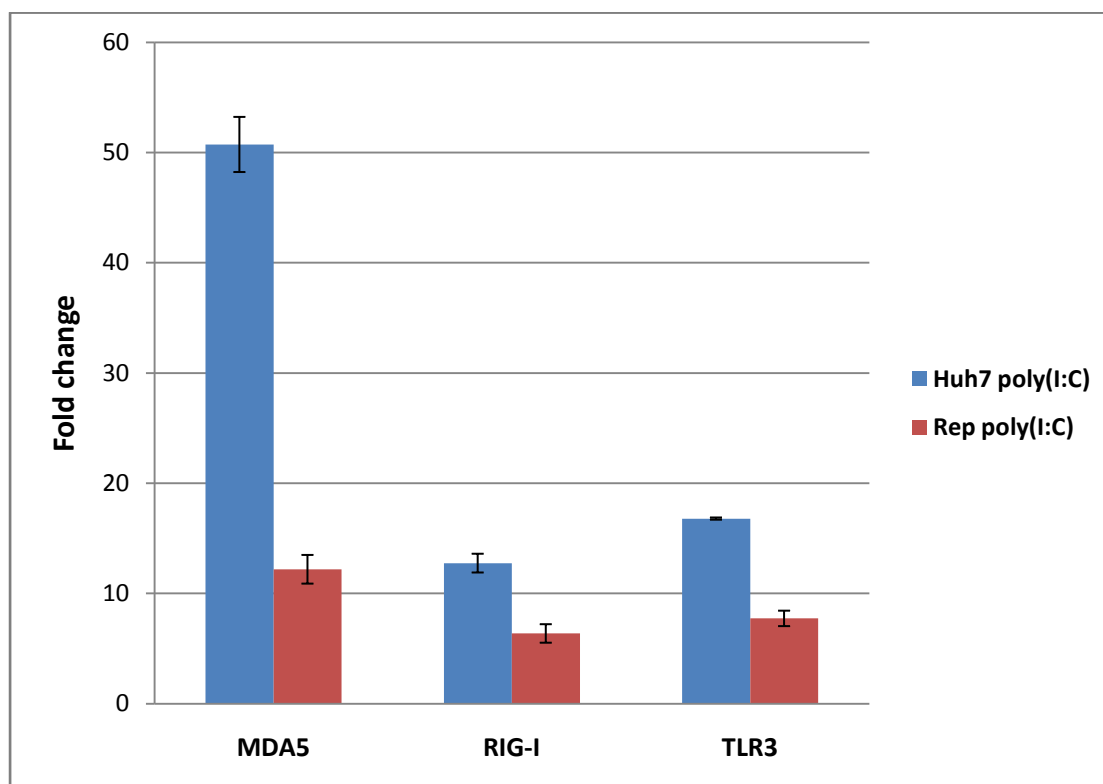


Figure 3.4: Fold change induction of RIG-I, MDA5 and TLR3 mRNA in Huh7 and Rep4 cells after stimulation with poly (I:C). RIG-I and MDA5 were measured after transfection with poly (I:C), whereas TLR3 was measured after extracellular stimulation with poly (I:C). Fold change was calculated by comparing expression data for the Rep4 cells to the naïve control Huh7 cells. The expression data for relative quantification was normalized using β -actin.

3.5. Expression level of signaling mediator genes in Huh7 and replicon cells:

RIG-I, MDA5, TLR3 and TLR7 signaling leads to the activation of NF- κ B and IRF3 transcription factors through pathways mediated by MAVS adaptors for RIG-I and MDA5, and TRIF for TLR3 and MyD88 for TLR7. To assess the consequences of pattern recognition receptors (PRRs) activation, the expression levels of components of the dsRNA response pathway were examined in Huh7 and replicon cells (Rep4). Upon transfection of Huh7 cells with poly (I:C), large increases in expression levels were observed for MAVS. TRIF expression levels were also stimulated by the presence of poly (I:C) in the medium but to a lesser extent. By contrast, transfection of Rep4 cells with poly (I:C) or adding in the medium showed that the expression of MAVS and TRIF mRNAs was significantly less compared to Huh7 (figure 3.5), similar to what was seen with the signaling receptors (figure 3.4). Taken together these data indicate that HCV non-structural proteins can inhibit the induction of the innate immune response pathway triggered by dsRNA.

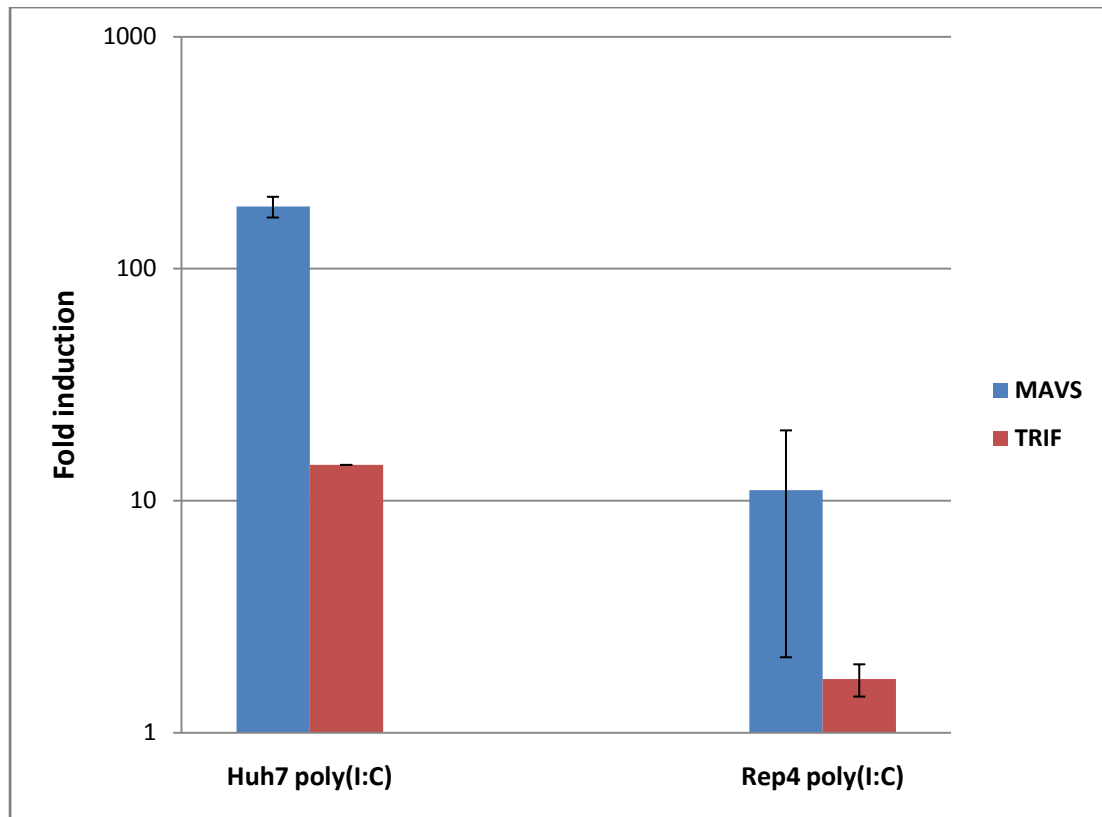


Figure 3.5: Relative expression of signaling mediators TRIF and MAVS mRNA in Huh7 and Rep4 cell and in response to poly (I:C) stimulation. MAVS was measured after transfection with poly (I:C), whereas TRIF was measured after extracellular stimulation with poly (I:C). The expressions levels were determined from total RNA using real time qRT-PCR and were normalized with β -actin.

3.6. HCV NS proteins affect IFN signaling via dsRNA dependent and ssRNA dependent pathways:

The effect of HCV on the upstream components of IFN signaling, TRIF and MAVS, was examined by analyzing the effect of expressing NS proteins in Rep4 cells (figure 3.5). However, there is also another important signaling pathway for IFN, in which signals for different TLRs are mediated by the adaptor protein, myeloid differentiation factor 88 (MyD88), and trigger the activation of transcription factors, such as NF- κ B, that are essential for the expression of proinflammatory cytokine genes. This pathway

also leads to the potent production of type I interferon (IFN) through the activation of IFN regulatory factor 7 (IRF7) upon stimulation of TLR 4, 7 and 8. The basal expression of MyD88 mRNA was up regulated by 2.5 fold in Rep4 cells (figure 3.6). To further study the effect of the presence of replicon on other TLRs through the MyD88 signaling pathway, Huh7 and Rep4 cells were treated with 10ng/μl of lipopolysaccharide (LPS). LPS is strong stimulator of TLR4 through MyD88 leading to the production of IFN- α . Expression of MyD88 was reduced in cells expressing NS proteins by stimulation with LPS (figure 3.6).

Taken together, these results indicate that NS proteins are responsible for the inhibition of IFN- α and IFN- β induction through dsRNA and ssRNA pathways by down regulation of adaptor proteins.

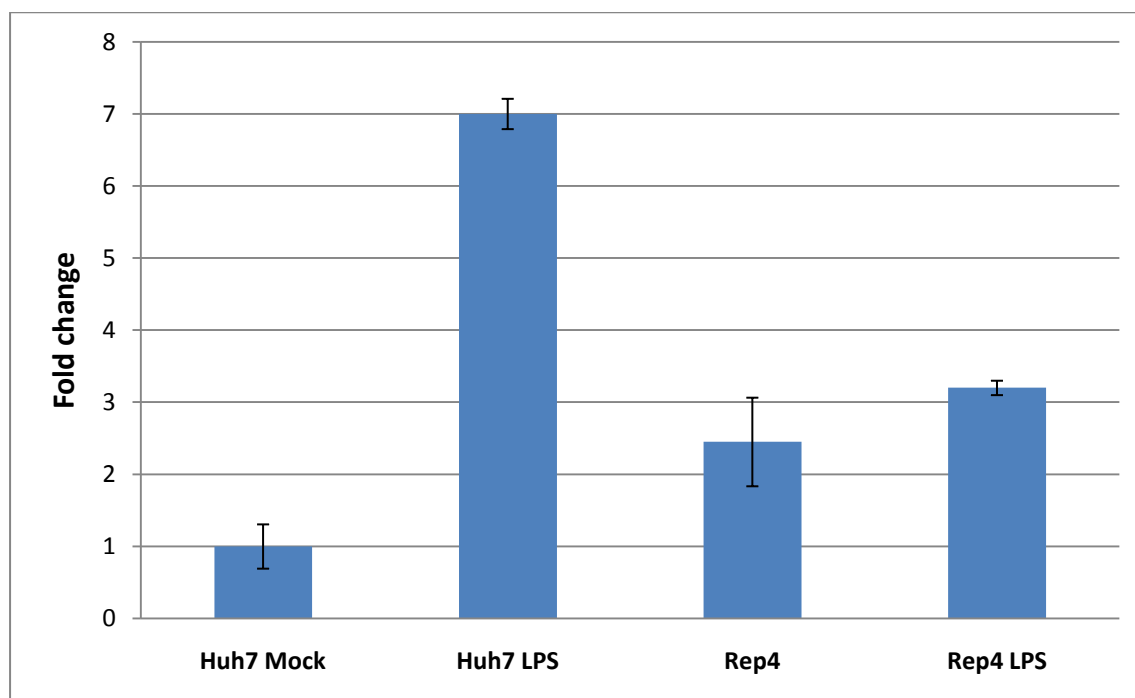


Figure 3.6: HCV NS proteins affect IFN signaling via ssRNA dependent pathways. MyD88 mRNA was measured in Huh7 and Rep4 and then both cell lines were stimulated with 10ng/μl LPS for 24 and expression MyD88 was measured by qRT-PCR. Expression were determined by real time qRT-PCR and normalized to GAPDH expression level.

3.7. Ablation of IFN pathway components by RNAi:

Like all RNA viruses HCV RNA replication involves the production of dsRNA in the cytoplasm of infected cells. Thus, the finding that dsRNA poly (I:C) induces IFN- β mRNA in Huh7, indicates that cytoplasmic sensors, such as RIG-I or MDA5, should also be able to sense HCV RNA. To examine which of these factors is required for the recognition of HCV RNA; RNAi was used to ablate individual genes in Huh7 cells using siRNAs corresponding to each of the target genes RIG-I, MDA5, TRIF, MAVS and MyD88 using duplex siRNAs. Additionally GAPDH and scrambled non-targeting siRNAs were used as a control to examine the efficiency of transfection. First, the efficiency of these siRNAs to knock down gene expression was tested for each single duplex by transfection in Huh7 cells with 20 μ M of each siRNA and lipofectamine 2000. 48 hours post-transfection, mRNA expression was examined by PCR and the inhibition level was determined by qRT-PCR. β -actin mRNA was used as an endogenous positive control for relative expression calculation. This showed that the target genes were significantly and specifically reduced in their expression levels by more than 80 % (figure 3.7).

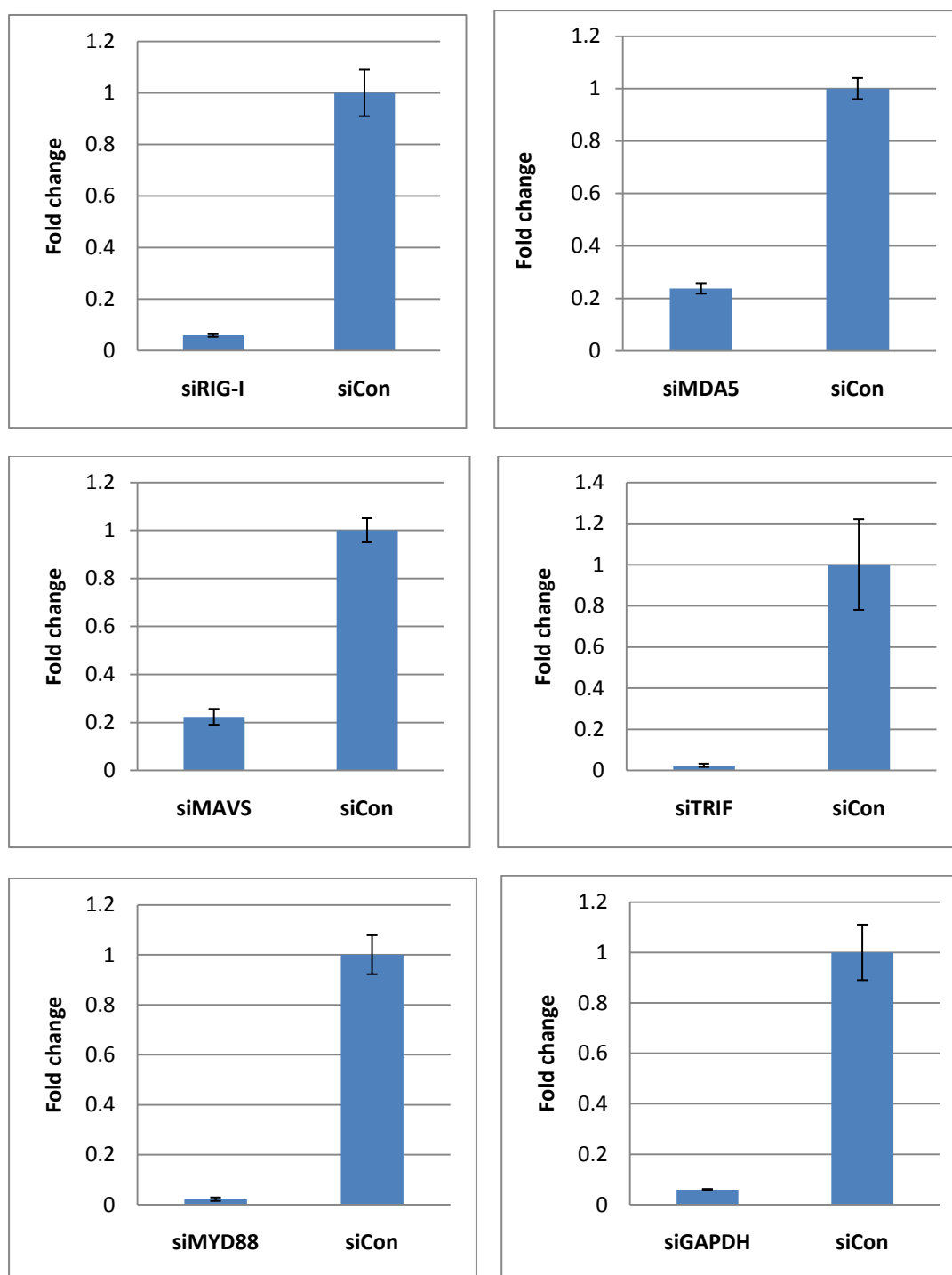


Figure 3.7: Quantification of siRNAs inhibition level. Huh7 cells were transfected with specific siRNA duplexes and 48 hours post transfection, the target genes were quantified by qRT-PCR. GAPDH siRNA was used to measure the efficiency of transfection. Control non-targeting duplex siRNA (siCon), with a scrambled sequence, was used as negative control. All mRNA levels were normalized against cellular β -actin.

Next, the cells treated with these siRNAs were used to measure their effect on IFN- β induction. Huh7 cells grown in 24-well plates were transfected with specific siRNAs for RIG-I, MDA5, MAVS, TRIF and MyD88 in addition to control scrambled siRNAs, 24h later; cells were mock-treated or stimulated with 5ng/ μ l poly (I:C) for 6h. 48 h posttransfection, quantitative RT-PCR was used for the detection of IFN- β and β -actin mRNAs in control and siRNAs transfected cells. The results showed that the inhibition of RIG-I or MDA5 by siRNAs reduced the ability of Huh7 to produce IFN- β transcripts (figure 3.8). Moreover, the inhibition of IFN- β resulting from MDA5 ablation was greater than RIG-I. This appears to correlate with the finding that MDA5 was the highest induced receptor in response to poly (I:C) transfection (figure 3.4). Taken together, these results indicate that MDA5 is the major sensor for poly (I:C), which agrees with the finding of other researcher groups. Since both intracellular receptors used MAVS as a signaling mediator, knockdown of this protein showed even more inhibition of IFN- β . By comparison, inhibition of TRIF had less effect than MAVS. Also, MyD88 knockdown showed a significant inhibition of IFN- β indicating the importance of other TLRs in the innate the immune response (figure 3.8).

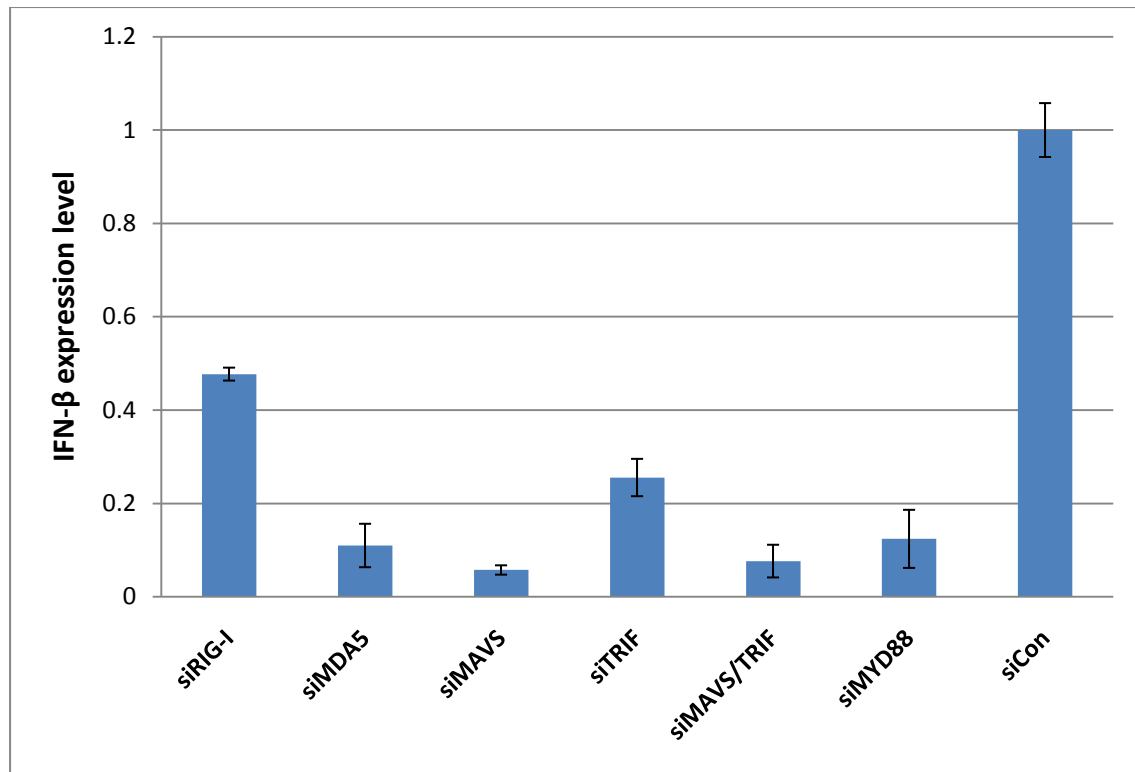


Figure 3.8: Inhibition of poly (I:C)-elicited production of IFN- β . Huh7 cells were grown in 24 well plates and transfected with control siRNA or siRNAs targeting RIG-I, MDA5, double knockdown RIG-I/MDA5, MAVS, TRIF and or MyD88. 48 hours later, cells were stimulated by transfection with 5ng/ μ l poly (I:C). After 48 h, the mRNA were analyzed by real time qRT-PCR for the IFN- β and normalized against β -actin.

3.8. Weak induction of the IFN- β RIG-I pathway in Huh7 cells by HCV

As seen previously, in figures 3.4 and 3.5; Huh7 cells respond strongly to dsRNA poly (I:C) stimulation by increased expression of the dsRNA pathway genes which induce the expression of IFN- β . These results led to a further examination of the role of the dsRNA recognition pathway for HCV and the host in response to virus infection.

To examine induction of IFN- β and RIG-I pathway components by HCV, Huh7 cells were infected with JFH-1 virus at a high (3.0) multiplicity of infection (MOI) and the expression of IFN- β mRNA and other components (RIG-I, MDA5, MAVS, TLR3, TLR7 and MyD88) was measured by qRT-PCR over a time course of infection for 6, 18, 24 and 48 hours. First to confirm the level of virus infection, HCV viral RNA was quantified by qRT-PCR during the time course of infection. As shown in figure 3.9A, after an initial lag period, the intracellular HCV RNA content increased exponentially. In contrast, there was a smaller change in the level of IFN- β (2.3 fold) and other dsRNA pathway expression genes RIG-I (3.2 fold), MDA5 (3 fold), MAVS (2.3 fold) and MyD88 (1.3 fold) at 48 post infection (figure 3.9B). HCV JFH-1 infection and poly (I:C) transfection of Huh7 cells were then compared for their ability to induce these genes over a similar time frame. As shown in figure 3.10, IFN- β and RIG-I pathway expression were highly induced in the Huh7 cells 6 h posttransfection with a maximum stimulation at 48 hours post infection in response to dose (5ng/ μ l) of poly (I:C). These results indicate that HCV infection of Huh7 cells does not trigger the intracellular dsRNA signaling pathway as efficient as poly (I:C) or that the virus may suppress these responses.

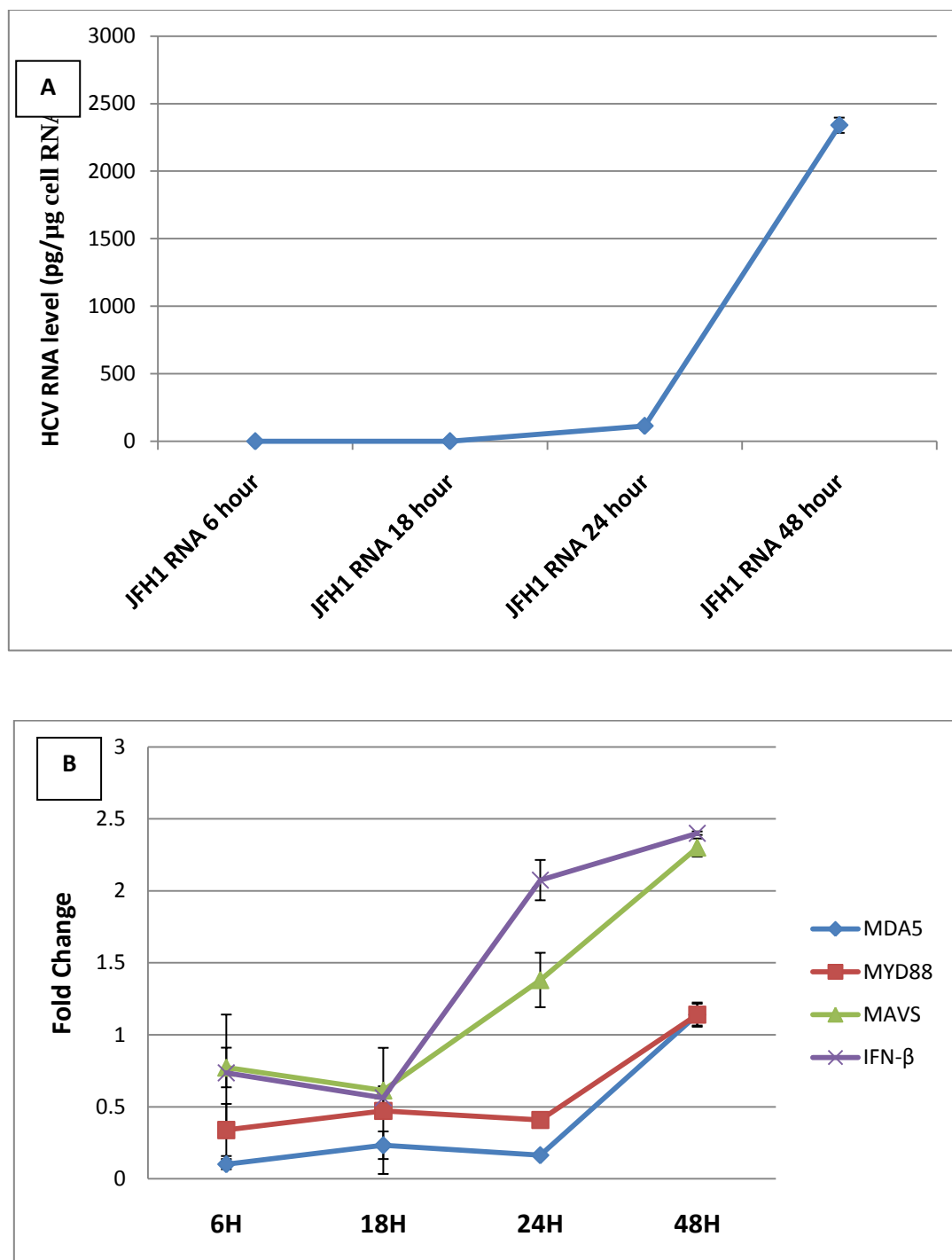


Figure 3.9: HCV infection induction of IFN- β or dsRNA response genes. (A) Quantification of intracellular HCV RNA by qRT-PCR analysis. Huh7 cells were infected with JFH-1 at a high multiplicity (MOI = 3). At the indicated times, total cellular RNA was extracted and HCV RNA were quantified by qRT-PCR. (B) The intracellular gene expression of IFN- β , MDA5, MAVS and MyD88. The expressions of target genes are presented as fold-induction relative to mock infected cells.

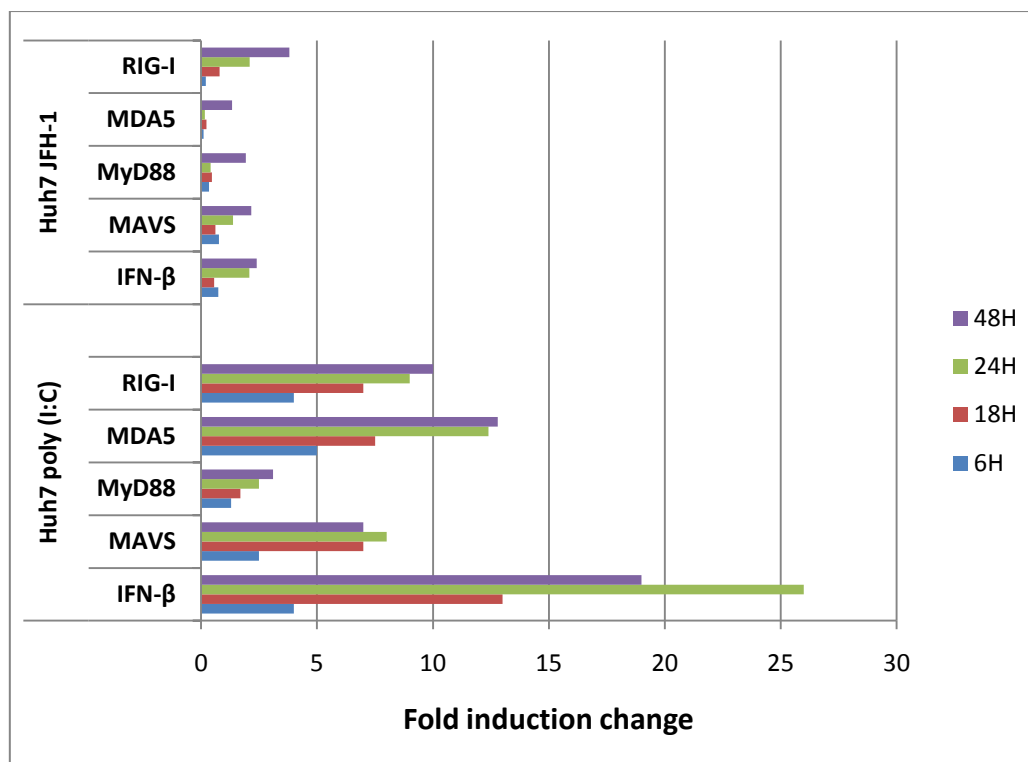


Figure 3.10: Comparison of HCV JFH-1 infection and poly (I:C) transfection on dsRNA response gene induction activated pathways in Huh7 cells. Huh7 cells were infected with JFH-1 or transfected with dsRNA (poly (I:C)) for the indicated times. IFN- β , MDA5, RIG-I, MAVS and MyD88 mRNAs were measured by qRT-PCR analysis and normalized on GAPDH.

To test the hypothesis, that HCV either fails to trigger or suppresses the intracellular dsRNA signaling pathway co-stimulation experiments were carried out. Huh7 cells were infected with JFH-1 for 48 hours and then stimulated intracellularly by transfection with 5ng/ μ l poly (I:C) or extracellularly by adding the same concentration to the infected cell medium for 24 hours. Total cell RNA was then extracted and qRT-PCR performed to detect various mRNA expression levels in comparison to Huh7 cells mock infected poly (I:C) treated cells. As seen in figure 3.11, Huh7 cells infected with HCV JFH-1 showed a significant decrease in the induction of IFN- β and other components after treatment with poly (I:C) compared to

uninfected cells treated with poly (I:C) (figure 3.11). These results indicate that HCV infection reduced the ability of Huh7 cells to activate the dsRNA and ssRNA recognitions pathway, which increases the ability of the virus to evade innate immune system and help to establish a chronic infection.

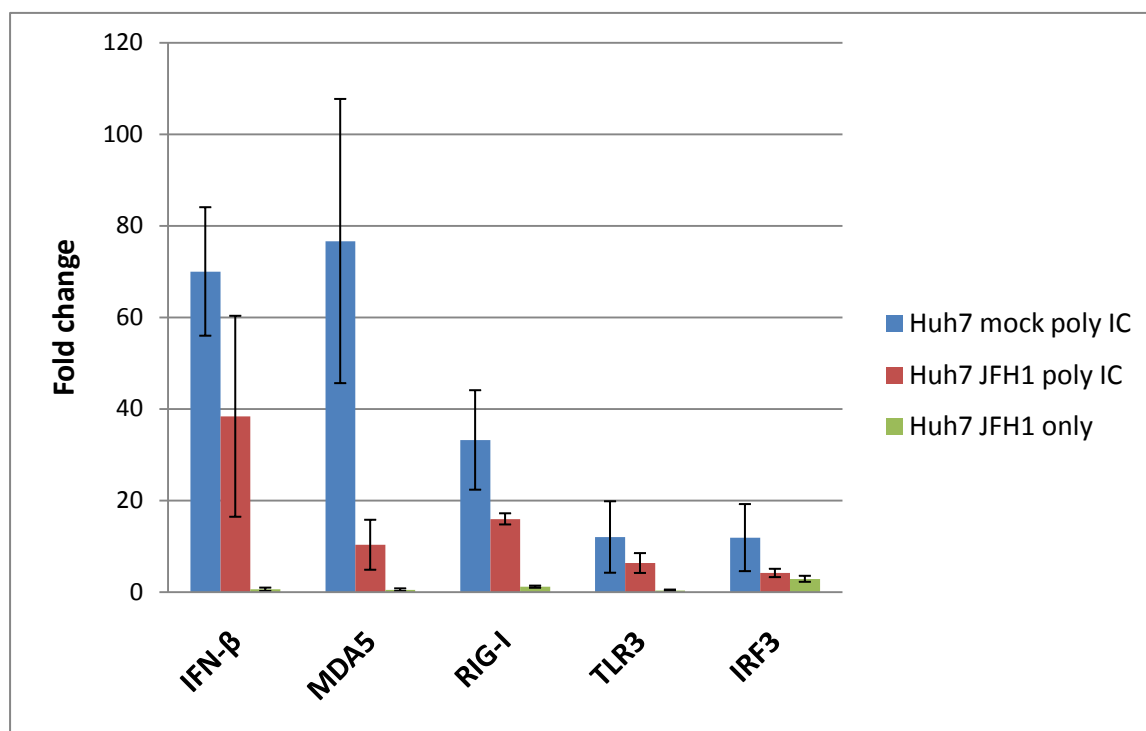


Figure 3.11: HCV suppresses the dsRNA recognition signaling pathway. Huh7 cells were infected with HCV JFH-1 for 48 hours and then treated with poly (I:C) followed by measuring the expression of IFN-β, RIG-I, MDA5, TLR3, MAVS and IRF3 48 hours later. These expression inductions of these genes were also measured in uninfected Huh7 cells treated with poly (I:C). Quantification of the gene expression was performed by qRT-PCR and normalized with GAPDH.

