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ROLE OF HCV F/ARF PROTEIN AND NAD(P)H OXIDASES IN THE RIG-I /MDA5-MEDIATED INTERFERON RESPONSE IN HEPATOCYTES

A dissertation submitted in partial satisfaction of the

requirements for the degree of

Doctor of Philosophy

in

Quantitative and Systems Biology

by

Seung Bum Park

Committee in charge:

Professor Patricia LiWang

Professor David Ojcius

Professor Masashi Kitazawa

Professor Kirk Jensen

2015

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2015

Dedicated to My Parents

For Their Unconditional Support and Love

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Abstract

ROLE OF HCV F/ARF PROTEIN AND NAD(P)H OXIDASES IN THE RIG-I /MDA5-MEDIATED INTERFERON RESPONSE IN HEPATOCYTES

Seung Bum Park

Doctor of Philosophy

University of California, Merced

2015

Advisor: Prof Masashi Kitazawa

Hepatitis C virus (HCV) is a major health problem which is affecting more than 170 million people worldwide. About 80% of HCV-infected patients develop chronic infections because they cannot clear the virus. Chronic infections increase the risks of progressing to severe liver diseases such as cirrhosis and hepatocellular carcinoma. HCV has several strategies to evade host interferon (IFN) responses. In this study, we hypothesize that a novel HCV viral protein, frameshift/alternate reading frame protein (F/ARFP) from HCV -2/+1 frame, contributes to the suppression of retinoic-acid-inducible gene-I (RIG-I)-mediated IFN induction. We found that F/ARFP suppressed type I IFN induction stimulated by HCV RNA pathogen-associated molecular pattern (PAMP) and synthetic double stranded RNA poly(IC). This suppression occurred independently of other HCV viral factors such as nonstructural protein NS3/4A. Point mutations in the full-length HCV sequence for introducing premature termination codons in the -2/+1 frame enhanced type I IFN induction. Taken together, we suggest that HCV F/ARFP suppresses type I IFN responses mediated by RIG-I. Additionally, we investigated the role of NAD(P)H oxidases (Noxs) in type I IFN pathway. Nox family enzymes function as important sources of regulated production of reactive oxygen species in cell signaling and regulation of gene expression. However, much remains to be investigated how Nox enzymes contribute to the immune response against virus infection. In this study, among seven members of Nox family, we hypothesize that Nox1 has a role in type I IFN pathway. We observed that the mRNA levels of Nox enzymes including Nox1, Nox4, Nox5, Duox1, and Duox2 were elevated by poly(IC). We showed that poly(IC)-triggered IFNβ1 mRNA induction was suppressed by DPI, a flavoprotein inhibitor, in Huh7 hepatoma cells. Further study suggested that Nox1 siRNA-transfected Huh7 cells decreased poly(IC)-stimulated IFNβ1 mRNA induction in Huh7 cells. Moreover, HCV replication was increased by Nox1 and Nox4 knockdown by corresponding siRNAs. Taken together, these data suggest that Nox1 may modulate type I IFN pathway. Therefore, our study may provide new insights into how HCV evades the host innate immune response and how Nox enzymes function as antiviral agents and points to possible new drug target for therapy.

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List of Abbreviations

HCV: Hepatitis C virus;

IFN: Interferon;

F/ARFP: Frameshift/alternate reading frame protein;

RIG-I: Retinoic acid-inducible gene I;

PAMP: Pathogen-associated molecular pattern;

Nox: NAD(P)H oxidase;

FDA: Food & Drug Administration;

CDC: Centers for Disease Control and Prevention;

AIDS: Acquired Immune Deficiency Syndrome;

HIV: Human immunodeficiency virus;

SVR: Sustained virological response;

DAA: Direct-acting antiviral drug;

IRES: Internal ribosome entry site;

UTR: Untranslated region;

SL: Stem loop;

RdRp: RNA dependent RNA polymerase;

NLS: Nuclear localization signal;

SR-BI: Scavenger receptor class B type I;

HVR: Hypervariable region;

ER: Endoplasmic reticulum;

TRIF: TIR-domain-containing adapter-inducing interferon-β;

PKR: Protein kinase RNA-activated;

DC-SIGN: Dendritic cell-specific intercellular adhesion molecule-3-grabbing nonintegrin;

ICAM-3: Intercellular adhesion molecule-3;

L-SIGN: Liver/lymph node-specific ICAM-3-grabbing integrin;

LDL-R: Low-density lipoprotein receptor;

ASGP-R: Asialoglycoprotein receptor;

CLDN1: Claudin-1;

OCLN: Occludin;

eIF: Eukaryotic initiation factors;

ELISA: Enzyme-linked immunosorbent assay;

RIBA: Recombinant immunoblot assay;

NAT: Nucleic acid testing;

IDU: Injection drug use;

ALT: Alanine transaminase;

NIH: National Institutes of Health;

RF2: Release factor 2;

NK: Natural killer;

PRR: Pattern recognition receptors;

MDA5: Melanoma differentiation-associated protein 5;

cGAS: Cytosolic GAMP synthase;

IRF: IFN-regulatory factor;

IKKε: IκB kinase-ε;

TBK1: TANK-binding kinase 1;

JAK1: Janus kinase 1;

TYK2: Tyrosine kinase 2;

STAT1: Signal transducer and activator of transcription 1;

ISGF3: Interferon-stimulated gene factor 3;

ISRE: IFN-stimulated response elements;

ISG: IFN-stimulated gene;

MHC: Major histocompatibility complex;

LCMV: Lymphocytic choriomeningitis virus;

MCMV: Mouse cytomegalovirus;

RLR: RIG-I-like receptor;

CARD: Caspase recruitment domains;

IPS-1: IFN-β-promoter stimulator 1;

TLR: Toll-like receptor;

LRR: Leucine-rich repeats;

TIR: Toll/IL-1R homology;

cDC: Conventional dendritic cell;

pDC: Plasmacytoid dendritic cell;

IRAK: IL-1R-associated kinase;

TRAF6: TNFR-associated factor 6;

NF-κB: Nuclear factor-κB;

DAI: DNA-dependent activator of IRF;

STING: Stimulator of interferon genes;

NLR: NOD-like receptors;

SOCS3: Suppressor of cytokine signaling-3;

ATP: Adenosine triphosphate;

ABC1: ATP-binding cassette transporter subfamily A;

ROS: Reactive oxygen species;

Duox: Dual oxidase;

Phox: Nox of phagocytes;

SOD: Superoxide dismutase;

MPO: Myeloperoxidase;

PTP: Protein tyrosine phosphatases;

PTEN: Phosphatase and tensin homolog;

MAPK-P: Mitogen-activated protein kinase phosphatase;

NOS: Nitric oxide synthase;

FPR: Formyl peptide receptor;

fMLF: N-formyl methionyl-leucyl-phenylalanine;

TNF: Tumor necrosis factor;

EGF: Epidermal growth factor;

VEGF: Vascular endothelial growth factor;

FGF-β: Fibroblast growth factor-β;

FA: Focal adhesion;

FAK: Focal adhesion kinase;

CGD: Chronic granulomatous disease;

GSH: Glutathione;

Keap1: Kelch-like ECH-associated protein 1;

Nrf2: Nuclear factor erythroid 2-related factor 2

VSV: Vesicular stomatitis virus;

EF: Elongation factor;

HA: Hemagglutinin;

qRT-PCR: Quantitative real time reverse transcriptase-polymerase chain reaction;

GAPDH: Glyceraldehyde 3-phosphate dehydrogenase;

RIPA: Radioimmunoprecipitation assay buffer;

PI: Propidium iodide;

HDAC1: Histone deacetylase 1;

siRNA: Small interfering RNA;

NLRC5: Nucleotide-binding oligomerization domain-like receptor family CARD domain containing 5;

FH: Fetal hepatocyte;

DPI: Diphenylene iodonium;

AEC: Airway epithelial cells;

RSV: Respiratory syncitial virus;

DENV: Dengue virus;

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CHAPTER 1

INTRODUCTION

My research during my Ph.D is divided into two main projects. In the first project, we hypothesize that F/ARFP may contribute to the suppression of the type I IFN induction. The study objective is to determine the biological function of the HCV -2/+1 frame, focusing on F/ARFP, using virus-producing JFH1 cell culture system. In the second project, we hypothesize that Nox1 functions as an antiviral regulator in type I IFN pathway. The study objective is to determine the biological function of the Nox enzymes, focusing on Nox1, using virus-producing JFH1 cell culture system. Therefore, as background information, HCV history and biology will be discussed in the beginning. Then, type I interferons will be covered because the first project is about the role of viral factor, HCV F/ARFP, in the type I IFN pathway and the second project is about the role of host factor, Nox1 enzyme, in the type I IFN pathway. Next, F/ARFP and Nox enzymes will be discussed in turn.

1.1 HCV History

In the 1970s, it was noticed that there is an unidentified agent causing transfusion-associated non-A, non-B hepatitis. [1] However, the cause of the non-A, non-B hepatitis was not defined until 1989 when Choo et al. isolated a cDNA clone derived from a blood-borne non-A, non-B viral hepatitis genome. [2] This group constructed random-primed complementary DNA library from non-A, non-B agent containing plasma. They showed that the complementary DNA clone was not derived from host chromosomal DNA, but from an RNA molecule in non-A, non-B hepatitis patient's blood samples.

After HCV was discovered, the Food & Drug Administration (FDA) approved the first HCV treatment in 1991. [3] Although interferon alpha (IFN-α) was already used for treatment of chronic non-A, non-B hepatitis in 1986 [1], the first-ever treatment for HCV was approved in 1991 and this was not an effective method to clear the virus from patients.

The two major avenues for HCV infection world-wide are associated with contaminated blood products through blood transfusions and drug injections. The United States began screening the blood supply from 1990 to prevent HCV from spreading further and believes that the HCV was removed from all blood banks by 1992.

According to Centers for Disease Control and Prevention (CDC), HCV infections have continuously declined since the HCV was discovered in 1989. In less than 10 years after the discovery, the annual number of new HCV infections had decreased by more than 80 percent. [4] The CDC pointed out that the reason for the dramatic decline in less than 10 years was due to new infection control practices and Acquired Immune Deficiency Syndrome (AIDS) education. Although HCV infection rates continuously declined, deaths due to HCV outnumbered deaths due to human immunodeficiency virus (HIV) in 2007 in the United States. [5]

Until 2011, the standard of care was a combination of pegylated interferon alpha and ribavirin. The combination of pegylated interferon alpha and ribavirin achieved a sustained virological response (SVR) of about 50%. However, SVR rates are dependent on different genotypes thus having differing values. For this reason, there has been a high demand for improving SVR rates. [6] From 2011, in addition to pegylated interferon alpha and ribavirin, two NS3-4A protease inhibitors (PIs) were added to the standard of care (**Figure 1-1**). Telaprevir and boceprevir are first-generation NS3-4A protease inhibitors that are effective against genotype 1. This increased SVR up to 60-70%. [6] Recently, new direct-acting antiviral drugs (DAAs) targeting not only HCV nonstructural protein, NS3, but also nonstructural proteins such as NS5A and NS5B were introduced as a new HCV treatment and this shows promising results. Daclatasvir, ledipasvir, and ombitasvir are examples of NS5A inhibitors. Sofosbuvir and dasabuvir are examples of NS5B inhibitors. [6]

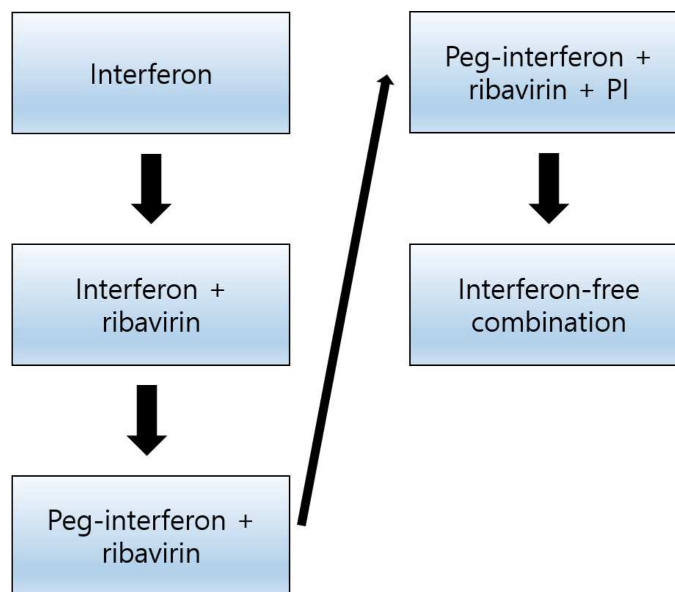


Figure 1-1. Changes in standard of care for HCV.

1.2 HCV Biology

1.2.1 HCV Genotypes

In the early 1990s, it was noticed that there was genetic heterogeneity of HCV and a number of different methods were employed to classify different variants of HCV. [7], [8] To study the epidemiology, evolution, and pathogenesis of HCV, it is important to classify the HCV genotypes. For instance, different genotypes react differently to interferon alpha treatments. Therefore, knowing genotype-specific differences is the beginning to understand and deal with HCV.

Currently, HCV is divided into six major genotypes. There are four determination factors to classify genotypes of HCV. 1. To identify three coding regions, core, E1, and NS5B of HCV 2. To align sequences with representative sequences from each genotype 3. Highest percentage of similarity gives tentative clade assignment 4. To analyze using DNADIST of Phylip (Kimura 2-parameter), bootstrap to confirm. [9]

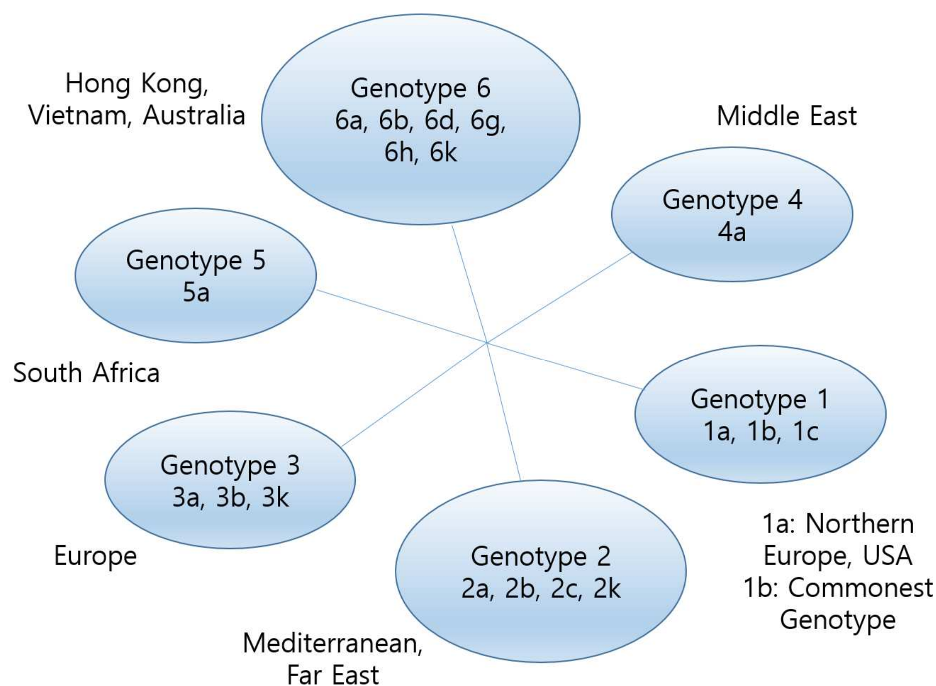


Figure 1-2. HCV genotypes and distribution.

Genotypes differ from each other by about 30% at the nucleotide level. Although there is some extent of heterogeneity of HCV, there are also conserved regions of the HCV genome within genotypes. The 5' untranslated region and the terminal 99 bases of the 3' untranslated region are the most conserved regions. However, the most variable region of the HCV genome is the hypervariable region of E2. [10] The VIIIth Report of the International Committee for the Taxonomy of Viruses (ICTV) classifies HCV and GB virus B as members of the *Hepacivirus* genus in the virus family, *Flaviviridae*. [11] ICTV divides 6 different groups of HCV and designates them as clades.

As for the geographical distribution of HCV genotype, genotype 1a is widely distributed in Northern Europe and USA (**Figure 1-2**). Genotype 1a distribution is believed to be associated with injection drug use. On the other hand genotype 1b is the most common genotype worldwide. It is found from older age people. Genotype 2a, 2b, 2c is found predominantly in older HCV infected individuals from Mediterranean countries and Far East. Like genotype 1a, genotype 3a is also widely distributed in injection drug users from Europe. Genotype 4a is found in Middle East and the distribution of this genotype is believed to be strongly associated with past medical treatment against the parasitic disease. Genotype 5a is found commonly only in South Africa. Finally, genotype 6a is distributed in injection drug users in Hong Kong, Vietnam, and more recently in Australia. [11]

1.2.2 HCV Genome Organization

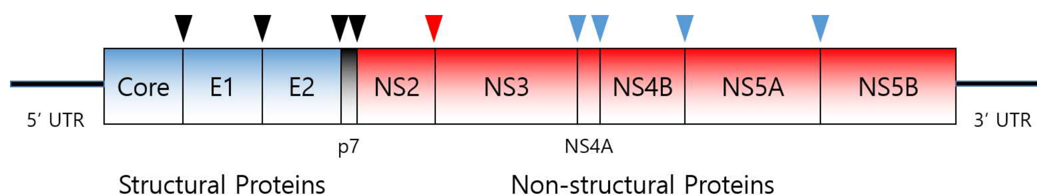


Figure 1-3. Hepatitis C virus genome organization. The 5’ UTR contains an internal ribosome entry site (IRES). The structural proteins are core, E1, and E2. The non-structural proteins are NS2, NS3, NS4A, NS4B, NS5A, and NS5B. HCV open reading frame encodes the single long polyprotein which is cleaved by cellular signal peptidases (indicated by black triangle) and by viral proteases (indicated by red and blue triangles). Core needs to be cleaved by cellular signal peptide peptidase for maturation. The junction between NS2 and NS3 is cleaved by NS2 autoprotease, while rest of the other junctions are cleaved by NS3 and NS4A. The core-coding region also produce alternate reading frame proteins by ribosomal +1 frameshifting. UTR: untranslated region.

HCV belongs to the flaviviridae family which is divided into three genera: flavivirus, pestivirus, and hepacivirus. Yellow fever virus is the type virus of flavivirus genus. On the other hand, bovine virus diarrhea and hepatitis C virus are the type viruses of pestivirus genus and hepacivirus genus, respectively. [12]

Flaviviridae family share basic structural features. They all have envelopes which are composed of a lipid bilayer. Inside the lipid bilayer envelope is the nucleocapsid which is composed of capsid protein. The flaviviridae genome is a positive stranded RNA found inside the nucleocapsid. The genome size of the flaviviridae family viruses ranges from 9.6kb to 12.3kb. [13]

HCV is a 9.6kb positive-stranded RNA virus which encodes about 3,000 amino acids polyprotein precursor. HCV RNA genome is flanked by untranslated regions (UTR), 5' UTR and 3' UTR which are important for polyprotein translation and RNA replication (**Figure 1-3**). [14] The 5' UTR of HCV is 341 nt long and is the most conserved region of the genome. [15] The 5' UTR contains six secondary structure domains which are termed stem loops (SLs). Among these, SLII, SLIII, and SLIV make up an internal ribosome entry site (IRES) which is important for the translation of HCV RNA. [16] In addition, a liver-specific microRNA, miRNA-122, is known to bind to SLI so that the interaction regulates HCV replication and translation. [17]–[20]

The 3' UTR is approximately 225 nt long and is composed of three different regions; a variable region of approximately 30-40 nt, a long poly(U/UC) tract, and a highly conserved 98 nt long 3' X tail. [21] The 3' UTR is also known to be essential for HCV replication. [22]–[25] Cheng et al. showed that the 3' UTR interacts with the NS5B RNA dependent RNA polymerase (RdRp) and this result suggests the potential role for RNA replication. [26] Specifically, the 3' X tail region and the 52 upstream nucleotide of the poly(U/UC) tract were considered to be important for RNA replication and the remaining sequence of the 5’ UTR seems to be involved in viral replication enhancement. [22], [24], [25], [27]

HCV RNA encodes 3,000 amino acids long polyprotein precursor which is encoded by the single open reading frame. During and after translation, the long polyprotein precursor is processed by cellular and viral proteases to form three structural proteins (core, E1, and E2), p7, and six nonstructural proteins (NS2, NS3, NS4A, NS4B, NS5A, and NS5B). The cleavage sites for host signal peptidases are located in between core-E1, E1-E2, E2-p7, and p7-NS2. The viral protease, NS2/NS3, cleaves in between NS2-NS3 and another viral protease, NS3/NS4A, cleaves in between NS3-NS4A, NS4A-NS4B, NS4B-NS5A, and NS5A-NS5B. For core maturation, the host signal peptide peptidases cleave the C terminus of core sequence to make 21kDa protein. [28]

Core is 191 amino acids long and is 23 kDa. Although core size varies from 17 kDa to 23 kDa, the form considered the predominant one is 21 kDa. [29] Core is a nucleocapsid protein and is important for the production of infectious viral particle. [30], [31] Core is involved in RNA binding, oligomerization. Core protein contains three distinct domains, an N terminal hydrophilic domain (120 amino acids), a C terminal hydrophobic domain (50 amino acids), and a signal peptide for the downstream envelope protein E1. [32]–[34] The N terminal hydrophilic domain contains many positive charges so that it can bind to negatively charged HCV RNA. It has been shown to have the nuclear localization signal (NLS) which allows core proteins to translocate into the nucleus. [35]–[37] The core proteins localized in the nucleus interact with nuclear molecules and may regulate the expression of cellular genes. It was shown that HCV core protein interacts with p300/CBP and enhances NF-AT1 transcriptional activity. [38] A C terminal hydrophobic domain associates with endoplasmic reticulum membranes, outer mitochondria membranes, and lipid droplets. [35], [39] Core protein has been suggested to interact with many cellular proteins that are important for the viral lifecycle. [40] It promotes hepatocyte growth in Huh7 hepatoma cells by transcriptional upregulation of growth-related genes. [41] It may also be involved in tissue injury and fibrosis progression. [42] The ARFP is encoded and produced by ribosome shifting within the core sequence. [43]

E1 and E2 are 33-35 kDa and 70-72 kDa glycoproteins, respectively. [44] E1 and E2 are on the HCV viral envelope and function as ligands for host cell receptors such as CD81, scavenger receptor class B type I (SR-BI) [45], [46] They both are heavily glycosylated with up to 5 and 11 glycosylation sites, respectively, and form a heterodimer. In addition, E2 contains hypervariable regions which differ up to 80% between HCV genotypes and subtypes in a same genotype. [47] Hypervariable region 1 (HVR1) is considered as a major neutralizing epitope. [48], [49] Although the HVR1 sequence is varied from different genotypes, the overall properties and conformation of the HVR1 are highly conserved. This suggests that HVR1 is a critical component for HCV lifecycle. [50] E2 has an important role in the early stages of virus infection since E2 is implicated in the interaction with one or more host receptors. [51], [52] Compared to E2, much more research is required for E1 to understand its biological functions. E1 is believed to play an important role in intracytoplasmic virus membrane fusion. [51], [52]

p7 is a 63 aa long integral membrane protein. [53] The p7 protein is a hydrophobic calcium ion channel protein which has two transmembrane domains spanning the endoplasmic reticulum (ER) membrane. [53] It is known that p7 protein is not required for HCV RNA replication, but it is important for producing infectious viruses which demonstrates a possible role of p7 in viral assembly and release. [54]–[56]

NS2 is 21-23kDa protein which is anchored in the ER membrane. [57] NS2 contains two internal signal sequences required for ER membrane association. [57], [58]. The C terminal of NS2 and the N terminal of NS3 contains protease domain that comes together to form a catalytically active

protease that cleaves in between NS2-NS3. [59], [60] NS2 has a short half-life and loses its protease activity soon after it is cleaved from NS3. NS2 is degraded by the proteasome in a phosphorylation-dependent manner. [61] NS2 can also interact with host cellular proteins such as the liver-specific pro-apoptotic CIDE-B protein, but little is known about its biological function with or without host cellular proteins. [62] Like p7, NS2 is not required for HCV RNA replication, but is important for producing infectious viral particles. [56], [63]

NS3 is a 69kDa protein and functions as a serine protease. Together with NS4A, NS3 cleaves in between NS3-NS4A, NS4A-NS4B, NS4B-NS5A, and NS5A-NS5B. A serine protease domain of NS3 is approximately a third N terminal and a helicase/NTPase domain is about two-thirds C terminal. [64]–[66] NS4A is a cofactor of NS3 protease activity. The central region of NS4A functions as a cofactor and allows its stabilization, localization at the ER membrane, etc. [67]–[69] In addition to the protease activity for HCV polyprotein itself, NS3/NS4A cleaves out the critical mediator of retinoic acid-inducible gene I/melanoma differentiation-associated protein 5 pathway, MAVS, from the mitochondria. It also suppresses the toll-like receptor 3 pathway by degrading of another important mediator, TIR-domain-containing adapter-inducing interferon-β (TRIF). [70]–[72] Therefore, NS3/NS4A protease functions not only for HCV polyprotein itself, but also for the critical host cellular mediators in innate immune pathway. The 442 C terminal amino acid region of NS3 protein functions as a helicase/NTPase. The NS3 helicase/NTPase has several activities such as RNA-stimulated NTPase activity, RNA binding activity, unwinding of RNA secondary structure. [73], [74] As a result, unlike p7 and NS2, NS3 is required for HCV RNA replication. [75], [76] In addition, it is involved in viral assembly. [77]

NS4B is an integral membrane protein which is 261 amino acids long and is localized in an ER or ER-derived membrane. [78], [79] NS4B contains at least four transmembrane domains and an N-terminal amphipathic helix which is involved in membrane association [78]–[80] NS4B serves as a membrane anchor for the replication complex and is responsible for the production of the membranous web which is considered to be the HCV RNA replication site. [80]–[82] It also appears to suppress cellular syntheses and modulate HCV NS5B RdRp activity. [83]–[85]

NS5A is a 56-58 kDa phosphorylated zinc-metalloprotein that has a critical role in virus replication and regulation of various cellular pathways. The first 30 amino acids of NS5A N-terminal region contains an amphipathic α-helix that is involved in membrane localization as well as in assembly of the HCV replication complex. [86]–[88] The fact that mutations in the NS5A sequence suppresses HCV replicon RNA replication suggests the critical role of NS5A in HCV RNA replication. [87], [89] Evans et al. showed that NS5A hyperphosphorylation disrupts the interaction with hVAP-33 and suppresses HCV RNA replication in the replicon model system. [90] Another study suggests that the NS5A phosphorylation status has an crucial role for the HCV life cycle through regulating replication complex from replication to assembly,

whereby hyperphosphorylated NS5A maintains the replication complex in an assembly-incompetent state. [91] In addition, NS5A can bind to NS5B, but the underlying mechanism how NS5A regulates NS5B RdRp activity remains to be elucidated. [92] Like NS3/NS4A protease, NS5A appears to suppress interferon pathway through binding to and inhibiting protein kinase RNA-activated (PKR) which is a critical antiviral molecule in the type I IFN pathway. [93]

NS5B is a 68kDa protein and is a RNA-dependent RNA polymerase. The C-terminal region of NS5B has an α-helical transmembrane domain. This domain is important for post-translational targeting to the cytosolic side of the ER, where the functional part of the protein is exposed. [94], [95] Like NS5A, NS5B also interacts with a number of cellular proteins. The C-terminal region of NS5B can bind to the N-terminal region of hVAP-33, and this interaction results in the formation of the HCV replication complex. [95], [96] In addition, NS5B appears to bind cyclophilin B, a cellular peptidyl-prolyl cis-trans isomerase that is critical for the efficient HCV replication. NS5B interacted with cyclophilin B shows efficient replication activity through enhanced its RNA binding activity. [97]

1.2.3 HCV Replication Cycle

HCV virion contains a single-stranded RNA genome inside the nucleocapsid which is composed of core protein. HCV virion size varies from 50 to 80 nm in diameter. The nucleocapsid is surrounded by the lipid bilayer which is embedded with E1 and E2 glycoprotein. [98], [99] The schematic flow of HCV replication cycle is shown in **Figure 1-4**.

There are several cell surface molecules that have been suggested to mediate HCV attachment and entry. CD81 is the most extensively studied cell surface molecule that is a 25 kDa and belongs to the tetraspanin or transmembrane 4 superfamily. [46] HCV E2 glycoprotein is believed to interact with CD81. Flint et al. showed that aa 480-493 and 544-551 in the truncated soluble form of E2 is involved in the binding to CD81. [100] In addition, another study suggested that aa 613-618 and the two HVRs (aa 384-410 and 476-480) are responsible for the binding. [101]

The scavenger receptor B type I has been proposed as another receptor for HCV attachment and entry. [45] SR-BI is a 509 aa glycoprotein and is expressed at high levels in hepatocytes and steroidogenic cells. [102], [103] A recombinant E2 glycoprotein of HCV genotypes 1a and 1b were shown to interact with HepG2 human hepatoma cells that do not express CD81. [45] However, antibody neutralization against SR-BI did not result in complete suppression of binding. This suggested that SR-BI is not the only cell surface molecule involved in HCV attachment. [104]

The dendritic cell-specific intercellular adhesion molecule-3-grabbing nonintegrin (DC-SIGN) and the liver/lymph node-specific intercellular adhesion molecule-3 (ICAM-3)-grabbing integrin (L-SIGN) are another candidates for HCV entry. [105]–[108] Ludwig et al. showed that both DC-SIGN and L-SIGN capture HCV pseudo-particles through E2 binding and induce a rapid internalization of virus-like particles. [109] However, this was not observed from another study. [107]

The low-density lipoprotein receptor (LDL-R) delivers lipoproteins into cells via receptor-mediated endocytosis. [110] It has been reported that virus-like particles bound with LDLs entered into cells via the LDL receptor. [111], [112] This suggested the potential role of LDL-R for HCV attachment and entry.

