HOST FACTOR REGULATION OF HEPATITIS C VIRUS REPLICATION IN
RODENT CELLS

by

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In memory of Mr. Tony An Lee (1931-2009).

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ABSTRACT

Hepatitis C virus (HCV) is a serious global health problem with an estimate of 170 million carriers worldwide. Most individuals exposed to this blood-borne pathogen develop chronic infection, which may result in severe liver complications as well as end-stage liver diseases including cirrhosis and hepatocellular carcinoma. Current treatment options are suboptimal with no effective vaccines available to date. Development of a readily accessible mouse model that is permissive to natural HCV infection is important to facilitate drug and vaccine discovery, and also to better understand the viral pathogenesis. The inherent difficulty is that HCV displays very limited tropism, infecting only livers from humans or chimpanzees. An attempt was made to elucidate the key determinants in rendering the murine intracellular environment permissive to HCV replication. The results revealed that deletion of the interferon regulatory factor-3 and overexpression of microRNA-122 can independently enhance viral subgenomic replication in murine fibroblasts, with microRNA-122 being the stronger determinant. Interestingly, the phenotype established by these genetic manipulations was insufficient to support full-length HCV genome replication. Murine hepatic cell lines, with or without microRNA-122 expression, were also non-permissive to genomic HCV replication, despite the fact that translation of viral RNA was observed. These results suggest that additional host-specific factor(s) are required to support replication of full-length HCV RNA. These studies provide insight on the essential factors capable of influencing permissiveness of rodent cells to HCV replication, and also suggest genetic modifications to be considered when modeling the complete viral life cycle in a rodent animal model.

LIST OF ABBREVIATIONS USED

2',5' [A]	2',5' oligoadenylate
2',5'-OAS	2',5' oligoadenylate synthetase
aa	amino acids
ADAR	dsRNA-dependent adenosine deaminase
AGO	argonaute protein
AIM2	absent in melanoma 2
Alb-uPA	albumin-urokinase type plasminogen activator
ALT	alanine aminotransferase
AP-1	activator protein 1
APOB	apolipoprotein B-100
APOE	apolipoprotein E
ARFP	alternate reading frame protein
ASC	apoptotic speck-like protein containing a CARD
AST	aspartate aminotransferase
ATF2	activating transcription factor 2
BCR	B cell antigen-receptor complex
bDNA	branched-chain DNA
BF	bright field
BHK 21	baby hamster kidney cells
BSA	bovine serum albumin
Bsd	blasticidin S deaminase
CARD	caspase-recruitment domain
Cardif	CARD adaptor inducing IFN-β
CAT-1	cationic amino acid transporter
CBP	CREB-binding protein
CD81	cluster of differentiation 81
cDNA	complementary DNA
CES1	carboxylesterase 1
CFU	colony forming unit
CLDN	claudin
CLEC4M	C-type lectin domain family 4 member M

Cntrl	control
CpG	2'-deoxyribo cytidine-phosphate-guanosine
CRE	*cis*-acting regulatory element
CREB	cAMP response element binding
DAA	direct-acting antiviral
DAI	DNA-dependent activation of IFN regulatory factors
DC	dendritic cell
DC-SIGN	dendritic cell-specific intercellular adhesion molecule-3-grabbing non-integrin
DC-SIGNR	DC-SIGN-related protein
DENV	Dengue virus
DF	dark field
DGCR8	DiGeorge syndrome critical region 8
DKO	double-knockout
DLC1	deleted in liver cancer 1
dsRNA	double-stranded RNA
EBV	Epstein-Barr virus
eIF-2	eukaryotic translation initiation factor-2
ELISA	enzyme-linked immunosorbent assay
EMCV	encephalomyocarditis virus
ER	endoplasmic reticulum
ERIS	endoplasmic reticulum IFN stimulator
Exp5	exportin-5
F	fusion protein
FADD	Fas-associated death domain protein
FL	full-length
FMDV	foot-and-mouth disease virus
GAF	IFN-γ-activated factor
GAG	glycosaminoglycan
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
GAS	IFN-γ-activated site
GBV-B	GB-virus B
GFP	green fluorescent protein
GLuc	*Gaussia* luciferase
GTP	guanosine triphosphate

H	hemagglutinin protein
HAART	highly active antiretroviral therapy
HBV	hepatitis B virus
HCC	hepatocellular carcinoma
HCMV	human cytomegalovirus
HCV	hepatitis C virus
HCVcc	cell-culture produced (derived) HCV
HEK 293	human embryonic kidney cells
HIN	hematopoietic IFN inducible nuclear protein
HIV	human immunodeficiency virus
HLA	human leukocyte antigen
HMG-I(Y)	high mobility group protein (non histone chromosomal) isoform I and Y
HSV-1	herpes simplex virus type 1
HVR	hypervariable region
IE	immediate-early protein
IFA	influenza A virus
IFI16	IFN-γ-inducible protein 16
IFN	interferon
IFN-α	interferon-alpha
IFN-β	interferon-beta
IFN-γ	interferon-gamma
IFN-λ	interferon-lambda
IFNAR	IFN-α/β receptor
Ig	immunoglobulin
IκB	inhibitor of NF-κB
IKK	IκB kinase
IKK-*i*	inducible IκB kinase
IL	interleukin
IL-1β	interleukin-1 beta
IL1-R	interleukin-1 receptor
IL-10Rβ	interleukin-10 receptor beta chain
IL-28Rα	interleukin-28 receptor alpha chain
IP-10	IFN-inducible protein 10

IPS-1	IFN-β promoter stimulator 1
IRAK	IL-1 receptor-associated kinase
IRES	internal ribosome entry site
IRF	IFN regulatory factor
ISDR	IFN sensitivity determining region
ISG	IFN-stimulated gene
ISGF3	IFN-stimulated gene factor 3
ISRE	IFN-stimulated regulatory element
IU	international units
i.v.	intravenous
JAK	Janus kinase
JEV	Japanese encephalitis virus
JFH	Japan Fulminant Hepatitis
kDa	kiloDalton
KO	knockout
KSHV	Kaposi's sarcoma-associated herpesvirus
LD	lipid droplet
LDL	low-density lipoprotein
LDLR	low density lipoprotein receptor
LGP2	laboratory of genetics and physiology-2
LPS	lipopolysaccharide
LRR	leucine-rich-repeat
L-SIGN	liver and lymph node-SIGN
LTA	lipoteichoic acid
LVP	lipo-viral particle
Mal	MyD88-adaptor-like
MAPK	mitogen-activated protein kinase
MAVS	mitochondrial antiviral signaling
MCMV	murine cytomegalovirus
MDA5	melanoma differentiation-associated gene 5
MEF	mouse embryonic fibroblast
Met-tRNAi	initiator methionine transfer RNA
MICB	major histocompatibility complex class I polypeptide-related sequence B
miR-122	microRNA-122

miRISC	miRNA-induced silencing complex
miRNA	microRNA
MITA	mediator of IRF-3 activation
MMTV	mouse mammary tumor virus
MOI	multiplicity of infection
MV	measles virus
Mx	myxovirus-resistance
MyD88	myeloid differentiation factor 88
NALP3	NACHT-, LRR- and PYD-containing protein 3
NAK	NF-κB-activating kinase
NANB	non-A, non-B
NBT/BCIP	Nitro-Blue Tetrazolium Chloride/5-Bromo-4-Chloro-3'-Indolyphosphate p-Toluidine Salt
NEMO	NF-κB essential modulator
NF-κB	nuclear factor kappa B
NHL	non-Hodgkin's lymphoma
NK	natural killer
NLR	nucleotide-binding domain leucine-rich repeat containing receptor
NLRC5	NLR family, CARD domain containing 5
NLRP3	NLR family, pyrin domain containing 3
NOD2	nucleotide-binding oligomerization domain 2
NPT	neomycin phosphotransferase
NS	non-structural
nt	nucleotide
NTPase	nucleotide triphosphatase
OCLN	occludin
OPN	osteopontin
ORF	open reading frame
PAMP	pathogen-associated molecular pattern
PBMC	peripheral blood mononuclear cell
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
PD-1	programmed death 1
PEG	pegylated

PFV-1	primate foamy virus type 1
PI3K	phosphatidyl-inositol 3-kinase
PIAS1	protein inhibitor of activated STAT1
PKR	dsRNA-dependent protein kinase
PKRBD	PKR binding domain
pol +	polymerase active
pol -	polymerase defective
poly(I:C)	polyinosinic:polycytidylic acid
PP2A	protein phosphatase 2A
ppp	triphosphate
pre-miRNA	precursor form of miRNA
pri-miRNA	primary transcript of miRNA
PRR	pattern recognition receptor
PUMA	p53-upregulated modulator of apoptosis
PYHIN	pyrin and HIN domain-containing protein
qRT-PCR	quantitative RT-PCR
RANTES	regulated upon activation, normal T cell expressed and secreted
RBV	ribavirin
RdRp	RNA-dependent RNA polymerase
rER	rough endoplasmic reticulum
RIG-I	retinoic acid-inducible gene-I
RIP1	receptor-interacting protein1
RISC	RNA-induced silencing complex
RLR	RIG-I-like receptor
RLU	relative light unit
RNAi	RNA interference
RNA Pol II	RNA polymerase II
RNA Pol III	RNA polymerase III
RNase III	ribonuclease III
RNase L	ribonuclease L
RSV	respiratory syncytial virus
RT	reverse transcription
S	structural
SARM	sterile alpha- and armadillo-motif-containing protein

SCID	severe combined immunodeficient
SEM	standard error of the mean
SENV	Sendai virus
SL	stem-loop
SNP	single nucleotide polymorphism
SOCS	suppressor of cytokine signaling
SR-A	class A scavenger receptor
SR-B1	scavenger receptor class B type I
ssRNA	single-stranded RNA
STAT	signal transducer and activator of transcription
STAT-C	specific targeted antiviral therapy for hepatitis C
STING	stimulator of IFN genes
SVR	sustained virological response
T2K	TRAF2-associated kinase
TAB	TAK1 binding protein
TAK1	TGF-β-activated kinase 1
TANK	TRAF-family-member-associated NF-κB activator
TBK1	TANK-binding kinase 1
TGF-β	transforming growth factor-beta
THBS1	thrombospondin 1
TICAM-1	TIR-domain-containing molecule 1
TIR	Toll/IL-1R homology
TIRAP	TIR-associated protein
TLR	Toll-like receptor
TM	transmembrane
TNF	tumor necrosis factor
TRADD	TNF receptor-associated death domain protein
TRAF	TNF receptor-associated factor
TRAM	TRIF-related adaptor molecule
TRIF	TIR-domain containing adaptor inducing IFN-β
TurboRFP	turbo red fluorescent protein
TYK2	tyrosine kinase 2
USP18	ubiquitin-specific peptidase 18
UTR	untranslated region

VACV	vaccinia virus
VISA	virus-induced signaling adaptor
VLDL	very low-density lipoprotein
VSV	vesicular stomatitis virus
WNV	West Nile virus
WT	wild-type
ZBP1	Z-DNA-binding protein 1

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CHAPTER 1   INTRODUCTION

1.1. The Hepatitis C Virus

1.1.1. General Background

The history of hepatitis C virus (HCV) studies stemmed in the 1970's from the search for a causative agent of post-transfusion non-A, non-B (NANB) hepatitis. In 1989, Choo and colleagues reported the first sequences of the virus by screening lambda phage complementary DNA (cDNA) expression libraries made from nucleic acids that were isolated from liver and plasma samples of experimentally infected chimpanzees (94). The discovery of the resulting HCV molecular clone (designated *5-1-1*), which expressed an antigen that was recognized only by serum antibodies from NANB hepatitis patients (264), marked the beginning of a whole new field of studies in deciphering the biological nature of this virus. Since that initial discovery, HCV was shown to be a blood-borne pathogen that caused both acute and chronic hepatitis. Following acute infection, it is estimated that approximately 80 % of patients develop chronic liver disease that puts them at risk for liver complications such as steatosis, progressive fibrosis, cirrhosis, and hepatocellular carcinoma (HCC) (285). The adept ability of HCV to establish persistent infections makes this virus one of the most important causes of chronic liver-related illnesses worldwide.

1.1.2. Taxonomy

HCV is classified as the sole member of the genus *Hepacivirus* within the *Flaviviridae* family, which also includes yellow fever virus, dengue virus (DENV), West

Nile virus (WNV), Japanese encephalitis virus (JEV), bovine viral diarrhea virus, and GB virus. Due to its error-prone RNA-dependent RNA polymerase (RdRp), a feature shared amongst positive-stranded RNA viruses, HCV shows extensive genetic variability. This is evident both at the level of the individual in the form of quasispecies variation within a single infection, and at the level of geographical regions that are each associated with their own significantly divergent isolates. HCV is currently classified into six main genotypes (identified as 1 through 6, with greater than 30 % sequence divergence between them) and each genotype is further divided into subtypes (designated by lowercase letters, with each subtype differing from one another by 10-30 %) (31). In contrast to HCV genotypes 1 and 2, which have a more or less worldwide distribution, genotype 3 viruses are mainly found in the Indian subcontinent and Southeast Asia; genotype 4 infections are predominantly observed in Africa and the Middle East; genotype 5 viruses are most prevalent in South Africa; and genotype 6 infections are common to the Southeast Asia region (31, 506). While the genetic disparity across genotypes may account for their biological discrepancies with respect to replication, mutation rates, disease manifestations, and treatment outcomes, these differences are not fully understood. Nonetheless, it is widely observed that genotype 3 HCV infections have a higher prevalence of steatosis (304) and individuals infected with HCV genotype 1 are typically poor responders to standard interferon (IFN)-based therapies (146, 210). As such, genotypes have been used as predictors of therapeutic outcome and a determining factor in assessing the duration of treatment, dose of antiviral therapy, and virological monitoring procedure necessary in the clinical management of hepatitis C (371).

1.1.3. Prevalence

It is currently estimated that approximately 170-300 million people worldwide (roughly 3 % of the present global population) are infected with HCV and the global burden of disease attributable to HCV-related chronic liver diseases is substantial (278). The pathogen causes significant morbidity, with an estimated 100,000 deaths per year as a direct result of complications involved in end-stage liver diseases (279). Consequently, HCV has been labeled as the most common cause of chronic hepatitis-induced liver failure and orthotopic liver transplantation in North America (72). Prevalence of HCV is estimated to range from 0.1-5 % with wide variation between geographical locations; some areas that are hyperendemic include countries such as Egypt (up to 20 %), China, Japan, and Taiwan, as well as regions of Northern Africa (11). Although the relative prevalence of the genotypes varies depending on the geographic region, genotype 1a and 1b are the most commonly encountered strains and account for approximately 60 % of global infections (506). The extent of global HCV incidence is difficult to assess, in part because systematic screening data is not always available from countries around the world, but also because acute, newly acquired, infections are often asymptomatic. Also, chronic liver disease may develop years after the initial onset of infection, and therefore most morbidity associated with chronic hepatitis C is usually realized during the development of these complications. Furthermore, the currently available diagnostic assays do not effectively distinguish between acute, chronic, and resolved infections, which may mask the degree of the viral epidemiology (11, 431). Thus, without an effective preventive vaccine program/therapeutic management strategy, as well as better

control of transmission spread, the extent of HCV incidence and the magnitude of global burden associated with it will likely continue to rise in the near future.

1.1.4. Risk Factors

The primary route of transmission for HCV is through percutaneous exposure to blood from infected individuals. Contaminated blood transfusions and needles have been the two major risk factors of contracting hepatitis C over the past few decades. After the implementation of screening tests in the 1990's, transfusion-associated HCV infection has been substantially decreased and is virtually eliminated in most countries that appropriately screen blood from donors (76). Today, the use of unsafe needles, whether in a medical setting or through illicit drug abuse, is the main source of HCV infections. Intravenous (i.v.) drug use through repetitive percutaneous injections is the major cause of hepatitis C in developed countries, with average prevalence rate among i.v. drug users between 68-80 % in countries such as USA and Australia (12, 124). In many developing nations, illicit drug use is also becoming an important source of HCV spread (495). However, nosocomial transmission remains also a major transmission route for the virus, accounting for up to 40 % of infections worldwide (190). In contrast, occupational, perinatal, and sexual (household or high-risk) transmissions remain low in incidence, and exhibit less geographical variation (11). Another particular group at risk for HCV infection and complications are individuals infected with another blood-borne pathogen, the human immunodeficiency virus (HIV). Many individuals infected with HIV, amongst which a significant proportion consists of i.v. drug users, are also infected with HCV (16-33 %)(431). In this case, HCV behaves much like an opportunistic infection, where co-

infection produces higher HCV titers and is associated with accelerated progression to liver cirrhosis (131, 442). Numerous human activities also possess the potential for percutaneous exposure to contaminated blood products, including tattooing, body-piercing, and religious or ritual practices, which might result in blood-to-blood contact and an increased risk in contracting HCV. Despite a decrease in the relative incidence of HCV infections worldwide with increased public education/awareness and improved diagnostic screening tests, the spread of HCV transmission is ongoing, albeit at much slower rate.

1.1.5. Pathogenesis And Clinical Manifestations

The most striking feature of HCV infection is its remarkable ability to induce a persistent chronic infection ultimately leading towards liver disease. In acute HCV infection, the virus incubation period ranges between 2-10 weeks, with viral RNA being detectable in the serum within 7-10 days after initial exposure (143, 203). Liver-associated serum enzymes, such as alanine aminotransferase (ALT) and aspartate aminotransferase (AST), become elevated weeks after the initial onset of infection, and are indicative of hepatic injury. Seroconversion, marked by presence of anti-HCV antibodies, becomes apparent approximately 50 days post-exposure (173). Clinical manifestations from acute HCV infection are usually asymptomatic or associated with a mild, non-specific, disease presentation such as abdominal pain, fatigue, and nausea. The risk for fulminant liver failure in acute hepatitis C is low (142). Whereas viremia will reach a stable level and remains fairly constant over a decade post-infection, serum ALT/AST levels usually tend to fluctuate and vary amongst individuals (464). Due to the

relatively late appearance of anti-HCV antibodies, as well as non-apparent and nonspecific symptoms associated with the initial exposure to the virus, acute hepatitis C is often unrecognized and difficult to diagnose.

In the natural course of hepatitis C, approximately 60-85 % of the infected individuals will become chronic carriers of HCV (51, 425). This is indicated by the presence of HCV RNA in the blood at least 6 months after the onset of acute infection. Only 15-40 % of infected individuals are able to achieve spontaneous virological resolution (51, 223, 425), with undetectable levels of HCV RNA and normalization of ALT and AST levels. Spontaneous viral clearance is usually observed in younger individuals (< 40 years), that are female, and that present symptomatic illnesses and vigorous innate/adaptive immune responses (98, 425, 464). Conversely, immunosuppressed males infected later in life, and presenting no jaundice or symptoms during the acute infection stage, tend to be at a higher risk for development of chronic HCV infection (87). These observations suggest that the immune status of the host may affect the clinical presentation of hepatitis C (as discussed in subsequent sections). Nevertheless, the vast majority of HCV-infected individuals establish a persistent infection, and over the course of 10-30 years following primary infection, 10-30 % of them will eventually progress to liver cirrhosis, and 1-7 % of these cirrhotic patients will develop HCC (464). Additional factors can accelerate progression to severe end-stage liver disease and HCC, including HIV/HCV and hepatitis B virus (HBV)/HCV co-infections (122, 123, 131, 442), as well as heavy alcohol consumption (40). Chronic hepatitis C has also been associated with various extrahepatic manifestations involving various organ systems, such as the endocrine, dermatologic, hematologic, lymphatic,

renal, and rheumatologic systems (19, 105, 132, 183, 523). These disorders include arthritis, cryoglobulinemia, glomerulonephritis, non-Hodgkin's lymphoma (NHL), porphyria, and type II diabetes. The mechanisms underlying these extrahepatic manifestations are not fully understood, however their clinical presentations have provided insight into the biology of the virus and its impact on the host with regard to the viral pathogenesis (as discussed later). The importance of these non-hepatic disorders is also highlighted by their potential to cause further disease burden to the natural course of chronic hepatitis C infection, which warrants considerations when providing antiviral treatments.

1.1.6. Viral Immune Interactions And Disease Progression

The variation within the pathogenesis of hepatitis C and the course of disease progression are thought to be largely associated with differences in the immune interactions that occur between the host and HCV. It is now known that HCV triggers and interacts with the host immune system at numerous different levels. The elicited host defense, particularly the intensity and quality of the adaptive immune response, as primed by the innate immune response of the host and intracellular antiviral innate immunity, determines the outcome of the infection. At the cellular level, HCV induces type I IFN production upon infection of hepatocytes. While the intrahepatic antiviral state provided by IFN can control or limit viral replication, HCV appears to use several mechanisms to subvert and attenuate the endogenous IFN response (discussed in later section). In the liver microenvironment, resident macrophages (Kupffer cells) are also known to play an important role in containment and clearance of foreign materials, and in recruitment of

natural killer (NK) cells and T cells during infections. NK cells can be frequently found in the liver, and their production of cytokines such as interferon-gamma (IFN-γ) can help to inhibit the viral infection and activate dendritic cells (DCs) and T cells responses (441). DCs, on the other hand, act as antigen presenting cells in the peripheral blood and tissues, and are important for producing type I IFNs and priming T cell responses. HCV proteins have been shown to influence the function and activation of Kupffer cells (121, 206), NK cells (468), and DCs (449). However, disparate results have been observed regarding the relative importance of these contributions to the outcome of HCV infection (305, 400, 520). While their specific role in hepatitis C remains to be determined, it is thought that their activities contribute to the control of viral pathogenesis. Alternatively, HCV may have also evolved measures to perturb their functions during the establishment of chronic infections.

There is a consensus of opinion and strong evidence regarding the role of T cell responses during HCV infection and the resulting effects on liver pathogenesis (67, 354). During the acute phase, the presence of a vigorous, multispecific (targeting multiple epitopes), and sustained HCV-specific CD4+ and CD8+ T cell response, is associated with a self-limited course of infection (1, 111, 281, 463). In an attempt to control the virus, CD8+ T cells serve an effector function in the clearance of the virus, whereas CD4+ T cells provide a helper function to maintain the CD8+ T cells; both are present in the liver and blood during the infection. The importance of these two T cell types in limiting HCV infection is further supported by depletion studies involving experimentally-infected chimpanzees. Persistent viremia is observed in the absence of CD8+ immune responses or CD4+ help, and protective immunity is achieved in the

presence of both (180, 433). However, during established chronic infections, HCV-specific CD4+ T cell responses are absent or weaker, while viral-specific CD8+ T cells can still be detected but are observed to be dysfunctional (112, 438, 472, 476). Several mechanisms have been proposed for the observed T cell failure during the establishment and response to chronic infections. These include lack of sufficient stimulation from innate immune cytokines produced by infected-hepatocytes (163), inefficient priming by antigen presenting cells (24), mutational escape from the virus (135), genetic impact of human leukocyte antigen (HLA) alleles (355), suppression by regulatory T cells (64), inhibition by the increased interleukin (IL)-10 secretion during HCV infection (238), and upregulation of the T cell inhibitory molecule programmed death 1 (PD-1) (27). Although the exact underlying mechanism in exhaustion of HCV-specific T cell responses remains to be clarified, it appears to be influenced by multiple factors.

With regard to the humoral response, although antibodies against HCV proteins can be detected in immunocompetent individuals at approximately 7-8 weeks post-infection, they do not provide protection from reinfection in either chimpanzees or humans (141, 271). Different mechanisms have been suggested for this inability to neutralize the virus (462), including the high rate of mutation in the viral glycoprotein E2 region, which may result in HCV variants that can escape recognition and binding of potentially neutralizing antibodies. Nevertheless, the role of the humoral response in mediating viral resolution remains poorly defined. Interestingly, chronic HCV infection causes B cell clonal expansion and secretion of non-HCV specific IgGs (immunoglobulin G) (389). This has been suggested to contribute to the increased manifestation of B cell-associated diseases such as mixed cryoglobulinemia (deposits of circulating

cryoglobulins) and NHL in chronic hepatitis C (274). From the provided evidence, it is understood that HCV stuns and evades the host adaptive immune system. In addition, HCV's high viral mutation rate allows for the constant modulation of immunogenic epitopes and production of rapidly evolving progenies, making it extremely difficult for antibodies, as well as B and T cells, to control or eliminate the infection.

Based on the above description of the current hepatitis C disease progression model, the natural history of HCV infection is largely defined by host immune interactions with the virus. It is thought that HCV efficiently establishes persistent infections by employing sophisticated strategies to subvert innate immunity of the cell as well as host innate and adaptive immune responses. As HCV incessantly escapes effective immune surveillance, the liver suffers from continuous damages due to ongoing ineffective cytotoxic T cell responses and the presence of viral factors, including proteins that appear to modulate apoptosis and steatosis (334). Persistently infected-hepatocytes produce inflammatory cytokines thereby further influencing local immunity through inflammation. They also secrete profibrotic molecules such as transforming growth factor-beta (TGF-β) that activates hepatic stellate cells to induce fibrogenesis. Over time, severe fibrosis can develop, which is then followed by liver cirrhosis and establishes a premise for the ensuing development of HCC. The end-stage liver diseases do not occur until approximately 20 years post onset of infection. These pathogenic features make HCV a remarkable pathogen at establishing an insipid but slow-progressing chronic infection.

1.1.7. Diagnosis

Advancements in molecular virological techniques have been instrumental in both the diagnosis of viral infections and in the monitoring of antiviral treatment efficacy. Serological tests for detecting anti-HCV specific antibodies (for instance anti-HCV core antigen) in either patient plasma or serum are based on third generation enzyme-linked immunosorbent assays (ELISAs) that detect antibodies directed against various HCV epitopes (90). Since up to 30 % of acutely infected patients can test negative for anti-HCV antibodies at the onset of symptoms (143) and a minority of chronic carriers of HCV could have normal levels of ALT and AST (73), serological tests and ALT/AST levels alone are not adequate to rule out the diagnosis of acute and chronic hepatitis C. At this time, the most reliable test for identifying HCV infection is by nucleic acid tests designed to detect HCV-specific RNA. This involves an amplification technique using polymerase chain reaction (PCR) and specific sequence detection using probe hybridization. Standard nucleic acid tests consist of qualitative assays, including qualitative PCR (AMPLICOR 2.0) and transcription-mediated amplification (VERSANT HCV RNA Qualitative Assay), as well as quantitative tests, such as branched-chain DNA (bDNA) amplification (Quantiplex bDNA 3.0), quantitative reverse-transcription PCR (MONITOR 2.0), and real-time PCR (TaqMan) (89, 424). For standardization purposes, the World Health Organization has established an international standard for universal quantification of HCV RNA units (90). The conversion factors allow comparisons and the use of any HCV RNA quantitative tests in a clinical setting (372). It is determined that 800 000 international units (IU)/ml corresponds to 2 million viral genome copies/ml, which is the threshold used in tailoring IFN-based treatment modalities (373, 424).

Qualitative tests have higher sensitivity with lower detection limits, ranging from 5-50 IU/ml and are used to confirm HCV infection. Standard quantitative tests are less sensitive (detection limit ranging 10-615 IU/ml) in comparison, but are useful for monitoring viral loads during treatment of hepatitis C. The TaqMan real-time PCR quantitative test achieves both sensitivity and specificity for quantitative and qualitative purposes, being able to detect between 10 IU/ml to 100 million IU/ml over a broad linear range (88, 424). Current HCV RNA diagnostic tests are therefore improving through the use of robust real-time PCR assay platforms.

The identification of HCV RNA in the absence anti-HCV antibodies with follow-up confirmation by seroconversion is strongly indicative of an acute HCV infection (90). On the other hand, the presence of both anti-HCV-specific antibodies and HCV RNA, with serum persistence of the viral nucleic acid for $\geq$ (greater than) 6 months, suggests diagnosis of chronic hepatitis C (88). One important aspect of HCV diagnosis is viral genotyping, which can be determined by both molecular RNA and antibody testing. Clinically, genotyping serves to guide treatment options and strategy, and also fulfill the purpose of prognosis and predicting treatment outcome. Another role that molecular diagnostic tools accomplish in the guidance of hepatitis C management is the monitoring of viral loads during antiviral therapy to reflect the virologic response to treatment. The current definition of successful treatment outcomes, in other words "viral clearance", is established as a sustained virological response (SVR). SVR is defined as the absence of detectable HCV RNA in the serum 24 weeks after the end of treatment using an HCV RNA assay with a lower limit of detection of 50 IU/ml or less (350). However, with the advent of more sensitive assays capable of detecting in the order of $\leq$ 2 IU/ml, the current

definition equating SVR to viral eradication has been challenged. The clinical occurrence of "occult infections" has been observed, whereby the HCV RNA persists at low copy number in peripheral blood mononuclear cells (PBMC) or in the liver tissues of patients who have achieved spontaneous recovery or SVR after anti-HCV therapy (380). Occult infection (< 200 virus genome copies/ml) falls below the range of standard detection tests, and has raised the need to implement more sensitive diagnostic assays in clinics as well as to revise the current definition of SVR (380). Despite these challenges, it is without doubt that these issues only further highlight the importance of accurate diagnostic tools and the critical role that they play in both the diagnosis and management of HCV infection.

1.1.8. Vaccines

There is presently no effective vaccine available to prevent HCV infection. Vaccination against HCV represents a difficult challenge due to several factors, including the propensity of the virus in promoting chronic persistent disease, the possibility of re-infection following re-exposure, and the highly mutable nature of the virus causing epitope heterogeneity (207). The observations of spontaneous recovery from HCV with robust and specific cell-mediated immunity (128), as well as the identification of neutralizing antibodies against divergent strains of the virus in the persistently-infected humans and convalescent chimpanzees (337), have created optimism regarding the development of a protective and therapeutic HCV vaccine. The initiative is further encouraged by several other findings: (a) antibodies directed against recombinant viral envelope glycoprotein can provide partial protection with reduced viral

loads after heterologous challenge in immunized chimpanzees (522); (b) a stimulatory vaccine directed against non-structural HCV genes is capable of protecting chimpanzees from acute hepatitis induced by challenge with heterologous virus (153); (c) recombinant HCV-like particles containing HCV structural proteins are capable of inducing strong and broad humoral and cellular immune responses in chimpanzees after infectious inoculum challenge (134). Ongoing research should reveal the immune correlates involved in the effective control of HCV, providing a model viral antigen that could be used as part of a protective vaccine. The absence of an effective vaccine also underscores the importance in understanding the viral-host interaction and highlights the need of a suitable research model for further investigation.

1.1.9. Standard Therapy For Hepatitis C

As vaccines against hepatitis C are not currently available, drug treatment remains the primary means of therapy in the treatment of HCV infection. The current standard treatment for chronic hepatitis C is a combination of pegylated (PEG)-interferon-alpha (IFN-α) and the broad-spectrum antiviral nucleoside analogue ribavirin (RBV) (350). Treatment decisions, including when to start or to prematurely end treatment, are dependent on multiple factors including the severity of the hepatic illness, prognosis of the liver disease, genotype of the virus, presence of contra-indications to therapy, and patient's willingness to undergo treatment (91). The end-point of treatment is at present determined by the achievement of SVR within 24 or 48 weeks of therapy depending on the genotype. The HCV genotype is systematically assessed prior to therapy, since it has been demonstrated to have a strong impact on treatment parameters (duration, dose, and

virological monitoring procedure) and on its likely success in managing the disease. The introduction of PEG-IFN-α, which has a prolonged biological half-life compared to conventional IFN-α, and its use in combination therapy with RBV, was considered a significant progress over previous IFN modalities (159). Unfortunately, the most frequently encountered HCV genotype, genotype 1, is also the most resistant to the current standard therapy, achieving SVR only in approximately 40-50 % of the infected individuals (159) compared to 70-80 % in patients infected with genotypes 2 or 3 (418). If patients infected with genotype 1 HCV do not show an early virological response (2-log viral load reduction or complete clearance of RNA) after 12 weeks of PEG-IFN-α with RBV, then the chance of treatment success is less than 1 %. In addition to the low SVR rate in the most prevalent genotype 1, the disadvantages associated with IFN therapies also include strict criteria for receiving therapy, considerable negative side effects (such as flu-like symptoms and depression), high cost, and low patient adherence. Furthermore, the reasons underlying the frequent failure of IFN-based therapeutic modalities are still poorly understood.

When standard therapies fail or are strongly contra-indicated, liver disease continues to progress over the course of 10 to 20 years. Once the liver has decompensated from advanced cirrhosis or HCC, the only treatment option available is liver transplantation following liver failure. However, a major problem associated with this surgery is the rapid reinfection of the graft (within days post-transplant) with selection for viral variants characterized by efficient entry into the new hepatocytes and poor neutralization by antibodies in the pre-transplant serum (139). In addition, liver transplantation is not always feasible, due to a combination of factors including donor

shortage, long waiting lists, and potential contra-indications to the procedure. The above observations suggest that alternatives to the current standard-of-care treatment for chronic hepatitis are warranted in order to better prevent the progression to end-stage liver diseases.

1.1.10. Novel Direct-Acting Antivirals Against HCV

Recent advances in our understanding of HCV, particularly in its biology, have led strong interest in the development of specific targeted antiviral therapy for hepatitis C (STAT-C). These agents, also called 'direct-acting antivirals' (DAA), consist of small molecule inhibitors that aim to cripple the HCV life cycle by specifically targeting viral enzymatic activities involved in HCV replication. The STAT-C list of compounds (recently reviewed in (376)) includes protease inhibitors such as boceprevir (SCH 503034) and telaprevir (VX-950), and polymerase inhibitors such as filibuvir (PF-00868554) and VCH-222. Many of these inhibitors are currently in preclinical development. The efficacy and proof of concept in abrogating virus production in using the STAT-C approach have been demonstrated in clinical trials (273). However, a major problem associated with these target-specific compounds that act on viral enzymes is the risk of the rapid development of resistance. Since HCV replicates at very high rates (356) and the viral RdRp has poor fidelity with no proof-reading function, a large number of variants are produced during the viral life cycle. Given the diversity in viral sequence, it is unlikely that a compound will exhibit equal effectiveness across all HCV genotypes, subtypes, and isolates (412). As such, the heterogenic pool that HCV generates during its replication will naturally include variants that will be less susceptible to these antiviral

agents. Thus, under drug selection pressure, the pool of viral sequences will have a high propensity to select for resistant variants capable of becoming dominant strains, even at some cost to overall viral fitness. Indeed, resistance has been characterized with almost every single STAT-C compound tested to date, making viral eradication especially difficult with the highly mutatable HCV (412).

1.1.11. Targeting The Host As Treatment Against Hepatitis C

An alternative approach to developing drug therapeutics is to target the host cell, rather than the virus itself. IFN therapy is part of this concept, whereby stimulation of the host antiviral innate immune response is used to limit the virus infection. The implication of this strategy and the importance of the host innate immunity in controlling HCV infection are further highlighted by recent genome-wide association studies of SVR to PEG-IFN-α/RBV combination therapy. Specifically, single nucleotide polymorphisms (SNPs) near the human *IL28B* gene, which encodes interferon-lambda (IFN-λ) 3 , are associated with outcome of treatment response as well as viral clearance in genotype 1 hepatitis C (171, 327, 394, 447, 458, 465). IFN-λ3 is classified in the family of type III IFNs (along with IFN-λ1 [*IL29*] and IFN-λ2 [*IL-28A*]), which are functionally similar to type I IFNs (161), but they activate antiviral immunity in fewer cell types because of the more restricted distribution pattern of the IFN-λ receptor (heterodimer composed of interleukin-28 receptor alpha chain [IL-28Rα] and the interleukin-10 receptor beta chain [IL-10Rβ]) (436). IFN-λ has been demonstrated to inhibit HCV replication (323, 365, 397) and is inducible by IFN-α (435). These genetic association and virological studies have recently spurred interest in developing therapies using IFN-λ against HCV infection.

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Early clinical trials with IFN-λ1 have shown promising results in chronic hepatitis C patients, displaying potent antiviral activities and fewer side effects compared to IFN-α (344). Future treatment regimens could therefore use IFN-λ as an alternate therapy to IFN-α-based modalities or in combination with IFN-α. In addition to IFNs, stimulators of components of the host cell innate immunity, such as Toll-like receptors (TLR) and the double-stranded RNA-dependent protein kinase (PKR) that sense common pathogenic signatures, have also been observed to inhibit HCV replication (205, 257, 283, 329, 399). This suggests feasibility in using ligands to these receptors as immunomodulatory agents in a complementary fashion to IFN treatment.

Besides host defenses that limit HCV infection, it is now known that the virological life cycle of HCV intersects with various other host cell pathways that are necessary for efficient viral multiplication. For instance, host factors that regulate and help HCV replication include cyclophilins that associate with HCV polymerase (191, 496), and heat shock proteins, which associate with viral non-structural proteins (174, 471). Indeed, inhibitors that target these host factors have been found effective in limiting the viral load (99, 152, 174, 470). Another example of host targeting is the recent application of antisense RNA molecule SPC3649 to sequester liver-specific microRNA-122 (miR-122) (276). The miR-122 plays a crucial role enhancing HCV replication (described later in more detail) and is a major tissue-specific microRNA (miRNA) that regulates a multitude of genes in the liver. Significant reduction of serum HCV RNA and cholesterol levels were observed in HCV-infected chimpanzees treated by intravenous infusion with SPC3649 for 12 weeks (276), and the antiviral effect was associated with concomitant improvements in liver histology. Remarkably, the viral pool of RNA within

the infected chimpanzees exhibited little genetic variability, with no variability observed in the miR-122 seed sites, and did not produce viral resistance over the course of the experimental treatment. Furthermore, no significant side effects were observed while the animals were under treatment (276). These data suggest that SPC3649 has strong potential as a therapeutic for use in treatment of chronic hepatitis C. From the above description, it is becoming clear that modulating the host in limiting the virus infection appears to be a foreseeable strategy with less risk of developing viral resistance. Clearly, an array of drugs with mechanistically different modes of action could complement and support each other, and allow development of tailored therapy specific to both genotype and patient responses to treatment. Future progress in developing effective treatments against chronic hepatitis C should involve a combination cocktail with therapeutics that target the host cell, the virus, and the interactions between them, to ultimately limit viral spread and maximize viral clearance.

1.1.12. Structural Biology Of HCV

The flaviviruses are enveloped virions composed of glycoproteins-inserted into a lipid bilayer, surrounding a nucleocapsid, and encompassing a positive sense single-stranded RNA (ssRNA) genome (295). HCV virions are spherical particles with a diameter of approximately 50-60 nm, based on filtration and electron microscopy (31). The core protein surrounds the viral genetic ssRNA and is encapsulated by host-derived membranes into which two envelope glycoproteins, E1 and E2, are embedded (FIG. 1A). The HCV genome is 9.6 kb long and encodes a single open reading frame (ORF) that is flanked by the two highly structured and conserved 5' and 3'untranslated regions (UTRs)

(FIG. 1B). There is an alternative ORF that overlaps the core gene, and is produced by ribosome shifting, generating a set of core-related proteins termed the alternate reading frame proteins (ARFPs) (69). The precise function of ARFPs is unclear, although RNA elements in this region may function in viral RNA translation and replication (331, 482). The 5' UTR contains signals important for both the translation and replication of the viral RNA. It includes six secondary structure domains namely stem-loops (SL) I-VI, and contains two tandem sites for binding of miR-122 (231) as well as an internal ribosome entry site (IRES) that mediates the cap-independent translation of a precursor polyprotein approximately 3000 amino acids (aa) in length. The 3' UTR has a tripartite structure and is known to be essential for HCV replication (510, 513, 515). Its composition starts with a variable sequence, followed by a poly(U/UC) tract, and finally ends with a highly conserved 3' X-tail that contains 3 SL structures (254). Besides its role in HCV RNA synthesis, the 3' UTR has also been observed to enhance the IRES-mediated translation of the HCV genome (437). Finally, in addition to those located in the 5' and 3' UTRs, *cis*-acting regulatory elements (CREs) have also been identified in the NS5B coding region as 3 SLs (5BSL3.1-5BSL3.3) (282, 469, 521). The 5BSL3.2 is shown to be absolutely required for viral RNA replication, and is thought to form a long-distance RNA-RNA base-pair interaction with the middle loop in the X-tail to mediate its effect (158).

The HCV polyprotein is co-translationally and post-translationally processed by both cellular and viral proteases to form three structural (S) elements (core [C], E1 and E2), one ion channel (p7), and six non-structural (NS) proteins (NS2, NS3, NS4A, NS4B, NS5A, and NS5B) (FIG. 1B). The structural proteins precede the p7 protein and are

located in the N-terminus of the polyprotein, whereas the remainder non-structural proteins are situated towards the C-terminus. Host signal peptidases allow release of the core, E1, E2, and p7. The core protein is further processed by the host signal peptide peptidase for maturation into a 21 kiloDalton (kDa) molecule, and is transported from the endoplasmic reticulum (ER) membrane to the surface of the lipid droplets (330), where HCV particle assembly is thought to occur (339).

The E1 (192 aa) and E2 (363 aa) envelope glycoproteins are extensively glycosylated, and they form a non-covalent heterodimer in the ER; this structure is believed to form the basic building block of the virus envelope. These two glycoproteins play crucial roles in mediating viral binding and entry, and E2 can associate with a number of cell-surface factors including cluster of differentiation 81 (CD81), scavenger receptor class B type I (SR-B1), and occludin (OCLN), all of which have been identified as putative receptors/co-receptors for HCV entry (48, 298, 386, 416). Within the E2, there are two hypervariable regions (HVRs), namely HVR1 and HVR2, which are viewed as hotspots for extreme sequence variability. Epitopes localized to these regions evolve rapidly, and are thought to be targets of neutralizing antibodies (144, 480), wherein adaptive immune pressure is considered to be primarily responsible for its high rate of mutation (145, 432).

The non-structural proteins, from p7 to NS5B, are presumed to be present in infected cells, but not in the mature virions. The p7 (63 aa) is a small hydrophobic protein that is classified in the viroporin family due to its similarity to other known ion channel proteins. Following its release from the polyprotein, p7 oligomerizes on the ER membrane to form ion-conductive pores (182, 370). While deletion of p7 does not appear

to affect HCV replication, it significantly abrogates infectious virion production, both in *in vitro* cell culture model (228, 439) as well as in intrahepatic transfection of chimpanzees (408). It is suggested that p7 likely participate in progeny virus assembly and release, although the precise mechanism of its action remains unclear.

The viral non-structural proteins are processed by two viral proteases, specifically the NS2/3 cysteine protease and the NS3/4 serine protease. The NS2/3 cysteine protease, which consists of the C-terminal domain of NS2 in conjunction with the N-terminal region of NS3, is responsible for the autocatalytic cleavage of the NS2-NS3 junction (197). It has a dimeric structure with composite active sites (308). Although NS2 is dispensable for viral replication, it is crucial for the production of infectious particles, both *in vitro* and *in vivo* (226, 228, 383).

The NS3 (69 kDa) is a multifunctional protein with serine protease activity provided by its N-terminus domain, and DExH/D-box RNA helicase as well as nucleotide triphosphatase (NTPase) activity produced by its C-terminus domain. The NS3 serine protease domain, which engages the NS4A polypeptide as a cofactor, is responsible for the cleavage of all of the remaining junctions in the non-structural portions of the polyprotein. This same protease activity also cleaves two cellular signaling molecules involved in the induction of the innate immune response, thereby blunting the host cell's antiviral response (described in later section). The C-terminal RNA helicase/NTPase activity of NS3 unwinds viral double-stranded RNA (dsRNA) or ssRNA regions in conjunction to ATP hydrolysis (453). Due to its vital enzymatic activities relating to viral protein processing and RNA replication (255, 272), the NS3

protein has long been a major target of antiviral therapeutics (protease and helicase inhibitors) development for the treatment of HCV infection.

The NS4A is 54 aa in size, and is required for efficient polyprotein processing. Besides functioning as viral protease cofactor, the NS4A provides stability to NS3, and is responsible for membrane anchorage of the NS3/4A complex via its transmembrane (TM) helix within the hydrophobic N-terminus (504). It also plays a role in viral replication by regulating NS5A phosphorylation (297).

The NS4B (27 kDa) is an integral membrane protein that is best recognized for its induction of intracellular membrane alterations, known as the 'membranous web'. This membranous vesicle compartment apparently acts as scaffold for the HCV replication complex and is presumably the site of viral RNA production (129). During this process, NS4B is thought to recruit other non-structural proteins of the replicase complex to this apparent site of RNA replication. Recently, binding of NS4B with HCV RNA has been reported (130), which further emphazises its role in viral RNA replication and highlights this viral protein as a possible novel therapeutic target (93).

The NS5A phosphoprotein exists in two forms: a basally phosphorylated form (56 kDa) and a hyperphosphorylated form (58 kDa) (236). The phosphorylation of the NS5A is mediated by cellular kinases, and although the exact role of the phosphorylation status is unclear, several lines of evidence suggest that it is involved in modulating viral RNA replication efficiency (20, 137, 351). This concept is further supported by the finding that adaptive mutations in the NS5A can significantly increase replication of HCV RNA subgenomes (59, 262, 302). More recently, it was reported that this viral protein can also participate in the assembly of infectious viral particles in cell culture (21, 461). Besides

being a factor in the production of HCV virions, the NS5A also harbors a potential 'Interferon Sensitivity Determining Region (ISDR)', in which sequence variations within this cluster may contribute to IFN-α resistance that is often observed with genotype 1 viruses (200).

The NS5B (68 kDa) is the RdRp for HCV. This enzyme possesses typical features shared by other polymerases including the classical right hand shape, finger, and thumb subdomains, but has a completely enclosed active site suggesting tight interactions between the finger and thumb subdomains (4, 70, 286). The NS5B is also membrane-associated, which is a characteristic that is dispensable for *in vitro* polymerase activity but critical for RNA replication in the cells (341). Since the structure of the NS5B has been extensively characterized, the viral enzyme has also emerged as a prime target in therapeutic development against HCV infection.

1.1.13. Viral Tropism

Thus far, the only identified susceptible hosts for HCV are the chimpanzees and humans. HCV virions primarily infect the hepatocytes, and the liver serves as the pathogen's primary tissue tropism as well as the site where most of the pathogenic effect occurs. Hepatocytes are highly differentiated and highly metabolically active cells that constitute the majority of the liver. Hepatocytes perform crucial functions, including major synthetic and metabolic activities, as well as important detoxification functions. The hepatic damage caused by HCV can inhibit the organ's normal functions and explain some of the clinically observed consequences, such as liver enzyme level fluctuation (ex. ALT and AST) and jaundice, as well as the most severe complications of disease

progression including fibrosis, cirrhosis, and HCC. Despite being hepatotropic, HCV RNA has been detected in PBMCs (including B cells, T cells, dendritic cells, and monocytes/macrophages) as well as in bone marrow cells and also in the central nervous system of hepatitis C patients (see review (54) and references within). Infection of immune cells is thought to be associated with immune disorders observed in chronic hepatitis C, and is also hypothesized to form the basis of occult infection, whereby the PBMCs serve as viral RNA reservoirs (379). However, it is unclear whether productive infection can occur in PBMCs. In cell culture, replication of HCV RNA has been observed in several non-hepatic cell types, including human embryonic kidney (HEK) 293 cells (9), B cells (445), T cells (256, 320), neuroepithelioma cells (150), cervical epithelial HeLa cells (244, 530), as well as cell lines of non-primate origin such as murine cells (82, 475, 530). This suggests that a non-hepatic and non-primate host setting can support HCV replication, although this has only been observed in cell culture and not all studies used an infectious HCV model. HCV displays very limited species tropism, and given the limited number of model systems that could be established with HCV (as explained later), it is recognized that host factors play an important role in determining permissiveness to HCV replication.

1.1.14. HCV Life Cycle

Significant efforts have been invested in deciphering the multitude of steps involved in the complete life cycle of HCV (FIG. 2). In the liver microenvironment, the viral life cycle begins with binding of the HCV E1/E2 glycoproteins to cell surface receptors, which leads to a receptor-mediated endocytosis event followed by pH-

dependent fusion and capsid release within an acidic endosomal compartment. Cellular factors implicated in HCV binding and entry include glycosaminoglycans (GAGs; such as heparan sulfate proteoglycans) (36, 259), low-density lipoprotein (LDL) receptor (LDLR) (3, 340), SR-B1 (39, 524), the tetraspanin family protein member CD81 (386), and tight junction proteins including claudins (CLDN-1, 6, and 9), (138, 332) and occludin (OCLN) (298, 387). Several studies have also indicated DC-SIGN (dendritic cell-specific intercellular adhesion molecule-3-grabbing non-integrin; CD209) and C-type lectin domain family 4 member M (CLEC4M; often referred as the liver/lymph node-specific L-SIGN which is also known as CD209L and DC-SIGN-related protein [DC-SIGNR]) as capture receptors for HCV particles through interaction with E2 (103, 168, 309). It is understood that HCV entry into the hepatocyte occurs as a multi-step process that successively engages entry factors in a time-point-specific manner. The current paradigm suggests that initial attachment probably occurs through interaction with GAGs, capture receptors such as DC-SIGN and L-SIGN, as well as lipoprotein receptors such as LDLR and SR-BI. This is followed by receptor attachment to CD81 which in turn forms a complex with SR-BI. The virus is then thought to be transferred to tight-junction proteins (CLDN-1, 6, and 9, and OCLN) that allow for the entry event via clathrin-mediated endocytosis. While the list of entry factors for HCV continues to grow, the exact role of most of these molecules and their precise coordination in mediating HCV entry remain to be elucidated. Furthermore, most of these molecules are not specific to the hepatocytes, and studies have suggested that the hepatotropism tendency of HCV may involve a balance of levels of the entry factors (258, 387), and/or the presence or absence of additional components that may render the surface of hepatocytes

receptive for HCV entry (398). Finally, recent reports suggest that HCV is capable of cell-to-cell transmission independent of the initial binding receptor CD81 (502). Further studies are therefore necessary to clarify what specific factors are involved in cell-free HCV entry and which are implicated in direct cell-to-cell spread.