3.9. siRNA knockdown of RIG-I and MDA5 receptors stimulates HCV replication:

The results obtained confirm that MDA5 is an important sensor for dsRNA. These results led to the further examination of the role of MDA5 in the host in response to HCV infection. Huh7.5 cells, which contain a defect in the RIG-I pathway were used to examine the role of MDA5 in the host response to HCV replication. To measure the signaling activity of Huh7.5, the cells were transfected with 5ng/μl of poly (I:C) and the transcription levels of IFN-β and MDA5, TLR3 and MAVS mRNAs were determined by qRT-PCR. This experiment showed that the signaling pathway mediated through MDA5 stimulated high levels of IFN-β induction in addition to the both MDA5 and MAVS mRNAs. TLR3 was stimulated to much lesser extent indicating that the stimulation of IFN-β is mainly activated through the MDA5 pathway (figure 3.12).

Importantly, HCV JFH-1 infection in Huh7.5 cells showed a weak signaling induction (figure 3.13). Taken together, this further confirms that HCV infection leads to the inhibition of the IFN pathway.

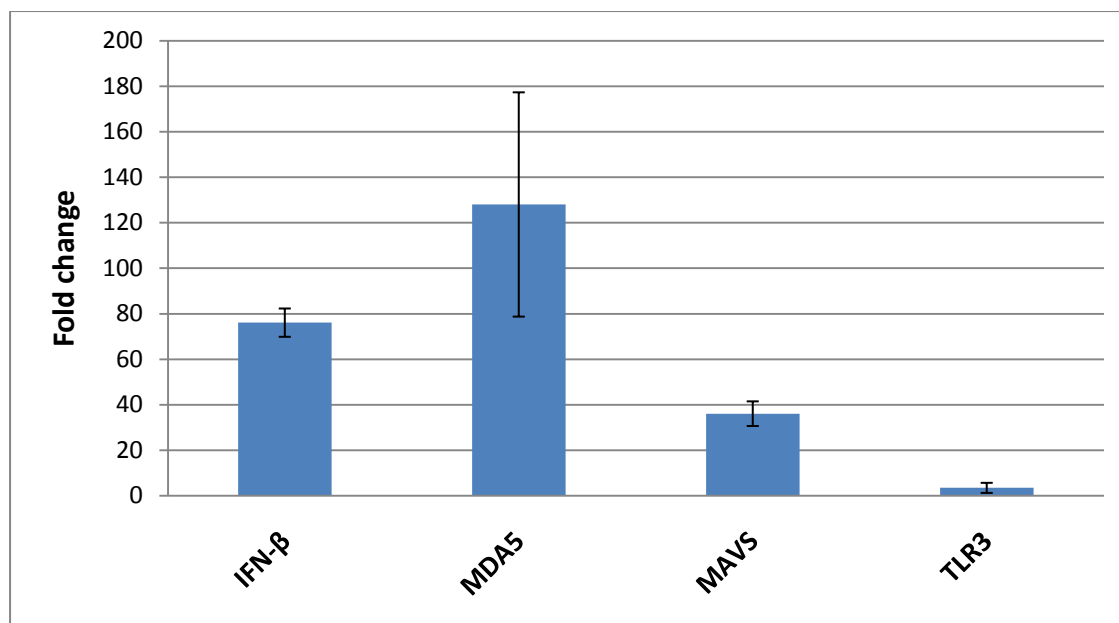


Figure 3.12: Poly (I:C) induction of antiviral responses in Huh7.5 cells. Huh7.5 cells were transfected with dsRNA poly (I:C) for 24 hours and IFN- β , MDA5, MAVS and TLR3 mRNAs were measured by qRT-PCR analysis and normalized with GAPDH.

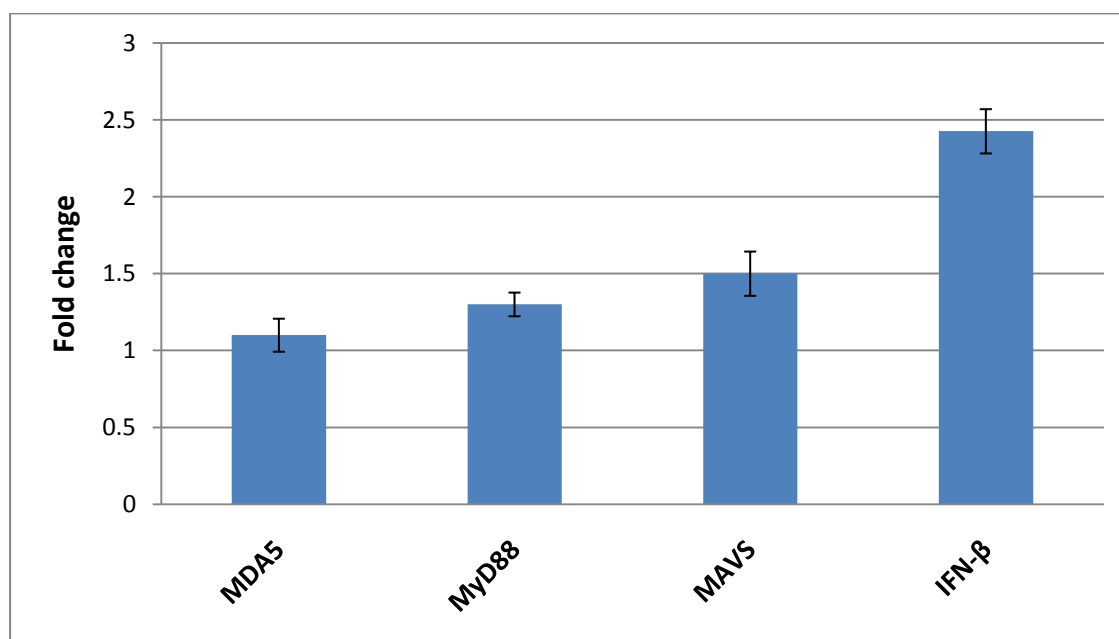


Figure 3.13: induction of IFN- β signaling pathway components in Huh7.5 cells by HCV infection. Huh7.5 cells were infected with JFH-1 at a high multiplicity (MOI = 3) for 48 hours and total cellular RNA was extracted and mRNA levels of IFN- β , MDA5, MAVS and MyD88 were determined by qRT-PCR. The expression of the target gene is presented as fold-induction relative to mock infected cells.

To further examine the role of MDA5 in IFN induction in response to HCV infection of Huh7.5 cells, MDA5 siRNA was used to transfect naïve Huh7.5 followed by HCV JFH-1 infection for 48 hours. The total cellular RNA was then isolated and HCV RNA and IFN- β transcripts were quantified by RT-PCR. HCV infection led to stimulation of IFN- β production. However, MDA5 ablation reduced IFN- β production significantly demonstrating an important functional role of MDA5 in the IFN- β response pathway (figure 3.14).

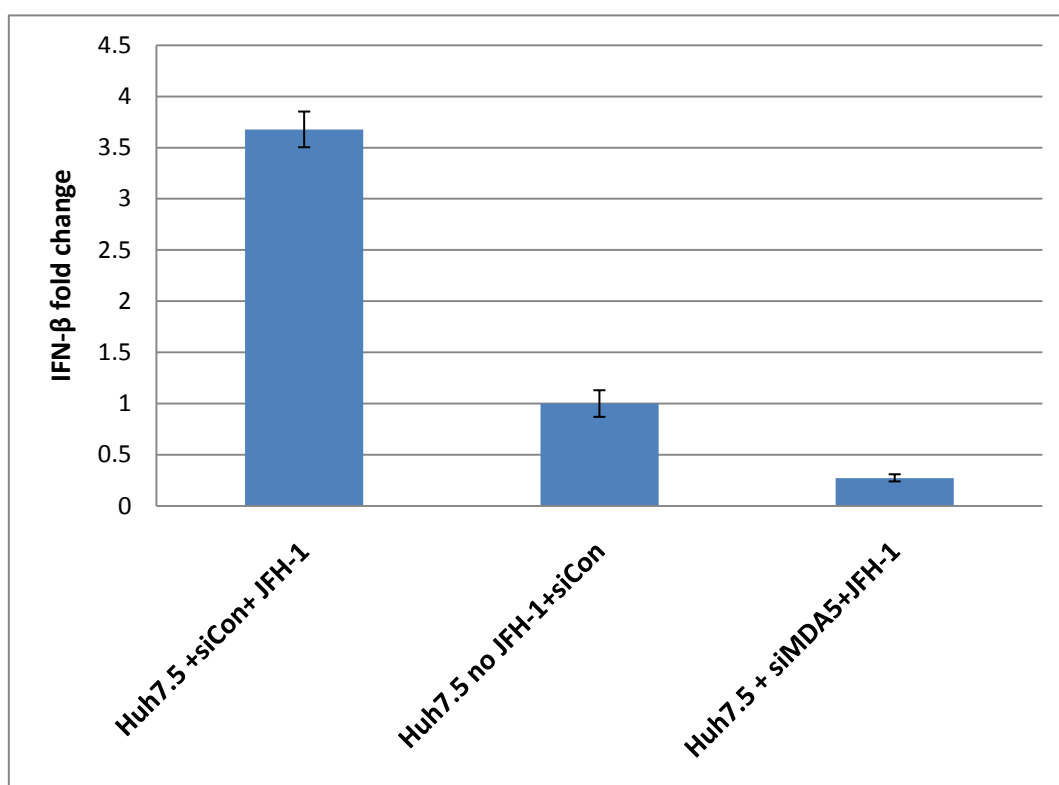


Figure 3.14: The role of MDA5 in the IFN- β response to HCV infection. Huh7.5 cells were transfected with control siRNA (siCon) or siRNAs targeting MDA5 (siMDA5), 48 hour posttransfection, cells were either infected with HCV JFH-1 or mock infected and incubated for 24 hours. IFN- β mRNA levels were measured by qRT-PCR.

HCV was previously shown to replicate at much higher titers in Huh7.5 than in the parental Huh7 cells (278). Although RIG-I has an important role in sensing HCV RNA, the results of MDA5 ablation in Huh7.5 cells show that it also has a role in the induction of IFN- β in response to HCV infection. RIG-I and MDA5 belong to the same family of DExD/H box helicases. However, MDA5 is known to preferentially recognize picornavirus RNA and poly (I:C). To further investigate the role of MDA5 in the host response to HCV replication, siRNA targeting MDA5 was transfected into Huh7.5 cells which were subsequently infected with HCV. 48 hours post infection the intracellular virus RNA levels and the virus release into the supernatant was measured. Importantly, the analysis of virus yield further revealed that MDA5 knockdown increased HCV production by more than 17 fold and intracellular replication was enhanced by more than two fold (figure 3.15). Taken together, these data suggest that RIG-I and MDA5 are both important mediators for HCV elicited induction of type I IFN in infected Huh7.5 cells and that the knockdown of RIG-I or MDA5 reduces IFN- β induction. RIG-I and MDA5 expression are both highly up-regulated during virus infection and are required for optimal sensing of virus infection.

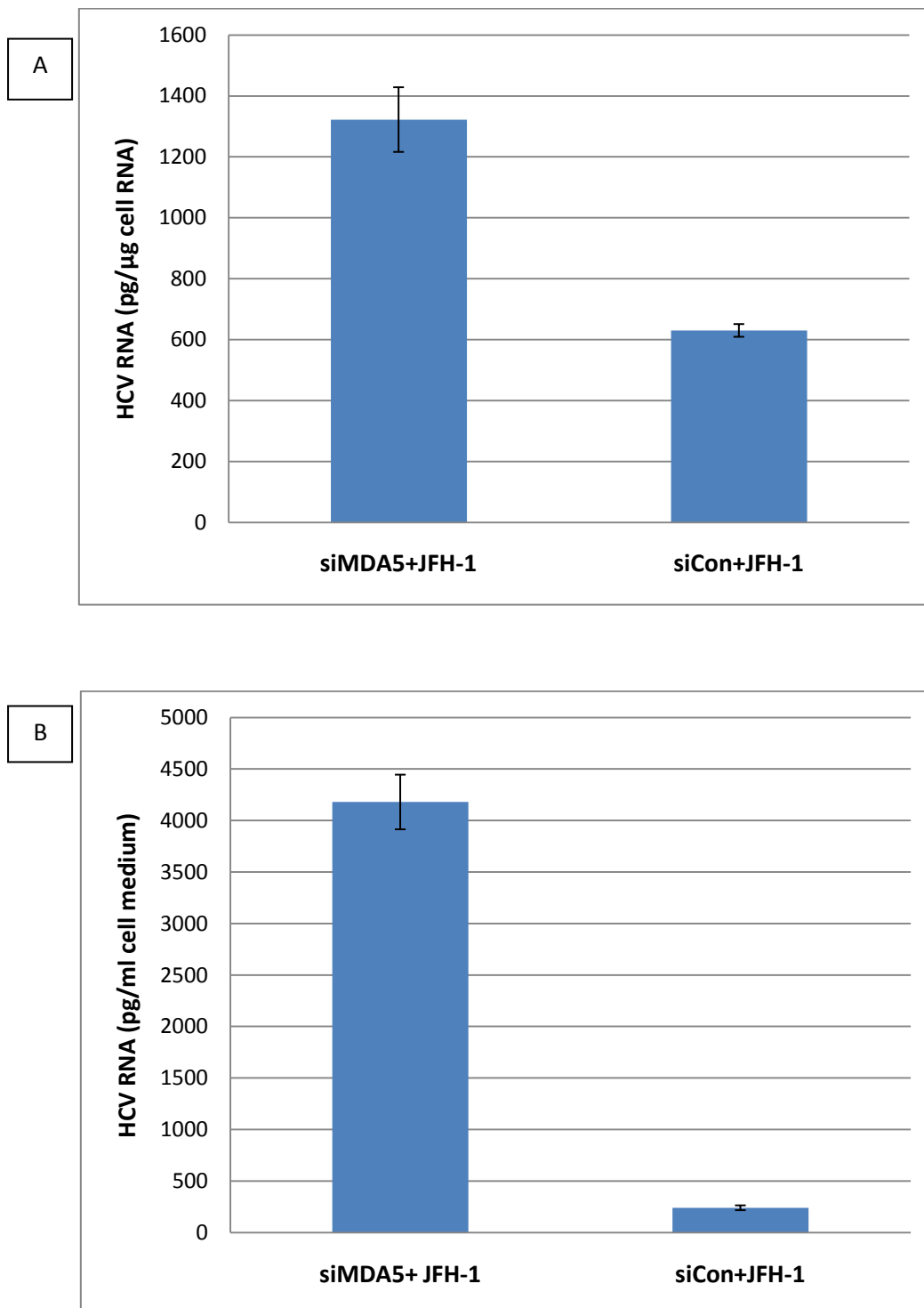


Figure 3.15: MDA5 mediated cellular restriction to HCV infection. Huh7.5 cells were transfected with control or (siCon) MDA5 (siMDA5) siRNA, 48 hours posttransfection, cells were infected with HCV-JFH-1 and 48 hours post infection the HCV RNA levels were determined by qRT-PCR. (A) Intracellular HCV RNA. (B) Virus released into the cell culture medium.

3.10. Effect of siRNA knockdown of IFN signaling components on HCV replication:

To examine the effects on HCV replication, siRNAs were also used to knock down the expression of MDA5 and other dsRNA and ssRNA signaling pathway components (MAVS, TRIF and MyD88) in naive Huh7.5 cells. Inhibition of these signaling mediators by siRNAs showed a corresponding increase in the level of HCV replication. There were four fold increases in HCV when MAVS was ablated, and also significant increases in HCV yields when either MyD88 or TRIF were targeted (figure 3.16).

Taken these results together indicate the innate immune response play a vital role in host response and virus replication.

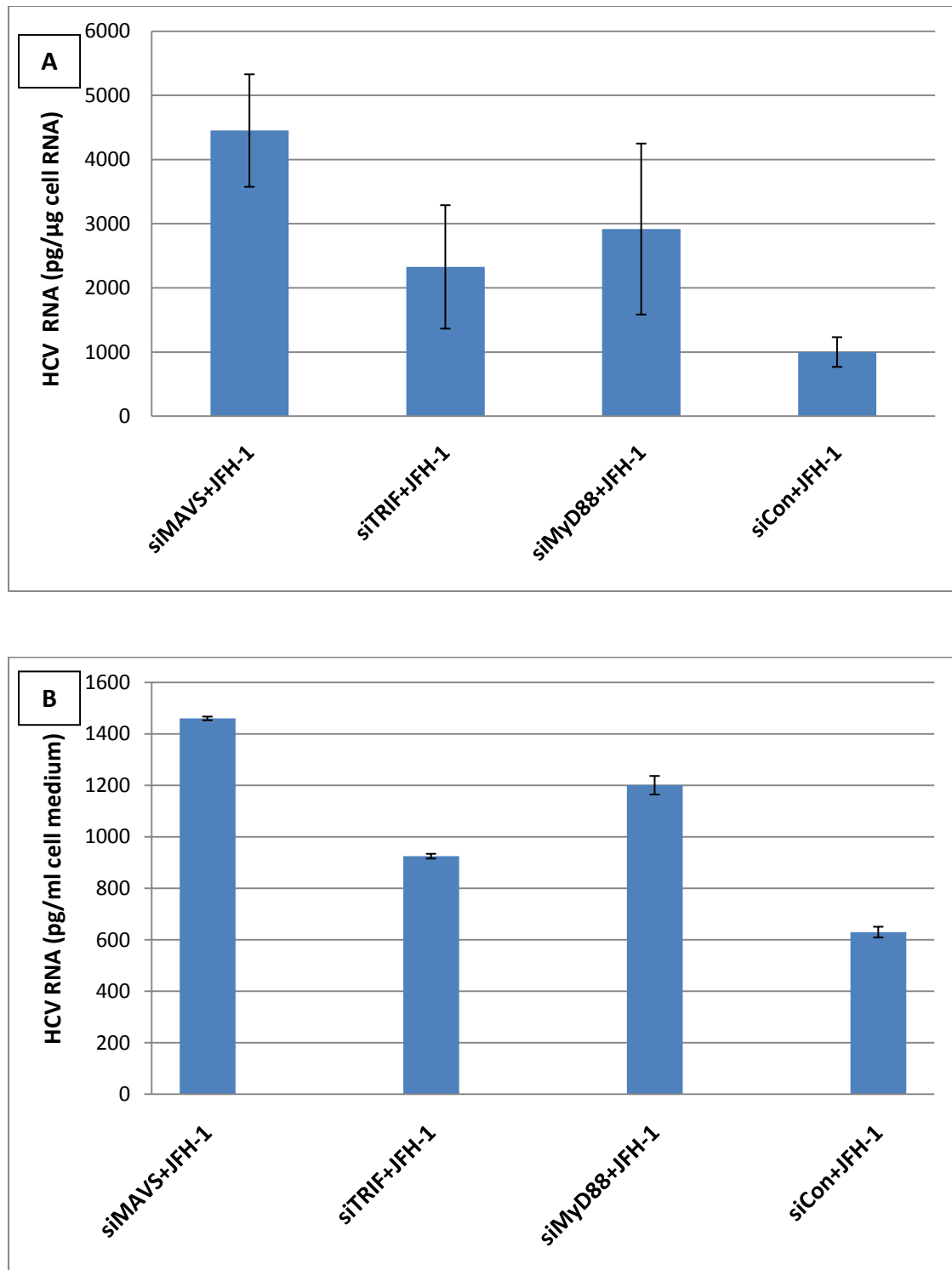


Figure 3.16: Effect of ablation of MAVS, TRIF and MyD88 on HCV replication. Huh7.5 cells were transfected with control siRNA (siCon) or siRNA which targeted the signaling mediators indicated and then infected with JFH-1 HCV. HCV quantified by qRT-PCR at 48 h after infection. GAPDH was used as a control. **(A)** Intracellular HCV RNA. **(B)** Virus released into the cell culture medium

CHAPTER FOUR:

4.0. Effects of HCV NS3 and NS/4A expression on type I IFN pathway components

4.1. Cloning HCV NS3 PCR product into an entry plasmid:

In order to generate HCV NS3 expression constructs in adenovirus vectors, it was first necessary to clone HCV genome sequences into entry vectors so that they could be modified and propagated as required. The pENTR Directional TOPO Cloning Kits (Invitrogen) utilize a highly efficient, cloning strategy to directionally clone a blunt-end PCR product into a vector for entry into the Gateway System. Blunt-end PCR products clone at greater than 90% efficiency. The TOPO vector contains attL1 and attL2 sites for site-specific recombination of the entry clone with a Gateway destination vector and Kanamycin resistance gene for selection in *E.coli* (section 8.2 appendix).

DNA fragments encoding each of the HCV NS3 and NS3/4A nonstructural proteins were generated from a full-length cDNA containing the whole genome of HCV clone of three different HCV genotypes. HCV genotype 2a, JFH-1 isolate, HCV genotype 1b Con1 isolate and HCV genotype 1a isolate H77. PCR primers were designed to amplify the region of interest and a four nucleotides “CACC” sequence necessary for directional cloning was added to the 5’ end of the forward primers. The overhang in the cloning vector (GTGG) complements the 5’ end of the PCR product, and anneals to the PCR product in the correct orientation, which allows the inserts to be cloned efficiently. ATG initiation codons were also added for the proper initiation of translation. After the PCR developing strategy, the primers were used to produce blunt-ended PCR products using thermostable, proofreading polymerase. The PCR reactions were carried out as described in the Materials and Methods using

ACCUAZYME proofreading polymerase (Accuzyme polymerase) and the PCR products were subjected to electrophoresis, to verify the quality and quantity of the PCR product (figure 4.1). Once a single discrete band of the correct size for each PCR was produced (figure 4.1), the PCR products were then subcloned into the pENTR/D-TOPO vector and TOP 10 competent *E. coli* were transformed with the recombinant vector into and cultured overnight in LB medium containing 50µg/ml kanamycin. The cDNA inserts were verified by DNA sequencing of the constructs to confirm that genes were cloned in the correct orientation.

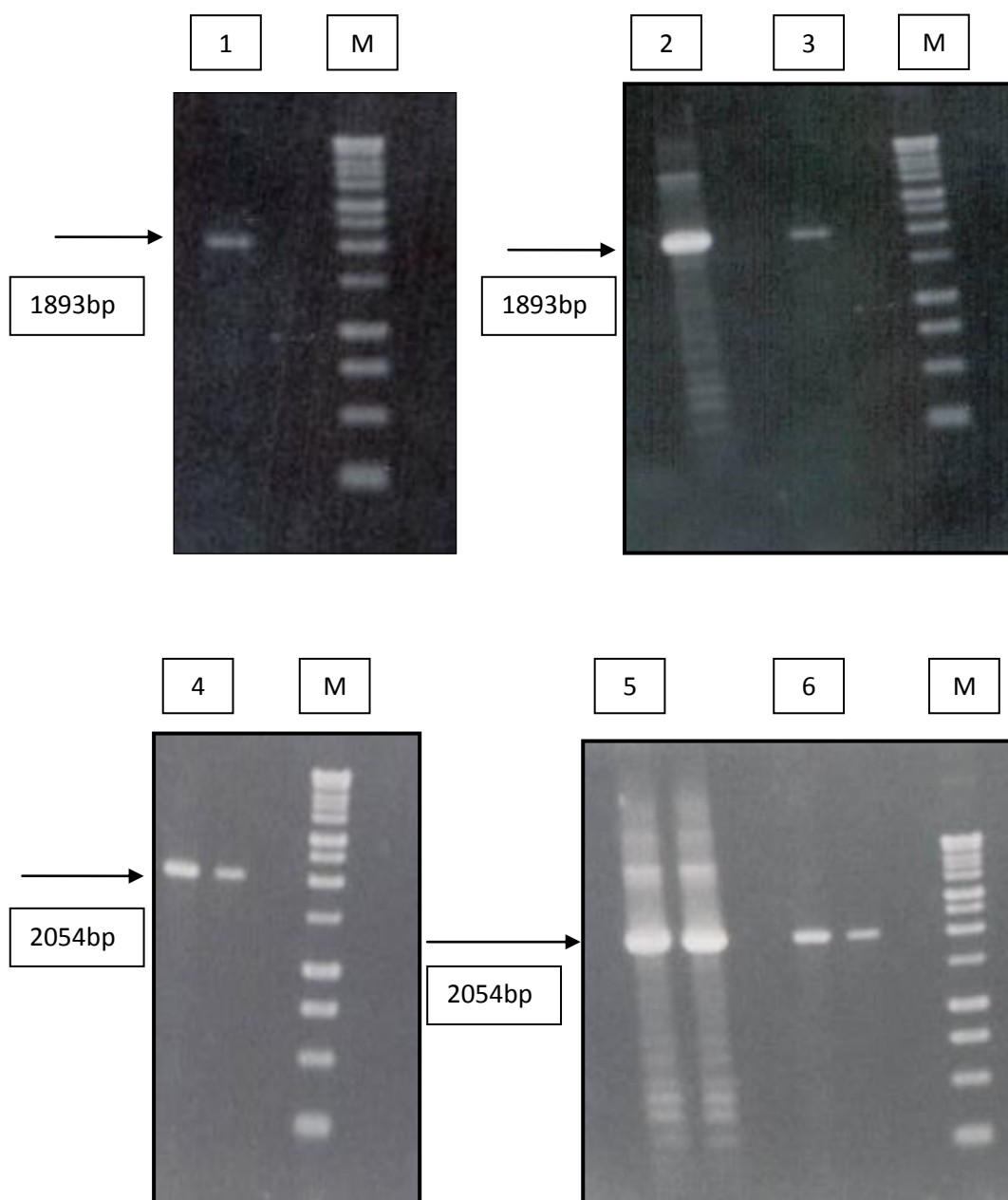


Figure 4.1: NS3 and NS3/4A amplification by PCR for cloning into the pENTR TOPO vector. ACCUAZYME proofreading polymerase was used and the PCR products were subjected to electrophoresis and stained with ethidium bromide. The single bands represent the correct product size (1893bp) of NS3 and (2054pb) of NS3/4A from different genotypes. Lane 1: NS3-1a, lane 2: NS3-2a, lane 3: NS3-1b, lane 4: NS3/4A-1b, lane 5: NS3/4A-2a, lane 6: NS3/4A-1a. All PCR products were run alongside with 1Kbp DNA marker ladders (M).

4.2. Virus production by transfection of pAd/CMV/V5-NS3 and NS3/4A into 293A cells:

Once the entry clones containing NS3 and NS3/4A genes were obtained, recombination reactions were carried out to transfer the NS3 and NS3/4A genes into the pAd/CMV/V5-DEST vector by means of the Gateway system using LR Clonase. pAd/CMV/V5-DEST (36.7 kb) destination vectors are adapted for use with the Gateway Technology, and are designed to allow high-levels of transient expression of recombinant fusion proteins in dividing and non-dividing mammalian cells using the ViraPower Adenoviral Expression System (Invitrogen). The pAd/CMV/V5-DEST vector is a replication-deficient adenovirus vector which contains the adenovirus serotype 5 (Ad5) encoding genes with an E1/E3 deletion mutation. Additionally it also contains the following elements (Left and Right Inverted Terminal Repeats (ITRs), encapsidation signal sequence, late genes) required for proper packaging and production of adenoviruses. Two recombination sites, attR1 and attR2 required for recombination cloning of the DNA sequence of interest from a pENTR entry clones were also present in the adenovirus genome. A ccdB gene located between the attR sites was used for negative selection. A human cytomegalovirus (CMV) immediate early promoter gave high-level constitutive expression of the gene of interest. An ampicillin resistance gene provided for selection in *E. coli* and chloramphenicol resistance gene (CmR) located between the two attR sites for counter selection. For detection of the recombinant protein, this vector contains a V5 epitope at C-terminal (appendix 8.2).

To carry out the LR recombination reaction, LR Clonase II enzyme mix (Invitrogen) was used. This enzyme contains the bacteriophage lambda recombination proteins Integrase (Int) and Excisionase (Xis), and the *E. coli* encoded protein Integration Host

Factor (IHF) which promotes in vitro recombination between attL- and attR-flanked regions on entry clones and destination vectors to generate attB-containing expression clones. A recombination reaction between the NS3 and NS3/4A pENTR Topo vectors and pAd/CMV/V5-DEST was performed as described in section (2.2.7.4) and the recombined plasmids were immediately transformed into Max efficient E.coli cells, and selected on LB plates containing 50µg/ml ampicillin. Resistant colonies were picked and grown and the plasmids were extracted. These were then tested for correct recombination of the inserts by DNA sequencing. Seven constructs of pAd/CMV/V5-DEST were produced and subsequently designated as pAdNS3-1a, pAdNS3-1b, pAdNS3-2a, pAdNS3/4A-1a, pAdNS3/4A-1b, pAdNS3/4A-2a and in addition to pAdLacZ which used as a control.

Once the correct size and sequence of the plasmid constructs were confirmed, they were then linearized by digestion with PacI and then transfected into subconfluent 293A cells with Lipofectamine 2000. Before transfection of pAdNS3 and pAdNS3/4A expression clones into 293A cells, the left and right viral ITRs must be revealed to allow proper viral replication and packaging by digestion of the vector with Pac I. This also removed the bacterial sequences (pUC origin and ampicillin resistance gene, 2000bp).

The 293A cell line was used to allow initial production, amplification and titration of the recombinant adenovirus. The 293A cells contain a stably integrated copy of E1 that supplies the E1a and E1b proteins required for adenoviruses production. 5µg of each plasmid was digested and then purified using a PCR purification kit (Qiagen). To test the complete digestion, the plasmids were run on 1% agarose gels (figure 4.2). The purified plasmids were then transfected into 293A cells. 48 hours post-transfection, the cells were transferred to T75 tissue culture plates until visible regions

of cytopathic effect (CPE) were observed approximately 6-8 days post-transfection. The infected cells were grown until a CPE approximately of 80% was observed and the adenoviruses were then harvested. This virus preparation was designated as P1 (first purification). From P1 virus stock, fresh 293A cells were infected to produce P2, and from this P3 were produced. Finally the virus stock were titrated by plaque assays and virus concentration determined between 10^8 - 10^9 plaque forming unit (pfu) as shown in table 4.1. Figure 4.3 summarizes the steps performed to produce the adenovirus constructs.

Constructs	Titre
AdNS3-1A	9.1×10^7 pfu/ml
AdNS3/4A-1A	3.6×10^8 pfu/ml
AdNS3-1B	3.2×10^7 pfu/ml
AdNS3/4A-1B	2.3×10^8 pfu/ml
AdNS3-2A	1.44×10^9 pfu/ml
AdNS3/4A-2A	9.6×10^8 pfu/ml
AdLacZ	4×10^9 pfu/ml

Table 4.1: Titration of recombinant adenoviruses by plaque assay: the constructed Ad NS3, NS3/4A and AdLacZ adenovirus vectors were titrated by plaque forming assay. Serial dilutions of virus were made in 2% FCS DMEM. An aliquot of 250 μ l of each dilution was added to a monolayer of 293A cells and incubated at 37°C for 2 h. Cells were then overlaid with 4% agarose in 2% FCS DMEM and incubated for 10 days. Plaque-forming units (pfu) were counted and calculated as detailed in section 2.2.7.8.

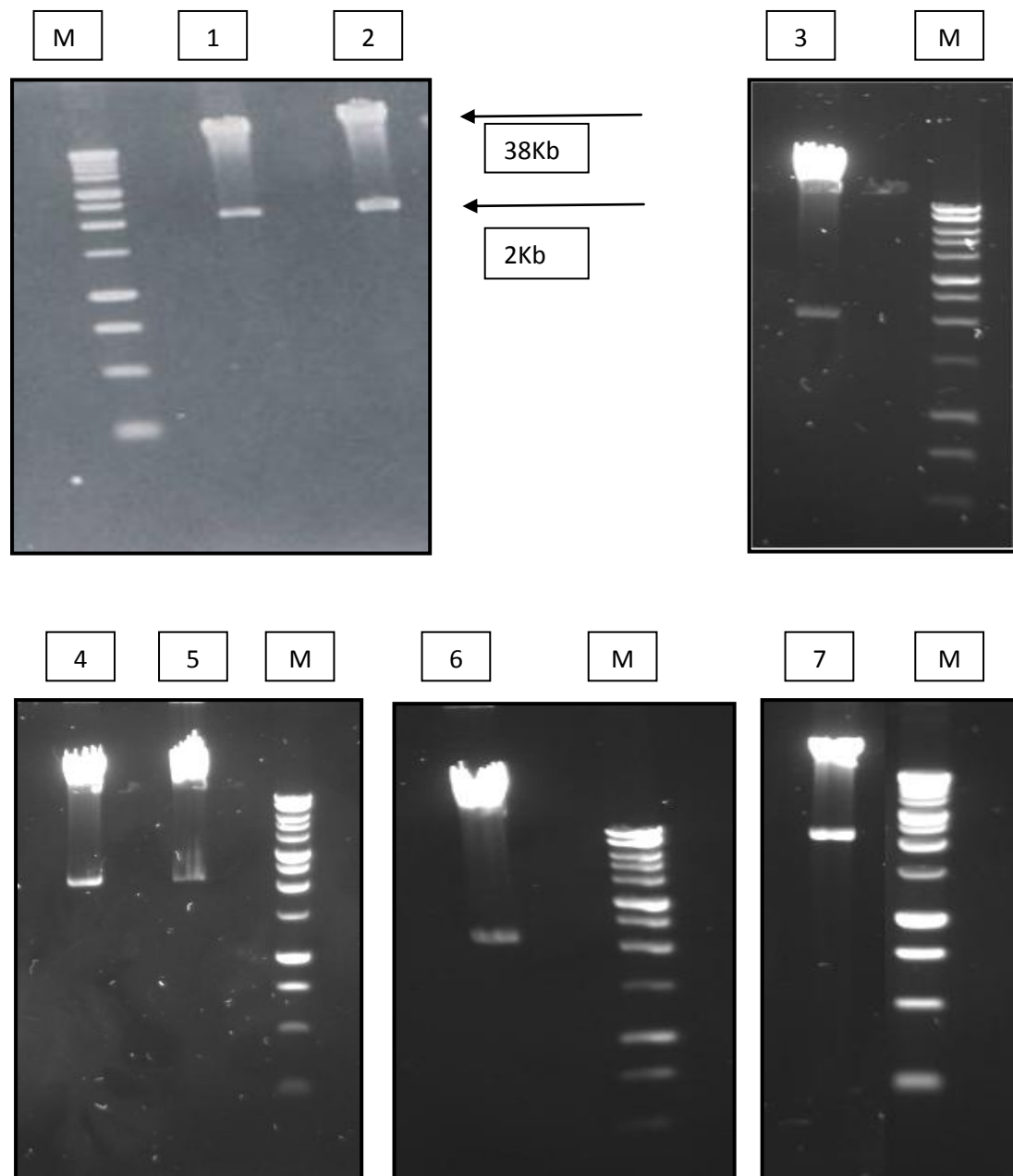


Figure 4.2: PacI restrictions digest of pAdNS3 and pAdNS3/4A recombinant adenovirus vectors. Digested products of each plasmid genotypes plus LacZ, as indicated were run alongside a 1 Kbp DNA marker ladder (M) on a 1% agarose gel and stained with SYBR safe. The top arrow showed linearized pAdNS3 and pAdNS3/4A (38.7 kb), while the below arrow showed the removed pUC origin and ampicillin resistance gene (2Kp). Lane 1: pAdNS3-1a, lane 2: pAdNS3-2a, lane 3: pAdNS3-1b, lane 4: pAdNS3/4A-1a, lane 5: pAdNS3/4A-2a, lane 6: pAdNS3/4A-1b, lane 7: pAdLacZ.

