Role of Hepatitis-C Virus Non Structural Proteins in the Induction of Insulin Resistance

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2009-NUST-DirPhD-V&I-42

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Dedicated

To

My Parents

TABLE OF CONTENTS

Chapter -3

Materials and Methods

Chapter -4

Results

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LIST OF ABBREVIATIONS

HCV	Hepatitis C Virus
HCC	Hepatocellular carcinoma
NS5A	Non-structural protein 5A
IRS	Insulin receptor substrate
GSK	Glycogen synthase kinase
FOX	Forkhead transcription factor
PEPCK	Phospho enol pyruvate carboxy kinase
G6P	Glucose-6-phosphatase
DGAT	Diacyl glycerol acyltransferase
ISDR	Interferon stimulated determining regions
IFN	Interferon
IRES	Internal ribosomal entry site
UTR	Untranslated regions
ORF	Open reading frame
PKR	RNA dependant Protein kinase
RdRP	RNA dependant RNA polymerase
PBMC	Peripheral blood mononuclear cells
MAPK	Mitogen activated protein kinase
ROS	Reactive oxygen species
ER	Endoplasmic reticulum
TNF	Tumor necrosis factor
CREB	cAMP response element binding protein
UPR	Untranslated protein
ATF	Activated transcription factor

eIF	eukaryotic initiation factor
ERAD	ER -associated degradation
NAFLD	Non-alcoholic fatty liver disease
HEV	Hepatitis E Virus
NANB	Non-B hepatitis virus
LDL	Low density lipoproteins
VLPs	Very low density lipoproteins
IVDU	Intravenous drug usage
T2DM	Type 2 diabetes mellitus
IR	Insulin receptor
MAPK	Mitogen activated protein kinase
JNK	Jun N-terminal kinase
ERK	Extracellular signal regulated kinase
RNS	Reactive nitrogen species
Nox	Nitrogen oxygen species
C/EBP β	CCAAT/enhancer binding protein-β
IRR	Insulin receptor-related receptors
PTPases	Protein tyrosine phosphatases
PDK	Phosphoinositide dependent protein kinase
DMEM	Dulbecco's modified eagle medium
FBS	Fetal bovine serum
PBS	Phosphate buffer saline
RIPA	Radio immunoprecipitation assay
BSA	Bovine serum albumin

LIST OF TABLES

LIST OF FIGURES

Abstract

Hepatitis C Virus is one of the lethal infections prevailing throughout the world. There are approximately 8.5 million individuals that are infected with this deadly virus in Pakistan. Hepatitis C virus is responsible for acute and chronic viral infection. Chronic hepatitis C virus infection causes persistent inflammation that leads to liver fibrosis, insulin resistance/type 2 diabetes mellitus, liver cirrhosis and finally hepatocellular carcinoma (HCC). There is strong evidence that insulin resistance has a major role in metabolic syndrome, and is a risk factor for increased liver fibrosis in patients with chronic hepatitis C virus infection. However the underlying mechanism of insulin resistance in chronic hepatitis C virus infection is not well known. The present study describes the molecular mechanism of HCV nonstructural protein 5A (NS5A) induced insulin resistance. In this study, we elucidated the molecular mechanism involved in HCV nonstructural protein 5A (NS5A) induced insulin resistance.

In the present study human hepatoma cell line Huh 7.5 was transfected with HCV NS5A (Huh 7.5/NS5A) as well as HCV (JFH-1) genomic RNA was transfected into Huh 7.5 cell line (Huh 7.5/HCV) to discern the effect of HCV and HCV NS5A protein upon modulation of insulin signaling pathway. Here, we demonstrated that an increased serine phosphorylation of insulin receptor substrate-1 ($pSer^{307}$) and Akt ($pSer^{473}$) in Huh 7.5/HCV infected cells compared to mock infected cells. Interestingly, the Huh 7.5/NS5A cell line showed an increased serine phosphorylation of $pSer^{307}$ IRS-1 and $pSer^{473}$ Akt, compared to the mock transfected cells, which is a critical step defining the downstream insulin signaling pathway.