Following successful HCV particle entry, the internalized clathrin-coated vesicles containing the virus is delivered to the early endosome. This event leads to endosomal acidification followed by fusion from within the endocytic compartment membrane and the uncoating of the virion (58). Upon release of the viral genome into the host cell, translation of the polyprotein is initiated via an IRES-directed, but cap-independent, mechanism (489), which is thought to occur at the rough endoplasmic reticulum (rER). During or shortly after the polyprotein processing (mediated by host cell peptidases and viral proteases), the replication complex is formed, presumably involving viral proteins, viral RNA, and host cell factors. Viral replication complexes are associated with altered host cell membranes, specifically the "membranous web" (175), and are also subject to intracellular transport involving ER, actin filaments, and the microtubule network (229, 270, 503). The NS5B protein begins synthesis of the complementary negative-strand RNA intermediate that then serves as a template to generate genomic positive sense RNA. The newly synthesized positive-strand RNAs are used for translation, employed in further RNA production, or become the genetic material for virion packaging.

Assembly of the nascent HCV particles is thought to occur through contact of the replicated genomes with the core protein on the cytosolic side of the ER membrane, and apparently employs lipid droplets (LDs) as potential scaffolds in the process (339). Both the core protein and NS5A participate in mediating the transfer of viral proteins and

genomic RNA from the replication complexes to these lipid droplets for virion assembly (21, 339, 429). Further maturation and release of progeny virions occurs through transfer of newly formed particles across the ER membrane, which enables these particles access to the secretory pathways involving the very low-density lipoprotein (VLDL) assembly system (recently reviewed in (230)). Although the precise mechanism of HCV virion morphogenesis and egress remains elusive, the process appears to involve interactions between various viral non-structural proteins and host cell factors pertaining to lipid and cholesterol transport. The association between HCV core protein with LDs is especially important, as it is required for infectious particle formation (66, 339). Through the VLDL secretory pathway, the resulting HCV progeny virions are associated with lipoproteins. The degree of lipidation of the secreted particles positively correlates with viral infectivity, with the most infectious low density particles coated in a mixture rich in triglycerides, apolipoprotein B-100 (APOB), and apolipoprotein E (APOE) (18, 170, 358, 364). These virions very much resemble VLDL particles, and have been designated as lipo-viral particles (LVPs) (18). Thus, HCV co-opts host lipid metabolism to facilitate its morphogenesis/egress. The nature of HCV virions acquiring a low-density lipoprotein-like configuration prior to release is also supported by the virus' use of lipoprotein receptors such as LDLR and SR-BI (a cholesterol transporter) during entry. The association between HCV and lipid metabolism/biogenesis also reflects the viral pathogenesis, since steatosis (an accumulation of LDs in the liver, also termed "fatty liver") and altered lipid profiles are common clinical consequences observed in chronic hepatitis C infection (348, 353).

HCV has a high rate of turnover. It is estimated that the half-life of the HCV virion is only 2-5 hours, and approximately 10^{10} to 10^{12} viral particles are produced and cleared each day in an infected individual (196, 356). The HCV NS5B RdPd has poor fidelity and lacks proof-reading capacity. The error rate is approximately 10^{-3} to 10^{-5} mutations per nucleotide during each round of genomic replication (33). Due to the error-prone nature and the outstanding high processivity of the HCV polymerase, a large number of HCV variants are produced continuously, forming a pool of quasispecies that supplies a constantly renewing population of closely related but genetically heterogeneous virions within the infected host. However, not all variants that are generated will replicate efficiently, because certain mutations will reduce viral fitness. Therefore, the pool is maintained with different viral subpopulations surviving at varying frequencies. Despite the "fitness cost" imposed on HCV replication, this inherent mechanism provides this pathogen with an advantage in the form of escape mutations from emerging humoral and cellular immune responses. Indeed, under host immune pressures, particularly driven by B and T cell immune epitopes in the HCV polyprotein, a dominant strain is constantly being selected for, from within the quasispecies pool. This strategy allows the virus to alter its antigenic epitopes thus escaping immunosurveillance (67, 145). The identification of antibodies- and T cells-selected HCV escape mutants in humans and chimpanzees (summarized in (395)) supports this notion, and is thought to be associated with the mechanism of viral persistence in chronic hepatitis C.

1.1.15. *In Vitro* Models For HCV

Development of experimental model systems for HCV has been challenging. Although the genome has been cloned since 1989, it was soon realized that the *in vitro* cultivation of this virus was experimentally difficult, and that the only *in vivo* model available and susceptible to natural HCV infection was the chimpanzee. Although infectious cDNA clones derived from chronic hepatitis C patients have been reported to infect chimpanzees (45, 253, 277, 509), none could be efficiently cultured *in vitro*. This scenario presented a serious roadblock to the study of the HCV life cycle and associated pathogenesis. Despite numerous attempts to cultivate this hepatotropic pathogen in various different cell types, including primary and immortalized hepatocytes (212-214, 401) as well as lymphocyte-derived cell lines (49, 106, 213, 242), most have been met with little success, yielding extremely low replication efficiency. Efforts have also been made to generate various chimeric viruses (312, 526) as well as surrogate models based on the related GB virus (43) as potential *in vitro* systems.

Ten years after its initial discovery, the first breakthrough for cell culture replication of HCV came with the development of a subgenomic replicon system, first reported in 1999 by Bartenschlager's group (303). Their approach was to generate self-replicating HCV subgenomes (Con1 isolates) that carried an antibiotic-resistance gene, which could be in turn be used for selection of cells permissive to its replication. The subgenomic replicon is composed of the UTRs, a selectable marker such as neomycin phosphotransferase (NPT) driven by the IRES in the 5' UTR, and a heterologous IRES (from encephalomyocarditis virus [EMCV]) that directs translation of the remaining polyprotein (from NS3 to NS5B). Successfully established cells harbor persistent

presence of the replicon RNA, as long as the cells are continuously maintained under antibiotic selection pressure. The bicistronic replicon system proved to replicate at very high efficiency and led to the identification of the Huh-7 hepatoma cell line as the most permissive cell type for HCV RNA replication. Since it was first described, several improvements and variations have been made to the HCV replicon system. These included identification of adaptive mutations that increase replication efficiency (59, 262, 302), variations within the marker cassette (such as using a luciferase gene) (262, 459), generation of genotype-specific (243, 514) or chimeric subgenomes (179, 284), and development of cell-based high-throughput screening assays (188, 531). The discovery of the HCV replicon system not only had a tremendous impact on our understanding of viral replication and virus-cell interactions, but also in providing a means of testing therapeutics against the HCV NS3 protease and NS5B polymerase.

Ensuing attempts were made to generate infectious virions using full-length replicons (60, 215, 384) or ribozyme-based constructs (194), which unfortunately either failed to produce infectious particles or exhibited virion production efficiencies that were suboptimal. Although HCV-like particles and pseudovirus (38, 151, 349) have been developed and allowed the study of viral entry into the cell, there was a long-standing urgent need for an available infectious model to examine other aspects of the viral life cycle. This quest was eventually answered by a second breakthrough that stemmed from the discovery of a high-replication molecular clone, JFH-1, discovered by Wakita's group (243). The JFH-1 (abbreviation for Japan Fulminant Hepatitis) genome was obtained from a "fulminant" hepatitis patient infected with genotype 2a HCV (245). The replication efficiency of subgenomes based on this HCV sequence was unprecedented,

yielding high replicative ability (a log order higher compared to Con1 replicons) without the requirement of adaptive mutations in Huh-7 cells (243). The inherent replication competency of this clone provided a foundation that became key to the development of complete cell culture system for HCV capable of supporting viral particle release. Transfection of Huh-7 and its derivatives with *in vitro*-transcribed full-length JFH-1 RNA, or recombinant chimeric genome of JFH-1 with another genotype 2a isolate, J6, produced viral particles that were infectious to naïve Huh-7 cells, chimpanzees, and chimeric mice with human liver xenografts (294, 296, 487, 528). Since the establishment of the infectious cell-culture produced HCV (termed HCVcc) model in 2005, the system has been improved to increase robust production of infectious particles. These include construction of chimeric JFH-1 genomes (intergenotypic and intragenotypic chimeras) (178, 383, 516), adaptation of virus variants by cell-culture passage (74, 246), introduction of mutations to HCV proteins (74, 115, 439, 516), and viral growth in highly permissive Huh-7 cell clones for HCV replication and entry (404, 528). All of these experimental studies have provided insight into our fundamental understanding of the HCV life cycle. In addition, several reporter virus systems based on the JFH-1 genome have been characterized (227, 228, 259, 324). To date, genotype 1a (H77) and genotype 1b (Con1) virus production systems have also been established (385, 517). The development of infectious cell culture model for HCV is regarded as an important milestone in the HCV field, and has enabled various fundamental and functional studies to be accomplished.

1.1.16. *In Vivo* Models For HCV

The chimpanzee remains the cornerstone and exemplar immunocompetent animal model for HCV infection. As described in previous sections, studies in chimpanzees have significantly contributed to our knowledge and efforts in the management of HCV over the past decades. These included identification of the virus, infectivity assays for *in vivo* modeling of molecular clones, analysis of viral kinetics and adaptive mutations, exploration of virus-host immunological interactions, and testing of vaccines and therapeutics. Despite being highly relevant in modeling human hepatitis C, experiments using chimpanzee as model system are usually resctricted due to ethical constraints, limited accessibility, high costs, and conservation issues due to its status as an endangered species. Alternative *in vivo* models to study HCV include using the tamarins (*Saguinus* species) (44) and the common marmoset (*Callithrix jacchus*) (71) as surrogate models for infection through the closely related GB-virus B (GBV-B), and also the treeshrew (*Tupaia belangeri*) as an alternate primate model (15). Small animal systems, such as rodents, would provide a much more amenable substitute as they could be genetically altered to more appropriately model human diseases. Nonetheless, it was soon realized that development of rodent models supporting natural HCV infection would be extremely complex, as mice are non-permissive to HCV growth. Several transgenic mouse systems have been developed, based upon the overexpression of specific HCV proteins, individually or together as polyprotein, in order to study its contribution to the viral pathogenesis. Some of these include HCV core, E1/E2, NS3/4A, and NS5A transgenic mice (see references (35), (224), and (261) for recent reviews). While transgenic models overexpressing viral proteins have helped to clarify some histologic

and pathogenic effects mediated by HCV components, they do not reflect the natural course of infection and the pathogenesis involved.

More recent small animal models consist of rodents transplanted with human liver or hepatoma cells. These include the immunotolerized rat engrafted with human hepatoma cells (507) and the HCV-trimera model (irradiated mice reconstituted with bone marrow cells from severe combined immunodeficient [SCID] mouse and transplanted with HCV-infected human liver) (216). However, viremia is low in these models, a shortcoming that makes it difficult to use for anti-HCV drug evaluation. The SCID/albumin-urokinase type plasminogen activator (Alb-uPA) mouse transplanted with normal human hepatocytes is currently the closest physiological model for modeling HCV infection (335). With the SCID background, the xenografted human hepatocytes are able to repopulate (up to 90 %) the mouse liver that has undergone considerable liver toxicity due to expression of the Alb-uPA transgene. This mouse, containing a chimeric human liver, was shown to be susceptible to HCV infection and generated high viral titers following inoculation with human serum from HCV-infected patients (335). The animals could also be infected with infectious viral clones (such as genotype 1a H77 and 2a JFH-1) as well as by different genotypes of the virus (198, 234, 252). The usefulness of this model is highlighted by its application in drug testing for several anti-HCV compounds (219, 251, 336, 481). While the chimeric human liver SCID/Alb-uPA xenograft mouse model represents an important achievement of small animal system for HCV, several disadvantages limit their widespread use. For instance, the immunodeficiency of these mice precludes proper immunological analyses unless reconstituted (through adoptive immunotherapy as an exmaple). Also, technical

challenges involved in the xenotransplantation, as well as the limited availability of human donor liver tissue, present difficulties associated with generation of this model. Thus, only a few laboratories around the world are able to produce and maintain these chimeric mice. Continuous improvements in developing mouse models for natural HCV infection are therefore still highly warranted.

It is now clear that the interaction between HCV and the host is extremely complex. Furthermore, it appears that multiple viral and host factors are involved in determining host permissiveness to the viral life cycle. Difficulties encountered in attempting to culture HCV in non-liver and non-primate cells, and the limited range of permissive hosts susceptible to viral replication, illustrate this notion. It is thought that beside viral determinants (such as adaptive mutations to enhance replicative ability), specific host factors shape the environmental setting necessary to allow the viral genome biogenesis to occur (32, 34, 301). Indeed, an exhaustive list of potential host factors has been identified in limiting or contributing to HCV replication (62, 343, 388). Clarifying the necessary host factors to support HCV life cycle in non-primate and non-hepatic cells is not only an important step in unraveling the biology of HCV, but also in providing a foundation towards simpler, and smaller, animal model for the virus. Two particular host cell components that can influence the success in establishing HCV replication are the innate immune response of the host cell and miRNA; they are the focus of the studies presented within this thesis and are discussed in the following sections.

1.2. Innate Immunity Of The Host Cell

1.2.1. Viral Sensors Of The Host Cell

The host cell innate immune system serves to provide cellular defense against foreign pathogens whose specific signatures or pathogen-associated molecular patterns (PAMPs) are recognized by an array of pattern recognition receptors (PRRs). These receptors are localized to the cell surface, in endosomes, or in the cytoplasm, and upon sensing foreign material the PRRs rapidly induce an anti-microbial response. The most well-recognized PRRs against viral signatures are the Toll-like receptors (TLRs; named after the Toll analogue in Drosophila) and the RNA helicases, which include retinoic acid-inducible gene-I (RIG-I) and melanoma differentiation-associated gene 5 (MDA5) (FIG. 3). Both arms of viral sensing culminate in the activation of proinflammatory cytokines as well as the type I IFN system, the latter being the universal defense mechanism of the host cell against viral infections.

1.2.1.1. *TLRs*

The TLRs play an important role in mediating antiviral effector mechanisms through triggering type I IFNs. They are expressed in most cell types, particularly in the immune cell populations. TLRs contain several extracellular leucine-rich repeats (LRRs) that directly or indirectly mediate pathogen recognition, and a cytoplasmic signaling domain homologous to that of the interleukin-1 receptor (IL1-R), termed the Toll/IL-1R homology (TIR) domain (68), which serves to recruit downstream signaling molecules. There are at least 13 members of the TLRs discovered so far in the mammals, although not all have been functionally characterized. Being transmembrane proteins, the TLRs

localize to cell surface (TLRs 1, 2, 4, 5, and 6) or within intracellular compartments such as the endosomes (TLRs 3, 7, 8, and 9), thereby providing the host cell with surveillance of the extracellular milieu and incoming material taken in by the cellular membranes (6).

Specific TLRs or subset of TLRs recognize distinct PAMPs from various microorganisms, ranging from bacteria to fungi, protozoa, and viruses. In particular, TLRs 2, 3, 4, 7, 8, and 9 have been shown to detect viral components. Although TLR2 and TLR4 are best known to be involved in the recognition of Gram-positive (lipoteichoic acid; LTA) and Gram-negative bacteria (lipopolysaccharide; LPS), respectively, they also sense several viral structural proteins. These include the respiratory syncytial virus (RSV) fusion (F) protein (266), mouse mammary tumor virus (MMTV) envelope protein (392), vesicular stomatitis virus (VSV) glycoprotein G (172), herpes simplex virus type 1 (HSV-1) (265) and human cytomegalovirus (HCMV) (102) glycoproteins, measles virus (MV) hemagglutinin (H) (52), HCV core protein (83, 97), and structural components from vaccinia virus (VACV) (26, 211). As TLR2 and TLR4 are expressed on the cell surface, it is thought that they aid in the sensing of viral infection during the process of virion membrane-associated entry events. Conversely, the endosomal TLRs 3, 7, 8, and 9 detect viral nucleic acids and are presumed to sense viral presence following internalization or virion lyses. TLR3 activation is mediated by dsRNA and its synthetic analogue polyinosinic:polycytidylic acid (poly[I:C]) (7). It can be activated by genomic viral dsRNA or replicative dsRNA intermediates, such as from reovirus (7) and HCV (491), respectively. The structurally conserved TLR7 and TLR8 are known to recognize specific sequence motifs in the guanosine- and uridine-rich ribonucleotides (119, 154, 164, 192), and also ssRNA from different viruses including

HIV (420), Sendai virus (SENV) (333), VSV (314), and Influenza A virus (IFA) (314). TLR9 recognizes the unmethylated 2'-deoxyribo(cytidine-phosphate-guanosine) (CpG) DNA motifs that are frequently present in microbial genomes including DNA viruses such a HSV-1 (263), HSV-2 (313), and murine cytomegalovirus (MCMV) (450). TLR9 can also be activated by synthetic CpG oligonucleotides (41). The compartmentalization of TLRs 3, 7, 8, and 9 not only allows rapid induction of innate immune response following uncoating of viral genetic material, but is also crucial in conferring detection of viral products upon uptake by phagocytic cells including dendritic cells and macrophages. This function can help drive antigen cross-presentation by the phagocytic cell populations (423). Therefore, TLRs also play an important role in the priming of the adaptive immune response.

1.2.1.2. *RLRs*

While cellular surface and endosomal compartments are monitored by TLRs, detection of viral presence in the host cell cytoplasmic space is achieved by PRRs sensing specific viral genetic materials. One of the most well known cytoplasmic viral sensors is the family of RIG-I-like receptors (RLRs), which are ubiquitously expressed in the cytoplasm of most cell types. Thus far, three members have been described, including RIG-I, MDA5, and laboratory of genetics and physiology-2 (LGP2). RIG-I and MDA5 consist of a DexD/H box RNA helicase domain, two N-terminal caspase-recruitment domains (CARDs), and a C-terminal repressor domain (519); both molecules mediate direct recognition of viral RNA species. LGP2 on the other hand lacks a CARD, and has been suggested to act as a regulator of RIG-I/MDA5 signal cascades (415, 483).

Although RIG-I and MDA5 share similar function in the cytoplasm, the exact nature of their substrates remains unclear. Furthermore, they seem to detect different viruses and RNA species. RIG-I appears to preferentially recognize ssRNA of various length with some partial double-stranded characteristics in conjunction to a terminal 5'-triphosphate (ppp) (42, 204, 240, 310, 381, 396, 421, 494). This 5'-ppp moiety is typically capped (5'-methylguanosine in mRNA) or processed upon maturation (such as in tRNA and rRNA) in cellular RNA species, and therefore is thought to allow discrimination between host self-RNA and viral non-self RNA. In addition, specific RNA sequences (uridine and adenosine-rich regions) within the RNA are also activators of RIG-I (407). While substrates for RIG-I can include RNA generated from *in vitro* transcription, viral replication, or from cleavage products produced by the host endonuclease RNase L (ribonuclease L) (322), RIG-I can also recognize short poly(I:C) (< 1 kb) which is a double-stranded polymer that does not contain 5'-ppp (240). In contrast, ligands for MDA5 in general consist of relatively long poly(I:C) (> 1 kb) and long viral genomic dsRNA intermediates independent of the 5'-phosphate (240). A recent study, however, showed that activation of MDA5 requires a higher-order structured RNA, or an "RNA web", which contains both ssRNA and dsRNA, rather than just long dsRNA (382). This observation suggests that recognition by MDA5 may involve a more complex mechanism.

Apart from RNA structures, RNA viruses are also differentially recognized by RIG-I and MDA5. Studies in cells and mice deficient in specific RLRs revealed that RIG-I plays a more important role in the recognition of paramyxoviruses (including SENV, Newcastle disease virus, MV, and RSV), VSV, and IFA (239, 241, 306). In

contrast, cells deficient in MDA5 respond normally to these viruses, but are unable to mount an antiviral response against picornaviruses such as EMCV, Mengo virus, and Theiler's virus. Furthermore, genomic RNAs of Hantaan virus, Crimean-Congo hemorrhagic fever virus, and Borna disease virus are also specific to MDA5 detection (184). While the flaviviruses JEV and HCV are recognized specifically by RIG-I, two other *Flaviviridae* members including WNV and DENV are detected by both MDA5 and RIG-I (157, 241, 306, 443). It is not completely understood why viruses are preferentially recognized by RIG-I or MDA5. However, the discrepancies observed in RNA species recognition with respect to the RNA structure, nucleotide composition, and length by the two RLRs is thought to reflect this differential preference in RNA virus detection.

1.2.1.3. *Other Viral Sensors: SR-As, DNA Sensors, and NLRs*

Several other PRRs may participate in mediating viral recognition, including PKR, class A scavenger receptors (SR-As), genomic DNA sensors, as well as the nucleotide-binding domain leucine-rich repeat containing receptors (NLRs). PKR is a dsRNA sensor that can be induced by IFN signaling and is discussed in later sections as an antiviral protein. The SR-As, which are located on the cell surface, have been suggested as PRRs for detection of microbial components (22). A recent report indicated that the SR-As also function as extracellular viral dsRNA binding receptors, whereby they mediate foreign dsRNA entry into cell for detection by cytoplasmic dsRNA sensors (TLR3, RIG-I, and MDA5), and as a result trigger intracellular IFN antiviral response (116). This finding is particularly interesting because it shows how viral dsRNA intermediates released from lysed infected-cells can be detected by surrounding healthy cells in the extracellular

milieu, but also explains the experimentally known antiviral effects of dsRNA treatment on cell surfaces. While the underlying mechanism is obscure, it remains to be determined whether the SR-As function merely as accessory sensors to other intracellular PRRs or if they can function as independent detectors of dsRNA.

Besides intracellular RNA sensors, cytosolic DNA sensors have also been reported, including DAI (DNA-dependent activation of interferon regulatory factors; also known as DLM-1 and Z-DNA-binding protein 1 [ZBP1]) and the PYHIN (pyrin and HIN [hematopoietic IFN inducible nuclear protein] domain-containing protein) family protein AIM2 (absent in melanoma 2) (422, 455). Upon DNA virus infections, DAI appears to trigger the IFN synthesis pathway (455), while AIM2 induces the production of interleukin-1 beta (IL-1β) via the inflammasome (393). Interestingly, recent findings also point to possible involvement of RLRs in sensing cytoplasmic DNA, although this effect seems to be indirect. In this novel pathway, microbial dsDNA is used as template for transcription by the RNA polymerase III (RNA Pol III) into 5'-ppp-containing dsRNA, which can potently activate RIG-I and trigger downstream type I IFN production (2, 92). Infections with adenovirus, HSV, and Epstein-Barr virus (EBV), were observed to provoke this RNA Pol III-RIG-I-mediated signaling. While the exact role of this pathway remains elusive, a recent study indicated that VACV E3 protein binds to the poly(A-U) RNA transcribed by RNA Pol III from the synthetic poly(dA:dT) dsDNA to prevent RIG-I activation (479). This observation emphasizes a role for this "indirect sensing" of cytosolic DNA in the surveillance of DNA viruses. However, exogenous DNA can still induce interferon-beta (IFN-β) that is not due to DAI or RNA Pol III. Recent identification of another intracellular DNA sensor, IFI16 (IFN-γ-inducible protein 16),

which is also a PYHIN protein, has been suggested to accomplish this link (474). IFI16 senses dsDNA from VACV and HSV-1, and mediates the IFN-β response via IFN regulatory factor (IRF)-3 and nuclear factor kappa B (NF-κB) activation. It remains to be determined what ligand specifically causes selective activation of these DNA sensors and how their signaling maps out during an antiviral response.

Finally, NLRs are typically known for their function in regulation of inflammatory and apoptotic responses, and as PRRs they have been mostly studied in the context of bacterial infections. NOD2 (nucleotide-binding oligomerization domain 2), which was previously believed to bind bacterial ligands, has been shown as a PRR that can also sense ssRNA from RSV and IFA to trigger IFN response via downstream adaptor molecule IPS-1 (IFN-β promoter stimulator 1) from RIG-I (402). Likewise, the cytosolic NLRC5 (NLR family, CARD domain containing 5) is an NLR that has recently been investigated for its role in innate immunity of the cell. It was observed that NLRC5 is inducible by poly(I:C) and SENV infection, and that siRNA-knockdown of this protein reduces SENV- and poly(I:C)-mediated type I IFN-dependent responses (352). Another NLR, NLRP3 (NLR family, pyrin domain containing 3; also known as NACHT-, LRR- and PYD-containing protein 3 [NALP3] and cryopyrin), has recently been shown to be implicated in the inflammatory response control of DNA and RNA viruses. In response to infections from adenovirus, VACV, and IFA, the NLRP3 inflammasome (consisting of NLRP3, its adaptor molecule, apoptotic speck-like protein containing a CARD [ASC], and caspase-1) is stimulated, resulting in cellular production of IL-1β (10, 114, 346). In these recent studies, however, the mechanism through which NLRs sense the viral

nucleic acids (directly or indirectly) remains undefined, and their relative contribution versus other known cytosolic sensors needs to be further examined.

1.2.2. Signaling of Viral Sensors

The primary outcome of signaling mediated by PRRs is the production of chemokines and cytokines involved in antiviral and inflammatory responses. Signaling mediated by TLRs and RLRs generally results in activation of IRFs and NF-κB, which induces cellular expression of type I IFN and proinflammatory cytokines (FIG. 3). Detailed signaling pathways for the host cell PRRs have recently been reviewed elsewhere (248, 457, 518). For simplicity, an overview of the main PRR signaling cascades for viral infection is described below with focus given to pathways leading to activation of key transcription factors, including IRF-3 and NF-κB.

1.2.2.1. *TLR Signaling*

TLR signaling involves four primary adaptor molecules to relay downstream signaling, including the myeloid differentiation factor 88 (MyD88), MyD88-adaptor-like (Mal, also known as TIR-associated protein [TIRAP]), TIR domain-containing adaptor inducing IFN-β (TRIF, also known as TIR-domain-containing molecule 1 [TICAM-1]), and TRIF-related adaptor molecule (TRAM). Selective use of these adaptors allows distinct activation of downstream gene products in response to the specific TLR ligand stimulation. The TLR signaling network is broadly characterized as MyD88-dependent or MyD88-independent. TLR3 specifically transmits its activation signals in a MyD88-independent fashion by using TRIF, whereas TLR7/8 and TLR9 engage only MyD88 as

an adaptor. TLR2 signals through MyD88 via Mal. TLR4 is unique in its ability to signal through both MyD88-dependent and MyD88-independent arms due to its differential use of the TRAM (to feed into TRIF) and Mal (for recruitment of MyD88) adaptors. A fifth adaptor molecule, SARM (sterile alpha- and armadillo-motif-containing protein), appears to be a negative regulator of the mitogen-activated protein kinase (MAPK) activation downstream of TRIF and MyD88 (375).

In the TLR3 signaling pathway, interaction of TRIF with tumour necrosis factor (TNF) receptor-associated factor 3 (TRAF3) activates the TRAF-family-member-associated NF-κB activator (TANK)-binding kinase 1 (TBK1), and the non-canonical inhibitor of NF-κB (IκB) kinase ε (IKKε, also known as inducible IκB kinase [IKK-i]). Activation of TBK1 (also known as NF-κB-activating kinase [NAK] and TRAF2-associated kinase [T2K]) and IKKε directly causes phosphorylation of IRF-3 at specific serine residues that leads to its homodimerization and translocation into the nucleus. TRIF also signals through TRAF6 and receptor-interacting protein1 (RIP1) to stimulate the TGF-β-activated kinase 1 (TAK1) complex (associated with TAK1 binding proteins [TAB]) for NF-κB activation. This occurs via the canonical IKK complex (IKKα/β/γ; IKKγ is also known as NF-κB essential modulator [NEMO]), which leads to NF-κB phosphorylation and nuclear translocation. Signaling through TAK1 complex also causes phosphoactivation of the MAPK pathway, which results in phosphorylation of c-Jun N-terminal kinases and p38 to activate the activator protein 1 (AP-1; also called c-Jun/activating transcription factor 2 [ATF2]). The transcription factors IRF-3, NF-κB, and AP-1 serve to initiate transcription of IFN and proinflammatory cytokines. These signaling cascades are also regulated by ubiquitination and deubiquitination processes.

Deubiquitination of TRAF3 (249) and TRAF6 (65) inhibits activation of IRFs and NF-κB, respectively. In addition, recruitment of the phosphatidyl-inositol 3-kinase (PI3K) by TLR3 is also essential for full IRF-3 activation. Following activation of TLR3, PI3K is recruited and stimulates the downstream kinase Akt to cause additional phosphorylation of IRF-3, which appears to be necessary for the interaction with the transcriptional co-activator cAMP response element binding (CREB)-binding protein (CBP) (411).

In contrast to TLR3, all other TLRs (including TLR2 and TLR4) can use the MyD88-dependent pathway that typically leads to activation of NF-κB and MAPKs for expression of proinflammatory cytokines. This is achieved by MyD88 triggering of downstream kinases including members of the IL-1 receptor-associated kinase (IRAK) family (IRAK4 and IRAK1), TRAF6, and TAK1. In addition, IRF-5 can also be stimulated by TRAF6 for induction of genes involved in the inflammatory response (456). While TLR2 is known to trigger MyD88-dependent NF-κB proinflammatory cytokines, a recent study however suggests that it may also selectively induce IRF-3- and IRF-7-dependent type I IFN in response to viral ligands (26). Whether this is due to the ligand specifically and directly activating downstream gene production, or the differential use of a co-receptor, remains to be elucidated. In pDCs, however, TLR7/8 and TLR9 are known to rely on MyD88 for induction of both proinflammatory gene products and type I IFNs. In this cell type, nucleic acid recognition by TLR7/8 and TLR9 causes MyD88 to form a complex containing IRAK1, IRAK4, TRAF3, TRAF6, IKKα, and a precursor of osteopontin (OPN). This complex then engages and phosphorylates the constitutively expressed IRF-7 (a specialized feature in pDCs that is not usually seen in other cell types), which leads to its activation, nuclear translocation, and subsequent transcription of IFNs.

Signaling via TRAF6 also causes NF-κB and IRF-5 stimulation for production of proinflammatory cytokines (248).

1.2.2.2. *RLR Signaling*

For RLR signaling, the mitochondria-located adaptor molecule IPS-1 (also known as mitochondrial antiviral signaling [MAVS], virus-induced signaling adaptor [VISA], and CARD adaptor inducing IFN-β [Cardif]) relays signal for both RIG-I and MDA5 via its CARD-like domain. Upon non-self nuclei acid recognition, RIG-I/MDA5 undergoes conformational change and engages IPS-1 for CARD domain interaction. Formation of the RIG-I/MDA5-IPS-1 complex on the mitochondria causes further assembly of proteins that initiates downstream signaling. Molecules recruited for this downstream signal relay include TRAF3, TRAF6, RIP1, TNF receptor-associated death domain protein (TRADD), Fas-associated death domain protein (FADD), caspase-8, and caspase-10. The assembled complexes activate TBK1 and IKKε for phosphorylation of IRF-3 and IRF-7, thereby inducing an IFN response; it also stimulates the kinase activity of the IKKα/β/γ complex which leads to NF-κB activation. Involvement of stimulator of interferon genes (STING) (also known as mediator of IRF-3 activation [MITA], endoplasmic reticulum IFN stimulator [ERIS], or MPYS [named after its N-terminal 'methionine-proline-tyrosine-serine' amino acid sequence]) in mediating the IPS-1-IRF-3 activation has recently been suggested, although the precise mechanism remains unclear (220, 225, 444, 527). Interestingly, STING has been shown to interact with the dsDNA sensor IFI16, and is required for IFN-β induction of the latter in response to its ligand stimulation (474). This suggests that, similar to TLR signaling, the different cytosolic DNA and RNA sensors

may share common mediators of signaling relay for induction of downstream antiviral response.

1.2.2.3. *NLR and DNA Sensor Signaling*

Finally, NLRs are implicated in proinflammatory cytokine production, including IL-1β and IL-18. They achieve this through triggering of the inflammasome, which is a complex composed of specific NLRs that upon stimulation, oligomerizes and associates with CARD-containing adaptor molecules as well as caspase-1 through the CARD–CARD interactions. The result from this interaction induces the self-cleavage and maturation of caspase-1, which then processes pro-IL-1β and pro-IL-18 to release their mature forms for secretion. The DNA sensor AIM2 utilizes this pathway for induction of the proinflammatory cytokines (393), whereas DAI implicates TBK-1-IRF-3 signaling to induce IFNs upon sensing foreign dsDNA (455).

Production of type I IFNs, proinflammatory cytokines (e.g. IL-1β and TNF-α), and chemokines (such as IP-10 [IFN-inducible protein 10] and RANTES [regulated upon activation, normal T cell expressed and secreted]) from signaling of the PRRs not only drive the antiviral response, but also serve to recruit and orchestrate the adaptive immunity by enhancing NK cell function, activating immature DCs, and priming T and B cells for their survival and effector functions. Thus the ability of the PRRs to impart the adaptive immunity is crucial for host defense against a variety of viral infections (235, 241, 260).

1.2.3. The Type I IFN Response

1.2.3.1. *Induction of Type I IFNs*

The IFNs, including type I (IFN-β and over 12 IFN-α subtypes depending on the species), type II (IFN-γ), and type III (IFN-λ with three subtypes), play an essential role in the host immune response and mediate critical functions against viral infections. The type I IFNs are the most commonly induced cytokines during a viral infection. These multifunctional proteins act in an autocrine and paracrine manner to activate host cell antiviral responses and also alert the surrounding cells of the viral invasion. As described previously, upon detection of viral signatures by PRRs, key transcription factors including IRF-3 are turned on (FIG. 3). The activated IRF-3 translocates to the nucleus and together with NF-κB, AP-1, and the nuclear architectural protein HMG-I(Y) (high mobility group protein [non histone chromosomal] isoform I and Y) assemble into a stereospecific enhanceosome complex on the IFN-β promoter; this results in further recruitment of other molecules, including the coactivators CBP and/or p300, to initiate transcription and synthesis of IFN-β (366). In most cell types including fibroblasts, IRF-3 is constitutively expressed in the cytoplasm, whereas IRF-7 is only expressed upon induction by IFNs (413). Without the need for *de novo* synthesis, activation of IRF-3 therefore allows direct production of IFN-β, thereby permitting the host cell to quickly respond to the viral threat. Expression of additional IFN-α subtypes and amplification of the IFN response occur upon signaling elicited by binding of IFN-β to its receptor. This second phase requires activation of latent transcription factors such as IRF-9 and those induced for expression by IFNs such as IRF-7 (414). In addition, both IRF-3 and IRF-7 can form homodimers or heterodimers with each other, which permits differential

activation of the IFN genes (199). The preferential activation of the IFN-β promoter by IRF-3 is due to its restrictive DNA binding ability, which is reflected by its selective induction of the IFN-stimulated regulatory element (ISRE)-containing genes (199). In contrast, IRF-7 has a broader DNA binding specificity and is capable of inducing both IFN-α and IFN-β efficiently, thereby contributing to the diversification of IFN-α subtypes and amplification of the primary IFN response. Thus, the observed biphasic IFN-α/β gene induction is attributed to the differential expression and use of the IRFs, and also to the binding specificity provided by these transcription factors. The broad spectrum activation feature from IRF-7 explains why the pDCs, which have constitutively expressed IRF-7, are potent at secreting IFN-α and quick in mounting antiviral responses, which is consistent with their role as immune sensors in viral infections (202). Furthermore, this characteristic of IRF-7 also explains its low expression in normal cells and its short half-life (approximately 30 min to 1 h) (414), possibly a protective mechanism for the cell to keep the full IFN response transient and to avoid harm from its overexpression.

1.2.3.2. *Antiviral Signaling of Type I IFNs*

Antiviral proteins are produced from signaling events mediated by the secreted IFN-α/β binding to its receptor, the IFNAR (IFN-α/β receptor) (477) (FIG. 4). Ligand stimulation of IFNAR transmits downstream signals via the Janus kinase (JAK)-Signal transducer and activator of transcription (STAT) pathway (419). Following binding of type I IFNs, activated JAK members (including JAK1 and tyrosine kinase 2 [TYK2]) phosphorylate STAT proteins (such as STAT1 and STAT2) at specific tyrosine and

serine residues. Phosphorylated STAT1 and STAT2 translocate to the nucleus and form a heterotrimeric complex termed 'the IFN-stimulated gene factor 3 (ISGF3)' with the latent protein IRF-9 (also known as p48 or ISGF3γ) to activate transcription of IFN-stimulated genes (ISGs). ISGF3 recognizes and binds to ISREs in the promoter of the target genes. Alternatively, STAT1 homodimer complex (also known as the IFN-γ-activated factor [GAF]) binds to IFN-γ-activated site (GAS) to induce a subset of ISGs.

Over 300 ISGs have been identified (113) and they provide the host with the means to hinder the virus life cycle as well as to limit virus spread and help eliminate virus-infected cells. Some of these antiviral proteins include PKR, 2',5' oligoadenylate (2',5' [A]) synthetase (2',5'-OAS)/RNase L, myxovirus-resistance proteins (Mx), ISG15, ISG56, and dsRNA-dependent adenosine deaminase (ADAR). Of particular importance is PKR, which is a serine/threonine kinase that is stimulated by binding dsRNA from viral genomes or replicative intermediates. PKR exists as a latent protein, and upon ligand-binding activation, it becomes autophosphorylated, which permits it to then phosphorylate the α subunit of the eukaryotic translation initiation factor-2 (eIF-2). Phosphorylation of eIF-2α causes it to competitively bind to the guanine nucleotide exchange factor eIF-2B, thereby rendering eIF-2B unable to mediate exchange for guanosine triphosphate (GTP) (167). As eIF-2α is a critical cofactor required for the recruitment of initiator methionine transfer RNA (Met-tRNAi) to ribosomes to form the translation pre-initiation complex, the lockdown of phosphorylated eIF-2α with eIF-2B prevents recycling of initiation factors and thus shuts down the host translation machinery. Due to the low abundance of eIF-2B with respect to eIF-2, a small quantity of phosphorylated eIF-2α can cause an immediate stop to translation, thereby effectively

preventing viral protein synthesis. Furthermore, PKR also affects various signal transduction cascades in response to cellular stress, such as from viral infections, and has been shown to affect transcription regulation and apoptosis (166).

Another potent antiviral effector is the IFN-inducible 2',5'-OAS system, which involves the endoribonuclease RNase L. Upon viral infection, binding of dsRNA stimulates 2',5'-OAS to produce adenosine oligomers (2',5' [A]) which interacts and triggers RNase L (403). The activation of RNase L results in the cleavage-mediated degradation of viral ssRNA and cellular mRNA, which prevents template feed-in for protein synthesis. As cleavage products from this endonuclease can also activate RIG-I (322), the RLR signaling response is amplified during an antiviral response, and can thus further increase the activity of RNase L. Consequently, a viral overload may also result in excessive cellular RNA degradation, which can trigger apoptosis to quickly eliminate the heavily viral-infected cell.

1.2.4. Induction And Evasion Of Host Cell Innate Immunity By HCV

Upon infection of the hepatocyte and interaction with immune cells, HCV triggers the host cell innate immunity via several of the PRRs for production of ISGs as described above. In particular, both RIG-I (307, 443) and TLR3 (491) have been shown to sense HCV RNA and contribute to the host cell's efforts in restricting its replication through induction of IFN-β. The poly(U/UC) tract at the 3' end along with the 5'-ppp in the HCV genomic RNA is thought to be the major HCV molecular recognition pattern by RIG-I (407) whereas the viral dsRNA intermediate is targeted by TLR3 (491). In addition, TLR7 (454, 525) and TLR2 (83, 97) are also implicated in detecting HCV ssRNA and

core protein, respectively. While HCV PAMPs activate the PRRs and induce antiviral/proinflammatory cytokines production, the virus has evolved multiple means to subvert the host cell innate immune response. Importantly, HCV NS3/4A is able to cleave TRIF (288) and IPS-1 (290, 338) to attenuate TLR3 and RIG-I signaling, respectively. The NS3/4A-mediated proteolysis of TRIF and the dislocation of IPS-1 from the mitochondria inhibit the activation of the common downstream IRF-3 and NF-κB transducers, thereby preventing type I IFN production and facilitating establishment of HCV replication (155, 156). Indeed, a dsRNA-activated innate immune response is observed to be severely compromised upon ectopic expression of the HCV NS3/4A in normal human primary hepatocytes (233). In agreement with these observations, pharmacological inhibition of NS3/4A is capable of restoring the disrupted RIG-I signaling cascade (155, 233, 307). Furthermore, HCV NS3 (a part of the NS3/4A) can also bind to TBK-1 as a competitive inhibitor, thus further contributing to interference with IRF-3 phosphoactivation (363). Implication of the HCV NS4B in disrupting the RIG-I signaling pathway has also been suggested (460). Besides TLR3 and RIG-I, TLR7 is targeted as well by HCV. Replication of HCV RNA reduces expression and function of TLR7 in hepatoma cells, although the exact mechanism remains enigmatic (84).

Apart from inactivating cellular surveillance and preventing production of type I IFNs, HCV also impedes the IFN signaling and the resulting antiviral responses, apparently through multiple mechanisms. HCV core protein can interact with STAT1 to inhibit is function (293). Moreover, presence of HCV and its core protein seem to increase cellular levels of the JAK/STAT signaling inhibitor SOCS3 (suppressor of cytokine signaling 3), which dampens the IFN response (63, 209). Several studies have

also provided evidence that HCV-induced ER stress upregulates the protein phosphatase 2A (PP2A), which in turn causes binding of STAT1 with its inhibitor PIAS1 (protein inhibitor of activated STAT1), hence further resulting in negative regulation of the IFN signaling cascade (96, 125, 126). Finally, a correlation has been drawn for a role of the ubiquitin-specific peptidase 18 (USP18, also known as USP43), which is an IFN-inducible ISG15 deconjugating enzyme, in the establishment of chronic hepatitis C. USP18 can negatively regulate the JAK/STAT pathway by interacting with IFNAR (in its IFNAR2 subunit) (321), and is highly expressed in livers with chronic HCV infections and in IFN-α treatment non-responders (86, 410). Consistently, silencing of USP18 by RNA interference (RNAi) potentiates the antiviral activity of IFN against HCV infection *in vitro* (390). How HCV directly enhances USP18 expression is still unknown.

At the antiviral response level, several reports also indicate evidence of HCV interfering with host antiviral proteins. For instance, HCV can antagonize PKR activity through its NS5A and E2 proteins (369, 378), and also its IRES-containing domains in the viral RNA (466). The importance of PKR in repressing HCV is illustrated by its antiviral effects on HCV replication *in vitro* (81, 237). Moreover, it is known that a correlation exists between response to IFN therapy and mutations located within the ISDR of the PKR-interacting NS5A (368). The 40-residue stretch in the ISDR, along with the 26-amino acid region adjacent to it, constitutes the PKR binding domain (PKRBD), which is implicated in binding and inhibition of PKR by NS5A (162). Interestingly, recent studies have provided additional insight into the mechanism by which HCV interacts with PKR. It was shown that although HCV infection can activate PKR and induce eIF-2α phosphorylation, the virus however exploits this alarm to

negatively control the antiviral functions of IFN (23, 165). HCV therefore diverts PKR away from its antiviral role, and use it to its advantage to instead attenuate both the IFN-triggered antiviral response and the IFN induction itself. Finally, another effector mechanism subdued by HCV is the 2',5'-OAS/RNase L pathway. The viral NS5A protein can bind to 2',5'-OAS and inhibit its activity (451). Furthermore, it has been previously proposed that HCV viral genomes from IFN-resistant genotypes (1a and 1b) exhibit mutations with poor recognition by RNase L, thereby escaping from the antiviral endonuclease activity (187).

From the above discussion, it is clear that host cell innate immunity plays an important role in restricting the HCV life cycle, and conversely, HCV possesses multiple strategies by which it subverts and evades the host antiviral response. These evasion mechanisms not only contribute to creating a permissive environment for viral replication, assembly, and secretion, but also aid the conversion of an acute phase disease into a chronic phase infection.

1.3. MicroRNA And HCV

1.3.1. Overview Of MiRNA

The miRNAs are a class of endogenously synthesized small non-coding RNA approximately 22 nucleotide (nt) in length, that regulate gene expression by targeting mRNAs for translational repression or degradation by cleavage. They are derived from precursors possessing a characteristic stem-loop or hairpin structure, and are evolutionarily conserved. These short duplex RNA sequences achieve gene regulation via imperfect ssRNA binding to the 3' UTR of target mRNAs that carry complementary sites

(149), and about 60 % of mammalian genes may be subjected to this form of regulation (160). Due to their regulatory function, miRNAs can help to shape host transcriptomes and proteomes, and thus play a pivotal role in cellular physiology. They are involved in the fine-tuning and modulation of host genes in a broad range of biological pathways in the eukaryotic cell, including cellular development, differentiation, proliferation, metabolism, maintenance, immunity, and death (13, 77, 417). Because of their understood role in numerous crucial cellular functions, dysregulation of miRNAs is associated with a variety of human diseases including cancer and virally induced illnesses (169, 176). Computational prediction and experimental analyses have indicated that a single miRNA can target multiple, potentially hundreds, of mRNAs (29), and several miRNAs can target the same gene (377). The miRNAs can thus selectively regulate spatio-temporal and global gene expression pattern of mRNAs in a host cell (77). Since their first discovery in *Caenorhabditis elegans*, over 1000 miRNAs have been identified within the human genome (miRBase, release 16, http://www.mirbase.org).

1.3.2. Biogenesis Of MiRNA

The biogenesis of miRNAs in animal cells involves 3 successive manufacturing stages, beginning with: (i) generation of a primary transcript of miRNA (pri-miRNA) from genes encoding them, (ii) partial processing of the pri-miRNA in the nuclei to form a precursor form of miRNA (pre-miRNA), and (iii) additional processing upon translocation to the cytoplasm to create a mature miRNA (FIG. 5). In the canonical pathway of miRNA biogenesis, the miRNA sequence is transcribed by RNA polymerase II (RNA Pol II) as long pri-miRNAs (in the order of hundred to thousand nucleotides)

that contain hairpin structure (501). The pri-miRNAs then undergo first round of processing in the nucleus by the "Microprocessor" (consisting of the ribonuclease [RNase] III enzyme Drosha and the nuclear protein DiGeorge Syndrome Critical Region 8, DGCR8 or "Pasha" in invertebrates) to form 60-70 nt pre-miRNA stem-loops. Following active transportation into the cytoplasm by the nuclear export factor Exportin-5 (Exp5), the pre-miRNAs are further processed by the RNase III enzyme Dicer complex to release an approximately 22 nt mature duplex of miRNA (28). This hairpin sequence of mature miRNA contains a functional "guide strand" complementary to the target mRNA, and a "passenger strand", which is degraded. The guide strand selectively associates with Argonaute (AGO) 2 protein to form the core of the RNA-induced silencing complex (RISC; also called the 'miRISC' or miRNA-induced silencing complex). Once the mature miRNA is loaded into the RISC, it guides the complex to the 3' UTR of the target mRNA for interaction, resulting in either repression of mRNA translation (following imperfect base-pairing) or cleavage and degradation of the mRNA (when perfect complementary binding occurs) (501). Binding specificity for the target recognition is thought to be mediated by the "seed" region of the miRNA (29). There are several other pathways for the biogenesis of miRNA that are essentially variations of the canonical mechanism. Although their precise function is unclear, it is thought that these alternative pathways to generation of miRNAs may serve to provide flexibility to the cell and to regulate activity of canonical miRNAs (501).

1.3.3. Involvement Of MiRNA In Viral Infections

1.3.3.1. *MiRNA In Antiviral Defense*

There are several scenarios in which the miRNA mechanism can be implicated in viral infections. Both viruses and host cells are known to encode miRNAs. This small RNA pathway can be involved in regulating host immune defenses to counter viral infections, or become corrupted by the pathogens to reprogram host cell mRNA network to their own advantage. In terms of host defense, miRNAs can impact host control of viral infections by modulating the development, maturation, survival, and function of immune cell populations involved in the adaptive and innate immunity (300). At the cellular level, miRNAs have been implicated in limiting viral replication. For instance, the miR-32 has been determined to contribute to the repression of the retrovirus primate foamy virus type 1 (PFV-1) in cultured human cells (280). The antiviral effect of miR-32 is apparently achieved by binding to partially complementary sites in the 3' UTR of PFV-1-derived mRNAs, and consequently results in the downregulation of PFV-1 replication. Similarly, mice deficient in Dicer, which results in disrupted miRNA biogenesis, are observed to be hypersensitive to VSV challenge (362). The phenotype was attributed in part to the loss of miR-24 and miR-93 expression, both of which can downregulate VSV mRNAs, thus highlighting their potential antiviral functions. As another example, stimulation of TLRs or infection with VSV upregulates miR-155 in macrophages (492). This inducible miRNA acts as a positive feedback regulator of antiviral type I IFN signaling by targeting SOCS1 (suppressor of cytokine signaling 1), the negative regulator of IFN, and in doing so attenuates viral replication (492). Likewise, several human miRNAs have been identified to target HIV 3' UTR (208), and TLR3 activation can also

trigger their expression to restrict HIV infection in monocytes and macrophages (493, 529). Finally, it has been established that IFN-β can induce and cooperate with several cellular miRNAs to limit HCV replication, and this mechanism may contribute to the antiviral effects of IFNs against this virus (374).