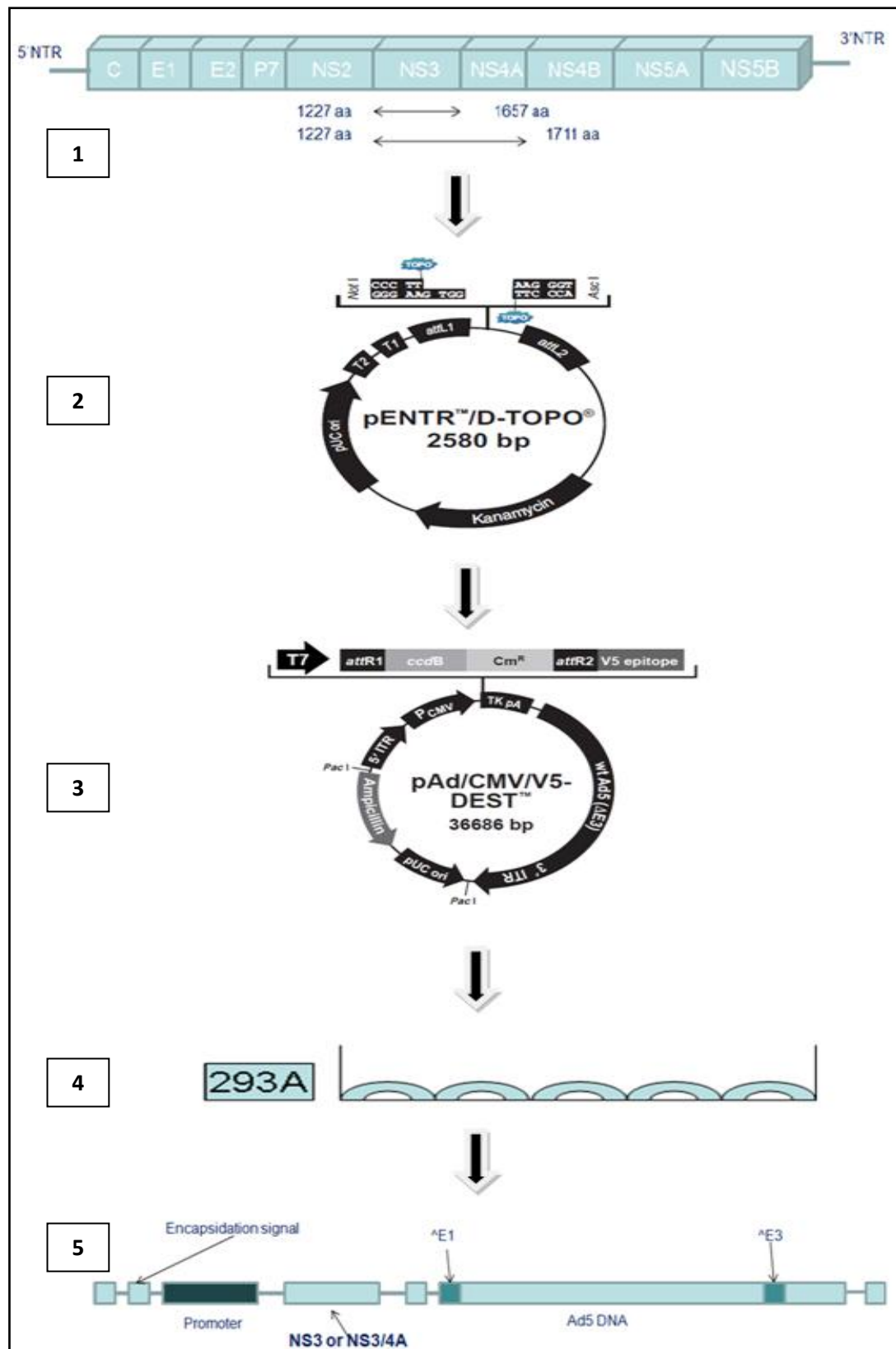


Figure 4.3: Construction of recombinant adenoviruses that express NS3 and NS3/4a proteins (1). HCV cDNA (genotypes 1a, 1b and 2a, the amino acids are indicated) was amplified and (2). cloned into the pENTR/D-TOPO (3) recombined into pAd/CMV/V5-DEST vector which contains homologous recombination between these two plasmids. This vector includes adenovirus sequences (Ad5), a CMV promoter, a V5 tag epitope (4). transfection into 293 cells led to (5). the formation of replication deficient recombinant adenoviruses that have the HCV genes of interest in the adenovirus genome.

4.3. Expression of recombinant NS3 and NS/4A proteins in Huh7 cells:

Recombinant adenovirus vectors containing coding sequence for HCV NS3, NS3/4a from genotypes 1a, 1b and 2a or the LacZ recombinant adenovirus control (Ad/LacZ) were incubated at 100–200 MOI with Huh7 cells for 72 hours. To determine the percentage of Huh7 cells expressing HCV NS3 or NS3/4A, confocal immunofluorescence staining was performed. Rabbit polyclonal anti-NS3 antibody (NS3-7) followed by FITC labeled donkey anti rabbit polyclonal antibody (data not shown) or anti-V5 tagged monoclonal antibody followed by Cy3 donkey anti-mouse antibody, were used to detect the recombinant proteins. The staining indicated that more than 90 % of the cells were transduced with the recombinant adenovirus vectors to express HCV NS3 or NS3/4A (figure 4.4). Control adenoviral vector-infected Huh7 cells did not show fluorescence with anti-NS3 or tag V5 antibodies.

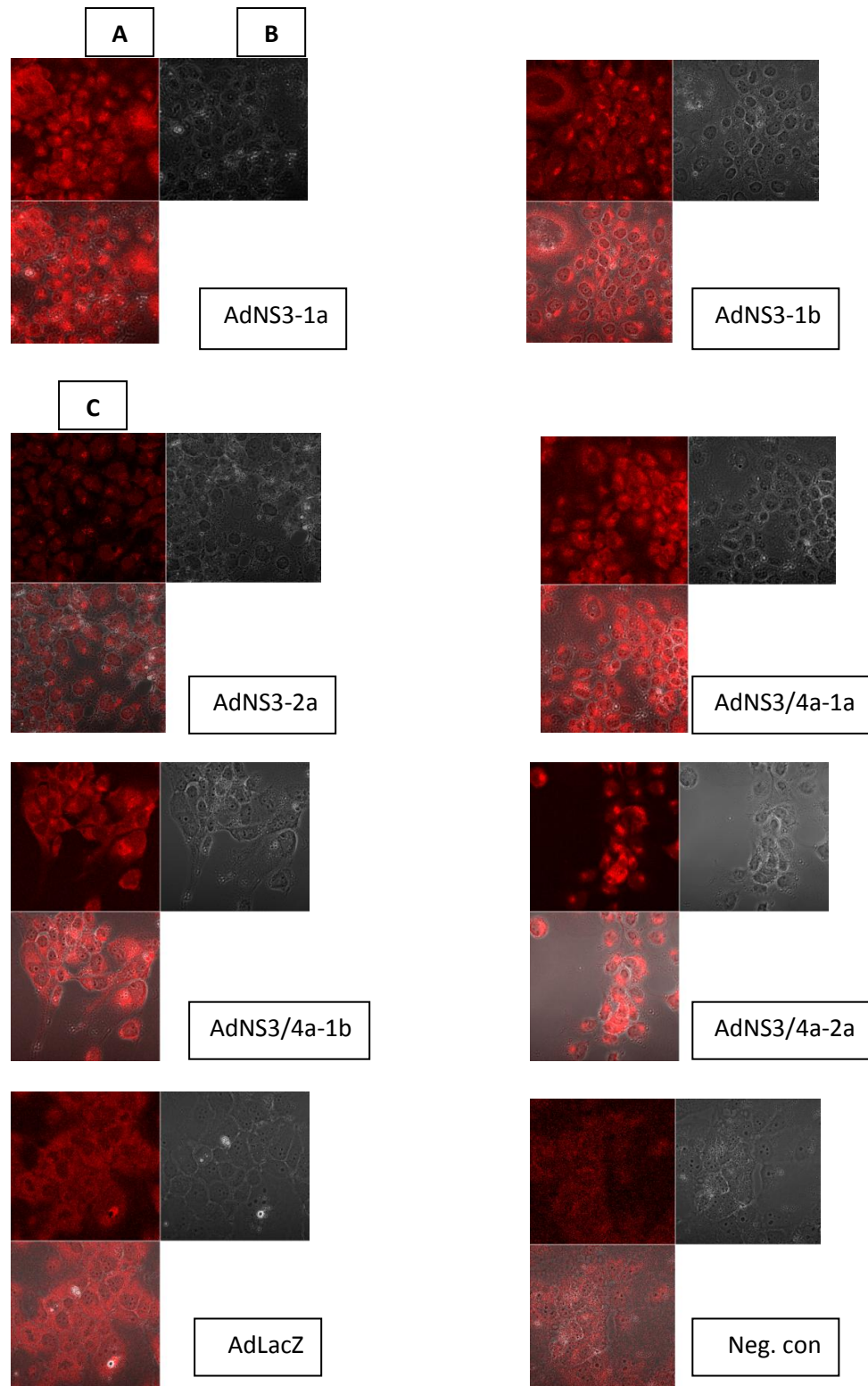


Figure 4.4: Confocal immunofluorescence staining of Huh7 cells expressing NS3 and NS3/4A proteins. Staining of V5 tagged peptide Cyc3 (red) with anti-V5 antibody to localize HCV-antigen expression. Each slide show expressed NS3 or NS3/4A from different genotype as indicated. **(A)** Fluorescence staining, **(B)** phase contrast and **(C)** merge.

The levels of expression of NS3 and NS3/4A in the transduced Huh7 cells were also quantified by performing real-time qRT-PCR analysis. For qRT-PCR reactions a specific set of primers for each NS3 genotype was used.

DNA was extracted from viral stocks and from infected cells. Huh7 cells were infected with all adenoviruses constructs for 72 hrs, then DNA extracted from virus stocks and Huh7 infected cells. The adenovirus titers were determined by qRT-PCR (figure 4.5). This provided the basis for optimizing the MOI, which was used for the subsequent experiments under the same infection conditions. In adenovirus infections in Huh7 cells MOIs ranged from 50 to 100 in experiments to produce consistent protein expression in 90% or more of cells.

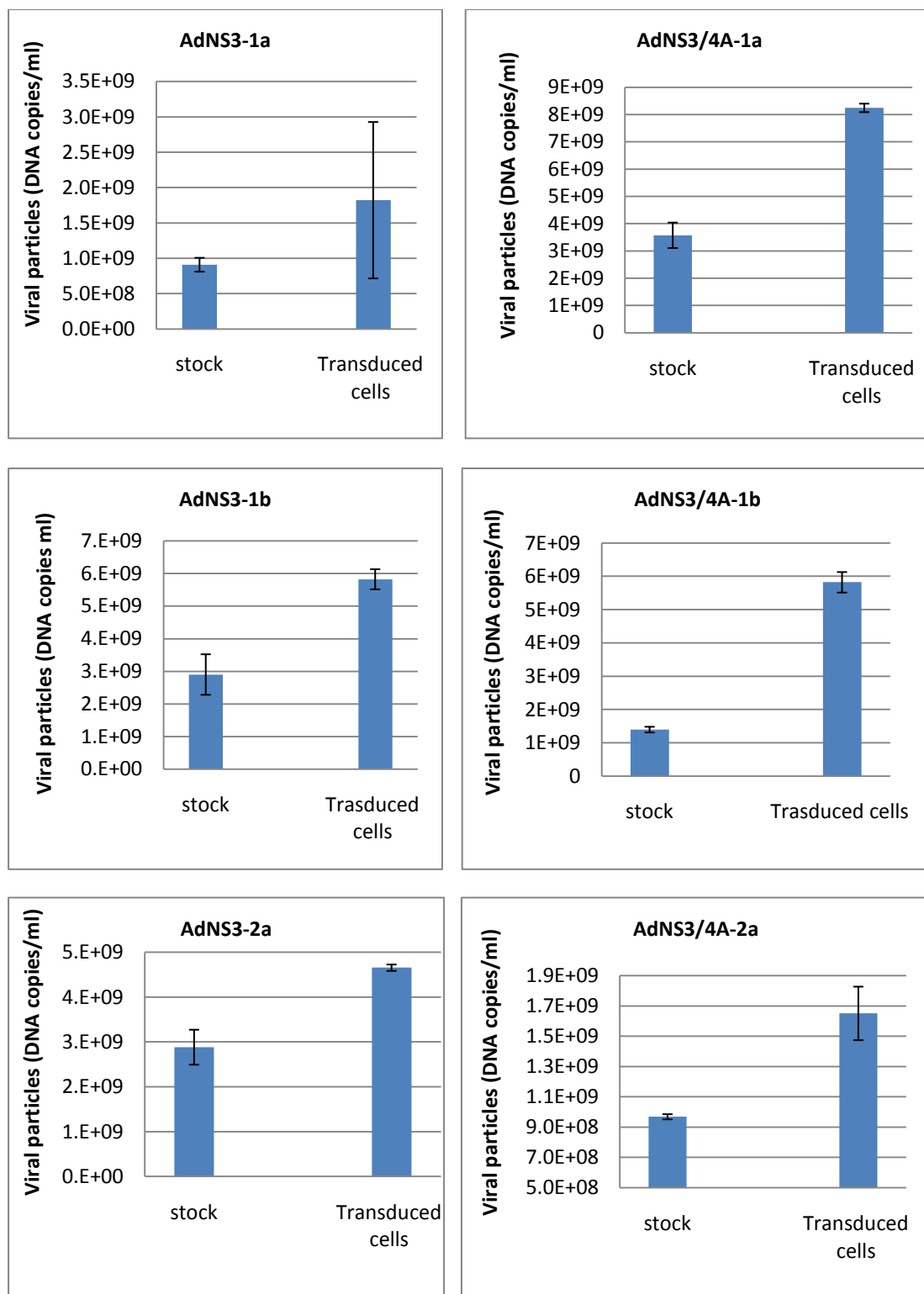
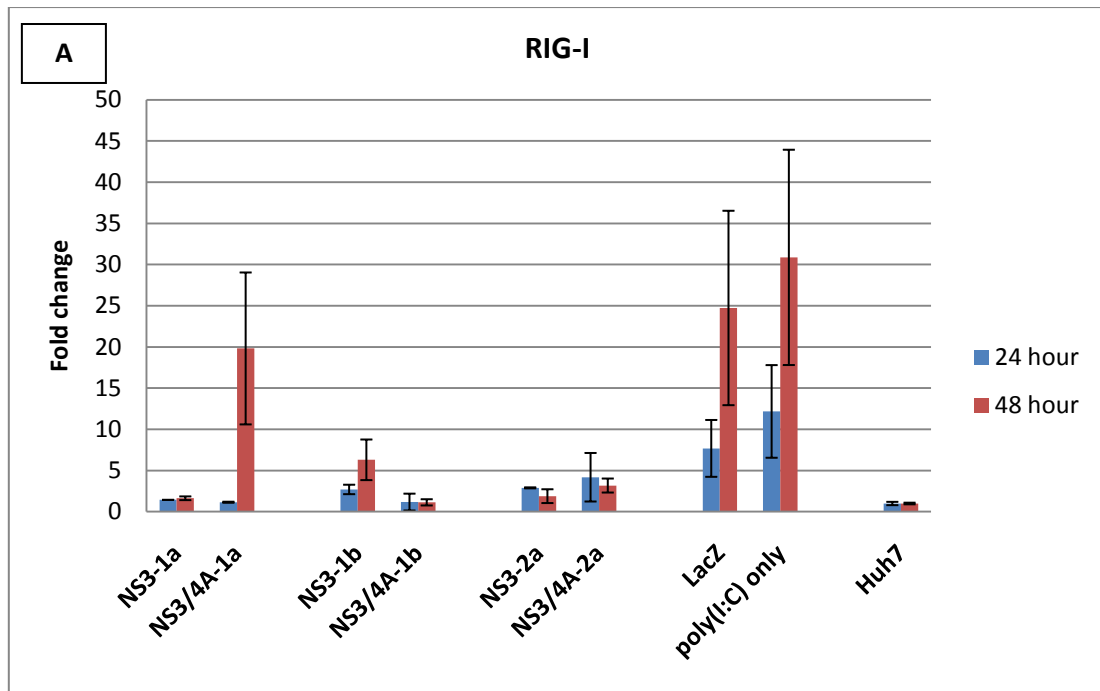
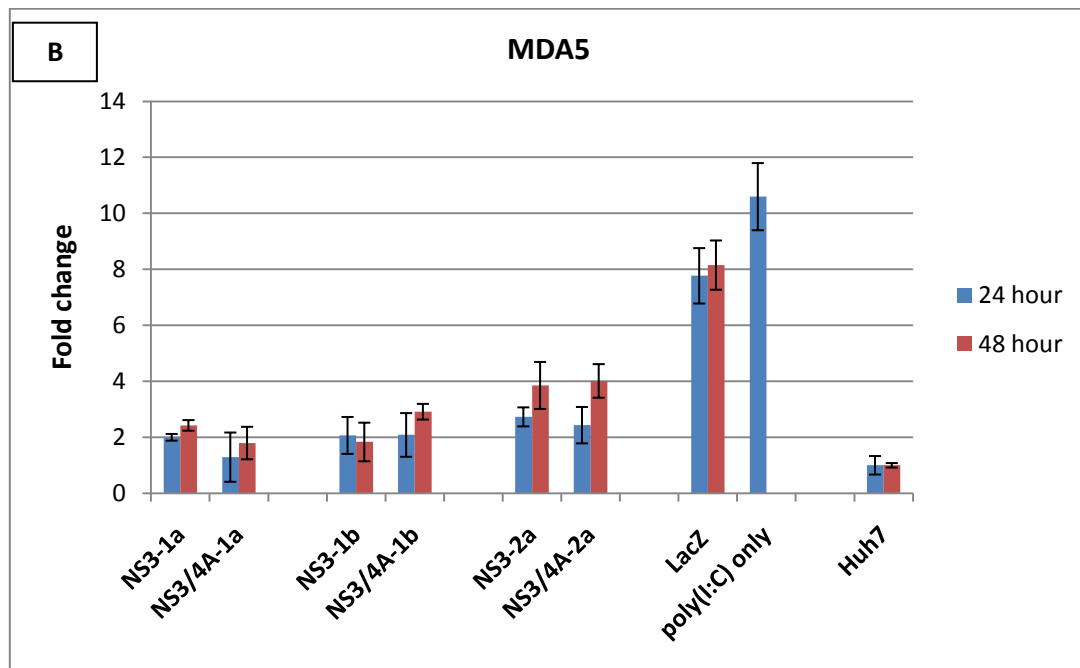


Figure 4.5: Quantitation of adenovirus vectors viral particles using qPCR. DNA was extracted from adenovirus stocks and transduced Huh7 cells. For each qPCR reaction a specific set of primers for each NS3 genotypes was used.

4.4. HCV NS3 and NS3/4A proteins reduce gene expression of IFN components:

Figure 3.4 and 3.5 showed that the presence of the HCV RNA replicon modulates the expression level of TLRs and RIG-I/MDA5 receptors and the mediator proteins MAVS and TRIF in Huh7 cells, and consequently reduced the ability of Huh7 to respond to dsRNA indicating a role of NS proteins in this inhibition. Many studies have shown that HCV NS3/4A serine protease inhibits IFN induction by disrupting the TLR-3 or RIG-I/MDA5 pathways by targeting two adaptor proteins, TRIF and MAVS, for proteolysis. To determine whether the NS3 protein and its cofactor NS4A could also inhibit at the transcription level, Huh7 cells were transduced with the AdNS3 and AdNS3/4A from genotypes 1a, 1b and 2a in addition to AdLacZ vector control for 72 hours. The cells were then stimulated with 5ng/μl poly (I:C) to induce an innate immune response via the dsRNA pathways. After 24 or 48 hours the effects on mRNA expression were measured for RIG-I, MDA5 and MAVS.

The results show that the HCV NS3/4A protein or NS3 alone could efficiently block the dsRNA signaling pathway by reducing many of the key signaling factors including RIG-I, MDA5 and MAVS (figure 4.6).



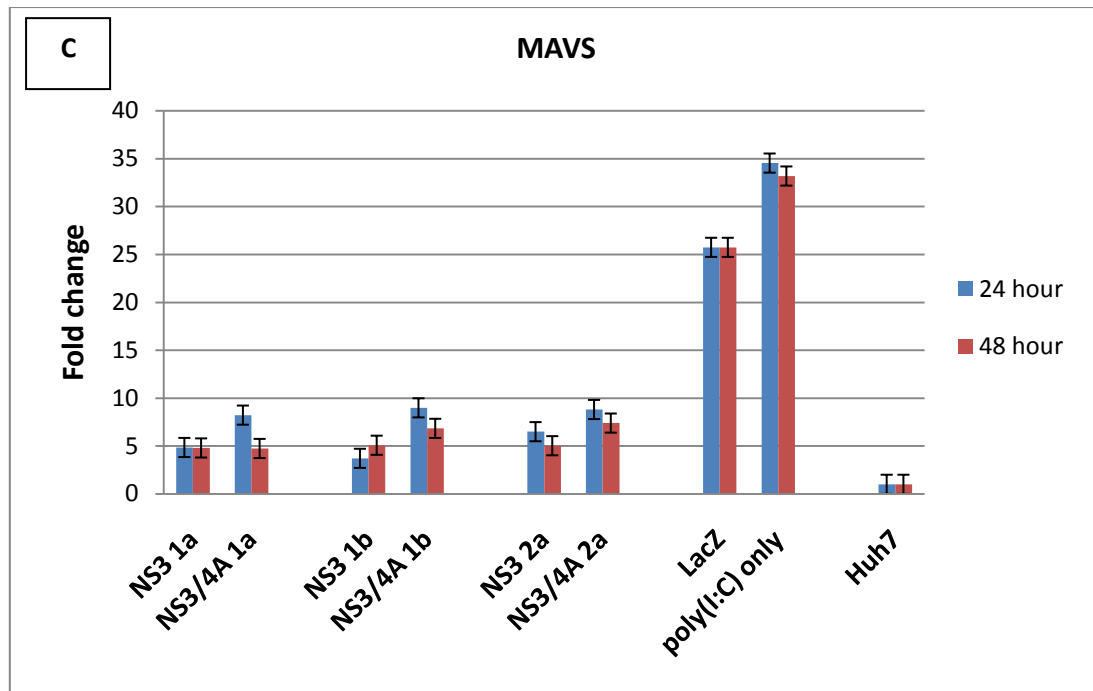


Figure 4.6: Effects of HCV NS3 and NS3/4A on RIG-I, MDA5 and MAVS expression. Huh7 cells were grown in 24 well plates followed by transduction with AdNS3, AdNS3/4A or AdLacZ for 72 hour. Cells were stimulated with poly (I:C) (5ng/μl) for 8 hours. Total RNA was extracted, and the mRNA expression: at 24 and 48 hour post transfection was determined by real-time PCR and normalized using β-actin mRNA (A) RIG-I, (B) MDA5 and (C) MAVS.

4.5. ssRNA Induced IFN-α Expression is blocked by the HCV NS3:

To investigate the role of HCV NS3/4A in inhibiting IFN-α promoter activation induced by MyD88, the signaling mediator for TLRs 4, 7 and 8, Huh7 cells were transduced with AdNS3 and AdNS3/4a viruses. Two days post infection cells were stimulated with 5ng/μl LPS for 24 and 48 hours. The results demonstrate that the HCV NS3/4A protein or NS3 alone could efficiently block the ssRNA signaling pathway by reducing the expression of MyD88 (figure 4.7).

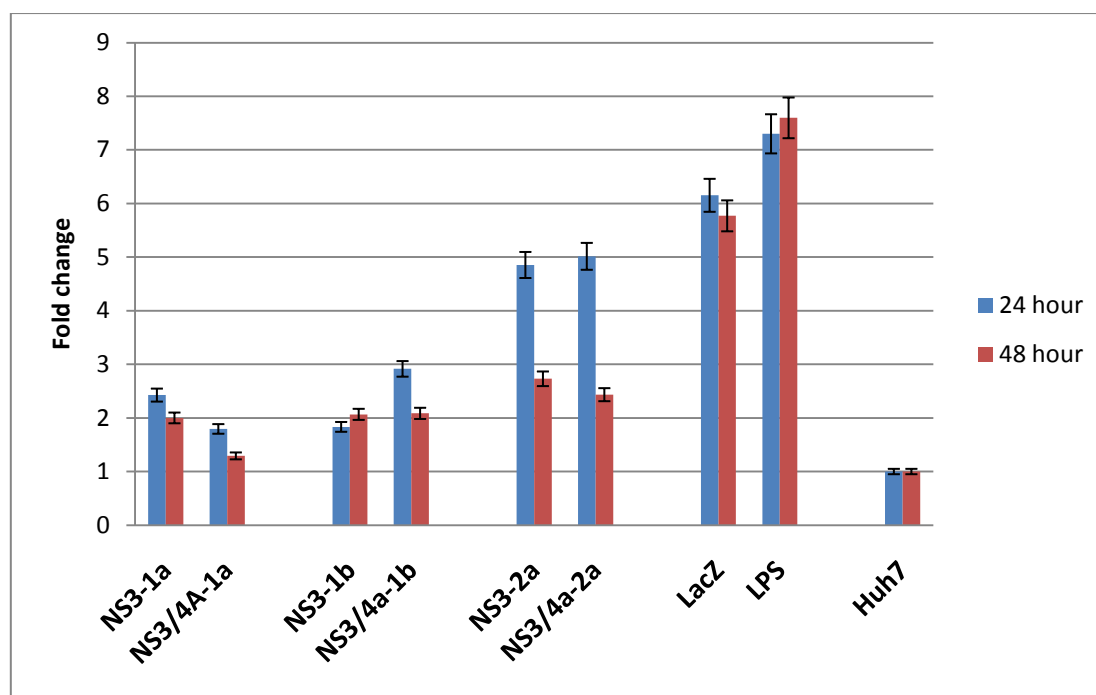
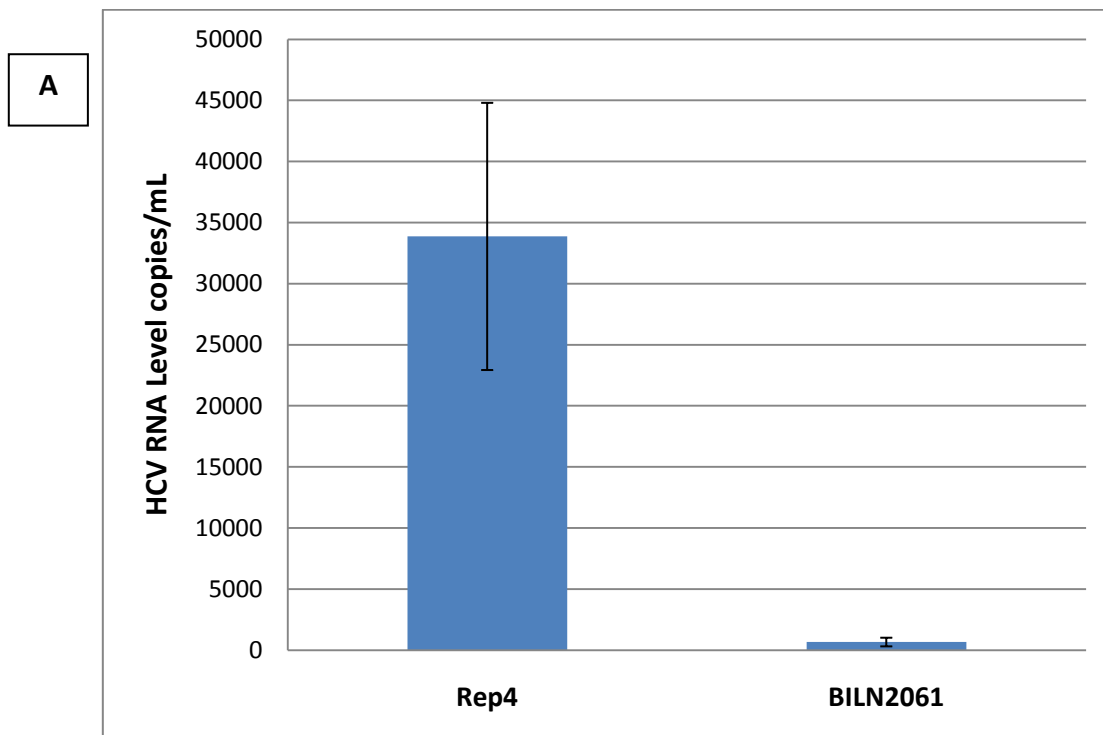


Figure 4.7: Effects of HCV NS3 and NS3/4a on MyD88 expression. Huh7 cells were grown in 24 well plates followed by transduction with AdNS3, AdNS3/4A or AdLacZ for 72 hour. Cells were then stimulated with 10ng/μl LPS. Total RNA was extracted and analyzed by real time RT-PCR for MyD88 mRNA expression at 24 and 48 hour post stimulation and normalized against the levels of β-actin mRNA.

4.6. Protease inhibitor rescue of RIG-I signalling:

To investigate the molecular mechanism of the antiviral activities of NS3/4A protease inhibitors in the type I IFN pathway, (10μM) of BILN 2061 was added to the culture media of HCV subgenomic replicons (Rep4) cells and Huh7 cells infected with HCV-JFH-1 for 24 hours incubation. To confirm the antiviral activity of the BILN 2061 compound, HCV RNA levels were determined by qRT-PCR. This showed, in agreement with many previous reports, that BILN2061 has potent antiviral activity against genotype 1b replicons (a 2 log reduction was observed), which indicate that these compounds have potent antiviral activity against HCV viruses (figure 4.8A). The effect of the compound on the expression of RIG-I, MDA5, IRF3 and MAVS

mRNAs expression was then measured, showing that BILN 2061 treatment of replicon cells reduced the expression levels of these genes to the level of naïve Huh7 cells (figure 4.8B). Additionally, BILN 2061 reversed the NS3/4A-mediated inhibition of RIG-I, MDA5 and MAVS expression in response to transfection with poly (I:C) as previously shown (figure 3.4). This failure to induce IFN- β promoter activation when NS3/4A is expressed is due to the cleavage of the RIG-I adaptor protein MAVS.