Glycogen synthase kinase-3 (GSK-3), the downstream target of Akt, is known to favor gluconeogenesis. Our results revealed a diminished phosphorylation level of GSK-3 in Huh 7.5/NS5A expressing hepatoma cells compared to the mock transfected cells, thereby favoring gluconeogenesis. Forkhead transcription factor (FOX-01) which is another important downstream target of insulin signaling pathway, was shown to undergo reduced phosphorylation level ($pSer^{256}$) in

Huh 7.5/NS5A expressing hepatoma cells compared to the mock transfected cells. Collectively, these findings suggest a molecular mechanism by which ectopic expression of Huh 7.5/NS5A modulates the insulin signaling pathway at post translational level.

There are several gluconeogenic and lipogenic markers lying downstream to the insulin mediated signaling molecules ($pSer^{307}$IRS-1, $pSer^{473}$Akt, $pSer^{256}$Fox-01 and GSK-3). In this study, we observed that Huh 7.5/HCV infected hepatoma cells as well as ectopic expression of Huh 7.5/NS5A leads to enhanced gluconeogenesis through up regulating the mRNA levels of gluconeogenic genes i.e. Phosphoenol pyruvate carboxy kinase (PEPCK) and Glucose-6-phosphatase (G6P) compared to their controls. In the similar way, an elevated mRNA level of Diacyl glycerol acyltransferase (DGAT), a key lipogenic marker, was also observed in Huh 7.5/HCV infected as well as Huh 7.5/NS5A expressing hepatoma cells compared to their controls.

Based on these results, we deduce a mechanism through which HCV NS5A is potentially capable of modulating the entire insulin signaling pathway at mRNA and post-translational level thereby paving a way towards insulin resistance, a metabolic syndrome.

INTRODUCTION

1.1 Background and Epidemiology of Hepatitis C Virus

Hepatitis C Virus (HCV) is one of the lethal blood borne pathogens targeting hepatocytes for its proliferation and was isolated for the first time in 1989 by the immunoscreening of patient's serum infected with non-A, non-B hepatitis (Farci *et al.*, 2002). It is estimated that approximately 200 million people are affected by this deadly virus (Dixit *et al.*, 2004). This virus is further categorized into six genotypes but the major ones are genotype 1-3. These genotypes are further divided into more than 50 subtypes that differ in their nucleotide sequences by 10-30% while a nucleotide variation of 30-50% is found among genotypes (Hoofnagle, 2002). HCV genotype 1 and 3 are tied to insulin resistance and steatosis respectively. In addition, the viral kinetics reveal that there is 2-3 fold increase responsiveness towards interferon in HCV genotype 2 and 3 as compared to the genotype 1 (Liang *et al.*, 2000)

The prevalence rate of HCV genotype varies with the geographical distribution and identification of the genotype may be helpful in the treatment of disease. However, in certain cases, patients may be infected with multiple HCV genotypes. So far, the maximum number of HCV infected cases is reported in Egypt that is thought to be because of extensive use of parenteral antischistosomal therapy (Frank *et al.*, 2000; Chayama and Hayes, 2011) .

As far as epidemiology of the HCV is concerned, it is linked with some etiological agents including use of un-sterilized needles, dental procedures and unsafe exchange of blood, blood products as well as sharing sharp items including razors etc (Idrees and Riazuddin, 2008). Among various genotypes of HCV, the epidemiological studies have shown that although all of these genotypes have worldwide distribution. However, HCV genotype 3a is prevalent in Pakistan, genotype 1a and 1b is predominant in United states and

Europe while HCV genotype 2 is predominant in Japan, North America and Italy (Zein *et al.*, 1996; McOmish *et al.*, 1994; Nousbaum *et al.*, 1995).