1.3.3.2. *Viral Explotation Of Host MiRNA*

Although miRNA can function in antiviral defense, some viruses have evolved to co-opt cellular miRNAs into their life cycle. This has been the case for HIV and HCV (discussed in the next section). In HIV infection, the establishment of latency (integration of provirus into host genomes without producing viral proteins) in a subset of memory resting CD4+ T cells allows the virus to evade immune responses and highly active antiretroviral therapy (HAART) (100). While the precise mechanism remains unclear, it has been reported that the cellular miR-28, miR-125b, miR-150, miR-223, and miR-382 are all apparently highly enriched in resting CD4+ T cells compared to activated CD4+ T cells (208). Analysis of target binding sites for these cellular miRNAs mapped to regions in the 3' UTR of HIV transcripts, suggesting that they could be recruited to mediate viral latency. This hypothesis was confirmed using inhibitors (antisense oligonucleotides) of these miRNAs on resting CD4+ T cells transfected with an infectious HIV clone, or derived from infected individuals undergoing HAART. HIV protein translation and viral production were found to be significantly increased upon neutralization of these miRNAs (208). Thus, HIV can engage host miRNAs to control its own protein expression and repress virion production, and in turn achieve immune evasion through establishment of a latent state in a subset of quiescent cells.

1.3.3.3. *Virus-Encoded MiRNA*

Considering the potent effects of miRNAs in programming mRNA expression patterns and its small/non-antigenic feature, they would appear instrumental to a virus in altering the host cell environment towards its own purpose. Indeed, viruses can encode miRNAs themselves that serve as regulators of virus and/or host gene expression to mediate viral pathogenesis or replication. While not all viruses encode miRNAs (for example HIV, HCV, and human papilloma virus do not), this strategy is most often seen in nuclear DNA virus family, particularly the herpesviruses, including HSV-1, HCMV, EBV, and Kaposi's sarcoma-associated herpesvirus (KSHV) (107, 176). Many of miRNAs encoded by these viruses are thought to help regulate and maintain viral latency. For instance, expression of viral DNA polymerase is inhibited by viral miR-BART2 in EBV infection to reduce viral genome amplification thereby promoting entry to latency (37). Similarly, KSHV-encoded miR-K3 can suppress lytic replication and gene expression to help establish latency (311). In addition, viral miRNAs from HSV-1 and HCMV also downregulate their own immediate early (IE) proteins which would favor a latency state over productive replication during infection (181, 473).

Besides targeting viral transcripts to determine transition between stages of the viral life cycle, viral miRNAs can also function in regulating host gene expression by targeting cellular mRNAs. This has been observed in the case of HCMV, whereby the viral miR-UL112 can cooperate with host miRNA to downregulate MICB (major histocompatibility complex class I polypeptide-related sequence B), which is known to be involved in NK cell killing of virus-infected cells, thus allowing viral immune escape (347). Another example is the targeting of host pro-apoptotic protein PUMA (p53-

upregulated modulator of apoptosis) by the EBV-encoded miR-BART5, which helps the virus to prevent cellular apoptosis (95). In addition, the KSHV-encoded miR-K12-11 is a functional orthologue of host miR-155, and therefore this viral miRNA is able to bind to targets of the cellular counterpart presumably to facilitate viral replication (177). Finally, several KSHV miRNAs also display complementarity to the angiogenesis inhibitor thrombospondin 1 (THBS1), which is involved in cell-to-cell adhesion (409). This targeting effect is speculated to contribute to KSHV-associated tumor formation. While the precise functional significance to some of the cellular mRNAs targeting by viral miRNAs remains unclear, it is thought that they may be utilized to escape from host antiviral strategies as well as to generate a favorable intracellular environment. Ultimately, the outcome from these effects would contribute to the virus' ability to induce oncogenesis in latency (176).

1.3.4. Role Of MiRNA In HCV Infection

Like many other viruses, HCV interacts with cellular miRNA. One such miRNA implicated in the HCV life cycle is miR-122, whose interaction with HCV represents another scenario of virus exploiting the cellular mechanism to its own advantage. The miR-122 is a liver-specific miRNA that is expressed in abundance (average of 66,000 copies per cell) and makes up approximately 70 % of all miRNAs in the liver (79, 80, 269). It is almost exclusively present in the liver, and is undetected in other tissues including lung, heart, brain, kidney, spleen, pancreas, small intestine, colon, testis, ovary, thymus, and skeletal muscle (79, 268, 269, 275, 427). The most well-known function of miR-122 in the mammalian liver is to regulate lipid and cholesterol metabolism (133,

136). It has been shown to be involved in cellular stress response particularly with the cationic amino acid transporter (CAT-1), wherein miR-122 represses CAT-1 mRNA (79) and this effect can be relieved upon amino acid starvation (50). Although the control of miR-122 expression is still unclear, its level has been shown to be upregulated by miR-370 (217) and attenuated by IFN-β treatment (374) (discussed later). The expression of miR-122 is also observed to be downregulated in rodent and human HCC, suggesting a link with hepatocarcinogenesis (267).

Interestingly, miR-122 has been identified as a host factor required for efficient replication and viral production of HCV (232, 391). In particular, miR-122 interacts with two seed sites that are located between stem-loops SL I and II of the highly conserved 5' UTR, which is a region involved in the regulation of both translation and replication (231). How miR-122 mediates its effect on HCV amplification remains unclear, but several studies have attempted to determine the underlying mechanism. For instance, it has been suggested that miR-122 causes accumulation of HCV RNA and thereby modulates rates of viral amplification (231, 232). On the other hand, it has been proposed that miR-122 stimulates viral translation by accelerating and enhancing the association of the small ribosomal subunit with the HCV RNA (195). In line with this notion, binding of miR-122 to HCV 5' UTR has been shown to relieve the viral IRES from the usually locked conformation, thereby permitting translation to occur (118). The closed configuration of the IRES is caused by annealing with immediate flanking sequences which normally results in inhibition of translation. Recent evidence also indicate that miR-122 promotes viral translation to yield efficient virion production (222), further emphasizing its function in mediating HCV polyprotein synthesis. However, the effects

on translation alone do not explain the extent of the viral amplification observed (222). While miR-122 does not appear to modulate the elongation phase of HCV RNA synthesis (484), it is likely that additional mechanisms are also responsible for the effects of miR-122 on HCV, such as enhancing viral RNA stability or facilitating *de novo* initiation of viral RNA synthesis, which can all contribute to viral genome amplification. Although the precise mechanism remains to be elucidated, the importance of miR-122 in HCV life cycle is highlighted by recent therapeutic targeting of miR-122 using an antisense inhibitor as an approach to decrease HCV viremia (276). Furthermore, the ability of miR-122 to confer enhanced permissiveness of HCV replication in non-hepatic cells suggests its role as a functional determinant of HCV viral tropism (78).

Besides miR-122, other miRNAs are also implicated in HCV infection. The miR-199a* has been shown to bind to SL II in the HCV 5'UTR and reduce HCV replication efficiency (345). It is unclear why the two miRNAs, miR-122 and miR-199a*, both being able to interact with HCV 5' UTR would have opposite effects on the viral replication. The miR-199a* is expressed at moderate levels in various human tissues but low quantities in the human liver (269, 345, 391). It may therefore contribute to suppression of HCV in non-hepatic tissue, whereas miR-122 specifies liver tropism to HCV. Another recently identified endogenous miRNA that interacts with HCV is miR-141. It is reported that intracellular levels of miR-141 is upregulated in HCV infection of primary hepatocytes (25). This appears to reduce the expression of the miR-141 target, the tumor suppressor gene DLC1 (deleted in liver cancer 1), whereby its depletion promotes cell proliferation. Results from this study also indicate that miR-141 is implicated in HCV replication, since antisense oligonucleotide depletion of miR-141 decreases viral RNA

levels. While the specific mechanism is unknown, the observations from this study suggest that HCV can modulate host miRNA expression to promote its replication/pathogenesis. These findings also provide a possible mechanism for HCV-induced hepatocarcinogenesis. Finally, as mentioned previously, a number of cellular miRNAs (miR-1, miR-30, miR-128, miR-196, miR-296, miR-351, miR-431, and miR-448) are induced by IFN-β treatment (374). These miRNAs have sequence-predicted targets within the HCV RNA, and were observed to be capable of attenuating viral replication. More importantly, IFN-β treatment causes significant reduction in miR-122 expression (374). These results suggest that viral transcript targeting by host miRNA and removal of the critical viral replication factor miR-122 contribute to IFNs' antiviral effects on HCV. These observations also emphasize how the host has evolved to utilize miRNAs in mediating its innate immune response and that the modulation process can occur both ways. From the above discussion, it is clear that there is significant interplay between the host miRNA system and HCV life cycle.

1.4. Hypothesis And General Research Objectives

Modeling the complete HCV life cycle in culture and in small animal models has been fraught with difficult challenges. The limited number of permissive host species and the inefficient replication in both hepatic and non-hepatic cells indicate that HCV relies heavily on specific host factors and control of innate immunity to achieve replication competency. Identifying the host factors implicated in replication of HCV in rodent cells is therefore a crucial step in uncovering host components required to model the HCV life cycle in a mouse. To date, the minimal essential factors and conditions required to

support HCV replication in rodent cells have not been elucidated. From previous experiments in the literature, it is clear that immunity of the host cell, mediated by type I IFNs, and the presence of liver-specific factors, such as miR-122, impart a significant effect on HCV intracellular replication. I hypothesized, that deletion of IRFs, which dampens host IFN responses, and providing a liver-specific factor, through expression of miR-122, would enable HCV RNA replication in non-permissive cells, such as the murine fibroblasts. Examining this hypothesis should help to determine the role of host factors in HCV replication in rodent cells. I have used murine fibroblasts as a naïve platform and HCV replicons (subgenomic and full-length) as HCV models to study the effects of host cell innate immunity (IRF-3 and/or IRF-9) and a liver-specific factor (miR-122) on HCV replication in rodent cells. In addition, experiments with murine liver cell lines were performed to further examine the involvement of liver-specific factors on the HCV life cycle. Results from these experiments should help determine which host factors are required for HCV replication in rodent cells, reveal potential therapeutic targets, and contribute to our understanding in overcoming species- and cell type-specific barriers. Furthermore, these studies could help provide a foundation for generating small animal models that would be useful for the development of antivirals and vaccines for the prevention and management of HCV.
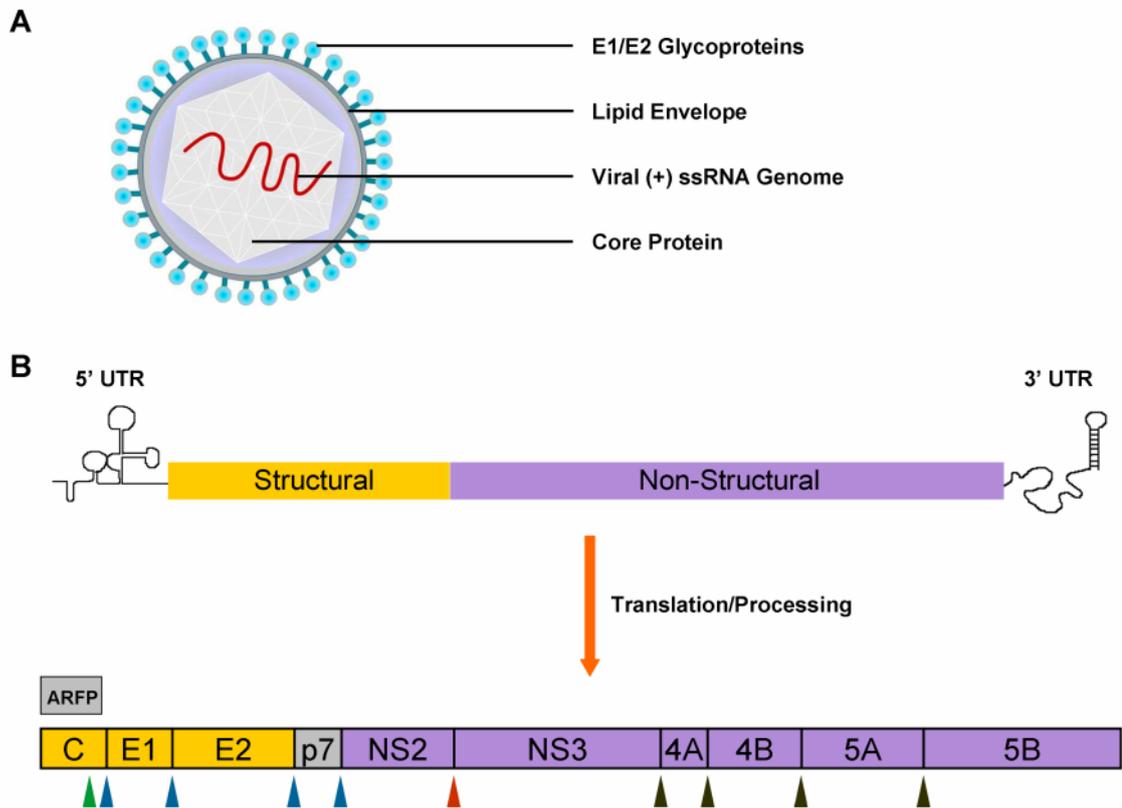
FIG. 1. Schematics of HCV virion and genome. **(A)** Structure of the HCV virion. **(B)** HCV genome contains 5' and 3' UTRs. The single ORF translates into a polyprotein that is processed by host and viral enzymes into 10 proteins including structural components (C, E1, E2), an ion channel (p7), and non-structural elements (NS2, 3, 4A, 4B, 5A, 5B). A translational frameshift may produce ARFP. Arrows indicate cleavage/processing sites mediated by: green = host signal peptide peptidase, blue = host ER signal peptidase, red = NS2/3 protease autocleavage, black = NS3/4A protease. See text for details. (Copyright © 2010 by Lin & Chin. All Rights Reserved.)
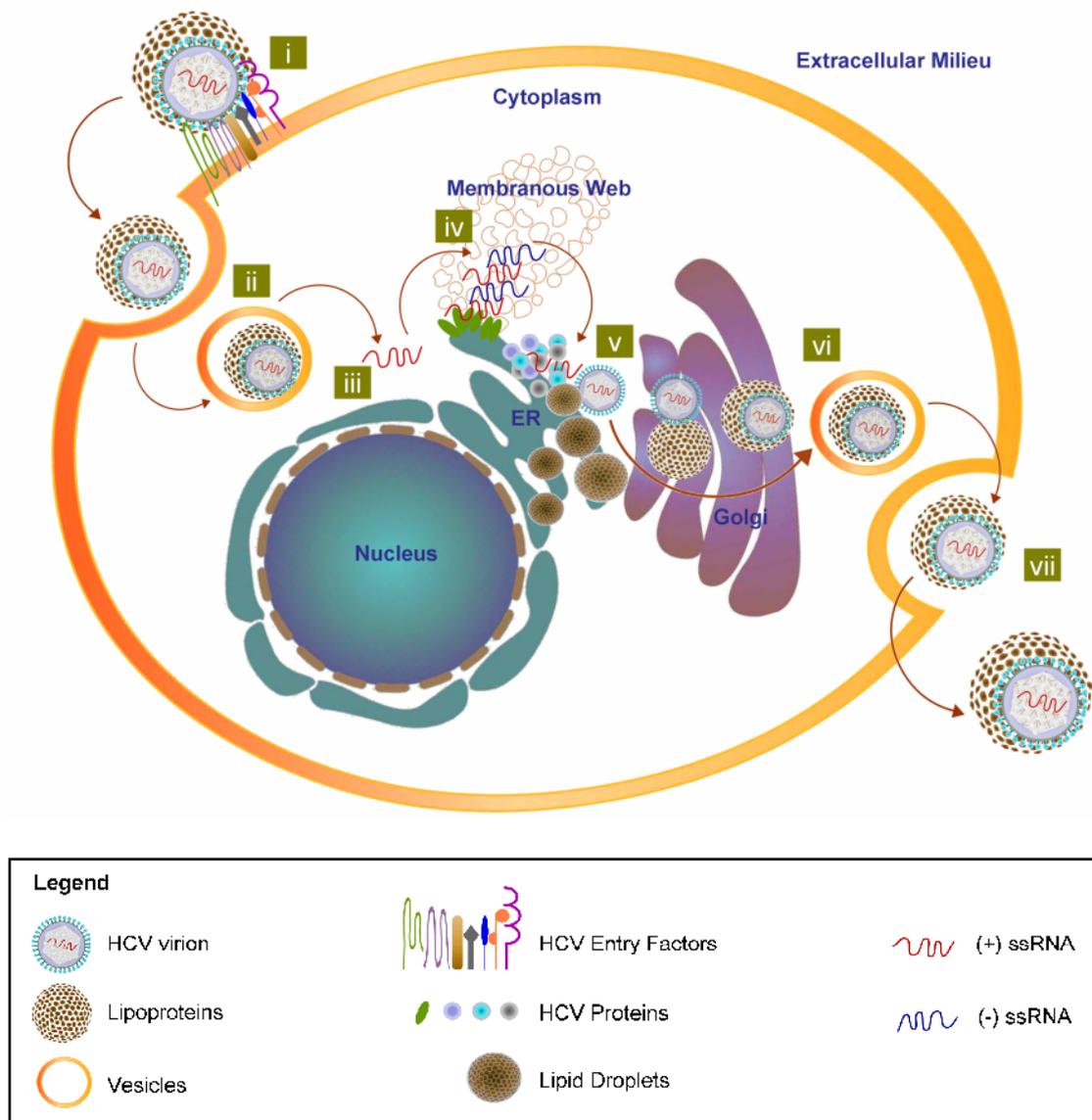
FIG. 2. Life cycle of HCV. (i) receptor binding, (ii) clathrin-mediated endocytosis, (iii) acidification/fusion-induced uncoating and release of viral genome, (iv) polyprotein translation/processing and viral replication, (v) virion assembly on lipid droplets, (vi) association with lipoproteins for maturation through the VLDL secretory pathway, and (vii) egress/release of progeny virions. See text for details. (Copyright © 2010 by Lin & Chin. All Rights Reserved.)
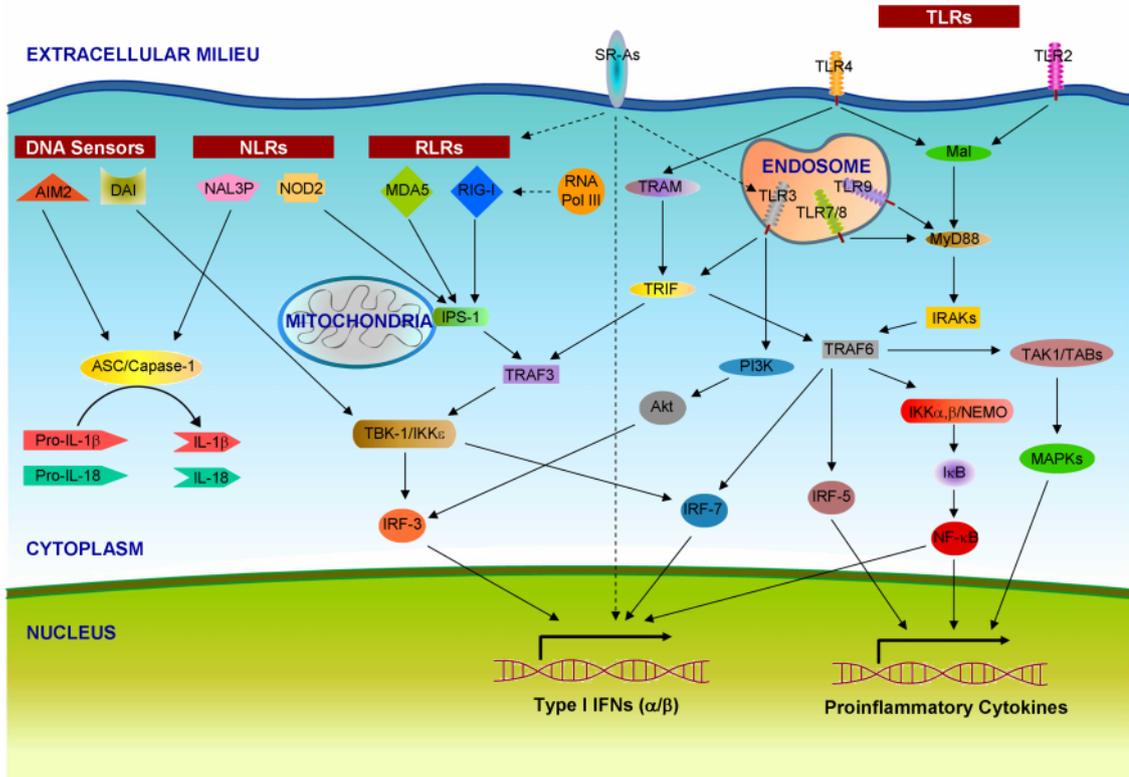
FIG. 3. Viral sensors and signaling pathways in innate immunity of the host cell. See text for details. (Copyright © 2010 by Lin & Chin. All Rights Reserved.)
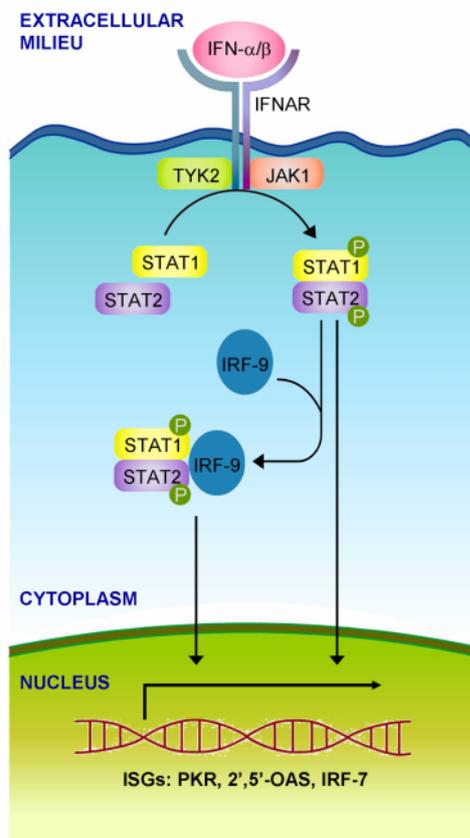
FIG. 4. Type I IFN signaling. See text for details. (Copyright © 2010 by Lin & Chin. All Rights Reserved.)
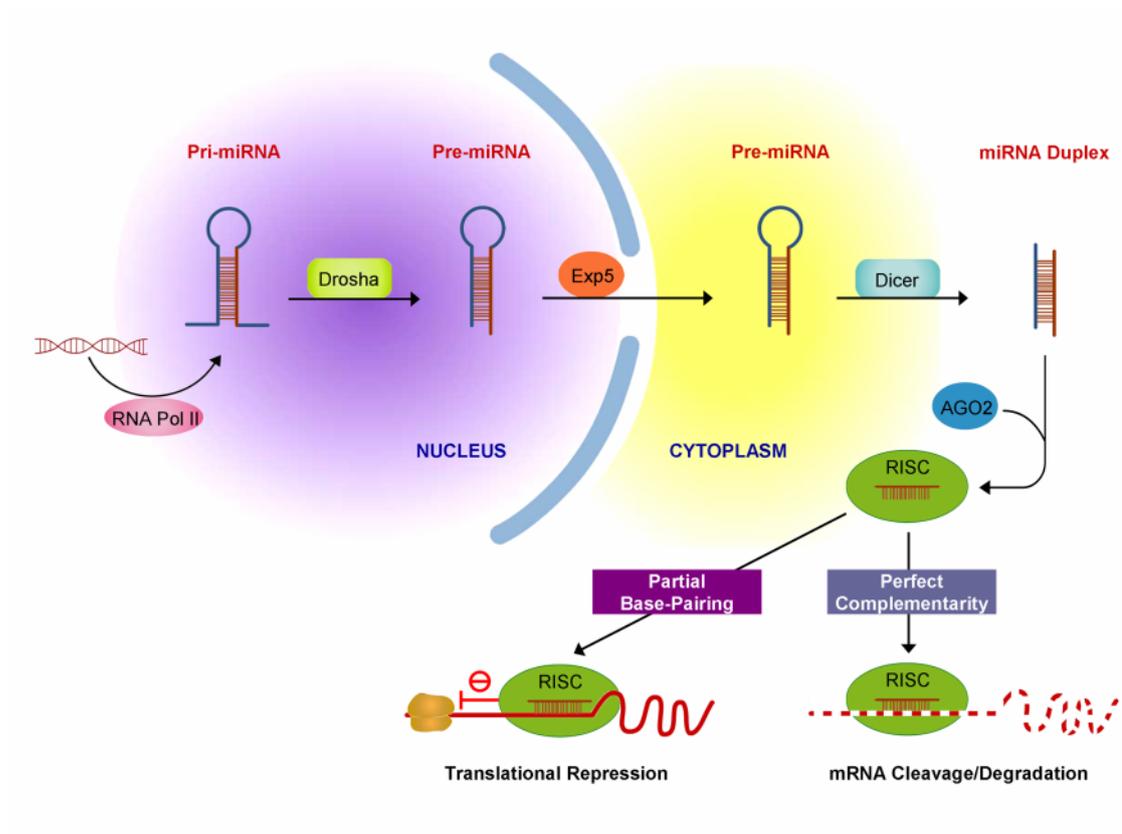
FIG. 5. Biogenesis of miRNA. See text for details. (Copyright © 2010 by Lin & Chin. All Rights Reserved.)

CHAPTER 2   MATERIALS AND METHODS

2.1. Cell Culture And Reagents

The human hepatoma Huh-7 and Huh-7.5 cells were kindly provided by Stanley M. Lemon (University of Texas Medical Branch, Galveston, TX, USA) and Charles M. Rice (Center for the Study of Hepatitis C, The Rockefeller University, New York, NY, USA), respectively, and were maintained in Dulbecco's modified Eagle's medium (Wisent; St-Bruno, QC, Canada) supplemented with 10 % fetal bovine serum (FBS; Wisent), 50 μg/ml gentamicin (GIBCO-Invitrogen; Carlsbad, CA, USA), and 0.5 μg/ml amphotericin B (GIBCO-Invitrogen). The C57BL/6 (B6) mouse embryonic fibroblasts (MEFs) from wild-type (WT), $IRF-3^{-/-}$ (IRF-3 deficient) and $IRF-3^{-/-}/IRF-9^{-/-}$ (IRF-3/9 deficient) mice (414) (originally obtained from Tadatsugu Taniguchi, University of Tokyo, Tokyo, Japan) were grown in alpha minimal essential medium (Wisent) supplemented with 10 % FBS, 50 μg/ml gentamicin, and 0.5 μg/ml amphotericin B. Murine liver cell lines FL83B (CRL-2390; normal liver), NMuLi (CRL-1638; normal liver), and Hepa 1-6 (CRL-1830; hepatoma) were obtained from from the American Type Culture Collection (ATCC; Rockville, MD, USA). NMuLi and Hepa 1-6 were maintained DMEM and FL83B was grown in DMEM/F12; the media were supplemented as described above. Cell lines carrying HCV replicons were maintained in the presence of blasticidin (InvivoGen; San Diego, CA, USA). MEFs were checked for deletion of IRF-3 and IRF-9 by standard RT-PCR. Mouse liver cell homogenate was kindly provided by Dr. Brent Johnston (Dalhousie University, Halifax, Canada). The miRNA-122 (miRIDIAN Mimic hsa-mir-122, C-300591-05) and negative control miRNA (miRIDIAN Mimic

Negative Control, CN-001000-01) were purchased from Dharmacon (Lafayette, CO, USA).

2.2. Plasmids And Replicons

The plasmids pSGR-Luc-JFH1 and its NS5B polymerase inactive control pSGR-Luc-JFH1/GND (459) were a generous gift from John McLaughlin (MRC Virology Unit, Institute of Virology, Glasgow, UK). To construct pSGR-Bsd-JFH1 and pSGR-Bsd-JFH1/GND, the plasmids pSGR-Luc-JFH1 and pSGR-Luc-JFH1/GND were digested with *Bgl*II and *Pme*I to remove the firefly luciferase gene, and then inserted with the blasticidin S deaminase (Bsd) gene amplified from pcDNA™6/V5-His A (Invitrogen) with *Bgl*II and *Pme*I sites at the 5' and 3' termini. The constructs were verified by sequencing. The full-length chimeric J6/JFH1 genome (FL-J6/JFH1) (294), Jc1FLAG2(p7-nsGluc2A) clone, and their respective polymerase defective control FL-J6/JFH(GND) and Jc1FLAG2(p7-nsGluc2A)/GNN, were generously provided by Charles M. Rice. Both FL-J6/JFH and Jc1FLAG2(p7-nsGluc2A) produce infectious cell culture-derived HCV (HCVcc) in Huh-7 cells and its derivatives. Jc1FLAG2(p7-nsGluc2A) is a full-length J6/JFH1 chimera harboring an intragenotypic breakpoint at the C3 position in NS2 (383), a FLAG epitope, and a *Gaussia* luciferase reporter (in tandem with the foot-and-mouth disease virus [FMDV] autoproteolytic peptide 2A) (324). The lethal mutation in the NS5B RNA polymerase active site (GlyAspAsp [GDD] to GlyAsnAsp [GND] or GlyAsnAsn [GNN]) destroys the replication ability of these full-length genomes.

2.3. *In Vitro* Transcription And RNA Preparation

HCV replicon RNA was transcribed *in vitro* using the MEGAscript T7 *in vitro* transcription kit (Ambion; Austin, TX, USA) according to manufacturer's instructions. For pSGR-JFH1 derivatives and the full-length constructs, plasmid DNA was digested with *Xba*I, followed by a single treatment with 1 unit/µg DNA Mung Bean Nuclease (New England Biolabs; Pickering, ON, Canada) as previously described (243, 294, 324). After RNA synthesis, the DNA template was removed by three repeated digests with 0.2 units/µl DNase I (Ambion), followed by standard phenol and chloroform purification. All RNA samples from cells were isolated using TRIzol Reagent (Invitrogen), treated with DNase I (Qiagen Inc.; Mississauga, ON, Canada) for the removal of genomic DNA, and purified by phenol/chloroform according to the protocols from the manufacturers.

2.4. RNA Transfection By Electroporation

For subgenomic replicons, cells were trypsinized, washed three times with cold Phosphate-Buffered Saline (PBS) (Wisent), and resuspended at a concentration of 1×10^7 cells/ml in PBS. A quantity of 10 µg *in vitro* transcribed RNA was mixed with 400 µl of 1×10^7 cells/ml of cell suspension in a 0.4 cm gap cuvette (Bio-Rad; Hercules, CA, USA) alone or in the presence of 50 nM miRNA (miR-122 or negative control), and immediately pulsed with Gene Pulser Xcell (Bio-Rad). The electroporation conditions used for Huh-7 cells were 270 V and 960 µF, and for MEFs were 400 V and 250 µF.

2.5. RNA Transfection By Nucleofection

For delivery of HCV replicon RNA into murine liver cell lines, a total of 4×10^6 cells were mixed with 10 μg *in vitro* transcribed RNA and transfected using the Amaxa Nucleofector system with the nucleofector solution V (Amaxa Biosystem; Cologne, Germany) according to the manufacturer's protocol. The program used for each cell line is as follows: FL83B = T-020, NMuLi = T-020, and Hepa 1-6 = T-028.

2.6. Transient-Replication Assay Using Firefly Luciferase

The transient-replication assay is based on previously described methods (262, 459). Briefly, cells were transfected by electroporation as described above using 10 μg of firefly luciferase replicon RNA in the presence or absence of 50 nM miRNA (miR-122 or negative control). Following dilution of the cells with 8 ml of media, 6-well plates were seeded, each with 0.5 ml aliquots of the electroporated cells. At 4 h and each subsequent day post-electroporation for up to 7 days, the cells were harvested and then assayed for luciferase activity using the Luciferase Assay System (Promega; Madison, WI, USA) and a luminometer (Promega). The luciferase levels at 4 h post-electroporation were used to correct for transfection efficiency. Values are expressed as the fold change in relative light units (RLU) with respect to the 4 h input (301). Independent experiments were plotted as mean ± standard error of means (SEM). Remaining cells from the electroporations were also seeded into a separate dish to harvest for RNA three days after the electroporation for detection of miRNA (see below).

2.7. Colony Formation Assay By Drug Selection

Cells were electroporated with subgenomic HCV RNA as indicated above. Electroporated cells were diluted with 10 ml of fresh medium and seeded at various dilutions depending on the experiment into tissue-culture dishes. After 24-48 h recovery, and every 3-4 days subsequently, the culture medium was replaced with fresh medium supplemented with 5-10 µg/ml blasticidin until colonies were visible (about 2-3 weeks depending on the cell type). Colonies were then characterized for HCV protein and genomic integration or fixed and stained with 0.1 % crystal violet (Sigma) for visualization. Colonies were also counted and expressed as colony forming unit (CFU)/µg of input HCV RNA with values presented as mean ± SEM in TABLE 1.

2.8. Detection Of HCV Protein By Immunohistochemical Staining

Detection of HCV protein by immunohistochemical staining was performed as previously described (294) with some modifications. Briefly, naïve cells or HCV RNA-induced colonies were fixed and permeabilized by methanol, treated with 3 % H_2O_2 for 30 min to inactivate endogenous enzyme activity, and then blocked for 1 h with 3 % bovine serum albumin (BSA). Immunostaining for NS5A was performed using the mouse monoclonal anti-NS5A 9E10 antibody (kindly provided by Charles M. Rice) and goat anti-mouse:alkaline phosphatase conjugate (Sigma), followed by development with the 1-Step Nitro-Blue Tetrazolium Chloride/5-Bromo-4-Chloro-3'-Indolyphosphate p-Toluidine Salt (NBT/BCIP) solution (Thermo Fisher Scientific/Pierce; Rockford, IL, USA). Nuclei were then counterstained with hematoxylin for photography. A mouse

IgG2 isotype control antibody (eBioscience; San Diego, CA, USA) was also included in the experiments.

2.9. Genomic Integration Analysis By PCR

Cellular genomic DNA was isolated from cells using DNeasy Blood & Tissue Kit (Qiagen Inc.) according to the manufacturer's instructions. A total of 200 ng of genomic DNA and 1 ng of plasmid DNA were used for detection of either Bsd gene or the HCV 5' UTR by standard PCR technique using the following primer sets: Bsd forward primer 5' ATGGCCAAGCCTTTGTCTCAAGA 3', Bsd reverse primer 5' TTAGCCCTCCCACACATAACCAGA 3' (399 bp); HCV 5' UTR forward primer 5' GCAGAAAGCGCCTAGCCAT 3', HCV 5' UTR reverse primer 5' CTCGCAAGCGCCCTATCAG 3' (244 bp). Control primers were: human β-globin forward primer 5' GAAGAGCCAAGGACAGGTAC 3', human β-globin reverse primer 5' GGAAAATAGACCAATAGGCAG 3' (408 bp) (110); and mouse glyceraldehyde-3-phosphate dehydrogenase (GAPDH) forward primer 5' ACCACAGTCCATGCCATCAC 3', mouse GAPDH reverse primer 5' TCCACCACCCTGTTGCTGTA 3' (646 bp).

2.10. Innate Immunity Gene Analysis By Reverse Transcription (RT)-PCR And VSV Infection Assay For Antiviral Response

WT and IRF-3 deficient MEFs were electroporated as indicated above with either 10 μg of luciferase replicon RNA (GND), 10 μg of polyinosinic:polycytidylic acid (poly[I:C]; Sigma), or mock-electroporated, followed by seeding into 6 cm dishes for RNA extraction or 12-well plates for vesicular stomatitis virus (VSV) infection.

For innate immunity gene analysis by RT-PCR, after a recovery incubation of 6 h, total cellular RNA was extracted from the samples as described in the above section. The purified RNA (2 μg) was then subjected to cDNA conversion by First-Strand cDNA Synthesis kit (GE Healthcare/Amersham Biosciences; Piscataway, NJ, USA) according to the manufacturer's protocol. Murine innate immunity genes were then PCR amplified with 10 % of the resulting cDNA using the following primers: ISG15 forward primer 5' TGGCCTGGGACCTAAAGGTGAAGA 3', ISG15 reverse primer 5' TGCACTGGGGCTTTAGGCCATACT 3' (432 bp) (361); ISG54 forward primer 5' GGAGAGCAATCTGCGACAGC 3', ISG54 reverse primer 5' GCTGCCCTGAGGAGTGTATC 3' (720 bp); ISG56 forward primer 5' GAGCCAGAAAACCCTGAGTA 3', ISG56 reverse primer 5' CCTCAGTTGGGAGATTTGTTGC 3' (381 bp); IFN-β forward primer 5' GCGTTCCTGCTGTGCTTC 3', IFN-β reverse primer 5' CCATCCAGGCGTAGCTG 3' (454 bp); IFN-inducible protein 10 (IP-10) forward primer 5' ATGAACCCAAGTGCTGCCGT 3', IP-10 reverse primer 5' CTGGGTAAAGGGGAGTGATG 3' (379 bp); and GAPDH forward primer 5' GTGAAGGTCGGTGTGAACGG 3', GAPDH reverse primer 5' GTGGCAGTGATGGCATGGAC 3' (542 bp). All PCR reactions were carried out with standard PCR technique.

For VSV infection assay, the electroporated cells were allowed to recover for 24 h prior to infection with a green fluorescent protein (GFP)-tagged VSV (VSV-GFP; Indiana serotype, a gift from Dr. Brian Lichty, McMaster University, Hamilton, ON) (440). As a control, mock-electroporated cells were also treated with 100 U/ml murine

IFN-β (Sigma) for 24 h. Following recovery, the cell monolayers were infected at MOI = 0.01 for 1 h before overlay with media containing 2 % FCS and 2 % methylcellulose. Pictures were taken between 24-48 h post-infection at 100× magnification (Leica Microsystems; Wetzlar, Germany).

2.11. Establishment Of Murine Cells Stably Expressing MiR-122

IRF-3 deficient MEFs were transduced by use of a lentivirus expression system (Thermo Fisher Scientific; Waltham, MA, USA) with a vector (pLemiR) expressing human primary-miR-122 (pri-miR-122) transcripts under control of the CMV promoter (Open Biosystems; Huntsville, AL, USA). The expression of the pri-miRNA transcripts allows interaction with the endogenous microRNA processing/regulatory pathways that will first yield precursor-miRNA (pre-miRNA) and subsequently mature miRNAs. The pre-miRNA is co-expressed with TurboRed Fluorescent Protein (TurboRFP) and a puromycin-resistance selectable marker which allows monitoring of miRNA expressing cells and generation of stable cell lines. The pLemiR pri-miR-122 vector was transfected into 293T cells using the Trans-Lentiviral™ GIPZ Packaging System (Open Biosystems) to produce a VSV-G pseudotyped virus according to the manufacturer's protocol. Puromycin selection and TurboRFP marker expression were used to exclude any non-transduced cells, and the successfully established cell lines that stably expressed miR-122 were termed 'IRF3 deficient (KO)-HmiR122 MEFs'.

2.12. TaqMan Probe Real-Time Quantitative RT-PCR (QRT-PCR) Detection Of MiR-122

To detect presence of miRNA, total cellular RNA was extracted from the different cells as well as samples in the transient replication assays as described above. The purified RNA (10 ng) was then subjected to cDNA conversion by TaqMan MicroRNA Reverse Transcription kit (ABI; Foster City, CA, USA) according to the manufacturer's protocol. The cDNA product was then assessed by real-time qRT-PCR using TaqMan MicroRNA Assays with primers and probes specific only for the mature sequences of miR-122 and the control miR-16 (ABI). Samples were run on an ABI 7900HT Sequence Detection System (ABI) and results were analyzed using the SDS software (ABI). Data from independent experiments (each containing samples in duplicate or triplicate) were normalized against the control miR-16 and then plotted with respect to control group in bar graphs as mean ± SEM.

2.13. RNA Replication And Infectivity Assays Using Full-Length HCV Reporter Genome

To examine replication of full-length HCV genome by reporter assay, cells were electroporated or transfected by nucleofection in the absence (mock) or presence of 10 μg polymerase active or defective Jc1FLAG2(p7-nsGluc2A) RNA using conditions described above. Transfected cells were seeded in 6-well plates following recovery in resuspension media. At 4 h, day 1, 3, 6, and 9 post-transfection, supernatants from electroporated cells were assayed for *Gaussia* luciferase activity using the BioLux™ *Gaussia* Luciferase Assay Kit (New England Biolabs) and a luminometer (Promega). Cell lysate was also obtained by treatment with cell-lysis buffer (Promega) for

comparison of supernatant versus cell lysate reporter activity. *Gaussia* luciferase levels at 4 h post-electroporation were used to correct for transfection efficiency. Values are expressed as the fold change RLU with respect to the 4 h input. Data from independent experiments were plotted as mean ± SEM. For monitoring translation of the reporter RNA, reporter activity measurements from the above experiment were also plotted into a bar graph as fold change of RLU with respect to values obtained from the respective mock transfection sample of the specific time-point indicated.

For infectivity assay, naïve cell monolayers seeded in 6-well plates were inoculated for 3 h with supernatants (0.5 ml; freeze-thawed and clarified by centrifugation) from day 6 post-transfection of the above experiments After infection, the monolayers were washed 3 times with PBS to remove any carry-over reporter *Gaussia* luciferase, and then overlaid with media. *Gaussia* luciferase activity was assessed during the next 72 h post-infection at 24 h intervals as described above. Absolute RLU values from independent experiments were plotted in bar graphs as mean ± SEM.

2.14. TaqMan Probe QRT-PCR Detection Of HCV RNA Replication

For assessment of full-length HCV replication by FL-J6/JFH1, cells were electroporated with 1 µg *in vitro* transcribed RNA (polymerase active or defective) as described above and seeded in dishes. Media was changed at 4 h post-transfection. At 4 h, and 1, 3, 6, and 9 days post-electroporation, cells were washed and harvested for total RNA extraction using Trizol as described in previous section. To detect HCV RNA, an input of 50 ng of total cellular RNA per sample was assessed by TaqMan probe-based real-time PCR analysis using an ABI 7900HT Fast Real-Time PCR System (ABI) with

primers and probe targeting the 5' UTR of the viral genome: forward primer 5' CGTACAGCCTCCAGGCC 3', reverse primer 5' CGGTTCCGCAGACCACTA 3', TaqMan probe 5' FAM-CTCCCGGGAGAGCCA-NFQ 3'. Reactions (20 µl) were assembled according to the manufacturer's protocol and data was analyzed using the associated ABI software. The HCV RNA loads were determined by comparison to 10-fold serially diluted *in vitro* transcripts in the qRT-PCR analysis.

2.15. Immunofluorescent Staining For HCV Protein In Infected Cell Monolayers

For assessment of HCV proteins in infection by FL-J6/JFH-derived HCVcc, cell monolayers were obtained in 4-well chamber slides and infected with day 6 supernatants from FL-J6/JFH1-electroporated cells by the same method described above as the reporter infectivity assay. After incubation for 72 h, the cell monolayers were fixed and permeabilized by methanol, and then blocked for 1 h with 3 % BSA. To detect HCV proteins, immunostaining for NS5A was performed using the mouse monoclonal anti-NS5A 9E10 antibody and an Alexa 488-conjugated goat anti-mouse secondary antibody (Invitrogen). A mouse IgG2 isotype control antibody (eBioscience) was also included in the experiments. Slides were mounted with cover-slips, and viewed/photographed using a fluorescence microscope (Leica Microsystems).

CHAPTER 3

DELETION OF INTERFERON REGULATORY FACTOR-3 AND EXPRESSION OF LIVER-SPECIFIC MICRORNA-122 FACILITATE REPLICATION OF HEPATITIS C VIRUS IN MOUSE FIBROBLASTS

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3.1. Abstract

Hepatitis C virus (HCV) infection causes significant morbidity, and efficient mouse models would greatly facilitate virus studies and the development of effective vaccines and new therapeutic agents. Entry factors, innate immunity, and host factors needed for viral replication represent the initial barriers that restrict HCV infection of mouse cells. Experiments in this paper consider early post-entry steps of viral infection, and investigate the roles of interferon regulatory factors (IRF-3, IRF-9) and microRNA (miR-122), in promoting HCV replication in mouse embryo fibroblasts (MEFs) that contain viral subgenomic replicons. While wild-type murine fibroblasts are restricted for HCV RNA replication, deletion of IRF-3 alone can facilitate replicon activity in these cells. This effect is thought to be related to the inactivation of the type I interferon synthesis that IRF-3 mediates. Additional deletion of IRF-9$^{-/-}$ to yield IRF-3$^{-/-}$/IRF-9$^{-/-}$ deficient MEFs, which blocks type I interferon signaling, did not increase HCV replication. Expression of liver-specific miR-122 in MEFs further stimulated the synthesis of HCV replicons in the rodent fibroblasts. The combined effects of miR-122 expression and deletion of IRF-3$^{-/-}$ produced a cooperative stimulation of HCV subgenome replication. MiR-122 and IRF-3 are independent host factors that are capable of influencing HCV replication and our findings could help establish mouse models and other cell systems that support HCV growth and particle formation.

3.2. Introduction

Hepatitis C virus (HCV) is a blood-borne pathogen that represents a serious public health burden and infects an estimated 170 million people or 3 % of the global

population (278). This virus establishes chronic infections in about 80 % of infected individuals, which may lead to severe liver complications, including cirrhosis and hepatocellular carcinoma (51). Current treatment options are suboptimal and there are no effective vaccines on the horizon. The differences in host response between patients who spontaneously clear the virus and those whose disease worsens are also not well defined. The only other natural host for HCV besides humans is the chimpanzee. A human liver xenograft model in a severely immunocompromised mouse (SCID) has been developed (335). However, there is still and urgent need for development of an efficient immunocompetent mouse model to facilitate drug and vaccine discovery and to better understand the virus-host interactions (35).

The 9.6 kb HCV genome contains a single continuous open reading frame (ORF) flanked by a 5' internal ribosomal entry site (IRES) and a 3' untranslated region (3' UTR). Translation of the viral genome yields a single polyprotein from this ORF is processed by host and viral proteases to yield 3 structural proteins (C, E1, E2), one ion channel (p7), and 6 non-structural proteins (NS2, NS3, NS4A, NS4B, NS5A, and NS5B) during the viral life cycle (342). Numerous studies modeling the HCV life cycle *in vitro* support the notion that viral replication is dependent upon specific cellular factors. The importance of the host is enunciated by the species and cell type restriction of HCV replication in culture, and the limitation of virion production to the human hepatoma Huh-7 cell line (404) which supports the complete viral life cycle (30). Genetic defects in the innate immune system have subsequently been identified as the factors affecting the efficiency of HCV replication in this cell line and its derivatives (287). More specifically, during host cell innate immunity, pattern recognition receptors (PRRs), such as Toll-like

receptor 3 (TLR3) and retinoic acid-inducible gene-I (RIG-I) recognize viral RNA signatures, trigger the downstream IRF-3 transcription activator to induce IFN-beta (IFN-β) synthesis, which finally activates IFN-stimulated genes (ISGs) of the antiviral defense program (247). Huh-7 and Huh 7.5 cells, which support the replication and assembly of HCV, have been shown to be deficient in TLR3 and in TLR3 and RIG-I, respectively (34, 287, 294, 443, 491, 528). IRF-3 deficient mice also have a poor IFN response and are more susceptible to virus infection in general (414).

The singular replication of HCV RNA in Huh-7 cells can also be attributed to another host factor, the liver specific microRNA 122 (miR-122). MicroRNAs (miRNAs) are short (~ 22 nt), non-coding, evolutionarily-conserved endogenous RNA species that normally regulate gene expression via mRNA cleavage or translational repression through binding to the 3' UTR of the target mRNA (28). The miRNAs are generated from two rounds of ordered cleavages, with primary transcripts termed primary-miRNA (pri-miRNA) being first processed to yield short stem-loop structures known as precursor-miRNA (pre-miRNA), which are finally cropped to produce functional mature miRNAs (28). Mature miRNAs then associate with other proteins to form RNA-induced silencing complexes (RISC) and through pairing with partially complementary sites in the 3' UTR of mRNAs, initiate the repression of their target mRNAs. Recent reviews have revealed that miRNAs participate in various cellular biological processes, including growth development, metabolism, cell proliferation, haematopoiesis, and apoptosis, and are functionally associated to a number of human diseases and cancers (13, 28, 417). While the effects of miRNA are general inhibitory, one particular miRNA, the liver-specific miR-122 (79), seems to positively promote HCV replication in the host cell and

has been suggested to contribute to the liver tropism of this virus (195, 232). Further evidence supporting this notion came with the observation that miR-122 can enhance HCV replication in non-hepatic human cells, suggesting that presence of miR-122 indeed plays an important role in sustaining HCV life cycle in hepatic cells (78).