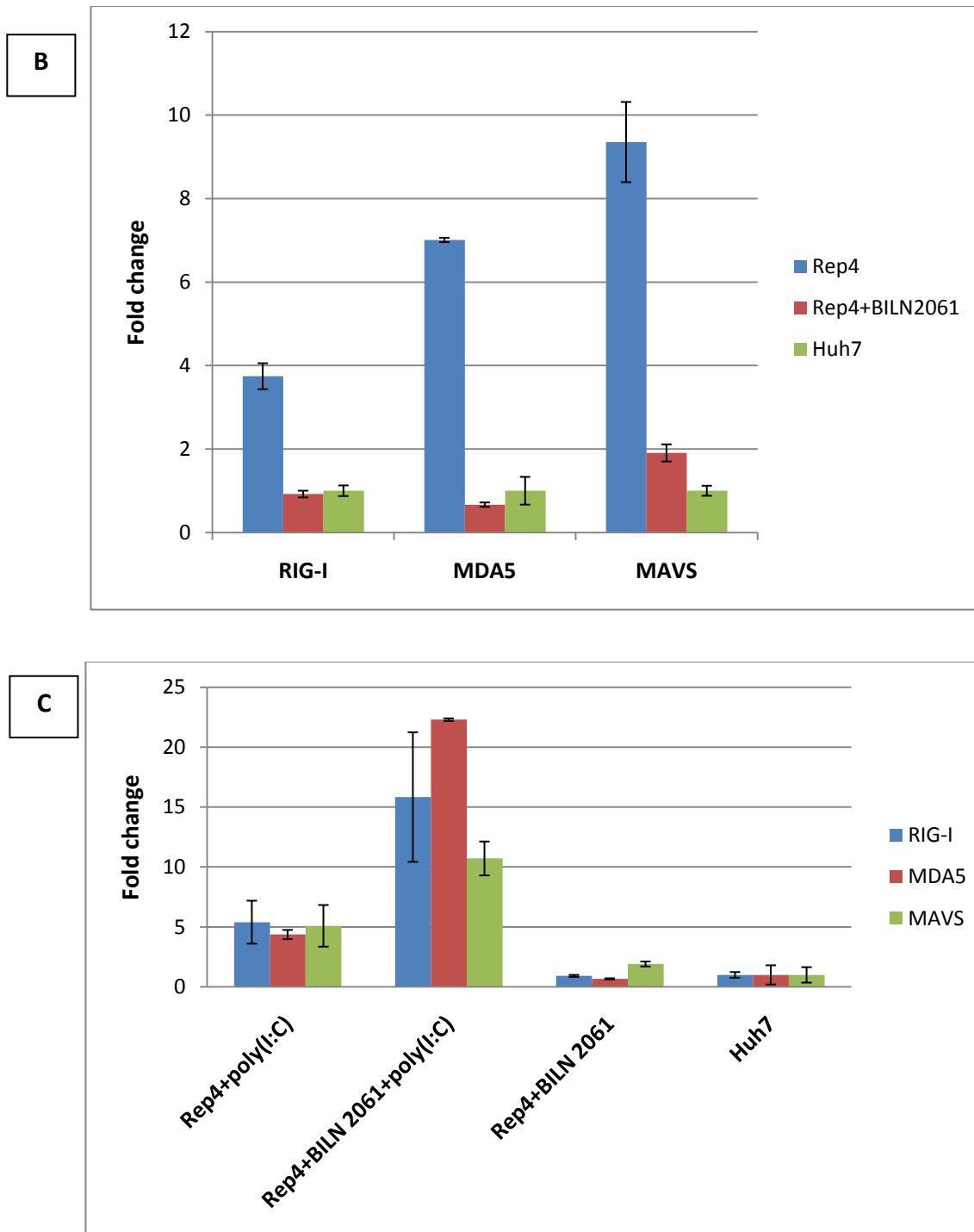


Figure 4.8: Rep4 inhibition in response to BILN 2061 protease inhibitor treatment. (A) Rep4 cells were treated with BILN 2061 and 24 hours post incubation the replicon RNA levels were determined by qRT-PCR. (B) Relative expression of RIG-I, MDA5 and MAVS mRNAs in Rep4 cell in response to BILN 2061. (C) Relative expression of RIG-I, MDA5 and MAVS mRNAs in Rep4 cell in response to BILN 2061 followed by poly (I:C) transfection. Expression levels were determined from total RNA using real time qRT-PCR and normalized with GAPDH.

CHAPTER FIVE:

5.0. Microarray Analysis of JFH-1 Infected Huh7 Cells

Gene Expression Analysis of Huh7 cells infected with HCV was conducted by Dr McGarvey's lab in collaboration with GlaxoSmithKline Lab in the USA. Huh7 cells were either infected with JFH-1 at a multiplicity of infection (MOI) of 3 or mock infected with an equal volume of concentrated conditioned growth medium. At 6, 12, 18, 24 and 48 hours post-infection, RNA was extracted, quality tested using an Agilent Bioanalyser and hybridized onto Human U133 Plus 2.0 Affymetrix microarray chips for fluorescence data acquisition. Raw microarray expression data was processed using Array Studio software (Omicsoft, USA) to generate values representing fold change in gene expression. An average of the triplicate values was used to calculate fold change and each value was assessed for its statistical significance using ANOVA (177). Heatmaps representing the microarray data were then generated using Multi Experiment Viewing (MEV) software available within the TM4 Microarray Software Suite. Hierarchical clustering by Pearson Correlation was used to cluster the genes in each heatmap (177).

5.1. HCV JFH-1 Infection alters Gene Expression in Huh7 Cell

The effect of acute HCV infection on host gene expression was investigated using Huh7 cells infected with JFH-1 for 6, 12, 18, 24 and 48 hours. Genes showing at least a 2 fold change in expression ($p \leq 0.05$) were considered to be significantly regulated by infection. The number of genes affected by infection increased over time as JFH-1 infection progressed through the HCV replication cycle. Interpretation of the

microarray gene expression data was performed using gene ontology analysis using the PANTHER Classification System. PANTHER identifies biological processes that are over-represented within microarray expression datasets. The biological processes significantly regulated by JFH-1 infection which are broadly related to cellular defense mechanisms, including immunity, interferon response and, cell cycle and apoptosis are shown in table 5.1.

Gene Symbol	Entrez Gene description	Affymetrix	Fold Change
JAK/STAT pathway			
KRAS	v-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog	1559203_s_at	2.290
MRAS	muscle RAS oncogene homolog	225185_at	2.460
PIAS2	protein inhibitor of activated STAT, 2	1555514_a_at	-2.200
PIK3R3	phosphoinositide-3-kinase, regulatory subunit 3 (gamma)	211580_s_at	-2.310
SOCS2	suppressor of cytokine signaling 2	203372_s_at	7.910
SOCS3	suppressor of cytokine signaling 3	227697_at	2.920
SOCS4	suppressor of cytokine signaling 4	1552792_at	2.350
STAT1	signal transducer and activator of transcription 1, 91kDa	M97935_5_at	-2.280
Interferon signaling			
IFNAR2	interferon (alpha, beta and omega) receptor 2	204786_s_at	-2.120
IRF1	interferon regulatory factor 1	202531_at	2.090
IRF9	interferon regulatory factor 9	203882_at	5.880
MX1	myxovirus (influenza virus) resistance 1	202086_at	3.880
STAT1	signal transducer and activator of transcription 1, 91kDa	M97935_5_at	-2.280
Cell cycle: G2/M DNA damage checkpoints regulation			

CCNB1	cyclin B1	214710_s_at	-5.230
CDC25C	cell division cycle 25 homolog C	217010_s_at	-2.300
GADD45A	growth arrest and DNA-damage-inducible, alpha	203725_at	2.950
SKP2	S-phase kinase-associated protein 2	203626_s_at	-2.320
TOP2A	topoisomerase (DNA) II alpha 170kDa	201291_s_at	-2.250
CCNE1	cyclin E1	213523_at	-2.110
HDAC4	histone deacetylase 4	228813_at	2.050
HDAC9	histone deacetylase 9	205659_at	4.120
Cell cycle: G1/S checkpoints regulation			
NRG1	neuregulin 1	206343_s_at	2.740
SKP2	S-phase kinase-associated protein 2	203626_s_at	-2.320
TGFB2	transforming growth factor, beta 2	228121_at	2.980
Cell cycle Arrest Proteins			
INHBE	inhibin, beta E	210587_at	18.01
CIP29	cytokine induced protein 29 kDa	229069_at	2.99
STC2	stanniocalcin 2	203438_at	20.5
CDKN3	cyclin-dependent kinase inhibitor 3	209714_s_at	-5.09
CDKN2C	cyclin-dependent kinase inhibitor 2C (p18, inhibits CDK4)	204159_at	-6.87

Table: 5.1. Microarray analysis showing changes in the expression of genes involved in the interferon signaling pathway and cell cycle in Huh7 cells infected with HCV JFH-1 (mRNA expression at 48 hours post infection).

5.2. HCV JFH-1 infection stimulates a pro-inflammatory host anti-viral response

Three families of proteins are known to negatively regulate the JAK/STAT pathway: one is the SH2-containing phosphatase (SHP) family, the second is the suppressor of the cytokine signaling (SOCS) family of proteins, and the third is the protein inhibitor of the activated STAT (PIAS) family of proteins. In human genomes, there are two SHPs (i.e., SHP-1 and SHP-2), eight SOCS (SOCS1 to SOCS7 and CIS), and four PIAS family consists of PIAS1, PIAS3, PIASx (also known as PIAS2) and PIASy (also known as PIAS4) (284). The induction of SOCS expression is Jak–STAT-dependent thus forming part of a negative regulatory mechanism.

Genes involved in multiple anti-viral signaling pathways increased in expression following JFH-1 infection including; the type I and II interferon responses (e.g. MX1 and OAS3), and genes involved in IFN induction through JAK-STAT (e.g. IRF1, IRF9). Increased expression of genes encoding negative regulators of the interferon response was also observed, (e.g. SOCS and PIAS). On the other hand down regulation of IFN receptors (IFNAR1 and IFNAR2) and signaling mediators STATs (STAT1 and STAT2) was shown.

There is evidence that SOCS-1 and SOCS-3 inhibit the IFN-induced signal cascade and in hemopoietic tumor cells, over expression of SOCS-1 or SOCS-3 inhibits the antiproliferative and antiviral activity of IFN- α/β and IFN- γ . In addition, many studies have shown a relationship between treatment failure and expression of the gene encoding SOCS3 in patients who did not respond to type I IFN treatment. Microarray analysis showed that expression of SOCS family mRNAs expression (table 5.1) was up regulated in response to JFH-1 infection. To validate these findings and examine

its role in IFN inhibition, infection of HCV JFH-1 in Huh7 cells was carried out in triplicate for 48 hours at an MOI of 3. Total RNA was isolated and cDNA synthesized to measure the expression of genes involved in IFN signaling in response to infection. In agreement with the microarray result, qRT-PCR showed significant induction of SOCS family (SOCS1, 2 and 3) of mRNA expression. Additionally other targets involved in IFN induction including IFNAR (1 and 2), STATs and PIAS family genes (PIAS1 and PIAS2) were down regulated. PCR analysis also confirmed that IFN effectors genes including MXA1, IRF9 and IRF1 and OAS3 were up regulated in response to HCV infection. Infection of Huh7.5 cells with JFH-1 also showed a similar effect on these genes (figure 5.1).

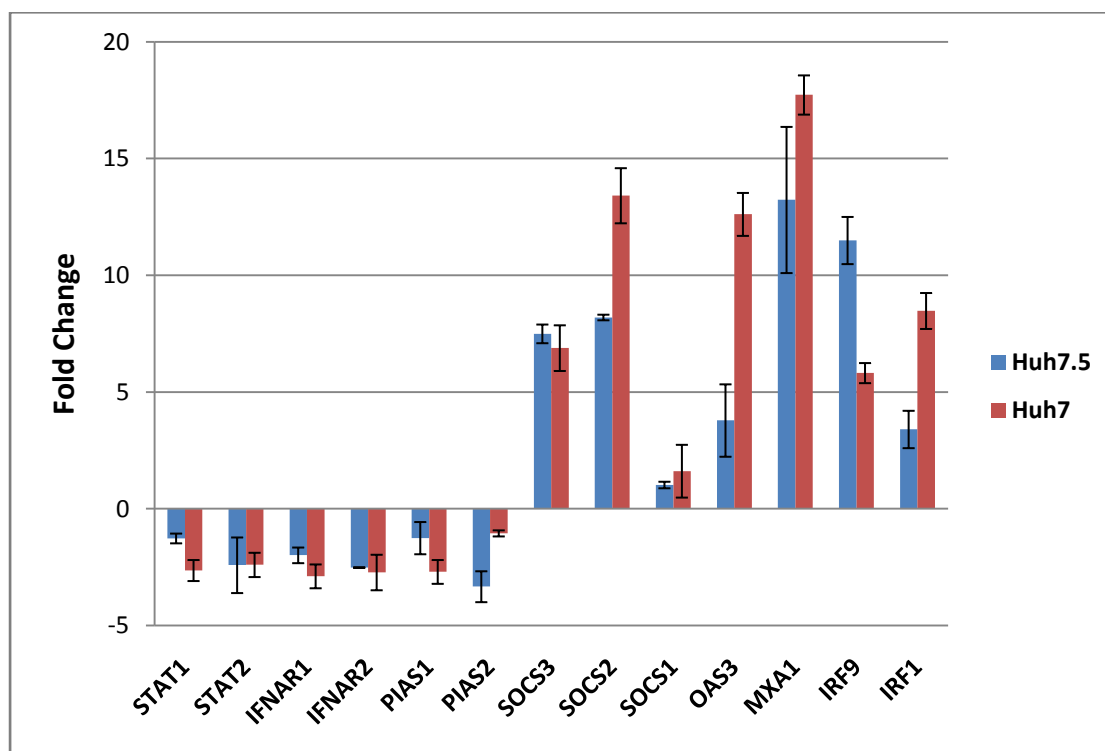


Figure: 5.1. Relative expression of interferon response and regulatory mRNAs in Huh7 and Huh7.5 cells in response to HCV JFH-1 infection. Expression levels were determined from total RNA using real time qRT-PCR and normalized with GAPDH gene.

5.3. Reduction of viral replication by suppression of SOCS3 expression:

Over expression of two families of negative regulatory proteins SOCS and PIAS inhibited the antiproliferative and antiviral activity of IFN- α/β (257, 258) and IFN- γ (259). In addition, many studies showed a relationship between treatment failure and expression of the genes encoding SOCS3 and PIAS1 in patients who did not respond to type I IFN treatment (261, 262). However, since PCR analysis showed that the PIAS family mRNAs were not up regulated compare to SOCS family mRNAs (figure 5.1), this led to further investigation of the role of SOCS proteins in viral replication and the IFN inhibition pathway. To investigate the role of SOCS3 in HCV antiviral mechanisms, siRNAs were used to suppress the expression of SOCS3 mRNA in Huh7.5 cells. Transfection of siRNA suppressed the SOCS3 expression level in Huh7.5 cells by around 80 % compared to cells transfected with scrambled siRNA (figure 5.2).

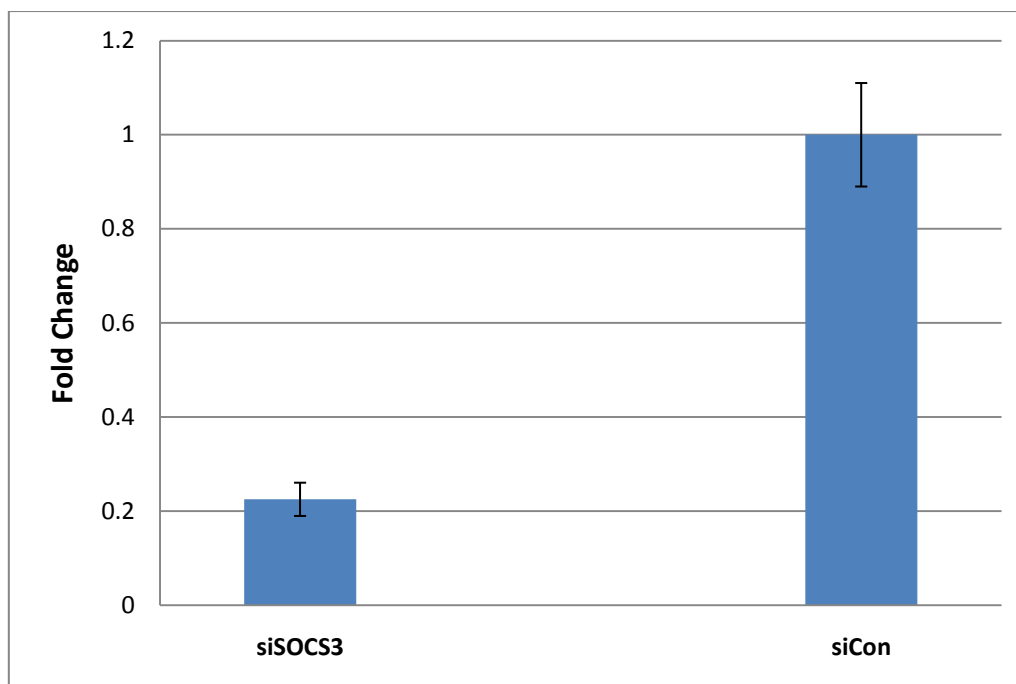


Figure 5.2: Inhibition of SOCS3 mRNA expression with siRNAs. Huh7.5 cells were transfected with specific siRNA duplexes and 48 hours post transfection, the target genes were quantified by qRT-PCR. Control non-targeting duplex siRNAs (siCon), with scrambled sequence, was used as negative control. mRNA levels were normalized against the cellular GAPDH gene.

To show the effect of reduced SOCS3 gene expression on HCV replication, Huh7.5 cells were transfected with 100 μ M of SOCS3 siRNAs for 24 hours followed by infection with HCV JFH-1. 48 hours post infection the level of SOCS3 knockdown and the HCV RNA level was determined by qRT-PCR. As seen (figure 5.3), a 60% reduction in SOCS3 mRNA level reduced the HCV RNA expression four-fold compare to negative controls transfected with scrambled siRNA (siCon). This result confirms that SOCS3 expression affects HCV replication.

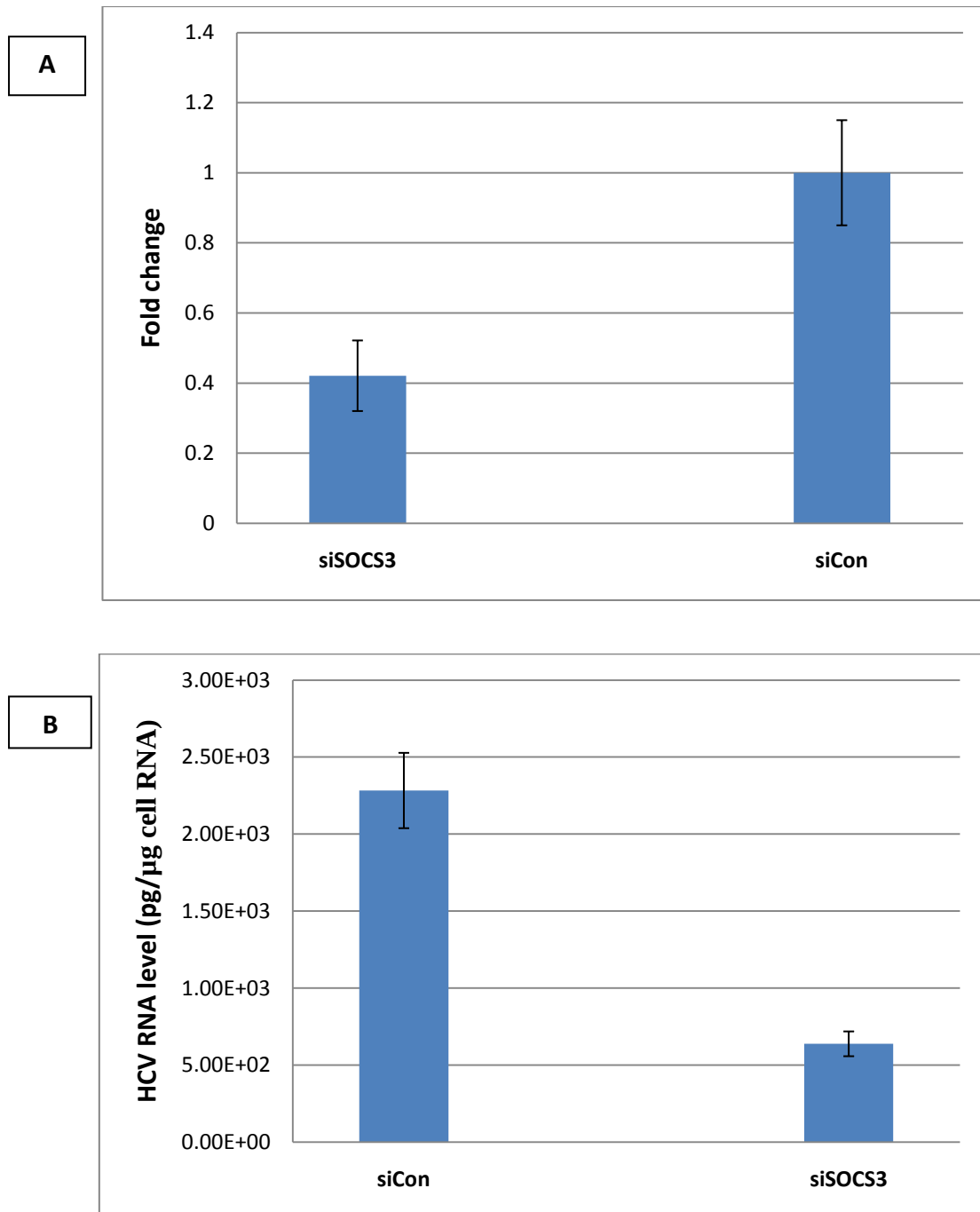


Figure 5.3: SOCS3 knockdown reduces HCV replication. Huh7.5 cells were transfected with control (siCon) or SOCS3 siRNA, 48 hour posttransfection, cells were infected with HCV-JFH-1 and 48 hours post infection the HCV RNA levels were determined by qRT-PCR. (A) quantitation of SOCS3 inhibition level on siSOCS3 and siCon transfected cells. (B) quantitation of JFH-1 RNA levels from total RNA.

To study the role of SOCS3 knockdown on IFN- α treatment for HCV infection, SOCS3 siRNAs were used to transfect the Huh7.5 cells for 24 hours before JFH-1 infection for 48 hours and then they were treated with IFN- α (25 IU/ml). HCV RNA levels were then determined at 24 hours after IFN- α treatment using qRT-PCR. As a control, Huh7.5 cells were similarly treated with scrambled siRNA and infected with JFH-1 and treated with IFN- α . This experiment showed that after treatment with IFN- α and siCon, the level of JFH-1 RNA was 7 fold lower than in Huh7.5 cell transfected with siSOCS3 (figures 5.4). Taken together, these results show that SOCS3 proteins play an important role in inhibition of the IFN pathway and provide evidence that increasing SOCS3 expression may be a mechanism by which a virus evades the host anti viral machinery and can increase its persistence.

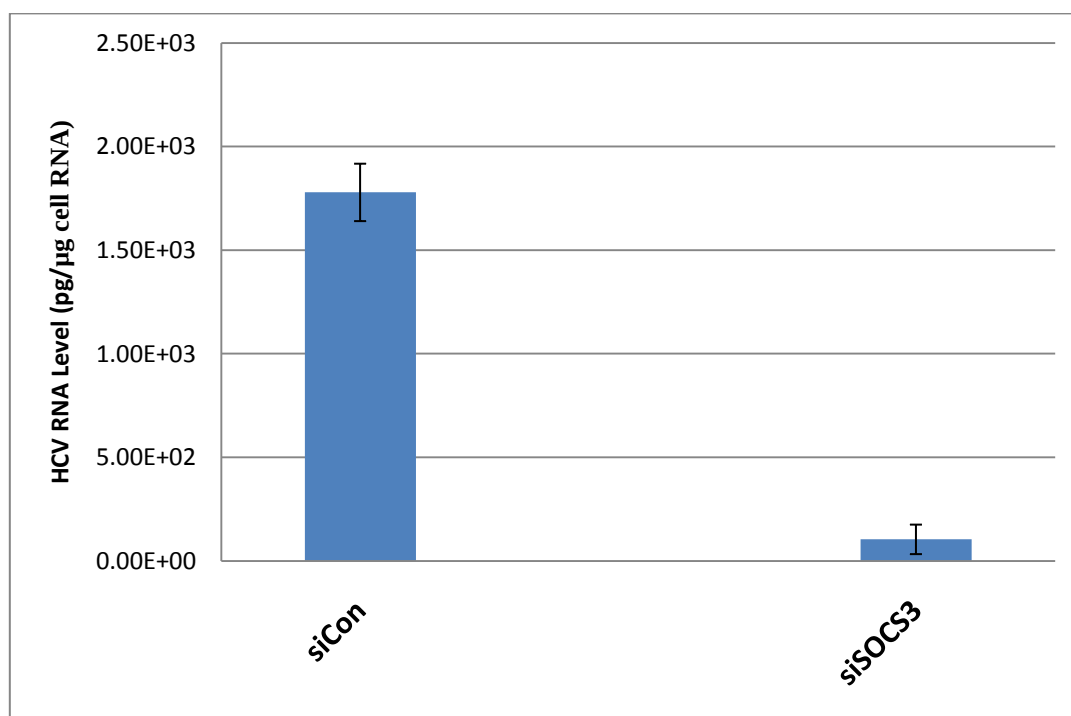


Figure 5.4: Reduction of viral replication by inhibition of SOCS3 expression and IFN- α combination treatment. Huh7.5 cells were infected with JFH-1 virus for 48 h, and then transfected with siRNAs, and treated with IFN- α . The level of viral replication was determined using qRT-PCR.

SOCS3 knockdown also showed a correlation between inhibition of STAT1 activation and induction of SOCS3 expression. Huh7.5 cells transfected with SOCS3 siRNA and infected with JFH-1 were used to measure STAT1 mRNA by qRT-PCR. As seen in (figure 5.5) inhibition of SOCS3 mRNA led to changes in the expression of STAT1 from a 1.2 fold down regulation to a 2.5 fold increase. This indicates that SOCS3 knockdown also increased STAT1 mRNA expression in JFH-1-infected cells (Figure 5.5). This indicates that IFN signaling through STAT1 correlates with SOCS3 induction and the inhibition of IFN signal related to synthesis of SOCS3.

Taken together, these data indicate that the regulation of interferon mediated antiviral responses by SOCS3 is important for HCV replication.

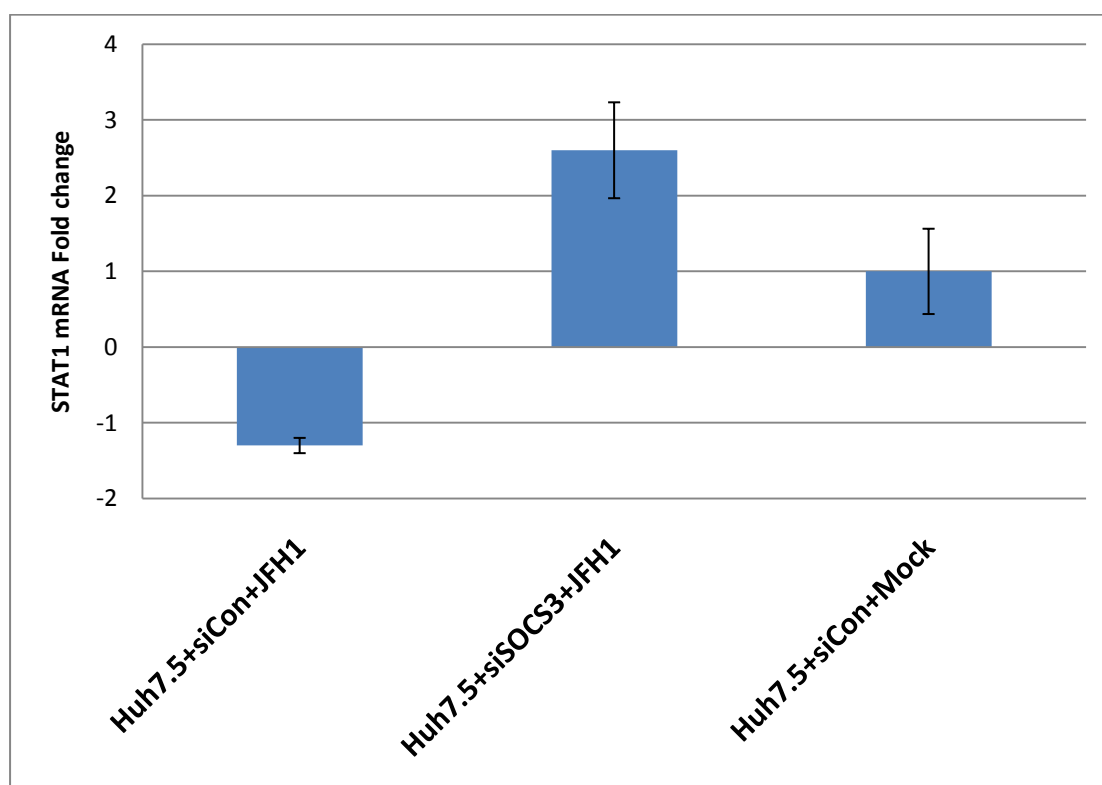


Figure 5.5: STAT1 induction correlates with SOCS3 inhibition. Relative expression of STAT1 mRNAs in Huh7.5 cells in response to HCV JFH-1 infection after SOCS3 knockdown by siRNA. Expression levels were determined from total RNA using real time qRT-PCR and normalized with GAPDH gene.

5.4. HCV infection regulates expression of genes that control apoptosis and the cell cycle:

Genes controlling apoptosis and the cell cycle arrest were significantly regulated by JFH-1 infection. Genes regulating cellular proliferation and cell cycle arrest were also significantly regulated following JFH-1 infection.

Transcriptional changes of genes involved in cell proliferation, included an increase in the expression of genes that promote cell cycle arrest (e.g. INHBE and STC2) and a decrease in the expression of genes required for cell cycle progression (e.g. CCNB1, CCNE1 and SKP2) (table 5.1). The majority of the differentially expressed genes associated with cell cycle regulation were involved with cell cycle checkpoint/arrest and subsequent induction of apoptosis, rather than actual progression through the cell cycle. Cyclin B1 plays an important role in control of the G2-M transition of the cell cycle, and in the regulation of mitosis. This gene was down regulated more than 5 fold during HCV infection. CDC25C and SKP2 play a key role in the regulation of cell division. Also GADD45- α and GADD45- β mRNA (growth arrest and DNA-damage-inducible, beta) were up regulated by significant levels (i.e. greater than 2 fold). GADD45- α and β are members of a group of genes whose transcript levels are increased following stressful growth arrest conditions and treatment with DNA-damaging agents. Furthermore, these findings, based on microarray analysis, were validated by real time qRT-PCR which showed the down regulation of different genes involved in cell cycle arrest (CCNB1, SKP2 and CDC25C) while GADD45- α and GADD45- β were up regulated in response to HCV JFH-1 infection. This was carried out in both Huh7 and Huh7.5 cells (figure 5.6).

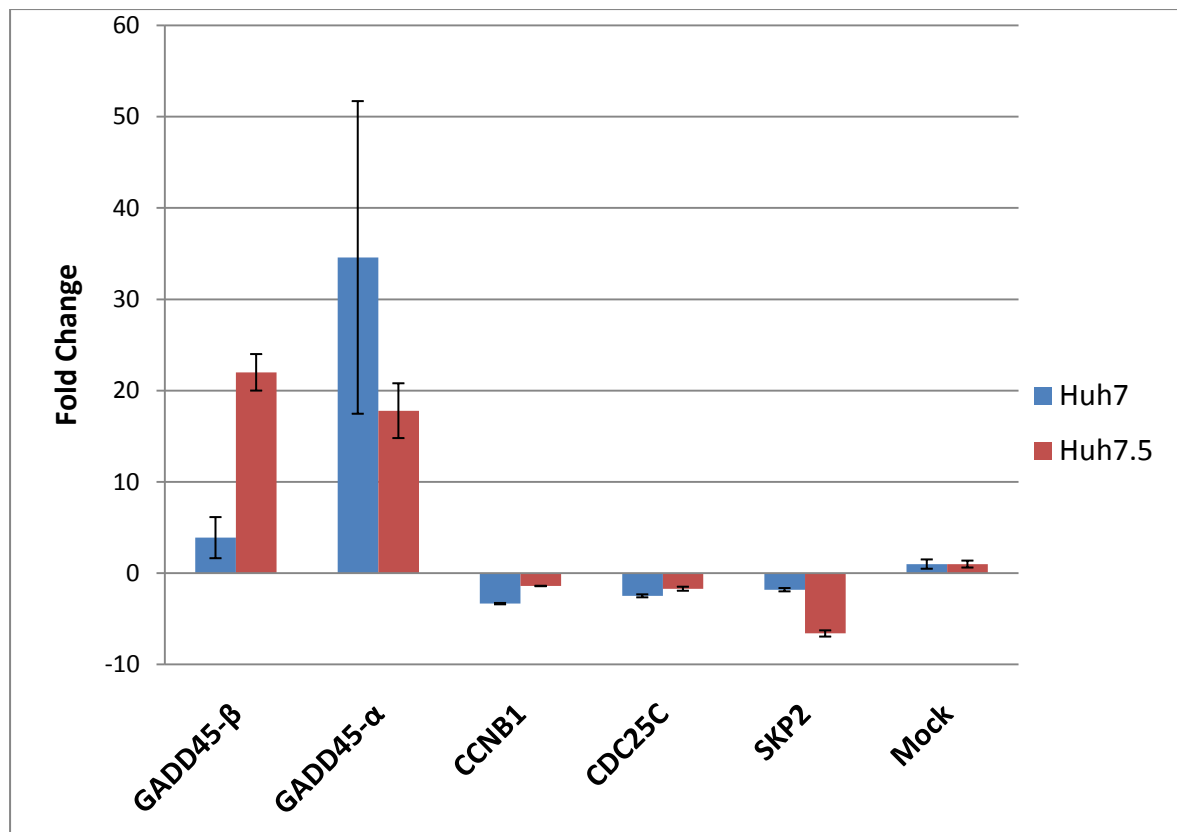


Figure 5.6: Relative expression of cell cycle regulatory mRNAs in response to HCV JFH-1 infection for 48 hours by comparison to mock infected cells. Expression levels were determined from total RNA using real time qRT-PCR using GAPDH as a control.

qRT-PCR was also used to investigate the level of expression of cyclin B1 and GADD45-β at 6, 18, 24 and 48 hours post-infection. Microarray and qRT-PCR data for the temporal regulation of expression for cyclin B1 and GADD45-β genes is shown in figure 5.7. GADD45-β was consistently transcribed at a high level from 12 to 48 hours post-infection (figure 5.7A). In contrast cyclin B1 expression decreased between 6 and 48 hours post-infection reached the maximum inhibition level (figure 5.7B).