HCV genotypes are often linked with the susceptibility to interferon (IFN) treatment. Many studies reveal the fact that HCV genotype 1 shows greatest resistance against interferon therapy as compared to other genotypes. Current treatment, combinatorial therapy of pegylated interferon and some antiviral drug like *ribavrin*, has improved the sustained virological response nevertheless response rate is also dependant on HCV genotype. Use of the pegylated interferon plus ribavirin among various HCV genotypes revealed that genotype 1 shows least sustained virological response as compared to HCV genotype 2-6 (Chayama and Hayes, 2011). In addition to the genotypes, an important predictor of IFN response is Interferon stimulated determining region (ISDR). While some other studies show that substitutions in ISDR as well as core protein are considered as significant predictors of IFN therapy outcomes (Enomoto *et al.*, 1995).

1.2 Hepatitis C virus and its classification

Hepatitis C virus was initially known as causative agent of non-A, non-B hepatitis virus that prevails worldwide. Later, it was named as Hepatitis C virus and classified as the sole member of genus *Hepacivirus* of family *Flaviviridae* before the identification of HCV homologue virus in dogs i.e. canine *Hepacivirus*. The classical members of this family are dengue virus, yellow fever and tick borne encephalitis virus. It is a positive stranded RNA virus that gets translated into structural and non-structural proteins (Kato, 2001; Bartenschlager and Lohmann, 2000; Kapoor *et al.*, 2011; Moradpour *et al.*, 2007).

1.3 Genome Organization of HCV

HCV genome is composed of about 9600 nucleotides that are flanked between 5´-and 3´-UTR regions. This much genome size of HCV encodes a single open reading frame (ORF) that encodes about 3010 amino acids and gets translated into structural proteins (E1, E2 and core protein) and non-structural proteins (NS2, NS3, NS4a, NS4b, NS5a and NS5b) (Farci *et al*., 2002; Bartenschlager and Lohmann 2000; Reed and Rice, 1998). The basic molecular organization of the HCV genome is similar to the members of family *Flaviviridae* yet certain features makes it different from the rest of family members therefore, put under separate genus *Hepacivirus* (Kato *et al.*, 1990; Reed and Rice 2000).

At the 5´-UTR region of the HCV genome, there is a string of 340 nucleotides acting as internal ribosomal entry site (IRES), facilitating the binding of ribosomes on the initiation codon of ORF and initiates translation (Wang *et al.*, 1993). On the other hand, 5´-UTR region has a highly variable sequences and favors replication of HCV *in-vivo* (Bukh *et al.*, 1999 and Kolykhalovk *et al.,* 2000). This 5´-UTR can be differentiated into four major domains i.e. domain I to IV. The domain I is involved in HCV replication likewise domain II. Novel research also lead to finding that HCV also exploits a cellular microRNA-122 (miR-122) for IRES mediated translation activity (Friebe *et al.*, 2001).On the other hand, 3´-UTR contains a short stretch of variable region containing about 80 nucleotides and a conserved sequence of 80 nucleotides. These conserved sequences are essential for the HCV replication *in-vitro* as well as *in-vivo* (Moradpour *et al.*, 2007) .

After the polyprotein is fully processed, gets cleaved into structural and non-structural proteins. The immediate product of this cleavage is the formation of core protein, an integral component of the nucleocapsid that is involved in various pathological conditions as well as modulates cellular processes (Chen *et al.*, 1997; Moriya *et al.*, 1998). There is an internal

signal sequence between Core protein and Envelope 1 (E1) that targets the nascent protein towards Endoplasmic membrane (ER) and imbalance homeostasis. Signal peptidase, an enzyme that mediates the catalysis of this signal peptide, yields 21kDa fully mature core protein, containing about 179 amino acids (McLauchlan *et al.,* 2002). The N-terminal domain of core protein forms α-helical proteins on the ER membrane and lipid droplets. The interaction of core protein with the lipid droplets is thought to be involved in modulating the lipogenic pathway, associated with steatosis and favors viral induced pathogenesis (Asselah *et al.,* 2006).