Besides the previous receptors, the asialoglycoprotein receptor (ASGP-R) has been also proposed to mediate binding and internalization of structural HCV proteins expressed in a baculovirus system. [113] A ubiquitously expressed cell surface molecule, glycosaminoglycan, claudin-1 (CLDN1), and occludin (OCLN) are also known to function as receptors for HCV entry. [114]–[116]

Once HCV virion is bound to its corresponding receptors, clathrin-mediated endocytosis occurs. [117] For fusion, a low pH is required in endosomes. [118], [119] HCV envelope glycoprotein E1 may function as a fusion triggering peptide because sequence analysis predicts the existence of a fusion peptide in its ectodomain. [51], [52] Although E1 is a good candidate for a fusion peptide, E2 is suggested as another candidate for a fusion peptide because it also shares structural homology with class II fusion proteins. [120], [121]

HCV RNA is translated from an IRES located in 5' UTR after HCV RNA is liberated into the cytoplasm. [122] IRES spans domains II to IV of the 5’ UTR and the first nucleotides of the core-coding sequence. The IRES mediates cap-independent internal translation of HCV polyprotein by cellular proteins such as eukaryotic initiation factors (eIF) 2 and 3 and viral proteins. [123]–[125] The IRES forms a stable pre-initiation complex with 40S ribosomal subunit without several translation initiation factors that are necessary for canonical translation. [126], [127] HCV proteins appear to downregulate IRES translational efficiency. Core, NS4A, and NS4B are the examples. [84], [128] Additionally, the HCV 3' UTR may also affect IRES-dependent translation, but this is controversial. For example, one study showed that the IRES-mediated translation efficiency could be enhanced by the HCV 3' UTR, while another study suggested that the IRES-mediated translation efficiency was unaffected by the HCV 3' UTR. [129], [130]

HCV RNA encodes a 3,000 amino acids long precursor polyprotein that is targeted to the ER membrane. The internal signal peptide located between the core and E1 sequences guides the precursor polyprotein to the ER membrane. A host signal peptidase first cleaves the signal peptide and generates the immature form of the core protein. [131] The signal peptide is

then further processed by a host signal peptide peptidase that is located in the ER membrane to yield the mature form of the core protein. [89] The host signal peptidase also cleaves the junctions in between E1-E2, E2-p7, and p7-NS2 in the ER lumen. The NS2/NS3 autoprotease cleaves out NS3 from NS2. NS3 and NS4A form a serine protease to catalyze cis-cleavage at the junction of NS3-NS4A and trans-cleavage at all the following downstream junctions. [132] The cleavage at the junction of NS2-NS3 cleavage is required for producing infectious viral particle. [133], [134]

It is known that a positive-stranded RNA virus rearranges intracellular membranes to form a replication complex. The HCV NS4B protein appears to induce the generation of a membranous web which is believed to be the HCV replication complex. [81], [82] The membranous web is derived from ER membranes. Shi et al. showed that HCV replication occurs in detergent-resistant membranes such as lipid raft domains. [135] Supporting this, lipid rafts are suggested to be implicated with the HCV replication complex. [96], [135], [136] The membranous web contains a number of small vesicles embedded in a membranous matrix, forming a multiprotein complex that has all of the nonstructural HCV proteins inside. [81] HCV replication is catalyzed by the NS5B RdRp. The positive strand RNA serves as a template to generate a negative strand RNA. The negative strand RNA then serves as a template to produce a number of the positive strand RNAs that are used for translation and progeny virus particle assembly. It is known that domain I of the 5’ UTR, the 3’ UTR, and the 3' end of the HCV NS5B-coding region are involved in the HCV RNA synthesis. [137] During HCV RNA replication, the NS3 protein is believed to function as a helicase to separate nascent and template RNA strands and unwind of RNA secondary structures. In addition, it is also capable of nucleic acid binding and 3' to 5' translocation coupled to hydrolysis of ATP. [138] The NS5A phosphoprotein consists of three domains. [139] Domain I and II are important for RNA replication and domain III is involved in viral assembly. [140], [141] Besides viral factors, liver-specific host microRNA miR-122 is shown to be implicated in efficient HCV RNA replication. [17], [142] Also, miR-122-HCV RNA complex is suggested to protect the 5' UTR from recognition by pattern recognition receptors such as RIG-I. [143]

HCV particle packaging seems to be induced by interaction of the core protein with HCV RNA. Supporting this, core is shown to bind positive-stranded RNA via stem-loop domains I and III and nt 23-41. [144], [145] Therefore, it may be possible that the core-RNA interaction plays an important role to turn on the HCV life cycle switch from replication to packaging, and vice versa. The N-terminal region of the core protein is responsible for capsid assembly, particularly the two clusters of basic residues. [146]–[150] Kunkel et al. showed that the core proteins efficiently self-assembled in the presence of a nucleic acid in bacterial systems. [149] In addition, the fact that the HCV envelope glycoproteins E1 and E2 associate with ER membranes suggests that virus assembly happens in the ER. [151] In the later steps, both ER and Golgi apparatus are involved in the HCV assembly because HCV structural proteins were found both in the ER and the Golgi apparatus. [152]

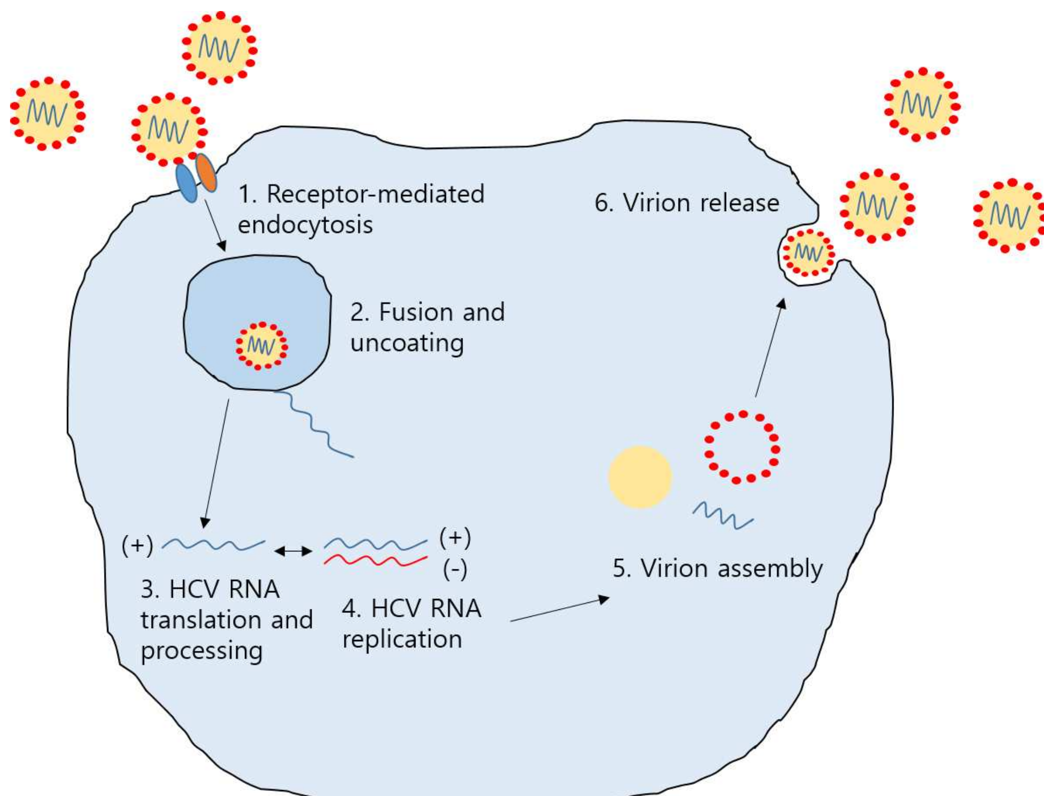


Figure 1-4. HCV replication cycle. HCV particle first interact with host receptors at the cell surface and enters into the cell by the receptor-mediated endocytosis. The HCV RNA is then released into the cytoplasm after HCV glycoprotein-mediated membrane fusion. The HCV RNA is translated to produce a single long polyprotein that is processed into 11 mature viral proteins. The HCV RNA is replicated and newly synthesized RNA is packaged into nucleocapsids. The mature virion is released from the cell.

1.2.4 HCV Transmission

Even after the discoveries of the hepatitis A virus and hepatitis B virus, not all of the strains of viral hepatitis were explained by these two viruses. Originally the third form of viral hepatitis was termed as non-A, non-B hepatitis. Later, it turned out that the major cause of non-A, non-B hepatitis resulted from HCV. After HCV was identified, methods for screening HCV in the blood supplies were developed. Initially, enzyme-linked immunosorbent assay (ELISA) was used until the introduction of recombinant immunoblot assay (RIBA) to screen for HCV in blood supplies. [153] These newly developed methods of screening resulted in the decrease of infection risk in 2002 from 7.7% to a 1 in 276,000 in blood transfusion. [154]

During an acute phase of infection, HCV RNA is present up to 6 weeks before anti-HCV antibodies are generated. Therefore, the screening tests using antibodies during this period show false negatives. Due to this, nucleic acid testing (NAT) was developed and used in developed countries to screen HCV in the early 2000s. [154] Newer screening methods with better approaches have dramatically lowered transmission of HCV after blood transfusions.

Transmission of HCV in low-income countries is still high in contrast to the rate of transmission in developed countries. [155] HCV transmission can be caused not only by whole blood transfusion, but also by blood products such as anti-D immune globulin. For example, administration of contaminated batches of anti-D immune globulin to Irish women caused hepatitis infection outbreaks with known dates of transmission, which enabled long-term evaluation. [156]

In resource-limited countries, iatrogenic HCV transmission is one of the main routes of HCV transmission. For example, Egypt has the highest HCV prevalence worldwide at 14.7% among people aged from 15 to 59. [157] It is widely believed that HCV transmission during the 1960s and 1970s were due to reusable syringes and multiple dose vials used to treat schistosomiasis, a parasitic disease. [158] Even now in present day, the main important route of HCV transmission is due to reusable syringes in countries where resources for disposable materials are limited. [159]–[161]

Worldwide, approximately 16 million people practice injection drug, a practice that is universally considered an important route of HCV transmission. [162] Conversely, iatrogenic and blood transfusion HCV transmission rate has declined as the rate of HCV transmission via injection drug use has become the main source of HCV transmission in developed countries.

In European Union countries, the prevalence of HCV ranged from 30 to 95% among injection drug use (IDU) males and 48 to 94% among IDU females. In addition, for gender unspecified group, the prevalence ranged from 33 to 98%. [163]

HCV transmission among heterosexual couples is rare, but it is now increasingly recognized among homosexual couples who have sex with men. [164]–[168] Terrault et al. studied 500 HCV-positive, HIV-negative individuals and suggested that the risk of heterosexual HCV transmission is about 1 per 190,000 sexual contacts. [169] However, the risk of HCV transmission in homosexual male couples were at a higher percentage.

The risk of HCV transmission from infected mothers to their child has been estimated to approximately 4-7%. However, mothers who are co-infected with HIV have a two to four fold higher risk when compared to HCV infection only. [169]–[171]

1.2.5 HCV Treatment

In the early period of HCV treatment history, IFNα alone was used to treat HCV infected patients. However, IFN treatment had some disadvantages such as side effects and the relatively low rate of SVR. The SVR rate to 24 weeks of IFNα only treatment was 6% and 48 weeks of IFNα only treatment slightly increased the SVR rate to 13-19%. [172], [173] The SVR rate showed some level of variation between HCV genotypes. HCV genotype 1 was harder to treat because the SVR rate for HCV genotype 1 was 2% with 24 weeks treatment and 7-11% with 48 weeks treatment. Therefore, people tested to increase the dose of IFNα and extend the treatment period to have a better SVR rate. However, trials for improving the SVR rate were not promising and encouraging, which resulted in the increasing demand for another antiviral agent. The nucleoside analogue ribavirin was started to use because of low effectiveness of IFNα only treatment.

In the early 1990s, some Swedish scientists tested ribavirin for the patients with chronic hepatitis C and observed that serum alanine transaminase (ALT) levels were significantly decreased for the patients with ribavirin only treatment. However, within 6 weeks of stopping treatment, ALT levels returned to baseline levels. [174] There was another study from National Institutes of Health (NIH) testing the effect of ribavirin treatment on HCV patients. [1] The patients with chronic hepatitis C were administered with ribavirin for 6 months and showed that serum ALT levels were decreased like in the Swedish study. But the ALT levels gradually returned to baseline levels with the exception of one patient. It was shown later that ribavirin has a modest temporary antiviral effect on HCV when it is treated alone. [175]

The result of ribavirin monotherapy led Swedish and Italian scientists to test combination therapy of both IFNα and ribavirin for patients with chronic hepatitis C. [1] The combination therapy was definitely effective because both patients who did not respond to IFNα monotherapy and who relapsed after cleared HCV RNA and achieved SVR after 24 weeks of the combination treatment. [1] As a result, the combination of IFNα and ribavirin was approved as the standard-of-care treatment for chronic hepatitis C in 1999.

Soon after the combination therapy was administered, the need for long-lasting IFNα rose because IFNα could not maintain as an active molecule in a body for long time. [175] Therefore, a polyethylene glycol moiety was attached to IFNα molecule and generated two kinds of pegylated-IFNs (IFNα2a and IFNα2b). These showed approximately 75 and 30 hours half-lives, respectively. The combination of pegylated-IFNα and ribavirin then became the new standard-of-care treatment for chronic hepatitis C.

Development of cell culture systems for productive infection of HCV has significantly advanced the field of molecular virology of HCV. The resolution analysis of the three-dimensional structure of HCV enzymes also allowed to identify the multiple steps of the HCV lifecycle as well as the multiple targets for drug discovery. As a result, two first-generation HCV NS3/4A protease inhibitors, telaprevir and boceprevir, were approved by FDA in 2011. These drugs were approved to use with pegylated-IFNα and ribavirin for the treatment of chronic HCV genotype 1. [176], [177]

Both telaprevir and boceprevir are oral drugs and act on HCV NS3/4A protease. [1] They both covalently binds to NS3/4A, but boceprevir forms a covalent but reversible complex with NS3/4A. Subsequently, both drugs potently suppress HCV replication, but chances to have resistance-associated variants rise when either drugs is used alone. Therefore, both agents are used with pegylated-IFNα and ribavirin, but regimens for telaprevir and boceprevir are different. Telaprevir is administered to patients for the first 12 weeks with pegylated-IFNα and ribavirin, followed by 12 to 36 weeks of pegylated-IFNα and ribavirin without telaprevir. However, for the case of boceprevir, patients are treated with pegylated-IFNα and ribavirin alone for the first 4 weeks, followed by up to 44 weeks of boceprevir, pegylated-IFNα, and ribavirin. Both agents, on the other hand, have some side effects such as anemia. It may be due to the combined effects of pegylated-IFNα, ribavirin and telaprevir (or boceprevir) on erythropoiesis. However, further studies should be performed to better define the effects of the NS3/4A protease inhibitors. [178] Soon after, second-generation NS3/4A protease inhibitors were developed to use for all genotypes. These second-generation NS3/4A protease inhibitors are not only effective against all genotypes, but also have less chances to have resistance-associated variants compared to first-generation agents. Simeprevir and paritaprevir, the second-generation NS3/4A protease inhibitors, were approved by the FDA in 2014. [6]

Another type of drugs such as nucleotide analogues, non-nucleoside inhibitors, and NS5A inhibitors emerged as alternatives for the NS3/4A protease inhibitors. [1] Nucleotide analogues are incorporated into the newly synthesizing HCV viral RNA and terminate the elongation acting on the HCV RdRp. Nucleotide analogues are effective against all genotypes and show less chances of rising resistance-associated variants. An example of nucleotide analogues is sofosbuvir which was approved by the FDA in 2014. [6] Non-nucleoside inhibitors for HCV RdRp bind to the enzyme and thus blocking its catalytic function. Currently, non-nucleoside HCV RdRp inhibitors are limited to use against genotype 1 and have a low barrier to resistance. Dasabuvir is an example of non-nucleoside HCV RdRp inhibitors. [6] Finally, NS5A inhibitors bind to NS5A protein and block its function to regulate HCV replication. [179] NS5A inhibitors not only suppress HCV replication, but also inhibit assembly and release of viral particles. [180], [181] Daclatasvir, ledipasvir, and ombitasvir are examples of NS5A inhibitors. [6]

1.2.6 Translational Frameshifting

Translational frameshifting is an alternative translation process that occurs rarely. In a conventional translation process, a protein is translated from the 5' end of an mRNA to the 3' end of the mRNA continuously. As a result, the protein is translated from the mRNA template with continuous blocks of 3 nucleotides that are read as one amino acid each. However, in some cases, ribosome changes the frame either 1 or 2 nucleotides in the leftward or rightward direction. This event can be programmed by the specific mRNA nucleotide sequences. The secondary or tertiary structures of mRNA may also affect to the translational shifting.

Compared to frameshift resulting from mutation, frameshift resulting from ribosomal frameshifting is controlled by a number of mechanisms. The most prevalent ribosomal frameshifting is the simultaneous slippage mechanism of -1 frameshifting. Sequence comparisons analysis revealed that there is a common structural motif for this mechanism. This is a heptameric sequence with X-XXY-YYZ (X, Y, Z can be any nucleotide), shown as codons in the upstream zero sequence. The simultaneous slippage frameshifting does not happen efficiently at all at the heptameric sites because the ribosome does not stall long enough for frameshifting to occur. In this frameshifting, a downstream secondary structure, a pseudoknot, induces frameshifting by forcing the ribosome pause at the heptameric sites. [182]–[184] It is shown that pseudoknots appear 6 nt downstream of the heptamer. [184] Although some pseudoknots can pause the ribosome, they do not stimulate frameshifting. [182]

Frameshifting reading +1 direction occur less common compared to -1 frameshifting. This +1 frameshifting by tRNA slippage, however, is found in many different species such as bacteria, yeast, and mammalian cells. [185]–[188] The prfB gene is the typical model of +1 frameshifting. This

frameshifting happens when a peptidyl-tRNA is bound to the ribosomal P site and the A site is empty, and the ribosome needs to pause at the frameshifting site. In prfB gene translation, if peptide release factor 2 (RF2), the protein product of prfB, is limiting, a UGA termination codon is not recognized at a high rate so that +1 frameshifting occurs. On the other hand, if RF2 is abundant, the UGA termination codon is recognized at a high rate so that translation terminates at the site and the +1 frameshifting does not happen. This is how RF2 level is autogenously controlled by frameshifting. [187], [189], [190]

There is another +1 ribosomal frameshifting. In this +1 frameshifting independent of tRNA slippage, frameshifting occurs during AGU codon decoding. The frameshifting site is the seven nucleotide sequence GCG-AGU-U. [186] In this frameshifting, instead of reading AGU codon, GUU codon is read after +1 frameshifting occurs. tRNA slippage is not involved in this mechanism since the tRNA to decode GCG could not base pair with the CGA codon. [186] This suggests that this frameshifting mechanism must not be related to peptidyl-tRNA slippage. [186] On the other hand, the frameshift mechanism for the production of the mammalian ornithine decarboxylase antizyme seems to be similar to this +1 ribosomal frameshifting. [185]

Another unusual frameshifting mechanism is translational hopping. This occurs during the bacteriophage T4 topoisomerase gene expression. [191], [192] When a peptidyl-$tRNA^{Gly}$ dissociates from a GGA codon and reassociates with a second GGA codon 50 nt downstream, translational hopping happens. [192] Three conditions are necessary for this frameshifting to occur, an in-frame UAG termination codon, a hairpin loop right next to the GGA codon, and a 14-amino acids sequence of the hopping site. The role of an in-frame UAG termination codon appears to be pausing the ribosome with the GCA in the P site. The other two conditions may be required to detach and/or reassociate the peptidyl-tRNA.

1.3 Type I Interferons

Among three distinct interferon families, the type I IFN family is composed of 13 IFNα subtypes (14 in mice), 1 IFNβ subtype, and other poorly defined type I IFN subtypes such as IFNε, IFNτ, IFNκ, IFNω, IFNδ, and IFNζ. [193] The type II IFN family encodes one gene product, IFNγ, which is mostly produced by T cells and natural killer (NK) cells. On the other hand, the type III IFN family includes four distinct subtypes, IFNλ1 (IL-29), IFNλ2 (IL-28A), IFNλ3 (IL-28B), and IFNλ4. [194], [195] These are known to function like the type I IFNs, but their activity is limited because their receptor is predominantly expressed only on the epithelial cells. [196] Therefore, immune cells are not responsive to IFNλ. [196], [197]

The type I IFN family is best characterized by their antiviral activity. This comprises inducing an antiviral state in both virus-infected cells and uninfected neighboring cells. Besides this, the type I IFNs mediate the innate and adaptive immune responses controlling viruses as well as bacterial infection.

1.3.1 Type I Interferon Gene Induction

Most of cells in the body produce the type I IFNs and the type I IFN gene induction results from the recognition of pathogens such as viruses and bacteria by pattern recognition receptors (PRRs). PRRs such as RIG-I and melanoma differentiation-associated protein 5 (MDA5) recognize RNAs derived from pathogens in the cytosol. Other cytosolic receptors that recognize foreign DNAs also induce the type I IFNs. These include DNA-dependent activator of IFN-regulatory factors and the recently identified cytosolic GAMP synthase (cGAS). [198], [199] In addition, NOD1 and NOD2 can also detect nucleic acids and induce the type I IFN genes. [200]–[203] TLRs, on the other hand, are localized on the cell surface or in the endosomal compartments. TLR3, TLR7/8, and TLR9 recognize dsRNA, ssRNA, and unmethylated CpG DNA, respectively. [204]

Downstream pathways from the PRRs mentioned above, all deliver signals to a couple of important transcription factors such as the IFN-regulatory factor (IRF) family. IRF3 and IRF7 are the fundamental transcription factors that stimulate the expression of the type I IFNs. The early stage of the type I IFN gene induction, IFNβ and IFNα4 genes are induced by IRF3. Subsequently, IRF7 is activated by the initial IFN production such as IFNβ and IFNα4 and results in the expression of more potent IFNα subtypes. [205], [206] It is believed that NF-κB is required as a cofactor in this pathway. [206] For the phosphorylation of IRF3 and IRF7, which is necessary for their activation, the kinases IκB kinase-ε (IKKε) and TANK-binding kinase 1 (TBK1) are responsible. Upstream of IKKε and TBK1, IPS-1 mediates signals from the cytoplasmic PRRs such as RIG-I and MDA5 to activate TBK1, whereas STING delivers signals from the cytosolic DNA sensors. [199] TLR3 uses TRIF to activate TBK1, but other TLRs such as TLR7 and TLR9 rely on another adaptor molecule MyD88 instead of TRIF.

1.3.2 Induction of Interferon-stimulated genes (ISGs)

All the type I IFNs bind to a receptor that is composed of the subunits IFNAR1 and IFNAR2. Once activated, IFNAR signals to the receptor-associated protein tyrosine kinases Janus kinase 1 (JAK1) and tyrosine kinase 2 (TYK2). Activated JAK1 and TYK2 phosphorylate signal transducer and activator of transcription 1 (STAT1) and STAT2 molecules resulting in the dimerization and nuclear translocation after interacting with IRF9. The heterotrimeric complex of STAT1, STAT2, and IRF9 is called the interferon-

stimulated gene factor 3 (ISGF3) complex. ISGF3 then translocates and binds to IFN-stimulated response elements (ISRE) in IFN-stimulated gene (ISG) promoters to induce the expression of ISGs. [207] In addition to the STAT1-STAT2 heterodimer pathway, there is another pathway mediated by the STAT1-STAT1 homodimer. STAT1 homodimer binds to γ-activated sequences. [207] Moreover, IFNα/β can also utilize other STATs such as STAT3, STAT4, STAT5A, and STAT5B. This diverse downstream pathway may result from the need to induce the broad range of genes including genes that encode not only antiviral effectors, but also antibacterial effectors, pro-apoptotic and anti-apoptotic molecules, and so on. [208]

1.3.3 Role for Adaptive Immune Response

The role of IFNα/β is not restricted to the intracellular antiviral response. IFNα/β function on both the innate and adaptive immune response. IFNα/β influence myeloid cells, B cells, T cells, and NK cells that ultimately support to resolve viral infection through enhancing immune response. It is shown that IFNα/β are involved in the adaptive immune responses by either activating dendritic cells or inhibiting these cells upon different circumstances. [209]–[212] Specifically, IFNα/β may activate immature dendritic cells by inducing the cell-surface expression of major histocompatibility complex (MHC) molecules and co-stimulatory molecules. These include CD80 and CD86 that are required for T cell stimulation. [213]–[215] IFNα/β also help dendritic cells to cross-present antigens from viruses such as vaccinia virus and lymphocytic choriomeningitis virus (LCMV). [216]–[218] In addition, IFNα/β promotes the migration of dendritic cells to lymph nodes, leading to T cell activation. [219], [220]

IFNα/β can also directly affect to both CD4+ and CD8+ T cells. Their functions include inhibition or stimulation for T cell proliferation, IFNγ production, and memory development. It seems that these effect is controlled by differential activation of STAT molecules.

NK cells are also regulated by IFNα/β. IFNα/β stimulate the function of survival of NK cells similarly to T cells. During both influenza virus and vaccinia virus infections, IFNα/β are necessary for the production of cytolytic effector molecules and IFNγ. [221], [222] However, during mouse cytomegalovirus (MCMV) infection, IFNγ is not produced by IFNα/β stimulation, while cytolytic effector molecules are expressed. [223]

B cells are important for the production of neutralizing antibodies that help resolving a number of different viral infections. Although some studies suggest that IFNα/β seem to impair the survival and development of B cells, IFNα/β may benefit B cells for many of their functions. [224]–[226] IFNα/β promote the activation of B cells and antibody responses. IFNα/β increase B7-2 expression in B cells and enhance antibody production in part by inducing B cell activation through B7-2. B7-2 is a co-stimulatory molecule

expressed in antigen presenting cells such as dendritic cells, macrophages, and B cells. It was shown that within the first 48 hours of influenza virus infection, B cell activation in the early stage results in upregulation of activation markers and change of the transcriptional response. [227]–[229]

IFNα/β, on the other hand, have detrimental effects during viral infection although they are important for antiviral immune response by ISG induction and adaptive immunity enhancement. IFNα/β induce immunosuppressive effects as well as inflammation and tissue damage during virus infection. [230], [231] Two studies suggest the harmful effects of IFNα/β. Both studies describe that IFNα/β increase the expression of immunosuppressive molecules such as PD-L1 and IL-10 which inhibit T cell activation and proliferation. In the studies, CD4+ and CD8+ T cell-mediated immune responses are improved when IFN-mediated signaling is blocked. [232], [233]

Taken together, IFNα/β have an important antiviral role during virus infections. However, they also contribute to immunosuppression as well as immunopathology.

1.3.4 Antiviral Pattern Recognition Molecules

There are a number of different antiviral pattern recognition molecules such as the RIG-I-like receptors, the Toll-like receptors, and the NOD-like receptors. They are located in different compartments of the cell, but ultimately results in the induction of type I IFNs.

The RIG-I-like receptor (RLR) family is composed of three cytoplasmic receptors, RIG-I, MDA5, and LGP2. [234], [235] RLRs share structural features such as two N-terminal caspase recruitment domains (CARDs), a central DEAD box helicase/ATPase domain, and a C-terminal regulatory domain.

The ligand of RIG-I is short dsRNA (up to 1kb). [236] Previously, it was considered that 50 triphosphate ssRNA was a RIG-I ligand [237], [238], but recent studies showed that 50 triphosphate ssRNA failed to stimulate RIG-I. [239], [240] This suggests that dsRNA, not ssRNA, is a ligand for RIG-I. In addition, a 50 triphosphate end is not required to activate RIG-I because both chemically synthesized dsRNAs with a 50 monophosphate end and those without a 50 phosphate end can stimulate RIG-I. [236], [241] It has been postulated that HCV RNA untranslated region with poly(U)- or poly(A)-rich sequences is responsible for the activation of RIG-I, but further studies are required to confirm whether specific RNA sequences are involved in the activation of RIG-I. [242]

MDA5 recognizes long dsRNA (more than 2kb). [236] A study using a dsRNA-specific nuclease confirmed that shortening the length of the poly(IC) converts the poly(IC) from a MDA5 ligand to a RIG-I ligand.

In contrast to RIG-I and MDA5, LGP2 lacks a CARD domain. Although the function of LGP2 is controversial, it has been considered that LGP2 functions as a negative regulator of RIG-I and MDA5 by reducing dsRNA accessibility or inhibiting RIG-I conformational changes. [243]–[245]

RIG-I and LGP2 have a large basic surface in the C-terminal regulatory domain which is responsible for the binding to dsRNAs. [241], [246], [247] However, MDA5 binding activity is much weaker than that of RIG-I and LGP2 although MDA5 has a large basic surface in the C-terminal regulatory domain. The weaker binding activity results from the extensively flat C-terminal domain of MDA5.

The RIG-I conformation is suggested to be regulated by ubiquitination. Both TRIM25 and Riplet function as E3 ubiquitin ligases that determine the K63-linked polyubiquitination of RIG-I. [248], [249] This K63-linked polyubiquitination activates RLR signaling pathway in contrast to the K48-linked polyubiquitination of RIG-I by RNF125. The K48-linked polyubiquitination of RIG-I results in the degradation of RIG-I by the proteasome. [250]

Finally, the CARD damain of RLRs interact with the N-terminal CARD-containing adaptor IFN-β-promoter stimulator 1 (IPS-1) for triggering downstream signaling cascades. [251] IPS-1 is located on the outer mitochondrial membrane and is known to be cleaved from the mitochondria by an HCV NS3/4A protease.

The Toll-like receptor (TLR) family recognizes pathogens outside of the cell and in intracellular endosomes and lysosomes. [252] TLRs share structural features such as N-terminal leucine-rich repeats (LRRs) and a transmembrane region followed by a cytoplasmic Toll/IL-1R homology (TIR) domain. Until now, ten TLRs have been discovered in humans and twelve have been identified in mice. TLRs have their own corresponding ligands derived from the molecular patterns of pathogens. Among TLRs, TLR3, TLR7, TLR8, and TLR9 recognize nucleic acids derived from viruses and bacteria. [252] Once activated, TLRs induce the type I IFNs as well as proinflammatory cytokines.

TLR3 detects virus-derived dsRNAs in the endolysosome. The crystal structure analysis of TLR3 complexed with dsRNA showed that dsRNA interacts with the regions in the TLR3 LRRs and this binding stimulates the dimerization of two TLR3 molecules. [253], [254] In contrast to TLR3, human TLR7 and TLR8 recognize ssRNA from RNA viruses. Besides ssRNA, human TLR7 and TLR8 also detect small purine analog compounds. In addition, human TLR7 recognizes RNAs from bacteria in conventional dendritic cells (cDCs). [255] TLR9, on the other hand, senses unmethylated DNA with CpG motifs derived from bacteria and viruses, although there is a study suggesting that the DNA sugar backbone of 20 deoxyribose also stimulate TLR9. [256]

TLR7 and TLR9 are highly expressed in plasmacytoid dendritic cells (pDCs) which produce large amounts of type I IFNs upon virus infection. To avoid negative effects of type I IFNs, TLRs are compartmentalized in the cell. TLRs such as TLR3, TLR7, and TLR9 that can recognize self-nucleotides are localized in the endoplasmic reticulum membrane. TLR1, TLR2, TLR4, TLR5, and TLR6 are present on the plasma membrane. [257] Upon virus infection, TLR3, TLR7, and TLR9 are recruited from the endoplasmic reticulum to endolysosomes, but the mechanism how they translocate remains to be elucidated. [258], [259] RNA ligands from invading viruses are recognized by TLR7 and TLR9 in pDCs after they are endocytosed or delivered to the endolysosome in the cytoplasm.