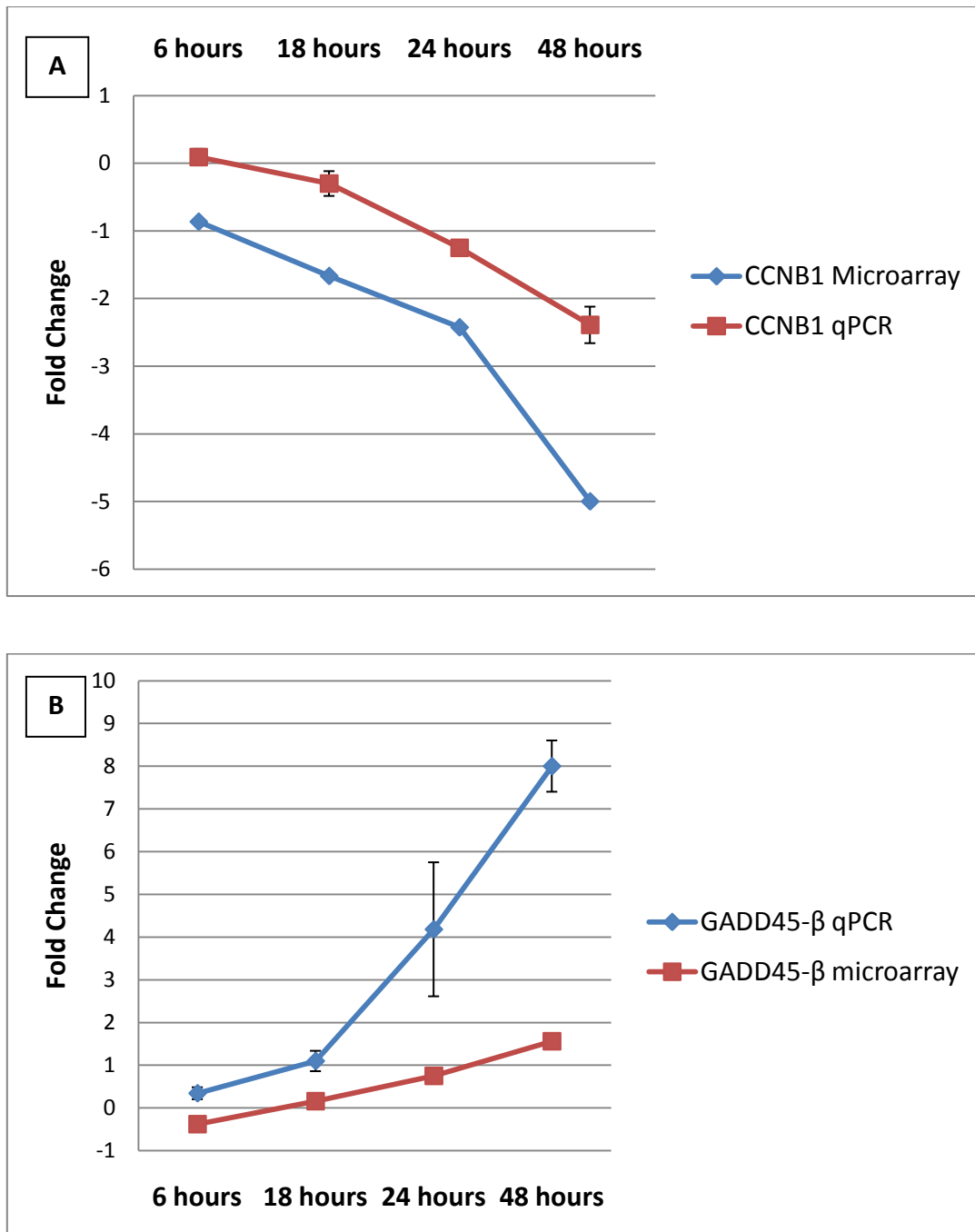


Figure 5.7: Temporal regulation of GADD45- β and cyclin B1 expression. qRT-PCR quantification of host gene expression at 6, 18, 24 and 48 hours post-JFH-1 infection of Huh7 cells was performed for (A) cyclin B1 (CCNB1) and (B) GADD45 β . Fold change in gene expression is shown for the qRT-PCR data (diamond) and the corresponding microarray data (square). The mean of triplicate infection samples are shown for each time point of the qRT-PCR and microarray data.

5.5. Analysis of cell cycle and apoptosis by propidium iodide staining using flow cytometry:

To further examine the role of HCV infection in cell cycle modulation and apoptosis, FACS analysis was used to measure the cellular DNA content in infected cells. This method is commonly used to measure the number of apoptotic cells by staining fragmented DNA with the propidium iodide (PI). PI is a fluorogenic compound that binds stoichiometrically to nucleic acids (285), so that its fluorescence emission is proportional to the DNA content of a cell (cellular RNA, is first removed by RNase treatment). Apoptotic cells stained with PI were analyzed with FACScan Becton Dickinson (BD). They displayed a broad hypodiploid (sub-G1) peak, which can be discriminated from the narrow peak of cells with normal (diploid) DNA content. Also, the distribution of cells in the various phases of the cell cycle indicates a proliferating cell population: the G1 and S (DNA synthesis phase), G2 and M-phase (mitosis). However, G2 and M phase, which both have an identical DNA content, cannot be discriminated based on a difference in DNA content (286).

Flow cytometry analysis was performed to determine if HCV infection was associated with the induction of apoptosis. Huh7.5 cells were infected with HCV JFH-1 for 72 hours at different MOIs (0.5, 1, 2, and 3 MOI). 72 post infection the cells were collected and the DNA was precipitated, and stained before FACS analysis. In addition, mock infected and non- treated cells were used as negative controls to discriminate true apoptotic nuclei from nuclear debris. These controls were used to set the acquisition parameters correctly and debris from necrotic cells was eliminated and gated-off by eliminating the corresponding region.

As seen in figure 5.8A, the DNA content distribution in Huh7.5 cells changed in response to JFH-1 infection. As the virus MOI increased, the percentage of cells in G2/M phase was increased with an increase of sub-G1 apoptotic peak (reaching a maximum of 20%), and those in S phase decreased in a dose-dependent manner. Also there was reduction in G0/G1 phase cell population compared to the mock infected cells. These results indicate that HCV infection causes inhibition of cell proliferation by inducing cell G2/M arrest, followed by apoptosis. This significant increase in the number of subdipliod cells stained with PI, which is a late stage of the process of apoptosis, indicates that there is a high level of apoptosis in HCV infected Huh7.5 cells (figure 5.8).

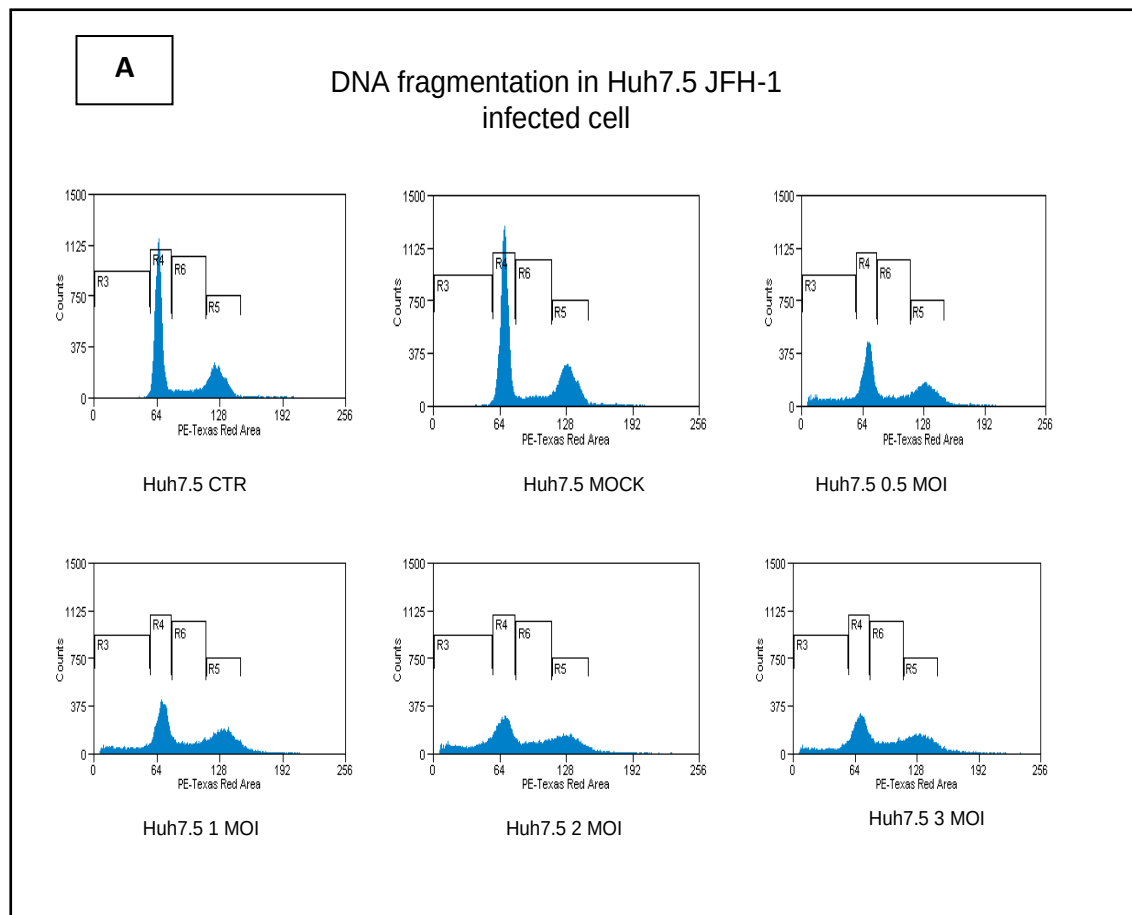
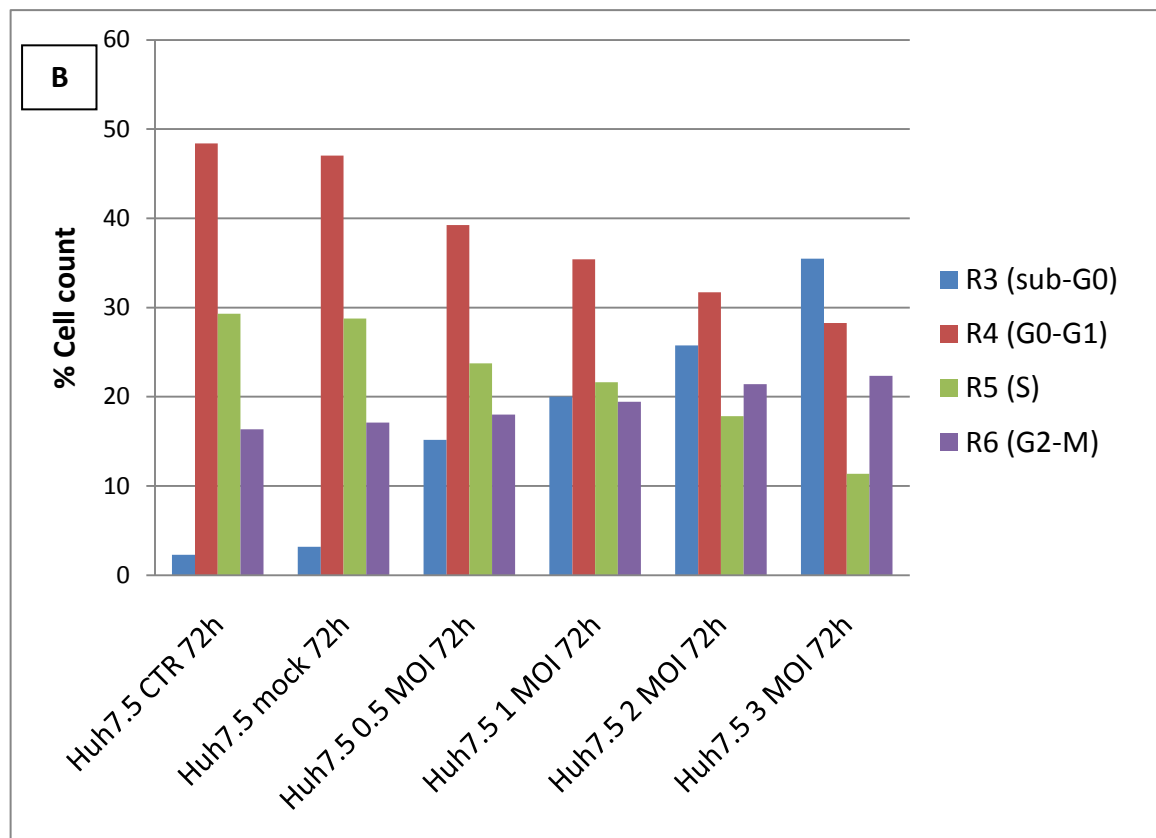


Figure 5.8: DNA fragmentation profiles of Huh7.5 cells. (A) untreated (CTR), mock infected (mock) or HCV infected cell at MOI of 0.5, 1, 2 and 3, representative graphs show DNA fragmentation by the appearance of a sub-G0 peak and changes in DNA contents G1 and S phase and G2/M cell phases. (B) Histograms showing the percentages of the cells from (A) at different phases at the cell cycle.

5.6. The role of RIG-I expression on cell cycle in HCV infected cells:

To determine whether RIG-I pathway plays a role in changes in cell cycle during HCV infection, similar experiments to those described in 5.5, were conducted using Huh7 cells. After 72 hour postinfection at similar MOI, FACS analysis was performed for DNA fragmentation staining. As seen in figure 5.9 the number of sub-G0 apoptotic Huh7 cells was significantly less compare to Huh7.5 cells (which have a defective RIG-I pathway) and the relative changes of in G1, S and G2/M were less marked compare to HCV infected Huh7.5 cells (figure 5.9). This suggests that the RIG-I pathway may play a role in linking innate responses via the IFN pathway and apoptosis and might define a role for HCV viral load.

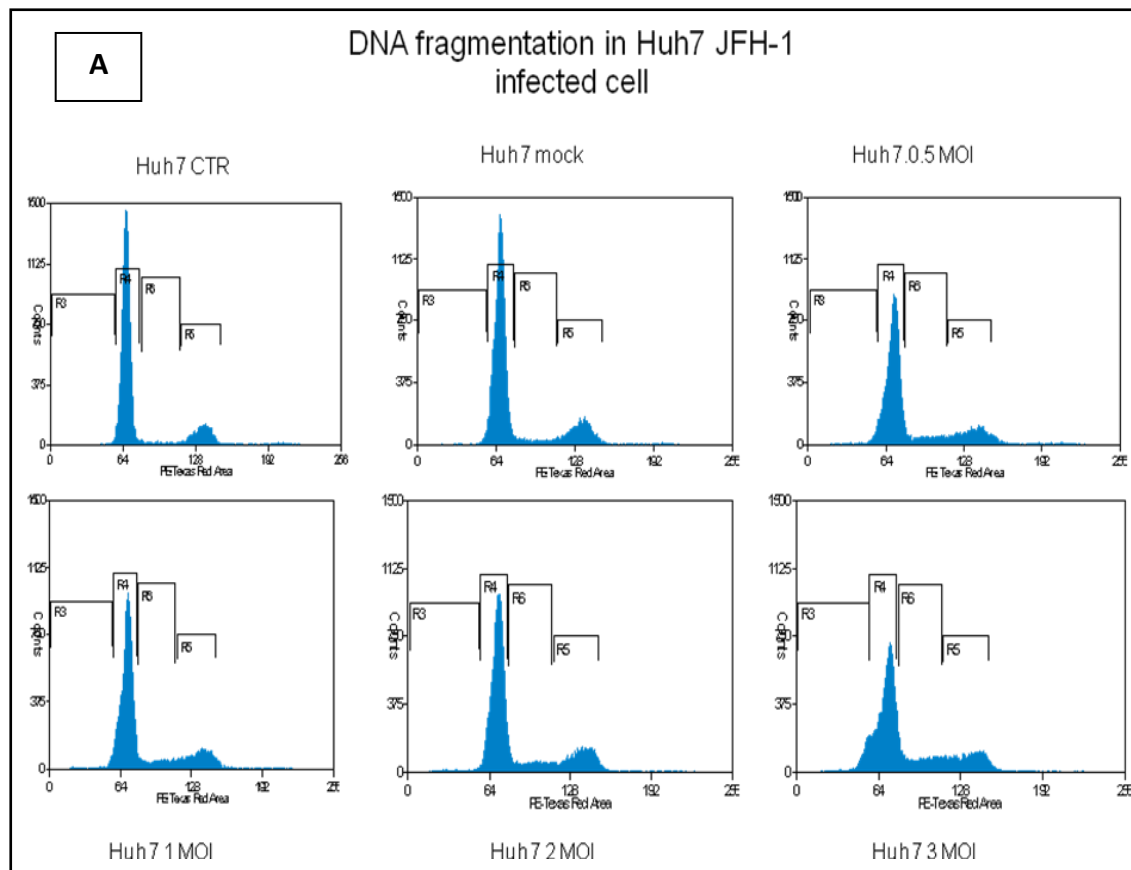
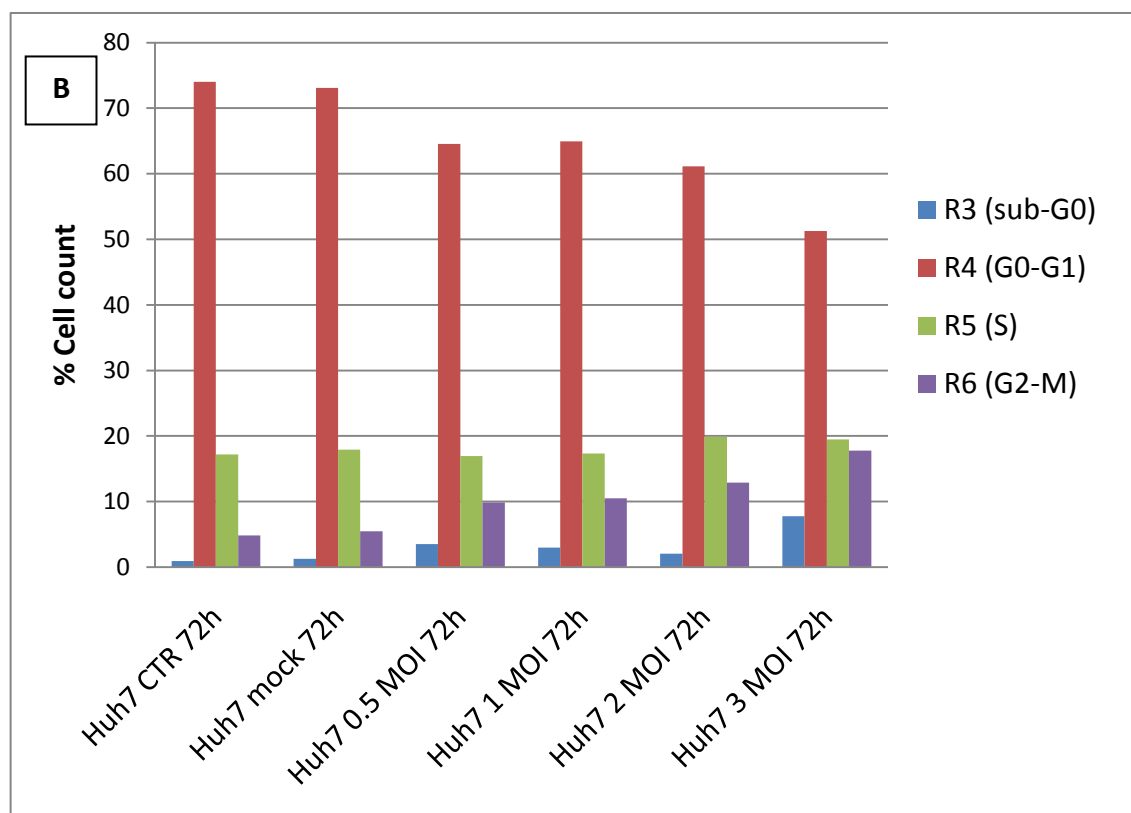
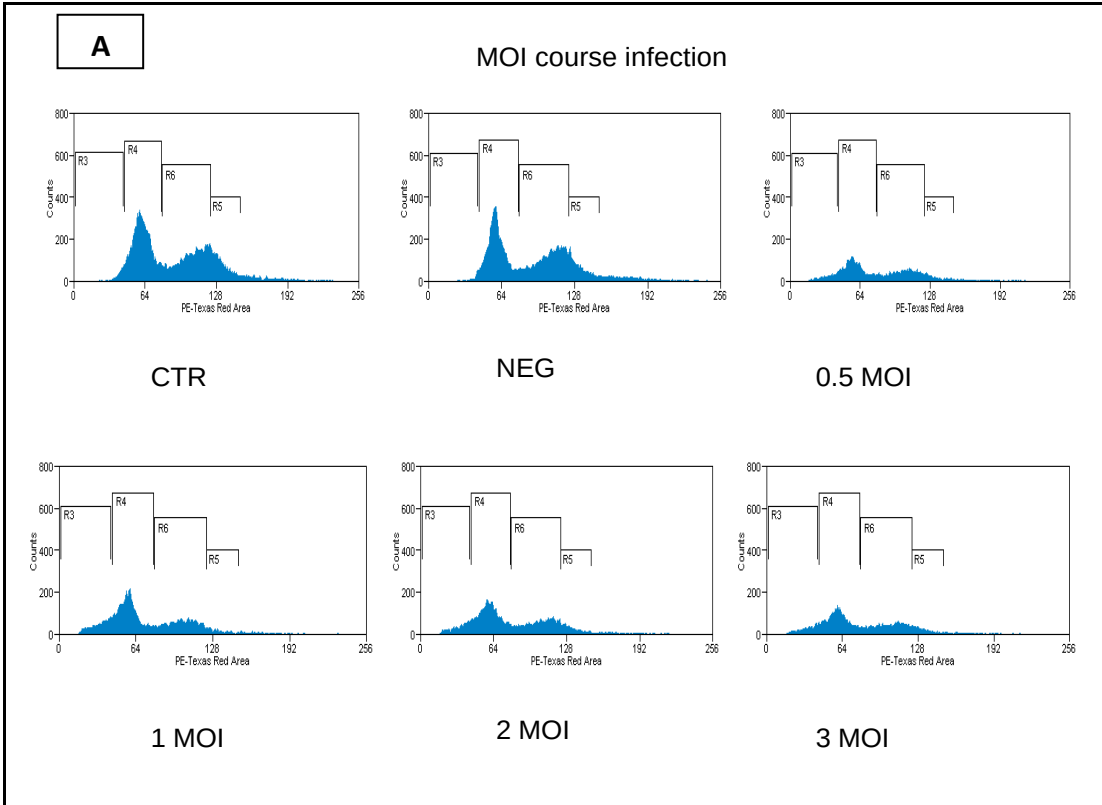
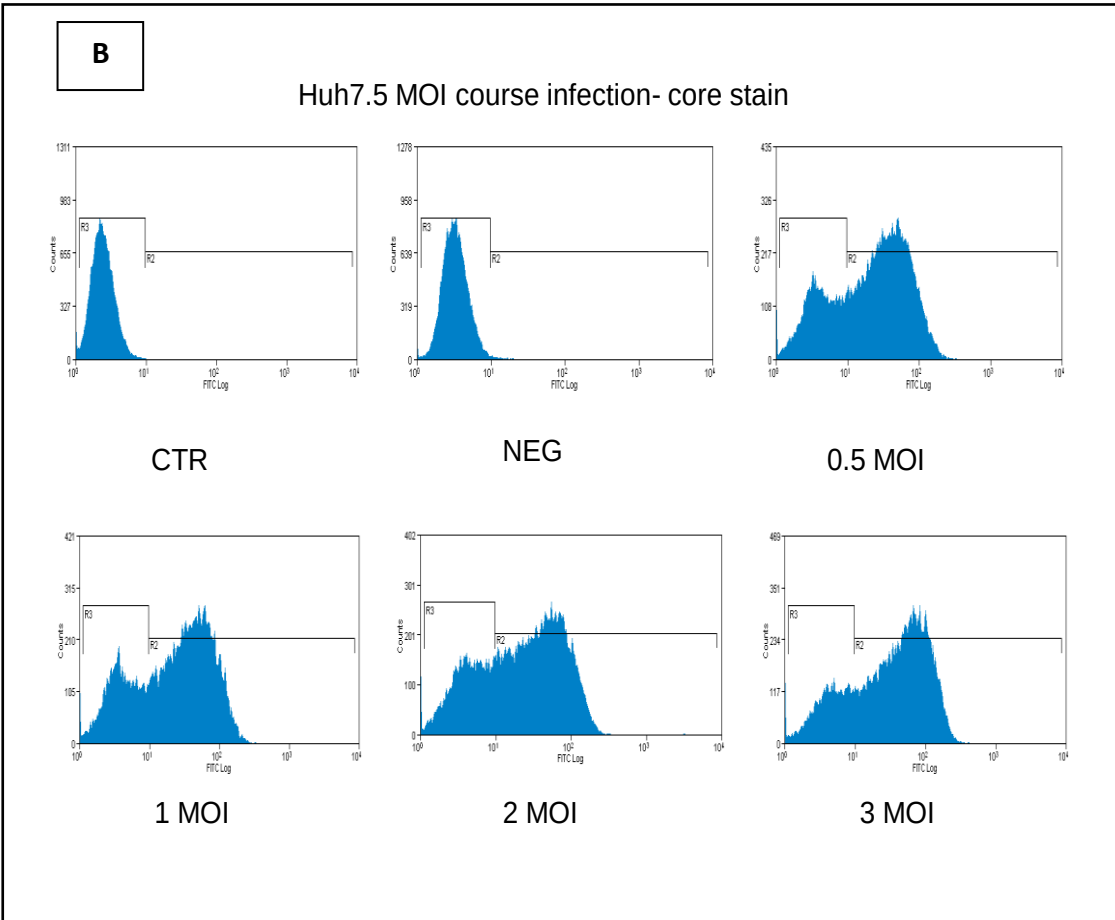


Figure 5.9: DNA fragmentation profiles of Huh7 cells. **(A)** untreated (CTR), mock infected (mock) or HCV infected cell at MOI of 0.5, 1, 2 and 3, representative graphs show DNA fragmentation by the appearance of a sub-G0 peak and changes in DNA contents G1 and S phase and G2/M cell phases. **(B)** Histograms showing the percentages of the cells from (A) at different phases at the cell cycle.

5.7. Apoptosis induction mediated by HCV JFH-1 infection correlates with intracellular HCV core levels:

To show if HCV infection is associated with increases in apoptotic cells, Huh7 and Huh7.5 cells were infected at MOIs of 0.5, 1, 2 and 3. 72 hours post-infection the cells were collected and analysed, as described before, using FACS analysis to quantitate the proportions of cells at different stages at the cell cycle including those that were apoptotic. The HCV viral load was also determined by measuring the intracellular core protein levels using specific anti-core monoclonal antibody. The results show that in Huh7.5 cells that as the number of infected cells increased there is an increase in the number of apoptotic cells (figure 5.10). The intracellular JFH-1 core reaches maximum levels when, more than 70% of the cells were positive for core and the number of apoptotic cells reached a maximum of around 20% (figure 5.10C). An increase in apoptosis coincided with peak levels of intracellular viral core, suggesting a causative link between the level of HCV replication and cell death and further indicates that the apoptosis correlates with the intracellular virus load.



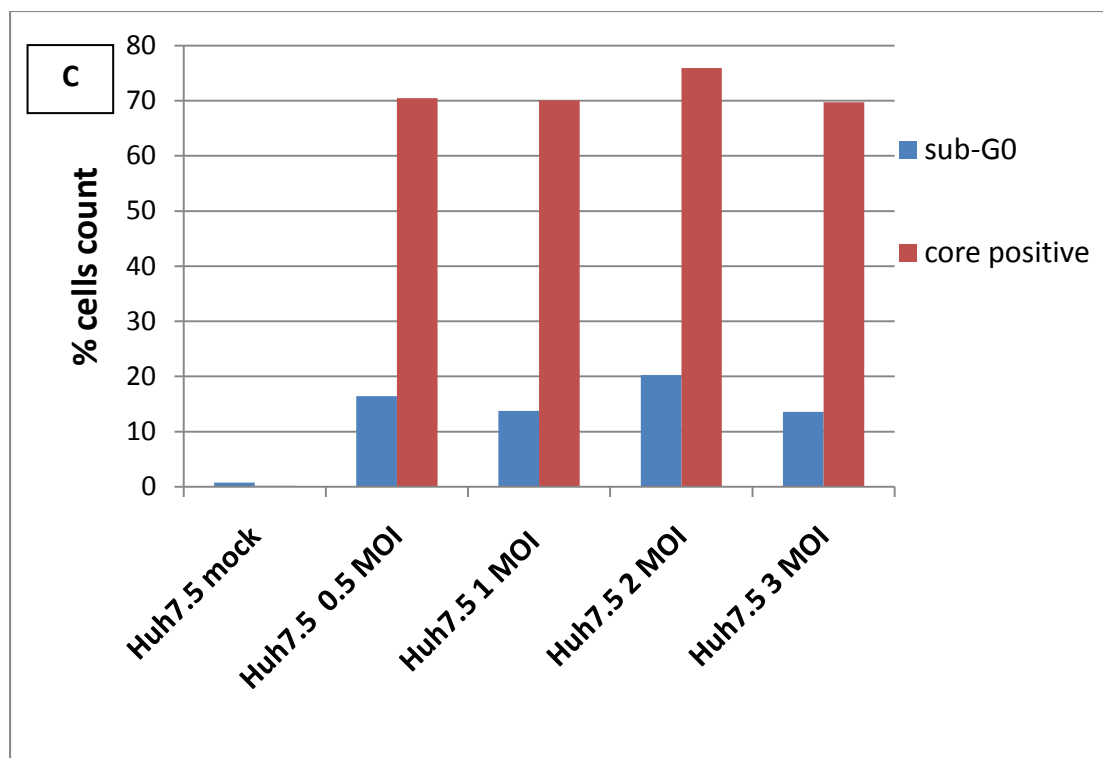
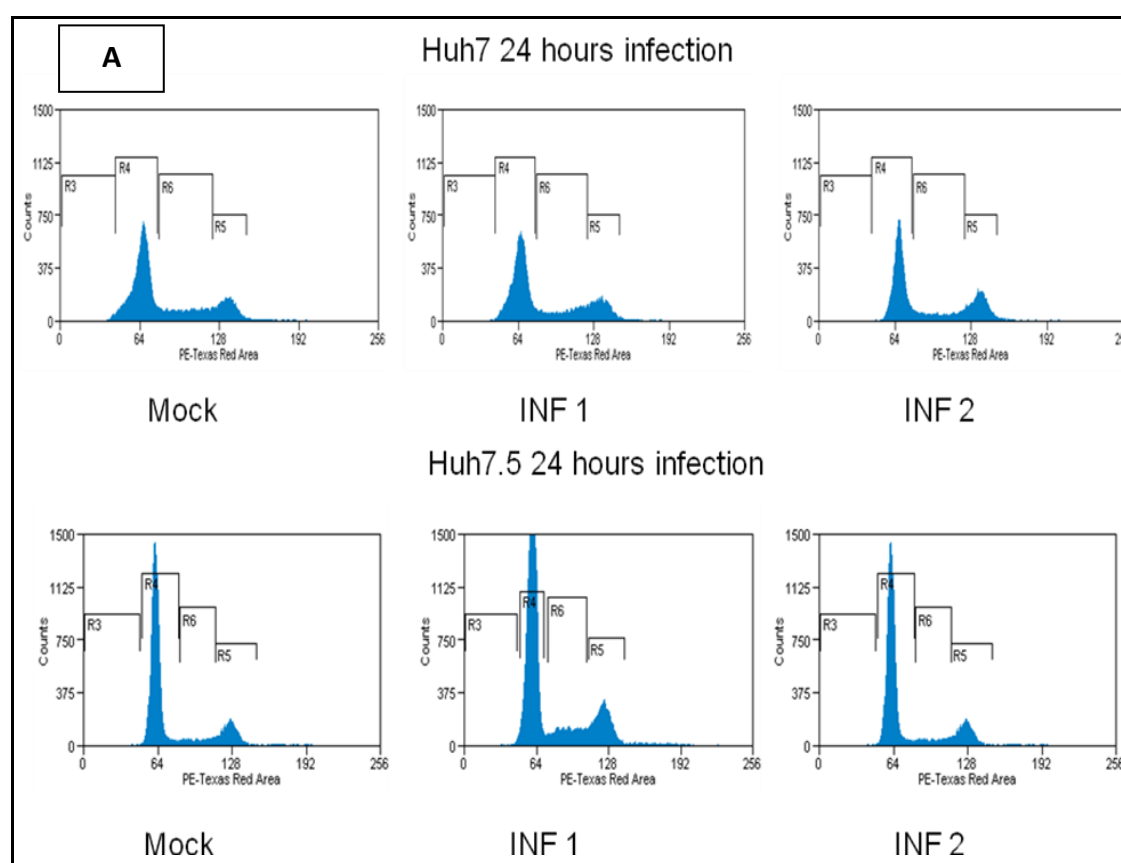
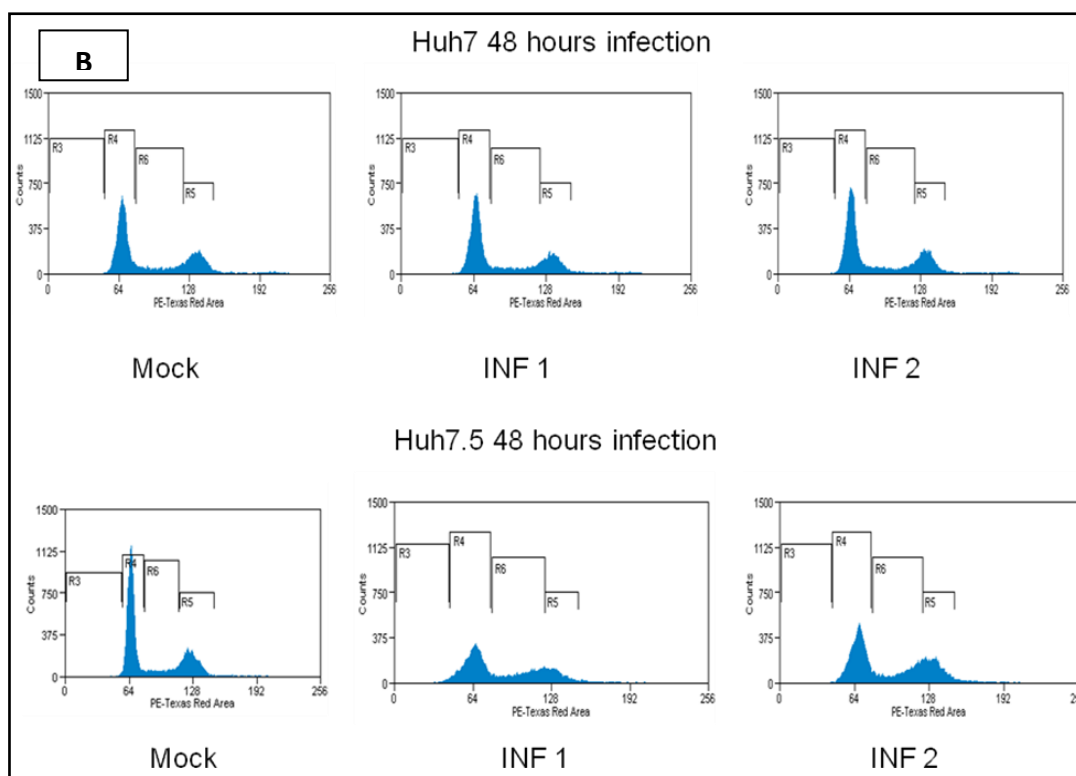
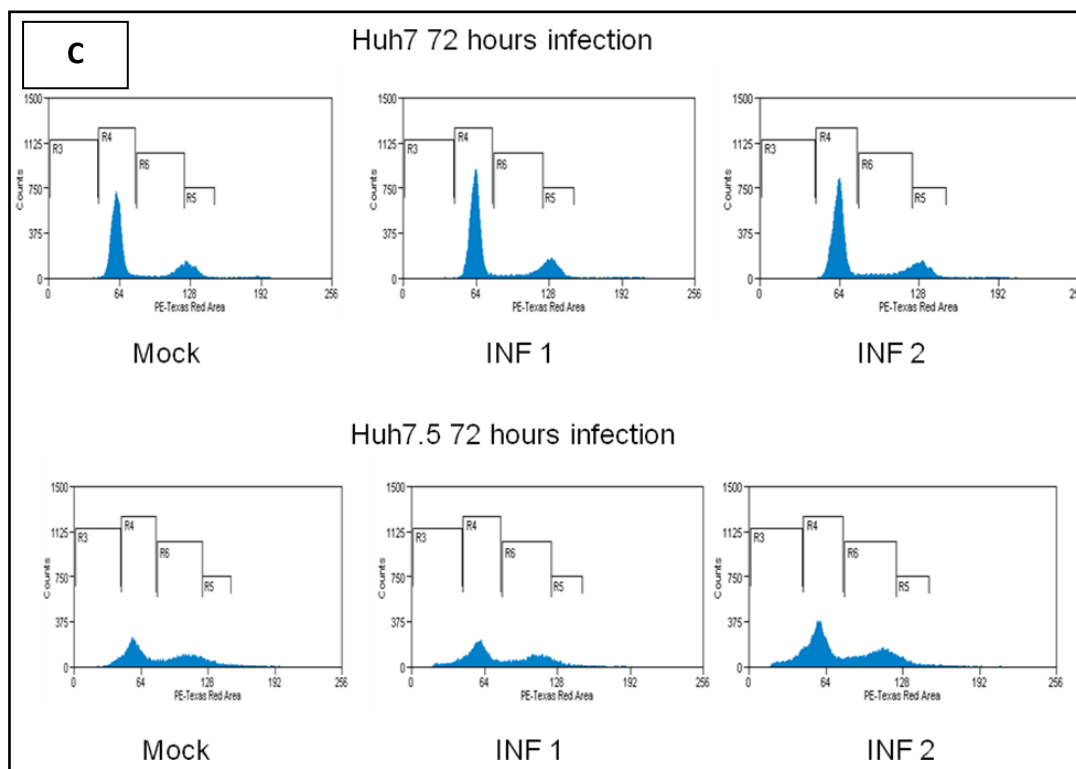


Figure 5.10: Apoptosis induction and cell death is correlated to HCV level. Huh7.5 cells were infected with JFH-1 at 0.5, 1, 2 and 3 MOI. The levels of apoptosis were quantitated by propidium iodide staining and flow cytometry analysis at 72 h post infection. Upper row demonstrate histograms of cell G1, S, G2/M and sub-G0 cells using PI staining in Huh7 cells, the lower row representative histograms of PI staining positive in Huh7.5 cells. (A) Graphs represent cells stained with PI indicating DNA fragmentation and apoptosis. (B): Cells were stained for virus core protein using specific monoclonal antibody. (C) Histogram represents percentage of apoptotic cells and HCV core protein positive cell.