While many of the host specific factors for HCV replication have been identified in the human liver, host components for viral replication in rodents are largely unexplored. In the attempt to facilitate HCV infection in small animal models, examination of specific intracellular determinants that support HCV infections have been initiated in several different mouse systems, including fibroblasts and hepatocytes, yielding greatly reduced replication when compared to human hepatoma Huh-7 cells (82, 315, 326, 475, 512, 530). Although the dsRNA binding protein kinase R (PKR) has been suggested as a potential innate immune factor regulating HCV replication in murine embryonic cells (82), the influence of other components of innate immunity remain to be tested. In order to clarify the host factors required to support HCV replication in rodent cells, focused on the roles of innate immune transcription factors, including IRF-3 and IRF-9, and the liver-specific miR-122 in influencing subgenomic viral RNA synthesis within murine fibroblasts. We provide evidence that both IRF-3 deletion and miR-122 expression can independently or in combination promote increased HCV RNA and viral protein production in mouse fibroblasts. Both transient luciferase reporter and drug-resistant subgenomic replicon assays have corroborated these results. These observations should provide insight into some of the post-entry factors that will have to be considered in generating a mouse model for HCV replication.

3.3. Results

3.3.1. Deletion of IRF-3 Supports Transient And Drug-Selected Replication Of Subgenomic HCV JFH1 RNA

The HCV JFH1 replicon is a robust RNA that requires minimal adaptation to replicate in either liver or non-hepatic cells (243, 244, 294), and has previously been shown to multiply in some mouse cells (82, 475). We subsequently used two subgenomic constructs based on this backbone to assess HCV replication, one engineered with the firefly luciferase reporter gene (SGR-Luc-JFH1) for transient replication assays, and another with blasticidin S deaminase gene (SGR-Bsd-JFH1) which confers resistance to blasticidin (FIG. 6A) for drug selection and cell colony formation assays. As expected, the HCV subgenomic RNA replicated robustly in Huh-7 cells (FIG. 6B and C) with a colony formation efficiency of $6.02 \times 10^3 \pm 0.32 \times 10^3$ CFU/µg RNA (TABLE 1). In contrast, wild-type (WT) MEFs were non-permissive for HCV transient replication, with no difference in luciferase reporter activity from either the replication competent or mutated control replicons (FIG. 6B). Similarly, subgenomic replicons expressing the Bsd resistance gene produced limited colony formation in WT MEFs grown in the presence of blasticidin (FIG. 6C and TABLE 1). Importantly, IRF-3 deficient MEFs were more supportive of HCV RNA replication in transient replicon luciferase reporter assays conducted 7 days post-electroporation (FIG. 6B). In addition, HCV replicons in IRF-3 null cells had a significantly higher replication activity (9-fold) in colony formation assays, compared to those performed in WT MEFs (FIG. 6C and TABLE 1). These observations indicated that deletion of the innate immune factor IRF-3 in mouse fibroblasts can favor HCV RNA replication.

Following binding of interferon (IFN) to its cell surface receptor, phosphorylated STAT proteins interact with IRF-9 to promote the expression of IFN-stimulated genes (ISGs). These include IRF-7 which activates the synthesis of more type I IFN (201). To determine whether deletion of IRF-9 in the IFN signaling pathway could further contribute to HCV replication, we assayed replicon activity in murine fibroblasts doubly deficient for IRF-3 and IRF-9 (IRF-3$^{-/-}$/IRF-9$^{-/-}$). Type I IFN production in these cells is greatly diminished and they are highly susceptible to viral infections (414). Surprisingly, IRF-3$^{-/-}$/IRF-9$^{-/-}$ MEFs exhibited no further increase in HCV replication when compared to that in IRF-3$^{-/-}$ cells (FIG. 6B). Thus, it appears when considering the type I IFN signaling pathway, IRF-3 deficiency alone is sufficient to promote HCV RNA replication in mouse fibroblasts. Of interest in both IRF-3$^{-/-}$ and IRF-3$^{-/-}$/IRF-9$^{-/-}$ cells, the NS5B polymerase defective mutant usually took longer (6 to 7 days) to produce baseline luciferase values compared the same replicon in WT MEFs (4 to 5 days), an observation likely due to diminished antiviral RNAse L activity present in the defective type I IFN system.

To further validate HCV replication in the cells used in the above experiments, colonies obtained in the dishes were assessed for HCV NS5A expression by immunohistochemical staining (FIG. 7). NS5A protein could be detected in the cytoplasmic and perinuclear regions of replicon cells. Mouse fibroblasts containing the HCV replicons were also stained with an IgG control antibody or compared with control cells that did not contain HCV RNA. To rule out genomic integration of cDNA from the template for the HCV replicon, PCR analysis was performed for the Bsd gene and HCV 5' UTR using genomic DNA from the pooled MEF colonies. None of the cells containing

HCV RNA or proteins yielded PCR signals that could be attributed to genomic integration of the blasticidin resistance gene or viral cDNA indicating that the MEFs were authentic replicon cell lines (FIG. 8). The immunochemical staining and PCR results provided correlation between the colony formation assays and transient luciferase reporter assays. The preceding results further demonstrated that HCV RNA could replicate in rodent cells and that the replication efficiency is enhanced by deletion of the gene for IRF-3.

3.3.2. HCV RNA Cannot Induce An Antiviral State In IRF-3 Deficient MEFs

The induction of type I IFNs and the ISGs can dramatically inhibit the replication capacity of HCV RNA (221). We postulated that the enhanced ability of HCV RNA to replicate in IRF-3 deficient murine cells was due to a diminished antiviral response. To validate this hypothesis, we examined the expression of IFN-β and several IRF-3-associated ISGs including ISG15, ISG54, ISG56, and IP-10 (16) in WT and IRF-3 null MEFs in the presence of HCV RNA. HCV RNA is recognized by RIG-I through the single-stranded RNA 3' poly-U/UC region (405) which subsequently triggers hepatic innate immunity through activation of IRF-3 (407). The non-replicative *in vitro*-transcribed single-stranded HCV GND RNA was used in our experiment and poly(I:C) was included as a control in experiments designed to trigger the antiviral response. HCV GND RNA or poly(I:C) were introduced into WT MEFs and IRF-3 deficient MEFs by electroporation, and the presence of ISG15, ISG54, ISG56, IFN-β, and IP-10 mRNA was assessed 6 h later by RT-PCR. The induction of an ISG response was completely absent in IRF-3 deficient cells (FIG. 9A). To determine whether the induction of IFN-β and

ISGs reflected the establishment of a true antiviral state within the MEFs, we performed a vesicular stomatitis virus (VSV) interferon bioassay by inoculating mouse cells with VSV-GFP after they had been induced for 24 h with either electroporated-HCV GND RNA or poly(I:C). As expected, the antiviral response triggered by HCV GND RNA was sufficient to block VSV infection in WT MEFs, but not in the IRF-3 null cells (FIG. 9B). Interestingly, the presence of poly(I:C) established an antiviral state in both WT and IRF-3 deficient MEFs, which confirms recent reports in the literature that poly(I:C) can trigger an IRF-3-independent antiviral response (117). In summary, our results suggest that HCV RNA can trigger an antiviral state in WT MEFs, and that IRF-3, type I IFN, and activation of downstream ISGs prevent the establishment of HCV replicons in these cells. Deletion of IRF-3 alleviates this situation and permits the synthesis of HCV RNA and expression of viral proteins.

3.3.3. Liver-Specific MiR-122 Promotes Subgenomic HCV Replication In WT And IRF-3$^{-/-}$ MEFs

While we observed that deletion of a host innate immune factor such as IRF-3 contributed to permissive HCV RNA replication, we speculated that other human liver-specific host factors could affect HCV RNA production in mouse cells. We therefore examined the effects of miR-122, which is known to promote HCV propagation in non-hepatic human cells (78). The mature sequence of the liver-specific miR-122 is actually conserved between humans and mice (79). We used this miR-122 in both transient luciferase and cell colony formation assays by co-electroporating cells with HCV replicon RNA and miRNA into the different mouse fibroblasts. The presence of exogenously introduced miR-122 in mouse fibroblasts was verified by TaqMan probe

qPCR, and miR-122 levels in mouse liver cells were assayed and included as a positive control. MiR-122 was detected only in the transfected MEFs or in the mouse liver cells, with levels in both cell types being comparable (FIG. 10A, bar graphs on the right of each panel).

In transient assays, input of miR-122 into Huh-7 cells, which normally express high levels of endogenous miR-122, did not further enhance HCV replication, suggesting that the stimulatory effect of miR-122 was saturated (FIG. 10A). However, our data demonstrated that introduction of miR-122 into mouse fibroblasts could promote HCV RNA synthesis even when IRF-3 was present in the cell (FIG. 10A). This enhancement of HCV replication by miR-122 appeared stronger than the stimulation elicited by IRF-3 deletion (FIG. 10A versus FIG. 6B). The same results were evident when HCV replicon activity was measured in MEF colony formation assays performed in the presence or absence of exogenously-introduced miR-122 (FIG. 10B and TABLE 1). Furthermore, the combination of the presence of miR-122 and the deficiency of IRF-3 was cooperative in MEFs, and produced even higher HCV replication in luciferase reporter and colony formation assays. Again, deletion of IRF-9 did not enhance HCV replication due to IRF-3 deficiency, even in the presence of the miR-122 (FIG. 10A). The levels of HCV replication obtained in the IRF-3 deficient MEFs containing miR-122 approached those obtained in Huh-7 cells, which seem to confirm that miR-122 expression and defects in innate immunity likely account for the robust replication of HCV in Huh-7 cells.

We also sought to clarify whether the enhancement of HCV replicon activity resulting from the presence of miR-122 was due to its effects on replication or the translation of the viral RNA. When we compared replicon luciferase activity from non-

replicating HCV GND subgenomic RNA in IRF-3 deficient MEFs either containing or not containing miR-122, we observed no difference (FIG. 10C). Similarly, IRF-3 deficient MEFs containing non-replicating HCV GND subgenomic RNA did not form any replicon colonies in the presence or absence of miR-122 (FIG. 10B). These results seemed to indicate miR-122 impacted viral RNA replication in order to increase replicon luciferase activity and colony formation. However, a role for miR-122 on translation and HCV protein synthesis cannot be completely ruled out.

Overall, our results suggest that the liver-specific miR-122 constitutes an important host factor for HCV replication in mouse fibroblasts, which also implies that the effect of miR-122 on HCV propagation in the liver is conserved in both humans and mice. Furthermore, while miR-122 can independently promote HCV RNA production, its effects are even more pronounced in the absence of the innate immune factor IRF-3.

3.3.4. Efficient Replication Of HCV Replicon RNA In IRF-3 Deficient Fibroblasts That Stably Express MiR-122

Based upon the previous observations, we attempted to establish HCV replication in IRF-3 deficient MEFs that stably expressed the human primary transcript for miR-122 (pri-miR-122). The pri-miR-122 was introduced into these cells with a VSV-G pseudotyped pLemiR lentivirus expression vector from Open Biosystems. Expression of the processed mature miR-122 was validated by TaqMan probe real-time qPCR specific only for the mature sequence of miR-122. In addition, expression of TurboRFP, which is also encoded in the lentivirus expression vector, and expression of the puromycin resistance indicated successful transduction into the target cell. We could detect mature miR-122 sequence in the transduced cells, demonstrating that the human precursor (pre-

miR-122) was cleaved in the mouse system. Although the levels of lentivirus expressed miR-122 levels were lower than those detected in transfected cells (FIG. 10A versus FIG. 11A, bar graphs), the transduced miR-122 effectively stimulated transient HCV RNA replication and replicon colony formation in these cells ('IRF3KO-HmiR122 MEFs'). Specifically, the IRF3KO-HmiR122 MEFs produced a firefly luciferase activity profile with a magnitude that more closely resembled that observed in Huh-7 cells than that in the parental miR-122-negative IRF-3 deficient fibroblasts (FIG. 11A versus FIG. 6B). Similarly, the number of cell colonies obtained with the IRF3KO-HmiR122 MEFs was 10-fold higher than that obtained with IRF-3 deficient fibroblasts alone and again approached the levels seen in the Huh-7 cells. The result obtained was also comparable to that previously observed during the transient introduction of exogenous miR-122 (FIG. 11B and TABLE 1). Authentic HCV replication and NS5A protein production was also verified in these miR-122-expressing fibroblast colonies (FIG. 11C) and genomic integration of the Bsd gene or HCV cDNA were not detected (FIG. 8). This cell model validates our observations that the deficiency of IRF-3 and the presence of liver-specific miR-122 provide a favorable environment for replication of HCV subgenomic replicon RNA in mouse fibroblasts.

3.4. Discussion

Restriction of HCV replication in mouse cells can be attributed to the presence or absence of primate- or organ-specific factors that affect virus attachment, entry, replication, packaging, and assembly. Determinants for HCV viral entry into rodent cells have previously been investigated (186, 315, 325, 367, 387, 499, 512) and many of the

limiting entry factors necessary for the uptake of the virus have been identified. Specifically, expression of the tetraspanin human cluster of differentiation 81 (CD81) and tight-junction protein occludin (OCLN) can overcome the receptor-entry block of HCV particles into mouse cells. Other entry factors such as claudin 1 (CLDN1), glycosaminoglycans (GAGs), low density lipoprotein receptor (LDLR), and scavenger receptor class B member 1 (SR-BI) are highly homologous to their mouse counterparts and still function in the rodent host (387). However, no infectious particles are released from rodent cell lines expressing human CD81 and OCLN indicating other host and tissue restriction factors prevail to inhibit virus replication and virion assembly (326, 475).

Host innate immunity is a determining factor for permissive infections and tropism in many viral systems (163, 328). Myxoma virus infections are specific for rabbits and tropism is mediated by interferon signal transducers and activators of transcription (STAT)-1-mediated type I IFN responses. Wild-type mice are normally resistant to myxoma virus but succumb to infection if the STAT-1 gene is deleted (490). Vaccinia virus virulence genes that target the antiviral protein PKR, including E3L and K3L, can allow the virus to replicate in HeLa and BHK 21 cells, but these cells are non-permissive to virus in which these genes have been deleted (46, 47). Another example of the importance of host innate immunity in determining host resistance is the mouse model for measles virus. Rodents are normally resistant to measles virus but transient infections can be achieved in mice expressing primate-specific receptors (CD46 and CD150) for the virus, albeit these infections were limited due to the presence of an intact innate immune system (61). However, greatly improved replication of measles virus can be observed if the CD46/CD150 mouse is bred into a STAT-1-deficient or interferon

receptor deficient background (147, 500). The preceding examples provide precedent for the importance of both receptors and innate immunity in determining whether a particular host is susceptible to virus infections.

It is known that HCV translation and genome replication can be influenced by host-specific factors that have been elucidated from experiments with non-liver and non-primate cells (9, 82, 326, 367, 475, 511, 512, 530). Although the growth and establishment of HCV replicons can be optimized for RNA synthesis through adaptive mutations within the viral genome in the host cell (59, 262, 301, 302), the presence and absence of these host-specific factors significantly influence HCV replication in a particular cell type (32, 34, 301). An appreciation for the impact of the host cell environment on HCV replication is emphasized by the narrow range of cell lines that are susceptible to viral replication. Through functional genetic and proteomic approaches, exhaustive lists of host factors influencing the HCV life cycle has been assembled (recently reviewed in (343), (62), and (388)). Components related to immunity (TRAF2, JAK1, cyclophilin A/B, lymphotoxin β) and the miRNA processing and effector system (miR-122, miR-199a, DICER1, RNA helicase) are some of the factors in these lists. Our findings that IRF-3 and miR-122 act as independent host factors influencing HCV RNA replication validates this approach and suggest that they may have roles in regulating HCV infections in a mouse genetic background.

The importance of host innate immunity in controlling HCV is underscored by the IFN-α-based therapies currently used for treating hepatitis C. It is also believed that the innate immune response helps determine the quality and strength of adaptive immunity and its effect on the outcome of an infection — resolution or the establishment of a

chronic persistent infection (128, 163, 448). HCV is very adept at crippling the host's innate immune system. For instance, the HCV NS3/4A protease cleaves the IFN-β promoter stimulator 1 (IPS-1) (307) and Toll-interleukin-1 receptor-domain-containing adapter-inducing IFN-β (TRIF) (288) adaptor proteins, thereby blocking and attenuating the RIG-I and TLR3-mediated activation of the IRF-3/type I IFN pathway (155, 156). Although HCV possesses these immune evasion strategies they may not be efficient enough to escape innate immune surveillance *in vitro*. We postulated that the relative potency of the host cell innate immune system could determine the efficiency of HCV replication *in vitro*. The immune characteristics of Huh-7 (TLR3 deficient) (287) and Huh-7.5 cells (TLR3 and RIG-I defective) (443) support this notion. Interestingly, defects in the Huh-7 cell lines leading to enhanced HCV replication all revolve around inactivation of IRF-3, thus emphasizing IRF-3's critical role in controlling HCV (156, 406, 508). On the other hand, several studies indicated only limited participation of this transcription factor in inhibiting the virus (8, 14) and permissiveness of Huh-7 to HCV replication (53). However, our results clearly demonstrated that the IRF-3 pathway and the subsequent activation of type I IFN and induction of ISGs play a principal role in controlling HCV in mouse cells. Deletion of IRF-3 seemed sufficient for these outcomes, since an additional deletion of IRF-9 leading to decreased production of IRF-7 in the IRF-3$^{-/-}$/IRF-9$^{-/-}$ MEFs did not further stimulate HCV replication. Previous questions as to the importance of IRF-3 in controlling HCV replication could be due to host species-specific differences in the immune proteins and alternative IRF-3/IRF-7 independent pathways that are present in some cell types (108, 109, 287).

The positive effect mediated by miR-122 on the growth of HCV is now widely accepted and has recently been shown to be a valuable therapeutic target for decreasing HCV viral loads in chimpanzees (276). Indeed, from our transient assays or colony formation studies, exogenous introduction of liver-specific miR-122 to mouse fibroblasts created a permissive environment for HCV replication irrespective of whether IRF-3 was expressed or not. However, HCV replication was still significantly enhanced in the IRF-3 deficient mouse fibroblasts when compared to the same replicon in wild-type fibroblasts. The presence of miR-122 and a crippled innate immune system likely explain why Huh-7 cells and its derivative cell lines (Huh-7.5, Huh-7.5.1) are the cell lines of choice for *in vitro* studies with HCV (60, 301, 528). More specifically, we could only observe an effect of miR-122 on HCV RNA replication and found that it did not appear to enhance translation and protein synthesis from a non-replicating subgenomic replicon. In this regard, the precise mechanism by which miR-122 enhances HCV production is still hotly debated. Both the regulation of viral RNA abundance/amplification (78, 231, 232, 428) and the stimulation of viral RNA translation (118, 195) have been proposed as possible targets of action for miR-122. However, the use of a bicistronic replicon system could complicate the debate due to the presence of the picornavirus (EMCV) internal ribosome entry site (IRES) that is used to control translation of the HCV non-structural proteins. The EMCV IRES is not recognized by miR-122 and although the reporter gene or drug selection marker are under control of the HCV IRES, the foreign IRES could prevent an enhancement of viral translation and production of HCV proteins (359). Nevertheless, we did observe that miR-122 facilitates the synthesis of HCV subgenomic replicons in mouse fibroblasts. Further investigation with a full-length HCV replicon solely under

control of the HCV 5' UTR-IRES may clarify the controversy between the enhancement of translation or replication. A recent report examined this issue using such a full-length replicon reporter, demonstrating that miR-122 promoted viral translation to yield efficient virion production (222). However, the authors concluded that miR-122 is likely involved in other aspects of viral genome amplification.

Our study is an important step in overcoming the HCV replication barrier in mice using a minimal number of factors to drive HCV replication in rodent cells. Information provided here could be a prelude to exploring viral packaging steps in murine host cells. The next step would be to express human-specific receptors for the virus (CD81 and OCLN) in IRF-3 deficient MEFs expressing miR-122 or IRF-3 deficient hepatocytes, and study virion packaging, assembly, and egress from the host cell. Additional host factors derived from the liver may have to be identified to facilitate the complete life cycle of HCV in mouse cells. These studies will eventually translate to the generation of efficient small animal models, and offer improvements over existing *in vivo* HCV systems. Knowledge related to these processes will also undoubtedly expose new targets for antiviral therapy.

3.5. Acknowledgements

3.6. Tables, Figures, And Legends

Cell Type	CFU/μg RNA
Huh-7	$6.02 \times 10^3 \pm 0.32 \times 10^3$
WT MEF	$4.20 \times 10^1 \pm 1.45 \times 10^1$
IRF-3 KO MEF	$3.91 \times 10^2 \pm 0.21 \times 10^2$
WT MEF + miR-122	$1.72 \times 10^3 \pm 0.34 \times 10^3$
IRF-3 KO MEF + miR-122	$4.43 \times 10^3 \pm 0.58 \times 10^3$
IRF3KO-HmiR122 MEF	$4.05 \times 10^3 \pm 0.51 \times 10^3$

TABLE 1. Colony forming efficiency induced by HCV SGR-Bsd-JFH1 RNA. Values are given in mean ± SEM from three independent experiments.
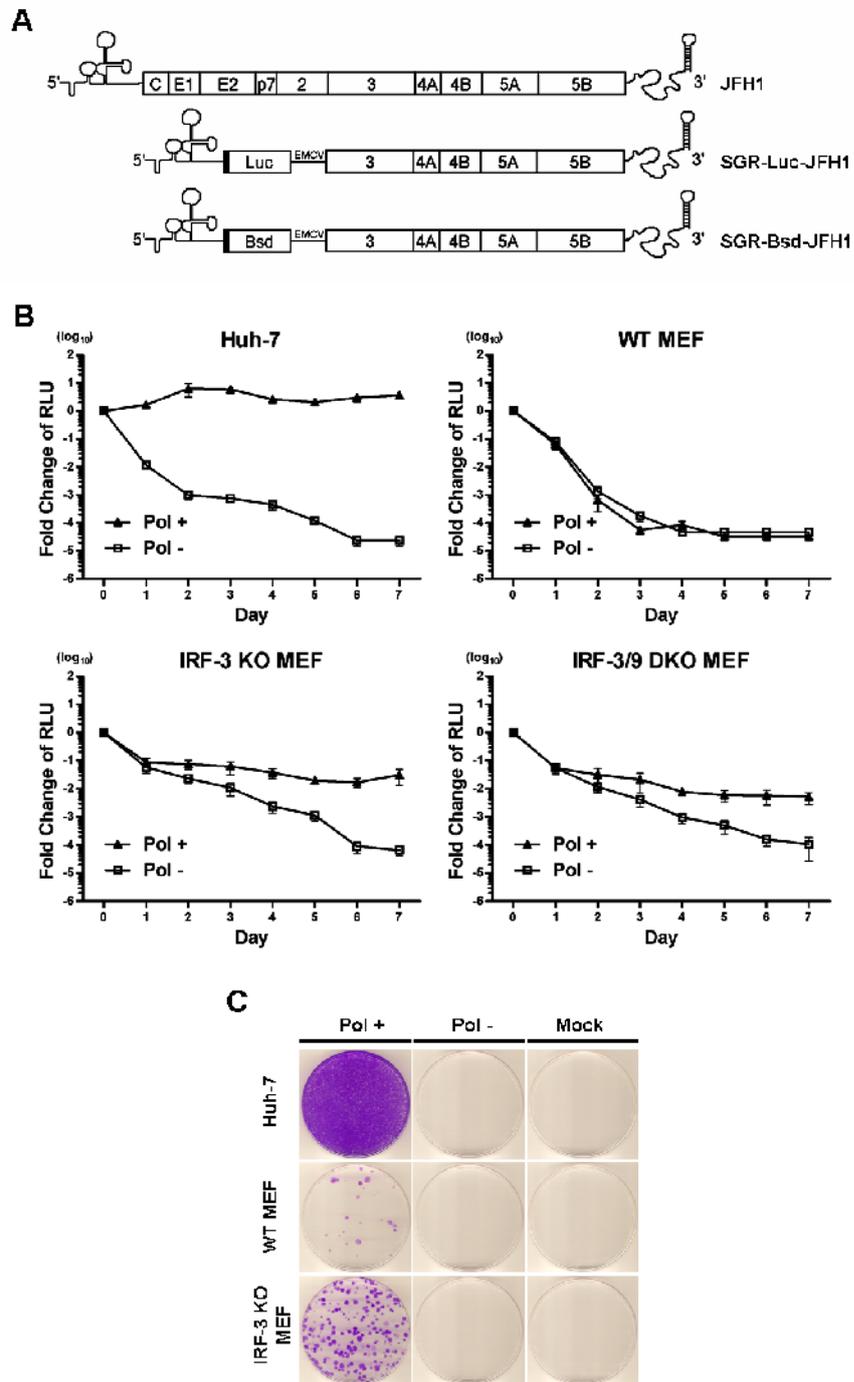
FIG. 6. IRF-3 deficiency facilitates replication of HCV replicon RNA in mouse fibroblasts. **(A)** Schematic representation (not to scale) of HCV JFH1 RNA genome,

subgenomic luciferase reporter JFH1 construct (SGR-Luc-JFH1), and subgenomic blasticidin resistance JFH1 construct (SGR-Bsd-JFH1). EMCV: encephalomyocarditis virus IRES; Luc: firefly luciferase gene; Bsd: blasticidin S deaminase gene conferring resistance to blasticidin. **(B)** Transient replication assays using the HCV SGR-Luc-JFH1 RNA in Huh-7 cells and WT, IRF-3 deficient (KO), and IRF-3/9 deficient (DKO) MEFs. Cells were electroporated with the HCV RNA, harvested at the indicated time-points, and assayed for luciferase activity. The luciferase reporter activity is indicative of HCV RNA replication. Bioluminescence is expressed in logarithmic scale units as the fold change of relative light units (RLU) normalized to the light emitted from input RNA determined at 4 h post-electroporation. Each point represents mean values from three independent experiments with error bars showing SEM. Pol + (solid triangle): polymerase-active HCV RNA; Pol - (open square): polymerase-defective (non-replicating; GND) control. **(C)** Colony formation assay using HCV SGR-Bsd-JFH1 RNA in Huh-7 cells and WT and IRF-3 deficient (KO) MEFs. Cells were electroporated with the HCV RNA, seeded onto culture plates at 1:2 dilution, and then selected with blasticidin for 2-3 weeks before staining with crystal violet. Representative images from three independent experiments are shown here. Pol +: polymerase-active HCV RNA; Pol- : polymerase-defective (non-replicating; GND) control; Mock: mock-electroporation with buffer.
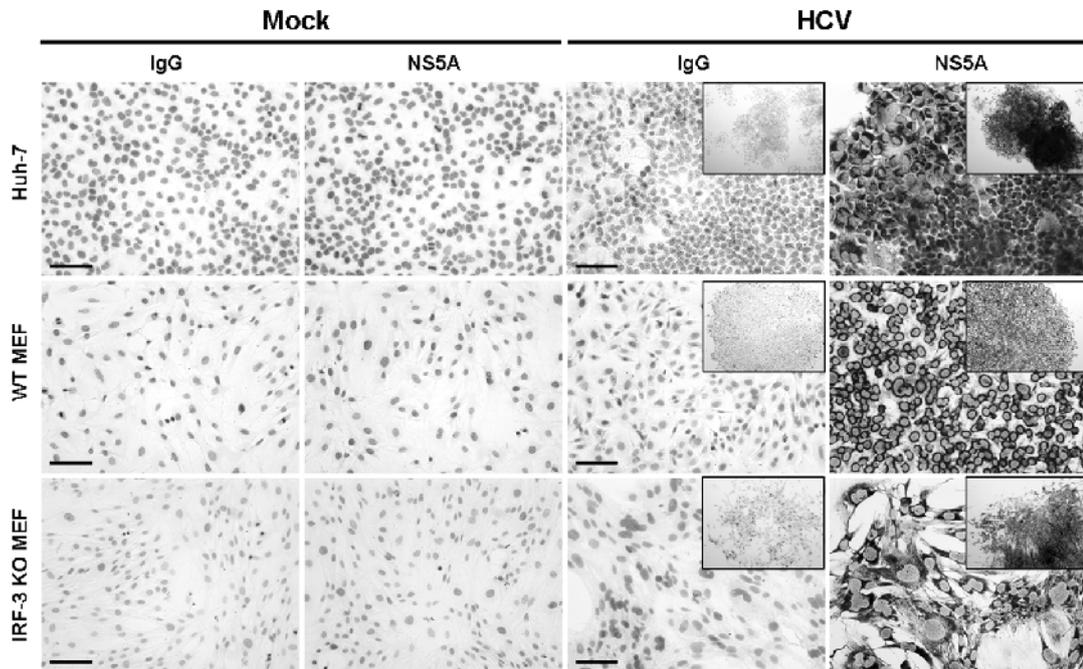
FIG. 7. Detection of NS5A protein in HCV replicon colony formation assays. Colonies obtained from blasticidin drug selection of HCV SGR-Bsd-JFH1 RNA in Huh-7 cells and WT and IRF-3 deficient (KO) MEFs were fixed and immunostained for NS5A with anti-NS5A primary or IgG control antibody. Antibody binding was determined with a goat anti-mouse secondary antibody conjugated to alkaline phosphatase. Binding of the secondary antibody was revealed by incubating the cells with NBT/BCIP substrate, which formed a brown precipitate. Nuclei were counterstained with hematoxylin. Naïve cells (Mock) from each cell type were stained for control. Magnification, 200×; inset showing colonies, 50×. Scale bars, 100 μm. Representative images from two independent experiments are shown.
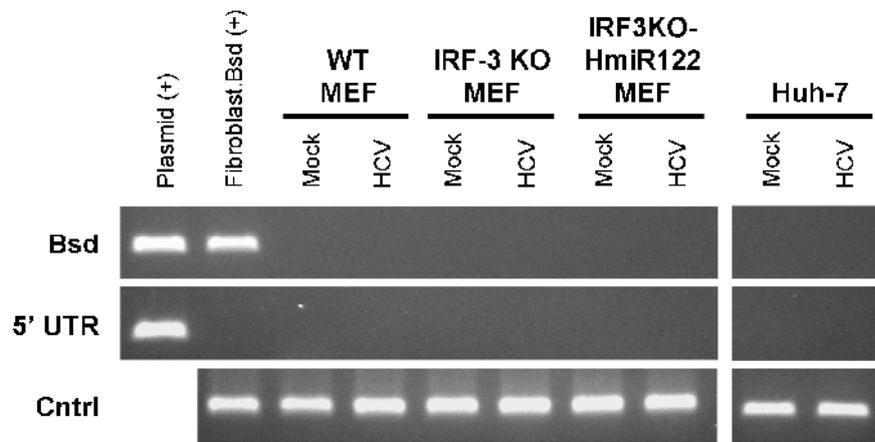
FIG. 8. Genomic PCR for Bsd gene or HCV 5' UTR. Colonies obtained from establishing HCV SGR-Bsd-JFH1 RNA in the different cell types were pooled and extracted for genomic DNA. PCR amplification using 200 ng of DNA with primers specific for Bsd and HCV 5' UTR were carried out to rule out genomic integration of HCV genetic material in the cells. Plasmid construct of HCV SGR-Bsd-JFH1 (1 ng) and murine fibroblast DNA with Bsd integration (Fibroblast.Bsd; 200 ng) served as positive controls. Human β-globin and mouse GAPDH were included as loading controls (Cntrl). Mock: naïve cells; HCV: HCV SGR-Bsd-JFH1-established replicon cells. Representative images from two independent experiments.
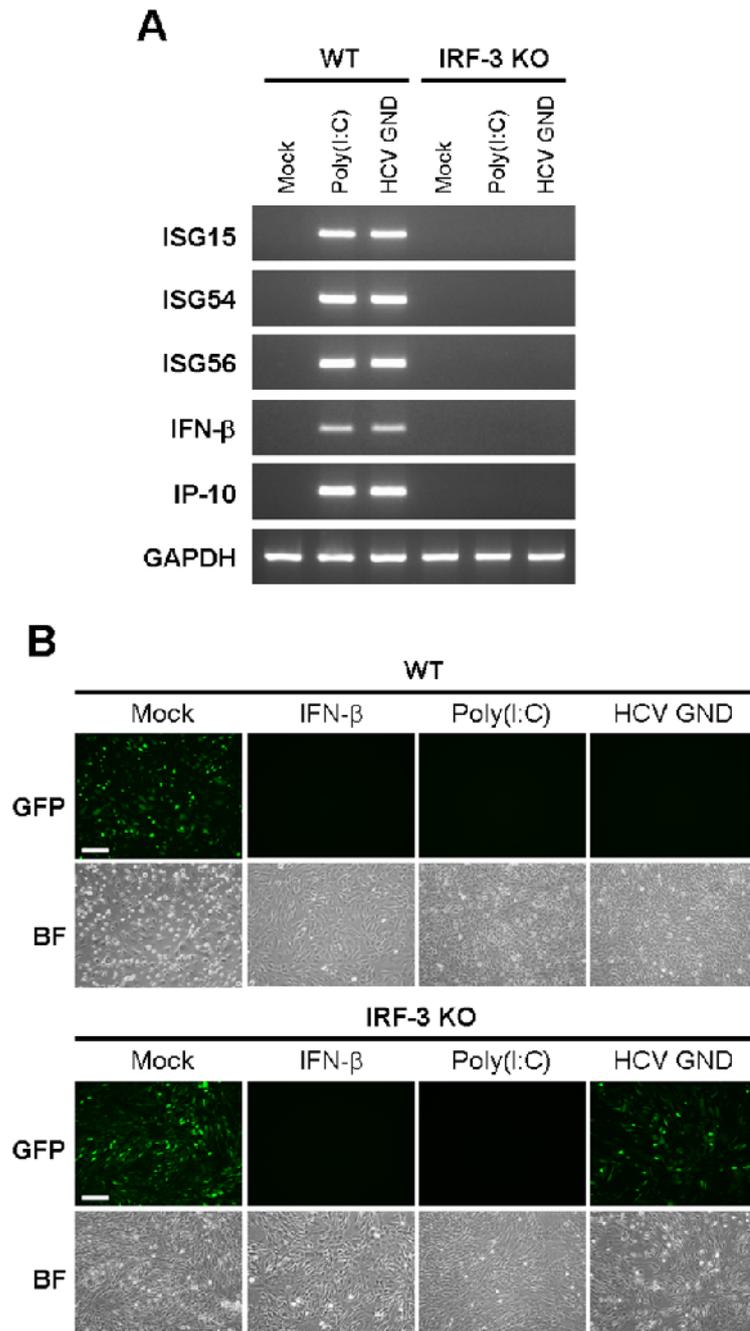
FIG. 9. HCV RNA does not induce an antiviral state against VSV in IRF-3 deficient (KO) MEFs. **(A)** RT-PCR analysis of IRF-3-associated interferon stimulated genes (ISG) in WT or IRF-3 deficient (KO) MEFs following mock-electroporation with buffer or

electroporation with 10 µg of non-replicating subgenomic HCV GND RNA or poly(I:C). Total RNA was harvested at 6 h post-electroporation and subjected to RT-PCR for the indicated genes with gene specific oligonucleotide primers. Representative images from two independent experiments are shown here. **(B)** VSV interferon bioassay on WT and IRF-3 deficient (KO) MEFs that were mock-electroporated or electroporated with 10 µg of HCV GND RNA or poly(I:C). 24 h following the electroporation, a VSV-GFP infectivity assay (MOI = 0.01) was carried out and images were taken between 24-48 h post-infection at 100× magnification. An IFN-β positive control on mock-electroporated cells was also included. GFP = green fluorescent protein, BF = bright field. Scale bars, 200 µm. Representative images are from two independent experiments.
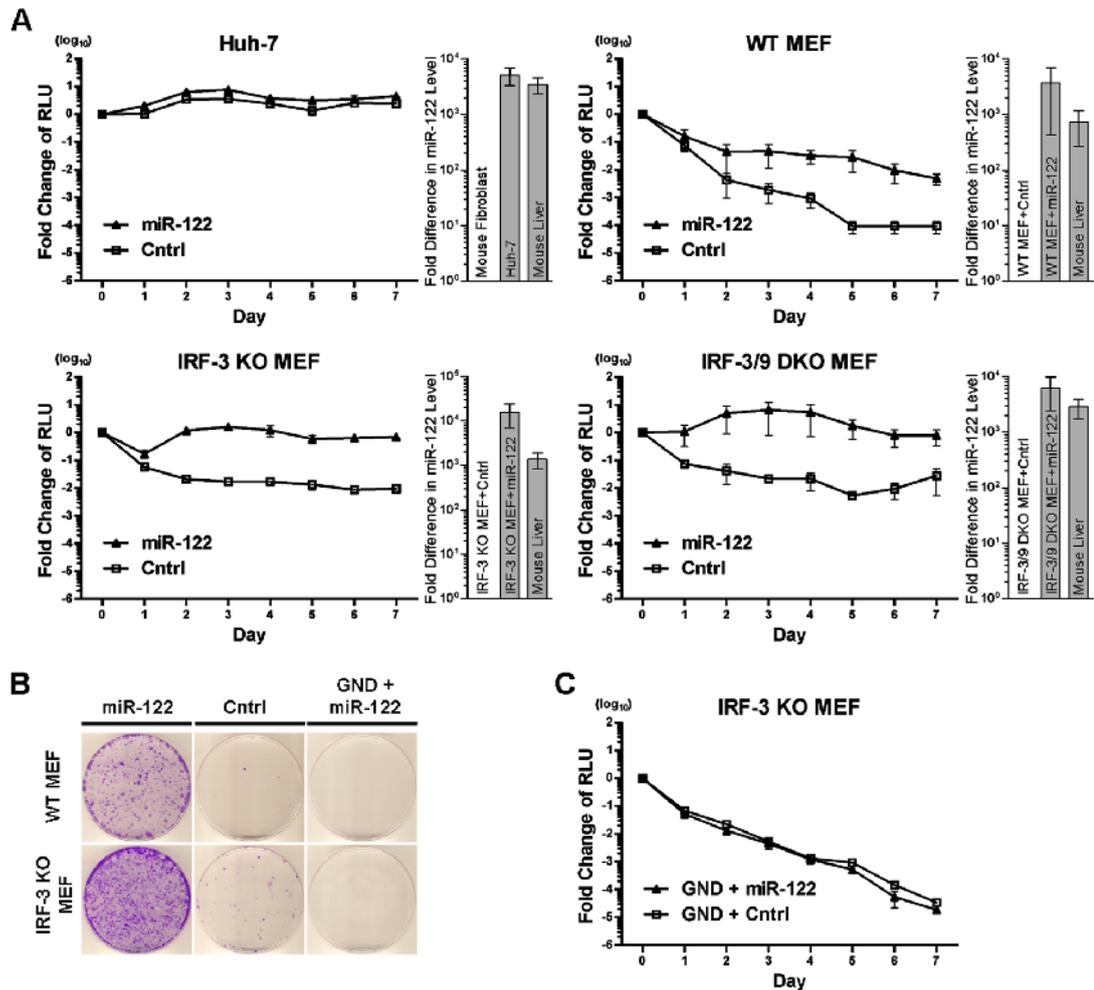
FIG. 10. The liver-specific miR-122 promotes HCV replication in mouse fibroblasts. **(A)** Transient replication assay using the HCV SGR-Luc-JFH1 Pol + RNA with miR-122 or negative control miRNA (Cntrl) in Huh-7 cells and WT, IRF-3 deficient (KO), and IRF-3/9 deficient (DKO) MEFs. Cells were co-electroporated with the HCV RNA in combination with miR-122 or negative control miRNA, harvested at the indicated time-points, and then assayed for luciferase activity. Data are plotted as in FIG. 6B, where each point represents mean values from three independent experiments with error bars showing SEM. Shown on the right of each panel is the TaqMan probe qPCR detection of

exogenously introduced mature miR-122 sequence in comparison to control miRNA and mouse liver; data from three independent experiments are plotted on log scale with error bars showing SEM. **(B)** Colony formation assay using HCV SGR-Bsd-JFH1 Pol + RNA with miR-122 or control miRNA (Cntrl), and Pol - RNA (GND) with miR-122 in WT and IRF-3 deficient (KO) MEFs. Cells were co-electroporated with the HCV RNA in combination with miR-122 or control, seeded at 1:10 dilution, and then selected with blasticidin for 2-3 weeks before staining with crystal violet. Representative images are from three independent experiments. **(C)** Transient replication assays using the non-replicating HCV SGR-Luc-JFH1 Pol - RNA (GND) with miR-122 or control (Cntrl) in IRF-3 deficient (KO) MEF. Data are expressed as in (A) with each point representing mean values from three independent experiment with error bars showing SEM.
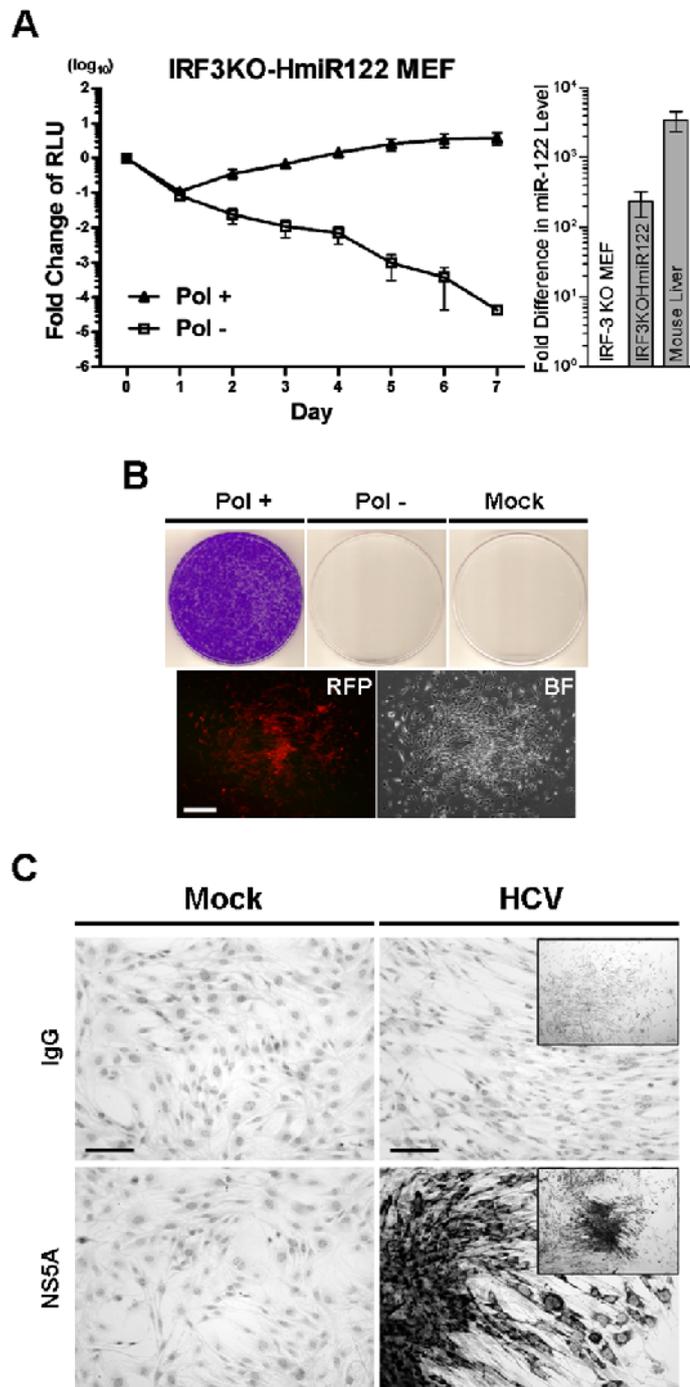
FIG. 11. HCV replicon RNA replicates efficiently in IRF-3 deficient MEFs stably expressing miR-122 that was introduced into cells by lentivirus transduction. **(A)**

Transient replication assay using the HCV SGR-Luc-JFH1 RNA in IRF-3 deficient (KO) cells stably expressing human liver-specific miR-122 (IRF3KO-HmiR122 MEFs). The assay was performed as in FIG. 6B. Each point represents mean values from three independent experiments with error bars showing SEM. Pol + (solid triangle): polymerase-active HCV RNA; Pol - (open square): polymerase-defective (non-replicating; GND) control. The bar graph on the right is the TaqMan probe qPCR detection of the mature miR-122 sequence in the IRF3KO-HmiR122 MEFs in comparison to parental IRF-3 KO MEFs and mouse liver from three independent experiments with the data plotted on log scale and error bars showing SEM. **(B)** Colony formation assay using HCV SGR-Bsd-JFH1 RNA in the IRF3KO-HmiR122 MEFs. The assay was performed as in FIG. 6C with electroporated cells seeded at a 1:2 ratio; shown here are representative images from three independent experiments. The established colonies express TurboRFP (bottom panels) due to miR-122 expression. The photomicrographs were taken at a magnification of 50× and the scale bar indicates 200 μm. Pol +: polymerase-active HCV replicon RNA; Pol -: polymerase-defective (non-replicating; GND) replicon control; Mock: mock-electroporation with buffer; RFP = red fluorescent protein; BF = bright field. **(C)** Detection of HCV NS5A protein in IRFKO-HmiR122 MEF colonies established with HCV SGR-Bsd-JFH1 RNA. The assay was performed as in FIG. 7 using primary mouse anti-NS5A antibodies, alkaline phosphatase goat anti-mouse secondary antibodies, and NBT/BCIP detection. Photomicrographs were taken at a magnification of 200× and the inset showing colonies was photographed at a magnification of 50×. Scale bars indicate 100 μm. Representative images are from two independent experiments.

CHAPTER 4

REPLICATION OF HEPATITIS C VIRUS FULL-LENGTH GENOME IN MOUSE CELLS REQUIRES ADDITIONAL HOST FACTORS BESIDES MICRORNA-122

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4.1. Abstract

Hepatitis C virus (HCV) establishes chronic infection that causes significant morbidity associated with end-stage liver disease. Generation of a convenient mouse model capable of supporting natural HCV infection could further facilitate the development of antiviral drugs and vaccines against the virus. HCV exclusively infects the livers of humans and chimpanzees. We have previously demonstrated that subgenomic replication of HCV is significantly enhanced in mouse fibroblasts by deletion of interferon regulatory factor (IRF)-3 pathway and the expression of the liver-specific microRNA-122 (miR-122). In this study we extend our investigation to the full-length HCV genome, by studying its ability to replicate in several murine intracellular environments. Surprisingly, murine fibroblasts deficient in IRF-3 and overexpressing miR-122 were non-permissive to full-length HCV RNA genome replication. Furthermore, murine liver cells, irrespective of miR-122 expression, were also non-permissive to HCV genome amplification. These observations suggest that additional host-specific factor(s) independent of miR-122 are required to mediate HCV genome replication. The relationship of subgenomic and genomic HCV RNA to the intracellular environment in terms of mediating viral replication is discussed.

4.2. Introduction

Hepatitis C virus (HCV) is a pandemic pathogen that chronically affects about 170 million people worldwide. The virus establishes persistent infections (hepatitis C) in exposed individuals that can lead to severe liver conditions such as cirrhosis and hepatocellular carcinoma. There is currently no vaccine available for prevention of

hepatitis C, and the standard treatment of pegylated interferon (IFN) in combination with ribavirin is often plagued by poor antiviral response and low patient compliance. The severity of chronic HCV infection has made it the most common indication for liver transplantation (72).

HCV possesses a positive single-stranded RNA genome that contains an open reading frame flanked by 5' and 3' untranslated regions (UTRs) that codes for a single polyprotein. Following translation in the host cell, the polyprotein is processed by viral and host proteases into 10 different proteins. The N-terminus region of the polyprotein consists of structural proteins, including core, envelope glycoprotein 1 (E1), and E2, followed by the ion channel p7, and then continues on towards the C-terminus with non-structural proteins. These include NS2, NS3, NS4A, NS4B, NS5A, and NS5B (342). NS2 is a cysteine protease that is responsible for cleavage between NS2 and NS3, whereas the NS3/4A serine protease complex mediates cleavage and release of the remaining regions of the polyprotein between NS3 to NS5B. Replication of HCV is driven by the viral RNA-dependent RNA polymerase (RdRp) NS5B and occurs in cytoplasmic regions known as the "membranous web", which is induced by NS4B expression (129, 175). The membranous web consists of endoplasmic reticulum (ER) membranes and lipid droplets (LDs), which function as the site of virion assembly (339). Interactions mediated by core and NS5A function to attract viral proteins and genomic RNA from the replication complexes onto the LDs to produce infectious virions (21, 66, 339, 429). The process of virus maturation and egress is thought to involve the very low-density lipoprotein (VLDL) assembly pathway (230).