TLR signaling pathway is divided into two downstream pathways depending on the presence of MyD88 or TRIF. MyD88 is utilized by most of TLRs except for TLR3. MyD88 interacts with IL-1R-associated kinase (IRAK)-4 which activates other IRAK family members such as IRAK-1 and IRAK-2. [260] Then the IRAKs translocate from MyD88 to TNFR-associated factor 6 (TRAF6). TRAF6 functions as an E3 ubiquitin protein ligase. TRAF6 together with an E2 ubiquitin-conjugating enzyme complex results in a lysine 63 (K63)-linked polyubiquitin chain on TRAF6 itself as well as the production of an unconjugated free polyubiquitin chain. [261] The unconjugated free K63 polyubiquitin chain activates downstream signaling molecules and subsequently translocates nuclear factor-κB (NF-κB) into the nucleus. NF-κB translocation induces proinflammatory cytokine genes expression. In case of TLR7 and TLR9, type I IFNs are also produced in a MyD88-dependent manner.

In contrast to most of other TLRs, TLR3 and TLR4 utilize TRIF-dependent signaling pathway. Upon stimulation with dsRNA, TLR3 recruits TRIF instead of MyD88. TLR4, on the other hand, triggers both MyD88-dependent and TRIF-dependent signaling pathway. TRIF interacts with TRAF3 and TRAF6. TRAF3 is necessary for inducing two IKK-related kinases, TBK1 and IKK-i (also known as IKKε) [262], [263] Subsequently, TBK1 and IKK-i phosphorylate IRF3 and IRF7. IRF3 and IRF7 dimers then translocate into the nucleus and stimulates expression of type I IFNs. [264]

Cytoplasmic pattern recognition receptors that recognize DNA is important for the expression of type I IFNs upon DNA virus infection. Infection with intracellular bacteria also induces type I IFNs. [265] It is shown that poly (dA:dT) is transcribed into dsRNA by polymerase III and the dsRNA is then detected by RIG-I resulting in the induction of IPS-1 pathway. [266]–[268] Besides this, a cytoplasmic DNA-binding protein, DNA-dependent activator of IRF (DAI), binds with TBK1 and promotes the expression of type I IFNs upon interaction with its DNA ligand. [269] Moreover, another cytoplasmic DNA-binding protein, stimulator of interferon genes (STING), is known to be located in the ER and translocates from the ER to the Golgi apparatus in response to dsDNA recognition. [270]–[272]

The nucleotide-binding oligomerization domain receptors (NOD-like receptors or NLR) family is composed of a central nucleotide-binding domain and C-terminal leucine-rich repeats. [273] The N-terminal regions have protein-binding motifs such as CARDs, a pyrin domain, and so on. Although most of NLRs and CLRs recognize ligands derived from bacteria, NOD2 is shown to be involved in the induction of type I IFNs upon 50-triphosphate RNA ligand recognition. [274]

1.3.5 HCV Factors for Inhibiting Type I Interferons

Although HCV can be recognized by several PRRs to alert an antiviral immune response, HCV has strategies to evade host immune response (**Figure 1-5**). Subsequently, about 80% of patients with acute HCV infection fail to clear the virus and develop a chronic infection. [275] In order to evade host innate immune response, HCV may have developed several mechanisms.

The best known viral protein that is responsible for innate immune evasion is the NS3/4A protease. NS3/4A protease cleaves IPS-1 at the outer membrane of mitochondria which is important mediator of type I IFN pathway activated by the RIG-I. [70], [276] The cleavage of IPS-1 by NS3/4A protease was also observed in the patients with chronic HCV infection. [70], [277] The IPS-1 cleavage affects not only RIG-I pathway but also PKR pathway because the dsRNA-dependent protein kinase PKR is activated by the binding of HCV dsRNA to PKR and IPS-1 plays a role in the PKR pathway which ultimately induces type I IFNs and early ISGs. [278]–[280] The role of PKR activation, however, is controversial because the phosphorylation of eIF2α by PKR kinase inhibits the translation of cellular mRNAs that contains host factors for HCV replication and at the same time IFNs and ISGs. [281], [282] NS3/4A also cleaves TRIF which is an important mediator for TLR3 signaling pathway. Like RIG-I pathway, TLR3 pathway also triggers the induction of type I IFNs. In addition, it is known that the cleavage site by NS3/4A, Cys372, contains high sequence homology to the NS4B-5A cleavage site of HCV polyprotein. [71], [283] Finally, NS3/4A inhibits the K63-linked polyubiquitination of RIG-I through targeting the E3 ubiquitin ligase Riplet. As a result, the interaction between RIG-I and TRIM25, TBK1 is suppressed. [284]

Two other viral proteins, E2 and NS5A, targets PKR and inhibits its downstream pathway though direct interaction. [285], [286] However, the detailed mechanism how E2 and NS5A inhibit PKR pathway remains largely unknown. NS5A also directly binds to 2'-5'-oligoadenylate synthase to block the subsequent antiviral response. [287] In addition, NS5A interacts with MyD88 in macrophages and suppresses the TLR pathway except for TLR3 pathway. Since it is believed that HCV does not infect macrophages, it remains to be explained the significance of this result. [288] It is shown that HCV E1 and E2 glycoproteins suppress the expression of RIG-I and TLR3, which again downregulate the expression of IFNs and ISGs that are induced by the two pattern recognition receptors, RIG-I and TLR3. [289]

On the other hand, the HCV core protein decreases the expression of ISGs by blocking JAK/STAT signaling pathway. [290] The HCV core protein directly binds to STAT1 to suppress its phosphorylation, resulting in the blocking of downstream pathway. [291]–[293] Moreover, the HCV core negatively regulate the JAK/STAT pathway by inducing the expression of the suppressor of cytokine signaling-3 (SOCS3). [294]

In addition, HCV NS4B is suggested to suppress the RIG-I pathway. Moreover, HCV NS2 protease is recently shown to inhibit host cell antiviral response by inhibiting IKKε and TBK1 functions. [295], [296] However, it needs to be confirmed by other studies to show the effect of NS2 and NS4B for the downregulation of IFN expression.

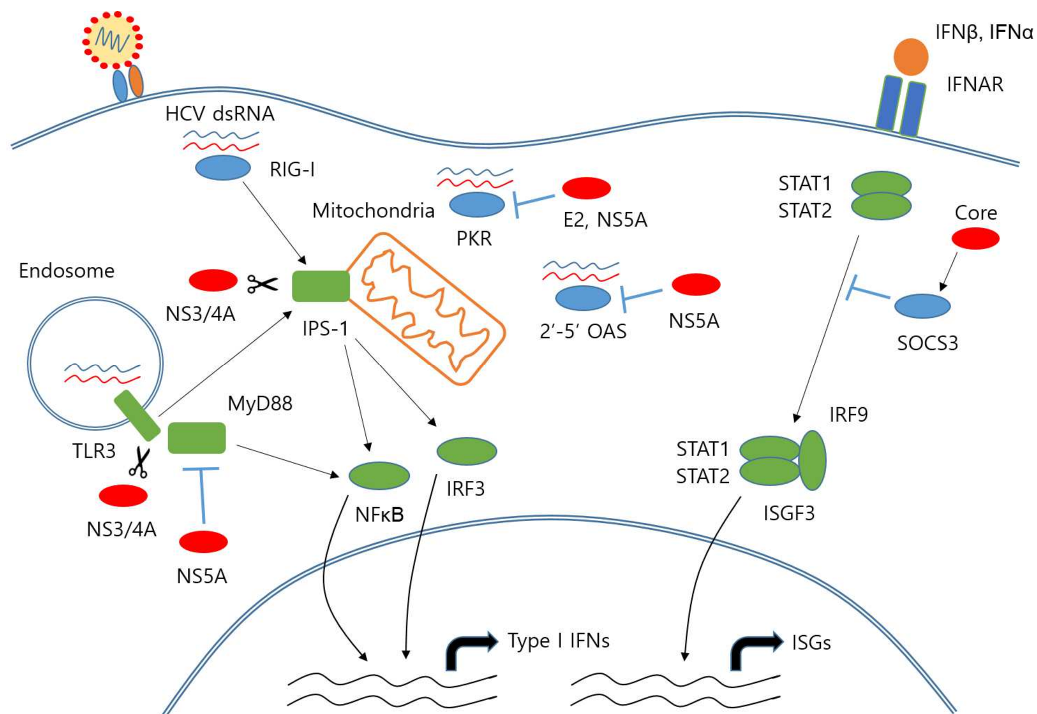


Figure 1-5. HCV factors for inhibiting type I interferons. HCV NS3/4A protease cleaves both IPS-1 and TRIF, resulting in the suppression of type I IFN expression. NS5A physically binds to MyD88 to inhibit TLR signaling. In addition, NS5A as well as E2 inhibits PKR activation. NS5A also interacts with 2'-5' OAS to block the subsequent antiviral pathways. Core induces the expression of SOCS3 to suppress the JAK/STAT pathway. IRF, IFN regulatory factor; ISG, IFN-stimulated gene; IPS-1, IFN-β promoter stimulator 1; MyD88, myeloid differentiation pro-inflammatory response 88; PKR, dsRNA-dependent protein kinase R; SOCS3, suppressor of cytokine signaling 3; STAT, signal transducer and activator of transcription; TRIF, TIR domain-containing adaptor inducing IFN-β.

1.4 Frameshift / Alternate Reading Frame Protein (F/ARFP)

1.4.1 Discovery of Alternate Reading Frame Protein

Figure 1-6. Variants of the alternate reading frame protein. White bar represents the protein product generated by 0 frame. Grey bar represents the protein product generated by -2/+1 frame. Number shows the codon number.

HCV encodes a long precursor polyprotein which is processed by cellular and viral proteases into 10 structural and nonstructural proteins. Besides this 10 viral proteins, a novel HCV protein has been identified in 1998 by Walewski et al. Although this protein has been discovered more than 10 years ago, little has been revealed about the biological role of this protein. This protein is known as F (Frameshift), ARFP (Alternate Reading Frame Protein), or core+1 protein (**Figure 1-6**).

In 1998, Walewski et al. identified a functional alternate open reading frame which is overlapping inside the core sequence at +1 frame. [43] This discovery resulted from a bioinformatics study of eight different HCV RNA sequences. In this study, specific nucleotide positions where mutations would occur due to the natural selection process were identified, but mutations were suppressed by restrictions. This suggests that HCV requires a conserved sequence at +1 frame within core region. [297] Walewski et al. also provided an evidence of the ARFP expression by designing ARFP peptides and using the peptides as antigens for the detection of certain antibodies in HCV patient serum samples. [43], [297] In 2000, Vassilaki et al. and Xu et al. independently reported the ARFP expression in a cell-free system, rabbit reticulocyte lysate or wheat germ extracts, and the discovery of a 16 kDa protein which is encoded from the alternate reading frame. In order to identify the mechanism of ARFP generation, site-directed mutagenesis and radioactive sequencing experiments were performed and revealed that this 16 kDa protein is translated from the AUG initiator codon, followed by a -2/+1 ribosomal frameshift in an adenosine-rich sequence within core codons from 8 to 11. [298], [299]

1.4.2 Different Forms of Alternate Reading Frame Protein

Since ARFP was discovered in 1998, there have been studies to identify the nature of the ARFP. However, there is no consensus on the nature of the ARFP because alternate translation initiation sites and different mechanisms for the expression of ARFP have been proposed. [300]–[303] This may result from the use of different HCV strains for the studies. In the early studies for ARFP, Vassilaki et al. and Xu et al used HCV-1 core coding sequences that have 10 consecutive adenosine-rich region within codons 8-11. Compared to this HCV strain, other HCV strains do not show corresponding frameshift-prone sequence within core codons 8-11. This may be the reason for the discrepancies for the ARFP expression studies as other HCV strains may not have a frameshift event at core codons 8-11.

Independent of a frameshift event at codons 8-11, another study revealed the existence of novel frameshifting starting from the internal AUG codons at 85/87. [304], [305] The initiation of internal translation at 85/87 produces shorter ARFP compared to the ARFP generated by frameshifting at codons 8-11. This shorter ARFP is known as core+1/S (short). In addition, codon 26 is also identified as the internal translation initiation site. [302] Finally,

Boulant et al. discovered another form of ARFP generated from E. coli by using HCV-1b strain core coding sequence. [301] This protein uses two different frameshift mechanisms independent of the frameshift event at codons 8-11. First +1 frameshifting occurs at codon 42 followed by -1 frameshifting at the stop codon 144. These studies suggest that there may be several translation initiation sites within the core coding sequence. It remains unclear whether all these different ARFP have same or similar functions or not.

1.4.3 Conservation of ARFP among HCV Strains

The length of the ARFPs differs from different genotypes, varying from 126 and 162 amino acids. HCV genotype 1a, 1b, 2a generate 162 aa, 144 aa, 126 aa ARFP, respectively. Other HCV genotypes show 126-155 aa long ARFP. [299] Compared to the core protein, ARFP amino acid sequence shows less conserved sequences among different HCV genotypes. [306]–[308] However, it is as well conserved as the E1 and NS2 proteins. [306]

Vassilaki et al. discovered that ARFP amino acid sequence from position 72 to 115 shows statistically significant similarity compared to the transmembrane domain of the adenosine triphosphate (ATP)-binding cassette transporter subfamily A (ABC1, aa 27–69) [305], [309] ABC1 is known to translocate cholesterol and phospholipids out of macrophages. Therefore ABC1 is responsible for lipoprotein metabolism in macrophages. [310] In addition, Shesheer Kumar et al. showed the existence of a binding site for LDL receptor class B in ARFP. [311] Further study for the role of ARFP in lipid metabolism may be interesting because HCV replication is associated with the modulation of lipid metabolism-related genes. [312]

1.4.4 Properties of Alternate Reading Frame Protein

ARFPs are highly basic with an isoelectric point of about 11.5. The isoelectric point of HCV-1a core protein is 11.46. [297] In addition, ARFPs are unstable as their half-lives are about 10 min. This was shown by the pulse-chase experiments in vitro or in cultured Huh7 human hepatoma cells. [313], [314] However, degradation of ARFP is strongly inhibited by proteasome inhibitors such as MG132 and lactacystin. Vassilaki et al. showed that core+1/S protein was detectable in the presence of proteasome inhibitors as well as in the absence of core proteins. [305] This suggests that not only proteasome inhibitor, but also core protein regulate the intracellular core+1/S level. Wolf et al. also confirmed the negative role of core protein on core+1/S level. [315] The fact that the introduction of core protein both in cis and in trans negatively impacts on core+1/S level suggests that this negative effect may not result not only from translation initiation competition, but also from posttranslational regulation.

The core+1/F protein generated from frameshifting at core codons 8-11 in genotype 1a has been detected in the cytoplasm of Huh7, HepG2 human hepatoma cells and in the perinuclear or in the endoplasmic reticulum membrane. [313], [314], [316] One study reported that the core+1/F is located in the nucleus in HEK293 human embryonic kidney cells. In addition, ARFP generated from internal translation initiation at codon 26 was proposed to be located in the mitochondrial area. [317]

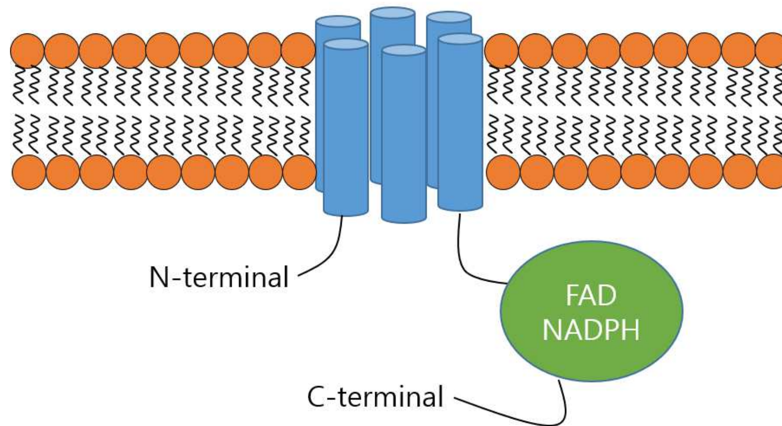
In terms of functional properties of ARFP, subgenomic HCV replicon system studies suggested that the ARFP is not necessary for viral replication because subgenomic HCV replicon lacks the entire structural region including ARFP encoding sequence. [318], [319] However, a role for ARFP in the natural hepatocyte infection cannot be excluded because current HCV infectious systems are using Huh7 or Huh7.5 cells that are recovered from curing the cells with interferon gamma or an RdRp inhibitor. [320], [321] The biological role of ARFPs may be overlapped with core protein because most of the experiments for studying core functions cannot exclude the possibility of ARFP production. Therefore, some of the core functions revealed so far may need to be attributed to the function of ARFP or the combined function of both ARFP and core protein.

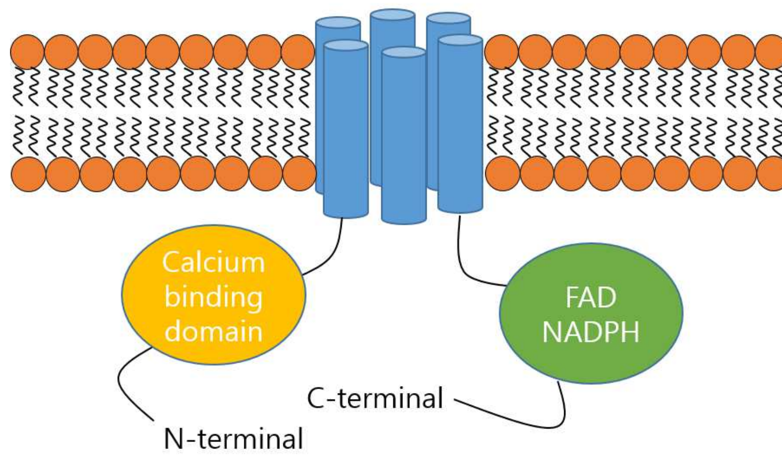
In order to identify the biological role of ARFP, there have been studies to reevaluate the functions of the core protein in the absence of ARFP production. Basu et al. suggested that the core+1/F is associated with host growth regulation as ARFP is involved in the p21 cdk inhibitor gene promoter repression. [322] Wu et al. showed that the core+1/F protein from HCV-1b (J4) increases the transcription and translation of c-Myc and decreases the transcription and translation of p53 in HepG2 cells. [323] The ARFP may also be involved in the production of fibrogenic chemokines such as IL-6, IL-8, and MCP-1 because higher prevalence of anti-ARFP antibodies is detected in HCV-infected patients with advanced liver disease and hepatocellular carcinoma. [324] Tsao et al. demonstrated that the core+1/F protein from HCV-1a (RH) interacts with the cellular prefoldin 2 protein, which results in the disturbance of the normal function of prefoldin complex. Consequently, nascent polypeptide chains cannot be folded properly. For example, an aberrant organization of the tubulin cytoskeleton was observed in the yeast two-hybrid system and in HEK293 cells. [325]–[327] This suggests that core+1/F may be involved in the establishment of viral persistence and chronic infection because microtubule formation may result in the decrease of HCV replication. [328]

The high conservation of the ARFP encoding sequence suggests that the function of the ARFP may be important for the virus survival and propagation. However, the biological role and significance of ARFP is not completely elucidated.

1.5 Nox/Duox Enzymes

1.5.1 Nox/Duox History





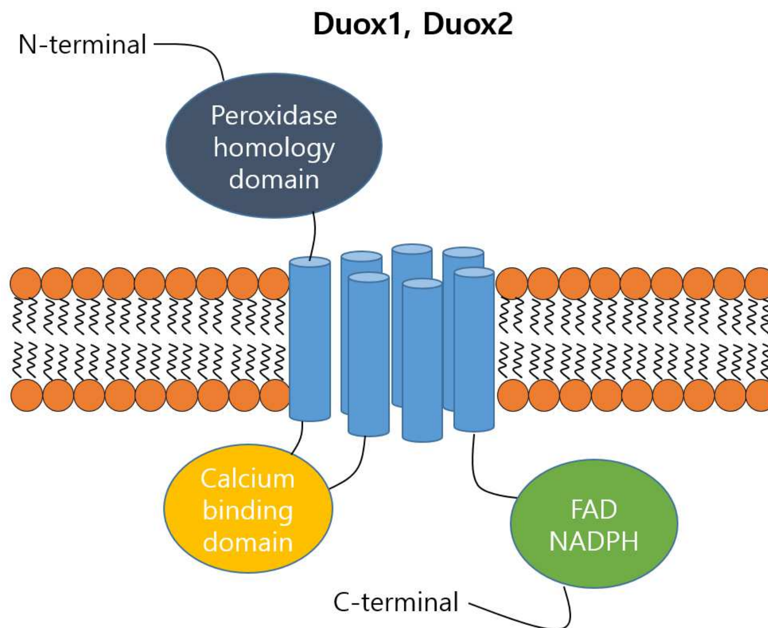


Figure 1-7. Structures of Nox/Duox family. NADPH oxidases have similar structural components that are necessary for transmembrane electron transfer from the cytosol to molecular oxygen. These oxidases have six (Nox1, Nox2, Nox3, Nox4, Nox5) or seven (Duox1, Duox2) transmembrane segments anchoring two heme groups and the C-terminal region contains NADPH and FAD binding sites. Nox5, Duox1, and Duox2 have an N-terminal region containing calcium binding domain. In addition, Duox1 and Duox2 have an extracellular N-terminal peroxidase homology domain.

It was discovered that cells utilize enzymes that produce reactive oxygen species (ROS) to regulate cellular ROS levels. These enzymes are designated to generate ROS as their sole biochemical function. [329], [330] The enzymes are called NADPH oxidases that are composed of seven members, Nox1, Nox2, Nox3, Nox4 Nox5, dual oxidase 1 (Duox1), and Duox2 (**Figure 1-7**). The enzymes generate ROS in a regulated manner in response to cytokines, growth factors, and calcium.

The first discovered Nox enzyme is the Nox of phagocytes (Phox) that processes the respiratory burst. [331] This oxidase is activated by the presence of microorganisms or inflammatory mediators mainly in neutrophils and macrophages. It is composed of the catalytic subunit gp91phox (alternatively known as Nox2) regulatory subunits p22phox, p47phox, p40phox, p67phox, and the small GTPase Rac. This enzyme requires the electron-donating pyridine nucleotide NADPH. It transfers electrons from NADPH to molecular oxygen to produce superoxide and other ROS.

There had been obstacles to measure ROS levels in various cell types other than phagocytes because many of the assays have the non-quantitative nature. Moreover, there is another problem that ROS levels in non-phagocytes are very low compared to the levels in an activated neutrophil. However, during the 1990s, sensitive assays were developed and allowed investigators to detect low amounts of ROS in various cell types.

In 1999, the first of the Nox homologues of gp91phox was identified and was named as Nox1. [329] When this enzyme was expressed in cells, it produced low levels of ROS, however, it generated high levels of ROS in the presence of novel regulatory subunits. Soon after Nox1 was discovered, the other Nox enzymes in the same family were found. It is now considered that many cell types express Nox enzymes that are responsible for a variety of cellular activity. ROS produced by these Nox enzymes are likely to participate in a limited range of reactions with biomolecules instead of having broad range of oxidations that may affect to inflamed tissues. It was shown that deliberate mechanisms were developed to harness the oxidations in specific manners at low levels of ROS. In these specific ways, the oxidations can be reversed and occur at certain residues like catalytic cysteine in the active site of tyrosine phosphatase and PTEN. [332], [333] The oxidations inhibit the function of these enzymes, however specific ways have also been evolved to reverse the oxidation. An example is that an oxidation in the active site of protein tyrosine phosphatase 1B can be reversed by cellular thiols such as glutathione and thioredoxin, resulting in the reactivation of the enzymes. [334]

The Nox enzymes can be categorized into three groups based on the presence of domains except for the gp91phox domain. [330] Nox1, Nox3, and Nox4 have similar size and structure to gp91phox. These enzymes oxidize NADPH in the cytoplasmic part of the membrane and reduces oxygen across the membrane to produce superoxide. In the next reaction, two molecules of superoxide generate hydrogen peroxide that can function as a substrate for peroxidases. On the other hand, Nox5 uses the basic structure of gp91phox, but has an additional amino-terminal calmodulin-like domain that contains four binding sites for calcium. As expected, Nox5 is activated by the calcium ionophore ionomycin. [335] Finally, the Duox enzymes have not only a calmodulin-like domain, but also an amino-terminal peroxidase-homology domain. Although this peroxidase-homology domain has an amino acid replacement at critical residues for enzymatic activity, it was shown that it can catalyze H_2O_2-dependent peroxidative reactions. [336], [337] Therefore, Duox seems to be able to both generate ROS and use its peroxidase-homology domain for enzymatic function. Its name, dual oxidase, came from its possible dual activity.

1.5.2 Reactive Oxygen Species (ROS)

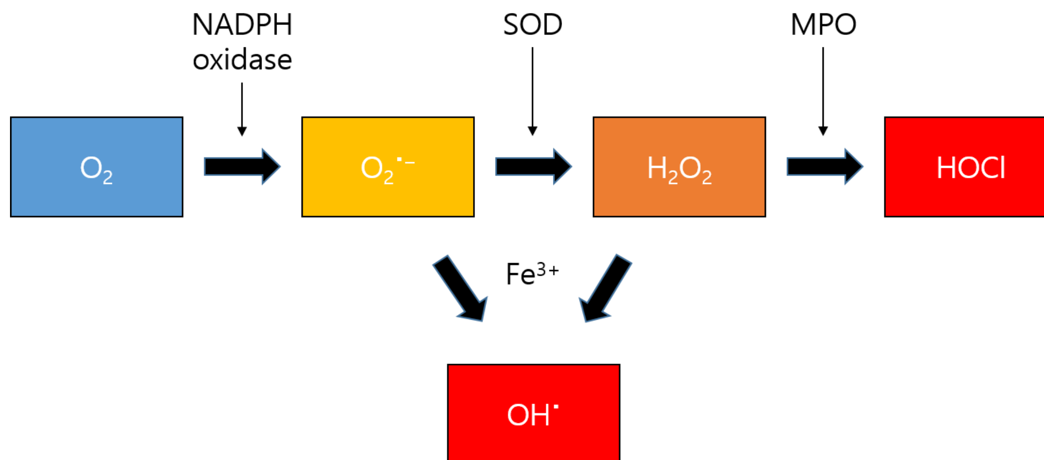


Figure 1-8. Reactive oxygen species. Superoxide ($O_2^{\bullet -}$) can be generated from the NADPH oxidase enzymes. Then, hydrogen peroxide (H_2O_2) is produced from dismutation reaction with two molecules of superoxide, which is facilitated by superoxide dismutase (SOD). When there is iron, hydroxyl radical ($HO^{\bullet}$) is generated from the reaction with superoxide and hydrogen peroxide. In inflamed areas, hypochlorous acid (HOCl) is formed from hydrogen peroxide and chloride by the myeloperoxidase (MPO). The color indicates the reactivity of the molecule. Blue, relatively unreactive; yellow, limited reactivity; brown, moderate reactivity; red, high reactivity.

ROS are highly electrophilic molecules such as superoxide (O_2^-), hydrogen peroxide (H_2O_2), and hydroxyl radical ($HO^{\bullet}$). ROS also includes secondary metabolites such as lipid peroxides, peroxynitrite ($ONOO^-$), and hypochlorous acid (HOCl) (**Figure 1-8**). ROS are short-lived and are produced by the reduction of oxygen. The overproduction of ROS results in oxidative stress which is involved in some kinds of diseases.

ROS can be divided into two groups in terms of reactivity, a radical group and a mild group. Superoxide, hydroxyl radical, hypochlorous acid, and peroxynitrite are examples of highly reactive ROS, while hydrogen peroxide is an example of mildly reactive ROS. Superoxide, on the other hand, can function as both a mild oxidant and a reductant upon different circumstances.

Radical forms of ROS are considered to cause damage to macromolecules, interfering biochemical function. This unstable form of ROS can bring a chain reaction that negatively modifies macromolecules such as certain proteins and nucleic acids. This feature can be used beneficially when it is confined well. A good example of this is that the radical forms of ROS mediate microbicidal killing in a phgolysosome. However, if it is not contained well, it can cause DNA strand breaks and oxidative damage to DNA bases, which will result in mutagenesis and cancer. Moreover, it is known that peroxidation of unsaturated lipids changes membrane structure, resulting in disrupting membrane properties and affecting cellular components recognition by immune system.

A physiological role for ROS has been suggested to be involved in many cellular activities such as cell signaling and transcriptional regulation. For example, hydrogen peroxide is generated in response to growth signals, suggesting its role for cell signaling. [338], [339] Consistently, hydrogen peroxide can be overproduced in transformed cells with mutated Ras. [340] Taken together, it is now clear that ROS are generated in response to a number of different ligands such as cytokines and growth factors. [341] ROS can also interfere with several signaling pathways which include ERK1, ERK2, NF-κB, AP-1, and JAK/STAT. [342]

The way how ROS affect cellular signaling pathways may be indirect in many cases, but there is a direct target for ROS on certain cysteine-containing proteins. [343] Certain cysteines can be targets for oxidative modification by ROS when proteins with the cysteines have exceptionally low pKa or specialized structural features that make them reactive. [344] An example of these cysteines are thiolate anions (Cys-S^-) at physiological pH. They can be easily oxidized by hydrogen peroxide. [345] However, an additional protein like peroxiredoxin may be needed because the direct reaction of hydrogen peroxide with the cysteine residues takes too much time to function for cellular signaling pathways. [346] Therefore, cysteine oxidation happens within the time required for the cellular signaling regulation. Moreover, it is shown that reactive cysteines reside in the enzyme's active site, where oxidation by ROS positively or negatively affects to its function. [345], [347]–[349] Examples of oxidation-regulated proteins are protein tyrosine phosphatases (PTPs), phosphatase and tensin homolog (PTEN), mitogen-activated protein kinase phosphatases (MAPK-Ps), and so on. [348]–[350] Oxidation of the thiol residues by hydrogen peroxide is reversible, which is defaulted by thioredoxin or glutathione. This allows for the oxidation by ROS to control a variety of cellular signaling pathways. On the other hand, unusually elevated ROS under pathological circumstances interfere with redox-sensitive signaling pathways.

In addition to direct effect on signaling pathways, unusually high ROS levels result in the production of highly reactive peroxynitrite that is generated by the reaction between nitric oxide and superoxide. This highly reactive peroxynitrite can directly react with a number of different biomolecules, which causes molecular damages to the biomolecules.

It has been considered that ROS are mainly produced by accidental mechanisms. The examples are ingestion of drugs or toxins that produces ROS through redox reaction. [351] In addition, gamma and UV irradiation as well as enzymatic processes can also initiate free radical reactions that produce ROS. An example of ROS production by enzymatic processes occurs in mitochondria during normal respiration. ROS production in mitochondria happens at several sites such as at sites of coenzyme Q reduction in complexes I and III. Recent studies suggest that respiring mitochondria release very low levels of ROS. [352] Although normal mitochondria produce low levels of ROS, it is believed that control of mitochondrial ROS level is important for preventing diseases. [353] In addition to mitochondria, there are a number of different redox enzymes such as xanthine oxidase, cytochrome p450, cyclooxygenase, lipoxygenase, and nitric oxide synthase (NOS).