Time course infections were also carried out for 24, 48 and 72 hours in Huh7 and Huh7.5 cells. Infections were carried out in duplicate for each infection and mock infected and untreated cells were used as controls. Cells were harvested as described before at different time and analysed for DNA staining and intracellular core protein staining. High levels of HCV core were detected from 48 hours post-infection and reached a maximum at 72 hours for both cell lines leading to apoptosis (figure 5.11 and 5.12). HCV JFH-1 infection cause a significant effect and perturbation of cell cycle phase of G1 and S phase which was observed at 48 hours post-infection (figure 5.12).





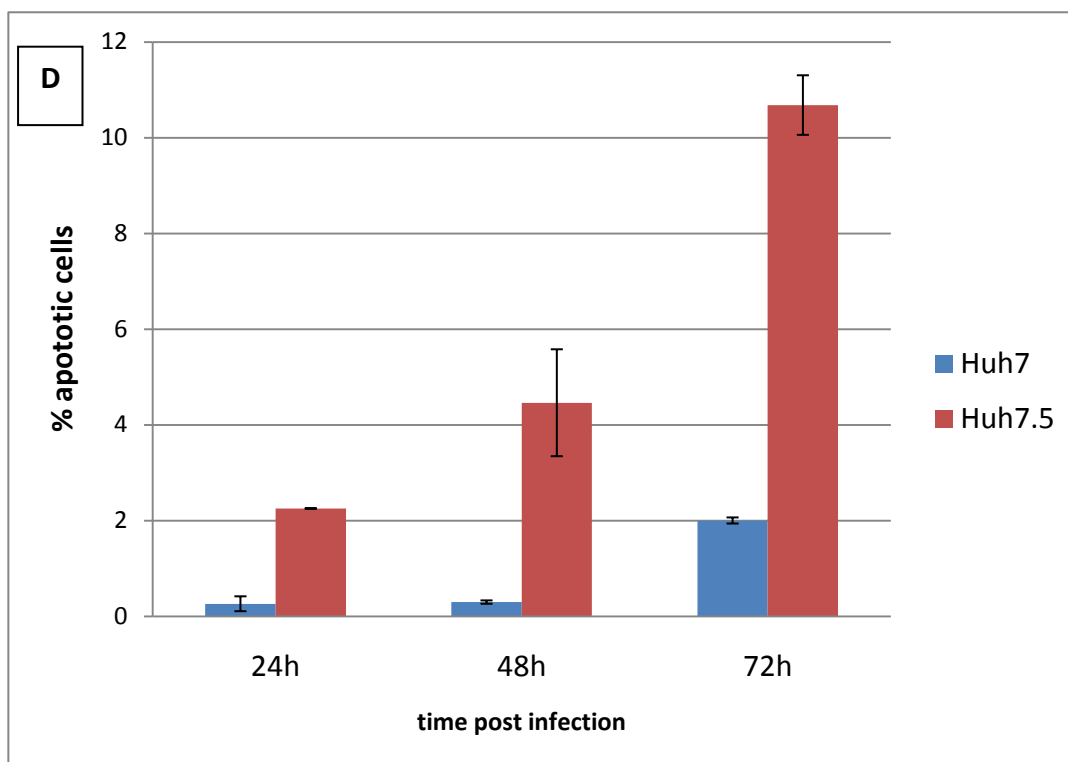
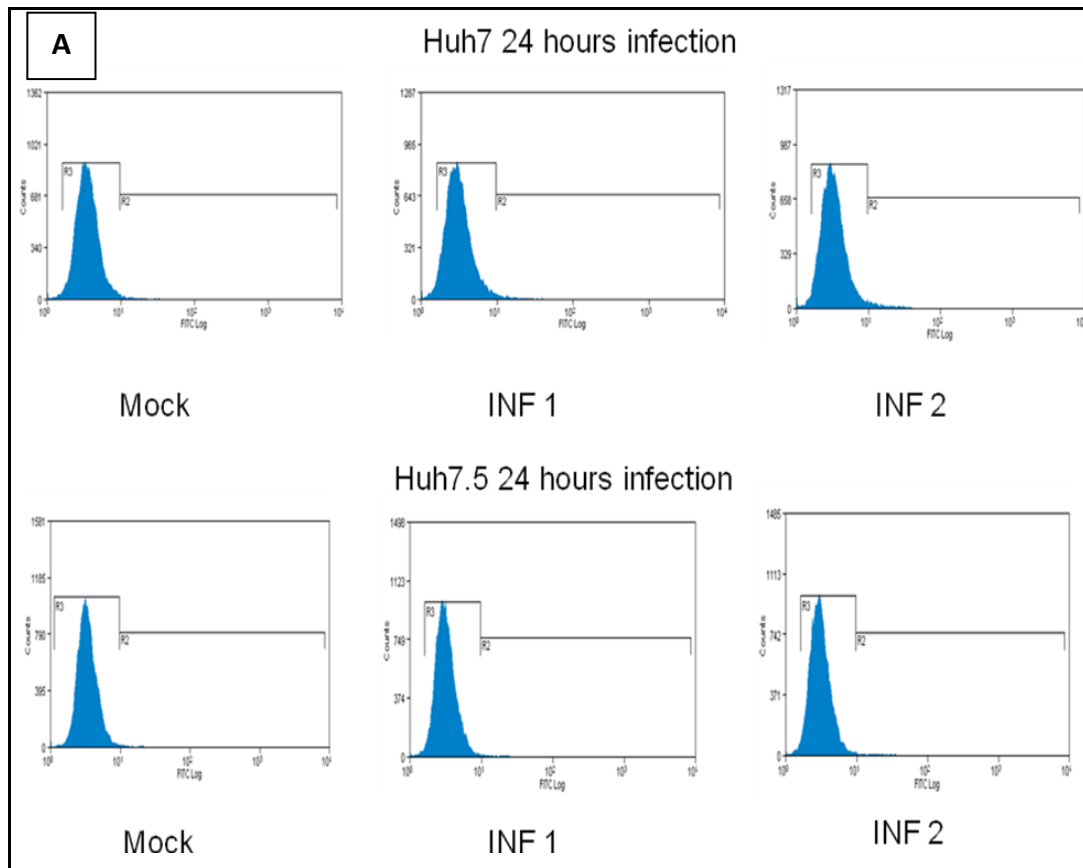
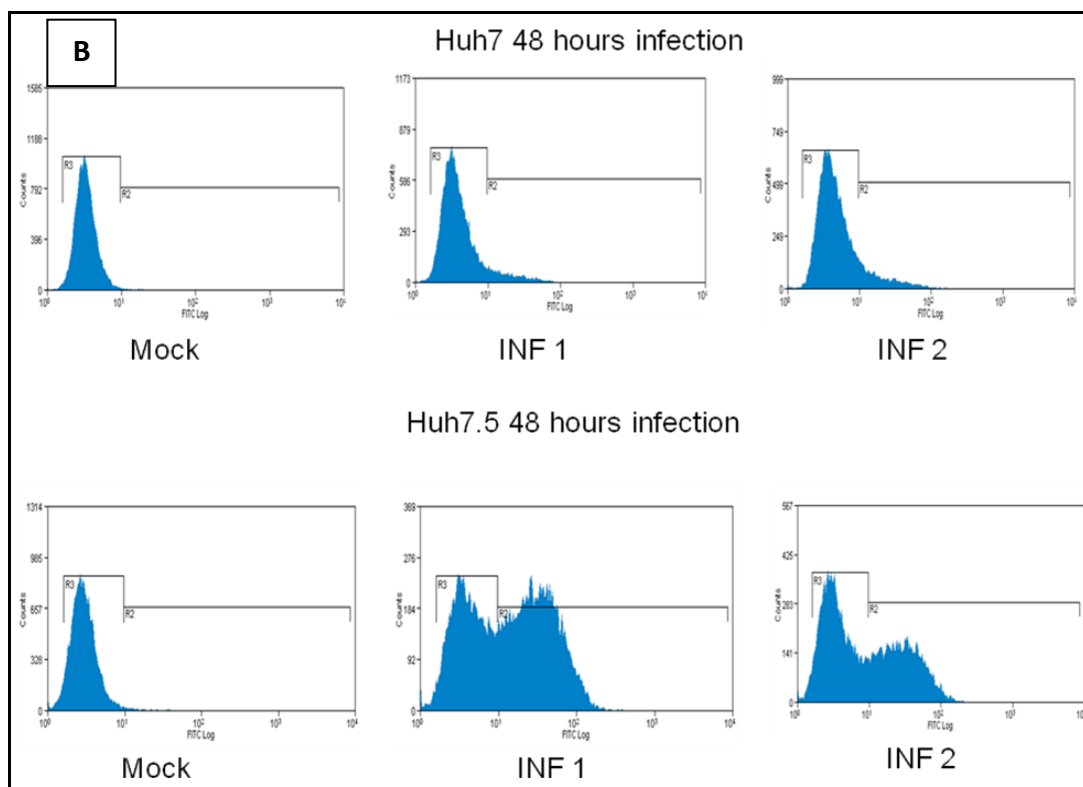


Figure 5.11: Effects of HCV JFH-1 infection on the induction of apoptosis. Huh7 and Huh7.5 cells were infected with JFH-1 (INF 1 and INF2) at MOI of 3 or mock infected. **(A)** DNA fragmentation profiles at 24h post infection, upper row Huh7 cells, lower row Huh7.5 cells. **(B)** DNA fragmentation profiles at 48h post infection, upper row Huh7 cells, lower row Huh7.5 cells. **(C)** DNA fragmentation profiles at 72h post infection, upper row Huh7 cells, lower row Huh7.5 cells. **(D)** Histogram representing percentage of apoptotic cells in Huh7 and Huh7.5. Infections were carried in duplicate (INF1 and INF2) and mean and $SD\pm$ of two infections were calculated.



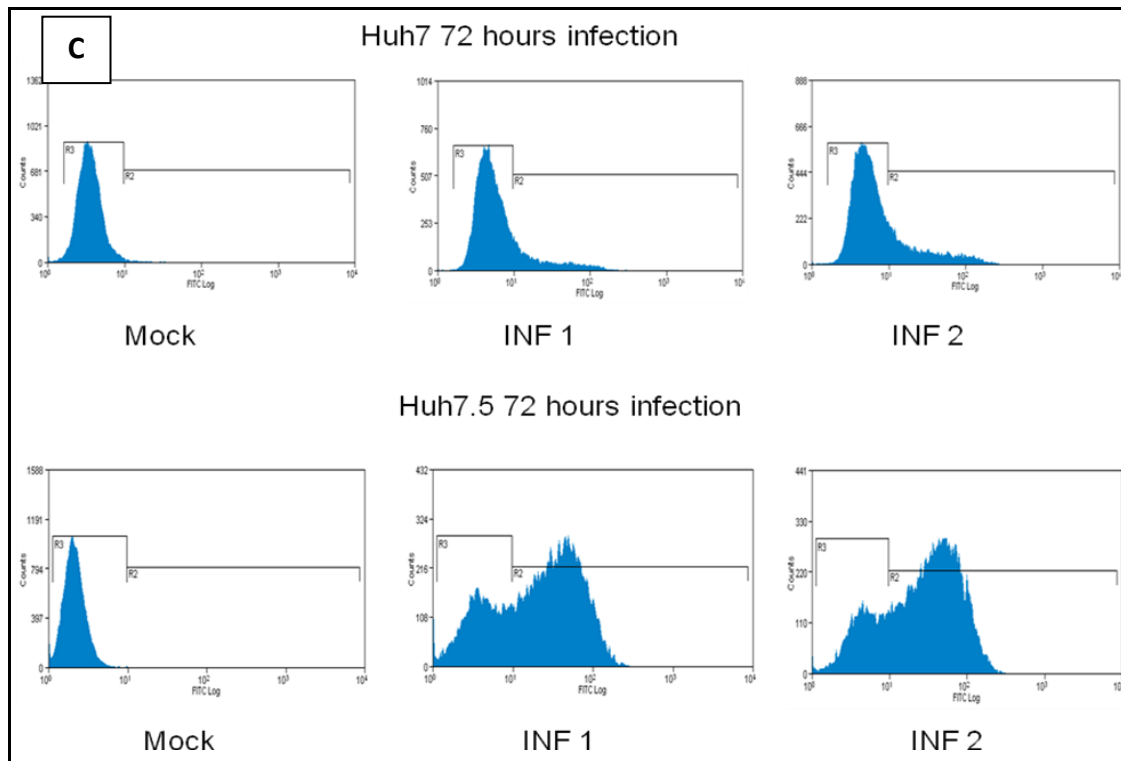
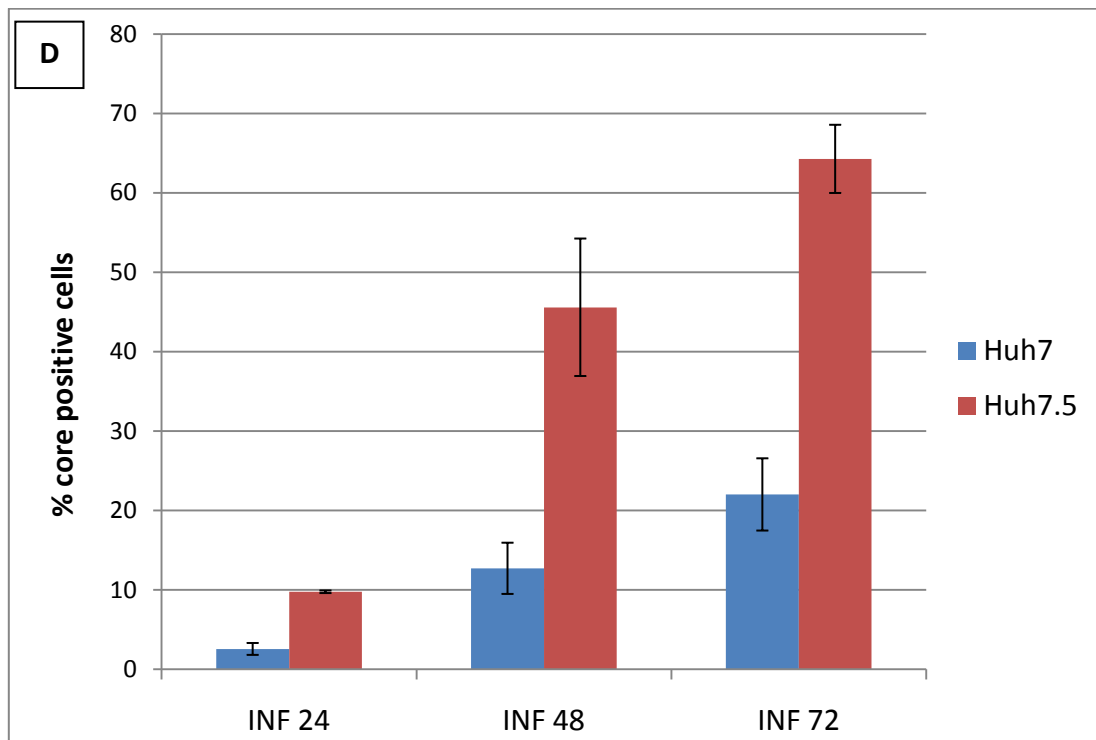


Figure 5.12: Quantitation of core expression level in HCV JFH-1 infected cells. Huh7 and Huh7.5 cells were infected with HCV JFH-1 at MOI of 3 (INF1 and INF2) or mock infected. Cells were stained for HCV core protein. (A) 24h post infection,

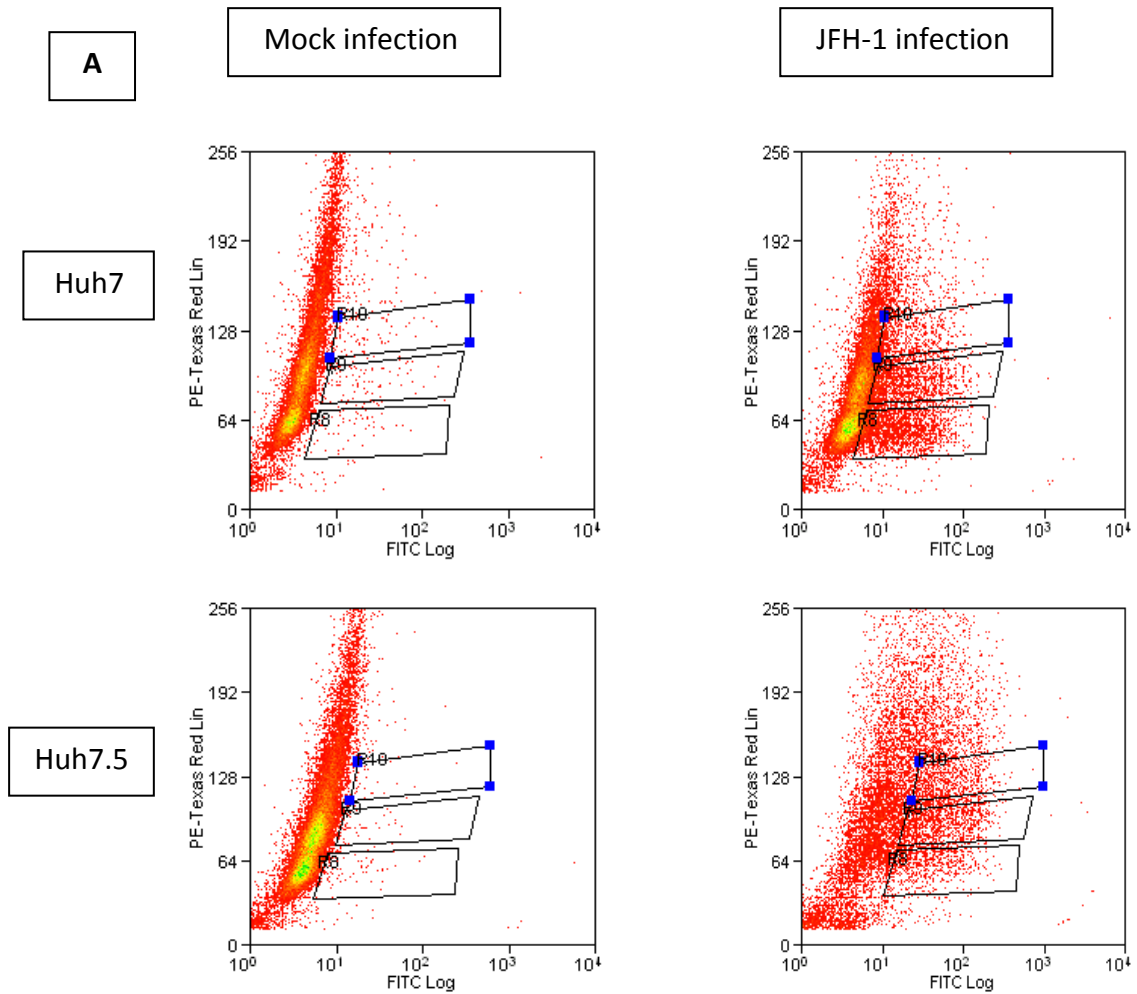
upper row Huh7 cells and the lower row Huh7.5 cells. **(B)** 48h post infection. **(C)** 72h post infection. **(D)** Histogram represents percentage (INF1 and INF2) of core positive cells in Huh7 and Huh7.5. Infections were done in duplicate and mean and SD $\pm$ of two infections were calculated.

Taken together the results indicate that cell cycle modulation and apoptosis induction correlate with intracellular viral replication significantly implicates the RIG-I pathway in the induction of apoptosis.

5.8. HCV arrest cell cycle in G phase promotes increase for viral replication:

The results of HCV JFH-1 infection indicate that there is an increase in the population of G2/M phase cells in virus-infected cells and a decrease in the number the G0/G1 or S phase cells. To investigate the effect of host G2/M arrest on HCV replication, the levels of virus replication and G2/M arrest was compared between Huh7 and Huh7.5 cells. HCV JFH-1 was used to infect Huh7 and Huh7.5 at equal MOI. Cells were harvested 72 h post infection, and infected cells (core positive) were assayed for their cell cycle profile using PI staining. These experiments showed that HCV can cause a G2/M arrest in Huh7.5 cells and that virus replicates at higher level at G2/M (figure 5.13 and appendix 8.4 for detailed analysis). These data suggested that HCV induces a G2/M phase arrest in infected cells to promote favorable conditions for viral replication.

To further investigate the induction of a G2/M phase arrest by HCV infection, cells should also be synchronized by thymidine for G1/S arrest and nocodazol for G2/M-phase synchronization.



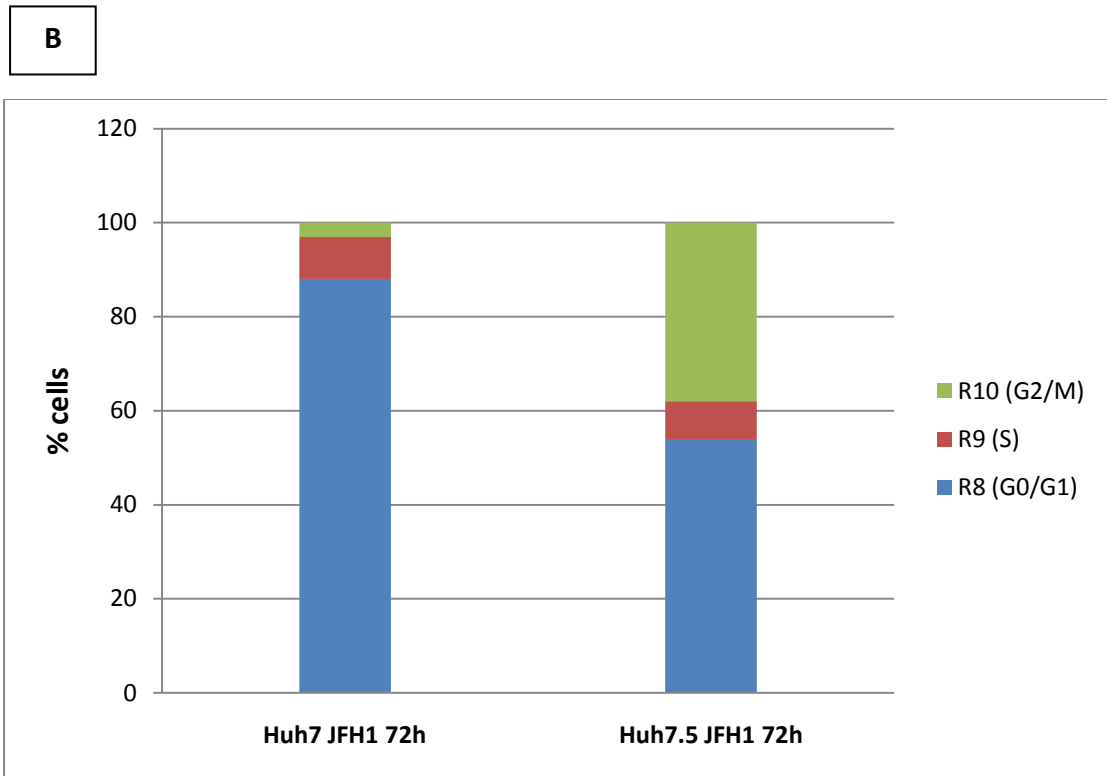
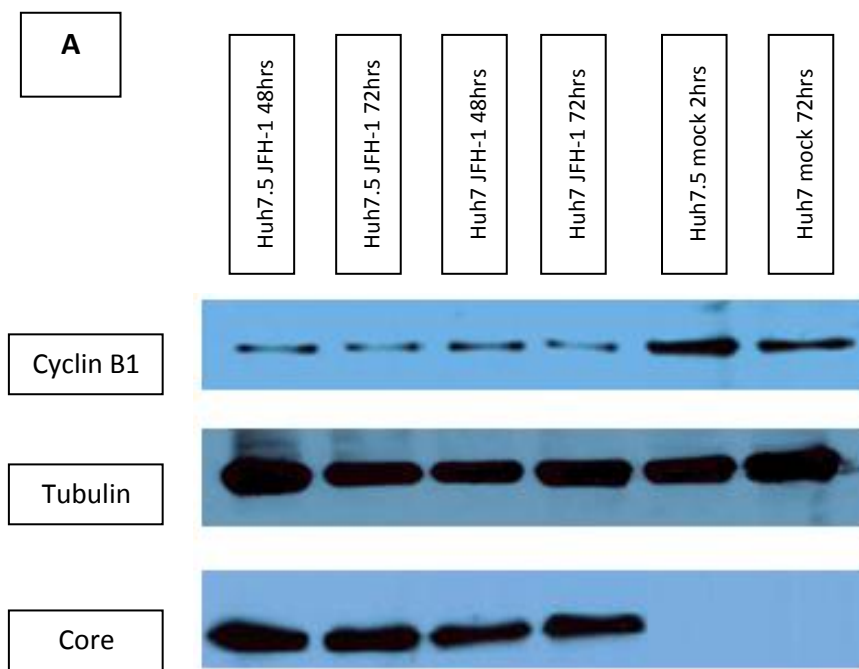


Figure 5.13: HCV infection mediated G2 cell cycle arrest: **(A)** Huh7 and Huh7.5 cells were either mock infected or infected with HCV JFH-1 (MOI = 3). Cells were harvested 72 h post infection and analyzed by double staining for DNA content and HCV core levels using anti-core antibody. **(B)** Percentage of core positive cells in Huh7 and Huh7.5 in different phases of the cell cycle (R8= G0/G1, R9= S and R10= G2/M). Infections were performed in duplicate and mean and $SD \pm$ of two infections were calculated.

5.9. HCV JFH-1 causes perturbations in cell cycle regulatory molecules and reduces cyclin B1 expression:

Cyclin B1 plays an important role in the control of the G2-M transition of the cell cycle and controls entry into mitosis. To determine whether HCV JFH-1 infection had an effect on the levels of cyclin B1 protein, Western blots were used to measure cyclin B1 protein level in HCV infected and mock infected Huh7 and Huh7.5 cells at 48 and 72 hours post infection. The level of tubulin was used to demonstrate equal loading of proteins on gels. This experiment was conducted in duplicate, and representative Western blot data are presented in (figure 5.14). To obtain a measure of JFH-1 infection, immunoblotting was also used to show the presence of core or NS3 protein, at 48 and 72 hours postinfection (figures 5.14 and 5.15). Western blot data indicated that there was no change in the level of tubulin between mock infected and infected cells at any time point. Between 48 and 72 h post infection the level of cyclin B1 was lower in infected Huh7 and Huh7.5 cells than in mock-infected cells (figure 5.14). Image J software were used to measure blot density. Analysis showed that there was fivefold less cyclin B1 in infected cells at 48 h postinfection compared to mock-infected cells. This indicates that HCV infection reduces the expression level of cyclin B1 protein required for mitosis, leading to cell cycle arrest and reduced cell proliferation.



B

Infected cells	Intensity reading
Huh7.5 mock	1553
Huh7 mock	254
Huh7.5 72h	259
Huh7.5 48h	502
Huh7 72h	290
Huh7 48h	460

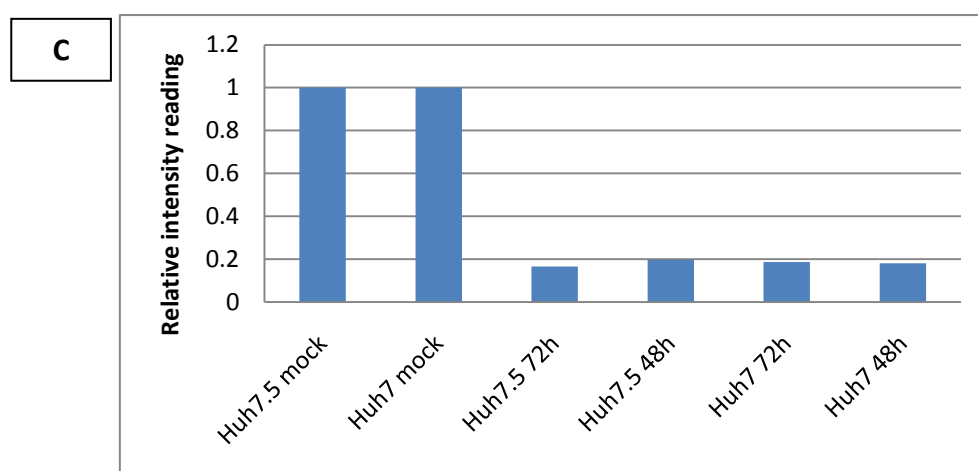


Figure 5.14: HCV infection decreases cyclin B1 expression levels in Huh7 and Huh7.5 cells. **(A)** Western blot analysis was performed at 48 and 72 hours post infection with JFH-1. Equal amounts of total cellular protein were separated by SDS-PAGE, transferred to nitrocellulose membranes, and blotted to detect cyclin B1, core and tubulin. **(B)** Representation of quantification of each band of cyclin b1 protein that appeared on the Western Blot was calculated using the Image-J program. Relative Intensity was calculated by dividing the absolute intensity of each sample band (on the table) of infected cells by the absolute intensity of mock. **(C)** Histogram presents relative intensity for each sample band.

The expression of GADD45- β in JFH-1 infected Huh7 and Huh7.5 cells was also examined. Western blot analysis revealed that GADD45- β was induced between 48 and 72 h post infection in Huh7.5 cells. In contrast in Huh7 GADD45- β accumulated lower levels at 48 hours but increased reaching high levels at 72 and 96 hours post infection (figure 5.15). This finding is consistent with previous results which showed that HCV infected Huh7.5 cells showed higher levels of apoptosis compared to Huh7 cells. In general, GADD45- β protein was induced at levels consistent with alterations observed for the expression mRNA by microarray and qRT-PCR analysis. These changes are also consistent with the cell cycle arrest finding of HCV infected Huh7.5 cell indicating the possible roles of cyclin B1 and GADD45- β in the induction of apoptosis and cell death by during HCV infection.