Animal modeling of viral infections continues to be an important cornerstone to antiviral drug development, vaccine trials, and the study of viral pathogenesis. The mouse represents an invaluable experimental tool since they are amenable to genetic manipulation and easy to handle. However, there is still a need for a readily available mouse model that supports natural HCV infection. This obstacle has hampered development and preclinical testing of antiviral treatments and preventive vaccines. The virus is known to possess species- and tissue-specific restrictions, and only infects livers of humans or chimpanzees. The chimpanzee is the only immunocompetent *in vivo* experimental system that is susceptible to exogenous HCV infections. However, use of this animal is limited due to ethical concerns, availability, and high cost of maintenance and handling. Despite the development of transgenic mice overexpressing viral proteins or SCID (severe combined immunodeficient) mice containing human liver xenografts (reviewed in (35)), these systems do not accurately recapitualate HCV pathogenesis *in vivo*. There is a need to develop a better HCV small animal model that will accelerate testing of therapeutics and vaccines against the virus.

Identifying the critical host factors that support HCV RNA replication in murine cells is essential for generating a mouse system that will support natural viral infections. To this end, we previously studied the ability of subgenomic HCV RNA (consisting of the 5' and 3' UTRs flanking NS3-NS5B) to replicate in mouse embryonic fibroblasts (MEFs). We demonstrated that innate immunity of the host cell and the liver-specific miR-122 are two independent factors that influence subgenomic RNA replication in the MEFs (292). Specifically, the IRF-3-mediated type I IFN response restricts subgenomic HCV RNA replication, while miR-122 significantly enhances its amplification in the

MEFs. Furthermore, the conditions created through IRF-3 deficiency and miR-122 overexpression rendered the MEFs highly permissive to replication of HCV subgenomes (292). The level of replication was close to that observed in the Huh-7 cell line, which is the most susceptible human cell type supporting replication of HCV *in vitro* (294). These studies indicated that the intracellular replication of subgenomic HCV RNA in the rodent cells was affected by similar host factors that were reflected in the Huh-7 cells. These cells contain a deficiency in innate immunity due to lack of Toll-like receptor 3 (TLR3) (287) and express high levels of miR-122 (79). This makes permissive HCV RNA replication possible (232, 491). However, the ability of full-length HCV genome to replicate in a murine cells lacking IRF-3 and expressing miR-122 was not assessed.

In continuation of our previous work, this article examines the replication of full-length JFH1-based genomes in IRF-3 deficient (KO) murine cells that stably express human liver-specific miR-122 (IRF3KO-HmiR122 MEFs). In addition, murine liver cell lines were also assessed for permissive HCV replication. Surprisingly, genome-length HCV RNA does not replicate in IRF3KO-HmiR122 MEFs, even in the presence of miR-122. Introduction of the full-length viral RNA into murine liver cell lines that contained miR-122 also remained non-permissive to viral replication, suggesting that additional human host factor(s) are required to support HCV genome replication in rodent livers. Our data suggest that other viral components besides NS3-NS5B mediate HCV genome replication. The results also indicate that there are differences between subgenomic and full-length HCV RNA replication in terms of their requirements for host cell factors, during the process of viral replication.

4.3. Results

4.3.1. IRF3KO-HmiR122 MEFs Do Not Support Full-length HCV RNA Replication

In an attempt to model the complete HCV life cycle in rodent cells, we utilized a full-length reporter HCV genome system to assess virus replication. The Jc1FLAG2(p7-nsGluc2A) (324) is a monocistronic reporter construct that contains a *Gaussia* luciferase (GLuc) gene in tandem with the FMDV autoproteolytic peptide 2A, and is flanked by p7 and NS2 (FIG. 12). Following translation of the HCV polyprotein, the GLuc reporter is thought to translocate to the ER lumen along with the p7 C-terminal transmembrane domain. GLuc is released from the polyprotein due to cleavage by a host signal peptidase at the ER and by the autocleavage catalyzed by the FMDV 2A peptide at the GLuc C-terminus. The reporter stays fused with FMDV 2A peptide and is secreted, whereas the remaining HCV polyprotein continues its normal processing. The Jc1FLAG2(p7-nsGluc2A) offers sensitive detection of HCV RNA translation through the release of secreted GLuc into the culture media. This "full-length" replicon can be used to measure RNA replication when compared to a polymerase defective GNN negative control. Furthermore, Jc1FLAG2(p7-nsGluc2A) can produce HCVcc (cell-culture derived HCV) particles containing the GLuc gene that can be used to monitor virion production.

Reporter RNA was introduced into our previously established IRF3KO-HmiR122 MEFs, whose deficiency in IRF-3 and overexpression of miR-122 had been demonstrated to support subgenomic HCV RNA replication. Huh-7 cells were also electroporated with the same RNA as a positive control. Culture supernatants were assessed for GLuc activity, and values were normalized for electroporation efficiency against the signals emitted from input determined at 4 h post-transfection. As expected, the Huh-7 cells were

permissive to full-length HCV genome replication. This was indicated by GLuc activity measured in the Huh-7 culture supernatant with progressive logarithmic amplification of the signal over the 9-day time-course, which is indicative of viral replication (FIG. 13A). On the other hand, the non-replicating reporter GNN genome, which contains a lethal mutation in the RdRp, remained at baseline throughout the incubation. Surprisingly, amplification of the reporter HCV genome did not occur in the IRF3KO-HmiR122 MEFs, which produced a flat baseline similar to the GNN negative control throughout experiment (FIG. 13B). We also examined the translation profile of input RNA by following the reporter signals from full-length replicons over time and comparing them to backgrounds from a mock transfection. Both the polymerase active GLuc reporter RNA and the inactive GNN mutant produced similar bioluminescence levels that were above the mock background in the IRF3KO-HmiR122 MEFs (FIG. 14A and B). This suggested that translation of the polyprotein did occur in these cells. To rule out the possibility that the reporter was not efficiently secreted into the media, lysates and culture supernatants from the above experiments were assayed and compared for GLuc activity. As indicated in FIG. 15, cell lysate signals for GLuc were lower than those in the supernatant for Huh-7 cells (FIG. 15A), which was expected since most of the GLuc should be secreted. However, the levels of the reporter activity remained at baseline levels in both the cell media and cell lysates from the IRF3KO-HmiR122 MEFs (FIG. 15B). Taken together, the above observations suggest that the IRF3KO-HmiR122 MEFs allow translation of the polyprotein, but do not support replication of the full-length HCV reporter genome.

4.3.2. Supernatant From IRF3KO-HmiR122 MEFs Containing Full-Length HCV RNA Is Not Infectious To Huh-7 Cells

We also tested whether the full-length Jc1FLAG2(p7-nsGluc2A) HCV RNA produced infectious viral particles after it was introduced into IRF3KO-HmiR122 MEFs by electroporation. Culture supernatants from day 6 of the above experiments were harvested, freeze-thawed, clarified, and then used to infect naïve Huh-7 and Huh-7.5 cells, which are highly permissive to HCV replication following entry. After infection, the cells were washed three times to remove non-adsorbed virus and left-over GLuc from the previous experiment. Consistent with previous results in the literature, infectious particles were obtained from day 6 culture supernatant of Huh-7 cells transfected with polymerase-active Jc1FLAG2(p7-nsGluc2A) RNA (FIG. 16A and C). This was evidenced by GLuc signals following 72 h infection of either naïve Huh-7 or Huh-7.5 cells with the electroporated cell media. In contrast, culture supernatants from GNN control or mock electroporations of Huh-7 cells did not produce viral particles. In addition, IRF3KO-HmiR122 MEFs were resistant to infection by reporter virus particles produced from Huh-7 cells, consistent with the fact that murine cells lack receptor and entry factors for HCV (FIG. 16E) (387). Supernatants from IRF3KO-HmiR122 MEFs transfected with full-length HCV reporter RNA also did not produce any GLuc signals above background in Huh-7, Huh-7.5, and naïve IRF3KO-HmiR122 MEFs (FIG. 16B, D, and F). Furthermore, culture supernatants from later time-points (day 9 and day 12) of IRF3KO-HmiR122 MEFs electroporations did not yield infection of naïve Huh-7 cells (FIG. 17A and B). These data are consistent with previous results, where the reporter genome did not replicate in the IRF3KO-HmiR122 MEFs. We concluded that infectious virus was not produced in IRF3KO-HmiR122 MEFs. Similar observations were noted in wild-type

MEFs as well, which were also non-permissive for Jc1FLAG2(p7-nsGluc2A) replication and virus production (FIG. 18A and B). However, translation of the HCV RNA genome was detected in the MEFs (FIG. 18C).

4.3.3. IRF3KO-HmiR122 MEFs Are Also Non-Permissive To HCV FL-J6/JFH1 Genome Replication And Do Not Produce Infectious Virions Upon Transfection

Since the IRF3KO-HmiR122 MEFs were incapable of supporting replication of the Jc1FLAG2(p7-nsGluc2A) reporter HCV genome, we needed to verify that inclusion of the reporter, itself, did not perturb viral replication in the murine cells. We chose to use the full-length HCV J6/JFH1 genome (FL-J6/JFH1), which is also capable of producing HCV virions in Huh-7 cells (294). The cDNA for J6/JFH1 does not contain a reporter gene (FIG. 12). IRF3KO-HmiR122 MEFs and Huh-7 cells were electroporated with FL-J6/JFH1 RNA, and cells were harvested at subsequent times and assayed for HCV replication using real-time qRT-PCR. While polymerase active FL-J6/JFH1 RNA replicated in the Huh-7 cells, the GND polymerase mutant RNA progressively declined (FIG. 19A). In the IRF3KO-HmiR122 MEFs, both the RNAs of wild-type and mutant J6/JFH1 HCV consistently decreased over time, indicating that the FL-J6/JFH1 also did not replicate in these cells (FIG. 19B). Supernatants from HCV RNA-transfected cells were also examined for infectivity on naïve Huh-7 cells. Only the supernatant from FL-J6/JFH1 RNA-electroporated Huh-7 cells produced virions that formed foci in naïve Huh-7 cells (FIG. 20). These results reflect a correlation in data from both reporter and non-reporter HCV genomes, whereby IRF3KO-HmiR122 MEFs are indeed non-permissive to full-length HCV RNA replication. Again, a similar scenario was observed with the wild-type MEFs, which does not support FL-J6/JFH1 RNA replication (FIG.

21A), nor does it produce any infectious virions that could infect naïve Huh-7 cells (FIG. 21B). Of note, the viral RNA was observed to decline more rapidly in the wild-type MEFs, consistent with the notion that they possess a more intact innate immune system than the IRF3KO-HmiR122 MEFs or Huh-7 cells (FIG.19 A and B versus FIG. 21A).

4.3.4. Absence Of HCV Genome Replication In Murine Liver Cell Lines That Contain MiR-122

The above studies provide evidence that the murine fibroblasts are non-permissive for full-length HCV RNA genome replication, even in the absence of IRF-3 and in the presence of miR-122 expression. This suggests that additional host factor(s), independent of these components, contribute to HCV RNA amplification in murine cells. The hepatotropic nature of HCV suggests that the intracellular environment of the liver may be an important factor. Therefore, murine hepatocytes should possess factors that favour replication of the HCV RNA genome. Consequently, we assayed 3 different murine liver cell lines for their ability to support HCV replication. The FL83B and NMuLi are cell lines established from normal murine liver, whereas the Hepa 1-6 is derived from a mouse hepatoma. We first monitored the levels of miR-122 in these cells. As shown in FIG. 22, miR-122 was detected in NMuLi and Hepa 1-6, albeit at lower levels than what was measured in IRF3KO-HmiR122 MEFs, Huh-7, and mouse liver tissue. In contrast, FL83B did not contain any detectable miR-122 expression just like normal murine fibroblasts which are miR-122-negative. Following addition of reporter GLuc RNA into the host cell by nucleofection, we did not observe genome replication in any of the murine liver cell lines tested, whether miR-122 was present or not (FIG. 23A). However, translation was observed for both the polymerase active and defective RNA genomes in

all three cell lines over the course of the experiment. This suggested that the HCV IRES (internal ribosome entry site)-mediated translation can occur in these mouse liver cells (FIG. 23B). The data in the Hepa 1-6 cells agrees with a previous report, which showed that this hepatoma cell line was capable of supporting HCV IRES-driven translation but not HCV JFH1 genome replication (367). Altogether, the above data indicate that replication of HCV genome is still blocked in the murine liver cells, whether they contain miR-122 or not. The data suggest that additional human liver factor(s) are required to promote the replication of full-length HCV RNA.

4.4. Discussion

Our previous study suggested that the IRF-3-mediated type I IFN response and liver-specific miR-122 play roles in regulating subgenomic HCV RNA replication in MEFs (292). In this study, however, we observed that the same murine fibroblasts (deleted for IRF-3 and overexpressing miR-122), were unable to support full-length HCV genome growth. Furthermore, murine liver cells with or without miR-122 expression, also did not promote HCV genome amplification. Translation, however, was observed for both polymerase active and inactive full-length RNAs in these cell lines, suggesting that HCV IRES activity was not inhibited. These observations indicate that HCV genome replication requires additional host-specific factor(s) independent of miR-122. At the same time, it also implies that these host-specific factors may not be mutually exclusive, and a specific combination might be required to facilitate genomic replication of HCV.

Subgenomic replicons serve as an *in vitro* model for HCV transcription and translation when introduced into a permissive cell. Although the structural proteins and

the non-structural p7 and NS2 are required for virion assembly and the production of infectious virus (228), they are dispensable for viral RNA synthesis because the subgenomes lacking the core to NS2 region of the full-length genome are capable of efficient replication in Huh-7 cells (60, 243, 303). However, it is becoming clear that in the context of a full-length viral genome, the concerted interplay of viral protein-protein interactions and host components contribute to HCV RNA amplification and the formation of virus particles. We speculate that presence of the core-NS2 region in the full-length genome affects the intracellular environment or site of HCV replication during the process of polyprotein synthesis and virus assembly. For instance, NS2 is increasingly being recognized as the master regulator of HCV life cycle. The autocleavage mediated by NS2 to liberate NS3 from NS2, is crucial for viral replication. This has been shown using subgenomic replicons containing NS2-NS5B and full-length cDNA clones harboring mutations in the NS2/3 junction site (255, 497). The NS2/3 protease can also regulate HCV IRES-dependent translation and replication mediated by the NS5B activity (430). Studies have indicated that NS2 can interact with various HCV structural and non-structural proteins including E1, E2, p7, NS3, NS4A, NS4B, NS5A, and NS5B (120, 250, 316, 426). Colocalization of NS2 with several of the viral proteins such as E1, E2, and NS5A, is found near lipid droplets (316) where the viral core protein is known to associate during virion assembly. The complex formation between NS2 and the other viral proteins are thought to play important role in the viral life cycle, particularly in mediating/guiding interactions between the envelope proteins and non-structural proteins as they participate in the assembly of infectious particles. Since NS2 has been observed to interact with several host factors to modulate host gene expression and the cellular

environment (498), it may serve as a scaffold for viral as well as cellular proteins during the assembly process. Its presence could account for the differences in replication seen between the NS3-NS5B subgenomic RNAs used in our previous investigation and the full-length genomes examined in the present study. For example, NS2 could dictate host restriction or species tropism by recruiting host factors that are attracted to the intracellular sites of viral replication and assembly. Examining the role of NS2 and its cellular partners will perhaps help shed some light on these issues.

Replication of subgenomic HCV RNA in murine cells has been previously observed by our laboratory and others (82, 292, 475, 530). Other attempts have also been made to replicate full-length HCV genome in murine hepatocytes using RNA transfection or infection with HCV particles. However, these endeavours yielded little success or produced efficiencies that were lower compared to Huh-7 cells (315, 367, 475, 512). Our previous study was the only one, so far, to make reference to miR-122 in rodent cells. We demonstrated miR-122's capacity to facilitate HCV subgenomic RNA replication in the normally non-permissive and non-hepatic murine cells (fibroblast). It should be noted that mouse and human mature miR-122 are identical. Most hepatoma cells are downregulated in expression of miR-122 and it is only fortuitous that Huh-7 cells retain high level of this miRNA, since other hepatoma cell lines like HepG2, Hep3B, and SK-Hep-1 do not (79, 104, 267, 467). While miR-122 may seem to be a strong determinant of HCV replication, additional host factor(s) define species tropism and facilitate genome-length HCV RNA synthesis. This was documented by the absence of full-length HCV replication in IRF3KO-HmiR122 MEFs and the murine liver cell lines, despite the presence of miR-122. We could not rule out the possibility that the lack of genomic RNA

replication was due to lower levels of the miR-122 present in the murine cell lines (FIG. 22). It is possible to increase these levels through exogenous miR-122 expression. However, given the strong effect of miR-122 on HCV RNA amplification, we would expect to see at least some level of replication in the NMuLi and Hepa 1-6 cell lines, if it were the sole factor involved. The results indicate that the presence of miR-122 is insufficient to promote viral genomic RNA synthesis and virus assembly.

Further studies will be required to uncover the additional host factor(s) implicated in supporting full-length HCV genome replication in the rodent cells. Experiments with shRNA/siRNA libraries and high-content robotic screening using permissive cell lines and methodical knockdown of gene function may reveal these factors. Unraveling the key determinants involved could help decipher the species-tropism barrier to HCV.

4.5. Acknowledgements

4.6. Figures And Legends

FIG. 12. Structures of HCV full-length genome and constructs used in this study. Shown are schematic representation (not to scale) of full-length HCV JFH1 RNA genome, chimeric FL-J6/JFH1 genome, and *Gaussia* luciferase reporter Jc1 (J6/JFH1 chimera) construct Jc1FLAG2(p7-nsGluc2A). The Jc1FLAG2(p7-nsGluc2A) reporter chimera bears an intragenotypic breakpoint in NS2. Shaded area in gray indicates genome portion based on the J6 backbone. FMDV 2A: foot-and-mouth disease virus 2A peptide; FLAG: FLAG epitope; GLuc: *Gaussia* luciferase reporter.
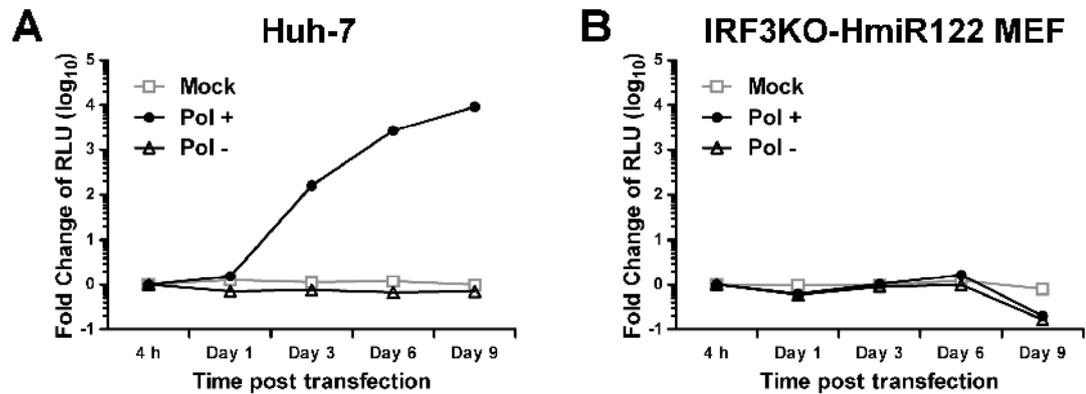
FIG. 13. IRF3KO-HmiR122 MEFs do not support full-length reporter HCV RNA replication. Huh-7 **(A)** and IRF3KO-HmiR122 MEFs **(B)** were electroporated with 10 µg of the reporter Jc1FLAG2(p7-nsGluc2A) RNA and the supernatant was harvested at the indicated time-points for reading *Gaussia* luciferase activity. The luciferase reporter activity is indicative of HCV RNA replication. Bioluminescence is expressed in logarithmic scale units as the fold change of relative light units (RLU) normalized to the light emitted from input RNA determined at 4 h post-electroporation. Each point represents mean values from three independent experiments with error bars showing SEM. Mock: mock electroporation (open grey square); Pol + (solid circle): polymerase-active HCV RNA; Pol - (open triangle): polymerase-defective (non-replicating; GNN) control. The error bars are sometimes barely visible in the graphs due to low variance and the logarithmic scale.
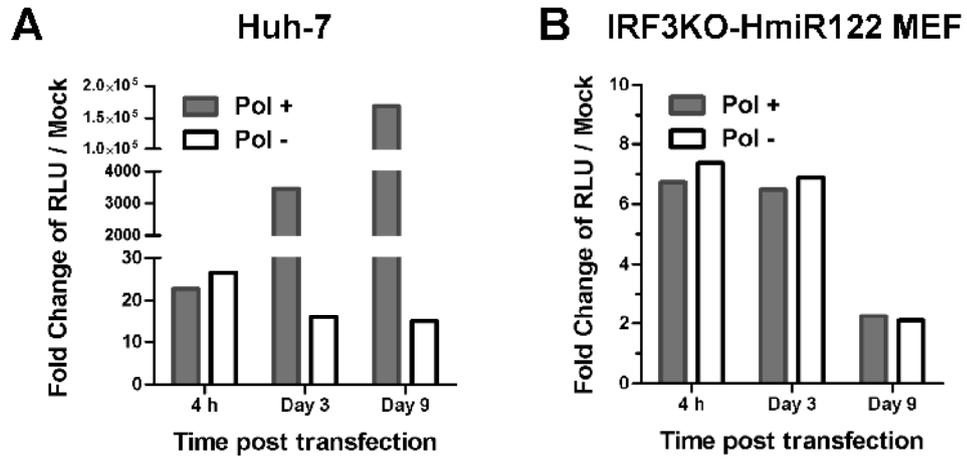
FIG. 14. Full-length reporter RNA is translated in IRF3KO-HmiR122 MEFs. To examine translation, measurements of reporter activity for Huh-7 **(A)** and IRF3KO-HmiR122 MEFs **(B)** from FIG. 13 were plotted as fold change of RLU with respect to values obtained from the respective mock transfection sample of the specific time-point indicated. Pol + (gray bar): polymerase-active HCV RNA; Pol - (open bar): polymerase-defective (non-replicating; GNN) control. Shown are representative data from three independent experiments.
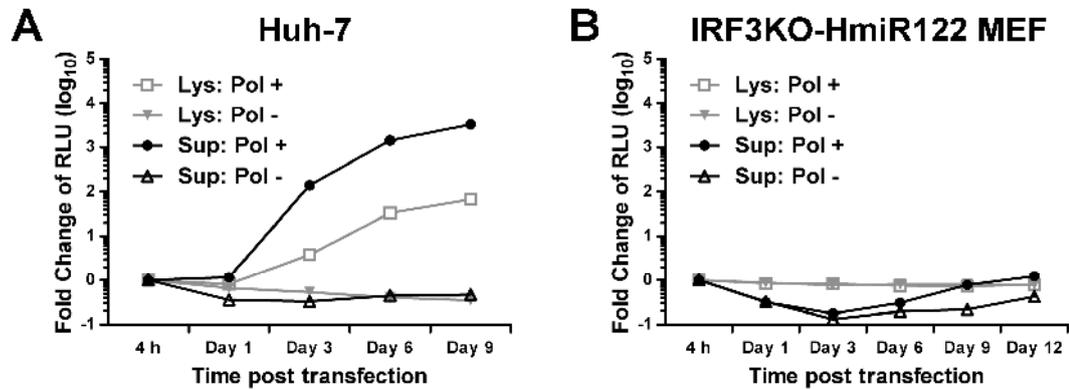
FIG. 15. Comparison of supernatant and cell lysate Gaussia luciferase activities in Huh-7 cells and IRF3KO-HmiR122 MEFs electroporated with reporter RNA. Huh-7 **(A)** and IRF3KO-HmiR122 MEFs **(B)** were electroporated with 10 μg of the reporter Jc1FLAG2(p7-nsGluc2A) RNA and both the supernatant and cell lysate were harvested at the indicated time-points for reading *Gaussia* luciferase activity. Bioluminescence is expressed in logarithmic scale units as the fold change of RLU normalized to the light emitted from input RNA determined at 4 h post-electroporation. Data shown are from one experiment. Lys: *Gaussia* luciferase activity assessed from cell lysate; Sup: *Gaussia* luciferase activity assessed from supernatant; Pol + (open grey square/solid circle): polymerase-active HCV RNA; Pol - (inverted solid grey triangle/open triangle): polymerase-defective (non-replicating; GNN) control.
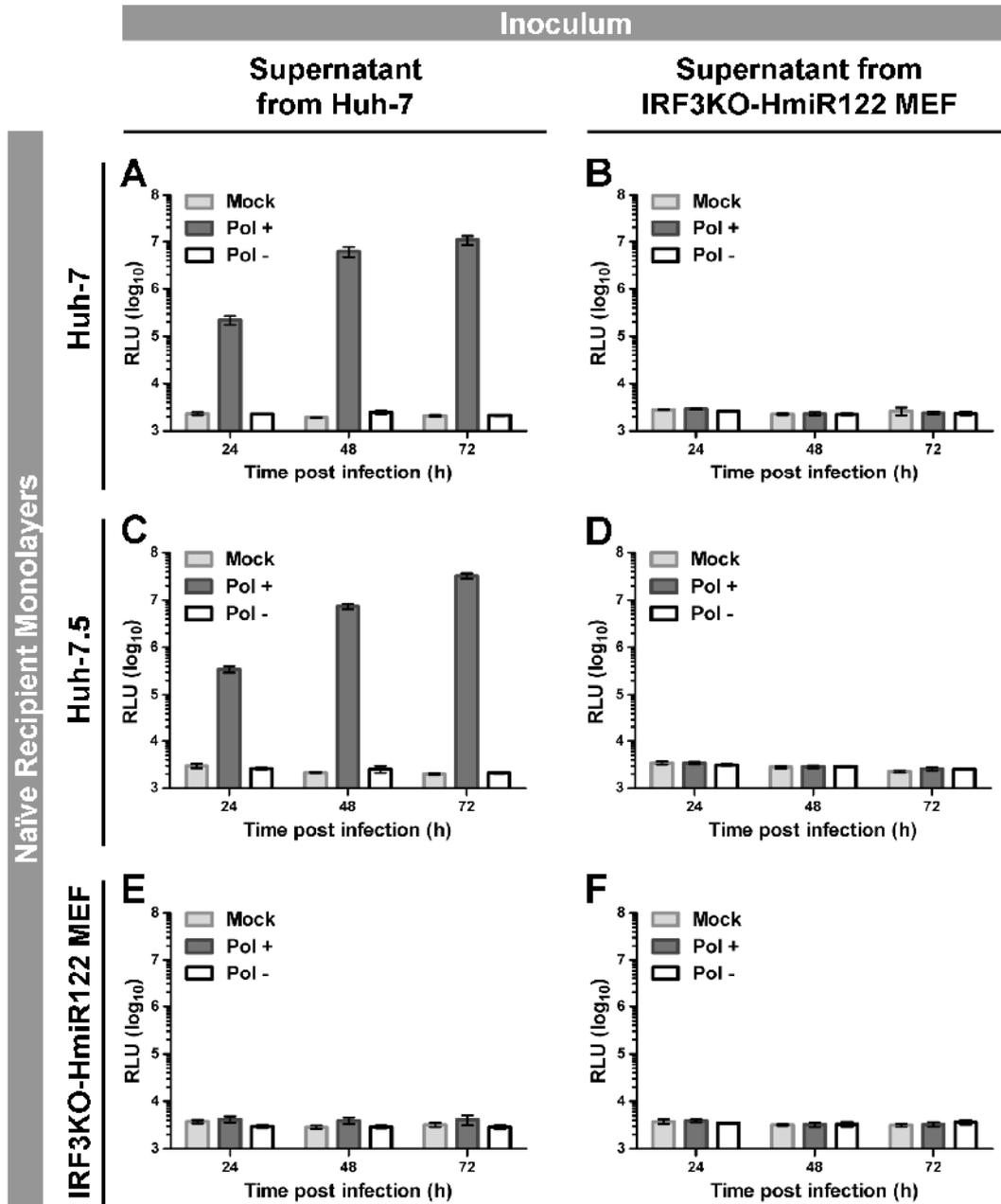
FIG. 16. Supernatant from HCV reporter RNA-electroporated IRF3KO-HmiR122 MEFs

is not infectious to Huh-7 cells. Supernatants from day 6 post-transfection of the

experiments from FIG. 13 were also harvested for infection assay. The harvested supernatants from transfected Huh-7 cells (A, C, E) and IRF3KO-HmiR122 MEFs (B, D, F) were freeze-thawed, clarified by centrifugation, and then used as inoculum to infect naïve monolayers of Huh-7 (A, B), Huh-7.5 (C, D), and IRF3KO-HmiR122 MEFs (E, F). The infection was carried out for 3 h before washing the monolayer 3 times with PBS and applying the overlay media. During subsequent incubation period, the supernatant from the respective monolayers was harvested at each indicated time-point and assessed for *Gaussia* luciferase activity. The luciferase reporter activity is indicative of HCV infection (i.e. virion was present in the inoculating supernatant). The readings were directly plotted as RLU in logarithmic scale. Each bar represents mean values from three independent experiments (starting from the electroporation) with error bars showing SEM. Mock: supernatant from mock electroporation (light grey bar); Pol + (dark grey bar): supernatant from polymerase-active HCV RNA electroporation; Pol - (blank bar): supernatant from polymerase-defective (non-replicating; GNN) control RNA electroporation. The error bars are sometimes barely visible in the graphs due to low variance and the logarithmic scale.
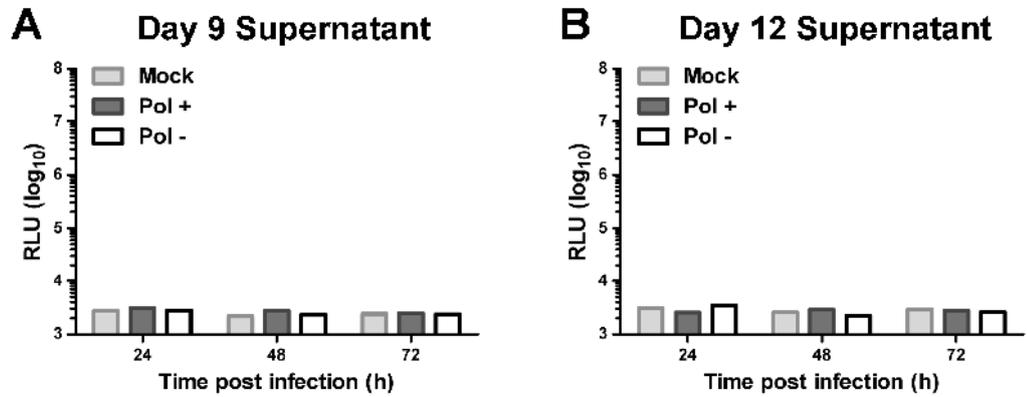
FIG. 17. Infection assay using day 9 and day 12 supernatants from reporter RNA-transfected IRF3KO-HmiR122 MEFs. Supernatants from day 9 **(A)** and day 12 **(B)** post-transfection of IRF3KO-HmiR122 MEFs in the experiment described in FIG. 13 were also used to infect naïve Huh-7 cells. The experiment was carried out as in FIG. 16. The readings were directly plotted as RLU in logarithmic scale. Each bar represents values from one experiment (starting from the electroporation). Mock: supernatant from mock electroporation (light grey bar); Pol + (dark grey bar): supernatant from polymerase-active HCV RNA electroporation; Pol - (blank bar): supernatant from polymerase-defective (non-replicating; GNN) control RNA electroporation.
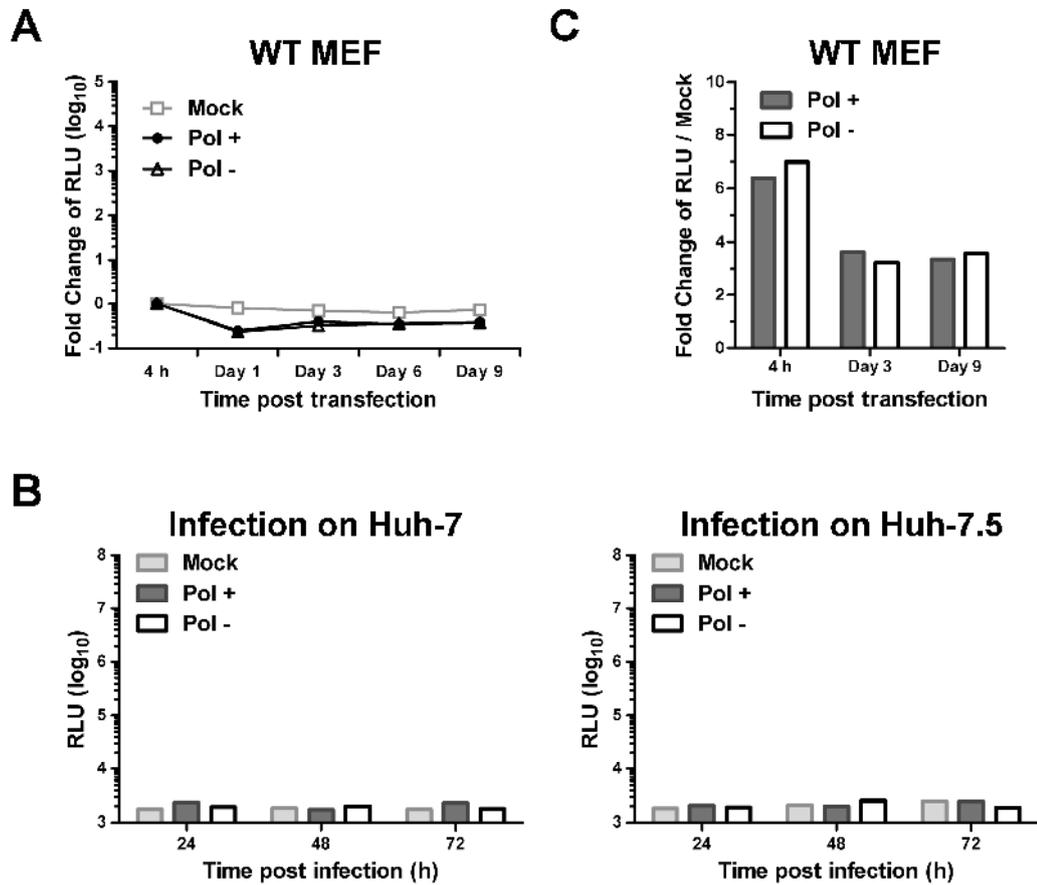
FIG. 18. Wild-type (WT) MEFs do not support full-length reporter HCV RNA replication and do not produce supernatant that is infectious to Huh-7 or Huh-7.5 cells. **(A)** WT MEFs were electroporated with 10 µg of the reporter Jc1FLAG2(p7-nsGluc2A) RNA and the supernatant was harvested at the indicated time-points for reading *Gaussia* luciferase activity as in FIG. 13. Bioluminescence is expressed in logarithmic scale units as the fold change of RLU normalized to the light emitted from input RNA determined at 4 h post-electroporation. Data shown are from one experiment. Mock: mock electroporation (open grey square); Pol + (solid circle): polymerase-active HCV RNA;

Pol - (open triangle): polymerase-defective (non-replicating; GNN) control. **(B)** Supernatant from day 6 post-transfection of the experiment in (A) was also harvested for infection assay as in FIG. 16 on naïve Huh-7 and Huh-7.5 cells. *Gaussia* luciferase readings were obtained at specific time-points post-infection, and directly plotted as RLU in logarithmic scale. Data shown are from one experiment. Mock: supernatant from mock electroporation (light grey bar); Pol + (dark grey bar): supernatant from polymerase-active HCV RNA electroporation; Pol - (blank bar): supernatant from polymerase-defective (non-replicating; GNN) control RNA electroporation. **(C)** To examine translation, measurements of reporter activity in (A) were plotted as fold change of RLU with respect to values obtained from the respective mock transfection sample of the specific time-point indicated. Pol + (gray bar): polymerase-active HCV RNA; Pol - (open bar): polymerase-defective (non-replicating; GNN) control. Shown are data from one experiment.
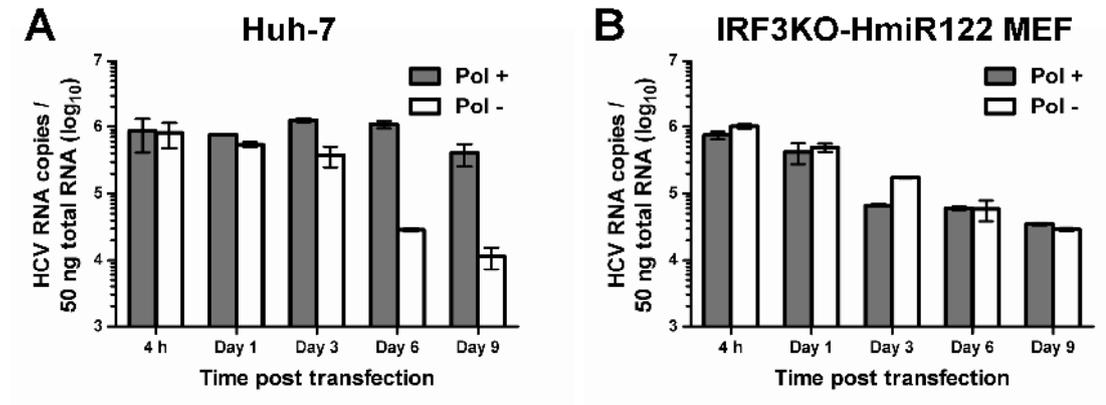
FIG. 19. IRF3KO-HmiR122 MEFs do not support full-length HCV J6/JFH1 RNA replication. IRF3KO-HmiR122 MEFs **(A)** and Huh-7 cells **(B)** were electroporated with 1 µg FL-J6/JFH1 polymerase active (Pol +) or polymerase defective (Pol -; GND) RNA before seeding in dishes, and total cellular RNA was extracted at the indicated time-points. An input of 50 ng of total cellular RNA per sample was quantified by TaqMan qRT-PCR analysis for the detection of HCV RNA. Data shown are mean values of triplicate measurements from two independent electroporation experiments with error bars showing SEM.
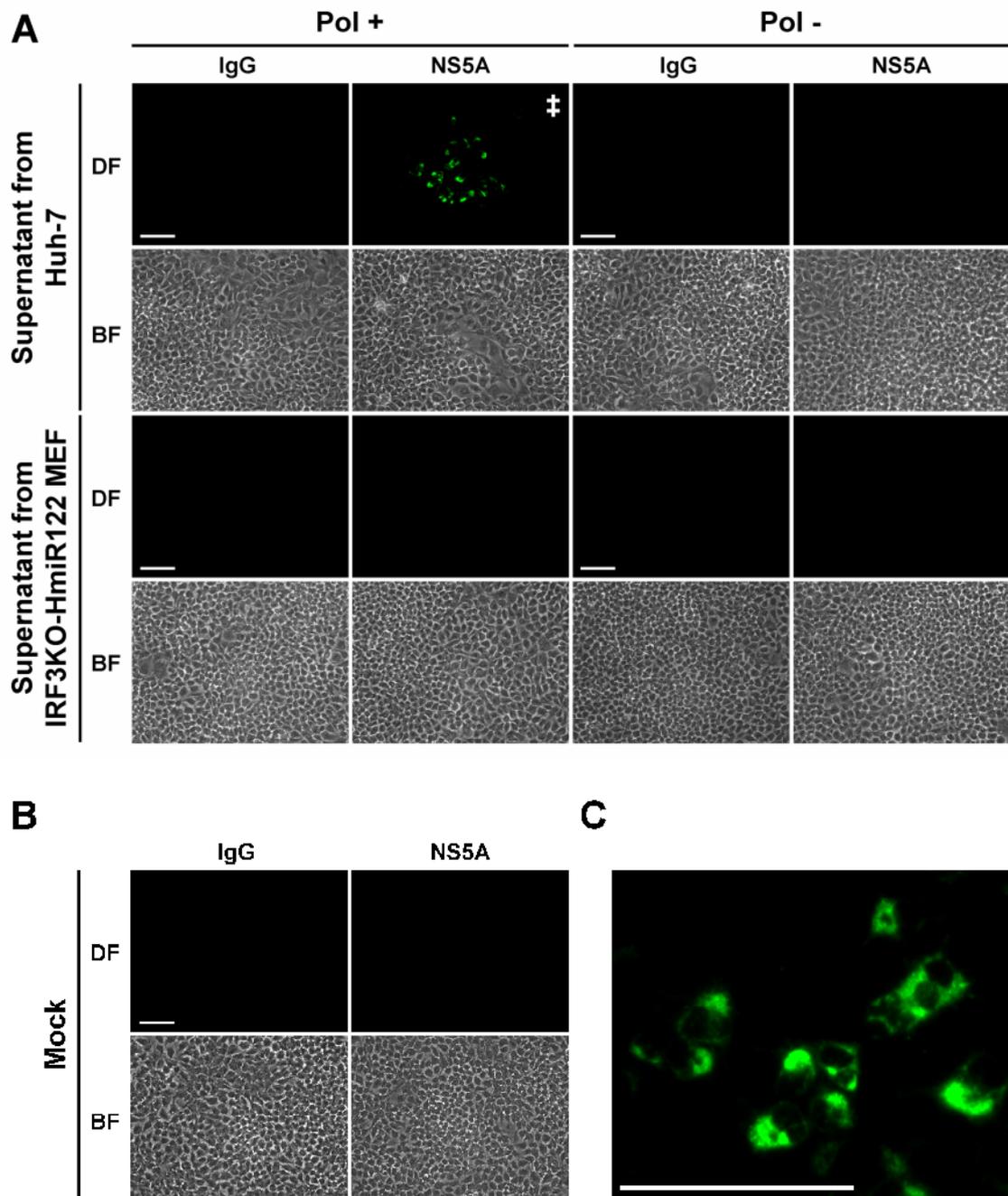
FIG. 20. Absence of HCV infection in Huh-7 cells using supernatant from FL-J6/JFH1 RNA-electroporated IRF3KO-HmiR122 MEFs. **(A)** IRF3KO-HmiR122 MEFs and Huh-7

cells were electroporated with 1 μg FL-J6/JFH1 polymerase active (Pol +) or polymerase defective (Pol -; GND) RNA and supernatant was harvested 6 days post-transfection. The harvested supernatant was freeze-thawed, clarified by centrifugation, and then used to infect naïve Huh-7 cells. The infection was carried out for 3 h before washing the monolayer 3 times with PBS and applying the overlay media. Following 3 days of incubation, the Huh-7 monolayer was assessed for HCV-infected foci expressing HCV proteins by immunostaining for NS5A with anti-NS5A primary or IgG control antibody. Antibody binding was determined with an Alexa-Fluor 488-conjugated goat anti-mouse secondary antibody. **(B)** Mock infection control immunostained with anti-NS5A or IgG control. **(C)** Magnified (6.67 times) image of ‡ showing typical cytoplasmic staining of NS5A. BF: bright field; DF: dark field. Magnification, 200×. Scale bars, 100 μm in each corresponding panels. Representative images from three independent electroporation experiments are shown.
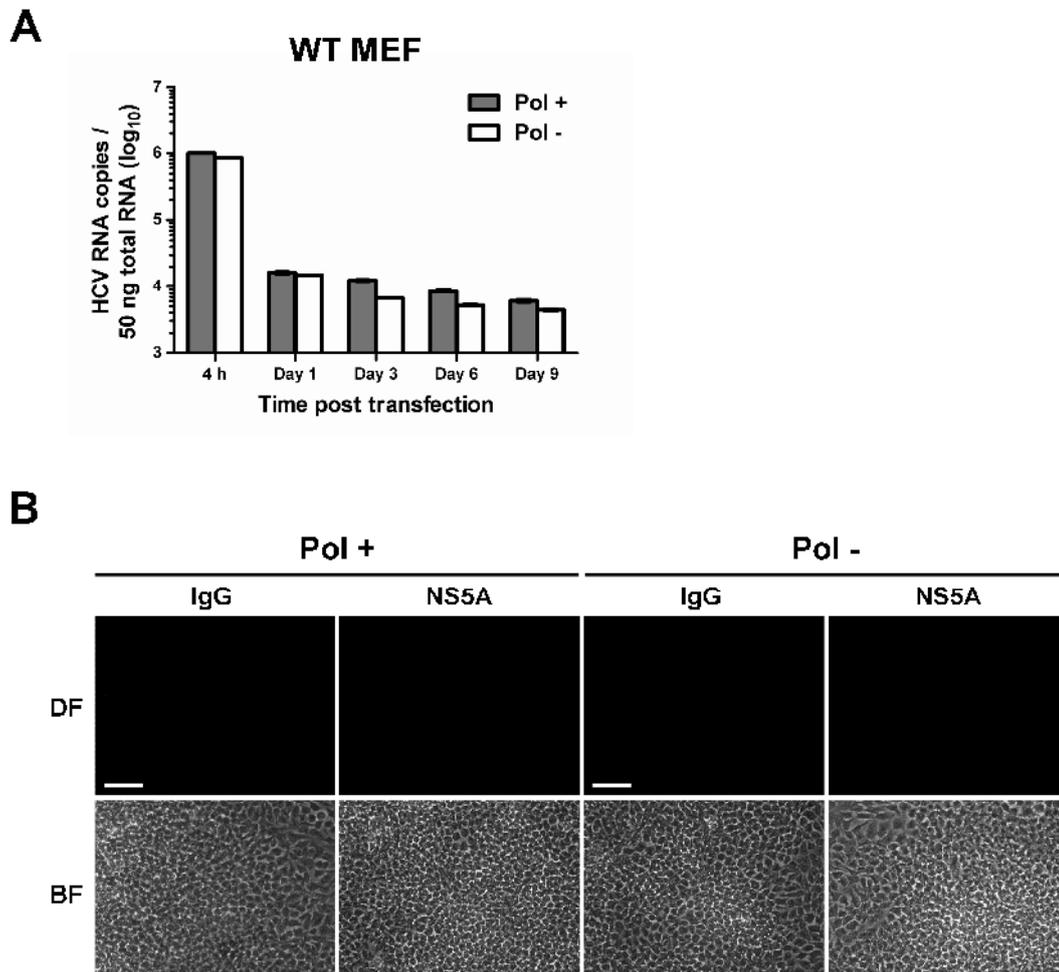
FIG. 21. WT MEFs do not support full-length HCV J6/JFH1 RNA replication and do not produce infectious supernatant. **(A)** WT MEFs were electroporated with 1 µg FL-J6/JFH1 polymerase active (Pol +) or polymerase defective (Pol -; GND) RNA and total cellular RNA was extracted at the indicated time-points for quantitation of HCV RNA by TaqMan qRT-PCR analysis as in FIG. 19. Data shown are mean values of triplicate measurements from one independent electroporation with error bars showing SEM for

the triplicate determinations. **(B)** Supernatant from day 6 post-transfection in (A) was harvested for infection assay and was carried out as in FIG. 20. HCV proteins were examined by immunostaining for NS5A with anti-NS5A primary or IgG control antibody. Antibody binding was determined with an Alexa Fluor 488-conjugated goat anti-mouse secondary antibody. BF: bright field; DF: dark field. Magnification, 200×. Scale bars, 100 μm. Images from one experiment are shown.
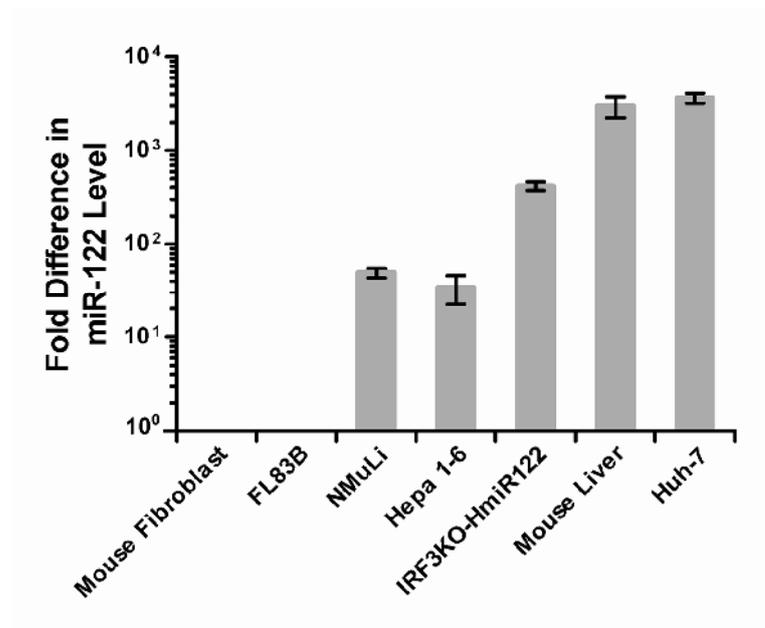
FIG. 22. Level of miR-122 expression in the murine liver cell lines. Total RNA were extracted from the cells and assessed by TaqMan probe qRT-PCR for the detection of mature miR-122 sequence. Results were plotted in comparison to the miR-122-negative mouse fibroblast on a log scale with error bars showing SEM. Data shown are from two independent experiments.
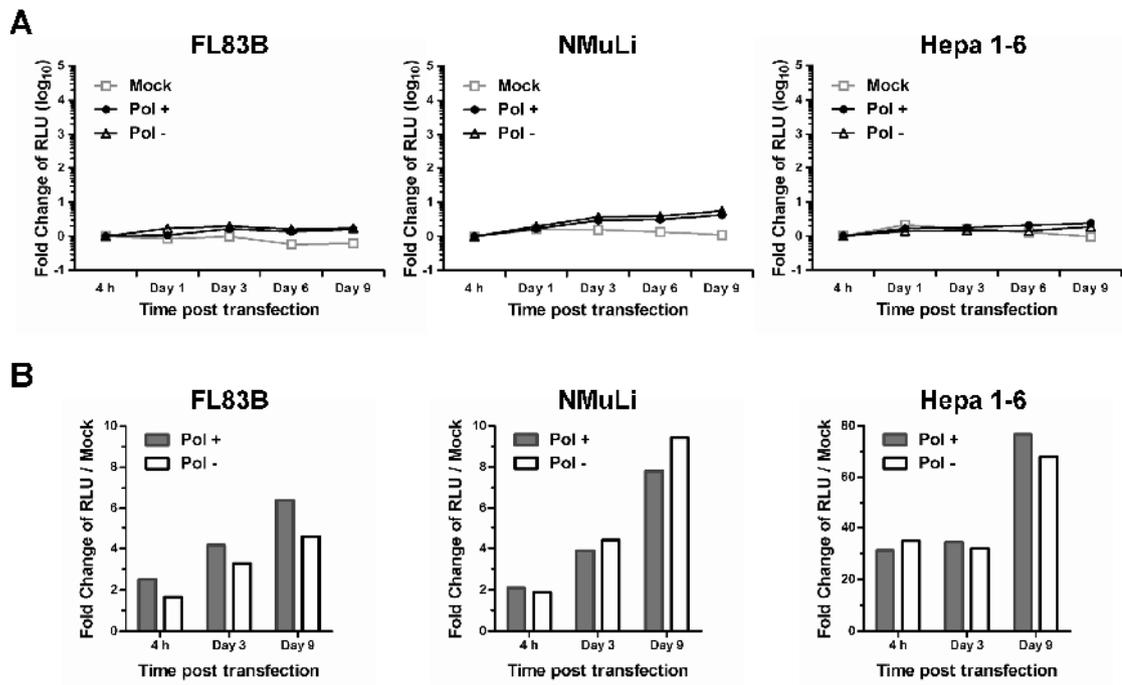
FIG. 23. Murine liver cell lines are non-permissive to full-length HCV reporter genome replication. **(A)** Cells were transfected by nucleofection with 10 μg of the reporter Jc1FLAG2(p7-nsGluc2A) RNA and the supernatant was harvested at the indicated time-points for reading *Gaussia* luciferase activity as in FIG. 13. Bioluminescence is expressed in logarithmic scale units as RLU normalized to the light emitted from input RNA determined at 4 h post-electroporation. Representative data from two independent experiments are shown. Mock: mock electroporation (open grey square); Pol + (solid circle): polymerase-active HCV RNA; Pol - (open triangle): polymerase-defective (non-replicating; GNN) control. **(B)** To examine translation, measurements of reporter activity from (A) were plotted as fold change of RLU with respect to values obtained from the respective mock transfection sample of the specific time-point indicated. Pol + (gray bar):

polymerase-active HCV RNA; Pol - (open bar): polymerase-defective (non-replicating; GNN) control. Shown are representative data from two independent experiments.