1.5.3 Biological Functions of Nox/Duox Enzymes

The G protein-linked receptor, the formyl peptide receptor (FPR), is identified as a key modulator for Nox2 activation in phagocytes. FPRs recognize bacterial proteins that are posttranslationally modified at their N termini with a formyl group. It was tested that the tripeptide N-formyl methionyl-leucyl-phenylalanine (fMLF) can be a model ligand for FPRs. FPR1 seems to have a high affinity to fMLF, while FPR2 may have a lower affinity to fMLF. [354] Consistently, FPR1-null mice were more susceptible to bacterial infection, suggesting the immune role of the receptor. [355]

FPRs are also expressed on non-phagocytic cell types. A study using mouse colonic epithelia confirmed that Nox1 activation is responsible for the production of ROS. A ligand for FPR1, annexin A1, was used to stimulate FPR1 in this study. When the ligand was treated for the Nox1-deficient mice, the ROS production was abolished. This suggests that Nox enzymes are involved in the production of ROS in non-phagocytes. [356]

In addition, there are other types of receptors that are able to stimulate ROS production. These receptors respond to various kinds of ligands such as a variety of hormones, cytokines, and growth factors. [357], [358] For example, one of the inflammatory cytokines, tumor necrosis factor α, can stimulate the tumor necrosis factor (TNF) receptors that physically interact with Nox1, subsequently generating ROS and triggering necrotic cell death pathways. [359] Moreover, TLRs and NLRs can also activate Nox-dependent ROS generation in the presence of pathogen-associated ligands.

During the 1990s, investigators have demonstrated that ROS are associated with cell division. Bae et al showed that Nox1 mediated the production of hydrogen peroxide by a mechanism involving PI3K and Rac1 in response to the epidermal growth factor (EGF). [360] In addition, hydrogen peroxide clearly stimulated mitotic pathways because it was demonstrated

that hydrogen peroxide generated by EGF stimulation could lead to reversible inactivation of protein-tyrosine phosphatase 1B. Oxidative inactivation of various protein tyrosine phosphatases seems to be a common biochemical reaction in the cells and is normally related to mitotic growth. [334] For example, oxidative inactivation of the tumor suppressor PTEN has been suggested to be related to growth and survival, especially in cancer. [333] Moreover, Nox1 overexpression seems to affect to the cell cycle and subsequently mitotic growth in lung epithelia. [361]

Nox-derived ROS seem to have a role in cell differentiation and development. In plants, ROS levels decide whether to pursue proliferation or differentiation in the root by a mechanism involving the transcription factor, UPBEAT1 (UPB1). [362] UPB1 can also directly control the expression of a range of peroxidases that regulate the balance of ROS. The control of UPB1 happens between the regions of cell proliferation and elongation where differentiation occurs. Therefore, if the UPB1 activity is disrupted, then it changes ROS level, which subsequently leads to a delayed cellular differentiation. In an invertebrate model study, similar result could be observed. ROS in a Drosophila model induce hematopoietic progenitors to pursue for differentiation. [363] Additional study in a Drosophila model demonstrated that control of the cellular redox state by the Nrf2/ARE complex induces proliferation in the midgut in response to oxidative stress. [364] Moreover, Keap1-null mice show a proliferative phenotype in squamous epithelia because Nrf2 is constitutively active. [365] Similar studies have been performed in mammalian systems. It was shown that Nox1 is associated with generation of the angiogenic vascular endothelial growth factor (VEGF), leading to the development of blood vessels. [366] In addition, Nox4 is involved in cardiomyocyte differentiation. [367] Moreover, both Nox1 and Nox4 are implicated in the vascular smooth muscle growth and differentiation that happen after arterial injury. [368]

Nox enzymes function for cytoskeletal reorganization and cell migration by several mechanisms. It is demonstrated that Nox1-produced ROS lead colon adenocarcinoma cells to migrate on collagen by controlling α2β1 integrin availability at the membrane. [369] Nox1 is also involved in vascular smooth muscle cell migration stimulated by fibroblast growth factor-β (FGF-β). [370] In lung endothelial cells, hyperoxia stimulates Nox4-derived ROS generation. When Nox4 is suppressed, cell migration and formation of capillary tubes in response to hyperoxia are inhibited. [371]

Cell migration requires the sophisticated mechanism to orchestrate remodeling of the actin cytoskeleton. This mechanism also needs changes in the proximity of the membrane edge to the extracellular matrix at specialized points termed focal adhesions (FAs). FA assembly is controlled by focal adhesion kinase (FAK) that is maintained in a dephosphorylated form by the redox-sensitive tyrosine phosphatases LMW-PTP and SHP-2. [372] Several endogenous stimulations such as growth factors and integrin binding with the epithelial basement membrane promote Nox1-derived ROS production at the FA, resulting in oxidative inactivation of the redox-sensitive

tyrosine phosphatases LMW-PTP and SHP-2 and ultimately initiation of cellular motility. [373] When mice are in a germ-free environment, they show decreased migration of intestinal epithelial cells along the crypt-villus axis. This suggests that intestinal microorganisms seem to be important for modulating epithelial growth and development. Therefore, taken together, ROS generation by intestinal microbiota causes reversible oxidation of redox-sensitive cysteines in LMW-PTP and SHP-2, FAK phosphorylation, and an increase of FAs, resulting in enhanced epithelial migration. [374]

Nox-derived ROS is also involved in programmed cell death. Some of the Nox isoforms and their regulatory subunits are known to be implicated with apoptosis. In pancreatic cells and endothelial cells, p47phox, p22phox and Nox1 are involved, respectively. [375], [376] In the presence of bacteria, Nox enzymes are responsible for the activation of autophagy. [377] Physical interaction between Nox1 and the TNF receptor mediate necrotic cell death in vitro. [359] On the other hand, Nox4-derived ROS in pancreatic cells and Nox1-derived ROS in colon adenoma and carcinoma are implicated in inhibiting apoptosis. [378], [379] Therefore, depending on the cell type and the Nox isoforms, Nox-derived ROS participate in either proapoptotic or antiapoptotic pathways.

Studies in non-mammalian organisms have revealed a role of Nox enzymes in the extracellular matrix modification. Peroxidases such as myeloperoxidase, lactoperoxidase, and the peroxidase domain of C. elegans Duox1 catalyze the cross-linking of tyrosine residues by hydrogen peroxide. [351], [359], [380], [381] In C. elegans, cross-linking by Duox1 leads to the stabilization of the nematode cuticle structure. [359] In sea urchin eggs, a Duox isoform catalyzes the cross-linking of the fertilization envelope initiated upon fertilization. [382] This cross-linking makes the fertilization envelope hard so that the access of additional sperm is blocked. Until now, Nox-stimulated tyrosine cross-linking of the extracellular matrix has not been reported in mammals. However, di- and trityrosine residues have been documented in regions of inflammation and in atherosclerotic plaques. [380]

The role of Nox enzymes in host immune defense is not only observed in animals including humans, but also in plants. Although the exact mechanisms how Nox enzymes function in host immune system are different, its immune role is evolutionarily conserved among plants and animals. For example, ROS generated by the Nox2 in phagocytes mediate microbicidal function by deriving toxic secondary products such as hypochlorous acid and peroxynitrite. [351] The importance of Nox-dependent ROS generation is clearly explained by chronic granulomatous disease (CGD). [383] CGD is a genetic disorder that has a defect in the catalytic or regulatory subunits of the Nox2 enzyme complex. As a result, phagocytes fail to produce ROS, leading to recurrent pyogenic infections and a failure to resolve inflammation that causes typical granulomatous lesions. Granuloma may be caused by the absence of anti-inflammatory ROS signaling in CGD, which results in changes of gene expression in neutrophils. [384]

The defensive role of Nox enzymes in lower animals appears to provide clues for studying the functions of Nox-derived ROS. In C. elegans, ROS derived from C. elegans Duox1 has a defensive role in the gut in presence of pathogens. [385] In the fly, Duox-derived ROS generation in the gut epithelia inhibits the proliferation of commensal microbes as well as happens upon pathogen infection. [386], [387] In addition, Nox1 and Duox2 are commonly observed in intestinal and respiratory epithelia in mammals. [357], [358], [388] In mammalian gastrointestinal and respiratory tracts, Nox enzymes generate hydrogen peroxide for lactoperoxidase in order to fight against pathogen infection. [389] The mechanism in which ROS from the epithelial Nox1 and Duox1 mediate an antimicrobial function is utilizing an oxidative inactivation of bacterial enzymes, inhibition of microbial signaling pathways, and the disruption of structural integrity of bacterial capsule. [390] Therefore, this suggests that Nox-derived ROS generation in epithelial barrier of the gut has been evolved to fight against microbes and their products.

On the other hand, plants have a different Nox-dependent defensive system because they lack circulating immune cells or epithelial barrier. When the host tissue is invaded by pathogens, calcium-dependent Nox enzymes are activated and trigger cross-linking of plant cell wall proteins, resulting in the enclosure of the invader. [391] In addition, Nox enzymes contribute to systemic signaling in response to damage. [392]

1.5.4 General Mechanisms of Oxidant Signaling

ROS were associated with the increase in phosphorylation of Tyr after growth factor-stimulated ROS burst which serves as evidence supporting the role of ROS in signal transduction. [338], [360] This increase of intracellular ROS was suggested to be necessary for downstream signaling. The ligand-stimulated ROS generation appears to depend on the activation of the Nox enzymes. [393] Moreover, the increase of Tyr phosphorylation after growth factor stimulation seems to be involved in the redox-dependent inactivation of protein Tyr phosphatases. [334] The PTPs act on a conserved reactive Cys residue that is located within the active site of the enzyme. Therefore, oxidation of the Cys residue leads to the inactivation of the enzyme. [394] In addition, ROS can also directly influence kinase signaling. [395] The Cys residue oxidation can be reversed by several molecules such as thioredoxin and glutathione (GSH). [396] Because of the reversibility of the Cys residues, specific Cys residues can function as redox-dependent switches. In addition, another amino acid residues, Met residues, can be oxidized and reduced like Cys residues and function as redox-dependent switches. [397]

The mammalian Kelch-like ECH-associated protein 1 (Keap1)-nuclear factor erythroid 2-related factor 2 (Nrf2) pathway is another evidence that shows Cys oxidation as a means of signaling regulation. [398] This pathway senses and responds to an intracellular increase of ROS levels. In this pathway, Nrf2 transcription factor is not the target of oxidation. The

interacting molecule with Nrf2, Keap1, has several reactive Cys residues and they are oxidized by increased intracellular ROS levels. Eventually, the Cys residue oxidation releases Nrf2 from Keap1 and leads to its translocation to the nucleus. [398]

1.5.5 Antiviral or Proviral Activities of ROS

The role of ROS regarding virus infection is controversial. The general role of ROS is considered to combat against intracellular pathogens such as bacteria and fungi. In line with this notion, there are evidences that ROS are used to combat against virus infection. In 1982, Rager-Zisman et al. showed that the respiratory burst was involved in vesicular stomatitis virus (VSV) clearance in a monocyte cell line. [399] In 1992, Huang et al. evaluated the role of superoxide anions in the establishment of antiviral state of the VSV-infected cells by transfecting with CuZnSOD. This study shows that superoxide anions participate in determining the sensitivity to type I IFN. [400] In 2006, Indukuri et al. suggested that IRF3 is activated by Nox enzyme(s) during respiratory syncitial virus infection. [401] In 2014, Olagnier et al. described that Dengue virus infection accumulated ROS generated by Nox enzymes to mount innate immune responses in DENV-infected dendritic cells. [402]

There are also evidences suggesting the proviral role of ROS. For example, in 1990, Roederer et al. showed that phorbol 12-myristate 13-acetate promoted HIV replication and N-acetyl-L-cysteine inhibited it in human peripheral blood or T cell lines. Phorbol 12-myristate 13-acetate is used for inducing oxidative burst and N-acetyl-L-cysteine is used as an antioxidant. [403] In 2010, Geiler et al. described that N-acetyl-L-cysteine reduced influenza A virus replication in human lung carcinoma. N-acetyl-L-cysteine inhibited production of CCL5, CXCL8, IL-6, and monocyte migration. [404] In 2011, Ye et al. showed that hydrogen peroxide promoted Kaposi's sarcoma-associated herpesvirus replication by activating ERK1/2 quinase, JNK, and p38. [405]

Therefore, depending on the viruses, cell types, and experimental conditions, the role of ROS regarding virus infection may be observed differently. As more data accumulate, the role of ROS in virus infections will be more understood.

CHAPTER 2

MATERIALS AND METHODS

2.1 HCV Constructs and Mutagenesis

JFH1 wild-type sequence and its replication-defective mutant, JFH1GND, were used. [406] Like H77c sequence, JFH1 wild-type sequence also contains the same frameshift signal which generates alternate reading frame proteins. The frameshift signal is at nt. 376 and 382 and contains other frameshift signals and internal translational initiation sites. [300]–[303]

Plasmids that are designed to code for JFH1 core sequence were generated. pCore, pCoreΔ, and pCoreΔ4 all code for JFH1 core sequence. However, pCoreΔ, and pCoreΔ4 contain premature termination codon(s) in the F/ARFP frame. In addition, pF and pFΔ were also generated to code for JFH1 F/ARFP sequence. (**Figure 2-1, Table 2-1**) These plasmids were generated by standard polymerase chain reaction (PCR) cloning followed by site-directed mutagenesis. The pcDNA3.1 plasmid vector was used to generate the plasmids mentioned above. This plasmid is under the control of eukaryotic elongation factor (EF) 1α promoter. PCR product from nt. 263-913 containing the JFH1 core sequence was inserted into the plasmid vector via *HindIII* and *XbaI* restriction sites (underlined). The primer sequences are as followed: Forward: 5'-CATGTAATCAATAAGCTT*GGGTTGCGAAAGGCC-TT*-3'; reverse: 5'-ATATTGCTAACGTCTAGA**TTA***AGCAGAGACCGGAACGGT* -3'. The core sequence is italicized and a stop codon introduced is shown in bold letters. Premature terminations in the -2/+1 frame do not affect to the amino acid sequence of core protein of the zero frame.

Core variant plasmids (**Figure 2-1, Table 2-1**) were generated by site directed mutagenesis of pJFH1-core using QuikChange XL Site-Directed Mutagenesis kit (Agilent). The primer sequences for site-directed mutagenesis are as followed: CoreΔ forward: 5'- *CCCGGGCGG-CGGCCAGAT**A**GT**A**GG- CGGAGTATACTTGTTGCC*-3', coreΔ reverse: 5'-*GGCAACAAGTATACTCCGCC**T**AC**T**ATCTGGCCGCCGCCCGGG*-3'. CoreΔ4 forward: 5'-*CGCCCAGAAGACGT**A**AAGTTCCCGGGCGGC*-3', coreΔ4 reverse: 5'-*GCCGCCCGGGAACTT**T**ACGTCTTCTGGGCG*-3'. Nucleotide substitutions are shown by underlined and bold letters. pF was also generated via site directed mutagenesis and it has a single nt. deletion at codon 10 (nt. 370) which makes the pF unable to express core protein. The primer sequences are as followed: Forward: 5'-*CCTCAAAGAAA_ACCAAA-AGAAACACC*-3', reverse: 5'-*GGTGTTTCTTTTGGT_TTTCTTTGAGG*-3'. Underline indicates the location of deletion. pHA-F contains hemagglutinin (HA) sequence combined with JFH1 core sequence harboring a single nt. deletion at codon 9 (nt. 366). In order to prevent core expression by -1/+2 frameshift, a premature termination codon in the core frame was introduced at codon 53, which substituted TCG with TAG. [300]

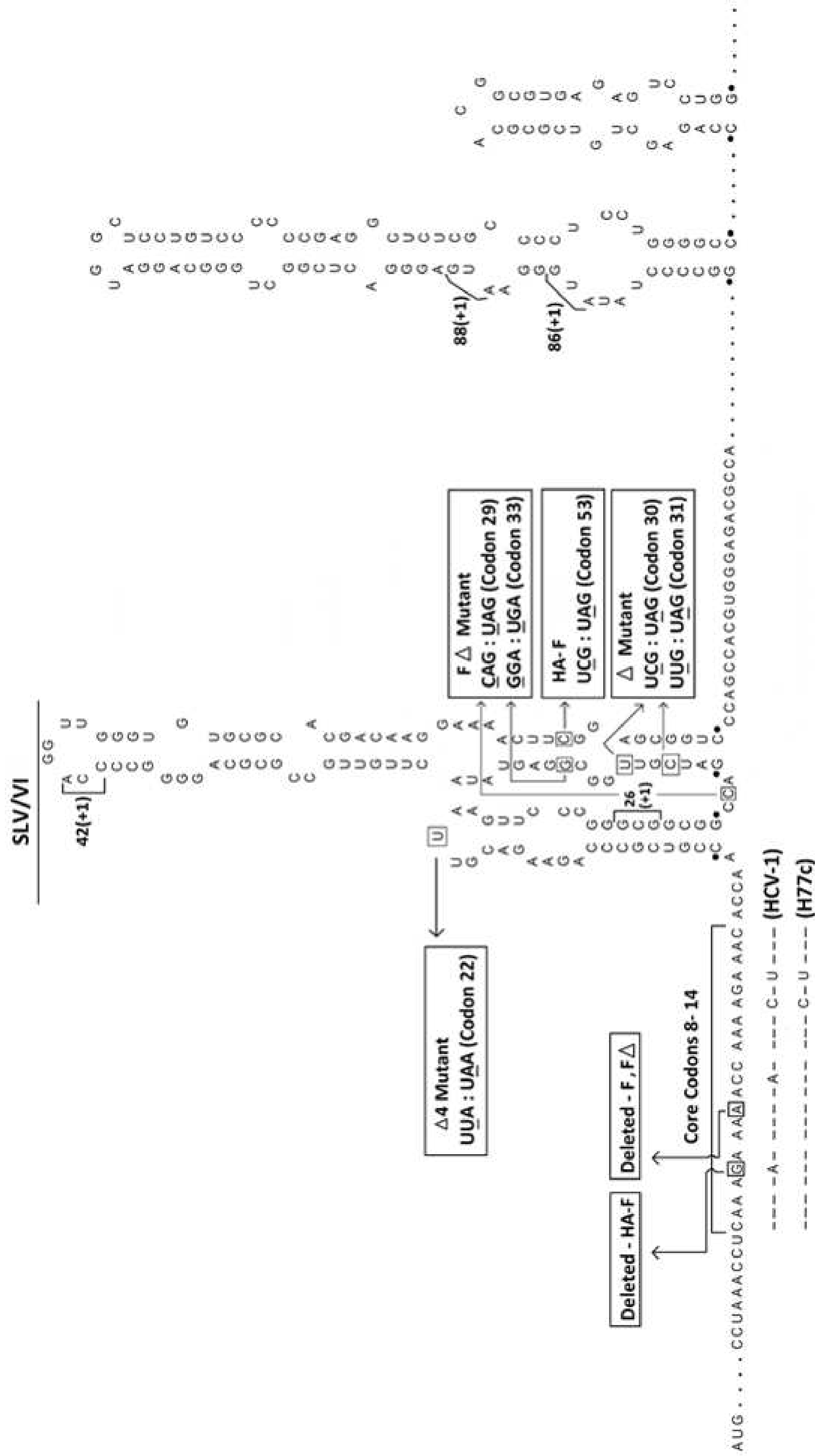


Figure 2-1. JFH1 constructs with mutations. Putative RNA elements necessary for F/ARFP expression and nt. substitutions introduced in JFH1 constructs are indicated. Numbers show nucleotide positions.

Construct	Mutation	Core	F/ARF	ARF/DS	Core+1/S	ARF/26(+1)	SLV/VI
JFH1wt & Core	None	+++	+ (by -2/+1 frameshift)	Normal	Normal	Normal	Normal
JFH1Δ & CoreΔ	-2/+1 frame stops at codons 30, 31	+++	None (truncated at 29th codon)	Normal	Normal	None (truncated at 29th codon)	Base of SL VI altered
JFH1Δ4 & CoreΔ4	-2/+1 frame stop at codon 22	+++	None (truncated at 21st codon)	Normal	Normal	Normal	Normal (Loop of SL V altered)
F	1 nt. deletion at codon 10	+ (by -1/+2 frameshift)	+++	Normal	Normal	Normal	Normal
FΔ	1 nt. deletion at codon 10, & 0 frame stop at codon 29, 33	None (truncated at 28th codon)	+++	None (truncated at 28th codon)	Normal	Normal	Base of SL VI altered
HA-F	1 nt. deletion at codon 9, & 0 frame stop at codon 53	None (truncated at 52nd codon)	+++	Normal	Normal	Normal	Base of SL VI altered

Table 2-1. JFH1 constructs. Location of nt. substitutions and expected effects on protein synthesis. +++ shows normal or relatively high expression, while + indicates reduced expression.

Along with core variant plasmids, JFH1 -2/+1 frame mutants (pJFH1Δ and pJFH1Δ4) were also generated. These contain premature termination codons in the -2/+1 frame that do not affect to the core amino acid sequence of the zero frame. (**Figure 2-1, Table 2-1**) The pGEM (Promega) plasmid vector was used to generate pJFH1Δ. PCR products from nt. 153 to 1349 were inserted into the plasmid vector via *HindIII* and *XbaI* restriction sites. The primer sequences used are as followed: Forward: 5'-ATCAAAGCTTaccggt*GAGTACACCGGAA*-3', reverse: 5'-GTTCGTCTAGAcgtacg*CCAGGATCATGGT*-3'. Underlined sequences indicate restriction sites. For pGEM-JFH1core generation, *AgeI* and *BsiWI* sites were used and indicated by underlined and lower case letters. HCV core sequences are italicized. To introduce premature termination codons in the -2/+1 frame at codons 30 and 31, QuikChange XL Site-Directed Mutagenesis kit (Agilent) was used. This produces the pGEM-JFH1coreΔ. The primer sequences used are as followed: Forward: 5'-*CCCGGGCGGCGGCCAGAT**A**GT**A**GGCGGAGTATACTTGTTGCC*-3', reverse: 5'-*GGCAACAAGTATACTCCGCC**T**AC**T**ATCTGGCCGCCGCCCG-GG*-3'. Bolded letters indicate the location of two point mutations. Next, nt. 153 - 1349 from pGEM-JFH1Δ using *AgeI* and *BsiWI* restriction sites are inserted into the full-length pJFH1. pJFH1Δ4, which has the same nt. substitution as coreΔ4, was generated by site directed mutagenesis using QuikChange XL Site-Directed Mutagenesis kit. The primer sequences are as followed: Forward: 5'-*CGCCCAGAAGACGT**A**AAGTTCCCGGGCGGC*-3', reverse: 5'-*GCCGCCCGGGAACTT**T**ACGTCTTCTGGGCG*-3'. Nucleotide substitutions are shown by underlined bold letters. Sequences explained above were confirmed by DNA sequencing from UC Berkeley DNA Sequencing Facility. The software Mfold was used to predict the RNA secondary structures of stem loops V and VI. [407] The predicted structures were consistent with the structure of stem loops V and VI shown by Tuplin *et al.* [408]

2.2 Transfection, Virus infection, and Tissues

Full-length genomic HCV RNA was transcribed in vitro as described. [409] T7 RNA polymerase (Promega) with RNase-free DNase I (GE Healthcare or Ambion) or using MEGAscript T7 High Yield Transcription Kit (Invitrogen) was used for in vitro transcription. The amount of JFHwt and mutant RNAs were measured by Nanodrop (Agilent Technologies) and the quality of in vitro transcribed RNAs were teste by formaldehyde agarose gel electrophoresis. In vitro transcribed RNAs with 7 - 10 μg were then electroporated into Huh7 and Huh7.5 human hepatoma cells in Opti-MEM or Dulbecco's minimum essential medium (Invitrogen) and the electroporated cells were cultured as described to generate infectious virus particles. [321], [409]–[411]

Plasmids were prepared by endo-free plasmid maxi kit (Qiagen, Inc.) and 0.025 - 10μg of plasmid DNA were used for transfection. For plasmid transfection, ProFection® Mammalian Transfection System (Promega) or

Lipofectamine LTX with Plus reagent (Invitrogen) was employed. For polyinosinic:polycytidylic acid (poly(IC)) and HCV RNA PAMP transfections, 5 μg of poly(IC) (GE Healthcare) using Lipofectamine LTX with Plus reagent (Invitrogen) was transfected. HCV RNA PAMP, 5' UTR (nt. 1 – 367) of Con1 (genotype 1b) sequence, was generated by primer-ligated polymerase chain reaction. [412]

Nox4 enzyme expressing Huh7-Nox4 cells were generated by the Huh7 cells which were transfected with human Nox4 complementary DNA via Lipofectamine 2000 (Invitrogen). 0.5 mg/mL of G418 (Invitrogen) was used for stable cell clone selection. Huh7-pcDNA cells were generated as control cells which were transfected with an empty plasmid vector. In order for continuous selection, 0.25 mg/mL of G418 was used while the cells were being maintained. The day before the experiment, G418 was not employed for the cell culture medium.

Infectious virus particles were collected from genomic HCV RNA-transfected cells and 1 – 2 mL of the cell culture medium containing infectious virus particles were inoculated into naïve Huh7 cells. [409] For control infection, mock transfections without any RNA or JFH1GND RNA transfection were employed and the cell culture medium from mock- or JFH1GND RNA-transfected cells were used for infection. The cells were cultured and collected at different times as shown in Results.

Human liver tissues with or without HCV infection (n=3 and 2, respectively) were obtained from the National Disease Research Interchange. These tissue samples are negative for immunodeficiency virus and hepatitis B virus. The liver tissue donors were between the ages of 49 and 65 and they did not have any liver-related deaths. [409] The study was approved by the Institutional Review Boards at Lawrence Livermore National Laboratory and University of California, Merced.

2.3 Determination of Viral and Cellular RNA Levels

Trizol Reagent (Invitrogen) was used for total intracellular RNA extraction from cells. Then, RNA concentrations was determined by Nanodrop. Quantitative real time reverse transcriptase-polymerase chain reaction (qRT-PCR) was followed to quantify mRNA levels. Power SYBR Green PCR Master Mix (Applied Biosystems) or EXPRESS One-Step SYBR® GreenER™ Universal (Invitrogen) was used. Data were calculated by ΔΔCt method and shown as fold difference based on the controls. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNA level was employed to normalize target gene RNA level. Primer sequences are shown in **Table 2-2**.

Table 2-2. List of qRT-PCR

Set	Gene	Direction	Sequence (5' to 3')
1	JFH1	Sense	TCTGCGGAACCGGTGAGTA
		Antisense	TCAGGCAGTACCACAAGGC
2	JFH1	Sense	CGGGAGAGCCATAGTGG
		Antisense	AGTACCACAAGGCCTTTCG
3	IFNβ1	Sense	CATTACCTGAAGGCCAAGGA
		Antisense	CAATTGTCCAGTCCCAGAGG
4	IFNβ1	Sense	CCAACAAGTGTCTCCTCCAAA
		Antisense	CCTCAGGGATGTCAAAGTTCA
5	IFNα2	Sense	TGAAAACTGGTTCAACATGG
		Antisense	TAATGGATCAGTCAGCATGG
6	IFNα21	Sense	GCCCTGTCCTTTTCTTTACTG
		Antisense	TCCTTTGTGCTGAAGAGATTG
7	IFNα8	Sense	CTTCAACCTCTTCAGCACAAA
		Antisense	AGGATGGAGTCCTCGTACATC
8	IFNλ1	Sense	GCTGGTGACTTTGGTGCTA
		Antisense	GAGATTTGAACCTGCCAATGTG
9	IFNλ2/3	Sense	CCACATAGCCCAGTTCAAGT
		Antisense	GCGACTCTTCTAAGGCATCTT
10	RIG-I	Sense	CTCTGCAGAAAGTGCAAAGC
		Antisense	GGCTTGGGATGTGGTCTACT
11	MDA-5	Sense	GTTTGGCAGAAGGAAGTGTC
		Antisense	GCTCTTGCTGCCACATTCTC
12	ISG56	Sense	GCTGATATCTGGGTGCCTAAGG
		Antisense	CTTGAGCCTCCTTGGGTTCG
13	TNFα	Sense	CCATGTTGTAGCAAACCCTCAA
		Antisense	GCTGGTTATCTCTCAGCTCCA
14	GAPDH	Sense	GGTGGTCTCCTCTGACTTCAA
		Antisense	GTTGCTGTAGCCAAATTCGTT

Set	Gene	Direction	Sequence (5' to 3')
15	Nox1	Sense	TTAACAGCACGCTGATCCTG
		Antisense	CTGGAGAGAATGGAGGCAAG
16	Nox2	Sense	GAACGTCTTCCTCTTTGTCTGG
		Antisense	GTGATGACAACTCCAGTGATGC
17	Nox3	Sense	AGGCCCTGTGGTCTTGTATG
		Antisense	ACATCTGTCAGGGCAGTTCC
18	Nox4	Sense	CCACAGACTTGGCTTTGGAT
		Antisense	GGCAGAATTTCGGAGTCTTG
19	Nox4	Sense	GAATCAATCAGCTGTGTTATGC
		Antisense	AGAGGAACACGACAATCAGCCTTAG
20	Nox4	Sense	CTGGAGGAGCTGGCTCGCCAACGAAG
		Antisense	GTGATCATGAGGAATAGCACCACCACCATGCAG
21	Nox5	Sense	CAGGATCTTTGCCTCTGAGC
		Antisense	GAAGAAGACCTGCACCTTGC
22	Duox1	Sense	GGACTCACTTCTCCCACCATTA
		Antisense	GAAGAGGAGGAGGTATGGGAGT
23	Duox2	Sense	AGCTGGCTGAGAAGTTCGAC
		Antisense	AAGGCAGGATACTGGAAGCA

On the other hand, HCV RNA level was also measured by qRT-PCR, but the RNA copy number was calculated by standard curve prepared with in vitro transcribed JFH1 RNA. [409], [411] Taqman One-Step RT-PCR Master Mix Reagent Kit (Applied Biosystems) was used. For minus sense HCV RNA quantification, only the forward primer was used in the reverse transcription step. Then, qPCR reactions were followed with the addition of reverse primer. Minus sense JFH1 RNA standards were generated by SP6 promoter insertion into the pJFH1 via XbaI restriction site. The plasmid was linearized with EcoRI restriction site and was transcribed by SP6 RNA polymerase (Promega) per manufacturer's protocol. [411] As negative controls, reactions without RNA and without reverse transcriptase were used. Northern blots confirmed intracellular HCV RNA titers. DNA probes for Northern blots were obtained from nt. 4128 – 8273 or 358 – 2816 of JFH1 by ScaI and APaLI restrictions sites, respectively. Northern blot images were taken by Cyclone Phosphorimager (Perkin Elmer).