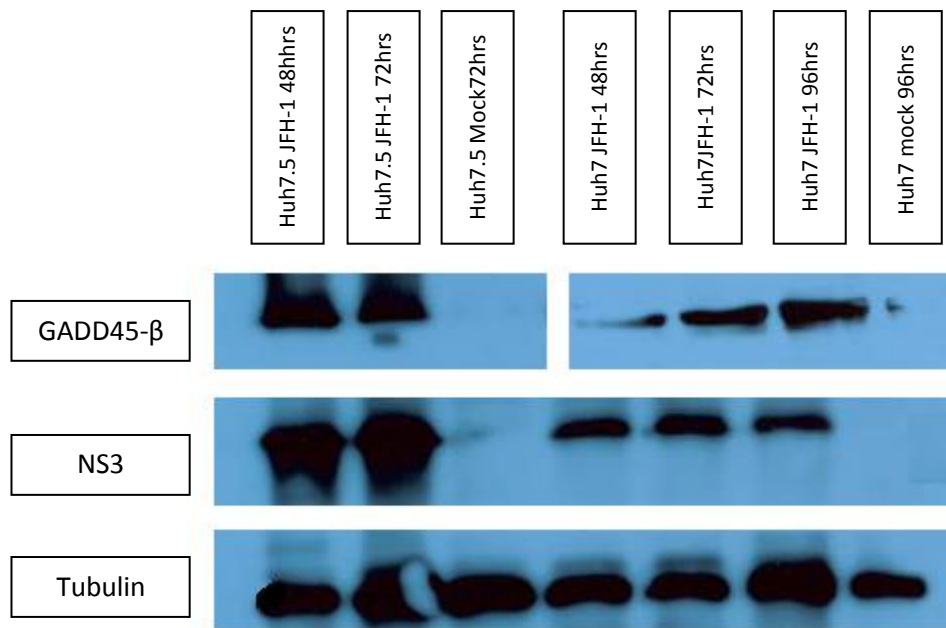


Figure 5.15: HCV infection increases GADD45-β expression levels in Huh7 and Huh7.5 cells. Western blot analysis of GADD45β, NS3 and tubulin performed at 48, 72 and 96 hours post infection with HCV JFH-1. Equal amounts of total cellular protein were separated by SDS-PAGE, transferred to nitrocellulose membranes, and blotted to detect GADD45β, NS3 and tubulin.

5.10. Colocalization of cyclin B1 with HCV JFH-1 Huh7.5 infected cells:

To further investigate how HCV infection influences cyclin B1. Immunofluorescence staining in HCV infected cells was carried out. Cyclin B1 (red color) was mainly detected in the nucleus with very low levels in the cytoplasm of the mock infected cells (figure 5.16, upper line). By contrast, the Cyclin B1 was mainly in the nucleo-cytoplasmic distribution of JFH-1 infected cells at 48h post infection, with only small quantities observed in the nucleus. These results indicate that HCV JFH-1 infection leads to a redistribution of the nuclear localized cyclin B1 to the cytosol thus initiating cell division and subsequently over expression GADD45-β leading to DNA fragmentation and cell death.

To examine its possible direct interactions of cyclin B1 with NS5A protein, HCV infected cells were double stained with anti-cyclin B1 and anti-HCV NS5A. Staining of NS5A proteins (green) was predominantly detected in the cytoplasm throughout the infection period. The merged images did not show colocalization of cyclin B1 and NS5A in the cytoplasm during infection (figure 5.16). However, the redistribution of the nuclear localized portion of cyclin B1 to the cytosol in JFH-1 infected cells did confirm that HCV infection alters the distribution of components involved in the regulation of the cell cycle. Further work should be carried out to reveal the molecular mechanisms involved in the alteration of cyclin B1 expression levels and location in infected cells.

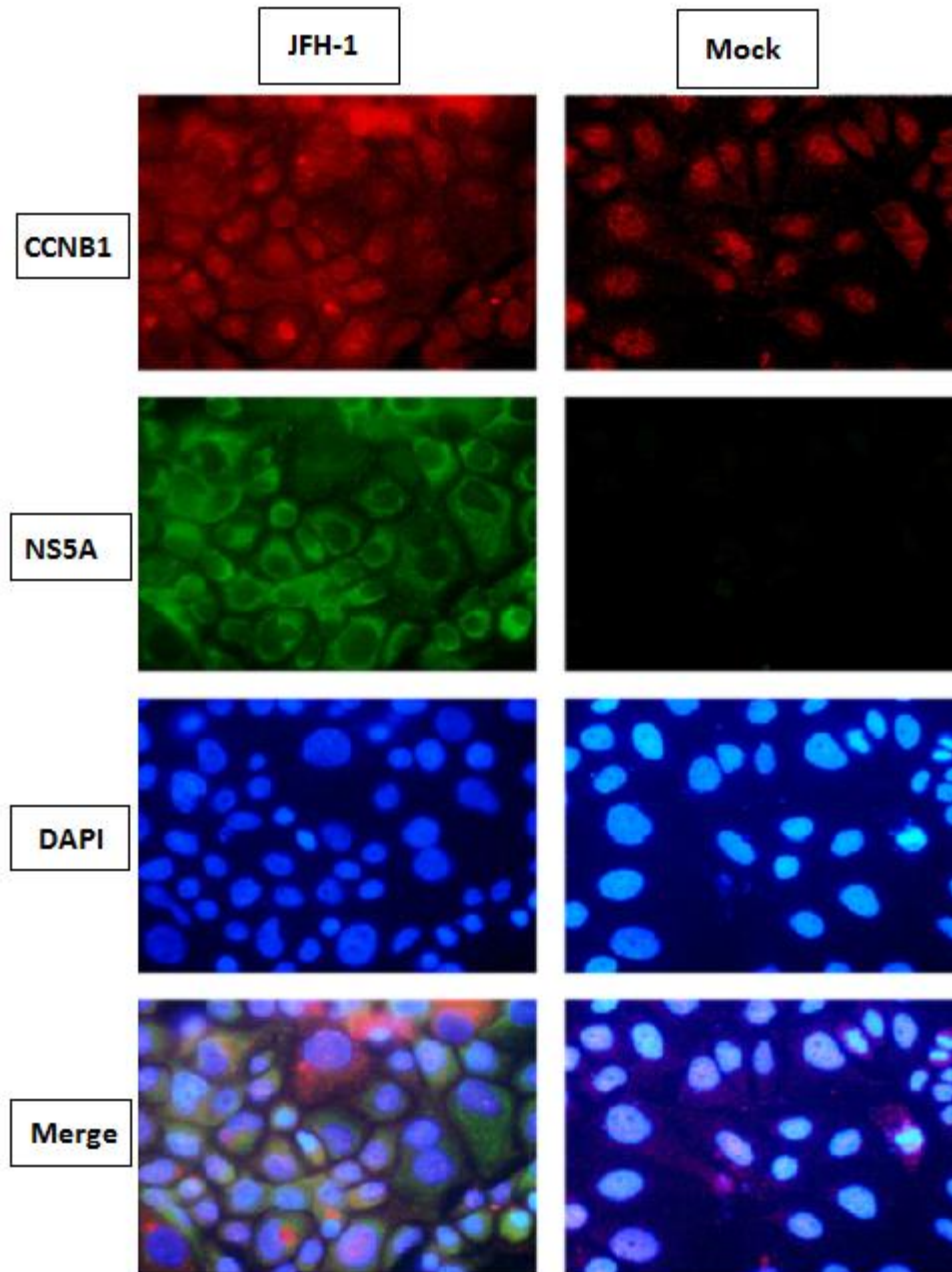


Figure 5.16: Colocalization of cyclin B1 and NS5A proteins in the JFH-1 infected cells. Double staining of cyclin B1 (CCNB1) (red) and NS5A (green) proteins in uninfected (Mock) or infected Huh7.5 cells. Cells were stained as for 72 hours post infection. Colocalization of both NS5A and cyclin B1 was evidenced by merged images (yellow).

6.0. Chapter six: Discussion:

The innate immune responses are the first line of defence against an invading pathogen. Interferon type I (IFN- α and IFN- β) responses are key to innate antiviral immune defences. Early immune or innate responses depend on the detection of viruses by TLRs and intracellular patterns recognition receptors sensing viral components. The intracellular pattern recognition receptors (PRRs), RIG-I and MDA5 are the main sensors for the induction of IFN- β mediated by dsRNA in Huh7 cells during HCV infection. This study investigated the effects of HCV on the expression of key proteins e.g. microbial PRRs: TLRs (3, 7 and 8), RIG-I and MDA5 and other signaling proteins e.g. TRIF, MAVS and MyD88, which are involved in the interferon response. The expressions were measured in the presence and absence of HCV NS proteins in RNA replicons and HCV JFH-1 infected cells and treatment with artificial dsRNA in Huh7 and subclone Huh7.5 cells. The measuring of the levels of mRNAs encodes these proteins using real time qRT-PCR.

To accurately quantify the mRNAs encoding these proteins, control amplicons which contain the target DNA sequence for the different genes were constructed to produce standard curves for the amplification of target DNA sequences. For each gene examined, PCR amplified DNA fragments of approximately 150 base pairs were synthesized and ligated separately into plasmid pCR2.1. In addition a target DNA sequence for β -actin was also used to compare any changes in the expression of the interferon pathway associated genes to those of a “housekeeping” gene.

Effects of HCV replicon on the expression of TLRs and RIG/MDA5 pathways:

First, comparisons of the basal expression levels in hepatocyte cell lines were performed between HCV RNA replicons in Huh7 cells and the parental Huh7 cells. In this project, it was found that Huh7 cells that contain HCV RNA replicons which showed upregulation of TLR3, TLR7, RIG-I, MDA5 and the signaling mediators TRIF, MAVS and MyD88 compared to normal Huh7 cells. These may indicate that nucleic acids sensors TLRs, MDA5 and RIG-I are functionally active in Huh7 and Huh Rep4 cells and work in concert to maintain the induction of the antiviral genes, while IFN functions to amplify and/or expand the response in an attempt to control viral replication.

TLR3 recognizes dsRNA that is generated during viral infection and plays an important role in host defenses against viruses. In this study, quantification of TLR3 expression levels was demonstrated to be very low in Huh7 and Rep4 cells. Treatment of Huh7 cells by adding poly (I:C) (i.e. non specific dsRNA), showed little change in TLR3 expression. Similarly, the TLR3 signaling mediator TRIF was also found to have very low levels of expression in Huh7 cells. This agrees with the results from other groups which have reported that Huh7 cells possess a weak or defective TRIF signaling pathway (270).

MDA5, RIG-I and MAVS, mRNAs basal expression were found to be expressed at high levels in Huh7 cells and upregulated up to four-fold in Rep4 cells (figure 3.3). This is consistent with the detection of dsRNA in Huh7 cells being mainly mediated by the intracellular receptors RIG-I and MDA5 and that provides a useful model to elucidate the individual importance of RIG-I and MDA5 in HCV infection. This was supported by showing that in Huh7 cells, expression of both

RIG-I and MDA5 respond to poly (I:C) (figure 3.4, 3.10) and demonstrated that the parental Huh7 cells could induce IFN expression in response poly (I:C). In contrast to Huh7 cells, the expression of TLR3, TRIF, RIG-I, MDA5 and MAVS mRNA transcription in response to transfection of poly (I:C) , showed lower levels in the Huh7 Rep4 cells, indicating strong inhibition of IFN expression in the presence of the HCV replicon (figures 3.4 and 3.5). In this study, expression of MDA5 showed more stimulation with poly (I:C) (50 fold) compared to RIG-I (12 fold), in Huh7 cells. This is consistent with previous findings on post-transcriptional activation of RIG-I and MDA5, in that RIG-I is activated mainly in response to RNA bearing a triphosphate at the 5' end and a polyuridine motif in the 3' untranslated region (UTR) of HCV, while MDA5 is activated primarily by poly (I:C) and picornaviruses.

TLR7 and TLR8 are receptors for ssRNA associated with viruses that enter cells through endocytosis and they induce interferon expression via MyD88. In humans, the expression of TLR7 is mainly confined to plasmacytoid dendritic cells and B lymphocytes. However, TLR7 mRNA has been detected in Huh7 cells, and HCV replicon cells showed a two fold increase (figure 3.3), indicating that expression of TLR7 is functionally active in hepatocyte cells. Several reports have indicated that TLR7 ligands such as imiquimod and SM360320 can inhibit HCV replication in hepatocytes via a type I IFN-independent mechanism in addition to its IFN-mediated activity (287). This raised the possibility that TLR7 stimulants may be able to control chronic HCV infection. TLR7 signaling is mediated primarily by MyD88, which triggers the activation of transcription factors, such as NF- κ B, through the activation of IRF7 which leads to the production of type I interferon (IFN) mRNA. HCV NS5A interacts with the death

domain of MyD88 through the IFN sensitivity-determining region (ISDR) spanning amino acid residues 240 to 280 and can impair cytokine production in macrophage cells (288). To activate MyD88, LPS was used as it is a strong stimulator of TLR4 which acts through MyD88 leading to the production of IFN- α . In this study, it was clearly demonstrated that the expression of MyD88 mRNA was reduced in HCV Rep4 cells in response to stimulation with LPS (figure 3.6).

Effects of HCV JFH-1 infection on the expression of TLRs and RIG/MDA5 pathways:

Comparisons between the basal expression levels in Huh7 cells and cells that have been infected with HCV, by qRT-PCR, showed low levels induction of IFN- β and the dsRNA recognition receptors, RIG-I and MDA5, and signalling mediators, MAVS, MyD88 and IRF3. In contrast, poly (I:C) strongly induces dsRNA recognition components and induces IFN- β mRNA transcription in Huh7 cells. These comparisons of infection and transfection were performed, beginning early after infection, before the HCV NS3/4A protease could cleave MAVS and abrogate this pathway.

The antiviral transcription activity in Huh7.5 cells was also examined as they lack an active RIG-I pathway. Huh7.5 cells transfected with poly (I:C) showed high levels of IFN- β , MDA5 and MAVS mRNAs. TLR3 mRNA transcription was induced to a much lesser extent indicating that in the absence of RIG-I the stimulation of IFN- β is likely to be activated through the MDA5 pathway (figure 3.12). By contrast, HCV JFH-1 infection in Huh7.5 showed a weak signaling induction (figure 3.13) similar to that shown in Huh7 cells. Together, the results of HCV JFH-1 infection of Huh7 and Huh7.5 cells showed that HCV infection leads

to the inhibition of the IFN induction pathway. Similar findings were observed in a recently published study which used Huh7.5 and TRIM25 permissive Huh7.25.CD81 cells (289). These results indicate the importance of the RIG-I/TRIM25 pathway in the control of HCV expression since both Huh7.25.CD81 and Huh7.5 cells, have defects in this pathway, at the level of RIG-I for Huh7.5 cells and of TRIM25 for Huh7.25.CD81 cells (289).

Loo et al, suggested that HCV could induce the IFN- β pathway in infected cells by the production of dsRNA replication intermediates, which would then be suppressed by NS3 protein when it reached sufficient expression levels (273). However, in this study, the RIG-I pathway in Huh7 cells, fails to trigger IFN- β induction as early as 6 hours after HCV infection and at 48 hours infection (figure 3.10). These results indicate that HCV may control IFN induction at the transcriptional level during infection in addition to the NS3/4A-mediated MAVS proteolysis cleavage.

To further investigate this, the effects of double-stranded RNA on altering gene expression levels in hepatoma cells was carried out using poly (I:C). JFH-1 HCV infected Huh7 cells and mock infected Huh7 cell were stimulated with poly (I:C). Huh7 cells infected with HCV JFH-1 showed a significant decrease in the induction of IFN- β and other components after treatment with poly (I:C) leading to reduced gene induction, confirming that HCV infection reduced the ability of Huh7 cells to activate the dsRNA and ssRNA recognition pathway. These results showed that HCV efficiently suppresses IFN- β pathway induction which increases the ability of the virus to evade the innate immune system and help to establish a chronic infection.

Ablation of Interferon Pathway Components by RNA interference;

To examine the roles of innate immune proteins of the interferon response pathway during HCV expression, siRNAs corresponding to each of the target genes RIG-I, MDA5, TRIF, MAVS and MyD88 were used to knock out individual or combinations of genes of the interferon pathway. The knockdown of TRIF followed by poly (I:C) stimulation showed a direct effect on IFN- β transcription, whereas, the knockdown of MAVS after poly (I:C) stimulation showed a significant inhibition of IFN- β . Additionally, double knockdown of TRIF and MAVS showed further clear reduction in IFN- β levels indicating a minor effect of TRIF on IFN- β induction. Some reports have claimed that NS3/4A did not respond to poly (I:C) in Huh7 cells because it was unable to cleave TRIF. TLR3's involvement in host defense against HCV remains controversial. TRIF was shown to be cleaved by purified single chain NS3/4A protease in vitro, whereas TRIF cleavage products in HEK 293 cells expressing NS3/4A have not been found. Dansako et al. demonstrated that the TLR3/TRIF signaling pathway was not affected in the non-neoplastic human hepatocyte PH5CH8 cells expressing NS3/4A (290). However, this could be due to low levels of expression of TLR3 and TRIF proteins in Huh7 and Rep4 cells and their relative unimportance in these cells.

Experiments also showed that even after ablation of MAVS and RIG-I in TLR3 deficient Huh7 cells, the levels of IFN- β were still detectable. It is therefore possible that additional proteins could be important for host responses to HCV infection. Loo et al demonstrated that infection by DEN2 and Reovirus triggered IRF3 in wt, RIG-/- and MDA5-/- cells (273). Taken together, these results could support the hypothesis that as yet undefined cytoplasmic components are also involved in innate immune signaling in HCV infected Huh7.5 cells.

The role of MDA5 in HCV infection:

Several recent reports have clarified the molecular pathways recognized by RIG-I and MDA5 during virus infection. RIG-I has been shown to trigger innate immune defenses during infection by paramyxoviruses, orthomyxoviruses, and rhabdoviruses, (vesicular stomatitis virus) (238, 250). In measles virus (MV) infection, RIG-I and MDA5 are both involved in virus recognition. The V protein plays an important role in MV growth by inhibiting the MDA5-mediated induction of the host IFN responses (291). Also herpes simplex virus-1 and adenovirus which are both DNA viruses, were shown to replicate to much higher titers in RIG-I mutant human hepatoma cells Huh7.5.1 than in the parental Huh7 cells (292). In contrast, MDA5 triggers innate immune defenses during picornavirus (250, 254) and rhinovirus (RV) infection (293). Other recent reports have shown that members of the *Flaviviridae* exhibit differential triggering of the innate immune responses. Japanese encephalitis virus and hepatitis C virus initiated a primarily RIG-I dependent response (250, 294), whereas West Nile virus and dengue virus triggered RIG-I- and MDA5-dependent signaling (295), suggesting that flaviviruses may have different features that can be discerned separately by RIG-I and MDA5 (295).

However, the importance of RIG-I in HCV replication was shown in the JFH-1 replication system in Huh7.5 cells which lack RIG-I. However, despite experimental evidence, the roles played by other individual pattern recognition receptors in the restriction of HCV infection are still not clearly understood. This study demonstrated that MDA5 has a role in triggering HCV elicited induction of IFN- β and virus replication, and that MDA5 silencing by siRNA increases the release of progeny virus. Moreover, this study indicates further roles of different

signaling mediators MAVS, TRIF and MyD88 in HCV replication. Taken together these results indicate that both RIG-I and MDA5 are involved in induction of IFN response in HCV infected cells and that several innate immune response components play a vital role in the host response to virus replication and consequently disease outcome.

Identification of novel innate response proteins:

To identify novel host genes involved in the innate host responses to HCV infection. Gene profiling by microarray analysis was used to identify mRNAs that are up regulated or down regulated during HCV infection. mRNAs were extracted from Huh7 cells infected with HCV JFH-1. Genes identified by microarray analysis were characterized from sequence databases and by RT-PCR. According to the microarray results, HCV infection showed a remarkable modulation of the pathways involved in the innate immune response. Quantitative PCR analysis of a number of the IFN-regulated genes identified from the microarray analysis demonstrated a good correlation with the gene expression data from the microarrays, although the ratios calculated from RT-PCR generally exceeded those obtained using microarrays.

Analysis of the expression of genes encoding proteins involved in interferon (IFN) signaling pathways and genes that regulated by IFN has shown that chronically infected HCV individuals show evidence of an IFN response. One of the main characteristics of HCV is its tendency to persist despite the massive type I ISG expression response that it induces in the liver (6, 170, 296). Gene expression analysis of liver tissue from HCV-infected mice also demonstrated an up-regulation of many known IFN-stimulated genes (ISGs). These included key genes associated with the IFN signalling pathway, such as IFNAR1, STAT1,

IRF1, IRF9, OAS3, and Mx1, (table 5.1). qRT-PCR confirmed these findings in HCV-infected cells indicating that HCV can induce ISG mRNA transcription, indicating that HCV does not completely block IFN signaling. These results confirm previous observations in vitro showing comparable induction of ISG mRNAs (297, 298), and are also consistent with the strong ISG mRNA induction in the liver during HCV infection (6).

No changes in gene expression were observed in infected cells of the antiviral response to HCV JFH-1 which occurs through the activation of the RIG-I/MDA5 pathway, indicating that viral attachment/entry does not significantly impact host gene expression. Similar results were obtained from HCV infection in Huh7.5 cells (299). In comparison to functional gene analysis conducted with other viruses, Influenza A infection showed, that in contrast, both RIG-I and MDA5 changes are observed during acute virus infection. In addition to both cytosolic recognition receptors, RIG-I and MDA5, TLR3, MAVS, MyD88 and TRIF were also upregulated (295, 300). However, in response to the highly lethal 1918 virus, RIG-I and MDA5 were not induced (300). Dengue virus also showed upregulation of TLR3, TLR8, RIG-I and MDA5 mRNA along with an increase in IFN- β expression (301). Moreover, intra-hepatic gene expression during HCV infection of chimpanzees showed that RIG-I was strongly induced. This discrepancy may be a reflection of the inherent differences in the host response to acute versus chronic viruses. Chronic viruses such as HCV may have evolved to cause minimal impact upon entry into cells in an effort to delay cellular changes that may trigger an immune response, as evidenced by the minimal effect on innate immune host gene expression even at 48 hrs post-HCV infection and by reduction of gene expression in response to poly (I:C) induction.

On the other hand, functional genomic analyses show that infection of influenza A and Dengue viruses typically trigger the rapid expression of immune system-related genes responsible for mediating transient immune responses that effectively resolve infection (300, 302). Importantly, many of these disease-related genes induced by pandemic influenza virus infection are present in the RIG-I-responsive genes (295). From all of this it might be concluded that the processes that trigger and control RIG-I/MDA5 signalling during virus infection and the products of RIG-I/MDA5 responsive genes could be important determinants of viral pathogenesis and disease outcome during HCV infection.

Alternative IFN inhibition by SOCS3

The results from the microarray analysis of host gene expression during HCV infection also showed an up-regulation of genes associated with the JAK/STAT pathway, including SOCS1, SOCS2, SOCS3, PIAS2, which play a role in regulating the Jak-STAT pathway. Many of the up-regulated ISGs in HCV-infected mice are also induced during the acute phase of HCV infection of chimps as well as chronically infected human patients. The SOCS family proteins are of particular interest as SOCS3 is expressed at high levels in patients who are nonresponsive to IFN treatment (261, 262, 303, 304). HCV genotype 1b infected patients exhibit higher level of expression of SOCS3 than genotype 2a-infected patients (304). It has been hypothesized that SOCS3 overexpression may explain why HCV genotype 1b-infected patients do not respond to IFN and suggested that reducing SOCS3 gene expression may be an approach to treating HCV infection (304). In this study, HCV JFH-1 infected cells were used to investigate the relationship between SOCS3 and HCV replication. HCV infection increases

SOCS3 mRNA levels and SOCS3 expression inhibits phospho-STAT1 expression, which impairs the IFN defense pathway. In addition, it has been previously reported that HCV core protein induces SOCS3 expression in cell lines causing impaired IFN and specifically STAT1 signaling (305). By contrast, recent work has shown that core did not alter SOCS3 levels (306). But from genotype 1 replicon cells and JFH-1 infection did cause overexpression of SOCS3 and inhibited IFN induced STAT1 phosphorylation (306). In agreement with this, the current study showed that JFH-1 infection causes upregulation SOCSs (1, 2 and 3) mRNA expression when measured by microarray and qRT-PCR. These results led Chao et al to suggest that the antiviral actions of SOCS3 are mediated by an independent pathway (306). Importantly, this study showed that knockdown of SOCS3 clearly caused an inhibitory effect on HCV replication. Also, SOCS3 knockdown leads to the restoration of the expression level of STAT1 mRNA (figures 5.3 and 5.5). Taken together, we conclude that HCV has evolved an additional mechanism to evade host innate immune responses, also providing a possible therapeutic candidate.

Analysis of the Effect of HCV NS proteins on the Interferon Pathway

The HCV NS3/4A protease can disrupt antiviral signaling in various hepatic cell lines. Both TRIF and MAVS adaptors of antiviral signaling pathways were identified as cellular substrates of HCV NS3/4A protease (270, 273, 292, 307). However, the effect of NS3/4A on the expression of genes involved in the recognition of dsRNA and ssRNA in primary human hepatocytes is not known. In this study, recombinant adenoviruses that express NS3 and NS3/4A from different genotypes were constructed and examined for the effects of viral proteins on dsRNA induction. The expression of the NS3 and NS3/4A proteins inhibits the

expression of dsRNA sensing receptors RIG-I, MDA5 as well as inhibiting MAVS. In this study, it was shown, that NS3 alone or in combination with NS4A could reduce the expression of the different IFN- β signaling component mRNAs and the inhibition efficiency of NS3 with NS4A was similar to NS3 alone. This was observed with NS3 and NS4A from the three different genotypes 1a, 1b and 2a. Furthermore, expression of NS3 and NS3/4A using the adenovirus vectors led to a reduction in MyD88 gene expression, which can cause a defect in the TLR7-mediated pathway for ssRNA detection. These results indicate that NS3 and NS3/4A proteins are responsible for the inhibition of IFN- α and IFN- β induction through dsRNA and ssRNA pathways.

HCV standard treatment is the combination of pegylated interferon and ribavirin. Efficacy of treatment is approximately 80% in patients infected by HCV genotype 2 or 3, whereas in patients infected by HCV genotype 1 the response to treatment is less than 50% (170, 308, 309). The NS3/4A protease can inhibit the innate immune response by reducing RIG-I, MDA5, MAVS and MyD88 expression. Thus, differences in NS3/4A activity could be related to viral pathogenicity. In this study, the inhibitory efficiency of NS3/4A from HCV genotypes 1b, 1a and 2a were analyzed. However this study the expression of the NS3 and NS3/4A proteins from genotypes 1a, 1b and 2a showed that was no significance difference in their ability to inhibit the transcription of RIG-I, MDA5, MAVS and MyD88. However, other studies have shown that the catalytic efficiency of NS3/4A protease is genotype dependent. The enzymatic activity of each variant protease were evaluated by engineering the HCV polyprotein NS5A/NS5B cleavage site into a bacteriophage λ , and this demonstrated a six-fold difference between

different genotypes (310). NS3/4A proteases from genotype 1b displayed the highest catalytic efficiency (310). Another study also showed that MAVS is cleaved by all HCV NS3/4A from all genotypes and that NS3/4A from genotypes 2 and 3 were more efficient in proteolysis than those genotypes 1 and 4 (311).

Potent inhibitors have been developed for both the viral NS3/4A protease and NS5B RNA-dependent RNA polymerase. Major in vivo antiviral responses have been demonstrated in early clinical trials with several protease inhibitors, including BILN 2061 and VX-950 (telaprevir). Some of these candidate antivirals are now advancing into larger phase III studies. To further confirm the role of NS3 protein in inhibition of type I IFN induction, BILN 2061 was used to study the effect of NS3 inhibition on dsRNA components expression restoration. The specific antiviral inhibitor BILN2061 blocked the NS3-mediated cleavage of MAVS and completely restored the dsRNA-activated antiviral response. In this study, treatment of replicon cells with BILN 2061 reduced the expression levels of RIG-I, MDA5, MAVS and MyD88 to the level of naïve Huh7 cells and reversed the NS3/4A-mediated inhibition of RIG-I, MDA5 and MAVS signaling in response to transfection with poly (I:C). These results indicate a possible role of NS3 protein in the inhibition of IFN induction mediated by dsRNA stimulation. However BILN 2061 also inhibits the replication of HCV and this could also lead to upregulation of ISG sensors. However, since the role of HCV NS3/4A proteins have been identified in IFN inhibition by direct targeting both TRIF and MAVS adaptors proteolysis (270, 273, 292, 307). The mechanism of the NS3/4A in the inhibition of IFN components at the transcription level, as demonstrated clearly in this study, is still not fully understood. In agreement, a recent study showed similar effects on the innate responses to intracellular dsRNA. It showed that

transcription of several innate response genes is severely compromised upon ectopic expression of the HCV NS3/4A protease in normal human primary hepatocytes, and treating cell with protease inhibitor BILN2061 completely restored gene expression (312).

Apoptosis and cell cycle regulation:

In this study, HCV infection showed direct cytopathic effects which indicate that HCV has the potential to mediate apoptosis. This suggests a role for HCV in liver injury during chronic HCV infection, in addition to the immune-mediated liver injury. The appearance of cytopathic effects may be due to the induction of cell death-related genes. The results of the microarray study, showed a reduction in expression of genes related to the cell cycle e.g. cyclin B1, CDC25C and SKP2 and related to growth arrest and DNA-damage e.g. GADD45- α and GADD45- β , were consistent with this. Furthermore this was confirmed by quantitation of sub-G0 cells as appeared in the FACS analysis results. The highest cell death rate coincided with peak viral loads, suggesting that intra-hepatic HCV RNA levels play a role in hepatocyte cell death. However, the role of viral load in HCV-liver disease remains controversial, Pal et al, demonstrated an association between the number of hepatocytes harboring replicating HCV and severity of fibrosis (313). Other studies have shown that HCV levels were associated with cholestatic fibrosis, a severe form of hepatitis (314) and accelerated HCV-induced allograft injury (315). In this study, we showed that, in vitro, the cell death rate is associated with viral replication.

Also in this study, a comparison between the parental Huh7 cells and Huh7.5 (which have a defective RIG-I) was performed. This comparison was necessary to explain the discrepancy in the level of hepatocyte cell death that occurred in vitro

and during chronic HCV infection. The levels of HCV replication in Huh7 cells are much lower than in Huh7.5 cells and there is an increase in the number of apoptotic cells in Huh7.5 cells. Additionally the level of HCV replication was associated with an increased expression of GADD45- β , as validated by qRT-PCR and at protein level as demonstrated by Western blotting. It might therefore be concluded that the inactivation of dsRNA signaling through RIG-I has an important role in apoptosis induction. This indicates that the balance of IFN mediated suppression of HCV replication and HCV-mediated regulation of host innate antiviral pathways are key factors for HCV control over innate antiviral signaling and shape the outcome of infection.

The ability of HCV to perturb the cell cycle and the consequent effects on virus replication are not well understood. Alteration of the host cell cycle by RNA viruses has not been described as extensively in the literature compared to DNA viruses or retroviruses. For example, human immunodeficiency virus type 1 (HIV-1) Vpr arrests cells in the G2 phase (316-318). This arrest leads to an increase in RNA transcription and virus production (317, 319). Similar observations were found in Coronavirus infection, which induces a G2/M phase arrest in infected cells to promote viral replication (320). However, studies based on single protein expression have shown that HCV core protein has a role in impairment of G1 to S phase transition by induction of p21 expression, a decrease in cdk2 activity (321), and direct interaction and suppression of CAK activity, and stabilization of cell cycle inhibitor p27 (322). In addition to microarray analysis and qRT-PCR (table 5.1 and figure 5.6), this study and another recently published study (299) have shown that gene expression profiling and flow cytometry analysis suggest that

HCV mediated apoptosis of Huh7.5 cells is linked to perturbations in cell cycle progression. However, the current study has also shown effects on cell cycle regulation in both Huh7 and Huh7.5 cells. Additionally, it was shown that there was an increase in GADD45- β protein expression, which is consistent with the findings that HCV infection causes cell death by DNA fragmentation. The cell cycle arrest caused by HCV infection led to increased virus production, as demonstrated by the increased levels of HCV core protein. Furthermore, the current study also showed that key points in the cell cycle were perturbed by HCV. In addition to the increase in GADD45- β expression a reduction of cyclin B1 expression and its redistribution in cell cytoplasm was also observed.

Perturbation of the cell cycle has been observed in other viral infections including HIV (vpr) (316-318), and HBV (x protein) (323). The role of the HCV proteins that are responsible for inducing the delay in cell cycle progression is not fully understood. The role of the NS5A protein was tested to see whether it associates with cyclin B1 in JFH-1 infected cells. No association between these proteins was observed. However, these results indicate that it would be very interesting to investigate the role of other HCV proteins in cell cycle control since it is linked to the development of chronic HCV infection and viral pathogenesis.

In conclusion, HCV infection controls IFN induction by reducing the transcription of the genes involved in the detection of dsRNA and ssRNA, RIG-I, MDA5, MAVS, TRIF and MyD88. However from the results of this study, it is not possible to distinguish whether this is due to direct and specific effects on expression of these genes or whether it is an indirect consequence of the

previously observed cleavage of MAVS, resulting in reduced expression of IFN- β and the ISGs, as the latter includes RIG-I, MDA5, MAVS, TRIF and MyD88. In agreement with other studies the work presented here also shows that HCV attenuates IFN signaling through the JAK-STAT pathway by the induction of SOCS3 protein. It was demonstrated that the HCV NS3 and NS3/4A protein inhibit the mRNA expression of dsRNA sensing components RIG-I, MDA5 and MAVS and also inhibit MyD88 mRNA expression. This results in the inhibition of IFN- α and IFN- β production which may contribute to viral persistence. It was also shown by knockdown of gene expression that MDA5 and the signaling mediators MAVS and MyD88 play an important role in the host response to HCV replication. Gene expression data and flow cytometry analysis indicate that HCV infection is associated with perturbations of the cell cycle which may lead to apoptosis and cell death. An increase in the number of apoptotic cells was also shown to be associated with HCV replication levels.