In addition to the core protein, envelope proteins (E1 and E2) play a pivotal role in the viral replication and pathogenic mechanisms. These envelope proteins are highly glycosylated proteins and form non-covalent complexes, essential for viral envelope formation. E2 is found to potentially interact and inhibits with PKR, an interferon stimulated gene, thereby decreasing the antiviral activity and favors viral replication and pathogenesis (Deleersnyder *et al.,* 1997 and Taylor *et al.,* 1999). This protein is mainly responsible for viral entry and infection mechanisms (Zhao *et al.,* 2005 and Mazzocca *et al.,* 2005). There is a signal sequence between E1 and E2 that needs to be cleaved for further protein processing. The elucidation of three dimensional structures of these proteins is the key to understand viral entry mechanisms (Dubuisson *et al.,* 2002).

Protein-7 (p7) is a short stretch of highly hydrophobic protein containing 63 amino acids located at the C-terminus of NS2. P7 is provided with two transmembrane proteins connected with a cytoplasmic loop and is directed towards ER lumen. Putative roles of p7 include cation channel activity, productive infection, virion maturation and egress.(Lohmann, Korner *et al.,* 1999), (Carrere-Kremer *et al.,* 2002; Griffin, Beales *et al.,* 2003)

NS2 is a small protein that interacts with its adjacent protein forming NS2/NS3 protease catalyzing site. Three residues (His143, Glu163 and Cys184) have been explicitly found to be involved in proteolytic activity (Grakoui *et al.*, 1993) .

NS3, a sequence of 630 residues, has dual role in HCV replication as it has serine protease activity at its amino terminal and helicase activity at its carboxy terminal (Bartenschlager *et al.,* 1993; Gwack *et al.,* 1996; Kolykhalov *et al.,* 2000; Wolk, *et al.,* 2000).

NS4a acts as a cofactor of NS3 as the proteolytic cleavage of HCV polyprotein by NS3 requires at least 14 residues of NS4a. HCV NS3/4a forms a non-covalent heterodimer that facilitates proteolytic cleavage and viral replication. This association of NS3/4a leads to the polyprotein processing at four distinct positions i.e. NS3- NS4a, NS4a- NS4b, NS4b-NS5A and NS5A- NS5b (Lin *et al.,* 1995; Zhu and Briggs, 2011). This makes N3/4a region as an important part of HCV genome as well as have great attraction towards drug targeting(Shiryaev *et al.,* 2012; Kwong *et al.,* 2011. NS4b is a highly hydrophobic protein that strongly interacts with lipid moieties and thereby favors viral replication (Palomares-Jerez *et al.,* 2012). It has also been found that HCV NS4b is critically involved in establishing viral host interactions that provoke HCV induced pathogenic mechanisms (Li, Yu *et al.,* 2012). As far as the topology of NS4b is concerned, it is categorized into three domains i.e. N-terminal domain (1-69), C- terminal domain (70-190) and a central transmembrane domain (191-261) (Gouttenoire *et al.,* 2009).

NS5A is a phosphorylated protein with 56 kDa (basal form) and 58kDa (hyperphosphorylated form) molecular weight. It is located in the cytoplasm where it is found to be in association with endoplasmic reticulum via its amphipathic α-helix and induces viral replication (Brass *et al.,* 2002; Bartenschlager and Lohmann, 2000). NS5A has a prominent role in HCV induced disease progression primarily because of interferon resistance