2.4 Western blot, ELISA, and Immunofluorescence Staining

For Western blot, cells were collected directly with radioimmunoprecipitation assay buffer (RIPA) or Laemmli buffer or sonicated with RIPA buffer or Laemmli buffer as previously described. [409] β-Actin level was used as a control. Western blot images were obtained by Kodak Digital Science Image Station 440CF or LI-COR Odyssey Infrared Imaging System. Monoclonal F/ARFP antibody was prepared against recombinant JFH1 F protein and kindly provided by Dr. James Ou from University of Southern California. IFNβ protein level in the cell culture medium was measured by ELISA using Human IFNβ ELISA kit from Interferon Source, Inc. For immunofluorescence staining, samples were fixed with 3.5% formaldehyde for 5 minutes and incubated with phosphate-buffered saline containing 1% (wt/vol) bovine serum albumin, 0.05% (wt/vol) NaN3, and 0.02% (wt/vol) saponin. Then, samples were incubated with primary and fluorophore-conjugated secondary antibodies in order and imaged by confocal laser scanning microscopy (C1, Nikon). [409] 100 μg/mL of RNase A was added when propidium iodide (PI) was used for nuclear staining. ImageJ was used for the quantification of images taken from confocal laser scanning microscopy.

2.5 Frameshift Reporter Assays

For the generation of frameshift reporter constructs, sense and antisense nucleotide sequences corresponding to the first 14 codons of the JFH1 core sequence were synthesized. (**Table 2-3**) Then, these sequences were fused into the zero frame, -2/+1 frame, and -1/+2 frame of the firefly luciferase-coding sequence via EcoRI restriction site. [299] The zero frame construct expresses the luciferase gene, while the -2/+1 frame construct expresses the luciferase gene when -2/+1 frameshift occurs. Negative controls which contained stop codons between the frameshift signal and the luciferase sequence were generated. (**Table 2-3**) All the sequences were confirmed by UC Berkeley DNA Sequencing Facility. These constructs were transfected into Huh7 cells and measured for frameshift efficiencies by Luciferase Assay System (Promega) and Sirius Single Tube Luminometer. [299]

Set	Gene	Direction	Sequence (5' to 3')
1	pJFH1FS-Luc(-1/+2, 0, -2/+1)	Sense	AATTC[a]aagctt[c]AACCTCAAACAGACACCATGAGCACAAATCCT AAACCTCAAAGAAAAACCAAAAGAAACG[a]
		Antisense	AATTC[a]GTTTCTTTTGGTTTTTCTTTGAGGTTTAGGATTTGTGC TCATGGTGTCTGTTTGAGGTTaagctt[c]G[a]
2	pJFH1FS(-2/+1NC)[d]	Sense	AATTC[a]aagctt[c]AACCTCAAACAGACACCATGAGCACAAATCCT AAACCTCAAAGAAAAACCAAAAGAAAC**GTAA**[b]G[a]
		Antisense	AATTC[a]**TTAC**[b]GTTTCTTTTGGTTTTTCTTTGAGGTTTAGGATTT GTGCTCATGGTGTCTGTTTGAGGTTaagctt[c]G[a]
3	pJFH1FS(-1/+2NC)[d]	Sense	AATTC[a]aagctt[c]AACCTCAAACAGACACCATGAGCACAAATCCT AAACCTCAAAGAAAAACCAAAAGAAACGA**TAA**[b]G[a]
		Antisense	AATTC[a]**TTA**[b]TCGTTTCTTTTGGTTTTTCTTTGAGGTTTAGGATT TGTGCTCATGGTGTCTGTTTGAGGTTaagctt[c]G[a]

Table 2-3. Frameshift signal sequences in frameshift reporter constructs.

[a] EcoRI sites used in the cloning are underlined.

[b] Modified sequences are bolded.

[c] Other restriction sites (HindIII) are shown in lower case letters.

[d] NC denotes negative control.

2.6 Nox Enzyme Activity Assays

For Nox enzyme activity measurement, Huh7 cells were first permeabilized in intracellular-like buffer (140 mM KCl, 1 mM $MgCl_2$, 10 mM glucose, 10 mM HEPES, 1 mM EGTA, 0.193 mM $CaCl_2$ for 100 nM free [Ca2+]i; pH to 7.4 using KOH) containing 40 μM digitonin at 30°C for 5 min. Then, SOD (200 μg/ml)-inhibited reduction of cytochrome c in an intracellular-like buffer containing 100 μM NADPH, 100 μM ATP, and 100 μM GTP was measured after 10 min pre-incubation with 5 - 30 μM DPI or DMSO. [413] 80 μM of cytochrome c was used for the reaction and the reaction occurred for 30 min at 37°C. Then, the reduction of cytochrome c (A550nm – A540nm) was analyzed. Data were normalized by total protein level. To determine the role of Nox1 and Nox4, Huh7 cells were transfected with Nox1, Nox4, and control siRNA for 72 hrs and then, they were proceeded for Nox activity assay.

2.7 Luciferase assays

pGL4.45[luc2P/ISRE/Hygro] Vector (Promega) and pIgK-IFN-luc (Addgene) were transfected into Huh7 cells to measure luciferase activities for the interferon-sensitive response element and IFNβ promoter, respectively. These cells were lysed with 1x Reporter Lysis Buffer and analyzed by Luciferase Reporter Assay Kit (Promega). [414] Luciferase activities were normalized by total protein level.

2.8 Subcellular Fractionation

Subcellular fractionation of nuclear and cytoplasmic fractions was performed to observe the localization of target proteins such as p-IRF3. NE-PER kit (Pierce) was used for the subcellular fractionation. Then, the fractions were further analyzed for Western blots. Calnexin and GAPDH were employed as cytoplasmic markers and lamin A/C and histone deacetylase 1 (HDAC1) were used as nuclear markers.

2.9 Small Interfering RNA (siRNA)

For the knockdown of several target genes, small interfering RNAs (siRNAs) were used. Huh7 cells were transfected with 40 nM of RIG-I siRNA (sense: 5'-GGAAGAGGUGCAGUAUAUUUU-3', antisense: 5'-AAUAUACUGCACCUCUUCCUU-3', Dharmacon), MDA-5 siRNA (sense: 5'-UAUCAUUCGAAUUGUGUCAUUUU-3', antisense: 5'-AAUGACACAAUUCGAAUGAUAUU-3', Dharmacon) or control siRNA (Dharmacon) using RNAiMax (Invitrogen). [409] Then, the cells were stimulated with HCV PAMP or poly(IC). Control and HCV-replicating cells were transfected with Nox1, Nox4, or control siRNA (40 nM, Smartpool siRNAs, Dharmacon) using RNAiMax (Invitrogen).

2.10 Statistics

Data were analyzed using Student's t test or one-way analysis of variance with post hoc comparisons, using SigmaPlot 11.0 (Jandel Scientific). A p value less than 0.05 was considered statistically significant. Data are shown as mean ± standard error of the mean. Experiments were performed in duplicates or triplicates and repeated up to six times.

CHAPTER 3

ROLE OF HCV F/ARF PROTEIN IN THE RIG-I/MDA5-MEDIATED IFN RESPONSE IN HEPATOCYTES

3.1 Introduction

HCV is a major health problem which is affecting more than 170 million people worldwide. [415] About 80% of HCV-infected patients cannot clear the virus and develop to chronic infection, which increases the risk for progressing to severe liver diseases such as cirrhosis and hepatocellular carcinoma. Until 2011, the standard of care was a combination of subcutaneous pegylated interferon alpha and oral ribavirin, which showed limited efficacy and severe side effects. The introduction of DAAs, two protease inhibitor drugs approved in 2011, has shown strong promise and marks a new era of HCV treatment. [173], [416]–[418] Newer DAAs are now being released and more DAAs are in various stages of preclinical and clinical development for new treatment options for HCV infected patients. [6]

HCV is an enveloped positive-strand RNA virus of the Flaviviridae family. The RNA genome of HCV is about 9.6kb and is flanked by nontranslated regions which are conserved and highly structured. The HCV RNA genome encodes a single polyprotein precursor of about 3,000 amino acids. [419] The HCV RNA genome contains PAMPs that are detected by RIG-I. [242] PAMP recognition by PRR results in the activation of RIG-I pathway, which eventually produces type I IFNs. HCV has several strategies to evade host IFN responses. [420] One example is that HCV NS3/4A protease cleaves IPS-1 and TRIF to suppress type I IFN signaling. However, the complete mechanisms how HCV evades hot IFN responses are not delineated.

In 1998, Walewski et al. identified a functional alternate open reading frame which is overlapping inside the core sequence at +1 frame. [43] Since ARFP was discovered in 1998, there have been studies to identify the biological role of the ARFP. Many of them suggest that ARFP may be involved in the establishment of chronic infection and hepatocellular carcinoma through different approaches. However, the biological function of HCV ARFP still remains largely unknown. The biological role of ARFPs may be overlapped with core protein because most of the experiments for studying core functions cannot exclude the possibility of ARFP production. Therefore, some of the core functions revealed so far may need to be attributed to the function of ARFP. The immune modulatory role of core protein led us to suspect that F/ARFP may be involved in the innate immune response. We then hypothesize that F/ARFP may contribute to the suppression of the type I IFN induction. Because several HCV proteins were shown to suppress the type I IFN response, we suspected that there may be more viral proteins suppressing the type I IFN response for the persistence of the viral life cycle.

Therefore, the study objective is to determine the biological function of the HCV -2/+1 frame, focusing on F/ARFP, using virus-producing JFH1 cell culture system. In order to study the role of F/ARFP, JFH1 strain, HCV genotype 2a (AB047639), was used. JFH1 strain produces infectious virus particles in cell culture, which provides us opportunity to study the role of F/ARFP in a relation to the host, Huh7 cell. In this project, we present evidence for a novel mechanism of interferon suppression by HCV F/ARFP. We suggest that HCV F/ARFP suppresses type I IFN responses mediated by RIG-I and affects the progression toward chronic infection which ultimately increases the risk for the hepatocellular carcinoma. Therefore, our study may provide new insights into how HCV evades the host innate immune response and points to possible new drug target for therapy.

3.2 Results

3.2.1 Suppression of Type I IFN Responses by HCV F/ARFP

Among many proposed functions of the HCV -2/+1 frame, we focused on the possible role for host immune response. [324], [421], [422] To test whether F/ARFP expression regulates type I IFN response, Huh7 human hepatoma cells were transfected with pHA-F, pFLAG-NS3/4A, and empty control plasmid (pcDEF or pEF). pHA-F has hemagglutinin (HA) tag followed by HCV F/ARFP sequence. pFLAG-NS3/4A has FLAG tag fused with HCV NS3/4A sequence. Western blot confirmed the expression of HA-F and FLAG-NS3/4A expression in the transfected cells through HA and FLAG tag proteins. (**Figure 3-1 (A)**)

Then, these cells were again transfected with in vitro synthesized HCV RNA PAMP (HCV 5' UTR or HCV 5' untranslated region) or synthetic dsRNA poly(IC). As we expected, Huh7 cells transfected with control plasmid (pcDEF or pEF) increased IFNβ1 mRNA. (**Figure 3-1 (B), (C), (D)**) However, Huh7 cells transfected with pHA-F significantly decreased IFNβ1 mRNA expression which was stimulated by poly(IC) and HCV UTR. (**Figure 3-1 (B), (C), (D)**) pFLAG-NS3/4A, a positive control, suppressed IFNβ1 mRNA expression as expected. For the cells transfected with both pHA-F and pFLAG-NS3/4A, further decrease of IFNβ1 mRNA was seen. (**Figure 3-1 (B), (C)**) pF also suppressed IFNβ1 mRNA induction which was stimulated by poly(IC) and HCV UTR. pF does not have the HA tag or single nt. substitution in the core-coding zero frame which gives stop codon and is present in pHA-F sequence. (**Figure 3-1 (D)**) These data indicate that F/ARFP can suppress type I IFN induction in human hepatoma cells when it is expressed alone or in combination with NS3/4A.

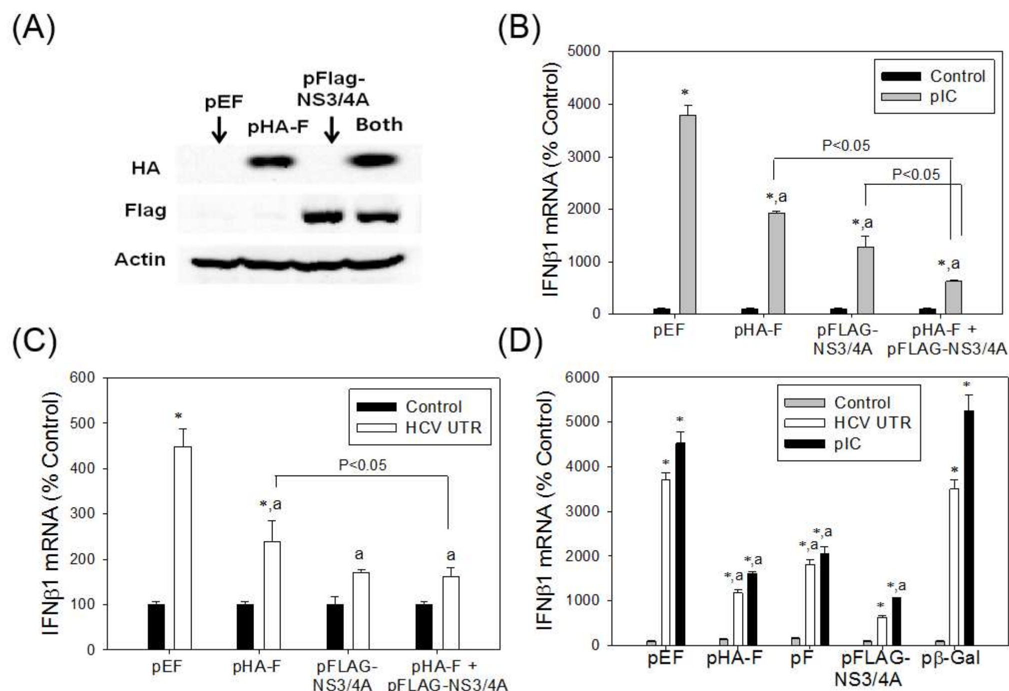


Figure 3-1. Suppression of type I IFN induction by HCV F/ARFP. (A) Huh7 cells were transfected with pEF (empty plasmid), pHA-F, pFLAG-NS3/4A, or pHA-F as well as pFLAG-NS3/4A. Then, Western blot tested whether HA, FLAG, and actin proteins were present in the transfected cells by anti-HA, FLAG, and actin antibodies. (B - D) Huh7 cells were transfected with indicated plasmids. After 48 hrs, the cells were again transfected with 5 μg of HCV UTR or poly(IC). Samples were collected 24 hrs after PAMP stimulation and were analyzed by qRT-PCR. For (B - D), star indicates statistically significant difference (P < 0.05) from corresponding controls (-PAMP group). Letter "a" indicates statistically significant difference (P < 0.05) from the corresponding pEF control for each -PAMP or +PAMP group. Lines with P values also indicate statistically significant difference between those samples. (For (B – D), n = 3)

3.2.2 Type I IFN Induction by HCV PAMP through RIG-I

To confirm the type I IFN induction stimulated by HCV PAMP through RIG-I signaling pathway, RIG-I siRNA or MDA5 siRNA was transfected in Huh7 cells. After 48 hrs, these cells were again transfected with HCV PAMP for 24 hrs to observe the IFNβ1 mRNA. Western blot confirmed the knockdown of RIG-I protein level in RIG-I siRNA transfected Huh7 cells. (**Figure 3-2 (A)**)

RIG-I siRNA transfected cells showed decreased level of IFNβ1 mRNA induction by HCV UTR as expected, while MDA5 siRNA transfected cells did not show significant change of IFNβ1 mRNA compared to the one of control siRNA transfected cells. (**Figure 3-2 (B)**) MDA5 siRNA, on the other hand, decreased IFNβ1 mRNA induction by poly(IC) (data not shown). These data suggest that HCV PAMP activates RIG-I signaling pathway which can be modulated by HCV F/ARFP as well as HCV NS3/4A.

(A)

(B)

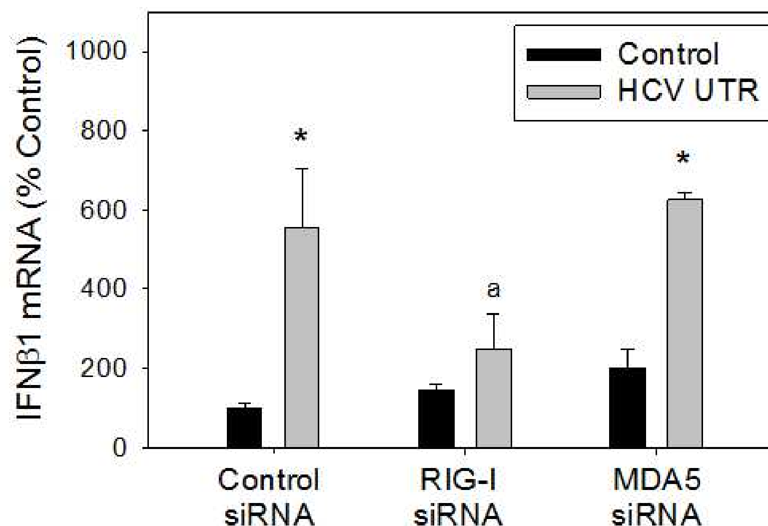


Figure 3-2. Type I IFN induction by HCV PAMP through RIG-I. (A) Huh7 cells were transfected with RIG-I or MDA5 siRNA and RIG-I protein level was analyzed by Western blot. (B) After 48 hrs, these cells were transfected with 5 μg of HCV RNA PAMP and analyzed for IFNβ1 mRNA after 24 hrs. Star indicates statistically significant difference ($P < 0.05$) from corresponding -PAMP control groups. Letter "a" indicates statistically significant difference ($P < 0.05$) from corresponding control siRNA groups for each -PAMP or +PAMP. All mRNA data were normalized by GAPDH mRNA and shown as percentage of controls. (n=3)

3.2.3 Suppression of Interferon Stimulated Genes (ISGs), Pro-inflammatory Cytokines by HCV F/ARFP

We also tested whether F/ARFP suppressed interferon stimulated genes and proinflammatory cytokines which were induced by RNA PAMPs. F/ARFP decreased ISGs including ISG15, ISG56, nucleotide-binding oligomerization domain-like receptor family CARD domain containing 5 (NLRC5), RIG-I, and PKR (also known as protein kinase R, interferon-induced, double-stranded RNA-activated protein kinase) which were stimulated by HCV RNA PAMP or poly(IC). (**Figure 3-3 (A)-(E)**) F/ARFP also suppressed proinflammatory cytokines such as TNFα induced by poly(IC). (**Figure 3-3 (F)**)

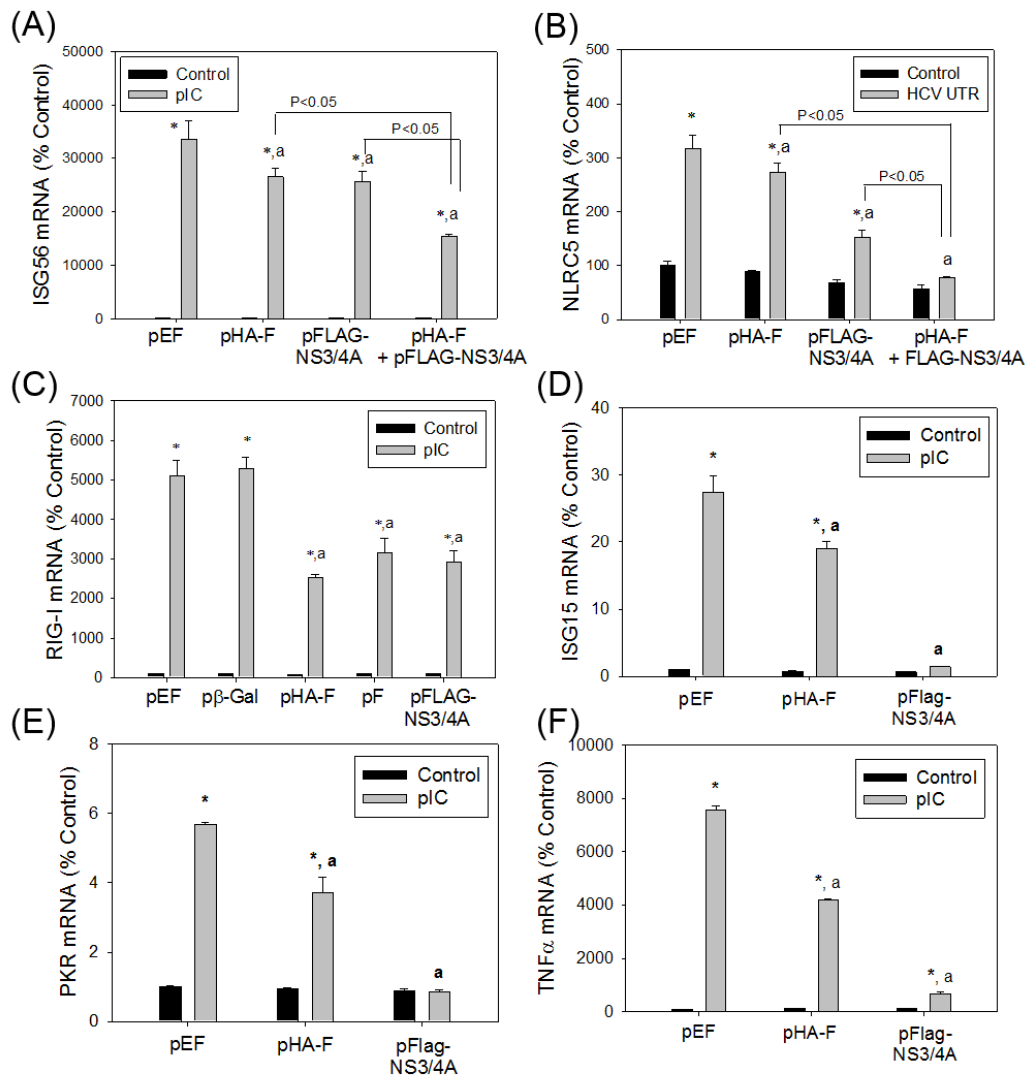


Figure 3-3. HCV F/ARFP suppresses ISGs and proinflammatory cytokines in Huh7 cells. (A - F) Huh7 cells were transfected with designated plasmids. After 48 hrs, they were transfected with 5 μg of HCV RNA PAMP or poly(IC). Samples were collected 24 hrs after stimulation by qRT-PCR. Data were normalized by GAPDH mRNA and shown as percentage of controls. Star indicates statistically significant difference (P < 0.05) from corresponding -PAMP controls. Letter "a" indicates statistically significant difference (P < 0.05) from the corresponding pEF control groups for each -PAMP or +PAMP. Lines with P values also indicate statistically significant difference (P < 0.05) between those samples. (n=3)

3.2.4 Suppression of Type III IFN by HCV F/ARFP

The biology of type III IFNs has recently started to be explained. Although there have been studies showing that type III IFNs are induced through mechanisms identical to the ones driving type I IFN expression, more recent studies have suggested that there are some important differences. [423]

We, therefore, examined whether F/ARFP suppressed type III IFN induction by RNA PAMPs. F/ARFP decreased IFNλ1 (or interleukin 29) and IFNλ2/3 (or interleukin 28A/B) mRNA elevation by poly(IC). (**Figure 3-4**). These data suggest that HCV F/ARFP participates in the suppression of type I IFN as well as type III IFN responses to intracellular RNA PAMPs in hepatocytes.

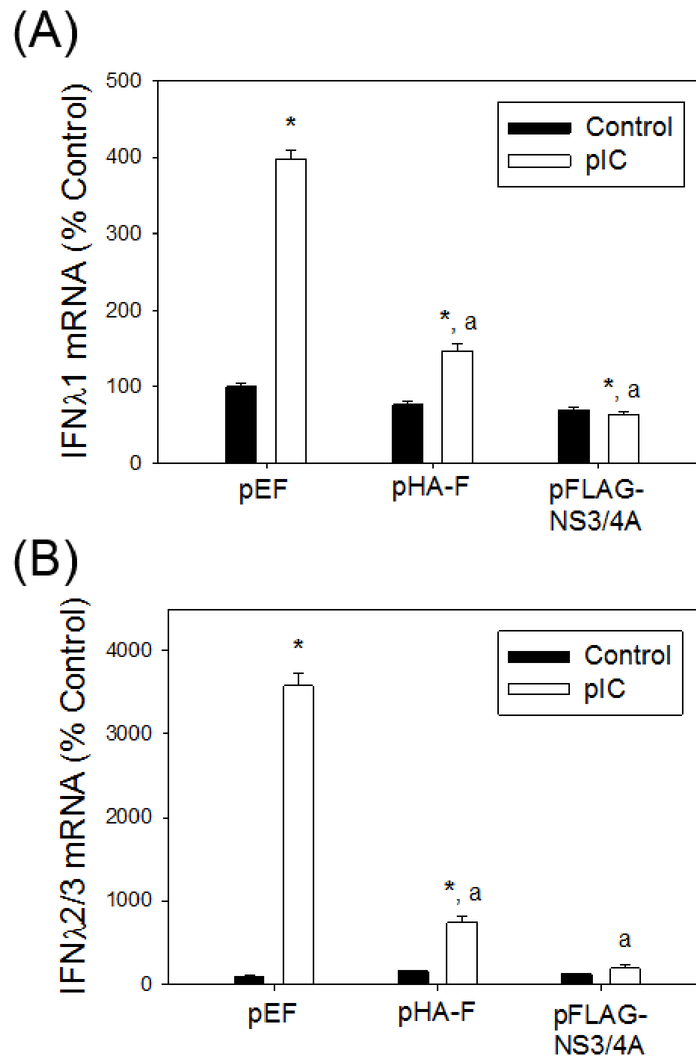


Figure 3-4. HCV F/ARFP suppresses type III IFN responses in Huh7 cells. (A - B) Huh7 cells were transfected with pHA-F or pFLAG-NS3/4A plasmids and, after 48 hrs, transfected with 5 μg of poly(IC). Samples were analyzed 24 hrs after poly(IC) stimulation by qRT-PCR. Data were normalized by GAPDH mRNA and shown as percentage of controls. Star indicates statistically significant difference ($P < 0.05$) from respective minus PAMP controls. Letter “a” indicates statistically significant difference ($P < 0.05$) from the corresponding pEF controls for each -PAMP or +PAMP group. Lines with P values also indicate statistically significant difference ($P < 0.05$) between those samples. (n=3)

3.2.5 Effects of HCV Core versus F/ARFP on IFNβ1 mRNA Elevation by HCV UTR

We tested whether F/ARFP suppression on type I IFN pathway was not due to HCV core protein which may have been produced by -1/+2 reverse frameshift in F/ARFP sequence. Data showed that the HCV UTR-stimulated IFNβ1 mRNA induction was significantly suppressed in Huh7 cells transfected with pHA-F, pF, or pFLAG-NS3/4A compared to pCoreΔ4. (**Figure 3-5**). This suggest that HCV RNA PAMP-stimulated type I IFN induction is mainly regulated by HCV F/ARFP and HCV core effect is minimal or negligible.

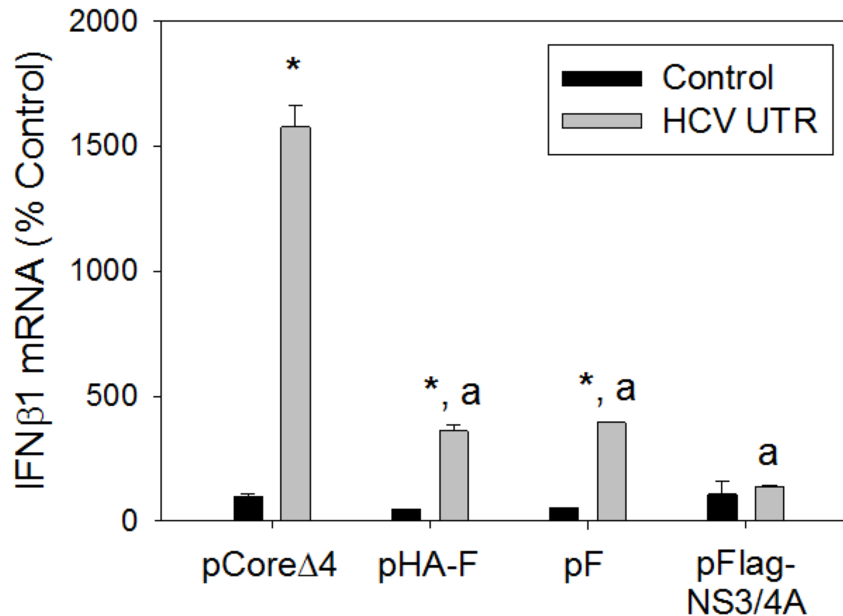


Figure 3-5. Effects of HCV core versus F/ARFP on IFNβ1 mRNA elevation by HCV UTR. Huh7 cells were transfected with pCoreΔ4, pHA-F, pF, and pFLAG-NS3/4A and analyzed for IFNβ1 mRNA by qRT-PCR. Data were normalized by GAPDH mRNA. Star indicates statistically significant difference ($P < 0.05$) from respective minus PAMP controls. Letter “a” indicates statistically significant difference ($P < 0.05$) from pCoreΔ4. (n=3)

3.2.6 Detection of HCV F/ARFP in Human Liver

We then evaluated HCV-infected human liver samples for HCV F/ARFP expression. As F/ARFP is derived from HCV core coding sequence and core may be produced by -1/+2 or -2/+1 frameshifts from the F/ARFP construct, we generated core and F constructs that only express core or F/ARFP. (**Table 2-1**) Therefore, pCoreΔ4 is designed to express only core without producing F/ARFP by introducing a stop codon in -2/+1 frame which does not affect to the amino acid sequence of the core coding zero frame. (**Table 2-1, Figure 3-6 (A)**) On the other hand, pFΔ is destined to express only F/ARFP without generating core protein by introducing stop codons in the zero frame which does not affect to the amino acid sequence of the F/ARFP coding -2/+1 frame.