Further characterisation of the roles of MDA5, MAVS, TRIF and MyD88 during HCV infection would help in understanding if the RIG-I/MDA5 pathways work independently or in synergistic manner. Additional genes including members of the SOCS family, IRFs and other candidate genes identified by microarray analysis may also be involved in the IFN response to HCV infection. Systematically targeting these by siRNA knockout should indicate if IFN- β can be still induced by other mechanisms and might indicate novel therapeutic targets. Further work is also needed to understand the mechanisms by which NS3/4A can suppress IFN transcription.

Transduction of Huh7 cells with NS3 and NS3/4A recombinant adenoviruses and then treating them with BILN2061, should show whether the NS3/4A protease is essential for the suppression of transcription related to IFN expression.

This study suggests a direct cytopathic effect of HCV through the induction of apoptosis and it investigated a number of candidate genes involved in ER stress. Future work will address the question of the role of the RIG-I/MDA5 pathways in linking innate immune responses and apoptosis. Furthermore, the effects of HCV infection on cell cycle modulation have not yet fully elucidated. However the transcription of several other genes (presented in table 5.1) involved in cell cycle regulation was also affected by HCV infection. It will be important to investigate their role in the regulation of the cell cycle and apoptosis during HCV infection.

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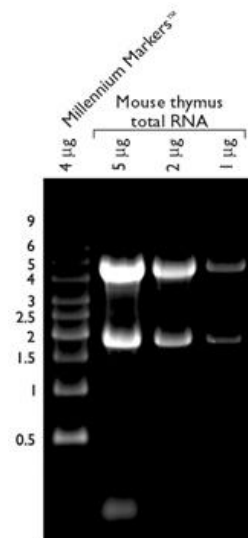
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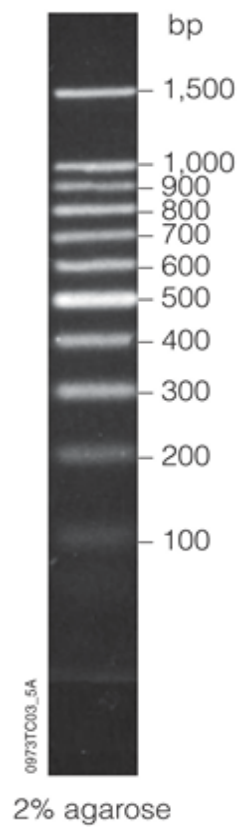
8.0. Appendix

8.1. Gel electrophoresis markers

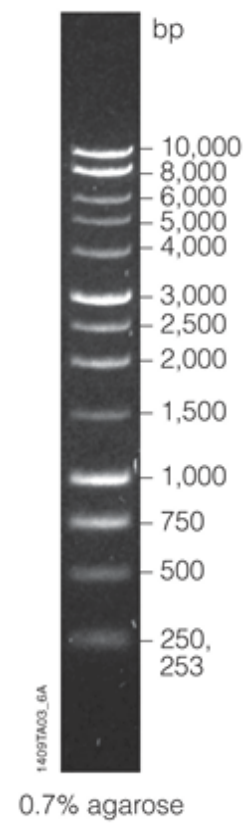
Ambion Millennium RNA ladder



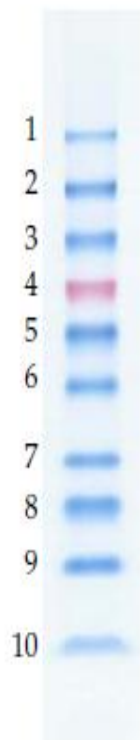
Promega DNA 100bp ladder



Promega DNA 100bp ladder



SDS-PAGE prestained molecular weight marker (Invitrogen)

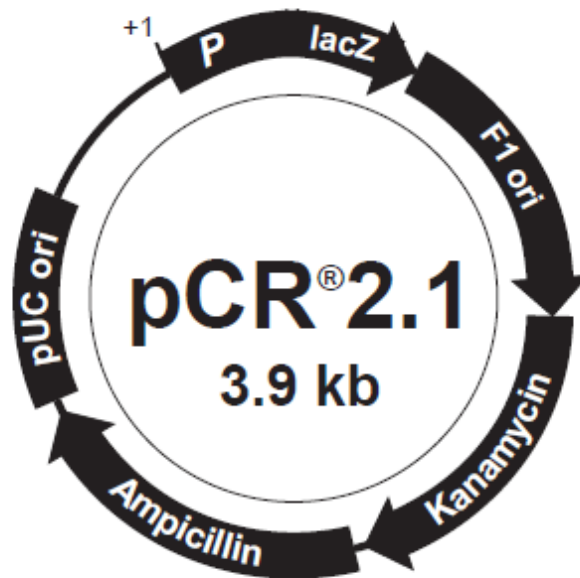


<i>Band number</i>	<i>Molecular weight</i>
1	180 kDa
2	115
3	82
4	64
5	49
6	37
7	26
8	19
9	15
10	6

Orientation band (pink)

8.2. Plasmid maps

I. pCR2.1 vector map:



Features of pCR[®]2.1

3929 nucleotides

LacZ gene: bases 1-545

M13 Reverse priming site: bases 205-221

T7 promoter: bases 362-381

M13 (-20) forward priming site: bases 389-404

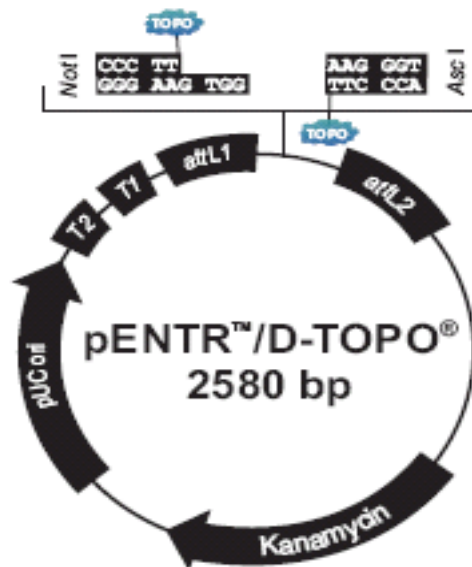
F1 origin: bases 546-983

Kanamycin resistance ORF: bases 1317-2111

Ampicillin resistance ORF: bases 2129-2989

pUC origin: bases 3134-3807

II. pENTR/D-TOPO vector map:



Features of pENTR/D-TOPO

2580 nucleotides

rrnB T2 transcription termination sequence: bases 268-295

rrnB T2 transcription termination sequence: bases 427-470

M13 forward (-20) priming site: bases 537-552

attL1 site: bases 569-668

TOPO recognition site 1: bases 680-684

Overhang: bases 685-688

TOPO recognition site 2: bases 689-693

attL2 site: bases 705-804

T7 promoter/priming site: bases 821-840 (c)

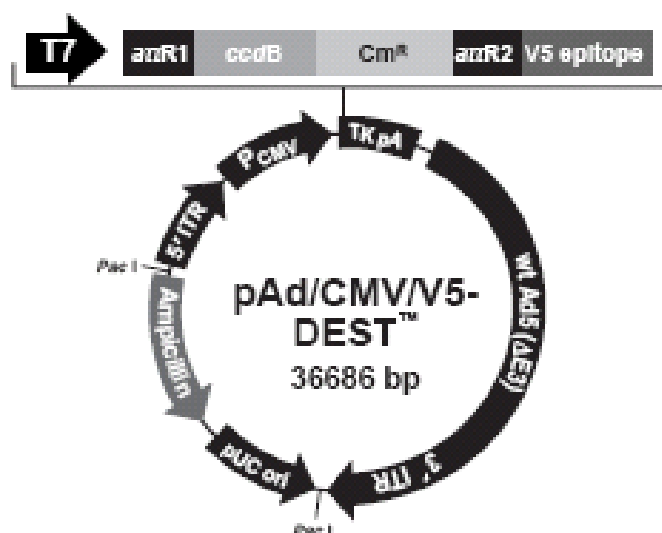
M13 reverse priming site: bases 845-861

Kanamycin resistance gene: bases 974-1783

pUC origin: bases 1904-2577

(C) = complementary strand

III. pAd/CMV/V5-DEST vector map:



Features of pAd/CMV/V5-DEST™

36686 nucleotides

Human Ad5 sequences (wt 1-458; includes 5' L-ITR and packaging signal): 1-

458pAd forward priming site: bases 361-384

CMV promoter: bases 728-1315

T7 promoter/priming site: bases 1359-1378

attR1 site: bases 1407-1531

ccdB gene: bases 1960-2265 (C)

Chloramphenicol resistance gene (*CmR*): bases 2607-3266 (C)

attR2 site: bases 3547-3671

V5 epitope: bases 3697-3738

TK polyadenylation signal: bases 3765-4036

Human Ad5 sequences (wt 3513-35935; E3 region deleted, includes 3' R-ITR): bases 4056-34604

pAd reverse priming site: bases 4059-4082

pUC origin: bases 34781-35442 (C)

Ampicillin (*bla*) resistance gene: bases 35568-36428 (C)

bla promoter: bases 36429-36527 (C)

Pac I restriction sites: bases 34610 and 36684

(C) = complementary strand

8.3. Quantifying of gene expression levels using real-time RT-PCR method:

I. Relative quantification

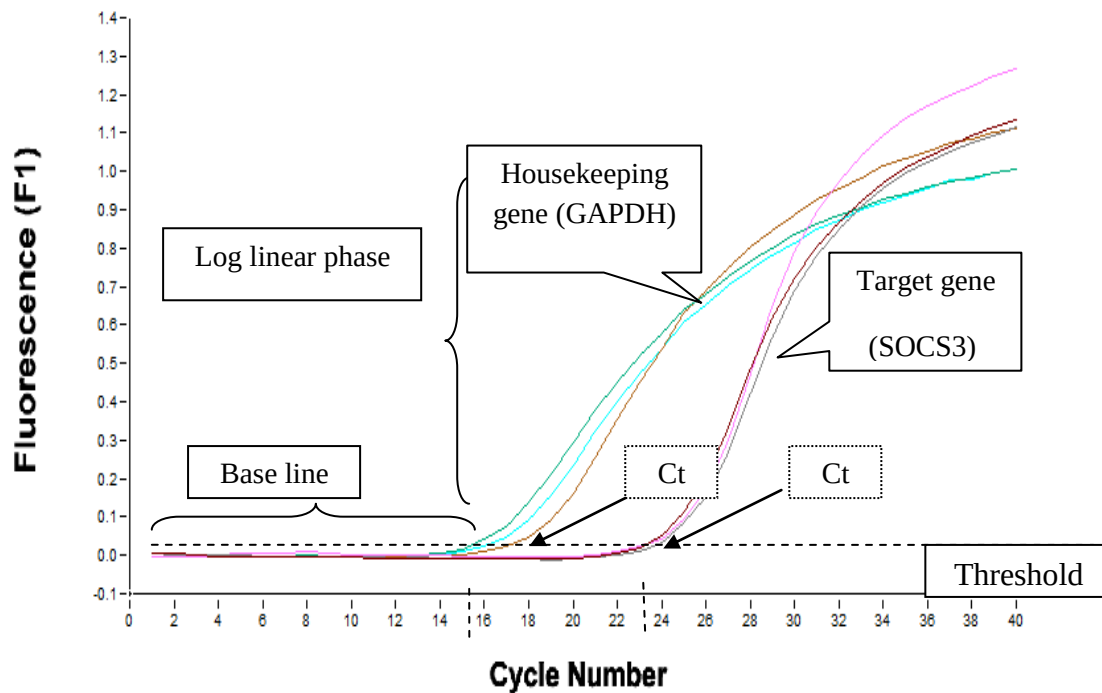
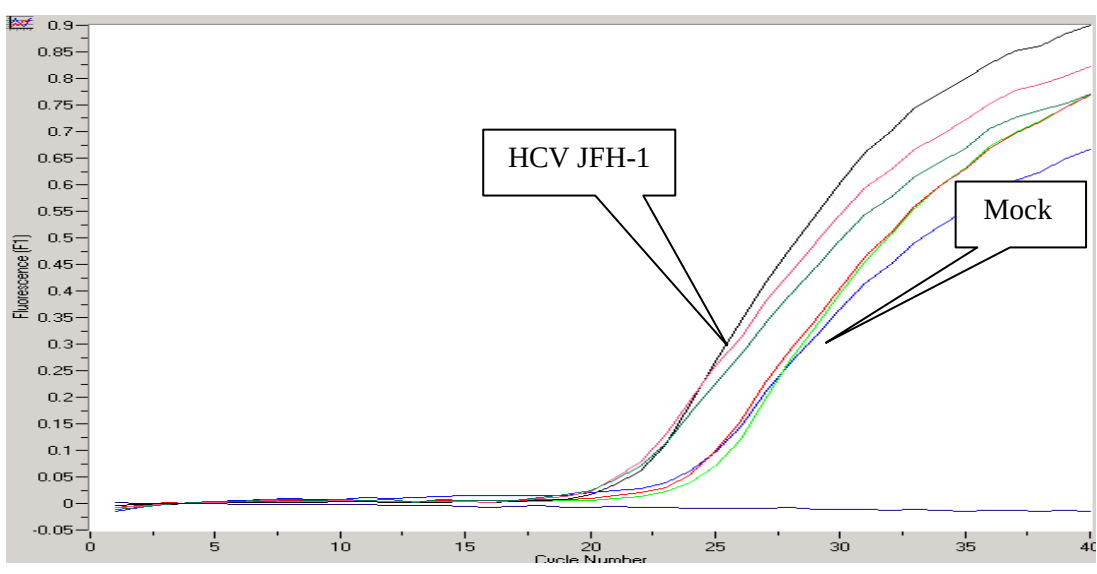


Figure 8.1: Example of real-time PCR using LightCycler 2.0 (Roche): the figure shows a linear plot of the amount of DNA (y axis) and the cycle number (x axis). Folds changes were calculated as detailed in section 2.2.2.11. The relative Ct method (or ΔCt) was used to assess relative changes in mRNA levels between two samples. Amplification of the example housekeeping gene (GAPDH) and the example target gene (SOCS3) was carried out in triplicate.

An example of employing the comparative Ct method:

A

	P...	Name	Calculated Concentrat...	Crossing Point
—	1	h7 GADD45b mock 1	0.000E+00	23.88
—	2	h7 GADD45b mock 2	0.000E+00	24.40
—	3	h7 GADD45b mock 3	0.000E+00	23.83
—	4	h7 GADD45b JFH1 1	0.000E+00	21.73
—	5	h7 GADD45b JFH1 2	0.000E+00	21.16
—	6	h7 GADD45b JFH1 3	0.000E+00	21.39
—	7	H2O		



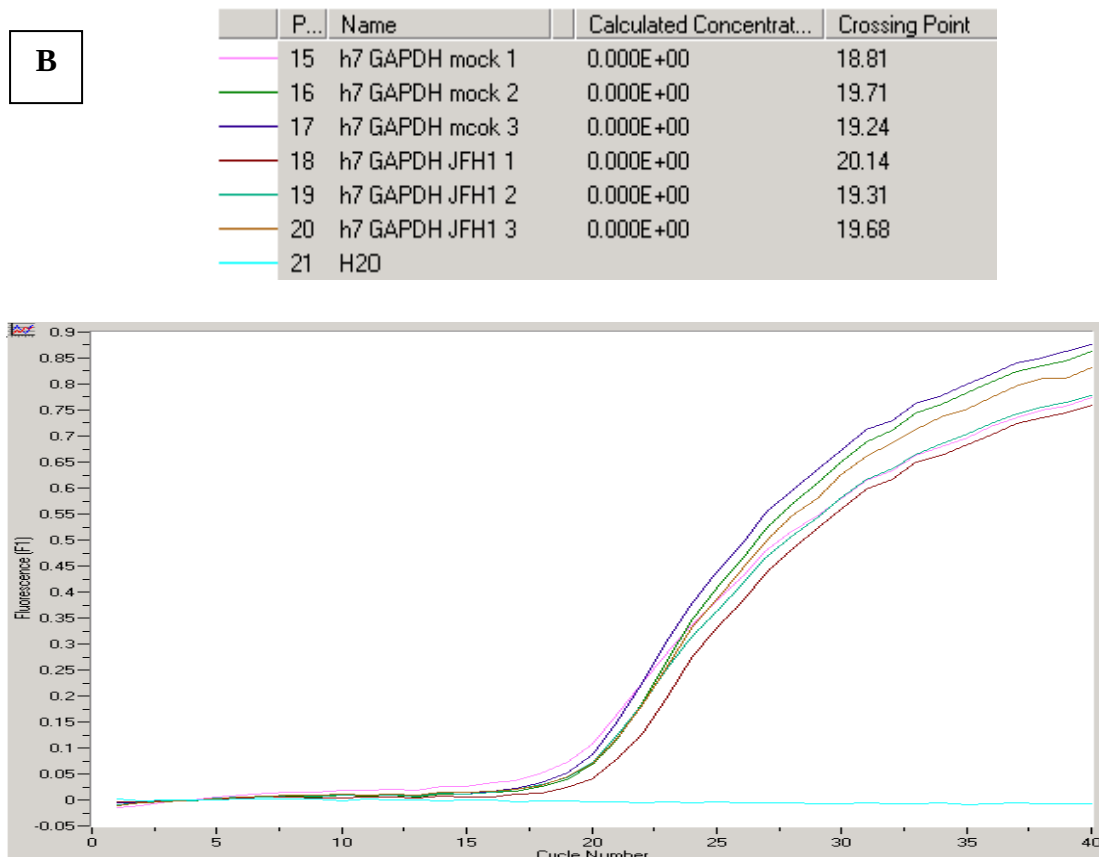
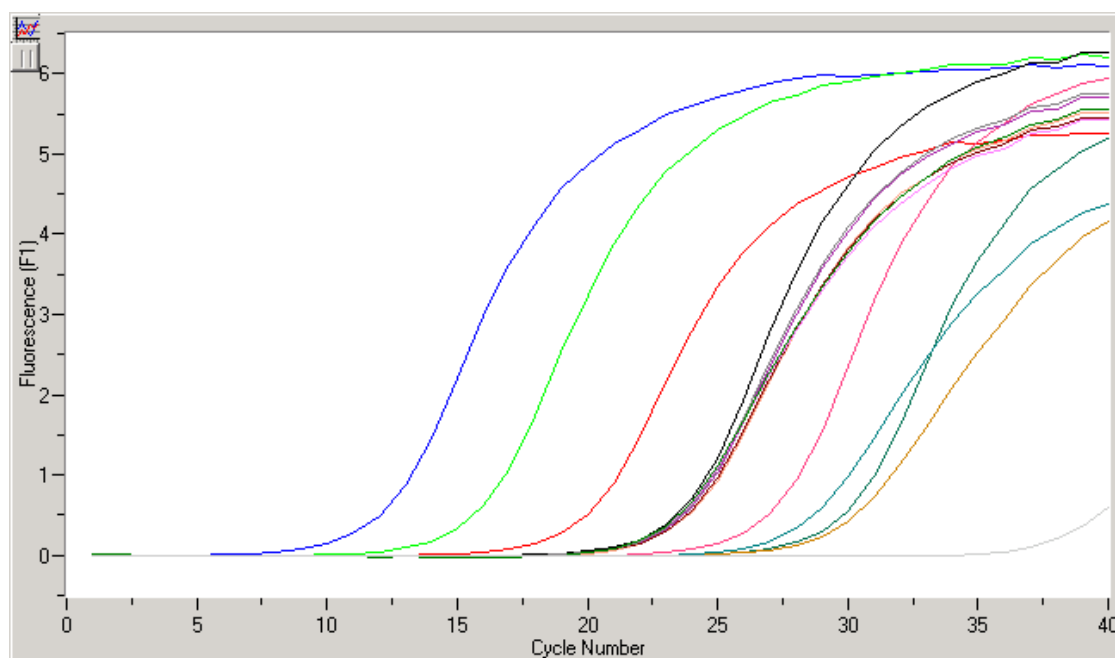


Figure 8.2: An example of employing the comparative Ct method by the comparison of the expression of innate immune responses target gene in mock infected vs. HCV JFH-1 infected Huh7 cells. (A). Amplification plots showing increases in fluorescence from target gene (HCV JFH-1 infected cells, in triplicate) and unknown samples (Mock infected cells, triplicate). (B) For controlling error in qRT-PCR, second PCR was performed for normalizing to an endogenous reference (GAPDH) gene.

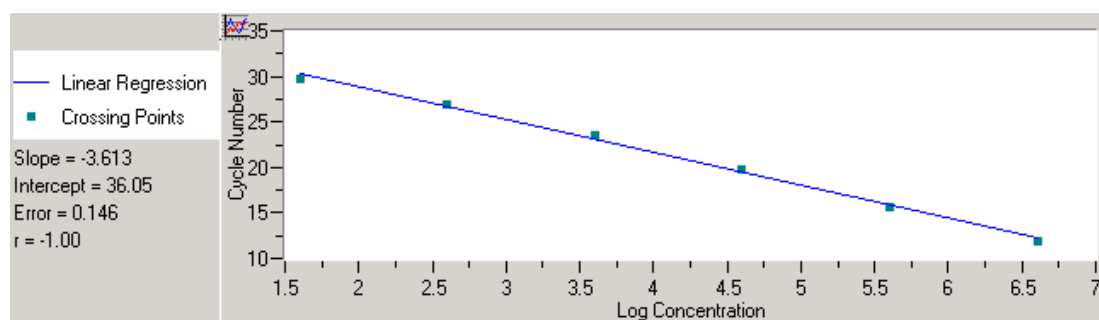
II. Absolute quantification

A

	P...	Name	Standard	Calculated Conc...	Crossing Point
1		std 1	4.000E+06	4.723E+06	11.94
2		std 2	4.000E+05	4.446E+05	15.65
4		std 3	4.000E+04	3.391E+04	19.68
5		std 4	4.000E+03	2.994E+03	23.49
6		std 5	4.000E+02	3.348E+02	26.93
7		std 6	4.000E+01	5.740E+01	29.70
8		7.5 mock		2.729E+01	30.87
9		7.5 mock		2.541E+01	30.98
10		7.5 siNEG		2.681E+03	23.67
11		7.5 siNEG		2.877E+03	23.56
12		7.5 siSOCS3		5.411E+01	29.79
14		7.5 siSOCS3		1.548E+02	28.14
15		7.5 siGAPDH		2.821E+03	23.59
16		7.5 siGAPDH		3.022E+03	23.48
17		7.5 JFH ONLY		3.713E+03	23.16
18		7.5 JFH ONLY		3.701E+03	23.16



B



C

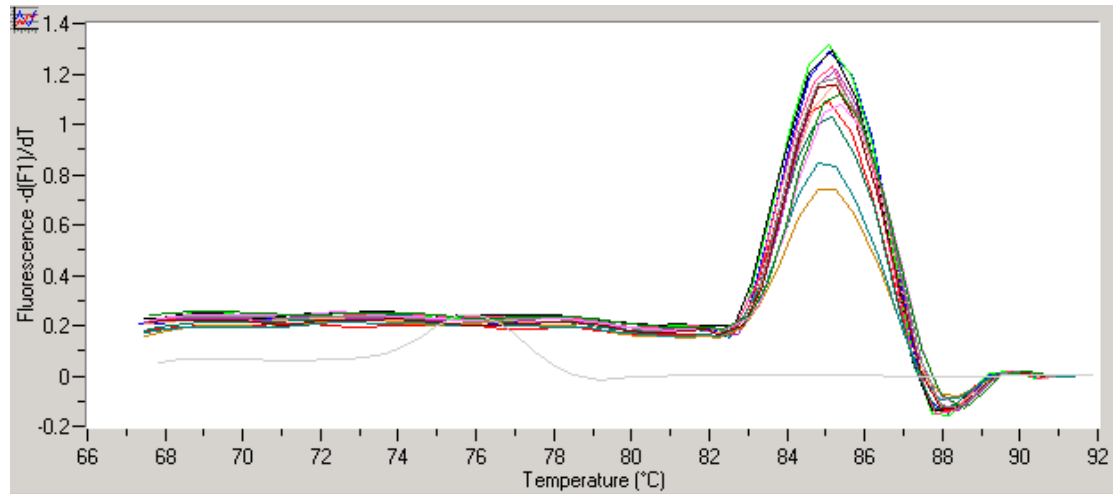


Figure 8.3: Example of an amplification plot obtained using a SYBR Green I assay (A) the standard curve method is used to assess the quantity of the transcript (JFH-1 genomic RNA). This requires that a template dilution series of an RNA standard is included (i.e. the same mRNA as the target RNA). Quantities interpolated from the resulting standard curve are used to calculate relative mRNA levels in each unknown sample. (B) The amplification plots were generated by ten-fold serial dilutions of an RNA sample containing 4ng of RNA. The standard curve generated from these amplification plots is linear, over 5 logs, with a slope = -3.613 and an $r = -1$. (C) Examination for non-specific amplicons e.g. primer dimers, and genomic DNA contamination identified by melting curve analysis, including shows only one peak corresponding to target amplicon.

8.4. Analysis of cell cycle using FACS by double staining for PI and HCV core protein:

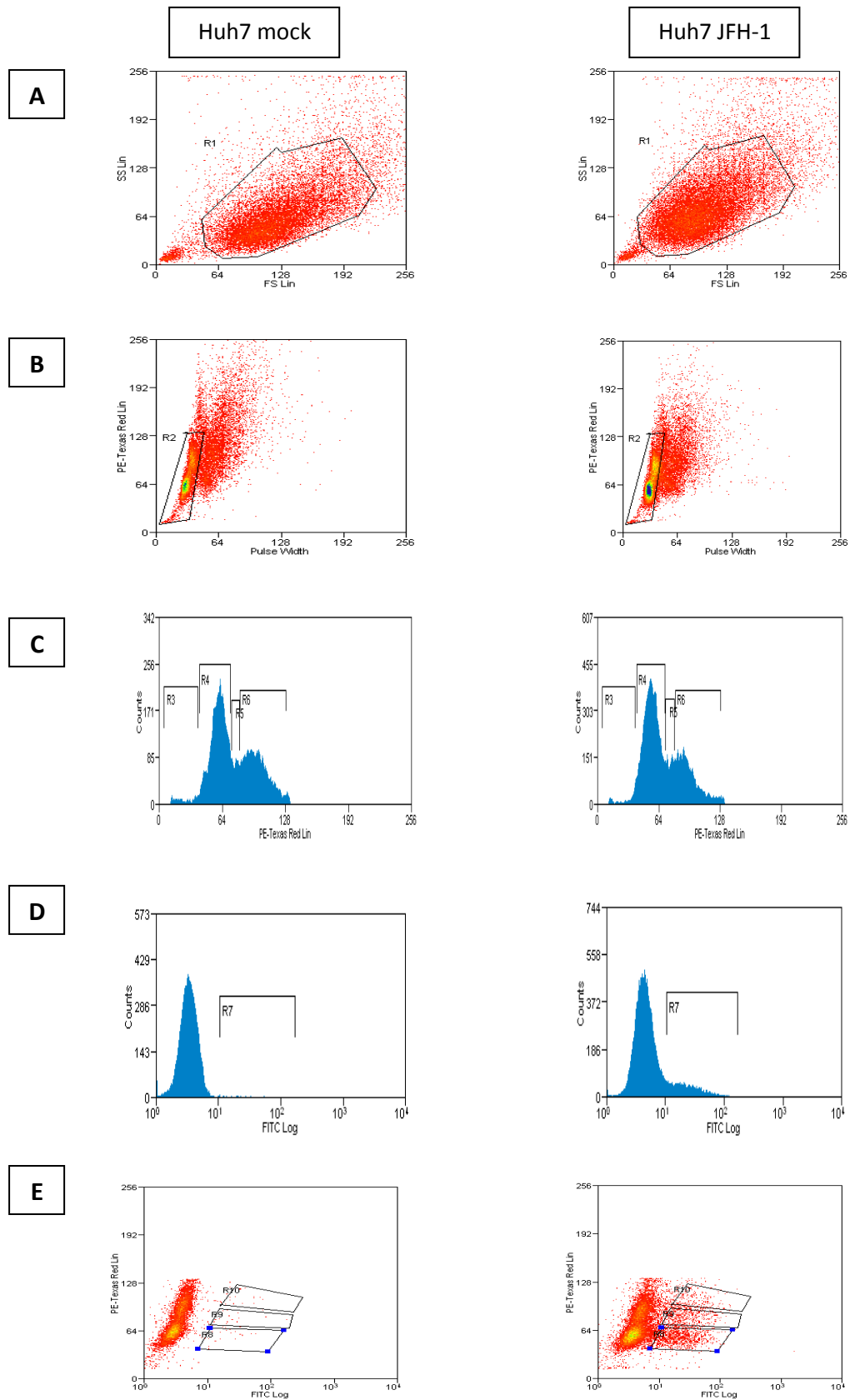


Figure 8.4: HCV infection mediated G2 cell cycle arrest results in higher levels of viral replication: Huh7 cells were either mock infected or infected with HCV JFH-1 (MOI = 3). Histogram of cell cycle phase analysis in mock and JFH-1 HCV infected cells (**A**), dot-plot analysis, of the selected population of gated cells is within the area bounded to eliminate the debris of necrotic cells. (**B**), doublet discrimination by a density plot of forward scatter versus DNA content, selected population of gated cells is within the area bounded. (**C**), cells were stained with PI and the cell cycle profiles at 72 hours post infection were shown. (**D**) cells were stained with anti-core antibodies and thus the infection efficiency was evaluated by percentage of core positive cells compared to the gated cells from B (**E**) The histograms were analyzed to determine the percentage of HCV core protein in each phase of the cell cycle.

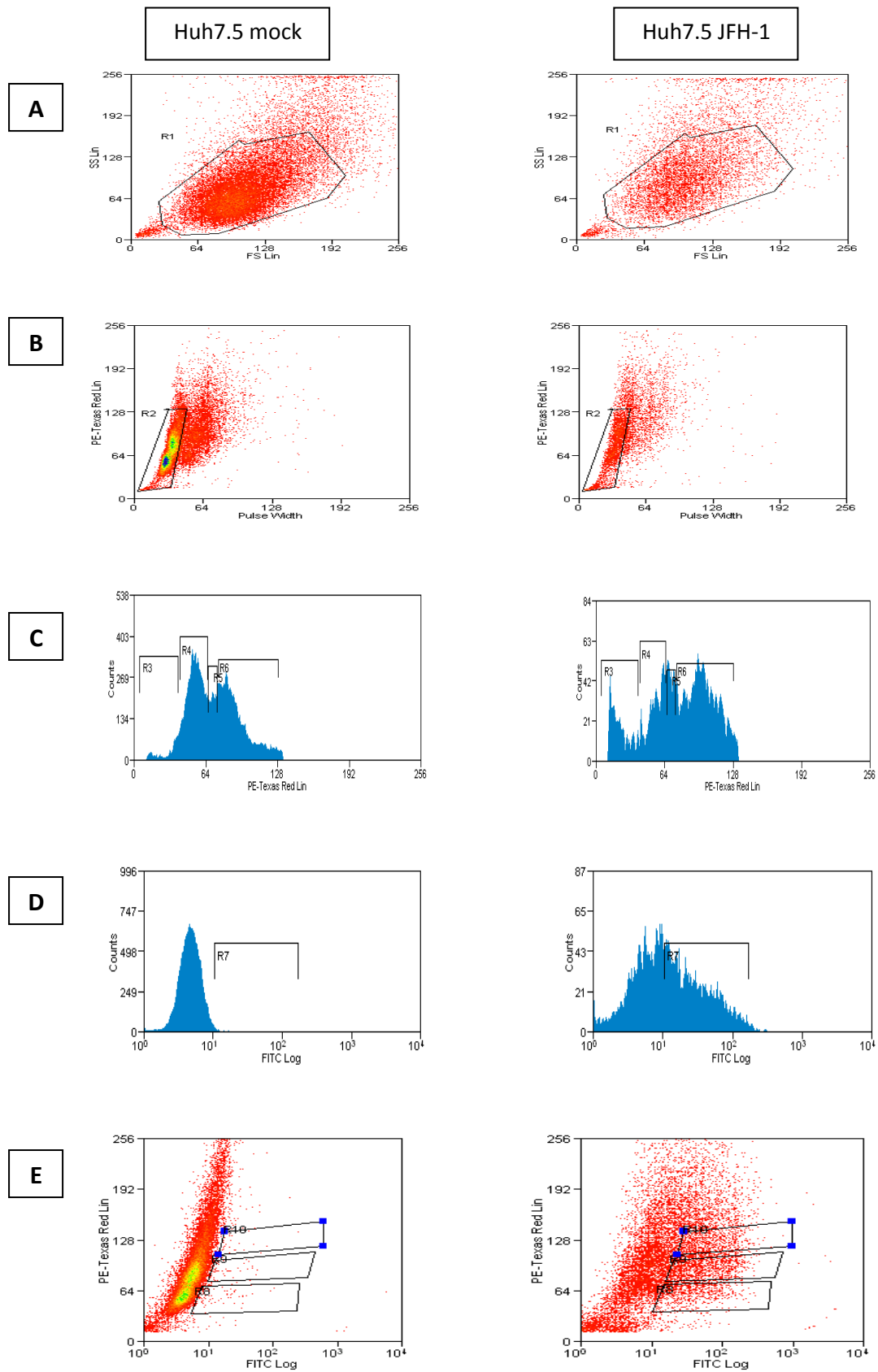


Figure 8.5: HCV infection mediated G2 cell cycle arrest results in higher levels of viral replication: Huh7.5 cells were either mock infected or infected with HCV JFH-1 (MOI = 3). Histogram of cell cycle phase analysis in mock and JFH-1 HCV infected cells (**A**), dot-plot analysis, of the selected population of gated cells is within the area bounded to eliminate the debris of necrotic cells. (**B**), doublet discrimination by a density plot of forward scatter versus DNA content, selected population of gated cells is within the area bounded. (**C**), cells were stained with PI and the cell cycle profiles at 72 hours post infection were shown. (**D**) cells were stained with anti-core antibodies and thus the infection efficiency was evaluated by percentage of core positive cells compared to the gated cells from B (**E**) The histograms were analyzed to determine the percentage of HCV core protein in each phase of the cell cycle.