mechanisms. PKR is a RNA dependant kinase that gets masked by NS5A, thereby downregulating interferon stimulated genes and leads to interferon resistance (Gale *et al.,* 1998). Furthermore, NS5A protein modulates intracellular signaling that are associated with viral infections (Waris and Siddiqui, 2003). Recent studies revealed the functional importance of NS5A as the key protein in HCV life cycle that acts as the molecular switch between HCV replication and its assembly, primarily because of its phosphorylation state that has strongly affects HCV replication(Moradpour *et al.,* 2007) . Phosphorylation of NS5A is a conserved feature among all *Flaviviridae* members, revealing its strong role in the viral life cycle. Cell culture based adaptive mutations revealed that phosphorylation state of NS5A is centrally located on serine residues (Evans *et al.,* 2004). According to a model proposed by Rice *et al.,*, mutations favoring hyperphopshorylation of NS5A blocks its interaction with human vesicle associated membrane protein- associated protein A (hVAP-A), a target for viral RNA replication, suggesting a condition where adaptive mutations act by blocking phosphorylation dependant dissociation of viral RNA complex (Evans *et al.,* 2004).

NS5b is an RNA dependant RNA polymerase (RdRp) that make use of negative stranded RNA as the template favoring the synthesis of negative stranded RNA (genomic RNA). NS5b of HCV share a common crystal structure of RdRp showing right hand with the palm, thumb and finger domains (Butcher *et al.,* 2001). Based on its polymerase activity, it is one of the important drug targets for anti-HCV treatment (Kronenberger and Zeuzem 2012) and (Di Francesco *et al.,* 2012).

1.4 HCV induced pathogenesis

HCV is a hepatocyte targeting virus that develops from acute to chronic infection with the projected outcome of about 85% patients developing chronic infection. The chronic infection then undergoes a variety of pathophysiological changes in the liver that eventually

turns into hepatocellular carcinoma (HCC). Chronic HCV is usually characterized by fluctuating Alanine transferase level (ALT) (Hoofnagle, 2002). In Pakistan, about 80% of HCV infected patients turn into chronic ones (Idrees and Riazuddin, 2009). After HCV gets entry into the host target cells, it modulates host machinery by way of gene expression, signaling, cell mediated death pathways and escapes from the host defense mechanisms thereby inducing pathogenesis (Chang *et al.,* 2008).

HCV infection is accompanied with a number of pathophysiological disorders including oxidative stress, ER stress, Steatosis, fibrosis, insulin resistance that eventually converge into hepatocellular carcinoma (Emerit *et al.,* 2000).

Oxidative stress is defined as the condition where there is an imbalance between oxidants and antioxidants level, triggering damage to the cells. The oxidants are naturally produced as a result of aerobic metabolism yet elevated levels are associated with the pathophysiological conditions(Sies, 1997). Oxidative stress is considered to be the key modulator in the induction of various HCV induced liver pathologies. HCV induced oxidative stress is characterized by a number of biomarkers found in the patient's serum, peripheral blood mononuclear cells (PBMC) as well as in the liver biopsies (Mahmood *et al.,* 2004). The experimental evidences reveal that oxidative stress and mitochondrial injury play pivotal role in liver injury induced by HCV infection and this may promote steatosis, fibrosis and hepatocellular carcinoma (Okuda *et al.,* 2002; Lonardo *et al.,* 2004). Both Structural and Non-structural proteins of HCV induces oxidative stress and the level of these antioxidants (like MnSOD, HO-1, catalase) varies with HCV proteins highlighting differential potential of structural and non-structural proteins in the induction of antioxidants (Abdalla *et al.,* 2005). Oxidative stress is initiated in response to the activated cellular kinases like JNK and p38 MAPK. During chronic HCV infection, MnSOD increases about five folds while there is no significant change upon the expression of CuSOD(Qadri *et al.,* 2004) . These cellular kinases

get activated in response to certain environmental stimuli including chemical and biological agents like viruses and initiates oxidative stress. As a natural mechanism, this burden has to be overcome by a group of molecules called antioxidants (Like MnSOD and CuSOD). However, in late stages of the disease like cancerous cells, there might be a decrease in the level of antioxidants due to the overburden of oxidants (Johnson and Lapadat, 2002; Kato *et al.,* 2000; Erhardt *et al.,* 2002; St Clair, Porntadavity *et al.,* 2002).