As shown in **Figure 3-6 (A)**, core protein was easily detected in Huh7 cells transfected with pCoreΔ4 using anti-core antibody. However, core protein was not observed in Huh7 cells transfected with pFΔ as well as pEF using anti-core antibody. F/ARFP, on the other hand, was detected in Huh7 cells transfected with pFΔ, but not with pCoreΔ4 as well as pEF. This suggests that core and F/ARFP antibodies did not cross-react significantly with F/ARFP and core, respectively. Using these antibodies, we evaluated HCV-infected human liver samples for HCV F/ARFP expression. HCV-infected human liver samples (n = 3) showed significant levels of F/ARFP compared to control human liver samples (n = 2) which were not infected with HCV ($P < 0.05$). (**Figure 3-6 (B)**)

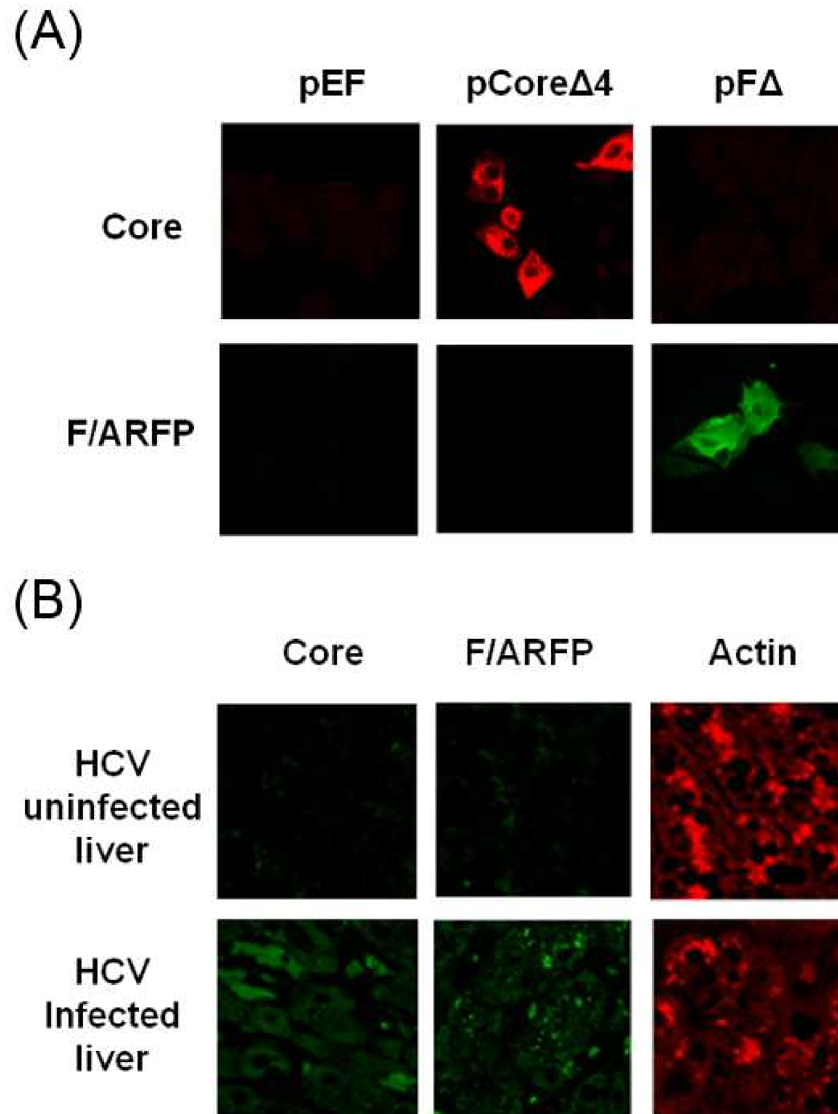


Figure 3-6. F/ARFP detection in human liver samples. (A) Huh7 cells were transfected with 2 μg of pEF, pCoreΔ4, or pFΔ and analyzed for HCV core and F/ARFP proteins by immunofluorescence using corresponding antibodies. (B) HCV-infected and HCV-uninfected human liver samples (n = 3 for infected, n = 2 for uninfected) were analyzed for HCV core, F/ARFP, and actin proteins by immunofluorescence using corresponding antibodies.

3.2.7 F/ARFP Level Comparison between Human Liver Samples and pHA-F-transfected Huh7 Cells

We compared the levels of F/ARFP in human liver samples and the ones in pHA-F-transfected Huh7 cells to identify the physiological level of F/ARFP expression in human liver infected with HCV. As shown in **Figure 3-7 (A)**, the average F/ARFP intensity measured by ImageJ software in human liver samples was comparable with the average F/ARFP intensity in Huh7 cells transfected with 0.5 µg of pHA-F. On the other hand, the core intensity calculated by ImageJ software in human liver samples was similar to the level in Huh7 cells transfected with 0.5 µg of pCoreΔ4. This gives us an idea of how much core protein and F/ARFP may be present in human liver infected with HCV.

We also tested whether F/ARFP suppressed type I IFN responses in a concentration dependent manner and whether these levels were comparable to the levels of F/ARFP in human liver samples. Data showed that F/ARFP suppressed type I IFN responses as a function of dose. (**Figure 3-7 (B)**) F/ARFP levels that suppressed poly(IC)-induced IFN response (i.e., 0.1 - 0.5 µg of pHA-F, per 3 x 10^5 cells) were comparable to the levels of F/ARFP oberved in human livers infected with HCV ($P > 0.05$, **Figure 3-7 (A), (B)**). Taken together these data suggest that HCV F/ARFP can decrease type I IFN responses at levels not significantly different from F/ARFP detection in vivo.

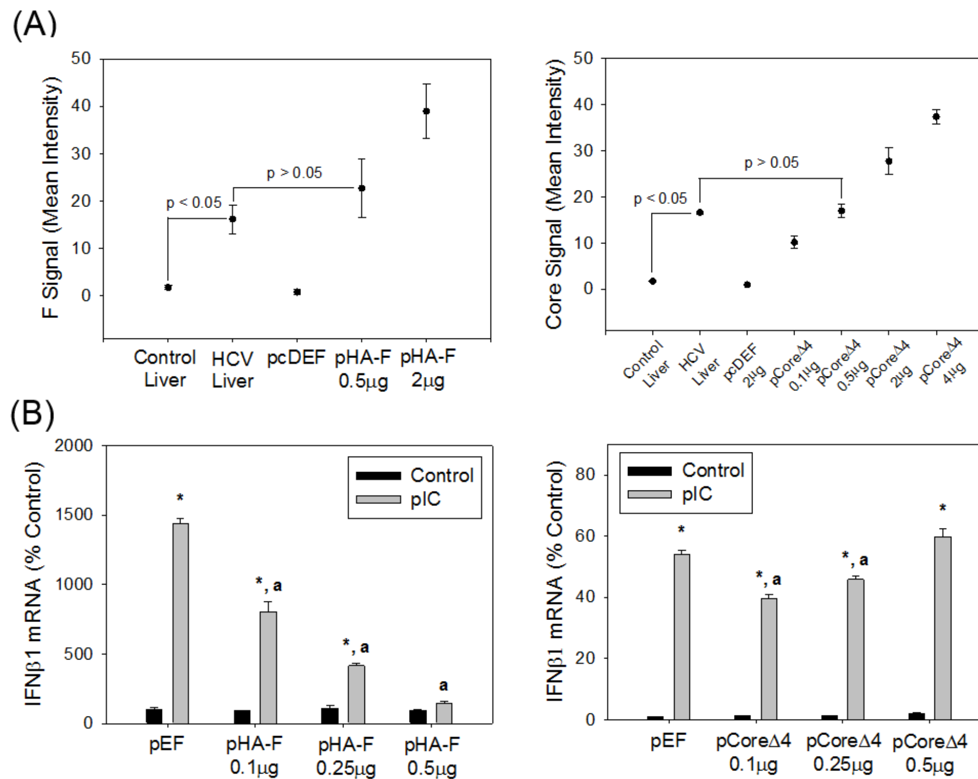


Figure 3-7. F/ARFP level comparison between human liver samples and pHA-F-transfected Huh7 cells. (A) HCV-infected and HCV-uninfected human liver samples (n = 3 for infected, n = 2 for uninfected) were analyzed for HCV core, F/ARFP proteins by immunofluorescence using corresponding antibodies. Immunofluorescence images were analyzed by ImageJ and shown as change in average intensities for each tissue. Lines with P values indicate statistically significance ($P < 0.05$) or no difference ($P > 0.05$) between the groups. (B) Huh7 cells (3×10^5) were transfected with different amounts of pHA-F plasmids. After 24 hrs, these cells were stimulated with poly(IC) and analyzed for IFNβ1 mRNA after another 24 hrs by qRT-PCR. Star indicates statistically significant difference ($P < 0.05$) from the control group. Letter “a” indicates statistically significant difference ($P < 0.05$) from the corresponding pEF control for each -poly(IC) or +poly(IC) group. Data were normalized by GAPDH mRNA. (For (B), n=3)

3.2.8 NS2, NS4B Expression in Huh7.5 Cells with HCV WT and Mutants

To test whether F/ARFP expression affect to the levels of HCV NS2 and/or NS4B, Huh7.5 cells were first electroporated with HCV wild-type (JFH1wt) and three mutants (JFHGND, JFH1Δ, JFH1Δ4). These were analyzed for HCV core, NS2, and NS4B proteins by Western blot using corresponding antibodies. JFH1GND is a mutant defective in replication and is used as negative control. JFH1Δ and JFH1Δ4 are mutants that only express core protein in core-coding sequence without producing F/ARFP by -2/+1 frameshifting in the same region.

As NS2 and NS4B were suggested to suppress IFN responses, the expression level or stability by F/ARFP expression would be important factors to evaluate. [295], [296] Data showed that NS2 and NS4B levels in Huh7.5 electroporated with JFH1wt, JFH1Δ, and JFH1Δ4 were comparable between groups. (**Figure 3-8**) This suggests that F/ARFP expression does not affect to the expression or stability of HCV NS2 and/or HCV NS4B.

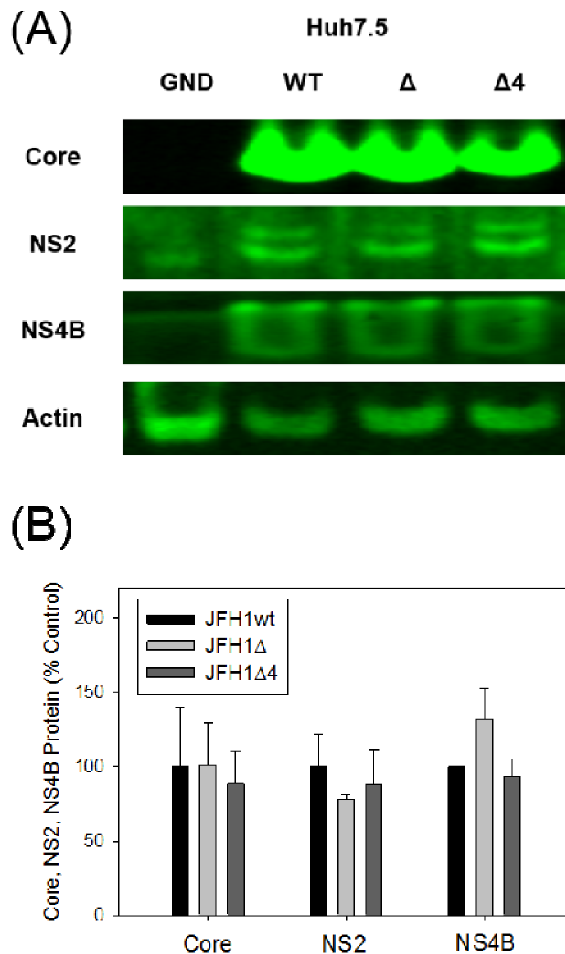


Figure 3-8. NS2, NS4B expression in Huh7.5 cells with HCV WT and mutants. (A) Huh7.5 cells were electroporated with HCV wild-type (JFH1wt) and three mutants (JFHGND, JFH1Δ, JFH1Δ4) and were analyzed for HCV core, NS2, NS4B, and actin proteins by Western blot using corresponding antibodies. This represents the results of two independent experiments. (B) The band intensity from the two independent experiments (n=2) was measured by ImageJ. Data are expressed as percentages with respect to JFH1wt controls. There is no statistically significant difference ($P < 0.05$) compared to corresponding controls.

3.2.9 Full-length JFH1 Alternate Frame Mutants and the Stability of Stem Loop V and VI

Next, we examined whether F/ARFP can regulate type I IFN pathway in the context of natural F/ARFP expression from full-length HCV in cell culture. As F/ARFP appears to be unessential for HCV replication, we generated full-

length JFH1 mutants that F/ARFP frame was disrupted. The predicted frameshift site (codons 8 – 14) and putative -2/+1 frameshift products of the JFH1 strain are shown in **Figure 1-5** and **Figure 2-1**. JFH1Δ has premature termination codons in the -2/+1 frame at codons 30 and 31 (UCG:UAG and UUG:UAG, respectively, **Figure 2-1**) that are designed to truncate F/ARFP at codon 29 without affecting the amino acid sequence of the zero frame. (**Figure 2-1, Table 2-1**) The nt. substitutions did not influence the synthesis of ARF/DS and Core+1/S, but affected the expression of ARF/26(+1). Although the nt. substitutions did not disrupt the overall structure of stem loops V and VI, there was slight destabilization of the base of SLVI which decreased ΔG of SLVI from -36 kcal/mol in the JFH1wt sequence to -30.40 kcal/mol in JFH1Δ based on RNA secondary structure prediction via Mfold. (**Figure 3-9**) On the other hand, JFH1Δ4 contained a single stop codon (UUA:UAA) in the -2/+1 frame at codon 22 which only disrupt the synthesis of F/ARFP without affecting to the synthesis of core, ARF/DS, Core+1/S, ARF/26(+1) or other potential protein products of the -2/+1 frame. (**Figure 2-1, Table 2-1**). JFH1Δ4 did not destabilize SLV or SLVI which is supported by the fact that ΔGs of SLV for both JFH1wt and JFH1Δ4 were not changed by the nt. substitution. (ΔG of -19.70 kcal/mol for both JFH1wt and JFH1Δ4 SLV by Mfold)

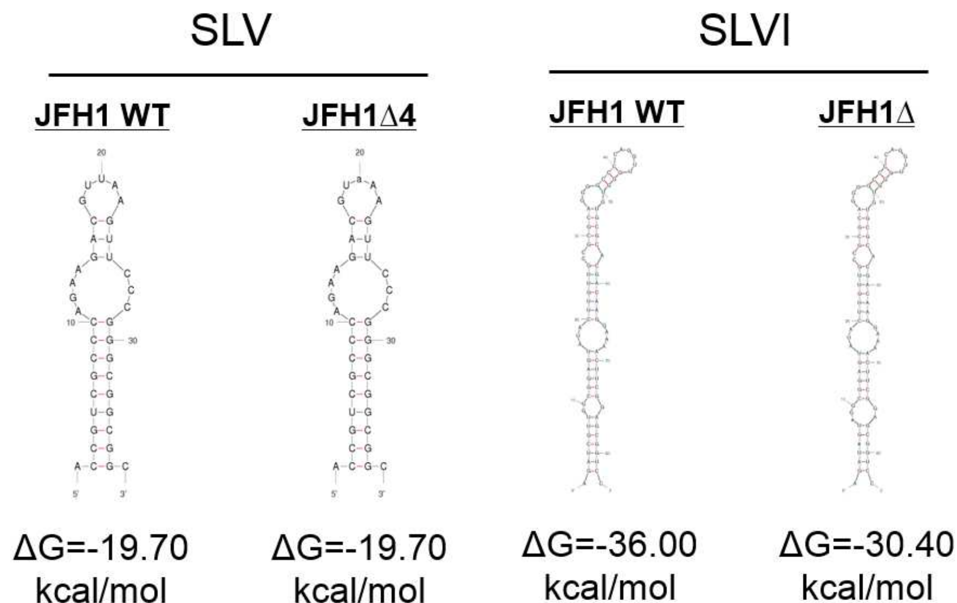


Figure 3-9. Full-length JFH1 alternate frame mutants and the stability of stem loop V and VI. To test whether the nt. substitutions for the JFH1 mutants affected the stability of SLV and SLVI, ΔGs for SLV and SLVI of JFH1wt, JFH1Δ, or JFH1Δ4 were measured by Mfold.

3.2.10 JFH1Δ and Δ4 Replication Measured by Intracellular HCV RNAs

To test whether the absence of F/ARFP affected the replication of JFH1 mutants, equal amounts of positive-sense JFH1wt or JFH1 mutant RNA transcripts were transfected into Huh7 cells. The cells were collected at different time points and analyzed for viral replication by qRT-PCR. Mock transfected cells or JFH1GND RNA-transfected cells were used as negative controls. JFH1wt replicated for at least 19 days in Huh7 cells. (**Figure 3-10 (A)**) For mock transfected Huh7 cells, viral RNA levels were below detection limit. For JFH1GND transfected Huh7 cells, on the other hand, viral RNA levels were between approximately 3 x 10^6 copies/μg (~2 – 3 % of JFH1wt) and below the detection limit at 24 hrs. As shown in **Figure 3-10**, both JFH1Δ and JFH1Δ retained the ability to replicate without significant difference compared to JFH1wt up to at least 19 days. For the evaluation of viral RNA levels, qRT-PCR as well as Northern blot were employed.

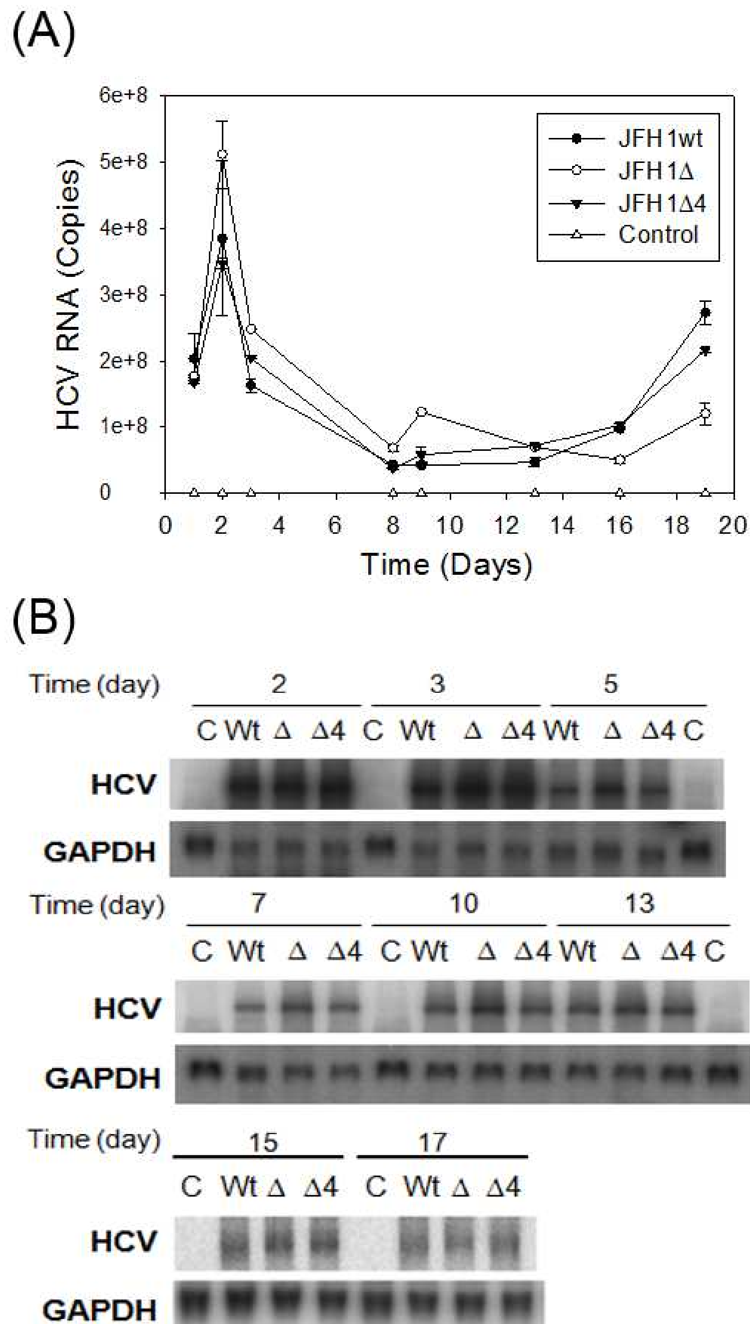


Figure 3-10. JFH1Δ and Δ4 replication measured by intracellular HCV RNAs. (A) Huh7 cells were transfected with JFH1wt, JFH1Δ, JFH1Δ4, or no RNA (mock) and analyzed for intracellular HCV RNAs by qRT-PCR. Data were shown as copies per μg total RNA. (B) Huh7 cells were transfected with JFH1wt, JFH1Δ, JFH1Δ4, or no RNA (mock) and analyzed for intracellular HCV RNAs by Northern blots. GAPDH mRNA was analyzed as control. (n=3)

3.2.11 JFH1Δ and Δ4 Replication Measured by Minus Sense HCV RNAs

We further tested the replication ability of both JFH1Δ and JFH1Δ4 by measuring intracellular minus sense HCV RNAs and intracellular HCV RNAs collected from the cells infected with the medium containing infectious viral particles. Both were the evidences to support the fact that active viral replication occurs in the cell.

As shown in **Figure 3-11**, both JFH1Δ and JFH1Δ4 showed the ability to replicate without significant difference compared to JFH1wt up to at least 19 days. For the evaluation of viral RNA levels, qRT-PCR was used. Together with the results from **Figure 3-10**, we found that JFH1wt and mutants generally replicated similarly except that the replication trends of JFH1Δ4 followed those of JFH1wt better than JFH1Δ did. (**Figure 3-10, Figure 3-11**) JFH1Δ showed slightly higher viral RNA levels than JFH1wt at earlier time points. (**Figure 3-10, Figure 3-11**) On the other hand, JFH1wt showed slightly higher levels of viral RNA than JFH1 mutants at later time points. When Huh7 cells were infected with JFH1wt and mutants, viral RNA levels were more similar between mutants than with JFH1wt. (**Figure 3-11 (B)**).

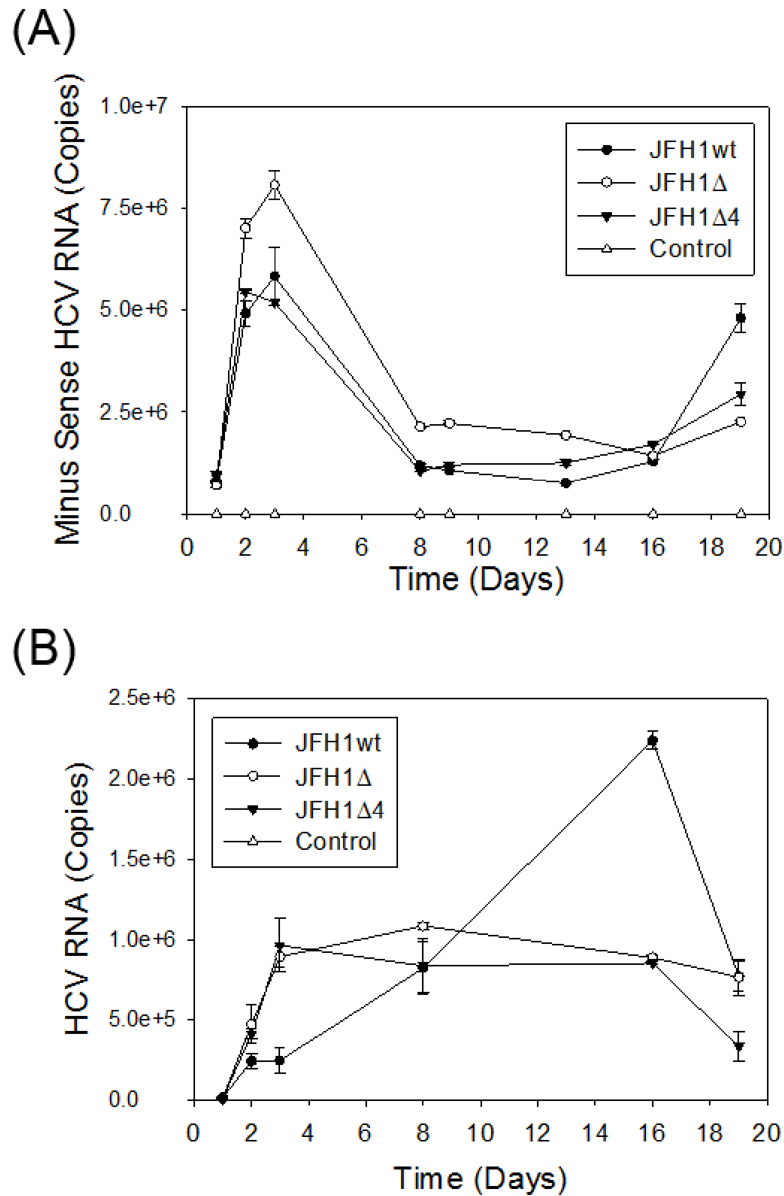
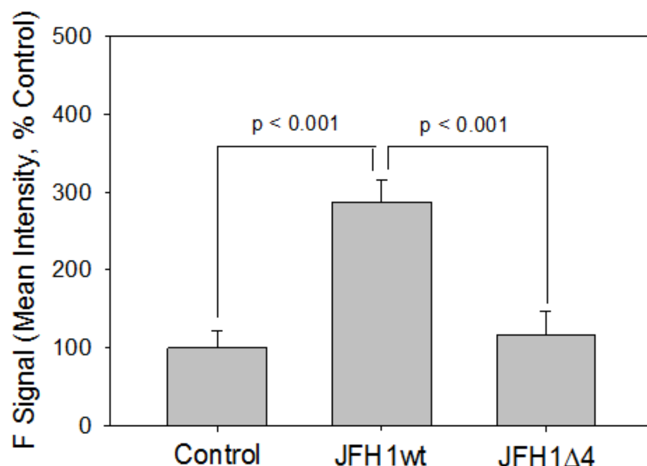


Figure 3-11. JFH1Δ and Δ4 replication measured by minus sense HCV RNAs. (A) Huh7 cells were transfected with JFH1wt, JFH1Δ, JFH1Δ4, or no RNA (mock) and analyzed for intracellular minus sense HCV RNAs by qRT-PCR. Data were shown as copies per µg total RNA. (B) Huh7 cells were infected with medium collected from HCV RNA transfected cells at times indicated and measured for HCV RNA level after 48 hrs by qRT-PCR. Data were shown as HCV RNA copies per µg total RNA. (n=3)

3.2.12 F/ARFP Signal Comparison between JFH1wt and Mutant

F/ARFP signal was measured in JFH1wt and JFH1Δ4 to check the relative level of F/ARFP using immunofluoresence analysis. As expected, higher levels of F/ARFP signal were observed in JFH1wt compared to JFH1Δ4, while the core signal was comparable between JFH1wt and JFH1Δ4. (**Figure 3-12**) On the other hand, frameshift efficiencies, measured by frameshift reporter constructs containing only codons 1 – 14 of JFH1 sequence, were 0.88 ± 0.05 % for the -2/+1 frameshift (versus the negative control containing stop codon, 0.15 ± 0.03, $P < 0.05$) and 0.81 ± 0.07 for the -1/+2 frameshift (versus the negative control containing stop codon, 0.065 ± 0.002, $P < 0.05$). (**Table 2-3**) Frameshift rates ranged from ~0.3 to ~0.9 % for the -2/+1 frameshift.



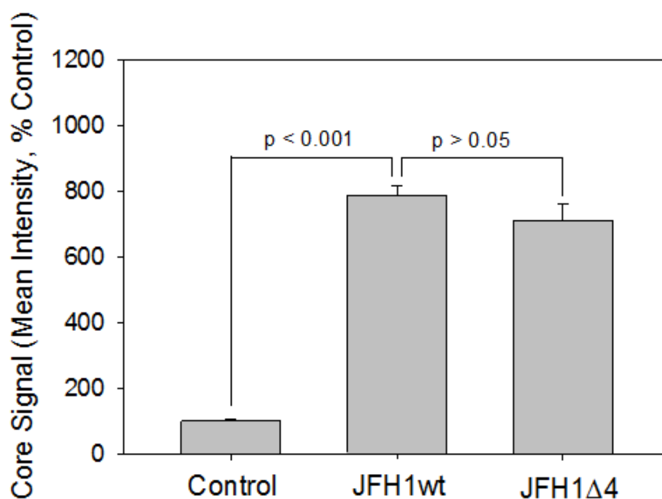


Figure 3-12. F/ARFP signal comparison between JFH1wt and mutant. Huh7 cells electroporated with JFH1wt and JFH1Δ4 were analyzed for core and F/ARFP proteins by immunofluorescence using corresponding antibodies and quantified using ImageJ. Lines with P values indicate statistically significant difference ($P < 0.001$) or no difference ($P > 0.05$) between samples. (For (A), n=5, for (B), n=3)

3.2.13 IFN Induction by the Full Length JFH1 F/ARFP Mutants

Then, we observed whether JFH1wt and the mutants showed different abilities to induce type I IFNs. Huh7 cells were transfected with JFH1wt or JFH1 mutant RNA transcripts and analyzed for IFNβ1 as well as IFNα mRNAs by qRT-PCR. JFH1wt did not increase IFNβ1 or IFNα8 mRNAs, however both JFH1Δ and JFH1Δ4 induced higher levels of IFNβ1 and IFNα8 mRNAs compared to JFH1wt. (**Figure 3-13 (A), (B)**) JFH1wt and mutants showed similar replication levels (**Figure 3-13 (A)**) and this was also observed in previous section. (**Figure 3-10, Figure 3-11**)

These experiments were mostly done within 48 – 72 hrs, but similar effects were shown at later time points. To measure the secreted IFNβ1 protein level in cell culture medium harvested from JFH1Δ and JFH1Δ4-replicating cells, ELISA was used. The result showed that there are modest increases in the amount of IFNβ1 in cell culture medium from JFH1Δ and JFH1Δ4-replicating cells compared to JFH1wt. (**Figure 3-13 (C)**) F/ARFP mutations, therefore, promoted the IFN response of Huh7 cells with replicating HCV and F/ARFP expression was sufficient to suppress IFN responses to HCV RNA PAMP in these cells. (**Figure 3-1, Figure 3-3**)

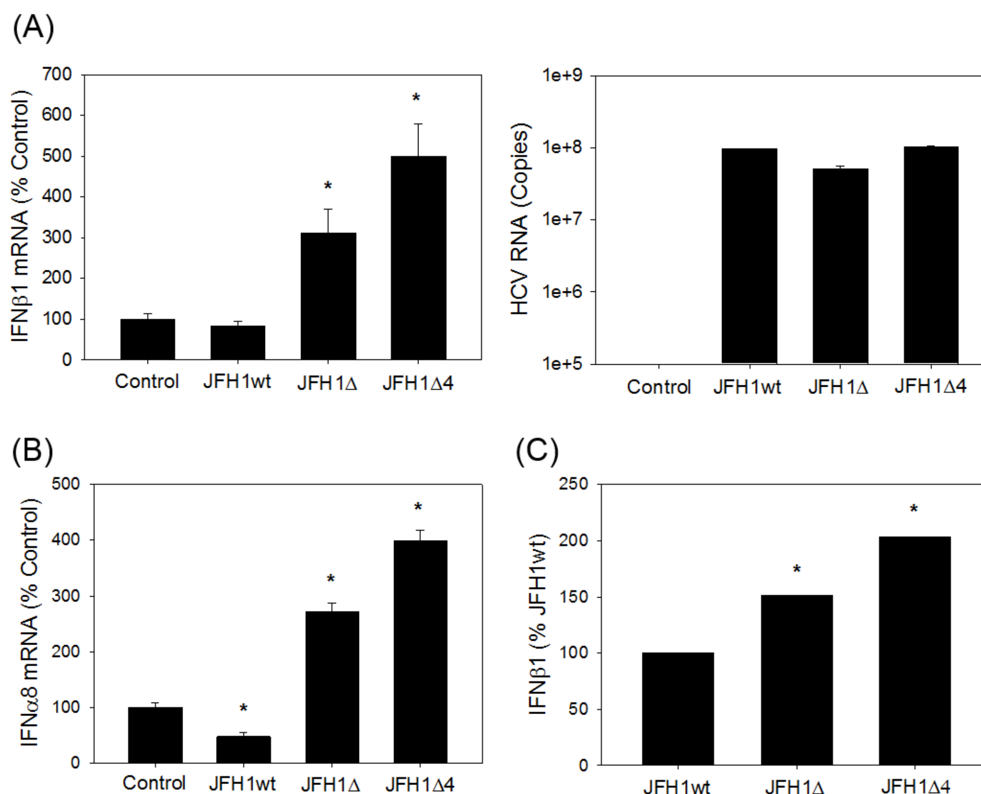


Figure 3-13. Induction of type I IFNs by HCV -2/+1 frame mutants. (A, B) Huh7 cells were transfected with JFH1wt, JFH1Δ, JFH1Δ4, or no RNA (mock) and analyzed for IFNβ1 mRNA, IFNα8 mRNA, or HCV RNA by qRT-PCR. (C) Cell culture media were analyzed for secreted IFNβ1 by ELISA. Star indicates statistically significant difference ($P < 0.05$) from controls. (For (A – B), n=3, for (C), n=2)

3.2.14 IFN Induction in Huh7 Cells Infected by the JFH1 Mutants

We further tested whether the type I IFN induction by HCV -2/+1 frame mutants could be observed in the Huh7 cells infected by the mutants. Cell culture medium harvested from the JFH1wt, JFH1Δ, JFH1Δ4, or no RNA (mock) transfected Huh7 cells were collected and used to infect naïve Huh7 cells. Cells were analyzed for IFNβ1 mRNA by qRT-PCR. Increased levels of IFNβ1 mRNA were observed in Huh7 cells infected with JFH1Δ and JFH1Δ4 compared to JFH1wt. (**Figure 3-14**) Therefore, together with the data from **Figure 3-13**, F/ARFP mutations promoted the IFN response of Huh7 cells both transfected and infected with HCV. (**Figure 3-1, Figure 3-3**).