CHAPTER 5   DISCUSSION AND FUTURE DIRECTIONS

To date there are approximately 200 million chronic infections of HCV worldwide, and without effective vaccines or therapeutics the morbidity of HCV-associated liver diseases is expected to increase over the next decade. Animal models remain an essential tool for understanding the process of viral pathogenesis, as well as a good predictive indicator of a novel therapeutic's efficacy prior to clinical trials. Thus, the creation of a small animal model system for HCV is not only important, but likely crucial for the development of effective antiviral therapeutics and preventive vaccines. The challenge has been that HCV only infects humans and chimpanzees, and this restricted host range has been indicative of the difficultly in generating cell culture and *in vivo* model systems for this virus. While chimpanzees have long been employed as a model animal system for HCV infection, they are expensive, constrained by ethical considerations, and are not readily available. In comparison, the mouse has proven to be an extremely useful and versatile tool for studying human diseases, and has been invaluable in the development of drugs and vaccines against many different pathogens. Due to the availability of well defined transgenic and knockout strains and their relative ease in manipulation, a mouse model for HCV would be valuable not only in characterizing the genetic elements involved viral replication, but also in testing potential immunotherapeutics and antiviral inhibitors *in vivo*. Previous attempts to generate a HCV rodent model have had limited success. Transgenic mice overexpressing HCV viral proteins exist, but cannot be used to study the full impact of HCV life cycle, nor can they be used for testing new antiviral therapies (35, 224, 261). SCID mice surgically

implanted with portions of human liver have been an important step forward in modeling HCV in non-primate animals (335), and have proven helpful in the testing of some anti-HCV compounds for safety and efficacy (252, 481). However, use of xenograft mice harboring chimeric human livers is limited by an arduous surgical protocol, a higher standard of maintenance, and limited availability. Thus, the limited availability of accessible mouse models in the two decades following the discovery of this virus, reveals the difficultly in generating small animal systems for HCV. The replication and propagation of this pathogen in rodent cells is apparently restricted due to a combination of different host factors that influence the permissiveness of cells to viral growth. The data presented in this thesis provides evidence that replication of HCV RNA in rodent cells is affected by host innate immunity and the presence of the liver-specific miR-122, but also additional unidentified host factor(s). Thus, I have considered a subset of elements and conditions required for HCV intracellular growth in cells of murine genetic background. These studies not only fundamentally change our understanding of HCV RNA replication in rodent cells, but also significantly contribute to building the foundation of an ideal HCV mouse model, one that is capable of supporting natural HCV infections.

The propensity of HCV to establish clinically persistent infections, while remaining resistant to *in vitro/in vivo* culturing, suggests that a complex host cell environment is required to support its life cycle. Viruses are obligatory intracellular parasites and require a suitable host to ensure their survival and propagation. Host factors are therefore critical to the viral life cycle, as the host cell provides the basic infrastructure and machinery for the production of progeny virions. As such, viruses have

evolved mechanisms to co-opt, gain control, and take advantage of key host components during the establishment of an infection. Besides overcoming innate immunity in the host cell, and reprogramming signaling pathways to prevent apoptosis and detection by the adaptive immune system, viruses must continuously adapt to their given cellular environment. An intricate balance between a permissive cellular environment and natural host immunity supports a virus' ability to establish chronic persistent infections. This is usually driven by specific virus-host interactions which limit the effects of innate and adaptive immunity. HCV is no exception to this paradigm, and research endeavours in deciphering this balance has highlighted the importance of several host factors that affect HCV replication. A global view of cellular expression profiles during *in vitro*/*in vivo* HCV replication via the means of transcriptome and proteome array analyses has identified several critical host components and pathways engaged by HCV during the completion of its life cycle (55, 56, 185, 289, 299, 357, 391, 434, 446, 452, 478, 488, 505). Furthermore, host factors that associate with specific viral proteins or RNA region have been uncovered (5, 189, 391). It is clear that the viral dependency on host machinery during HCV infection is intricate and extensive.

Each stage in the life cycle of HCV is intimately associated with multiple host molecules, and the virus employs a variety of strategies to establish chronic infections in hepatocytes. An association with lipoproteins allows the HCV virions to be camouflaged as VLDL-like particles, and through use of the lipoprotein receptor (LDLR, SR-BI), the virus is captured onto the cell surface and transferred over a cascade of higher affinity receptors and co-receptors (CD81, CLDNs, OCLN) before entering the cell. N-linked glycans on viral glycoproteins E1 and E2 also increase initial virion interaction with cell

surface GAGs to promote virion capture. At the same time these carbohydrates provide a "shield" against neutralizing antibodies, thus decreasing viral immunogenicity (140, 193). The internalization process makes use of host membrane trafficking systems including clathrin-mediated endocytosis and transport via the actin cytoskeleton (101). Following viral genome release and translation at the rER, the viral structural and non-structural proteins engage several host factors to establish control of the host cell machinery. Host membranes derived from the ER are specifically targeted by HCV proteins to induce subcellular compartmentalization which promotes genome amplification and virion production. The formation of HCV replication complexes also utilizes the host actin/microtubule system (229, 270, 503). The innate immune system of the cell is inactivated through mechanisms such as IPS-1 disruption, which provides a safe environment for virion production. Several intracellular components are co-opted to facilitate viral replication/translation, including miR-122, cyclophilins, carboxylesterase 1 (CES1), and several heat shock proteins (57, 62). Furthermore, HCV proteins have also been demonstrated to interact with and modulate host kinases, growth factor signaling, and processes relating to oxidative stress, as well as apoptotic and autophagosomal pathways (see APPENDIX B) in order to ensure full control of cellular metabolism, the cell cycle, and cellular fate (62, 291). Finally, lipid/cholesterol biogenesis is exploited by HCV, such as through modulation of CES1 (57), during virion assembly and egress. Several of these host cell interactions with HCV in the hepatocyte have been considered in the studies presented in this thesis. As shown in CHAPTER 3, functional inhibition of the host cell innate immunity through the deletion of the IRF-3 gene created a more permissive environment in the murine fibroblasts for HCV subgenomic RNA replication

when compared to the wild-type. Furthermore, expression of the liver-specific miRNA, the miR-122, significantly enhanced viral subgenomic RNA multiplication in MEFs. These observations suggest that host factors required to achieve competency for HCV RNA replication in rodent cells are similar to those identified within the human host. However, data from CHAPTER 4 also indicates that the replicative ability of the complete HCV genome in rodent cells will necessitate additional unidentified, potentially human, host factor(s). This was demonstrated by the inability of mouse fibroblasts (established with conditions sufficient for the replication of viral subgenomes) and murine liver cells to support full-length HCV genome growth. Thus, there could be an additional layer of species-specific component(s) engaged in clinical HCV replication, which may also help to determine the highly restricted primate tropism seen with this virus. The non-liver naïve murine fibroblast platform developed in CHAPTER 3 will be a useful tool for further elucidating these unidentified essential elements. Through the further introduction of key liver or species-specific molecules to this platform, it may be possible to clarify the minimal essential elements required to support genome-length HCV replication in rodent cells.

The findings presented in this thesis also provide a link between tissue and species-specific host factors and the high degree of tissue and species tropism evident in the case of HCV. More importantly, it highlights the importance of a liver-specific environment (and the presence of miR-122) in promoting HCV replication and translation. According to the current paradigm in the field, the requirement of cells to possess specific host factors that promote viral entry (CD81, SR-BI, CLDN, OCLN), replication/translation (miR-122 and additional host elements), and viral

packaging/egress membrane (components of the lipid/cholesterol pathway) are likely to be the primary determining factors of HCV tropism. Notably, expression of miR-122 and the presence of the lipid/cholesterol pathway are especially prominent characteristics of hepatocytes. It appears that efficient and productive infection can only occur if all of these factors are present, while any missing component may result in an abortive infection. This specificity observed *in vitro* is reflected in clinical setting, whereby HCV remains almost exclusively a hepatotropic virus. However, it has also been documented that HCV can replicate in extrahepatic sites independently of miR-122 *in vitro*, as demonstrated within this thesis and elsewhere, in HEK 293 cells (9, 244), HeLa cells (244, 530), cells of neural origin (75, 150, 486), and murine fibroblasts (82, 292, 475) (see CHAPTER 3). Furthermore, as indicated previously HCV lymphotropism has been observed clinically, as well as infection of PBMCs and several lymphotropic lines *in vitro* through the use of patient sera-derived wild-type HCV (54, 256, 320, 380, 445). Healthy PBMCs and immortalized immune cell lines such as THP-1, U937, BJAB, Daudi, Raji, Jurkat, and MOLT-4 do not contain any detectable miR-122 (Liang-Tzung Lin, personal observation). These observations of extrahepatic replication would suggest that a subpopulation of the virus may possess the capability of using non-liver-specific factors to facilitate infections in non-hepatocyte host cells outside the liver.

The notion for the existence a lymphotropic strain or subpopulation of HCV, and its associated lymphotropism, is becoming more apparent, especially in the clinical setting. The existence of these infections has been largely characterized through the development of more sensitive RNA detection methods as explained in the INTRODUCTION. The lymphotropic phenotype is particularly interesting since chronic

hepatitis C infection is strongly associated with B cell lymphoproliferative disorders, and a subpopulation of HCV has been shown to infect B lymphocytes (127, 218). Importantly, HCV is increasingly being recognized as a cause of mixed cryoglobulinemia (incidence rate of 10-70 % in HCV-infected patients) and non-Hodgkin's lymphoma (2-4 fold risk associated with chronic hepatitis C) (17, 85, 148, 360, 485). A previous report has indicated that B cell infection by HCV leads to a mutator phenotype, with enhanced mutations in many somatic genes including Ig genes, proto-oncogenes, and tumor suppressor genes such as p53 (318). Furthermore, the nucleotide substitution patterns in these mutations are similar to those found in HCV-associated lymphoma, suggesting a possible common mechanism relating to their occurrence (318). While the underlying mechanism remains unclear, it has been proposed that engagement of the B cell antigen-receptor complex (BCR) by HCV antigens (such as E2) may modify BCR-associated signaling pathway and induce hypermutations in the Ig genes (317, 319). Persistent viral presence is thought to induce multiple mutations over time, and may contribute to HCV lymphomagenesis. However, the differential contribution from lymphotropic versus hepatotropic strains of HCV in influencing dysregulation of B cells is currently unknown. Moreover, whether a hepatic infection can affect HCV-induced lymphatic pathogenesis, or vice-versa, is also elusive. It would be interesting to examine the relative contributions of persistent viral replication from hepatotropic and lymphotropic strains of HCV in their ability to influence the B cell activation status and proliferation. Furthermore, a comparative analysis of various hepatotropic and lymphotropic strains may also help to shed some light on the biological determinants of their tropism. A recent study suggested that HCV variants residing in B cells of chronic hepatitis C patients have a different IRES

translation capacity than liver-produced variants isolated from within patient plasma (127). In particular, the B cell-specific HCV IRES sequences exhibited poor translation efficiency in Huh-7 cells and primary hepatocytes, but not B cell lines including Daudi and Raji. These observations provide some insight to how subpopulation of HCV may be better adapted for propagation in immune cells such as B lymphocytes, which could be an important additional viral tropism factor. At the same time it suggests that the specific setting of the intracellular environment (lymphocytic or hepatic) could also pre-determine the selection of the different viral variants in the current paradigm of HCV tropism. It remains to be investigated whether other sequences in the viral genome or specific steps in the intracellular HCV life cycle (such as viral replication and packaging) may also contribute to ways of determining the cell-type tropism for the lymphotropic and hepatotropic viral strains. An understanding of host cell tropism in primates with regards to these subpopulations of HCV might provide insight to identifying determinants that influence HCV replication in rodent cells. Since miR-122 is not normally expressed in the immune cell populations, it also remains to be examined whether this miRNA has an impact on replication capacity of the lymphotropic variant of HCV. If the miR-122 can indeed upregulate lymphotropic HCV replication, exogenous introduction of this miRNA in PBMCs could be clinically useful, to rescue occult HCV infection for better assessment of spontaneous or therapeutic-induced viral clearance in the management of hepatitis C.

An understanding of the host factors involved in completing the HCV viral life cycle is important not only for clarifying viral pathogenesis, but also in uncovering potential therapeutic targets in order to hinder viral replication. Modulating the host

offers an alternative to the direct targeting of the virus, which has the persistent inherent risk of selecting for resistant mutants. In this sense, one can modulate cellular components or responses in order to shift the balance towards the host in controlling the viral infection. Through the course of evolution, viruses have adapted to utilize specific host components in its life cycle. Targeting the host alters an environment to which the virus has long evolved to take advantage of, and therefore could prevent access to essential components necessary for the virus to complete key steps in its life cycle. This would potentially result in an abortive infection, and is especially attractive in the case of HCV due to its high degree of host tropism and interrelating dependence on host cell-specific factors. Environmental changes imply a significant modification in the activities of the virus requiring a long time to adapt, and at higher cost of viral fitness. Conversely, changes induced by drugs targeting viral proteins are often considered less costly and easier for the virus to overcome, and are constantly provided for by HCV's diverse heterogenic pool and continuous mutations in the virus' genetic composition. The selective pressure induced by host components is thus more difficult to cope with for the virus. The difficulty in adapting HCV to other tissues/species suggests this domain as an attractive therapeutic target to which the virus is unlikely to be able to easily overcome. As mentioned earlier, in light of HCV's mutation rate, virtually all of the soon-to-be-implemented STAT-C therapeutic drugs for treating chronic hepatitis C have been shown to select for resistant HCV strains (412). The approach in targeting the host could help overcome this difficulty. Findings presented in this thesis, as well as those of other laboratories, have shown the influence of host cell innate immunity on HCV suppression through IRFs-induced type I IFNs, and the key role of miR-122 in promoting HCV

amplification. These host factors can be therapeutically targeted, as reflected by the current standard IFN therapy for hepatitis C as well as the recent therapeutic trial of a novel miR-122 inhibitor (276). Restricting viral engagement of the host intracellular environment and also direct targeting of viral proteins, through a combination drug cocktail containing host modulatory compounds (immunomodulatory molecules and miR-122 inhibitor) and virus-specific drugs, would be ideal to control or abolish the viral infection. The latter would include inhibitors targeting the HCV NS5B polymerase to limit viral genome multiplication within a host cell, and also inhibitory agents against the NS3/4A protease to impede viral polyprotein processing as well as to restore the IPS-1-mediated innate immunity (376). Additional antiviral compounds can include GAG competitors that would neutralize entry by targeting the GAGs-interacting viral glycoproteins (Lin *et al.*, submitted; see APPENDIX C). The application of such combinatorial therapy could help control HCV infection and may reduce viral loads in otherwise therapeutically resistant non-responders.

It is now clear host factors play a central role in creating a permissive intracellular milieu necessary for efficient HCV replication. These factors are also important in contributing towards determining HCV cell-type and species-specific tropism. The studies presented in this thesis significantly contribute to our knowledge of HCV replication in rodent cells, and provide direction towards the creation of a rodent *in vivo* model of HCV infection. Further studies should build on the murine platform generated in this thesis, and help uncover other necessary host components that would dictate viral replication competency, as well as factors essential for virus egress in the rodent genetic background. These would include experiments in deciphering host interactions with the

viral NS2 in full-length HCV genome replication, and cellular lipid/cholesterol pathways engaged by HCV to promote virion morphogenesis/egress in the mouse genetic background. High-throughput screening of shRNA/siRNA libraries using permissive cell lines and methodical knockdown of gene function may help elucidate these functional interactions. In addition, heterokaryon fusion between different intracellular environments (such as Huh-7 cells with IRF3KO-HmiR122 MEFs or murine liver cells) could be used to deduce positive and restrictive host factors to HCV replication in addition to the shRNA/siRNA knockdown experiments. Finally, strategies involving *trans*-complementation with core-NS2 can be used to rescue virion production, and clarify the host components involved in this process, in murine cells established with subgenomic HCV systems. Assembly of minimal key elements/conditions (integral to viral entry, replication/translation, and assembly/egress) should provide the necessary means for HCV to complete its life cycle in rodent cells. Transgenic mice containing all these factors/conditions should generate a mouse model capable of supporting a natural infection of this virus (FIG. 24).
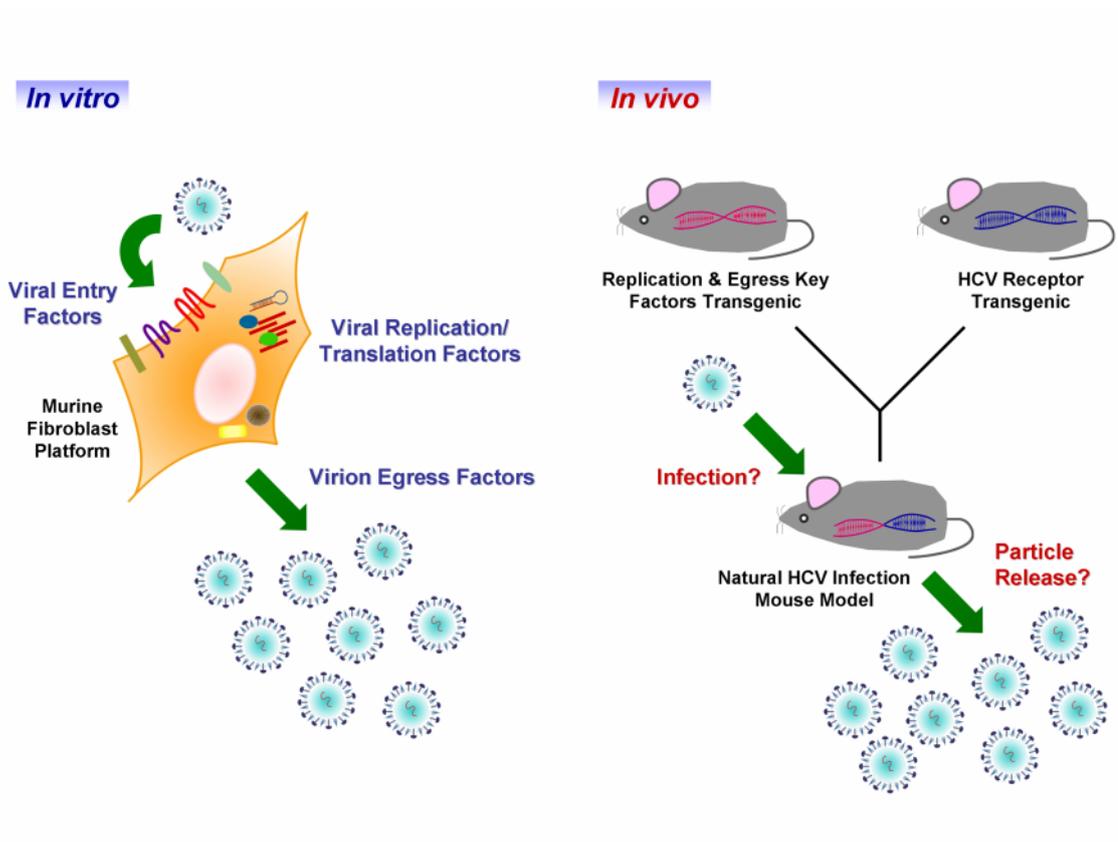
FIG. 24. Proposed strategy for generating natural HCV infection mouse model. (Copyright © 2010 by Lin & Chin. All Rights Reserved.)

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APPENDIX A

LIST OF SELECTED PUBLICATIONS AND COPYRIGHT PERMISSIONS

1. **Lin LT**, Noyce RS, Pham TN, Wilson JA, Sisson GR, Michalak TI, Mossman KL, Richardson CD. Replication of subgenomic hepatitis C virus replicons in mouse fibroblasts is facilitated by deletion of interferon regulatory factor 3 and expression of liver-specific microRNA 122. **J Virol. 2010 Sep;84(18):9170-80.**

2. **Lin LT**, Dawson PW, Richardson CD. Viral interactions with macroautophagy: a double-edged sword. **Virology. 2010 Jun 20;402(1):1-10. Review.**

3. Vivithanaporn P, Maingat F, **Lin LT**, Na H, Richardson CD, Agrawal B, Cohen EA, Jhamandas JH, Power C. Hepatitis C virus core protein induces neuroimmune activation and potentiates Human Immunodeficiency Virus-1 neurotoxicity. **PLoS One. 2010 Sep 21;5(9):e12856. [Open Access License, No Permission Required]**

4. **Lin LT**, Chen TY, Chung CY, Noyce RS, Grindley TB, McCormick C, Lin TC, Wang GH, Lin CC, Richardson CD. Hydrolyzable Tannins (Chebulagic Acid and Punicalagin) Target Viral Glycoprotein-Glycosaminoglycan Interactions to Inhibit Herpes Simplex Virus Type 1 Entry and Cell-to-Cell Spread. **Submitted to J Virol. (JVI01492-10).**

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APPENDIX B

<u>**REVIEW ARTICLE**</u>

VIRAL INTERACTIONS WITH MACROAUTOPHAGY: A DOUBLE-EDGED SWORD

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<u>Abstract</u>

Autophagy is a conserved eukaryotic mechanism that mediates the removal of long-lived cytoplasmic macromolecules and damaged organelles via a lysosomal degradative pathway. Recently, a multitude of studies have reported that viral infections may have complex interconnections with the autophagic process. These observations strongly imply that autophagy has virus-specific roles relating to viral replication, host innate and adaptive immune responses, virus-induced cell death programs, and viral pathogenesis. Autophagy can supply internal membrane structures necessary for viral replication or may prolong cell survival during viral infections and postpone cell death. It can influence the survival of both infected and bystander cells. This process has also been linked to the recognition of viral signature molecules during innate immunity and has been suggested to help rid the cell of infection. This review discusses interactions between different viruses and the autophagy pathway, and surveys the current state of knowledge and emerging themes within this field.

1. Introduction

Macroautophagy (hereafter referred to as autophagy) is a tightly regulated and evolutionarily conserved mechanism for the sequestration, lysosomal degradation, and recycling of discrete intracellular portions of eukaryotic cells, facilitating the removal of materials not typically degraded by the ubiquitin-proteosomal pathway. Regulators of this process include hormones and growth factors that suppress autophagy during cellular growth, as well as intracellular levels of nutrients, oxygen, and energy, allowing the pathway to act as a defense mechanism against inducers of cellular stress (Pattingre et al., 2008; Wullschleger, Loewith, and Hall, 2006; Yang et al., 2005). Perturbations in autophagy have been correlated with numerous pathological conditions, including oncogenesis and cancer progression, neurodegenerative disorders, liver disease, myopathy, and cardiac disease, highlighting the importance of this pathway in human health and cellular homeostasis (Levine and Kroemer, 2008; Meijer and Codogno, 2006; Mizushima et al., 2008). Autophagy has been shown to play an important role in the pathogenesis of several viral infections and is suggested to act as both an inducer and effector of innate and adaptive immune responses against intracellular pathogens, including viruses. Currently, evidence suggests that viruses have evolved a diverse array of countermeasures to contend with this pathway; some inhibit autophagy and are negatively affected when this interference is abrogated, while others appear to subvert it to their own ends and respond positively when it is induced. However, still other viruses are seemingly unaffected by autophagy, and do not appear to regulate the pathway through any apparent mechanism(s). This review seeks to provide both a synopsis of currently known and suspected viral interactions with the autophagy pathway, and to stimulate a critical discussion concerning the central trends that have been suggested within this field of research.

2. Overview Of The Mechanisms And Regulation Of Autophagy

For the sake of brevity, only an overview of the mechanisms and regulation of the autophagy mechanism will be provided. The reader is referred to more detailed reviews concerning specific aspects of autophagy, such as the formation and maturation of the autophagosome (Longatti and Tooze, 2009; Mizushima, 2007; Noda, Fujita, and Yoshimori, 2009; Reggiori and Klionsky, 2005; Xie and Klionsky, 2007; Yang et al., 2005; Yoshimori and Noda, 2008), regulatory mechanisms of this pathway (Meijer and Codogno, 2004; Pattingre et al., 2008; Wullschleger, Loewith, and Hall, 2006; Yang et al., 2005), roles in innate antigen recognition and MHC antigen presentation (Delgado et al., 2009; Delgado and Deretic, 2009; Levine and Deretic, 2007; Münz, 2006; Orvedahl and Levine, 2009; Schmid and Münz, 2005; Virgin and Levine, 2009), and the relationship between autophagy and regulated cell death (Codogno and Meijer, 2005; Ferraro and Cecconi, 2007; Kroemer and Levine, 2008; Maiuri et al., 2007; Scarlatti et al., 2009). The core process of autophagy is the *de novo* synthesis of a double membrane-bound vesicle capable of fusing with an endosome or lysosome, which ultimately leads to the catabolic degradation of the encapsulated cargo (FIG. 1). In mammals, this process begins with the expansion of a small, flat membrane sac of uncertain origin (termed the isolation membrane or phagophore). As autophagy-related (Atg) proteins are recruited to its surface, this membrane sac elongates and curves until the ends merge to form a double-membrane-bound vesicle (autophagosome). Atg proteins are then recovered or disassociate from the autophagosome, and the completed structure fuses with an endosome (amphisome) or lysosome (autolysosome). A brief overview of mammalian genes of particular significance in the regulation and execution of autophagy is provided in Table 1.

Central in the regulation of autophagy are two key proteins: mTOR and Beclin-1 (Pattingre et al., 2008; Sinha and Levine, 2008; Wullschleger, Loewith, and Hall, 2006). mTOR, a conserved serine/threonine kinase, is a component of protein complexes that integrate cellular signals relating to growth factors, nutrient and energy status, and cellular stress (Wullschleger, Loewith, and Hall, 2006). Important activators of mTOR include the class I PI3K-Akt/PKB signaling pathway and high concentrations of specific amino acids; high AMP/ATP ratios and hypoxia inactivate this pathway (Arsham and

Neufeld, 2006; Beugnet et al., 2003; Pattingre et al., 2008; Wullschleger, Loewith, and Hall, 2006). Activated mTOR suppresses autophagy, enhancing the accumulation of cellular bulk by limiting lysosomal digestion. Downstream of mTOR, Beclin-1 is at the heart of a regulatory complex for the class III PI3K hVps34, whose activity is essential during autophagosome formation. Activators, such as UV radiation resistance associated gene (UVRAG), Bax-interacting factor-1 (Bif-1), and activating molecule in Beclin-1-regulated autophagy-1 (Ambra-1) associate with the Beclin-1 complex and enhance PtdIns(3)P production, while the Bcl-2 family anti-apoptotic proteins such as Bcl-2 and Bcl-xL bind to Beclin-1 and act in an inhibitory fashion (Pattingre et al., 2008; Sinha and Levine, 2008). The Bcl-2-related inhibition of autophagy is abrogated by stress-activated c-Jun N-terminal protein kinase 1 (JNK1) phosphorylation and competition from other BH3 binding domain-containing proteins, providing one of several direct mechanistic links between autophagy and apoptosis (Sinha and Levine, 2008; Wei et al., 2008a,b).

Numerous other cellular factors have been shown or are hypothesized to regulate autophagy, many of which have importance in viral infections. The eukaryotic initiation factor-2 alpha (eIF2α) and the starvation-responsive general control nonderepressible-2 (GCN2) eIF2α kinase are both indispensable for starvation-induced autophagy, suggesting that other eIF2α kinases with important roles in antiviral defense, such as double-stranded RNA (dsRNA)-dependent protein kinase (PKR) and PKR-like ER kinase (PERK), may also upregulate this pathway in response to cellular stressors (Kouroku et al., 2007; Tallóczy et al., 2002; Tallóczy, Virgin, and Levine, 2006). Immune signaling molecules can modulate autophagy; type II interferon-γ (IFN-γ) and tumor-necrosis factor-α (TNF-α) are stimulatory, while the TH2-type cytokines interleukin-4 (IL-4) and IL-13 are suppressive (Deretic, 2005; Levine and Deretic, 2007). Certain pathogen-associated molecular patterns (PAMPs) trigger autophagy through their cognate pattern recognition receptors (PRRs), including Toll-like receptor 3 (TLR3), TLR4, and TLR7, although the molecular mechanism(s), physiological function(s), and range of PRRs that induce this pathway are all areas of continuing research (Delgado et al., 2009; Delgado and Deretic, 2009; Orvedahl and Levine, 2009). The p53 protein possesses a dual role in the regulation of autophagy dependent upon its localization; cytoplasmic p53 represses autophagy and must be degraded for autophagy to proceed, whereas nuclear p53 appears

to induce it (Maiuri et al., 2009; Tasdemir et al., 2008). Many additional cellular factors, including extracellular signal-regulated kinase (Erk1/2) activation (Pattingre, Bauvy, and Codogno, 2003; Shinojima et al., 2007; Wang et al., 2009), intracellular release of calcium (Gordon et al., 1993; Høyer-Hansen and Jäättelä, 2007), increases in reactive oxygen species (ROS) (Djavaheri-Mergny et al., 2007; Djavaheri-Mergny et al., 2006; Scherz-Shouval and Elazar, 2007; Scherz-Shouval et al., 2007), and endoplasmic reticulum (ER) stress (Ding et al., 2007; Kouroku et al., 2007; Ogata et al., 2006; Yorimitsu et al., 2006) have also been shown to trigger the pathway to autophagy.

While numerous experimental approaches including electron microscopy, LC3 lipidation (aggregation and modification), and protein degradation studies, amongst others, can be employed to identify or quantify autophagy in higher eukaryotes, a few warnings should be considered regarding the specific challenges these methods present. First, as autophagy is a process with numerous components, steps, and phases, it is important to clarify whether a given assay measures a step within this pathway (such as LC3 lipidation) or its overall physiological consequence (the aim of protein degradation studies). Since several viruses have been shown to modulate autophagy at multiple stages with varying effects, it is often necessary to combine assays examining both induction and maturation in order to make accurate observations. Second, while autophagy is a responsive cellular process, capable of fluctuation, most assays capture this dynamic process at a single static moment in time. This can pose challenges, as cellular populations are frequently asynchronous and the effects of a viral infection may vary during the course of the virus' life cycle. Finally, as different cell types have been shown to display different autophagy responses, it can be difficult to make direct comparisons between different virus-cell systems. Particular caution should be used when studying viruses, like HIV-1, which infect more than one cell type. For further information regarding the limitations of current autophagy assays, readers are advised to consult excellent reviews on this subject (Klionsky et al., 2008; Mizushima, 2004).

3. Viral Interactions With The Autophagy Pathway

Many viruses have been shown to evade, subvert, or exploit autophagy, seemingly to insure their own replication or survival advantage, while others are apparently unaffected by this intrinsic pathway and fail to modulate it by any detectable way. The following discussion concerning viral infection, autophagy, and host immunity has been structured on four emergent themes that have been identified in the research published to date; autophagy as 1) a mechanism for membrane remodeling, 2) a digestive defensive response, 3) a means of cellular surveillance, and 4) a cellular fate-determining process. A comprehensive summary of viruses for which studies have been undertaken is provided in Table 2.

3.1. Autophagy Is A Mechanism For Remodeling Internal Membranes Associated With Viral Replication.

For many viruses, the production of progeny virion is intimately associated with host cell membranes or cytoskeletal elements. As such, many viruses are known to subvert host endosomal and secretory pathways in order to induce host membrane alterations that can then support viral replication and assembly (Miller and Krijnse-Locker, 2008). A number of viruses have been shown to replicate in, or in close association with, multi-membraned vesicles that possess many of the characteristics of autophagosomes. Given the nature and location of these vacuoles, there is strong evidence that autophagosomes may serve as a site of viral replication during some infections and that the autophagy pathway might therefore be exploited by viruses to enhance virion production. Supporting this assertion, the membranes associated with viral replication are often derived from the ER, which has also been suggested as a source of the autophagic double-membrane (Mijaljica, Prescott, and Devenish, 2006). Indeed, the similarities between autophagic vacuoles and some virally-induced membrane alterations has lead to increased suspicion, and in some cases proof, of a connection between positive-stranded RNA viral replication and the autophagy mechanism.

A link between poliovirus (PV)-induced double-membrane vesicles and autophagy has long been suggested, and is often cited as the classic example of viral exploitation of the autophagy pathway. Ultrastructural and biochemical analyses have

revealed that PV induces massive rearrangements in intracellular membranes, resulting in clusters of double membrane-bound vesicles capable of supporting viral replication complexes (Bienz, Egger, and Pasamontes, 1987; Rust et al., 2001; Schlegel et al., 1996; Suhy, Giddings, and Kirkegaard, 2000). These PV-induced vesicles display the classic autophagosomal marker LC3, which has been hypothesized to be directly recruited by viral proteins (Jackson et al., 2005; Taylor and Kirkegaard, 2007). Subsequent studies have further shown that additional secretory vesicle trafficking molecules are also recruited during the formation of these membranous replicative vesicles (Belov et al., 2007), and that the association of these vesicles with the microtubule network aids in the non-lytic release of PV virion particles (Taylor et al., 2009). Hence, it has been postulated that PV factors initiate elements of both the interrelated secretory trafficking and autophagy pathways to ultimately create a membranous structure capable of supporting both viral replication and virion egress. Besides PV, several other picornaviruses also appear to subvert the autophagy machinery to promote their replication. The group B Coxsackieviruses, CVB3 and CVB4, induce autophagosome formation, and biochemical inhibition of this pathway negatively impacts virion production (Wong et al., 2008; Yoon et al., 2008). Similarly, the enteropathogen EV71 has been shown to trigger autophagy *in vitro*, and that this induction can significantly increase viral yield (Huang et al., 2009). Apart from the *Picornaviridae* family, two Dengue virus (DENV) serotypes (DENV-2 and DENV-3) have also been shown to interact with the autophagy machinery to promote their replication (Khakpoor et al., 2009; Lee et al., 2008; Panyasrivanit et al., 2009). Interestingly, the stage of the autophagic process with which DENV is associated varies depending upon the viral serotype involved; DENV-2 translation/replication complex has been shown to specifically locate with pre-lysosomal fusion amphisomes, while DENV-3 requires further vesicle maturation and post-lysosomal fusion vacuoles (autolysosomes) to efficiently produce virions (Khakpoor et al., 2009; Panyasrivanit et al., 2009). The autophagy pathway also appears to be critical in the replication of another *Flaviviridae* member, namely the hepatitis C virus (HCV), which has also been shown to induce autophagosome formation (Ait-Goughoulte et al., 2008; Dreux et al., 2009; Sir et al., 2008; Tanida et al., 2009). In the case of HCV, autophagy is thought to be responsible for providing membranous support structures during the initial translation and *de novo*

replication of HCV RNA following infection, but subsequently contributes little to virus maturation once an infection is stably established in the host cell (Dreux et al., 2009; Tanida et al., 2009). Finally, basal levels of autophagy have recently been shown to enhance macrophage-tropic human immunodeficiency virus (HIV) viral protein processing and virion production *in vitro*, suggesting a role for autophagy in HIV biosynthesis in this cell type (Kyei et al., 2009).

While these results are strongly suggestive that viruses can subvert or induce autophagy in order to create advantageous membrane alterations, other research has demonstrated that this effect is not universal. Not all viral infections that induce membrane alterations, including some which are closely related to the examples provided above, modulate or are affected by the autophagy pathway. The replication of human rhinovirus 2, a picornavirus that shares many similarities with PV, is not affected by drugs that either inhibit or induce autophagy, and does not elicit the formation of LC3-positive vesicles (Brabec-Zaruba et al., 2007). Contradictory observations have been made for the *Coronaviridae* members, murine hepatitis virus (MHV) and severe acute respiratory syndrome-coronavirus (SARS-CoV); some studies have suggested that endogenous LC3 co-localizes with SARS-CoV and MHV vacuole-associated replicase proteins (Prentice et al., 2004a; Prentice et al., 2004b), yet other studies have had difficultly corroborating these results (de Haan and Reggiori, 2008; Snijder et al., 2006; Stertz et al., 2007). In line with these observations, MHV viral replication and release have been shown to be comparable in cells with both normal and defective autophagy mechanisms (Zhao et al., 2007). Likewise, the viral production kinetics of vaccinia virus, which has been shown to utilize double-membrane vesicles for its replication, was similar in wild-type, Atg5$^{-/-}$, and Beclin-1-deficient cells (Zhang et al., 2006). Thus, subversion of autophagy as a mechanism for inducing membrane alterations may be either cell-type- or virus-dependent. These results suggest that considerable care should be exercised prior to concluding that autophagy is the mechanism responsible for membrane alterations observed under microscopy to ensure that they are biochemically and mechanistically connected to this pathway. While the evidence to date strongly indicates a positive role for autophagy in the replication of some viruses, the observed variation, particularly amongst closely related viruses, suggests that other mechanisms may exist for inducing

similar alterations in host cells. One should approach these studies carefully with the correct diagnostic tools for autophagy.

3.2. Autophagy Can Be A Mechanism For Defense: The Digestion Or Elimination Of Unwanted Viral Intruders.

As the previous section has indicated, viruses may induce autophagy for their own replicative advantage; yet, the same process may also confine viral replication complexes within vesicles as an innate defense against infection (Wileman, 2006). Furthermore, autophagy has been documented to function as a host cell defense against some intracellular pathogens. This process, which has been termed xenophagy ('to eat what is foreign'), and results in the autophagic-lysosomal destruction of invading pathogens. Several bacterial pathogens including *Mycobacterium tuberculosis* (Gutierrez et al., 2004), group A *Streptococcus* (Nakagawa et al., 2004), *Shigella flexneri* (Ogawa et al., 2005), *Legionella pneumophila* (Amer and Swanson, 2005), and *Yersinia pestis* (Pujol et al., 2009) have been shown to either actively inhibit or be degraded through xenophagy. Subsequently, this process has been demonstrated to be particularly important in restricting bacterial escape and survival. Just as xenophagy restricts certain bacterial pathogens, autophagy may capture replicating viruses or newly assembled virions within their host cells, and eliminate them through sequestration and lysosomal degradation (Kirkegaard, Taylor, and Jackson, 2004; Levine, 2005; Wileman, 2007).

The initial hypothesis that autophagy might function as an antiviral defense mechanism was suggested when the autophagy effector and regulatory protein Beclin-1 was shown to be an antiviral effector in mice infected with the neurotropic Sindbis virus (SINV) (Liang et al., 1998). Over-expression of Beclin-1 protected mice from fatal SINV encephalitis, reducing neuronal apoptosis and decreasing SINV viral replication (Liang et al., 1998). The antiviral and anti-apoptotic effects attributed to Beclin-1 in SINV infection *in vivo* suggested that autophagy may function as a defense against other viral infections. Since these initial observations were first published, further evidence has shown that autophagy may function as a defense against viral infections.

Similar effects to those observed with SINV infection have been demonstrated in HSV-1 encephalitis (Orvedahl et al., 2007). In murine fibroblasts and neurons, mutant

HSV-1 deficient in ICP34.5, a viral Beclin-1-interacting protein, but not wild-type virus, induced autophagy upon infection in a PKR-dependent manner (Alexander et al., 2007; Orvedahl et al., 2007; Tallóczy et al., 2002; Tallóczy, Virgin, and Levine, 2006). This viral induction of autophagy resulted in the observed localization and xenophagic degradation of virions within autophagosomes (Alexander and Leib, 2008; Alexander et al., 2007; Tallóczy, Virgin, and Levine, 2006). However, the exact significance of increased mutant virions within double-membraned vesicles and xenophagy is questionable, since suppression of autophagy through the use of Atg5$^{-/-}$ mouse embryonic fibroblasts (MEFs) did not significantly alter the replication efficiency of either wild-type or ICP34.5 mutant HSV-1 *in vitro* (Alexander et al., 2007; Jounai et al., 2007). In the case of HSV-1, it is thought that while ICP34.5 expression can inhibit autophagy in infected cells, the prevention of PKR-mediated translational arrest, rather than regulation of autophagy, may be the pivotal determinant of HSV-1 replicative efficiency in cell culture (Alexander et al., 2007). In contrast to these *in vitro* observations, the suppression of autophagy *in vivo* by ICP34.5 appears to be critical in HSV-1 pathogenesis, since Beclin-1-binding-deficient ICP34.5 mutant viruses are neuroattenuated with regards to lethal encephalitis in mice (Orvedahl et al., 2007). Interestingly, this HSV-1 mutant's virulence can be reconstituted if the infection is conducted in mice deficient for the antiviral effector PKR (Orvedahl et al., 2007). The observed discrepancies between *in vitro* and *in vivo* results may be due to cell-type dependent differences, and/or the effects of other HSV-1 proteins on the autophagy pathway (Alexander and Leib, 2008).

The observation that a HSV-1 viral protein that abrogates autophagy is necessary *in vivo* to observe certain pathogenic effects, highlights the potential antiviral function of this pathway. This may be particularly true in the case of neurotropic viruses, since modulation of autophagy has been suggested to influence the development of certain neurological diseases, (Itzhaki, Cosby, and Wozniak, 2008; Orvedahl and Levine, 2008). Along these lines, it has been noted that feline, simian, and human immunodeficiency viral infections *in vivo* can indirectly induce deficits in neuronal autophagy, and that this effect may contribute to the neuro-inflammatory pathology observed in these diseases (Alirezaei et al., 2008; Zhu et al., 2009). Thus, the dysregulation of autophagy by certain

neurotropic viruses may not only interfere with their lysosomal clearance, but may also have a significant impact in terms of their pathogenic effect.

One remaining question though is whether xenophagy truly functions as an antiviral effector mechanism, since only HSV-1 viral particles have been observed microscopically within the confines of autophagosomes. Furthermore, this effect may be virus-specific rather than a general defensive mechanism of viral clearance. While it is tempting to speculate that xenophagy, as demonstrated within the field of bacteriology, exists as a general host defense mechanism for the clearance of all intracellular pathogens, including viruses, the limited amount of direct evidence available at this time suggests that caution is warranted. In particular, care should be exercised in differentiating between the incidental inclusion of virion or viral components within autophagosomes due to either their uptake by background or stress-induced activation of this pathway, and enhanced, autophagosome-driven clearance of these materials.

The HSV-1 ICP34.5 protein is known to antagonize PKR by dephosphorylating eIF2α (He, Gross, and Roizman, 1997), and, as discussed above, also blocks the induction of autophagy in a PKR-dependent fashion (Orvedahl et al., 2007; Tallóczy et al., 2002; Tallóczy, Virgin, and Levine, 2006). As many viruses employ numerous countermeasures for disrupting the IFN and PKR antiviral systems, these viral disturbances may also have significance in modulating downstream autophagic processes. Indeed, unless a virus can inhibit autophagy it is subject to the effects of immune surveillance which harnesses the autophagy machinery (discussed in **Autophagy May Function In Security Surveillance: A Watchdog For Foreign Molecules** section).

3.3. Autophagy May Function In Security Surveillance: A Watchdog For Foreign Molecules.

In addition to xenophagy, autophagy may function as an antiviral pathway in a less direct fashion, sampling and delivering cytoplasmic material to cellular compartments (endosomes and lysosomes). This process may play a significant role in the activation of innate and adaptive immune responses to foreign pathogens. Research has shown that autophagy is involved in the delivery of various antigens (viral, self, and tumor origin) to MHC class II antigen-presenting molecules, which can in turn lead to the

activation of CD4+ T lymphocytes (Levine and Kroemer, 2008). As an example, the delivery of Epstein-Barr viral antigens to MHC-class-II-loading compartment (also known as late endosomes), prior to CD4+ T cell stimulation, has been shown to utilize the autophagy mechanism (Paludan et al., 2005). Constitutive autophagy in immune and epithelial cells has also been observed to participate in the delivery of LC3-tagged influenza matrix proteins to MHC class II-associated endosomal compartments, which resulted in enhanced antigen presentation to CD4+ T cells (Schmid, Pypaert, and Münz, 2007). Furthermore, the importance of autophagy-mediated class II antigen presentation was recently demonstrated in the regulation of HSV-1 pathogenesis by viral ICP34.5 (Leib et al., 2009). As well, it has been shown that the immunization of mice with influenza A (IFA)-infected cells exhibiting enhanced autophagy facilitates more robust MHC class I antigen presentation to the CD8+ T cells (Uhl et al., 2009). This evidence suggests that autophagy may contribute to MHC class I/II-specific viral antigen presentation, aiding the induction of adaptive immune responses. These studies also raise the exciting possibility to that it may be feasible to exploit autophagy to deliver viral antigens and enhance MHC class I/II presentation for the development of novel vaccines and adjuvant therapies (Schmid, Pypaert, and Münz, 2007).

The autophagy machinery has also been suggested to play a role in innate immunity by delivering PAMPs to their associated PRRs, including the TLRs. TLRs play critical roles in detecting bacterial and viral signatures and in eliciting appropriate host cell defenses such as IFNs and inflammatory cytokines against bacterial and viral infections (Takeuchi and Akira, 2007). It has been recently shown that the delivery of viral nucleic acids to endosomal TLRs in plasmacytoid dendritic cells (pDCs) can occur through the autophagosomes (Lee et al., 2007). While most TLRs reside on the cell surface, a subset involved in the detection of viral gene products, including TLR3, 7/8, and 9, are located in endosomal compartments and aid in the detection of endocytosed viral material (Barton, 2007). Autophagosomes could theoretically facilitate the sequestration, delivery, and detection of cytoplasmic viral nucleic acids, thereby helping to elicit a classical IFN response (Levine and Deretic, 2007). Indeed, autophagy has been shown to mediate ssRNA virus recognition through TLR7 during vesicular stomatitis virus (VSV) and Sendai virus (SENV) infection. It contributes to the production of

interferon-α by pDCs *in vitro* and *in vivo* (Lee et al., 2007). Hence, this intrinsic pathway may play an important role in the detection of viral antigens and in the induction of the subsequent IFN response in pDCs.