During the course of chronic HCV infection, Reactive oxygen species (ROS) is generated that disrupts the downstream signaling mechanisms and favors the disease progression. As far as ROS is concerned, it is known to be upregulated in response to the deregulated Ca^{2+} signaling in endoplasmic reticulum (ER). Several HCV proteins (Structural and Non-structural proteins) have been found to oxidative stress via upregulation of ROS, thereby favoring the disease progression like development of insulin resistance (Burdette *et al.,* 2010; Waris *et al.,* 2001; Tardif *et al.,* 2005) . *In-vitro* studies highlighted the potential role of HCV structural proteins in the induction of oxidative stress, and as a counter mechanism, there is an upregulation of Tumor necrosis factor-α (TNF- α). This gene has been found to be involved in the modulation of downstream insulin signaling mechanisms by favoring phosphorylation of Insulin receptor substrate-1 (IRS-1) (Banerjee *et al.,* 2008).

HCV targets the hepatocytes and tends to proliferate on the Endoplasmic reticulum membrane. This membranous organelle is provided with numerous scaffoldings, paving a way for the transportation of newly synthesized proteins. It is recognized that both HCV and HBV induce ER stress by mode of transcriptional activity of cAMP responsive element binding protein (CREB) which, in turns, modulates metabolic pathways like insulin resistance. Cells greatly sense the metabolic perturbations through their signaling mechanisms and responds accordingly to overcome the burden, whenever, cells monitor ER stress, there is an upregulation of the untranslated protein response (UPR). The UPR then

triggers the activation of ATF4 and ATF6 pathways leading to the activation of several molecular chaperones like GRP78 and GRP94 to favor the folding of this membranous organelle. It is also evident that UPR causes the upregulation of PERK, a transmembrane protein kinase, phosphorylating eukaryotic initiation factor 2-α (eIF-2α) and inhibits the translation, thereby preventing the lease of misfolded or damaged protein. Furthermore, UPR can also degrade the synthesized proteins via proteosome dependant degradation in a process called ER-associated degradation (ERAD) (Harding and Ron 2002; Christen *et al.,* 2007; Harding *et al.,* 1999; Meusser *et al.,* 2005).

Endoplasmic reticulum stress is believed to be the initial step in the HCV induced pathogenesis. The results have shown that HCV can induce oxidative stress through the metabolic disturbances of intracellular Ca^{2+} levels, thereby favoring the translocation of STAT-3 and NF-kB into the nucleus and favors oxidative stress (Gong, Waris *et al.,* 2001).

Insulin resistance is a complicated metabolic disorder that refers to the pre-diabetic phase with the modulation of insulin signaling at various cellular checkpoints like insulin receptors, Insulin receptor substrate (IRS) and impairment of homeostasis. (Parvaiz *et al.,* 2011). Impaired glucose tolerance and insulin resistance are the leading causes of morbidity and mortality rate worldwide. As a result of this disorder, fat and liver muscles become insensitive to insulin and leads to a condition where there is increased blood glucose level. The results have shown that insulin resistance is a complex array of signaling disorder involving modulation at the insulin receptor level, downstream signaling molecules as well as hormonal dysfunction (Saltiel and Kahn, 2001) .

Patients infected with HCV are at more risk (25%) towards the development of insulin resistance than that of HBV infected patients (10%) (Bugianesi *et al.,* 2005) and (Lecube *et al.,* 2006).The ultimate outcome of HCV infection is hepatocellular carcinoma

that can be the synergistic or alone effect of a numbers of predictors like insulin resistance, alcoholism and hepatitis infection. (Hassan *et al.,* 2002). In another study, diabetic patients have been found to be at more risk (2-3 folds) of developing Hepatocellular carcinoma than the rest of its causative agents (Davila *et al.,* 2005). It is well established that hepatocellular carcinoma can be the result of alcoholism or Non-alcoholic fatty liver disease (NAFLD). Among NAFLD, insulin resistance represents the hallmark of this disease and affects core organs if the body like liver, pancreas, brain and kidney(Kahn, 1978). One of the hallmarks of HCV infections is the development of insulin resistance, yet elucidation of the complete mechanism is still needed. HCV has been shown to induce insulin resistance primarily through interaction with Insulin receptor substrate 1 (IRS-1) and impaired downstream AKT signaling (Del Campo and Romero-Gomez, 2009).