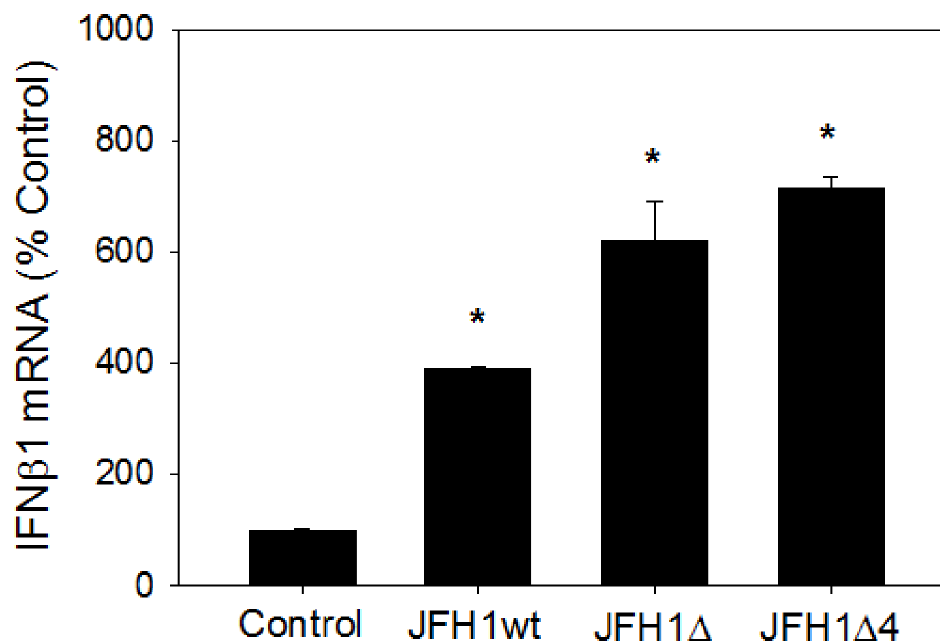


Figure 3-14. IFN induction in Huh7 cells infected by the JFH1 mutants. Cell culture medium harvested from the indicated viral RNA transcript-electroporated Huh7 cells were used to infect naïve Huh7 cells. Cells were collected and analyzed for IFNβ1 mRNA by qRT-PCR. Data were normalized by GAPDH mRNA and expressed as percentage of controls. Star indicates statistically significant difference ($P < 0.05$) from controls. (n=2)

3.2.15 Role of RIG-I Signaling Pathway

It is known that HCV RNA PAMP stimulates IFN pathway through RIG-I in Huh7 cells. (**Figure 3-2**) [410] To examine whether JFH1 mutants acted on this signaling pathway to promote IFN induction, we compared the IFNβ1 mRNA level of Huh7.5 cells, which are defective of functional RIG-I because of T55I mutation. [321], [410] Huh7.5 cells transfected with JFH1Δ and JFH1Δ4 showed the decreased IFNβ1 mRNA level compared to Huh7 cells. (**Figure 3-15 (A)**) We also used RIG-I siRNA to test whether the IFN response enhancement in Huh7 cells by the F/ARFP mutations in JFH1Δ or JFH1Δ4 was dependent on RIG-I. Both IFNβ1 and RIG-I mRNAs were increased in the Huh7 cells transfected with JFH1Δ4 compared to JFH1wt and RIG-I siRNA suppressed JFH1Δ4-associated IFNβ induction. (**Figure 3-15 (B)**) These data suggest that IFN modulation by alternate reading frame mutants are involved in the RIG-I signaling pathway.

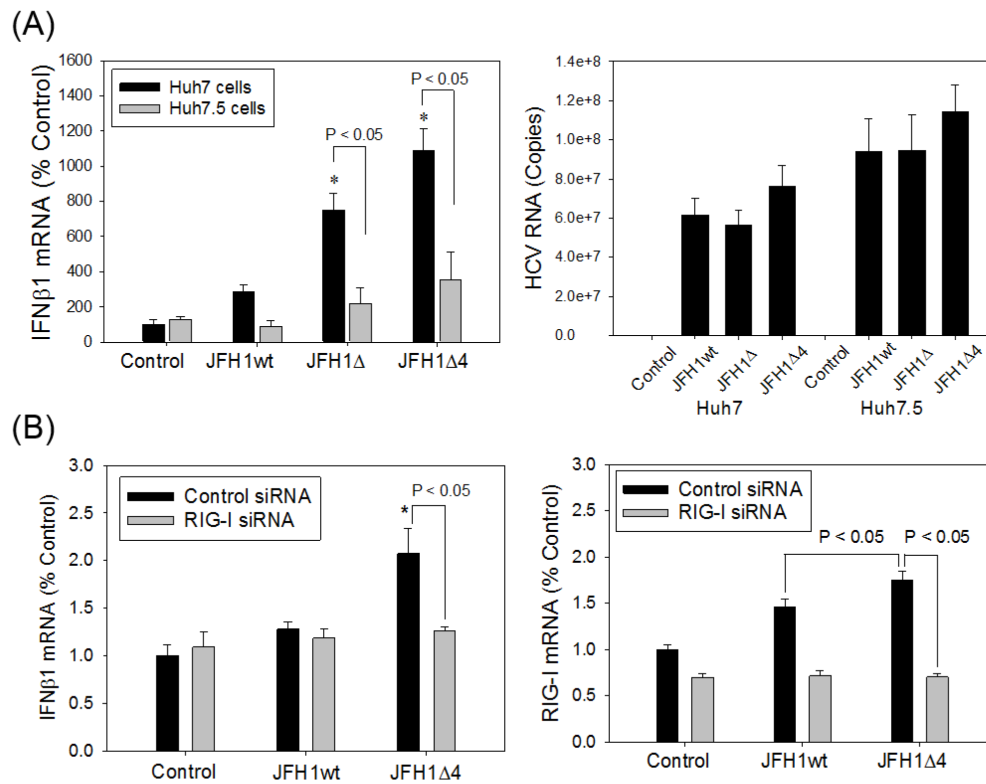


Figure 3-15. Role of RIG-I in the modulation of IFNβ1 by HCV -2/+1 frame mutants. (A) Huh7 and Huh7.5 cells were transfected with JFH1wt, JFH1Δ, JFH1Δ4, or no RNA (mock) and analyzed for IFNβ1 mRNA after 72 hrs by qRT-PCR. (B) Huh7 cells were transfected with RIG-I siRNA for 48 – 72 hrs and then electroporated with JFH1wt, JFH1Δ4, or no RNA (mock). After 24 – 48 hrs, these cells were collected and analyzed for IFNβ1 and RIG-I mRNAs by qRT-PCR. Data were normalized by GAPDH mRNA. Star indicates statistically significant difference ($P < 0.05$) compared to controls. Lines with P values indicate statistically significant difference ($P < 0.05$) between those groups. (n=3)

3.2.16 Antiviral Function of IFN and the JFH1 F/ARFP Mutants

To examine whether the antiviral function of IFNα was influenced by the -2/+1 mutants, we evaluated the effects of exogenous IFNα on JFH1wt, JFH1Δ, and JFH1Δ4 replication in Huh7 cells. As shown in **Figure 3-16**, JFH1Δ and JFH1Δ4 showed similar sensitivity to exogenous IFNα compared to JFH1wt. Both JFH1wt and JFH1 mutants were not resistant to exogenous IFNα as HCV RNA levels were decreased by IFNα treatment. (**Figure 3-16**) Northern blot also confirmed the decreases of HCV RNA levels. (data not shown)

Together with the data from **Figure 3-15**, HCV F/ARFP suppresses IFN production by aiming RIG-I pathway in Huh7 cells, without substantially affecting the antiviral functions of IFNα.

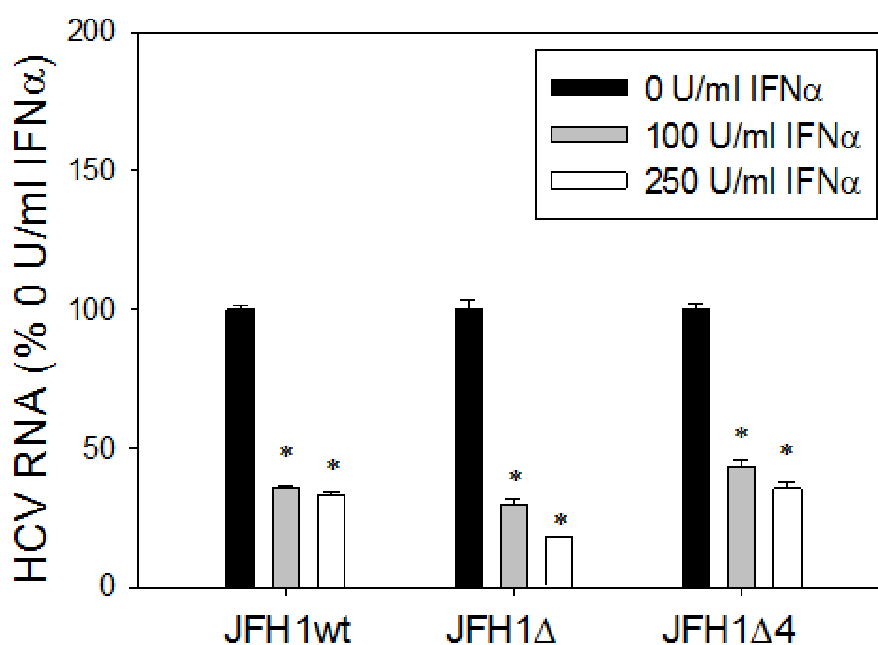


Figure 3-16. Antiviral function of IFN and the JFH1 F/ARFP mutants. JFH1wt, JFH1Δ, and JFH1Δ4-replicating Huh7 cells were treated with 0, 100, or 250 U/mL of exogenous IFNα (NIAID Reference Reagent Repository and Sigma Aldrich) with daily change of cell culture medium and IFNα for 72 hrs. Then, samples were collected and analyzed by qRT-PCR. Data are expressed as percentages with respect to 0 U/ml IFNα controls. Star indicates statistically significant difference ($P < 0.05$) compared to controls. (n=3)

3.2.17 Trans-complementation of JFH1Δ4 with HCV F/ARFP

Next, we tested whether IFNβ1 mRNA induction by JFH1Δ4 was suppressed by adding F/ARFP back. Huh7 cells were transfected with pCoreΔ or pF plasmid and analyzed for IFNβ1 mRNA after electroporating with either JFH1wt or JFH1Δ4 RNA. pCoreΔ contained the same nt. substitutions (stop codon) in the F/ARFP frame as JFH1Δ. JFH1Δ4 induced higher IFNβ1 mRNA level over JFH1wt, but the increase was significantly diminished by pF compared to pCoreΔ. (**Figure 3-17**) Data were normalized by intracellular JFH1 RNA in **Figure 3-17** and showed similar trends when they were not normalized by JFH1 RNA (data not shown). The trans-complementation of JFH1Δ4 with F/ARFP strongly suggests that the enhancement of IFN induction by the F/ARFP mutant results from the absence of F/ARFP.

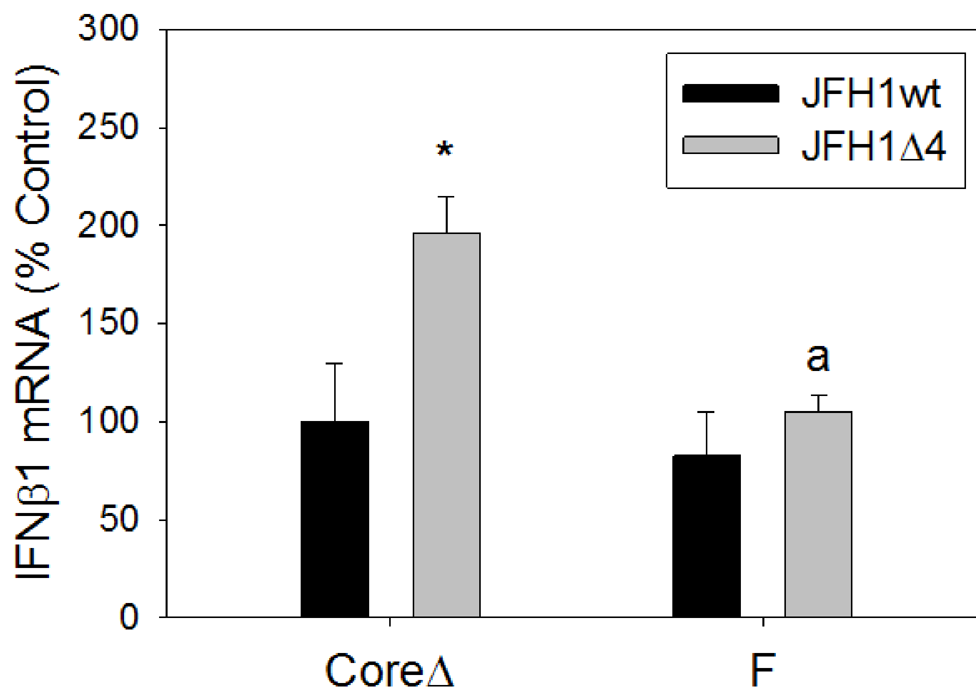


Figure 3-17. Effects of HCV core versus F/ARFP on IFNβ1 mRNA elevation by JFH1Δ4. Huh7 cells were transfected with pCoreΔ or pF and were electroporated with JFH1wt or JFH1Δ4 RNA. These cells were analyzed for IFNβ1 mRNA by qRT-PCR. Data are normalized by JFH1 RNA and GAPDH mRNA levels and shown as percentage of pCoreΔ / JFH1wt transfected control. Star indicates statistically significant difference ($P < 0.05$) from JFH1wt for each plasmid group. Letter “a” indicates statistically significant difference ($P < 0.05$) from pCoreΔ. (n=4)

3.2.18 HCV F/ARFP Translocation by Poly(IC) Stimulation

Finally, we observed whether HCV F/ARFP was translocated into the nucleus by poly(IC) stimulation in Huh7 cells transfected with pHA-F or pFLAG-NS3/4A. Immunofluorescence analysis showed that F/ARFP translocated into the nucleus by poly(IC) in Huh7 cells transfected with pHA-F, while NS3/4A localization did not change by the poly(IC) stimulation in Huh7 cells transfected with pFLAG-NS3/4A. Immunofluorescence was performed using anti-HA or anti-FLAG antibodies. Previously, subcellular fractionation experiment showed that p-IRF3 level in the nucleus was not decreased in the pHA-F-transfected Huh7 cells by poly(IC) stimulation (data not shown). Taken together, this suggests that F/ARFP appears to translocate into the nucleus by HCV PAMP stimulation and may regulate the type I IFN response in the nucleus.

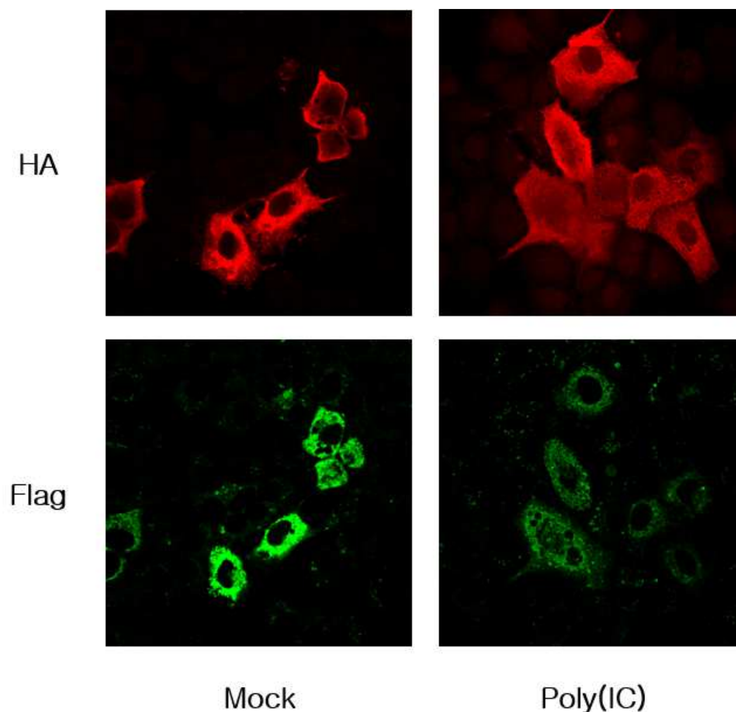


Figure 3-18. HCV F/ARFP translocation by poly(IC) stimulation. Huh7 cells were transfected with pHA-F or pFLAG-NS3/4A for 48 hrs and were again transfected with poly(IC). After 16 hrs, samples were analyzed for HA-F and FLAG-NS3/4A proteins using anti-HA or anti-FLAG antibodies by immunofluorescence.

3.3 Discussion

In this project, we suggest that F/ARFP expression is sufficient to suppress type I and III IFN as well as proinflammatory cytokines responses to RIG-I/MDA5 PAMPs in Huh7 cells. Additionally, mutations affecting the HCV F/ARFP induced IFN responses in Huh7 cells. JFH1Δ4 which has a single nt. substitution promoted the IFN response to HCV and JFH1Δ which has a couple of nt. substitutions also showed similar effects as JFH1Δ4. Moreover, the fact that trans-complementation of JFH1Δ4 with F/ARFP diminished the IFN response enhancement by JFH1Δ4 supported the suppressive role of F/ARFP on type I IFN signaling pathway. Taken together, these data suggest that F/ARFP involved in the suppression of the RIG-I/MDA5-mediated IFN responses in hepatocytes.

There are several viral factors that suppress RIG-I signaling pathway. These factors include NS3/4A, NS2, as well as NS4B. [295], [424]–[426] F/ARFP, therefore, is likely to cooperate with these factors to further downregulate IFN responses during HCV infection. The nt. substitutions introduced in pCoreΔ, pCoreΔ4, and pF plasmids are located in outside the known HCV RIG-I PAMP regions. [410], [412] Therefore, the nt. substitutions we generated may not affect to the PAMPs. It was recently shown that the amino acids 4 – 14 deletion of HCV core abrogate the suppression of Newcastle disease virus-induced IRF-3 activation by HCV core [427] and further studies may require to confirm whether this was due to the deletion of frameshift signal.

F/ARFP is a basic protein and has been difficult to study because of its short half-life as well as low levels of frameshift rates. Also, the frameshift rates can change in terms of the metabolic context of the cell which is not explained well. [313], [428]–[430] For the unstability of F/ARFP, the secondary RNA structure region appears to be involved. The secondary RNA structure is known to regulate HCV frameshifting. [300], [429] Despite of its short half-life and low frameshifting level, F/ARFP expression has been reported from the majority of hepatitis C patients who showed F/ARFP-reactive antibodies. In addition, in vitro translation of core and genomic HCV RNAs and frameshift assays using Huh7 cells also confirm the F/ARFP expression in the corresponding experimental models. [297], [299]–[301], [431] In this project, we detected F/ARFP expression using anti-F/ARFP antibody in the cells transfected with F/ARFP-expressing plasmid, HCV-infected human liver samples, and JFH1wt-replicating cells. (**Figure 3-6, Figure 3-12**) We showed that the cells transfected with F/ARFP-expressing plasmid produce F/ARFP within the range detected in HCV-infected patient liver.

Our mutational analysis suggests that F/ARFP regulation on the IFN response requires a sequence downstream of codon 29. (**Figure 2-1, Table 2-1**) The effects of Δ4 mutation and F/ARFP production further suggest that other alternate reading frame protein products and SLV/VI are not involved in the suppression of IFN responses by F/ARFP we observed. However, additional studies may require to confirm whether these factors as well as unknown alternate reading frame protein products participate in the suppressive effects on IFN. [300]–[303], [432]

In this project, we showed that the HCV F/ARFP mutants replicated at similar or increased levels to JFH1wt. This allowed us to study the biological role of F/ARFP in terms of complete viral replication. Moreover, this is consistent with the previous studies reporting that the -2/+1 frame of HCV core coding region appeared to be unessential for HCV replication. [318], [433], [434] Small increase observed from JFH1Δ replication can be explained by slight change of SLVI base or absence of cytoskeletal disruption by alternate reading frame products in JFH1Δ, but not JFH1Δ4. [326], [433], [434] Also, IFNs produced by Huh7 cells transfected with JFH1 mutants were not greatly higher than the ones produced by Huh7 cells harboring JFH1wt. The amount of secreted IFN difference did not appear to be significantly influential to the HCV replication because of the presence of multiple HCV viral factors that counteract against the functions of IFN. [420] In addition, standard dideoxy sequencing of fourteen RT-PCR clones produced from JFH1Δ4 RNA collected at day 26 showed no change from the Δ4 mutation we introduced. This suggests that there were no revertants arisen. However, further studies would be required to examine HCV F/ARFP functions in type I IFN pathway modulation using systems that generate physiological levels of IFN besides generating HCV.

The mechanisms of how F/ARFP interferes with IFN responses are yet unknown. However, our study suggests that the step(s) affected by F/ARFP occur at or around IRF-3 in the RIG-I/MDA-5 signaling pathway. RIG-I/MDA-5 signaling pathways are modulated by several mechanisms such as ligand interactions, phosphorylation, ubiquitination, protein degradation, and change of signaling protein expression. IFNβ1 mRNA transcription is regulated by the harmonious cooperation of IRF3, NFκB, and activating transcription factor 2 (ATF2)/c-Jun (alternatively AP-1). [435] Recently, it was shown that HCV alternate reading frame protein products decreased hepcidin transcription through an AP-1 binding site, suggesting that type I IFN as well as proinflammatory cytokine expression can be affected because of AP-1 participation for both the type I IFN and proinflammatory cytokine production. In our study, we observed that p-IRF3 levels in the nucleus had not decreased in the pHA-F-transfected Huh7 cells by poly(IC) stimulation in the subcellular fractionation experiment. (data not shown) Also, HCV F/ARFP was translocated into the nucleus by poly(IC) stimulation in Huh7 cells transfected with pHA-F. (**Figure 3-18**) Therefore, this suggests that F/ARFP appears to translocate into the nucleus by HCV PAMP stimulation and may regulate the type I IFN response in the nucleus by targeting an AP-1 binding site.

Moreover, there are other studies suggesting that F/ARFP modulates p21 expression and some of the NFκB-regulated gene expression. [322], [324] In addition, F/ARFP was shown to interact with the host proteasome [429], suggesting the possibility of the target protein degradation by F/ARFP. The suppression of type III IFNs, IFNλ1 and IFNλ2/3, mRNAs by F/ARFP (**Figure 3-4**) is consistent with the literature showing that type III IFNs are induced through mechanisms identical to the ones driving type I IFN expression. [423] Also, F/ARFP did not significantly affect to the amount of NS3/4A. (**Figure 3-1**).

F/ARFP decreased RIG-I mRNA (**Figure 3-3**) and the RIG-I mRNA level was not changed with F/ARFP expression. The RIG-I mRNA levels were similar in JFH1 F/ARFP mutants lacking PAMP stimulation, suggesting that this is likely due to the result of IFN suppression. Recently, it was shown that the presence of F/ARFP antibody was involved in decreased CpG-induced IFNα production by peripheral blood mononuclear cells and plasmacytoid dendritic cells in choronic HCV patients, suggesting the possible suppressive role of F/ARFP on another PRR signaling pathway. [436] Together, further studies are necessary to explain the precise mechanism of RIG-I/MDA-5 pathway suppression by F/ARFP.

This study suggests another mechanism utilized by HCV to evade host IFN responses and an important role of the alternate reading frame protein in the regulation of host innate immunity by HCV. It will be beneficial to know how HCV F/ARFP cooperates with other viral and host factors to counteract against the host IFN responses during HCV infection. Therefore, our study provides new insights into how HCV evades host innate immune response and points to possible new drug target for therapy.

CHAPTER 4

ROLE OF NAD(P)H OXIDASES IN THE RIG-I/MDA5-MEDIATED IFN RESPONSE IN HEPATOCYTES

4.1 Introduction

The first line of defense against viruses largely depends on restriction of virus replication by pathogen recognition receptors such as RIG-I and MDA5 that transmit signals to induce antiviral genes. Although recent studies have increased our understanding of how antiviral signaling mechanisms operate in various systems, much remains to be known concerning molecular mechanisms involved in the transduction of signals from viral recognition to antiviral gene expression. The activation of transcription factor IRF3, NFκB, and AP-1 prompts the cells to enter an antiviral state through the expression of type I IFN genes which modulate protein synthesis, growth arrest, and apoptosis. For IRF3, NFκB, and AP-1 activation, a set of phosphorylation events are required to occur, which results in the nuclear translocation of IRF3, NFκB, and AP-1.

ROS such as superoxide and hydrogen peroxide are considered to function as cellular switches for signaling cascades which regulates gene expression involved in cell proliferation, apoptosis, and immune responses. [437], [438] ROS control signaling cascades via redox-sensitive phosphorylation. Examples of oxidation-regulated proteins are protein tyrosine phosphatases, phosphatase and tensin homolog, mitogen-activated protein kinase phosphatases, and so on. [348]–[350] Oxidation of the thiol residues by hydrogen peroxide is reversible, which is defaulted by thioredoxin or glutathione.

There are seven members of this family, Nox1-5 and Duox1-2, which show tissue and cell-type specific expression patterns. [388] Nox2 was the first-identified member of this family and is well known for its defensive role against bacterial and fungal pathogens. [439] Aside from the established roles in innate immune functions of macrophages and other cells, NAD(P)H oxidase family enzymes function as an important source of regulated production of ROS in cell signaling and regulation of gene expression.

Previous studies in our lab indicate that Nox enzymes also play a role in HCV pathogenesis. However, the biological role in viral infections of Nox enzymes still remains largely unknown. Here, we hypothesize that Nox1 functions as an antiviral regulator in type I IFN pathway because the preliminary data showed that Nox1 gene expression was increased by poly(IC) stimulation. Therefore, the study objective is to determine the biological function of the Nox enzymes, focusing on Nox1, using virus-producing JFH1 cell culture system. In this project, we present evidence for an antiviral role of Nox1 enzyme in type I IFN pathway. We suspect that Nox1 enhances type I IFN responses initiated by RIG-I and ultimately decreases the HCV persistence in the infected cells. Therefore, our study may provide new insights into how Nox enzymes function as antiviral agents and points to possible new drug target for therapy.