More recently, evidence supporting the involvement of autophagy in the TLR-related detection of viral PAMPs has increased with the finding that TLRs themselves can induce autophagosome formation. This suggests a potential feedback circuit involving autophagy in the pathogen-triggered immune response. Lipopolysaccharide (LPS)-induced autophagy has been demonstrated in both murine and human macrophages, and requires the TRIF (Toll-interleukin-1 receptor domain-containing adaptor-inducing IFN-β)-dependent TLR4 pathway, whose downstream components include the receptor-interacting protein (RIP1) and the p38 MAPK (Xu et al., 2007). While the physiological importance of this TLR-mediated induction is currently unclear, it suggests that TLR4 can induce autophagy, which may help to limit the spread of pathogenic infections. More importantly, it appears that there is cross-talk between the two pathways and that the autophagic machinery may be regulated by TLRs. TLR3 (dsRNA recognition), TLR7/8 (ssRNA recognition), and TLR9 (recognizes dsDNA with unmethylated CpG motifs) can identify viral signatures (Kawai and Akira, 2006). TLR4 can also recognize mouse mammary tumor virus (MMTV) envelope protein (Rassa et al., 2002) and the respiratory syncytial virus (RSV) fusion protein (Kurt-Jones et al., 2000). In addition, TLR2 recognizes the envelope proteins of HSV-1 (Kurt-Jones et al., 2004) and human cytomegalovirus (Compton et al., 2003), as well as the measles virus hemagglutinin (Bieback et al., 2002). As many of these TLRs share downstream adaptors TRIF and myeloid differentiation primary response gene (88) (MyD88) (Kawai and Akira, 2006), it is possible that apart from TLR4, other TLRs may also trigger autophagy.

On the other hand, autophagy has also been suggested to suppress the innate immune response to VSV infection. The retinoic-acid-inducible gene I (RIG-I)-mediated recognition of VSV in murine fibroblasts is inhibited by overexpression of Atg12-Atg5 (Jounai et al., 2007). Furthermore, MEFs deficient in Atg5 or Atg7 are resistant to VSV infection, more sensitive to polyinosinic:polycytidylic acid (poly:IC) treatment, and produce higher type I IFN responses following either treatment (Jounai et al., 2007; Tal et al., 2009). This evidence suggests that autophagy's effects are likely both cell type- and

PRR-dependent, and further demonstrates the intricate nature of the relationship between autophagy and host cell innate immunity.

3.4. Viruses Can Modulate Autophagy To Determine Cell Fate By Either Postponing Or Hastening Cell Death.

Autophagy is involved with many biological pathways linked to cellular stress, and the signals that drive the activation of autophagy may ultimately decide the fate of the cell. Indeed, it has been suggested that autophagy possesses a dual role in mediating cell survival and cell death. As a survival mechanism, autophagy sustains cell viability during brief periods of cellular stress, either by providing metabolic substrates in times of shortage, or by removing damaged organelles in order to prevent apoptosis. On the other hand, autophagy has also been shown to induce cellular death under certain circumstances, which has been characterized as a form of programmed cell death (type II PCD) that differs from the more classical apoptotic (type I PCD) and necrotic forms of cell death (Levine and Yuan, 2005). Inhibition of autophagy can trigger apoptosis under starvation conditions (Boya et al., 2005), whereas cells deficient in the pro-apoptotic Bax and Bak proteins undergo autophagic cell death upon treatment with strong pharmacological agents such as etoposide (Shimizu et al., 2004). More importantly, based on autophagy's dual nature with regards to cell fate, it has been suggested that this pathway might have important roles in the development of cancer and tumor suppression (Baehrecke, 2005; Gozuacik and Kimchi, 2007; Kondo et al., 2005; Levine and Yuan, 2005) (Brech et al., 2009; Eisenberg-Lerner et al., 2009; Maiuri et al., 2009; Morselli et al., 2009; Scarlatti et al., 2009).

Recent studies indicate that autophagy may function as a pro-survival mechanism during viral infections. Autophagy has been suggested to extend the survival time of human parvovirus B19-infected erythroid cells during viral expansion (Nakashima et al., 2006). Furthermore, protection against SINV-induced neuronal apoptosis and encephalitis in mice is conferred by concurrent overexpression of Beclin-1, but not anti-apoptotic Bcl-2 (Liang et al., 1998). The hepatitis B virus (HBV)-encoded transcriptional transactivator protein X (HBx) has been shown to upregulate the expression of Beclin-1 and stimulate autophagy (Tang et al., 2009), an effect that was also observed in HBV-

associated hepatocellular carcinomas (HCC) (Song et al., 2004). Interestingly, HBx has long been suggested to be tumorigenic (Kim et al., 1991), while Beclin-1 is commonly regarded as a haplo-insufficient tumor suppressor gene (Qu et al., 2003; Yue et al., 2003). Hence, autophagy could be up regulated by HBV to prolong cell survival, and this process may help initiate the development of HCC through an as yet uncharacterized mechanism.

As previously indicated, autophagy has been theorized to induce a novel form of programmed cell death, particularly under circumstances when apoptosis is impaired. Hence, while autophagy has been generally characterized as a pro-survival mechanism, certain viruses may counter-intuitively block this process in order to prolong the survival of infected cells. The Kaposi's Sarcoma-Associated Herpesvirus (KSHV)-encoded viral Bcl-2 (vBcl-2) protein inhibits autophagy through its interaction with Beclin-1 (Pattingre et al., 2005) and similar effects have also been observed with the closely related murine γ-herpesvirus-68 (γHV-68)-encoded vBcl-2 molecule M11 (Ku et al., 2008; Sinha et al., 2008). However, in addition to their effects on autophagy, vBcl-2s from γ-herpesviruses also protect infected cells from apoptosis (Choi et al., 2001; Moore and Chang, 2003). While some authors have suggested that vBcl-2 help γ-herpesviruses escape autophagic degradation (Pattingre et al., 2005), given the powerful anti-apoptotic role of vBcl-2 it is difficult to gauge the relative importance of these effects. More recently, the KSHV viral FADD-like interleukin-1-β-converting enzyme (FLICE)/caspase-8-inhibitory protein (vFLIP) was shown to suppress autophagy by interfering with the processing of LC3, and that this effect abrogated autophagy-associated cell death in infected cells (Lee et al., 2009).

Interestingly, viral infections have also been documented to trigger autophagy specifically as mechanism of inducing cell death in uninfected bystander cell populations. During HIV infection, expression of HIV envelope glycoproteins (gp120 and gp41, collectively termed "Env") induces autophagy in uninfected bystander CD4+ T lymphocytes, which results in a novel cell death program with characteristics of both type I and type II PCD (Espert et al., 2006). Thus, it has been suggested that this autophagy-mediated phenomenon may be significant in contributing to general CD4+ T cell losses during the course of HIV infections (Espert et al., 2006; Espert et al., 2007; Levine and

Sodora, 2006). Similarly, HIV gp120 could also enhance autophagy in uninfected neuronal cells, leading to neuronal cell death and potentially contributing to HIV-associated dementia (Spector and Zhou, 2008). Interestingly, in plants, autophagy has been shown to protect bystander non-infected cells from cell death induced by Tobacco mosaic virus (TMV) infection (Liu et al., 2005).

The observed differential regulation of autophagy by viral infection in controlling cellular fate provides some indication to the potential complexity of this intrinsic pathway. It is important to note that it is inherently difficult to distinguish between autophagy as a mechanism of virally induced cell death and a cellular response to infection. Furthermore, there is potential that this may be a pathway whose biology is commonly modified in laboratory cancer cell lines used in virology research (Baird et al., 2008). For example, autophagy has been suggested to regulate the death of brain tumor and prostate cancer cell models infected with engineered adenoviruses (Alonso et al., 2008; Ito et al., 2006; Jiang et al., 2007; Rajecki et al., 2009; Ulasov et al., 2009; Yokoyama et al., 2008), but conflicting results have also suggested that this response may actually be an attempt at survival (Baird et al., 2008). Moreover, as characteristics of both type I and type II PCD are often seen in conjunction, it is often difficult to differentiate whether the observed phenomenon is an executioner mechanism or a futile attempt at cell survival. Nonetheless, from the above discussions it is clear that autophagy can contribute in the regulation of cellular fate, and as such may be specifically regulated by viruses to promote their life cycle.

4. Discussion

Questions regarding how viral infections interact with, and are impacted by, the autophagy pathway are increasingly gaining prominence within virology. While this relatively new area of research has produced a number of exciting results, limits in our understanding of the mechanism, its regulation, and in the available investigative tools continue to present challenges, as would be expected in any emerging area of research. One complexity for researchers is that the regulation of autophagy is intimately interconnected with cellular growth and survival, as well as numerous cellular stress responses. Hence, considerable caution should be exercised before an effect on viral replication is directly attributed to this pathway. Furthermore, the natural complexity and diversity of viral pathogens and their host cells has resulted in numerous conflicting observations, making it difficult to draw clear and concise conclusions regarding the general role of autophagy in viral infections.

Despite these and other challenges, there is increasing evidence that this ancient cellular process is a significant factor in the fate of numerous different viral infections. During poliovirus infection, autophagy promotes viral replication through its most primitive function: the ability to induce membrane remodeling and vesicle formation. The processes of xenophagy and immune surveillance via cytoplasmic sampling suggest that autophagy may have evolved to act as a defense against intracellular pathogens that viruses must now contend with. Finally, autophagy's interconnections with cell death programs ingrain it in processes that ultimately determine cellular fate, suggesting that viruses may modulate it to influence cell survival and ensure their own reproductive advantage. Thus, autophagy may be implicated in the biological pathogenesis and/or replicative success of many viral infections through a wide variety of possible mechanisms, and can exhibit multiple distinct and cell type-dependent roles in the course of a single viral infection. This is best illustrated in the case of HIV, where the autophagy machinery is: 1) downregulated in virally-infected CD4+ T cells (Espert et al., 2009; Zhou and Spector, 2008), which may constitute a strategy for avoiding autophagosome-mediated degradation and/or endosomal detection in order to enhance virion production; 2) engaged in HIV-infected macrophages to promote virion production (Kyei et al., 2009); and 3) activated in bystander neurological and immunological naïve cells to commit cell

death by type II PCD, thereby contributing to HIV pathogenesis (CD4+ T cell depletion and neuronal death associated-dementia) (Espert et al., 2006).

The revelation that autophagy can function in immune defense of the host cells, particularly relating to innate immunity, suggests reciprocal regulation (through TLRs and IFN response) aiding in the subsequent development of an adaptive immune response (through enhancement of MHC presentation) to clear pathogens in infected tissues. Further research has the potential to reveal unknown protein interactions and could lead to the development of new pharmacological therapies for treating various virally induced diseases and associated cancers. As new research tools emerge, continuing research will help to clarify the role of autophagy in viral replication, host immune responses, and viral pathogenesis. The intricacies of viral interactions with this ancient and highly conserved pathway strongly suggest that autophagy can function in a myriad of ways, some of which appear contradictory at first glance. Depending upon the circumstances, autophagy can be pro-survival or pro-death, can enhance viral replication or aid in the development of the antiviral response. Autophagy is a double-edged sword that can cut both ways. Clearly a virus would prefer to be on autophagy's good side!!

Acknowledgements

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Tables, Figures, And Legends

Gene	Important Interactions	Protein Function / Chacteristics
Formation of Autophagosomes		
ULK1 (ATG1)	Atg13, FIP200 (Atg17)	Ser/Thr kinase activity important for function; target(s) unknown. Downstream of mTOR signalling. Potentially involved in Atg9 cycling.
Beclin-1 (ATG6)	hVps34, Bcl-2/ Bcl-xL, UVRAG	Structural regulator of class III PI3 kinase hVps34. Contains BH3-like domain that is downregulatory when occupied.
hVPS34	Beclin-1, mTOR	Class III PI3 kinase; resulting PtdIns(3)Ps recruit Atg16L multimer/Atg18 to phagophore. Conflictingly activates mTor in response to amino acids.
ATG9	Atg2, Atg18	Transmembrane protein. Transits between phagophores and trans-golgi/late endosomes. Possible role(s) in protein recycling and/or membrane transit.
ATG12	Atg5, Atg16L	Covalently bound to Atg5 via mechanism similar to ubquitination.
ATG7	LC3, Atg12	Functionally similar to E1 ubiquitin activating enzyme (E1-like). Activates C-terminal glycine of both Atg12 and LC3.
ATG10	Atg12, Atg5	Functionally similar to E2 ubiquitin conjugating enzyme (E2-like). Accepts activated Atg12 and conjugates to internal lysine of Atg5.
ATG5	Atg12, Atg16L	Covalently bound to Atg12; conjugation allows Atg5 to associate with Atg16L.
ATG16L	Atg5-Atg12	Associates with Atg12-Atg5 and dimerizes. Present on outer surface of expanding phagophore; aids membrane curvature and LC3 recruitment (E3-like). Recycled.
ATG4	LC3	Cysteine protease; exposes C-terminal glycine on LC3 prior to lipidation. Subsequently recycles LC3 from outer membrane of autophagosome.
ATG3	LC3, Atg7	Functionally similar to E2 ubiquitin conjugating enzyme (E2-like). Conjugates LC3 with phosphatidylethanolamine (PE) phospholipid.
MAP1LC3 (ATG8)	Atg4	Experimental marker of induction. Cytosolic form (LC3-I) conjugated to PE, becoming membrane-associated (LC3-II). Possible role(s) in membrane expansion, autophagosome transit, and lysosomal fusion. Partially recycled by Atg4.
Regulation of Autophagy		
PI3K (class I)		Produces PtdIns(3)p that activates the Akt/PKB-mTor pathway.
PTEN		Phosphatase that counteracts PI3K by dephosphorylating PtdIns(3)p.
AKT/PKB	PDK1, Tsc 2	Ser/Thr kinase. Activated by PDK1 in the presence of PtdIns(3)p. Inactivates Tsc 2.
REDD1/REDD2		Transcriptionally up regulated in response to hypoxia. Inactivates mTor pathway.
AMPK	LKB1, Tsc2	Activates Tsc2, leading to the induction of autophagy when the AMP/ATP ratio is high.
TSC2	Tsc1, Rheb, Akt/PKB, AMPK	GTPase-activating protein (GAP) with Tsc1; inactivates Rheb. Akt/PKB interferes with function, as does Erk1/2. AMPK enhances activity.
Rheb	Tsc1/Tsc2, mTor	Small GTPase. Activates mTor via binding kinase domain in GTP-dependent fashion. Tsc1/Tsc2 GAP activity converts to inactive, GDP-bound form.
mTOR	Rheb, raptor, mLST8	Key regulator of cellular growth. Autophagy induced when mTor inactivated. Ser/Thr kinase. Forms two protein complexes; mTORC1 associated with autophagy.
Anti-apoptotic Bcl-2 family	Beclin-1	Inhibit autophagy via binding with BH3 motif on Beclin-1. JNK1-mediated phosphorylation disrupts interaction and associated inhibition.
BH3-only Bcl-2 family	Anti-apoptotic Bcl-2 family	Competitively bind with anti-apoptotic Bcl-2 family members, interfering with their association with Beclin-1. Stimulate autophagy.
JNK1	Anti-apoptotic Bcl-2 family	Phosphorylates anti-apoptotic Bcl-2 family members, inhibiting interaction with Beclin-1. Activity induces autophagy.
UVRAG	Bif-1, Beclin-1	Interacts with Beclin-1's coiled-coil domain, strengthening Beclin-1/hVps34 interactions; promotes autophagy. Possible additional role in lysosome fusion.
p53		Controversial/contradictory role(s) in autophagy. P53-dependent autophagy observed experimentally. However, cytosolic p53 is inhibitory (mechanism unknown).
DRAM		Transmembrane lysosomal protein transcriptionally induced by p53. Stimulates autophagy. Necessary for both p53-dependent autophagy and apoptosis.

TABLE 1. Significant genes in the mammalian autophagy pathway.

Virus	Known Interaction(s) with Autophagy	Key References
Adenovirus	Contradictory results; autophagy may enhance or detract from engineered adenovirus-induced cell death; differences possibly cell-type/virus specific.	(Ito et al., 2006) (Baird et al., 2008)
Coronavirus	Contradictory evidence. May induce or subvert autophagy membrane remodeling. Autophagy may enhance viral replication.	(Prentice et al., 2004a) (Zhao et al., 2007)
Coxsackievirus	Autophagosome formation enhances CVB3/CVB4 replication *in vitro*. Autophagy affects virally-induced apoptosis.	(Wong et al., 2008) (Yoon et al., 2008)
Cytomegalovirus	Inhibits autophagy through unidentified mechanism.	(Chaumorcel et al., 2008)
Dengue Virus	Induces autophagy. Disruption reduces corresponding viral titers. Membrane co-localization observed with serotype-specific differences.	(Lee et al., 2008) (Panyasrivanit et al., 2009) (Khakpoor et al., 2009)
Drosophila C Virus	Induces formation of COPI-dependent vesicles. Interactions with autophagy mechanism unknown.	(Cherry et al., 2006)
Enterovirus 71	Induces/co-localizes with autophagic membranes. Induction increases viral titer. Mechanism unclear.	(Huang et al., 2009)
Epsten-Barr Virus	Autophagy may aid EBV MHC-II antigen presentation, but contradictory evidence exists. Possibly part of proliferation regulation.	(Paludan et al., 2005) (Lee and Sugden, 2008)
Hepatitis B Virus	Encodes transcriptional transactivator HBx, resulting in increased expression of Beclin-1. Sensitizes cells to autophagy signals.	(Tang et al., 2009)
Hepatitis C Virus	Induces autophagy; inhibits maturation. Knockdown of autophagy genes or ER stress response limits replication. Autophagy may aid HCV antigen presentation/TLR detection in some cell types.	(Sir et al., 2008) (Dreux et al., 2009)
Herpes Simplex Virus Type I	Viral encoded ICP34.5 antagonizes pathway through inhibition of PKR/eIF2a induction, as well as Beclin-1 binding. Autophagy effects of minimal importance *in vitro*, but essential for neurovirulence *in vivo*.	(Tallóczy, Virgin, and Levine, 2006) (Alexander et al., 2007) (Orvedahl et al., 2007) (English et al., 2009)
Human Immunodeficiency Virus	Autophagy-related mechanism part of envelope protein-induced bystander T-cell death and HIV dementia. Pathway dysregulated in some infected cell types, but some components identified as necessary host co-factors.	(Espert et al., 2006) (Alirezaei et al., 2008) (Zhou and Spector, 2008) (Espert et al., 2009) (Kyei et al., 2009)
Influenza Virus	Induces autophagosome formation; inhibits maturation. Interference decreases viral yield. May enhance MHC-II antigen presentation.	(Gannagé et al., 2009) (Zhou et al., 2009)
Kaposi's Sarcoma-Associated Herpesvirus	Encodes viral homolog of Bcl-2 that inhibits autophagy through binding interactions with Beclin-1's BH3 domain. Viral FLIP suppresses autophagy through inhibition of Atg3.	(Pattingre et al., 2005) (Ku et al., 2008) (Lee et al., 2009)
Parvovirus	Induces formation of autophagosome-like vesicles, possibly to extend host cell survival during viral replication process.	(Nakashima et al., 2006)
Poliovirus	Induces formation of double membrane, LC3-positive vesicles. Aids viral replication and release. Viral 2BC protein triggers LC3 lipidation.	(Jackson et al., 2005) (Taylor and Kirkegaard, 2007)
Rhinovirus	Does not induce or modulate autophagy. Replication unaffected by induction or inhibition of pathway.	(Jackson et al., 2005) (Brabec-Zaruba et al., 2007)
Sendai Virus	In pDCs, autophagy enhances delivery of viral nucleic acids to endosomes for TLR7 recognition.	(Lee et al., 2007)
Sindbis Virus	*in vivo* up-regulation of autophagy via Beclin-1 over-expression reduces fatal encephalitis in mice.	(Liang et al., 1998)
Tobacco Mosaic Virus	Autophagy necessary to restrict virally-induced programmed cell death responses to the site of infection.	(Liu et al., 2005)
Vaccinia Virus	Induces vesicle formation, but mechanism independent of autophagy.	(Zhang et al., 2006)
Varicella-Zoster Virus	Induces autophagy. Role in infection unclear.	(Takahashi et al., 2009)
Vesicular Stomatitis Virus	Autophagy enhances endocytic detection in pDCs, but may diminishes cytosolic detection/antiviral responses in other cell types.	(Jounai et al., 2007) (Lee et al., 2007) (Shelly et al., 2009)

TABLE 2. Brief summary of known interactions between autophagy and viral infections.
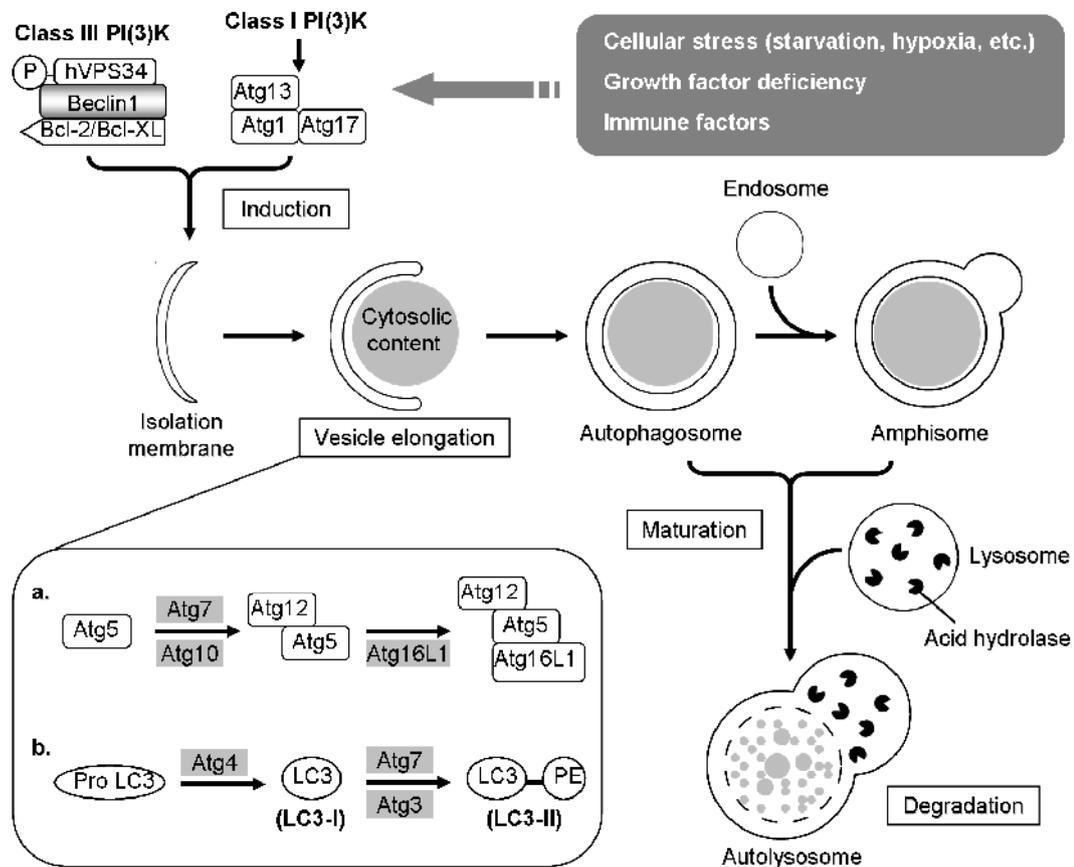
FIG. 1. Overview of the autophagy process. In response to cellular stimuli such as starvation and immune signals, the class I PI(3)K (phosphoinositide 3-kinases)-induced Atg1 complex and a class III PI(3)K complex involving Beclin-1 activate downstream ATG proteins in a series of steps that guide the induction, elongation, maturation, and degradation of the autophagosome. Two ubiquitin-like conjugation systems involving Atg12 (a) and LC3 (b) direct the vesicle elongation of the isolation membrane, which forms a crescent shape to sequester the cytoplasmic cargo. Upon completion, the autophagosome then undergoes the maturation step through a series of remodeling processes including fusion with endosomes/lysosomes. Fusion with lysosome helps the autophagosome mature into an autolysosome in which the autophagic vacuole along with its content is degraded.

APPENDIX C

HYDROLYZABLE TANNINS (CHEBULAGIC ACID AND PUNICALAGIN) TARGET VIRAL GLYCOPROTEIN-GLYCOSAMINOGLYCAN INTERACTIONS TO INHIBIT HERPES SIMPLEX VIRUS TYPE 1 ENTRY AND CELL-TO-CELL SPREAD

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Abstract

Herpes simplex virus type 1 (HSV-1) is a common human pathogen that causes life-long latent infection of sensory neurons. Non-nucleoside inhibitors that can limit HSV-1 recurrence are particularly useful in treating immunocompromised individuals or cases of emerging acyclovir-resistant strains of herpes virus. We report that chebulagic acid (CHLA) and punicalagin (PUG), two hydrolyzable tannins isolated from the dried fruits of *Terminalia chebula* Retz. (*Combretaceae*), inhibit HSV-1 entry at non-cytotoxic doses in A549 human lung cells. Experiments revealed that both tannins targeted and inactivated HSV-1 viral particles, and could prevent binding, penetration, cell-to-cell spread, as well as secondary infection. The antiviral effect from either of the tannins was not associated with induction of type I interferon-mediated responses, nor was pretreatment of the host cell protective against HSV-1. Their inhibitory activities targeted HSV-1 glycoproteins since both natural compounds were able to block polykaryocyte formation mediated by expression of recombinant viral glycoproteins involved in attachment and membrane fusion. Our results indicated that CHLA and PUG blocked interactions between cell surface glycosaminoglycans and HSV-1 glycoproteins. Furthermore, the antiviral activities from the two tannins were significantly diminished in mutant cell lines unable to produce heparan sulfate and chondroitin sulfate, and could be rescued upon reconstitution of heparan sulfate biosynthesis. We suggest that the hydrolyzable tannins CHLA and PUG may be of value as competitors for glycosaminoglycans in the management of HSV-1 infections, and that they may help reduce the risk for development of viral drug resistance during therapy with nucleoside analogues.

Introduction

Herpes simplex virus type 1 (HSV-1) is an alpha-herpesvirus that typically causes mucocutaneous lesions in oral, perioral, and other mucosal sites in the body (56). The virus commonly uses the oropharyngeal mucosa as a port of entry, and following primary infection, establishes a lifelong latent state in the host's trigeminal ganglia sensory neurons. Sporadic recurring infections occur when HSV-1 is reactivated by various stimuli, such as sunlight, immunosuppression, menstruation, fever, or stress (22). While primary or reactivated HSV-1 infections can be subclinical or manifested by mild and self-limited diseases, severe cases of this viral infection may lead to complications such as keratoconjunctivitis, meningitis, and encephalitis (2, 4). Importantly, corneal HSV-1 infection can lead to stromal keratitis, which remains one of the leading causes of blindness in developing countries (35). More aggressive diseases due to HSV-1 are common in immunocompromised individuals (2, 4, 22). To date, no treatment has been identified that eradicates or resolves latent infections by this ubiquitous pathogen.

HSV-1 viral entry into cells is initiated by interaction of viral envelope glycoproteins (gB and gC) with host cell surface proteoglycans (PGs) conjugated to glycosaminoglycans (GAGs) containing heparan sulfate (HS) or chondroitin sulfate (CS) moieties. These initial interactions are sufficient for viral adsorption but not viral entry (65). Subsequently, higher affinity interaction of gD with its receptors including herpesvirus entry mediator (HVEM, member of tumor necrosis factor [TNF] receptor family), nectin-1 and nectin-2 (two members of the immunoglobulin superfamily), and/or 3-*O*-sulfated HS, leads to fusion of the viral membrane with either the plasma or endosomal membranes of the cell through further interactions with gB, gH, and gL (28, 55, 65). Initial interaction of HSV-1 with GAGs ensures a highly efficient infection, but infection of cells deficient in HS or CS can still be achieved via the high affinity receptors, albeit at lower efficiency. Following transport of the viral capsid to the nucleus, where the HSV-1 genome is released, viral products are then expressed in a sequential and coordinated fashion, and are divided into three groups of virus-specific proteins designated as immediate early (α) (ICP0 and ICP4), early (β) (ICP8, UL42, and thymidine kinase), and late (γ) (gB, gC, gD, and gH) phase proteins (71). Although

cellular innate immunity is activated upon virus infection, HSV-1 can produce one or more proteins that counteract the host antiviral response (44, 47, 48).

Most anti-herpes drugs target the viral DNA polymerase, and include nucleoside or pyrophosphate analogues. Acyclovir (ACV), a guanosine analogue, has been the most important clinical drug for the prophylactic or therapeutic treatment of HSV infections, and represents the gold standard for anti-HSV therapy (3, 60). However, extensive use of this drug has led to clinical problems with the emergence of ACV-resistant virus strains, particularly in immunocompromised patients, including those who have had transplantation surgery or have been infected by the human immunodeficiency virus (HIV) (8, 20, 23, 46, 68, 77). Subsequent management of ACV-resistant patients using a different class of DNA polymerase-targeting inhibitor, such as foscarnet, has also been hindered by drug resistance (20, 57). There is a need to identify alternative antiviral therapies with different modes of action to improve the treatment and control of HSV infections, especially in immunocompromised individuals.

Terminalia chebula Retz. (*T. chebula*), a member of the *Combretaceae* family, is a traditional medicinal plant that is native to India and Southeast Asian countries. The dried ripened fruit of *T. chebula* (Fructus Chebulae), often referred to as "myrobalans", contains antioxidants (14) and is commonly used as a broad spectrum medicinal agent for the treatment of dysentery, asthma, cough, sore throat, bloody stools, and diseases of the heart and bladder (29). *T. chebula* is rich in tannins, which are polyphenolic secondary metabolites found in higher plants (26, 30, 34). Tannins are characterized by their relatively high molecular weight (500 to 20,000 daltons) and the unique ability to form insoluble complexes with proteins, carbohydrates, nucleic acids, or alkaloids (26, 53, 61). The hydrolyzable class of tannins possesses structures that generally consist of gallic or ellagic acid esters conjugated to a sugar moiety (27, 34). These polyphenols have high affinity for proteins and polysaccharides, and are thought to be the major bioactive compounds found in the leaves and the fruit of *T. chebula*.

Antiviral activities from hydrolyzable tannins are well documented and are generally thought to target viral adsorption to the host cell membrane (for HSV and HIV), as well as reverse-transcriptase activity of HIV (reviewed in (7) and (61)). We have previously identified several tannins of various plant sources that exhibit potent antiviral

activities against HSV-1 and HSV-2. These include 1,3,4,6-tetra-O-galloyl-beta-D-glucose (75), casuarinin (9), *ent*-Epiafzelechin-(4α→R8)-epiafzelechin (16), excoecarianin (15), geraniin (75), hippomanin A (74), prodelphinidin B-2 3'-O-gallate (10), prodelphinidin B-2 3,3'-di-O-gallate (11), pterocarnin A (12), and putranjivain A (13). Studies from other laboratories have also reported a series of tannins and related compounds capable of inhibiting HSV infections (24, 54, 62, 69, 70). These earlier reports provide strong precedent for our studies and suggest that the tannins constitute an excellent focus for antiviral discovery, particularly in the field of HSV therapeutics.

Identification of multiple drugs that can act on different phases of the viral life cycle can be particularly useful in managing HSV-1 infection or reactivation in either immunocompromised individuals, or cases of ACV resistance. To pursue this goal, we extended our previous studies and concentrated our efforts on four chemically-defined hydrolyzable tannins (Fig. 1), including chebulagic acid (CHLA), chebulinic acid (CHLI), punicalagin (PUG), and punicalin (PUN), which are present in *T. chebula* (37, 38, 76). Although an effect against HSV-1 has been previously reported for CHLA, the mechanism of its activity was not elucidated (69). In this study we report that two of the tannins tested, specifically CHLA and PUG, were found to be most effective against HSV-1. Detailed studies into their inhibitory action revealed that both drugs specifically target HSV-1 particles, block virus entry into the cell, inhibit cell-to-cell spread of the virus, and reduce secondary infection from released virions. The antiviral mechanism is attributed to the binding of CHLA and PUG to viral glycoproteins that interact with cell surface GAGs. Their ability to effectively control viral entry and spread, underscore the potential of these two hydrolyzable tannins for treating HSV-1 infection/recurrence.

Materials And Methods

Chemicals And Reagents

Dulbecco's modified Eagle's media (DMEM) and fetal calf serum (FCS) were supplied by Wisent Scientific (St-Bruno, QC, Canada). Gentamicin and fungizone were purchased from GIBCO-Invitrogen (Carlsbad, CA, USA). Acyclovir (ACV, Acycloguanosine) was obtained from Calbiochem (EMD Biosciences, Darmstadt, Germany). Foscarnet (FOS, Sodium phosphonoformate tribasic hexahydrate), dimethylsulfoxide (DMSO), and *in vitro* toxicology assay kit (XTT-based) were purchased from Sigma (St.Louis, MO, USA).

Test Compounds

Fructus Chebulae and dried leaves from *T. chebula* were commercially obtained from Uchida Wakanyaku Co. (Tokyo, Japan) and an herbal market in Ping-Tung, Taiwan, respectively. Prior to extraction, both materials were anatomically authenticated by Chung-Ching Lin. CHLA, CHLI, and PUG were extracted from Fructus Chebulae, and PUN was derived from the leaves of *T. chebula*. The tannins were isolated and purified as described previously (43, 44). Before use, the structure of each compound was further confirmed by HPLC-UV/ESI-MS analyses, and their purities were checked using HPLC with photodiode array detection (HPLC-PDA) as previously reported (37, 38). CHLA, CHLI, PUG, PUN, and ACV were dissolved in DMSO. FOS was dissolved in sterile ddH$_2$O. All compounds were diluted with cell culture medium before use. The final concentration of DMSO in the drug solution was < 1 % at the effective doses used.

Plasmids

The pCAGGS/MCS vector and its derivative plasmids expressing HSV-1 gB (pPEP98), gD (pPEP99), gH (pPEP100), and gL (pPEP101) (57) were generously provided by Drs. Patricia G. Spear and Richard Longnecker (Northwestern University, Chicago, IL, USA).

Cells And Viruses

Vero (African green monkey kidney cells, ATCC CCL 81), HEL (Human embryonic lung fibroblast, ATCC CCL 137), and A549 (human lung carcinoma, ATCC CCL-185) cells were obtained from the American Type Culture Collection (ATCC; Rockville, MD, USA) and cultured in DMEM supplemented with 10 % FCS, 50 μg/ml gentamicin, and 0.5 μg/ml fungizone at 37 °C in a 5 % CO_2 incubator. Mouse L cells (provided by Dr. Bruce W. Banfield, Queen's University, Kingston, ON, Canada) and its mutant derivative cell lines gro2C (25) (obtained from Drs. Gary H. Cohen and Roselyn J. Eisenberg, University of Pennsylvania, Philadelphia, PA, USA) and sog9 (5) were cultured as above. Sog9-EXT1 cells were established as previously described (43) by transfecting sog9 cells with plasmid expressing the exostosin-1 (EXT1) gene and selecting in media containing 700 μg/ml G418. HSV-1 KOS strain (a gift from Dr. James R. Smiley, University of Alberta, Edmonton, AB, Canada), HSV-1 KOS strain with green fluorescent protein tag (HSV-1-GFP; provided by Dr. Karen L. Mossman, McMaster University, Hamilton, ON, Canada) (45), and vesicular stomatitis virus with green fluorescent protein tag (VSV-GFP; Indiana serotype, a gift from Dr. Brian D. Lichty, McMaster University, Hamilton, ON, Canada) (67) were propagated in Vero cells. HSV-1-GFP and VSV-GFP exhibit similar infectivity as their non-tagged wild-type counterparts. Virus titers were determined by standard plaque assay on A549 cells. Overlay media containing 0.1 % Gamunex (purified clinical human IgGs, provided by Dr. Andrew C. Issekutz, Dalhousie University, Halifax, NS, Canada) or 2 % methylcellulose were used for determination of viral titer for HSV-1 and VSV, respectively. The basal medium for the antiviral assays consisted of DMEM plus 2 % FCS with antibiotics.

Cytotoxicity Assay

The cytotoxic effects of CHLA, CHLI, PUG, and PUN on the different cell types used in this study were measured by the calorimetric XTT (2,3-bis[2-methoxy-4-nitro-5-sulfophenyl]-5- phenylamino)-carbonyl]-2H-tetrazolium hydroxide) assay as described previously with some modifications (16). Briefly, cells were seeded in 96-well plates (1 × 10^4 cells per well) and incubated overnight to form a monolayer. Increasing concentrations of the test compounds were then applied to the culture wells in triplicate.

After incubation at 37 °C for 72 h, the medium on the plate was discarded and the cells were washed twice with phosphate buffered saline (PBS). A volume of 100 μl assaying solution from the *in vitro* XTT-based toxicology assay kit was added to each well. The plates were incubated for another 3 h to allow XTT formazan production. The absorbance was determined with an ELx800 Microplate reader (Bio-Tek, VT, USA) at a test wavelength of 492 nm and a reference wavelength of 690 nm. Data were calculated as percentage of surviving cells using the following formula: cell viability (%) = At / As × 100 %, where 'At' and 'As' refer to the absorbance of the test compounds and the solvent control (DMSO), respectively. The concentration of 50 % cellular cytotoxicity (CC_{50}) of the test compounds was determined as the drug concentration that yielded 50 % cell death as previously described (19).

Antiviral Plaque Assay And Drug Dose-Response Analysis

A549 cells seeded in 12-well plates (5×10^5 cells per well) were treated with serial dilutions of the test compounds for 15 min at 37 °C, and then challenged with HSV-1 (50 PFU/well) for 1 h. The inoculums and drugs were subsequently removed from the wells, and the cells were washed with PBS twice and overlaid again with different dilutions of the test compounds. After further incubation for 48 h, the supernatant was removed, and the wells were fixed with methanol and stained with Giemsa stain solution (Sigma). Viral inhibition (%) was calculated as: [1 − (No. of plaques)$_{exp}$ / (No. of plaques)$_{control}$] × 100 %, where (No. of plaques)$_{exp}$ indicates the plaque counts from virus infection with test compound treatment and (No. of plaques)$_{control}$ indicates the number of plaques derived from virus-infected cells with control treatment (HSV-1 with DMSO only) (9). The 50 % effective concentration (EC_{50}) for antiviral activity was defined as the concentration of antiviral compound that produced 50 % inhibition of the virus-induced plaque formation (18).

For dose-response determination, A549 cells seeded in 96-well plates were infected with HSV-1-GFP (MOI = 1) in the presence or absence of the test compounds at various concentrations (0, 10, 20, 40, and 60 μM) for 24 h. The plates were then scanned by the Typhoon 9410 variable mode imager (Amersham Biosciences; Baie d'Urfe, QC, Canada) and the data was analyzed by ImageQuant TL software (Amersham Biosciences).

Viral infection (%) was calculated as: $[(\text{Fluorescence})_{exp} - (\text{Fluorescence})_{cell\ control}]$ / $[(\text{Fluorescence})_{virus\ control} - (\text{Fluorescence})_{cell\ control})] \times 100\ \%$, where $(\text{Fluorescence})_{exp}$ indicates the GFP expression value from the virus-infected wells with drug treatment, $(\text{Fluorescence})_{cell\ control}$ signifies the GFP expression value of the cell control (DMSO only), and $(\text{Fluorescence})_{virus\ control}$ indicates the GFP expression value derived from virus-infected cells with control treatment (HSV-1-GFP with DMSO only). Values were obtained from three independent experiments with each sample assay performed in triplicate. A standard curve was also generated to ensure linear correlation between virus infection and GFP expression at the MOI assessed.

Assays For Effect Of Tannin Treatment At Different Times

The effect of drug addition over time was assessed according to a previously reported method with some modifications (45). To assess the effect of pretreating cells with tannins, A549 cell monolayers seeded in 12-well plates were treated with CHLA (60 μM) and PUG (40 μM) for 24 h (long term) or 1 h (short term), and then washed with PBS before challenge with HSV-1 (50 PFU/well) in DMEM containing 2 % FCS. To study the effect of adding tannins and virus concurrently, A549 cells were treated simultaneously with HSV-1 (50 PFU/well) and CHLA (60 μM) or PUG (40 μM). After incubation for 1 h at 37 °C, the virus-drug mixture was removed and cells were washed prior to overlay with media. To evaluate whether the tannins had any effects post viral entry, A549 cells were challenged with HSV-1 (50 PFU/well) for 1 h, and after removing the virus inoculum, infected cells were washed and subsequently overlaid with media containing CHLA (60 μM) or PUG (40 μM). For the continuous drug treatment, cells were pretreated for 1 h with the tannins, challenged with HSV-1 in the presence of the drugs, and overlaid with media containing the test compounds after viral entry. For all the above experiments, viral plaques were stained and counted following a total incubation of 48 h post-infection (p.i.). DMSO (0.1 %) treatment was included as control in each condition.

Vesicular Stomatitis Virus (VSV) Plaque Reduction Assay For Host Innate Immunity

To evaluate whether CHLA and PUG induced host innate immune response, a VSV plaque reduction assay was performed. Briefly, A549 cells were seeded in 12-well plates (5×10^5 cells per well) and then pretreated with CHLA (60 μM), PUG (40 μM), IFN-α from human leukocytes (1000 U/ml, Sigma), or with media and DMSO (0.1 %) controls for 24 h. Cell monolayers were washed with PBS twice and subsequently infected with VSV-GFP at MOI = 0.01 for 1 h before applying the overlay media containing 2 % FCS and 2 % methylcellulose. The plates were scanned by Typhoon 9410 variable mode imager to visualize fluorescent plaques at 48 h p.i.

Viral Inactivation Assay

A viral inactivation assay was performed as previously described with some modifications (45). HSV-1 (1×10^4 PFU/ml) was mixed with CHLA (60 μM) or PUG (40 μM), and incubated at 37 °C for 1 h. The test compound-virus mixture was then diluted 50-fold (final virus concentration 50 PFU/well) with DMEM containing 2 % FCS to yield a sub-therapeutic concentration of the drug, and the virus inoculums were subsequently added to monolayers of A549 cells seeded in 12-well plates. As a comparison, HSV-1 was mixed with test compounds, diluted immediately to 50-fold (no incubation period) and added onto A549 cells for infection. The 50-fold dilution served to titrate the drugs below their effective doses and prevent meaningful interactions with the host cell surface. Following adsorption for 60 min at 37 °C, the diluted inoculums were discarded and cells were washed with PBS twice. An overlay medium (DMEM containing 2 % FCS) was applied to each well and the plates were further incubated at 37 °C for 48 h before being subjected to plaque assay as described above. Viral plaques were counted and plaque numbers obtained from infections in the presence of drug compounds were compared to the 0.1 % DMSO control.

Viral Attachment/Binding Assay

To evaluate viral attachment by plaque assay (45), A549 cells were grown to confluence in 12-well culture plates and pre-chilled at 4 °C for 1 h. Medium was

aspirated and the cell monolayers were then infected with HSV-1 (50 PFU/well) in the presence or absence of CHLA (60 μM) and PUG (40 μM). DMSO (0.1 %) was used as a negative control and heparin (100 μg/ml, Sigma) was included as a positive control. After an additional 3 h of incubation at 4 °C, the medium was aspirated to remove any unadsorbed virus, and the cell monolayer was washed with PBS twice before overlay with media containing 2 % FCS. Following incubation for 48 h at 37 °C, viral plaques were stained and counted.

For direct binding assay by flow cytometry analysis, A549 cells (1×10^6 cells per well) were first dissociated using cell dissociation buffer (Sigma). Cells were infected with HSV-1 (MOI = 1) in the presence or absence of CHLA (60 μM) or PUG (40 μM) for 3 h at 4 °C. DMSO (0.1 %) was used as a negative control and heparin (100 μg/ml) was included as a positive control. Cells were subsequently washed twice with ice-cold FACS buffer (1×PBS, 2 % FCS, and 0.1 % sodium azide), blocked with 5 % FCS for 30 min on ice, and then stained with a fluorescein isothiocyanate (FITC)-conjugated polyclonal rabbit anti-HSV-1 antibody (1:500; Dako Canada, Inc.; Mississauga, ON, Canada). Stained samples were washed twice with FACS buffer and then fixed with 1 % paraformaldehyde (PFA) before being subjected to standard flow cytometry analysis. Normal rabbit serum was included in the experiments as an isotype control (1:250; Abcam). Data acquisition and flow cytometry analysis were performed on a Cyan flow cytometer (Dako Canada, Inc).

Viral Penetration Assay

The viral penetration assay was performed as previously described (45) with minor modifications. A549 cell monolayers grown in 12-well plates were pre-chilled at 4 °C for 1 h and subsequently incubated with HSV-1 (100 PFU/well) for 3 h at 4 °C to allow for viral adsorption. The infected cell monolayers were then incubated in the presence of CHLA (60 μM), PUG (40 μM), heparin (100 μg/ml), or DMSO (0.1 %) for an additional 20 min at 37 °C to facilitate HSV-1 penetration. At the end of the incubation, extracellular virus was inactivated by citrate buffer (pH 3.0) (18) for 1 min, and then cells were washed with PBS and overlaid with DMEM containing 2 % FCS. Following 48 h of incubation at 37 °C, viral plaques were stained and counted.

Effect Of Tannin Addition On Viral RNA Expression At Different Times Post-Entry

The effects of tannin addition on HSV-1 RNA expression within the cell was performed by reverse-transcriptase (RT)-PCR analysis. A549 cells were infected with HSV-1 (MOI = 1) for 1 h, and then treated with low pH citrate buffer (pH 3.0) to inactivate extracellular viral particles. Cells were then overlaid with media containing CHLA (60 μM), PUG (40 μM), or DMSO control (0.1 %). At 4, 8, and 12 h p.i., total cellular RNA was isolated using TRIzol Reagent (Invitrogen), treated with DNase I (Qiagen Inc.; Mississauga, ON, Canada) to remove genomic DNA, and purified by phenol/chloroform according to the protocols from the manufacturers. Aliquots of 1 μg RNA were used to generate cDNA with a High Capacity cDNA Reverse Transcription Kit (ABI; Foster City, CA, USA). The cDNA (10 %) was then subjected to standard PCR amplification using the following primers against HSV-1 immediate-early (ICP27), early (TK), and late (gD) genes, and also against the cellular glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene: ICP27 forward primer 5'-GCCGCGACGACCTGGAATCG-3', ICP27 reverse primer 5'-TGTGGGGCGCTGGTTGAGGATC-3' (216 bp); TK forward primer 5'-AGGTATCGCGCGGCCGGGTAG-3', TK reverse primer 5'-ATGGCTTCGTACCCCTGCCA-3' (533 bp); gD forward primer 5'-ATGGGAGGCAACTGTGCTATCC-3', gD reverse primer 5'-CTCGGTGCTCCAGGATAAAC-3' (250 bp); GAPDH forward primer 5'-GCCTCCTGCACCACCAACTG-3', GAPDH reverse primer 5'-ACGCCTGCTTCACCACCTTC-3' (349 bp). For comparison, A549 cells were also infected with HSV-1 (MOI = 1) in the presence of CHLA (60 μM), PUG (40 μM), or DMSO (0.1 %) at 0 time (co-addition). After incubation for 1 h at 37 °C, the inoculums and tannins were removed, and the cells were washed with PBS before being overlaid with DMEM containing 2 % FCS. Again at 4, 8, and 12 h p.i., total cellular RNA was harvested and subjected to RT-PCR analysis as described above.

Effect Of Tannins On HSV-1 Secondary Infection And Cell-To-Cell Spread

To assess the effect of the tannins on secondary viral infections, confluent A549 cells were seeded in 12-well plates and then infected with HSV-1-GFP (200 PFU/well)

for 1 h. After virus adsorption and penetration, extracellular virus was inactivated by citrate buffer (pH 3.0) for 1 min followed by PBS washes. The cells were then treated with CHLA (60 μM), PUG (40 μM), heparin (100 μg/ml), or DMSO (0.1 %) following one round of HSV-1 replication (12 h). Any increase in plaque formation due to released virions from secondary infections could be monitored over the subsequent incubation period. At 24 and 48 h post the initial infection, the plates were scanned using a Typhoon 9410 variable mode imager and fluorescent viral plaques were counted using the ImageQuant TL software to determine whether any new plaques were formed due to virus progeny. Values were obtained from three independent experiments with each sample being performed in duplicate and plotted on a bar graph.

For the analysis of virus cell-to-cell spread, experiments were performed as described above, except that the drugs were added to HSV-1-GFP-infected-cells at 24 h p.i. The overlay media also contained neutralizing Gamunex antibodies (0.1 %), which were used to prevent secondary infection from released HSV-1 virions. The antibodies do not affect lateral spread of virus between cells via intercellular junctions. This allowed the monitoring of cell-to-cell spread of virus in the presence and absence of tannins. Drugs were added at 24 h p.i. and a visual comparison of viral plaque size was performed at the end-point of the experiment (48 h p.i.). Photomicrographs were taken at 100× magnification (Leica Microsystems; Wetzlar, Germany) between 24 and 48 h p.i.

Virus-Free Cell Fusion Assay

To examine whether the compounds interacted with HSV-1 glycoproteins to inhibit glycoprotein-mediated fusion events, a virus-free cell fusion assay was performed (57). A549 cells were seeded in 6-well dishes and transfected with plasmid DNA expressing the individual HSV-1 glycoproteins (gB, gD, gH, and gL) or with the control vector using Arrest-In (Open Biosystems; Huntsville, AL, USA). The total amount of plasmid DNA per well was 4 μg. After 6 h of incubation at 37 °C, the transfection mixture was discarded and the cells were washed with PBS before treatment with CHLA (60 μM), PUG (40 μM), heparin (100 μg/ml), or DMSO (0.1 %). After incubation for another 24 h, cells were fixed with methanol and then stained with Hoescht dye (Sigma) or Giemsa stain solution. Photomicrographs were then taken at 200× magnification

(Leica Microsystems). Polykaryocytes with > 10 nuclei from the dishes were also counted.