4.2 Results

4.2.1 Nox/Duox mRNA Induction by Poly(IC)

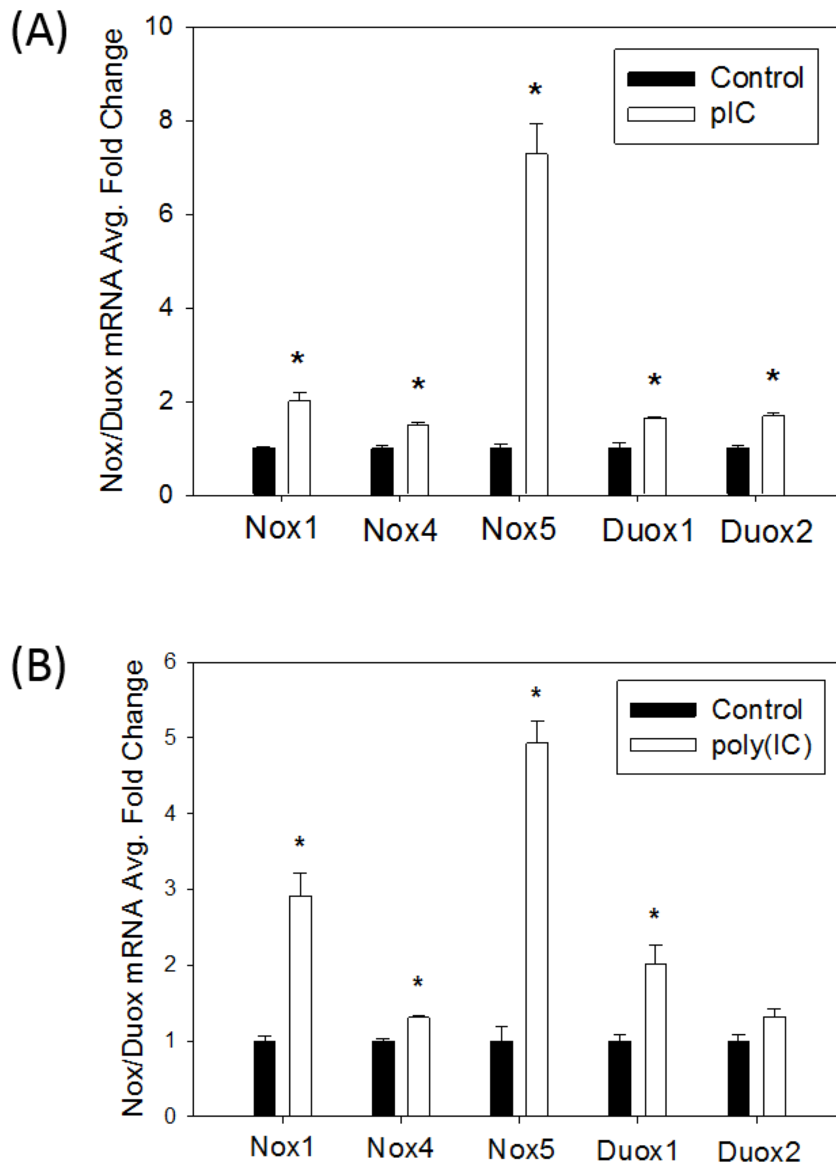
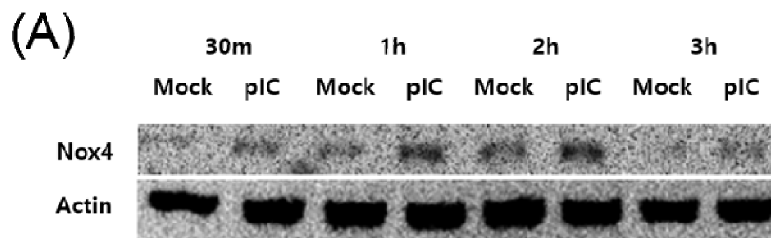


Figure 4-1. Nox/Duox mRNAs are increased by poly(IC). Nox/Duox mRNA levels were measured in (A) Huh7 and (B) telomerase-reconstituted primary human fetal hepatocyte (FH) cells by qRT-PCR after stimulation with poly(IC) for (A) 48 hrs and (B) 24 hrs. Data are normalized by GAPDH mRNA. Star (*) indicates statistically significant difference ($P < 0.05$) from the corresponding control. (n=3)

To test the role of Nox/Duox enzymes in viral infection, we first measured Nox/Duox mRNA level in poly(IC) stimulated Huh7 human hepatoma cells. Results showed that poly(IC), a synthetic double stranded RNA which mimics viral PAMP, elevates Nox1, Nox4, Nox5, Duox1, and Duox2 mRNA levels in Huh7 cells (**Figure 4-1 (A)**). Similar effects were seen in non-hepatoma hepatocytes such as telomerase-reconstituted primary human fetal hepatocytes (FH) cells (**Figure 4-1 (B)**). This suggests that Nox/Duox enzymes can be induced during viral infection and have a role in viral innate immune response.

4.2.2 Nox4 Protein Induction by Poly(IC)

We also measured Nox4 protein level in poly(IC) stimulated Huh7 human hepatoma cells. Results showed that poly(IC) elevates Nox4 protein level in Huh7 cells in early time points after poly(IC) stimulation (**Figure 4-2**). In another experiment, Nox4 protein elevation was also observed 6h and 24h after poly(IC) stimulation. This suggests that Nox4 enzyme can be induced during viral infection and have a role in viral innate immune response.



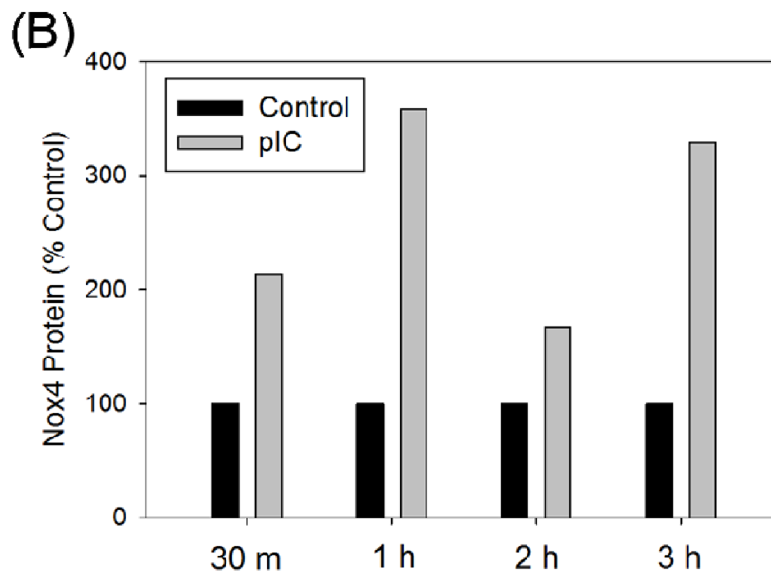


Figure 4-2. Nox4 protein is increased by poly(IC) in Huh7 cells. (A) Nox4 protein level was measured in Huh7 cells by Western blot after stimulation with poly(IC) for 30m, 1h, 2h, and 3h. Actin level was used as loading control. This is one representative result of two independent experiments. (B) The band intensity from (A) was measured by ImageJ. (n=1)

4.2.3 IFNβ, TNFα mRNA Level in DPI Treatment

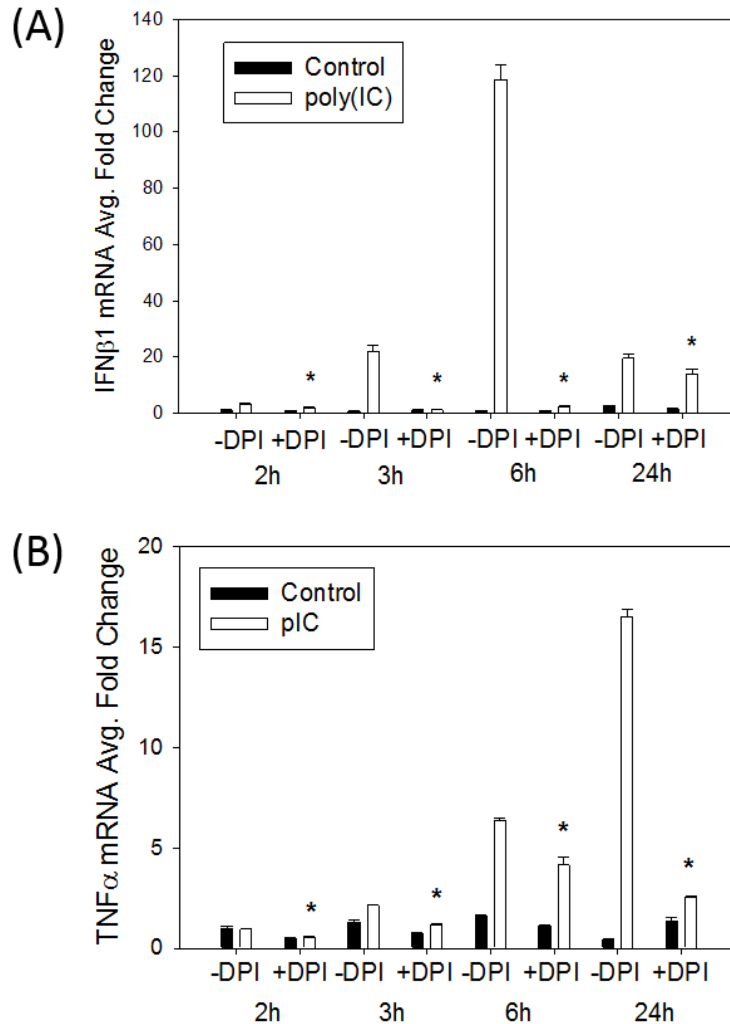


Figure 4-3. IFNβ mRNA level is decreased with DPI treatment. IFNβ1 mRNA expression was assessed by qRT-PCR in Huh7 cells that were pretreated with DPI (diphenylene iodonium) for 30 min and then transfected with poly(IC). Star (*) indicates statistically significant difference ($P < 0.05$) from the corresponding partner. (n=3)

Then we measured poly(IC)-stimulated IFNβ1 mRNA level in DPI pre-treated Huh7 cells in order to see whether Nox/Duox enzymes have a role in type I IFN signaling pathway. DPI is well known for its inhibitory effect on flavoproteins such as Nox/Duox enzymes. [440] If Nox/Duox enzymes function in type I IFN pathway as signal modulators, changes in IFNβ1 mRNA level would be observed. DPI suppressed the poly(IC)-triggered IFNβ1 mRNA induction (**Figure 4-3 (A)**). Similarly, DPI also downregulated the poly(IC)-induced TNFα mRNA elevation (**Figure 4-3 (B)**). TNFα, which is one of proinflammatory cytokines, is regulated by NFκB and/or AP-1 transcription factor(s). This suggests that Nox/Duox enzymes may modulate type I IFN pathway as well as NFκB and/or AP-1 pathway.

4.2.4 IFNβ, TNFα mRNA Level in Nox1 siRNA Transfected Huh7 Cells

Since the effect of DPI treatment suggested possible role of Nox/Duox enzymes in type I IFN pathway as well as NFκB and/or AP-1 pathway, we then started to narrow down specific Nox/Duox proteins that may be involved in this pathway. We first tested Nox1 because our recent studies showed that both Nox1 and Nox4 enzymes play an important role in viral infections including HCV. Also, there are evidences that Nox1 may function as a host defense oxidase. [409]

We measured IFNβ mRNA level in Nox1 siRNA transfected Huh7 cells that are subsequently stimulated with poly(IC). Nox1 siRNA diminished poly(IC) stimulated IFNβ mRNA increase in Huh7 cells, compared to non-targeting control siRNA (**Figure 4-4 (A)**). Similarly, Nox1 siRNA suppressed poly(IC)-triggered TNFα mRNA induction (**Figure 4-4 (B)**). Nox1 mRNA level was decreased by Nox1 siRNA (**Figure 4-4 (C)**). This suggests that Nox1 enzyme may function in the RIG-I/MDA5-mediated IFN signaling pathway as well as NFκB and/or AP-1 pathway and supports a role of Nox proteins in host innate immune response.

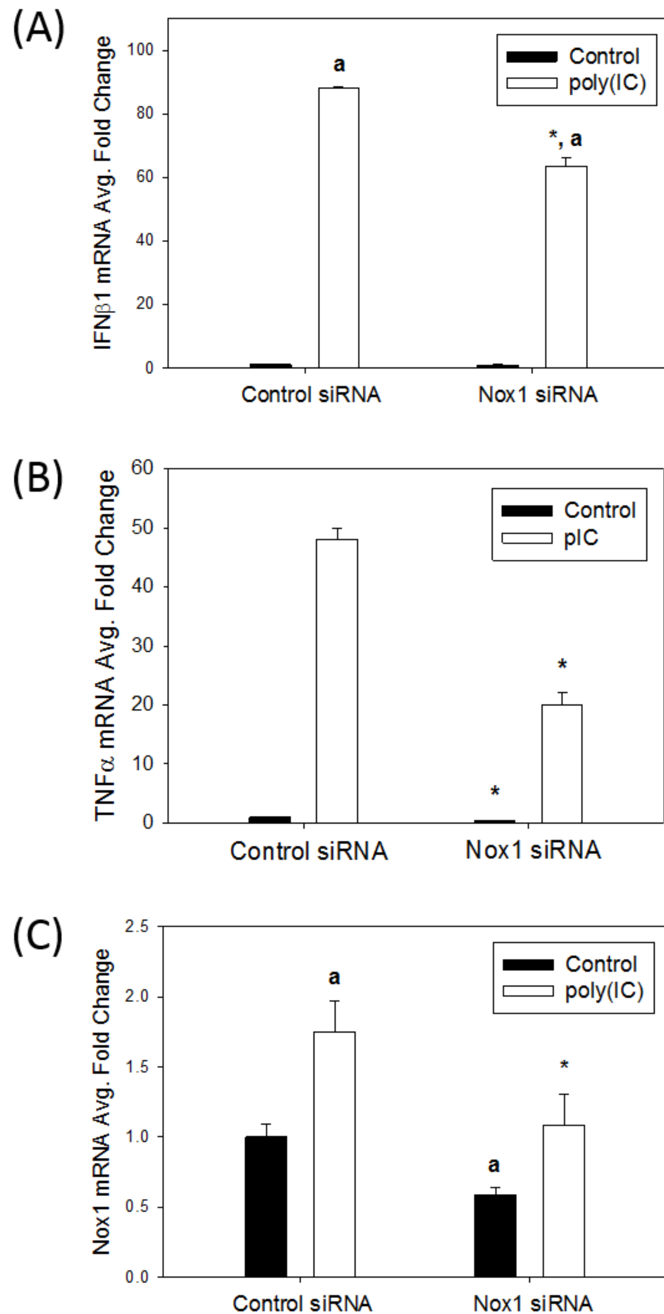


Figure 4-4. Type I IFN and proinflammatory cytokine modulation by Nox1 protein. (A, B) IFNβ1 and TNFα mRNA level were quantified by qRT-PCR in Nox1 siRNA transfected Huh7 cells stimulated by poly(IC). (A) IFNβ1 mRNA, (B) TNFα mRNA, and (C) Nox1 mRNA levels are shown. Data were normalized by GAPDH mRNA. Letter "a" indicates statistically significant difference ($P < 0.05$) from the corresponding control siRNA for each -poly(IC) or +poly(IC) group. (n=3)

4.2.5 Nox5 mRNA Level in HCV Transfected Huh7 Cells Treated with IFNα, IFNγ

Then we tested the effect of IFNα or IFNγ treatment in the presence of HCV in Huh7 cells. As it is known that Nox/Duox expression is regulated by IFNγ, our result showed that Nox5 mRNA is increased by IFNγ, but not by IFNα. In addition, the increase was amplified by the presence of HCV (**Figure 4-5**). This suggests that Nox5 can be induced during viral infection and have a role in viral innate immune response.

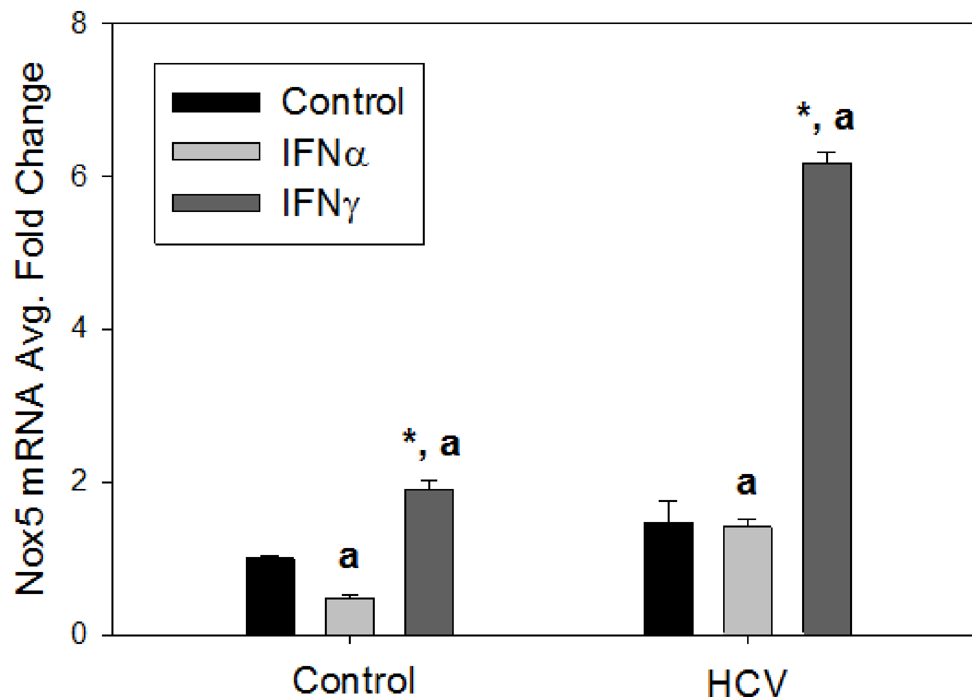


Figure 4-5. Nox5 mRNA induction in JFH1 transfected Huh7 cells treated with IFNα, IFNγ. Nox5 mRNA was assessed by qRT-PCR in JFH1wt transfected Huh7 cells. These cells were incubated with IFNα or IFNγ for 72 hrs before collection. Data are normalized by GAPDH mRNA. Letter “a” indicates statistically significant difference ($P < 0.05$) from the corresponding control. (n=3)

4.2.6 Upregulated HCV Replication by Nox1, Nox4 Knockdown

We also measured HCV replication in Nox1, Nox4 siRNA transfected Huh7 cells. Nox1, Nox4 siRNA transfected Huh7 cells were electroporated with HCV JFH1 strain, and incubated up to day 5. Results showed that HCV replication was increased by Nox1, Nox4 knockdown (**Figure 4-6**). At day 2, HCV replication is 14 fold increased by Nox1 knockdown and 2.5 fold elevated by Nox4 knockdown. This suggests that Nox1 and Nox4 may function as antiviral factors since HCV replication is significantly increased by Nox1, Nox4 knockdown.

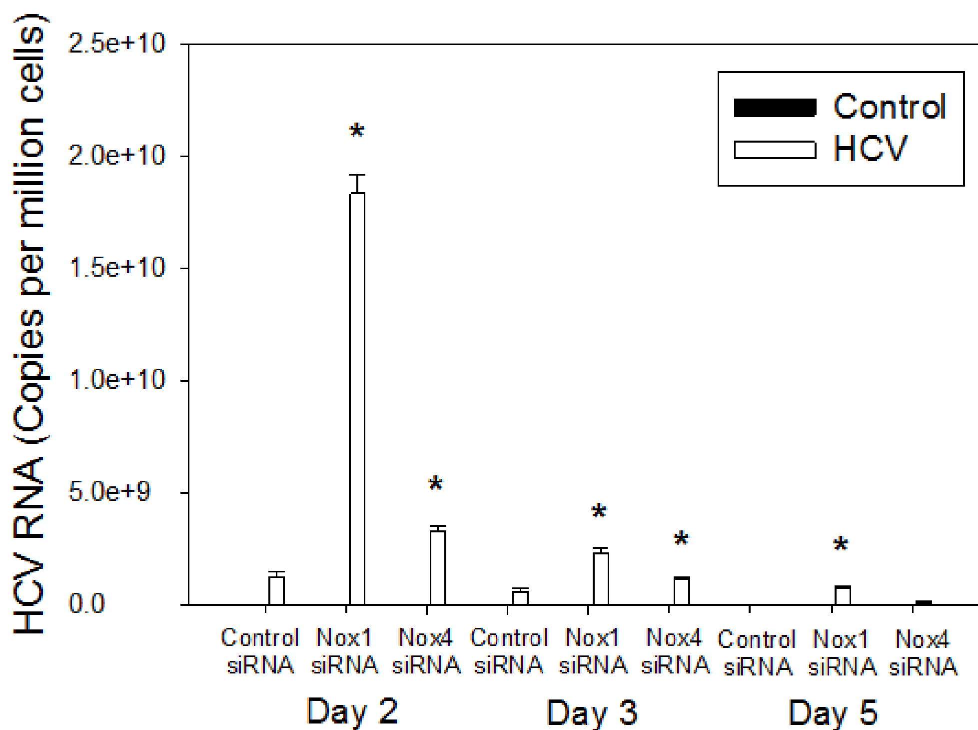


Figure 4-6. HCV replication is increased by Nox1, Nox4 knockdown. HCV RNA level was measured in Nox1, Nox4 siRNA transfected Huh7 cells by qRT-PCR. Star (*) indicates statistically significant difference ($P < 0.05$) from the corresponding control siRNA group. (n=3)

4.3 Discussion

In this project, we suggest that Nox/Duox enzymes in Huh7 cells function in the RIG-I/MDA5-mediated IFN signaling during HCV infections. In our study, the mRNA levels of Nox/Duox enzymes including Nox1, Nox4, Nox5, Duox1, and Duox2 were elevated by poly(IC), a synthetic double stranded RNA which mimics viral PAMP. (**Figure 4-1**) This trend was consistent with the protein level of Nox4 which was increased by poly(IC) at early times. (**Figure 4-2**) Nox/Duox mRNA induction by poly(IC) was not only observed in Huh7 human hepatoma cells, but also in telomerase-reconstituted primary human fetal hepatocytes. Together, this led us to hypothesize that Nox/Duox enzymes can be induced during viral infection and may modulate type I IFN pathway.

Previously, numerous studies regarding innate immune role of Nox/Duox enzymes are mainly focused on Nox2. It was shown that Nox2 had an important antibacterial role by generating large amounts of ROS, known as oxidative burst, in phagocytic cells. [441] However, Nox/Duox involvement in antiviral innate immune defense has not been vigorously studied until recently. Previously, Nox2 was shown to mediate HIV Tat-induced JNK activation and cytoskeletal rearrangement in HUVEC. [442] Also, Nox2 was suggested to mediate Paramyxoviridae virus-induced NF-kB activation and downstream proinflammatory cytokines production in airway epithelial cells (AEC). [443] More recently, Soucy-Faulkner et al. suggested that Nox2 and ROS are required for the host cell to induce an efficient RIG-I-mediated IRF-3 activation and downstream antiviral IFNβ1 and ISG56 (or IFIT1) gene expression. In addition to Nox2, Nox1 was also shown to be involved in the process of viral infection. Nox1 mediated Rhinovirus-induced barrier dysfunction in polarized airway epithelial cells. [444] Interestingly, Nox appears to be involved in IRF3 activation following TLR4 stimulation by LPS in U373/CD14. [445] In this study, Nox4 seems to play a role in an ASK1/p38 pathway that results in the accumulation of IRF3. Although it was not specified which Nox was involved, there have been studies suggesting that other Nox and/or Duox may also have a role during virus infection. Other reports suggested that IRF3 was activated by Nox enzyme(s) during respiratory syncitial virus (RSV) infection [401] and Dengue virus (DENV) infection accumulated ROS generated by Nox enzymes to mount innate immune responses in DENV-infected dendritic cells. [402]

In our study, we showed that poly(IC)-triggered IFNβ1 mRNA induction was suppressed by DPI, a flavoprotein inhibitor, in Huh7 cells. (**Figure 4-3**) Further study suggested that Nox1 siRNA-transfected Huh7 cells decreased poly(IC)-stimulated IFNβ1 mRNA induction compared to non-targeting control siRNA-transfected Huh7 cells. (**Figure 4-4**) Moreover, HCV replication was increased by Nox1 and Nox4 knockdown by corresponding siRNAs. (**Figure 4-6**) Taken together, these data suggest that Nox1 and/or Nox4 enzymes may modulate type I IFN pathway. Our study may provide new insights about antiviral roles of Nox/Duox enzymes because there have been few studies regarding Nox1 and Nox4.

Nox enzymes are well known sources for cellular ROS generation in response to a number of stimuli. Over the past years, there have been many studies to explain the biological function of Nox enzymes in the context of different biological processes including cell proliferation, apoptosis, proinflammatory responses, and cellular switches that regulate signal transduction pathways. [437], [438] In our study, except for the Nox1 and/or Nox4 participation in type I IFN pathway, these Nox enzymes are also involved in the NFκB and/or AP-1 pathway because Nox1 siRNA diminished poly(IC) stimulated IFNβ1 and TNFα mRNA increase in Huh7 cells compared to non-targeting control siRNA. (**Figure 4-4**) Therefore, Nox/Duox enzymes appear to have a role in type I IFN pathway as well as proinflammatory cytokine pathway.

Although there are several evidences suggesting that Nox/Duox enzymes may regulate type I IFN pathway as well as proinflammatory cytokine pathway, much remains to be explained how these enzymes modulate the signaling pathways. Recently, a role of ROS in RIG-I/MDA5 signaling pathway was explained in Atg52/2 MEF cells that are defective in autophagy process. ROS increased RIG-I/MDA5-mediated cytokines production in these cells. [446] However, it is not yet clear how this ROS-dependent mechanism is associated with RIG-I/MDA5 signaling pathway regulation in detail. It was shown that Nox2 knockdown changed phosphorylation of multiple phosphoacceptor sites including Ser386, Ser396, and Ser398 in the C-terminal region of IRF3. Also, previous studies demonstrated that NOX2 was necessary for TBK1 catalytic activation and IKKε expression. [447], [448] Therefore, ROS generated by Nox/Duox enzymes may be directly or indirectly involved in the phosphorylation of multiple phosphoacceptor sites of IRF3 as well as other mediators to regulate IRF3, NFκB, and/or AP-1 pathways.

CHAPTER 5

CONCLUSION & FUTURE DIRECTIONS

5.1 Conclusion & Future Directions

In this projects, we suggest that F/ARFP expression is sufficient to suppress RIG-I/MDA5-mediated type I IFN pathway in Huh7 cells and at least Nox1 enzyme among seven Nox/Duox family modulates RIG-I/MDA5-mediated type I IFN pathway in Huh7 cells. Additionally, F/ARFP was shown to suppress type III IFN as well as proinflammatory cytokine pathways and Nox1 was also suggested to participate in proinflammatory cytokine pathways.

There are several viral factors that suppress RIG-I signaling pathway. These factors include NS3/4A, NS2, as well as NS4B. [295], [424]–[426] In this project, therefore, we suggest that F/ARFP is likely to cooperate with these factors to further downregulate IFN responses during HCV infection. The mechanism how F/ARFP interferes with IFN responses are not yet known. However, our study suggests that the step(s) affected by F/ARFP occur at or around IRF-3 in the RIG-I/MDA-5 signaling pathway. Type I IFN expression is regulated by the harmonious cooperation of IRF3, NFκB, and AP-1. [435] Recent study showing that HCV alternate reading frame protein products decreased hepcidin transcription through an AP-1 binding site suggests that both type I IFN and proinflammatory cytokine expression can be affected by F/ARFP because AP-1 participates both the type I IFN and proinflammatory cytokine production. We observed that p-IRF3 level in the nucleus was not decreased in the pHA-F-transfected Huh7 cells by poly(IC) stimulation in the subcellular fractionation experiment. Also, HCV F/ARFP was translocated into the nucleus by poly(IC) stimulation in Huh7 cells transfected with pHA-F. Taken together, we suggest that F/ARFP appears to translocate into the nucleus by HCV PAMP stimulation and may regulate the type I IFN response in the nucleus by targeting an AP-1 binding site.

This study suggests another strategy used by HCV to evade host IFN responses and an important role of the alternate reading frame protein in the regulation of host innate immunity by HCV. It will be beneficial to know how HCV F/ARFP cooperates with other viral and host factors to counteract against the host IFN responses during HCV infection. Therefore, our study provides new insights into how HCV evades host innate immune response and points to possible new drug target for therapy.

For future studies, luciferase assay will be used to measure transcriptional activity of IRF3, NFκB, and AP-1. Once a target pathway is determined, immunoblots using phospho specific antibody targeting a phosphorylated mediator in IRF3, NFκB, and AP-1 pathway will be performed to confirm whether the pathway is involved in the F/ARFP suppression. Immunofluorescence and translocation experiments will be used for

transcription factors such as IRF3, NFκB in the presence and absence of F/ARF protein. To study the physical interaction(s) between F/ARFP and the possible target(s) in type I IFN pathway or proinflammatory cytokine pathways, immunoprecipitation as well as immunofluorescence staining using F/ARFP with or without HA tag will be employed.

For the project about Nox enzyme, we suggest that Nox/Duox enzymes in Huh7 cells function in the RIG-I/MDA5-mediated IFN signaling during HCV infections. We showed that poly(IC)-triggered IFNβ1 mRNA as well as TNFα mRNA induction were suppressed by DPI in Huh7 cells. Also, Nox1 siRNA-transfected Huh7 cells decreased poly(IC)-stimulated IFNβ1 mRNA as well as TNFα mRNA induction compared to non-targeting control siRNA-transfected Huh7 cells. Moreover, HCV replication was increased by Nox1 and Nox4 knockdown by corresponding siRNAs. Taken together, these data suggest that Nox1 and/or Nox4 enzymes may modulate IRF3 as well as NFκB and/or AP-1 pathways. Our study may provide new insights about antiviral roles of Nox/Duox enzymes because there have been few studies regarding Nox1 and Nox4.

Although there are several evidences suggesting that Nox/Duox enzymes may regulate type I IFN pathway as well as proinflammatory cytokine pathway, much remains to be explained how these enzymes modulate the signaling pathways. Recently, a role of ROS in RIG-I/MDA5 signaling pathway was explained in Atg52/2 MEF cells that are defective in autophagy process. ROS increased RIG-I/MDA5-mediated cytokines production in these cells. [446] However, it is not yet clear how this ROS-dependent mechanism is associated with RIG-I/MDA5 signaling pathway regulation in detail. It was shown that Nox2 knockdown changed phosphorylation of multiple phosphoacceptor sites including Ser386, Ser396, and Ser398 in the C-terminal region of IRF3. Also, previous studies demonstrated that NOX2 was necessary for TBK1 catalytic activation and IKKε expression. [447], [448] Therefore, ROS generated by Nox/Duox enzymes may be directly or indirectly involved in the phosphorylation of multiple phosphoacceptor sites of IRF3 as well as other mediators to regulate IRF3, NFκB, and/or AP-1 pathways.

This study suggests another innate immune role of Nox/Duox enzymes in non-phagocyte cells for mounting antiviral state for the virus infected cells. It will be beneficial to know how Nox/Duox enzymes modulate the type I IFN as well as proinflammatory cytokine pathways to counteract against the viral propagation in the infected cells. Therefore, our study provides new insights into how Nox/Duox enzyme(s) modulate(s) host innate immune response and points to possible new drug target for therapy.

First, which Nox and/or Duox is(are) involved and which Nox and/or Duox positively or negatively regulate the pathway will be continuously studied. As our study indicated that Nox1 may function as a positive regulator in type I IFN pathway, the role of Nox1 will be determined and then the role of Nox4 will be studied next. To test the role of Nox1 and Nox4 enzymes in type I IFN pathway, knockdown or overexpression test will be used by corresponding

siRNAs or plasmids, respectively, as well as Nox inhibitor compounds. After knockdown or overexpression for Nox1 and Nox4 enzymes, poly(IC) or HCV infection will be followed to measure type I IFNs as well as ISGs, proinflammatory cytokines mRNA or protein expression. Also, whether Nox1, Nox4 proteins and activity levels are increased by RNA PAMPs will be observed. ROS levels will be measured by activity assays such as lucigenin-enhanced chemiluminescence, dihydroethidium, and cytochrome c reduction assays. These assays will measure NADPH-dependent ROS generation such as hydrogen peroxide or superoxide anion. The mechanism of how Nox1 and Nox4 enzymes regulate the RIG-I/MDA-5 pathway will be identified. Nox1 and Nox4 enzymes may modulate the redox status for nearby environment which in turn regulates the phosphorylation of mediator(s) in type I IFN pathway and proinflammatory cytokine pathways. Therefore, immunoblots will be used to measure phosphorylation status of phosphor-mediators in type I IFN pathway and proinflammatory cytokine pathways after Nox1 or Nox4 gene is knocked down with corresponding siRNAs. Immunofluorescence and translocation experiments will be performed for transcription factors such as IRF3, NFκB and AP-1. Luciferase assay using IRF3, NFκB, or AP-1 promoter containing plasmids will be used for measuring transcriptional activity of those transcription factors.

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