Effect Of Tannins Using Plaque Assays In GAGs-Deficient Cell Lines

For antiviral analysis in GAGs mutant cell lines, confluent mouse L, gro2C, sog9, and sog9-EXT1 cells in 12-well dishes were infected with HSV-1-GFP, at 200 PFU/well respective to viral titers obtained in each cell line, in the presence of CHLA (60 μM), PUG (40 μM), or DMSO (0.1 %). The total amount of virus used on the different mutants was within an equivalent MOI of 1 respective to the titer determined on the parental mouse L cells. Following incubation for 1 h at 37 °C, the virus-drug mixture was removed and the cells were washed with PBS before overlaying with DMEM containing 2 % FCS. After further incubation for 48 h, fluorescent plaques were scanned as described above and counted using the ImageQuant TL software. Data was expressed as % viral infection = [(No. of plaques)$_{exp}$ / (No. of plaques)$_{control}$] × 100 %.

Results

Inhibition Of HSV-1 Infection By The Hydrolyzable Tannins

Chebulagic acid (CHLA), chebulinic acid (CHLI), punicalagin (PUG), and punicalin (PUN) (Fig. 1) have been reported to exhibit antiviral activities. We investigated whether these hydrolyzable tannins could inhibit HSV-1 infection. In order to ensure that the tannin concentrations were not cytotoxic, a toxicity analysis was carried out in A549 cells by using the XTT cell viability assay. Our results indicated that these four tannins did not have apparent cytotoxic effects below 100 μM in A549 cells while a dose-dependent cytotoxic effect was seen when concentration > 100 μM was used (data not shown). The 50 % cellular toxicity indices (CC_{50}) of CHLA, CHLI, PUG, and PUN were 316.87 $\pm$ 9.01, 330.83 $\pm$ 9.07, 318.84 $\pm$ 4.98, and 310.85 $\pm$ 1.99 μM, respectively (summarized in Table 1). For comparison, toxicity in primary human fibroblasts (HEL) cells was also assessed, and similar results were observed (data not shown).

We then evaluated the antiviral effects of these four natural compounds against HSV-1 infection using a plaque assay. Acyclovir (ACV) and foscarnet (FOS) were used as positive controls and DMSO was included as a negative control. FOS is the treatment of choice in the clinical setting if resistance develops against ACV. All four tannins could inhibit viral plaque formation, following inoculation of 50 PFU, in a dose-dependent manner, and their 50 % effective concentration (EC_{50}) values were 17.02 $\pm$ 2.82 (CHLA), 20.85 $\pm$ 2.40 (CHLI), 10.25 $\pm$ 1.13 (PUG), and 21.33 $\pm$ 1.77 μM (PUN) (Table 1). The selectivity index (SI), which measures the preferential antiviral activity of a drug in relation to its cytotoxicity, was calculated according to their CC_{50} and EC_{50}. The SI indices of CHLA, CHLI, PUG, and PUN were 18.62, 15.87, 31.11, and 14.57, respectively (Table 1). Given their higher SI values, CHLA and PUG were chosen for subsequent analyses.

To obtain a more accurate dose-response curve for these two hydrolyzable tannins, A549 cells were infected with HSV-1-GFP (MOI = 1) in the presence of the tannins, and fluorescent signals were quantified. The HSV-1-GFP was susceptible to the antiviral effects of the tannins. Both CHLA and PUG displayed anti-HSV-1 activity in a dose-dependent manner (Fig. 2), and the concentrations of CHLA at 60 μM and PUG at 40 μM,

which provided near complete protection against the virus infection at a MOI = 1, were chosen for all subsequent experiments.

Antiviral Activities Of CHLA And PUG Depend Upon The Presence Of HSV-1, And Inhibition Is Not Due To Activation Of Host Cell Innate Immunity

To better understand the antiviral mechanism and the stage of HSV-1 infection affected by these two *T. chebula* tannins, we added the compounds at different times of the virus life-cycle (pre-entry, entry, and post-entry). In order to study pre-entry, more specifically the effect of the compounds on the cell itself prior to virus addition, A549 cells were pretreated with CHLA and PUG for long term (24 h) or short term (1 h) periods and then washed prior to HSV-1 infection. For effects on the viral entry stage, virus and the drugs were simultaneously applied to the cells. To investigate events following virus entry, A549 cells were first infected with HSV-1 for 1 h and then treated with the tannins. For comparison, the tannins were also maintained throughout the entire experimental period.

Pretreating A549 cells with CHLA and PUG (both long term and short term) did not protect against HSV-1 infection. Both tannins were effective in preventing plaque formation when added during virus adsorption, immediately after viral entry, and throughout multiple cycles of virus replication (Fig. 3). The data indicate that HSV-1 infection is severely impaired only if the drugs are present at the time of infection or during viral spread, and that it is unlikely that the antiviral activity is due to direct effects on the cells (such as masking cellular receptors or entry factors for HSV-1). To confirm that neither of the tannins activated host cell innate immunity and induced production of antiviral cytokines such as interferons (IFNs), a VSV plaque reduction assay was performed. VSV replication is extremely sensitive to cellular IFN production. IFN-α, a potent inhibitor of VSV, was included as a positive control. In line with the above observation, neither CHLA nor PUG pretreatments protected A549 cells from VSV infection, whereas IFN-α treatment produced an intact cell monolayer (Fig. 4). Moreover, neither of the tannins induced IFN-stimulated genes in the A549 cells (data not shown). The above results suggest that the anti-HSV-1 activities produced by CHLA and PUG: (1) are unlikely to be mediated by effects through binding to the cellular receptors for HSV-1

or in triggering antiviral innate immunity, and (2) absolutely requires the presence of HSV-1.

CHLA And PUG Block HSV-1 Entry By Inactivating Virus Particles And Preventing Virus Attachment And Penetration Into A549 Cells

In order to evaluate the antiviral mechanism of CHLA and PUG, we investigated their effects on the virus particles themselves. Tannin compounds were pre-incubated with virus particles and then diluted to a sub-therapeutic concentration prior to infection. Both CHLA and PUG could interact with virus particles, irreversibly, to prevent infection (Fig. 5A). This suggests that CHLA and PUG can bind to virus particles and neutralize virus infectivity.

We next assessed the ability of CHLA and PUG to affect viral attachment and penetration. By plaque assay, both of these tannins completely inhibited HSV-1 attachment to A549 cells in the initial viral binding period at 4 °C and resulted in protection of the cell monolayer (Fig. 5B). A direct binding assay was also performed using virus-specific antibodies to detect bound HSV-1 particles. CHLA and PUG completely prevented HSV-1 binding to the cell surface (Fig. 5C). Heparin, a competitive HSV binding inhibitor, was included as a positive control in both experiments. These results suggest that CHLA and PUG might interact with viral glycoprotein(s) and/or cellular receptor(s) during the virus attachment phase. To further assess the effects of CHLA and PUG on virus penetration step, HSV-1 particles were allowed to first bind to A549 cells at 4 °C, and were subsequently allowed to fuse with and penetrate the host cell membrane by shifting the temperature to 37 °C in the presence or absence of the tannins. As shown in Fig. 5D, CHLA retained most of its antiviral activity even during the viral penetration phase and PUG could completely abrogate virus penetration into the A549 cells, resulting in a protected monolayer. In contrast, heparin, which is effective at blocking HSV-1 adsorption, did not prevent subsequent virus penetration (40). The data indicate that CHLA and PUG impair viral receptor attachment and penetration functions during the HSV-1 infection.

CHLA And PUG Do Not Affect HSV-1 Replication Following Entry But Limit Secondary Viral Infection And Cell-To-Cell Transmission

The observation that CHLA and PUG inhibited HSV-1 plaque formation when treatment was initiated immediately after the virus had entered the cell, suggested that the two tannins may block HSV-1 replication cycle or inhibit HSV-1 secondary infection and/or cell-to-cell spread in the ensuing incubation period. To specifically address these possibilities, we first evaluated the effects of CHLA and PUG on HSV-1 mRNA expression following virus entry. A549 cells were infected with HSV-1 for 1 h, then extracellular virus was inactivated by citrate buffer treatment and washed away, and CHLA, PUG, or DMSO was subsequently added to the cells. For comparison, CHLA and PUG were also added simultaneously with HSV-1. Total cellular RNA was isolated from all samples at various time points following viral infection. Our results clearly indicated that CHLA and PUG did not affect HSV-1 mRNA expression following virus penetration, since levels of immediate-early (ICP27), early (TK), and late (gD) viral gene transcripts were unaffected by the compounds (Fig. 6A). On the other hand, both tannins suppressed HSV-1 mRNA synthesis when added together with the virus at the same time (Fig. 6B). These findings suggest that neither CHLA nor PUG inhibit HSV-1 transcription and replication following penetration of the host cell.

We next examined whether CHLA and PUG inhibited HSV-1 secondary infection and/or cell-to-cell spread. A fluorescent plaque assay was performed using HSV-1-GFP. After viral inoculation of A549 cells, CHLA and PUG were added to the overlay media at 12 or 24 h p.i. in the presence or absence of HSV-1 neutralizing antibodies, and fluorescent viral plaques were quantified or photographed over the subsequent course of infection (total of 48 h post initial challenge). In this assay, neutralizing antibodies coat viral particles released from infected cells and prevent secondary infection of surrounding uninfected cells. Thus, the only route of cell-to-cell transmission in the presence of neutralizing antibody is via intercellular junctions between infected and uninfected cells. Furthermore, prior to drug addition (12 and 24 h p.i.), HSV-1 was permitted to complete at least one round of replication cycle, allowing drug effects on post-entry infection to be monitored in the ensuing incubation period. As expected, in the absence of neutralizing antibodies, there was an increase in viral plaques in the DMSO control group due to

secondary infections (Fig. 7A). However, addition of CHLA, PUG, or heparin substantially reduced the number of viral plaques formed and limited its increase in comparison to DMSO (Fig. 7A). Similarly, with respect to viral spread, treatment with CHLA and PUG prevented viral plaque growth. Incubation with the tannins yielded plaques with considerably reduced size compared to the DMSO control after the 48 h of infection (Fig. 7B). Interestingly, heparin, which has limited inhibitory activity on HSV-1 post-attachment, also exhibited some protective effect against viral spread albeit at a lower efficiency compared to the tannins. Taken together, the data indicated that once HSV-1 entered the cells and completed at least one cycle of infection (12-24 h), any subsequent *de novo* infections and viral spread via intercellular contacts were restricted upon addition of CHLA and PUG. Tannin-mediated inhibition of viral attachment and fusion, as observed earlier (Fig. 5B-D), confirmed these results, and may be responsible for their effects in neutralizing secondary infections and restricting cell-to-cell spread, respectively.

CHLA And PUG Target HSV-1 Glycoproteins That Mediate Glycosaminoglycan-Specific Interactions

HSV-1 viral glycoproteins are known to mediate HSV-1 binding, internalization, and cell-to-cell spread. From the preceding data, it appears that the hydrolyzable tannins CHLA and PUG target viral glycoprotein(s) which would explain the need for virus to be present during inhibition and their effect on virus entry and spread. In an attempt to elucidate the underlying mechanism, we checked whether the two tannins interacted with HSV-1 glycoproteins in order to block entry-associated events. Using a virus-free system, we overexpressed HSV-1 glycoproteins that have been shown to mediate cell fusion (occurs during entry and cell-to-cell spread) by transfecting the individual gB, gD, gH, and gL genes into A549 cells followed by treatment with the tannins. Expression of all four genes induced cell fusion resulting in polykaryocyte formation (> 10 nuclei) which is absent following transfection with the empty vector control. The two tannins and heparin each blocked polykaryocyte formation, suggesting that CHLA and PUG interact with HSV-1 glycoproteins to prevent virus attachment, entry, and cell-to-cell spread (Fig. 8).

Several HSV-1 glycoproteins are known to interact with cell surface GAGs. To further explore the virus-host interactions that are being targeted by the tannins, we used a series of cell lines known to possess defects in surface HS and CS synthesis. The relative infectivities of the HSV-1 (KOS) on the mutant cells compared to the parental mouse L cells (100 %) are about 3.3 % for gro2C and 0.3 % for sog9 (data not shown), similar to previously reported range (5). Reconstitution of HS synthesis through overexpression of the EXT1 gene in sog9 cells (sog9-EXT1) restores the sensitivity to HSV-1 infection about a log fold higher than in the naïve sog9 cells (data not shown) (41). To evaluate the effects of the drugs under the various GAGs expression, an amount of HSV-1 at 200 PFU/well respective to titers obtained in each cell line was used to infect the different monolayers. The mutants gro2C and sog9 would therefore require a much higher viral dose compared to sog9-EXT1 and L cells. The total amount of virus used on the mutant cell lines, however, was within an equivalent MOI of 1 respective to the titer determined on mouse L cells, which is in the protective range of the tannins' antiviral effects (data not shown). While both CHLA and PUG effectively protected the parental mouse L cells (which produce both HS and CS), the tannins' antiviral effect weakened when HS synthesis is lost (gro2C cells), and is significantly impaired when both GAGs are deficient (sog9 cells) (Fig. 9). Overexpression of the EXT1 gene in sog9 cells (sog9-EXT1), which restores HS synthesis only, without affecting CS deficiency, rescued both drugs' inhibitory effects (Fig. 9). These observations strongly suggest that the two tannins target interactions between HSV-1 glycoproteins and GAGs (HS and CS). CHLA inhibition also appeared to be more sensitive to cell surface deficiency in GAGs compared to that of PUG.

Taken together, the data indicate that CHLA and PUG function as GAG competitors to inhibit the initial events of HSV-1 infection (adsorption, penetration) and the cell-to-cell spread of virus. The interaction of HSV-1 glycoproteins with cellular GAGs plays a critical role in viral infections, and the hydrolyzable tannins could offer a primary means of defense against HSV-1 infections.

Discussion

There is currently no cure that completely resolves latent infections caused by alpha-herpesviruses. The development of small molecules capable of inhibiting infection by reactivated virus represents an attractive therapeutic strategy, particularly in immunocompromised individuals who are often at risk of generating ACV-resistant HSV-1 strains. In a search for such molecules, we report that CHLA and PUG, two hydrolyzable tannins isolated from the fruits of *T. chebula*, effectively inhibited HSV-1 infection in A549 cells, without significantly reducing cell viability. In addition, our results suggest that CHLA and PUG specifically targeted HSV-1 particles by binding to viral glycoproteins that interact with cellular GAGs, rendering the virus incapable of adsorbing, penetrating, and spreading throughout the cell monolayer. These features underscore the potential of tannins as HSV-1 entry inhibitors.

Our data show that entry events, including primary/secondary infection, viral attachment/penetration, and cell-to-cell spread are inhibited only when the tannins and HSV-1 glycoproteins are in contact with each other. Pretreatment of host cells with the tannins, followed by washes to remove unadsorbed compounds, had no effects upon HSV-1 replication. This indicated that masking cell surface receptors or entry factors for HSV-1 by the tannins is unlikely. A direct virus binding assay using flow cytometry revealed that the tannins blocked viral attachment to the host cell. While CHLA and PUG could inactivate the HSV-1 particles, we do not believe that a direct lysis effect of the viral membrane is responsible for their effects, since HSV-1 infection of GAG-deficient mutant cell lines was still observed, even in the presence of these compounds (Fig. 9). Given their large molecular weight (CHLA = 954 MW and PUG = 1084 MW) and high affinity for proteins and sugars, the two hydrolyzable tannins are thought to bind to HSV-1 glycoproteins on the infectious virions making them inert, impairing glycoprotein function, and preventing successful attachment and entry of the host cell. These tannins could also bind to viral glycoproteins on the infected-cell surface, rendering them unavailable to mediate the cell-to-cell spread of virus.

HSV-1 entry into epithelial cells, which express the cellular receptors (HS, HVEM, nectin-1, and nectin-2) for HSV (65), requires an ordered and concerted effort from the viral glycoproteins. Specifically, the glycoproteins gB, gC, gD, gH, and gL

interact with host cell receptors and are involved in penetration of the plasma membrane through a membrane fusion process (28, 55, 65). While viral entry and spread require a particular combination of viral surface proteins, several HSV-1 glycoproteins are repeatedly involved in both processes. Importantly, gB and gD function in viral binding and fusion, and are also engaged during cell-to-cell transmission in cultured epithelial cells (21, 28, 31, 36, 55, 58, 59, 65). The associations between viral glycoproteins that mediate HSV-1 attachment and entry represent a complex scenario when considering CHLA and PUG and their mechanism of action. The candidate targets of the tannins likely involve viral glycoproteins that interact with host cell surface GAGs and participate in adsorption, membrane fusion, and cell-to-cell spread. The observation that both tannins blocked virus attachment to cells, as did heparin, suggests that interaction of gC and gB with heparan sulfate proteoglycans (HSPGs) is targeted. However, the drugs also prevented virus internalization into cells in the post-binding phase. At this point, the interaction with HSPGs should be irrelevant, since virions now interact with a gD receptor and become resistant to removal by heparin (40) (Fig. 5D). One explanation is that the tannins bind to gB and block its interaction with HSPG while also interfering with its subsequent role in membrane fusion during virus entry (in which gC is not involved). Alternatively, the tannins may be impeding the activity of additional glycoproteins (gD and/or gH/gL) or working by some other mechanism(s) to prevent successful entry into the A549 cells. Finally, there is the possibility that viral glycoproteins may still be accessible to the tannins, even when these viral proteins are bound to the host cell or are expressed in the intercellular junctions. This could explain why the considerably larger heparin (17,000-19,000 MW) can bind free gB, but is unable to interact efficiently with the shielded glycoprotein which is needed in order to inhibit viral penetration or cell-to-cell transmission. Additional binding experiments using glycoprotein-deficient HSV-1 mutants as well as soluble recombinant HSV-1 could help elucidate the targeting specificity of the tannins. We speculate that the two natural compounds can bind to all GAG-interacting glycoproteins including gB, gC, and/or gD, and neutralize their functions. The ability of CHLA and PUG to effectively block virus membrane penetration, as well as virus attachment, could explain their higher efficacy in restricting the spread of HSV-1 compared to the inhibitory effects of heparin.

In the case of HSV-1, the interactions between several of its glycoproteins and cell surface GAGs are critical for ensuring efficient viral entry as well as viral spread (5, 25, 42, 50, 51, 63, 64, 66). CS can confer susceptibility to HSV-1 infection in the absence of HS (6, 25), but the latter carbohydrate moiety is still the preferred substrate for viral attachment. Absence of GAGs renders cell surfaces relatively resistant to HSV-1 binding. The lack of such interactions alters the kinetic and method of entry, whereby the virus relies on alternate mechanism via high affinity receptors to gain access into the cell. Earlier studies have shown that the HS- and CS-deficient sog9 cells are insensitive to inhibitory effect of soluble HS on HSV-1 infection (5). We observed that while the absence of HS alone in gro2C cells weakened the tannins' inhibitory effects, the loss of both HS and CS on the surface of sog9 cells also significantly reduced their antiviral activity, by about 60-100 % (Fig. 9). Overexpression of the EXT1 gene, which restores HS biosynthesis in EXT1 mutant sog9 cells, rescued the antiviral effects from both CHLA and PUG to > 90 %. Although both hydrolyzable tannins seemed to behave in a similar manner, it was noted that CHLA inhibition was more significantly affected by the deficiency of GAGs compared to PUG. This observation could be rationalized by differences in the two tannins' structures, and by different binding capacities/affinities to the viral glycoproteins, with CHLA being more selective against the GAG-interactions.

All four hydrolyzable tannins investigated in this report are composed of a glucopyranose core linked with galloyl derivatives, including hexahydroxydiphenoyl (HHDP; C-C coupling between galloyl moieties), gallagyl, and chebuloyl units (Fig. 1) (37, 76). Only CHLA and PUG possess the HHDP unit, with an (R) configuration (linked to the glucose core at the 3,6-positions) and (S) configuration (linked via the 2,3- or 4,6-positions of their glucose residue), respectively (34, 76). The anti-herpes activities of hydrolyzable tannins are thought to be dependent on the number of galloyl or HHDP groups, irrespective of the sugar core (69). Structures containing HHDP unit have also been valuable pharmacophores for inhibiting HIV enzymatic activities (19, 73). Indeed, anti-HIV activities from CHLA, CHLI, PUG, and PUN have been reported to prevent binding of recombinant HIV gp120 to CD4 and to exert inhibitory effects on HIV-1 RT and integrase (1, 49, 72). The ability of these natural agents to inhibit both HSV-1 and

HIV-1 underscores their potential value in the treatment of acquired immunodeficiency syndrome patients who also exhibit HSV-1-related symptoms.

Use of these tannins could improve the prognosis of anti-HSV-1 therapy in immunosuppressed individuals and help to reduce the risk of ACV-resistance by lowering the ACV-dose required. Since *Fructus Chebulae* contains both CHLA and PUG, inclusion of purified extracts from this plant in topical creams or microbicides would be a feasible method for controlling recurrent HSV-1 infections. Future studies will determine whether these natural products are effective against additional members of the herpesvirus family and other enveloped viruses. Our preliminary studies have shown that both CHLA and PUG inhibit the growth of HSV-2 and human cytomegalovirus (Lin, L.-T. and Chen, T.-Y., unpublished data). Other viruses known to use GAGs as entry factors, such as measles virus and human respiratory syncytial virus, are also inhibited by these tannins, reinforcing our discovery that these compounds act as GAG competitors that inhibit viral glycoprotein-cell receptor interactions (Lin, L.-T. and Chen, T.-Y., unpublished data). Further studies with the tannins derived from *T. Chebula* may provide new ways to inhibit recurrent HSV-1 infections and control the re-emergence of this virus in immunocompromised patients.

Acknowledgements

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Compounds	CC_{50}^{a} (µM)	Anti-HSV-1 effects	
		EC_{50}^{b} (µM)	SI^c
CHLA	316.87 ± 9.01	17.02 ± 2.82	18.62
CHLI	330.83 ± 9.07	20.85 ± 2.40	15.87
PUG	318.84 ± 4.98	10.25 ± 1.13	31.11
PUN	310.85 ± 1.99	21.33 ± 1.77	14.57
ACV	> 2000	14.45 ± 1.71	> 138.41
FOS	> 2000	183.37 ± 25.18	> 10.91

a. Cytotoxic effects were evaluated by XTT assay to determine the concentration of 50 % cellular cytotoxicity (CC_{50}) of test compounds.
b. Antiviral effects were evaluated by plaque assay to determine the effective concentration that achieved 50 % inhibition (EC_{50}) against HSV-1 infection.
c. Selectivity index (SI) = CC_{50} / EC_{50}.

Values shown are mean ± SEM (standard error of the mean) from three independent experiments with each treatment in triplicate.

TABLE 1. Cytotoxicity and anti-HSV-1 activity of CHLA, CHLI, PUG, and PUN in A549 cells.
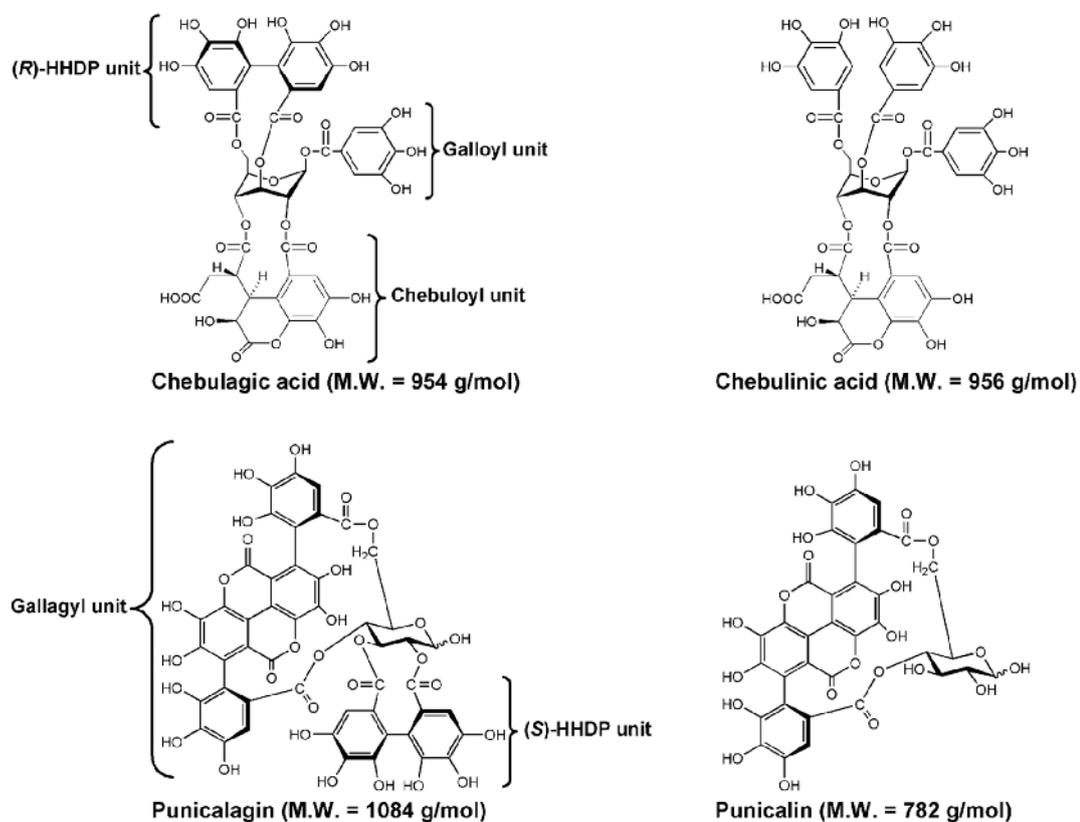
FIG. 1. Chemical structures of chebulagic acid (CHLA), chebulinic acid (CHLI), punicalagin (PUG), and punicalin (PUN). Components of the tannins including galloyl, hexahydroxydiphenoyl (HHDP), gallagyl, and chebuloyl units are indicated.

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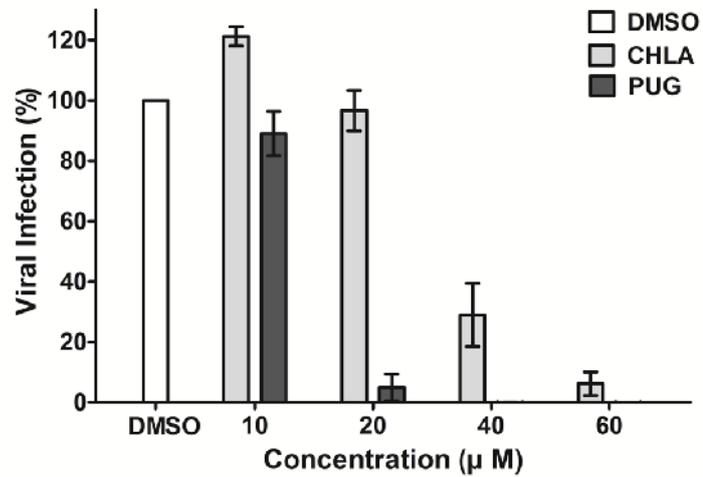
FIG. 2. Dose-response for inhibition of HSV-1 infection in A549 cells by CHLA and PUG. A549 cells were seeded into 96-well plates and then infected with HSV-1-GFP (MOI = 1) in the presence or absence of the tannins at various concentrations (0, 10, 20, 40, and 60 μM) for 24 h. DMSO (0.1 %) served as negative control. Viral infection was quantified by measuring GFP fluorescence using a Typhoon 9410 variable mode imager. Data shown are the mean ± SEM of three independent experiments with each tannin treatment being performed in triplicate.
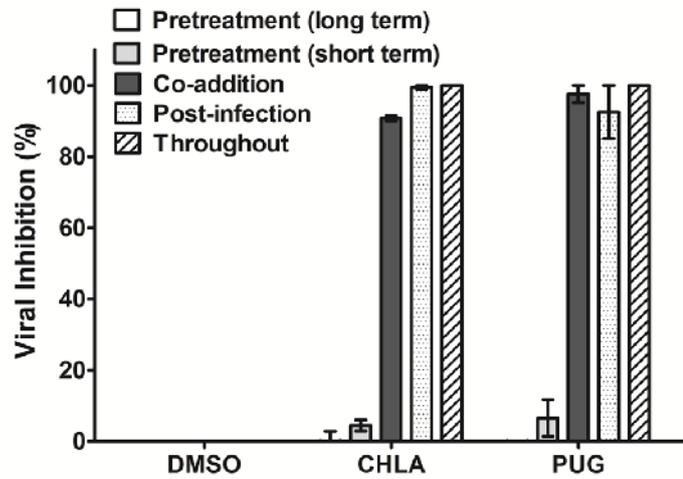
FIG. 3. Effect of time of CHLA and PUG addition on plaque formation by HSV-1. A549 cells were seeded in 12-well plates and then treated with CHLA (60 μM) or PUG (40 μM) at various stages of HSV-1 infection (50 PFU/well). For pretreatment, A549 cells were incubated with the test compounds for 24 h (long term) or 1 h (short term) and then washed once before infecting with HSV-1. For the co-addition experiment, the tannins and virus inoculum were added simultaneously to cells, incubated for 1 h, and then washed. In the post-infection experiment, the cells were infected with HSV-1 for 1 h, washed, and then subsequently treated with the tannins. In addition, cells were also incubated in the continuous presence of the test compounds from pretreatment to post-infection stages. After an additional 48 h of incubation, viral plaques were stained and counted. DMSO (0.1 %) was included as control. Data shown are mean ± SEM of three independent experiments with each treatment performed in duplicate.
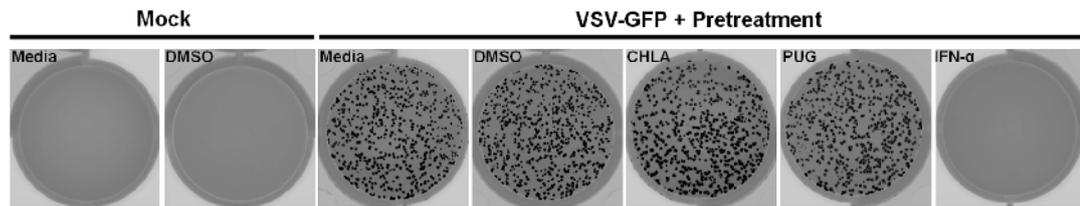
FIG. 4. CHLA and PUG do not induce IFN-mediated immune response against VSV infection. A549 cells were seeded in 12-well plates and then pretreated with CHLA (60 μM), PUG (40 μM), IFN-α (1000 U/ml), or with media and DMSO (0.1 %) controls for 24 h. Cell monolayers were washed with PBS twice and then infected with VSV-GFP (MOI = 0.01) for 1 h before applying overlay media containing 2 % FCS and 2 % methylcellulose. After an additional 48 h of incubation, the plates were scanned for visualization of fluorescent plaques. Data shown are representative images from one of two independent experiments.
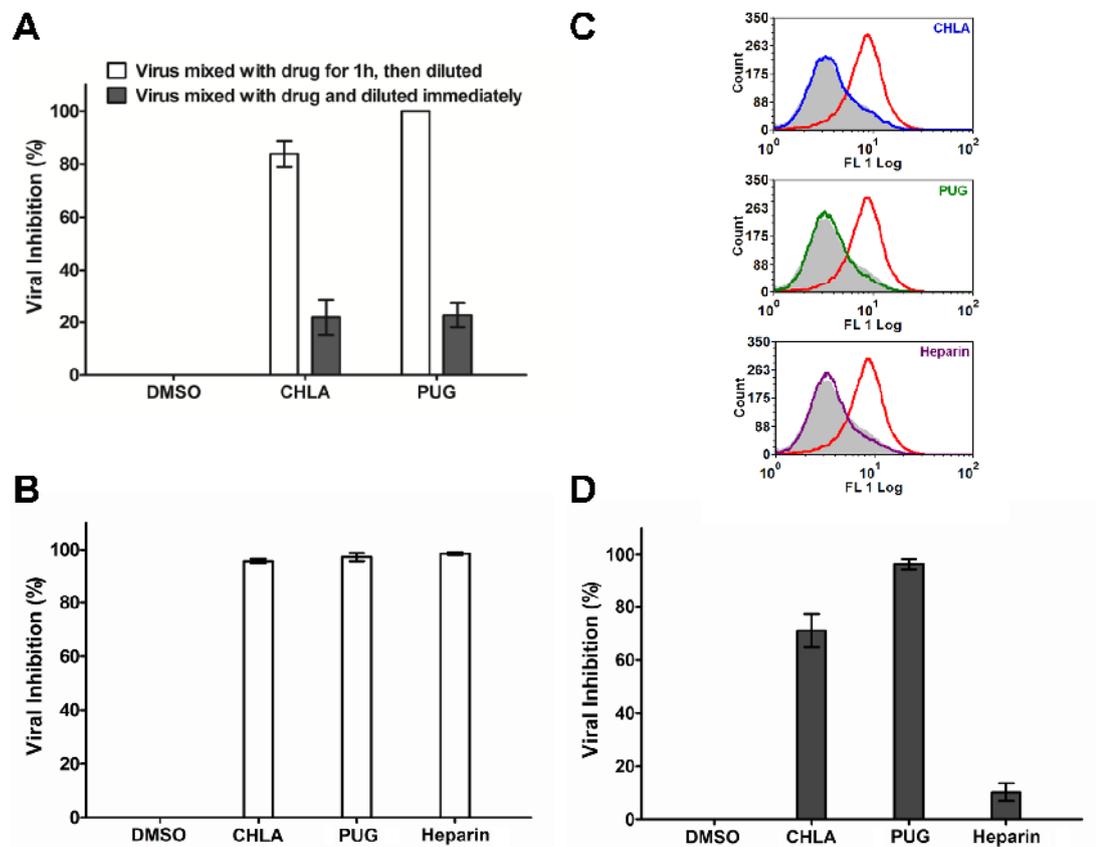
FIG. 5. CHLA and PUG inhibit HSV-1 entry by inactivating viral particles and preventing virus binding and internalization into A549 cells. (A) Viral inactivation assay. HSV-1 (1×10^4 PFU/ml) was mixed with CHLA (60 μM) or PUG (40 μM) for 1 h at 37 °C, and then diluted 50-fold (final virus concentration 50 PFU/well) before infecting A549 cells. As a control, the same amount of virus was also mixed with the tannin, but diluted immediately and applied to the A549 cells. After a 48 h incubation period, viral plaques were stained and counted. DMSO (0.1 %) was included as a negative control. Data shown are mean ± SEM of three independent experiments with each treatment performed in duplicate. (B) Viral attachment analysis by plaque assay. A549 cells were pre-chilled at 4 °C for 1 h and then inoculated with HSV-1 (50 PFU/well) in the presence of CHLA (60 μM), PUG (40 μM), or heparin (100 μg/ml) and DMSO (0.1 %) controls for another 3 h at 4 °C. Unadsorbed virus was then removed by washing the cell monolayer with PBS twice. After 48 h of incubation at 37 °C, viral plaques were stained

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and counted. Data shown are mean ± SEM of three independent experiments with each treatment performed in duplicate. (C) Viral binding assay by flow cytometry analysis. Dissociated A549 cells were infected with HSV-1 (MOI = 1) in the presence or absence of 60 μM CHLA or 40 μM PUG for 3 h at 4 °C. Cells were then washed, blocked, and stained with FITC-conjugated polyclonal rabbit anti-HSV-1 antibody (1:500). Stained samples were washed and fixed with 1 % PFA before being subjected to standard flow cytometry analysis. DMSO (0.1 %) was used as a negative control and heparin (100 μg/ml) was included as a positive control. Color indication for different treatments: grey = mock + DMSO, red = HSV-1 + DMSO, blue = HSV-1 + CHLA, green = HSV-1 + PUG, purple = HSV-1 + heparin. Data shown are representative of three independent experiments. (D) Viral penetration analysis by plaque assay. A549 cells were pre-chilled at 4 °C for 1 h before inoculation with HSV-1 (100 PFU/well) for 3 h at 4 °C. Then, cells were treated in the presence or absence of CHLA (60 μM), PUG (40 μM), or heparin (100 μg/ml), and further incubated for an additional 20 min with the temperature shifted to 37 °C to facilitate viral penetration. At the end of the incubation, extracellular virus was inactivated by citrate buffer (pH 3.0) for 1 min, then washed with PBS twice before overlaying with medium. After 48 h of incubation at 37 °C, viral plaques were stained and counted. DMSO (0.1 %) was included as negative control. Data shown are mean ± SEM of three independent experiments with each treatment performed in duplicate.
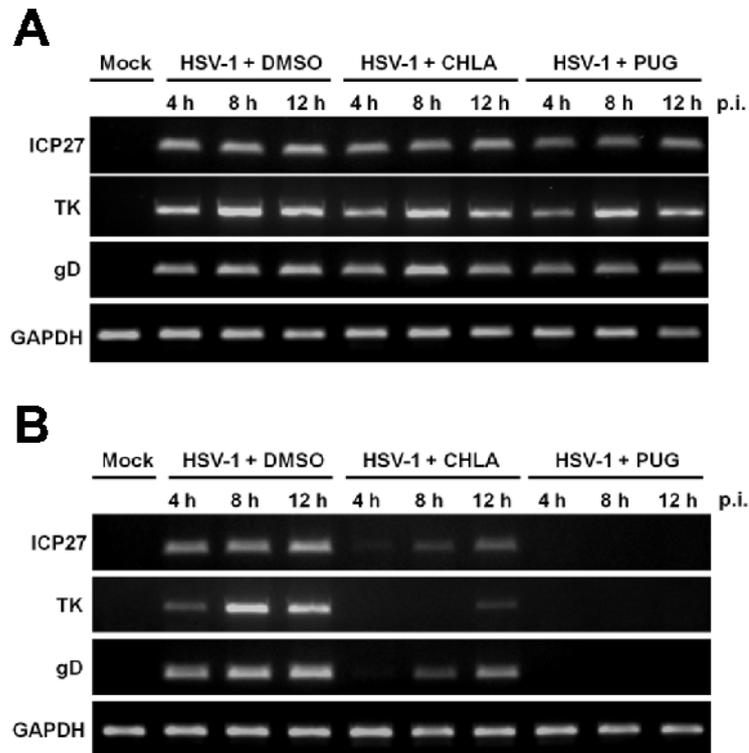
FIG. 6. CHLA and PUG do not affect HSV-1 transcription or replication following entry into the host cell. (A) A549 cells were infected with HSV-1 (MOI = 1) for 1 h, treated with low pH citrate buffer (pH 3.0) to inactivate non-internalized extracellular viral particles, and subsequently overlaid with media containing CHLA (60 μM), PUG (40 μM), or DMSO control (0.1 %). At 4, 8, and 12 h p.i., total cellular RNA was isolated, subjected to first-strand synthesis by reverse-transcription, and then amplified by standard PCR procedures with primers against HSV-1 immediate-early (ICP27), early (TK), and late (gD) gene products. GAPDH was included for control. (B) A549 cells were co-incubated with HSV-1 (MOI = 1) and CHLA (60 μM), PUG (40 μM), or DMSO control (0.1 %) for 1 h. Cells were washed with PBS before applying overlay media without the tannins. Total cellular RNA was isolated for RT-PCR analysis as in (A). Representative data shown are from one of two independent experiments.

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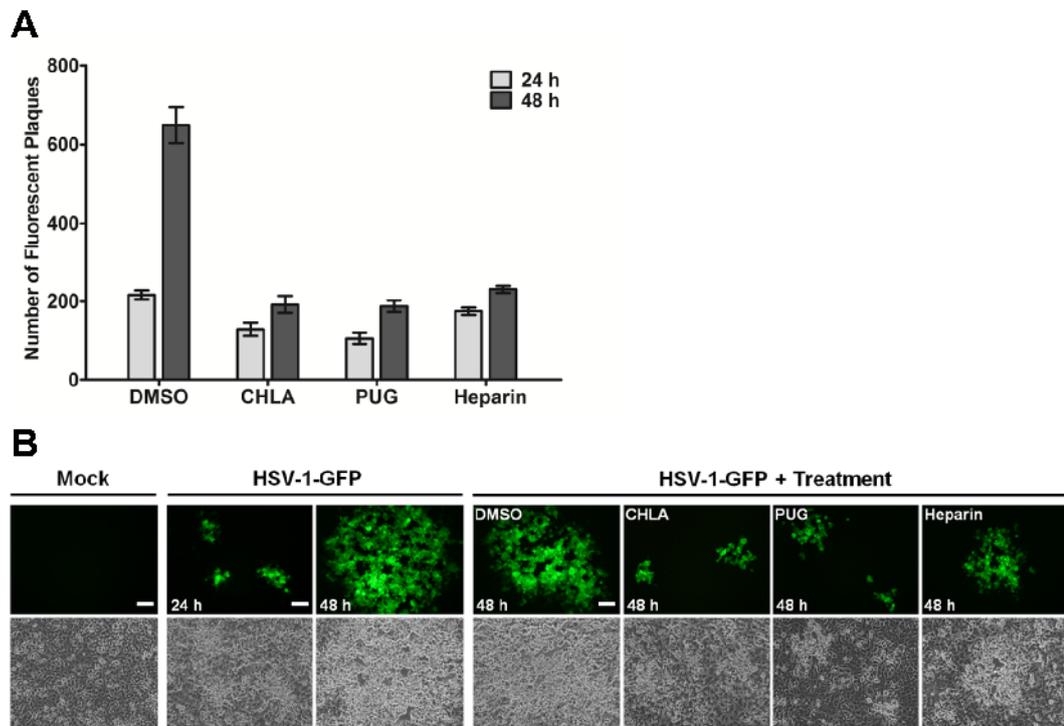
FIG. 7. CHLA and PUG can limit HSV-1 secondary infection and cell-to-cell spread of the virus. A549 cells were infected with HSV-1-GFP (200 PFU/well) for 1 h, then treated with citrate buffer (pH 3.0) to inactivate non-internalized extracellular viral particles. Cells were overlaid with (A) media or (B) media containing 0.1 % neutralizing antibody. Following an incubation period of 12 h (A) or 24 h (B) p.i., infected cells were treated with CHLA (60 μM), PUG (40 μM), heparin (100 μg/ml), or DMSO (0.1 %), before further incubating for a total of 48 h post the initial infection. Over the course of infection subsequent to the drug addition, the plates were (A) scanned and quantified for fluorescent viral plaques or (B) photographed using an inverted fluorescence microscope at 100× magnification. (A) Number of fluorescent plaques counted between 24 and 48 h p.i. with drug treatment initiated at 12 h post viral challenge. Data shown are mean ± SEM of three independent experiments with each treatment performed in duplicate. (B) Comparison of viral plaque size between 24 and 48 h p.i. with drug treatment initiated at 24 h post viral challenge; scale bars: 100 μm. Representative images are from one of two independent experiments.
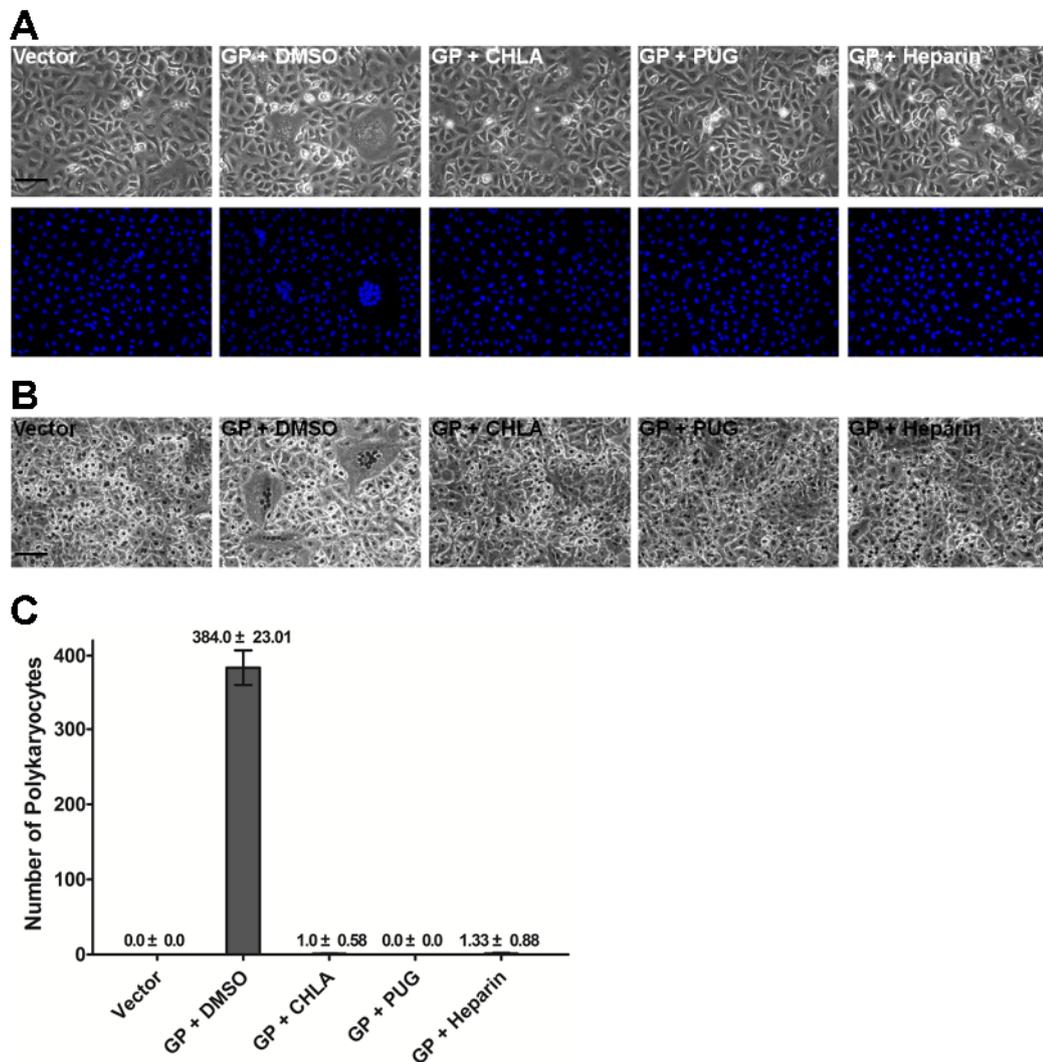
FIG. 8. CHLA and PUG can prevent HSV-1 glycoprotein-mediated cell fusion events. A549 cells were transfected with plasmids expressing the individual HSV-1 glycoproteins (gB, gD, gH, and gL). After 6 h of transfection, cells were washed with PBS and treated with CHLA (60 μM), PUG (40 μM), heparin (100 μg/ml), or DMSO (0.1 %). After further incubation for 24 h, cells were fixed with methanol and stained with Hoescht dye (A) or Giemsa (B). Photomicrographs were then taken at 200× magnification: (A) shows phase (upper panels) and the respective fluorescence pictures displaying the Hoescht dye-stained nuclei (bottom panels); (B) shows the Giemsa-stained cells of similar experiment. Representative pictures shown are from one of three independent experiments. Vector: empty vector; GP: HSV-1 glycoproteins; scale bars:

100 μm. (C) The total number of polykaryocytes (> 10 nuclei) from each treatment was quantified. Data shown are the mean ± SEM of three independent experiments.
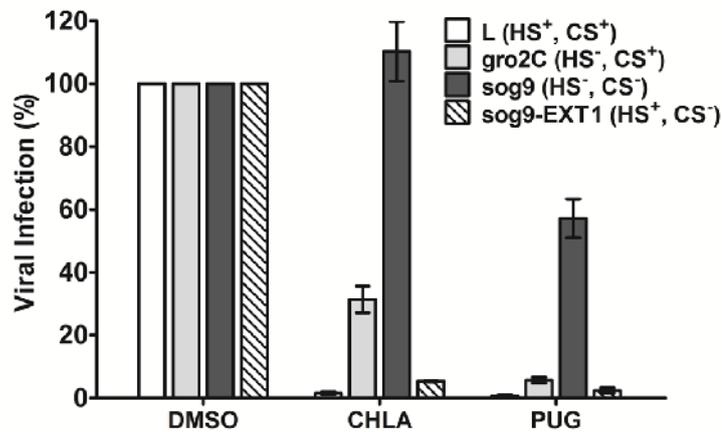
FIG. 9. Anti-HSV-1 effects mediated by CHLA and PUG are impaired in GAG-deficient cells and are rescued by restoration of heparan sulfate biosynthesis through EXT1 gene expression. Confluent cells in 12-well plates were infected with HSV-1-GFP (200 PFU/well respective to viral titer determined in each cell line) concurrently in the presence or absence of CHLA (60 μM), PUG (40 μM), or DMSO (0.1 %) for 1 h. The plates were washed with PBS before applying overlay media. After an additional 48 h of incubation, fluorescent viral plaques were scanned and quantified. Values obtained were compared against each cell line's respective DMSO control for HSV-1 infection which was considered to be 100 %. Respective status of the heparan sulfate (HS) and chondroitin sulfate (CS) GAG synthesis in the different cell lines are indicated in parentheses. Data shown are the mean ± SEM of three independent experiments with each treatment being performed in duplicate.