HCV infection has the capacity to modulate the immunological responses like cryoglobulinemia, glomerulonephritis and thyroiditis. Based on that, HCV induced insulin resistance was related to immunological disorders like immune reaction against β-cell of pancreas. However, several studies could not found a significant difference among control and HCV patients (with and without diabetes) for the islets cell antibodies (Lecube *et al.,* 2006; Knobler *et al.,* 2000; Betterle *et al.,* 2000). The plasma glucose level maintains itself within a range of 4-7mM in normal ones. This glucose level is effectively achieved by the coordinated activity of hormones like insulin. Insulin favors the uptake of glucose from muscles, fat tissues and liver by stimulating glycolysis, lipogenesis and protein synthesis with the concomitant inhibition of metabolic pathways like gluconeogenesis, lipolysis and glycogenolysis. Contrary, dysfunction of this hormone triggers the elevated blood glucose level that leads to a condition known as insulin resistance. In the similar way, it has been studied that HCV infection can modulate insulin signaling with the concomitant elevation of

glucose level by inhibiting its uptake, thereby leading towards insulin resistance (Saltiel and Kahn, 2001; Banerjee *et al.,* 2008).

1.5 Aims of the study

HCV Core protein has been studied with reference to the insulin resistance mechanisms. Previous studies have shown that among non-structural proteins, NS5A is the most potent protein that can damage host cellular machinery for viral and disease propagation, can favor oxidative stress and ER stress and interferon resistance. However, there is no conclusive study so far that describes potential role of NS5A in the development of insulin resistance and elucidates its mechanism. This study is a novel in the sense as it clearly depicts the interaction of NS5A with the cellular proteins and how this protein modulates signaling molecules as well as modulation of gluconeogenic and lipogenic pathways for the development of insulin resistance. To further discern the effectiveness of this protein in the context of insulin resistance, we transfected hepatocytes with HCV (JHF-1) as well HCV NS5A of two genotypes i.e. genotype1a and 2a so that a better understanding can be made that could pave a new way towards translational study in the future.

1.6 Objectives of the Study

The objectives of present work are:

- Quantification of targeted genes in HCV infected hepatocytes.
- To analyze transcriptional expression of key genes involved in insulin resistance in HCV NS5A transfected hepatocytes.
- To determine the effect of HCV NS5A on Key insulin signaling molecules using western blot analysis.

LITERTATURE REVIEW

2.1 Viral Hepatitis

The term viral hepatitis is coined for the viruses targeting hepatocytes (liver cells), therefore, also known as hepatotropic viruses. This group of viruses is not new rather it is as old as the beginning of mankind. It includes a diverse group of viruses named as Hepatitis A virus (HAV), Hepatitis B virus (HBV), Hepatitis C virus (HCV), Hepatitis D virus (HDV), Hepatitis E virus (HEV), Hepatitis F virus (HFV) and Hepatitis G virus (HGV). After infection, they can move from acute to chronic infections. As very little is known about HFV and HGV, so hepatitis A through E will be discussed here.

Among this group, HBV and HCV have the highest morbidity and mortality rate yet HCV seems to be more fatal as the mortality rate is projected to increase by about 3 folds in the coming decade. Nature and epidemiology of the hepatotropic viruses leads to much interest in health control programs worldwide (Szabo *et al*., 2003) and (Yen *et al*., 2003). Each of these viruses has been discussed here in detail below.

2.1.1 Hepatitis A Virus

Hepatitis A virus (HAV) was discovered in 1973 and classified under the family *picornaviridae* (Feinstone *et al*., 1975). HAV is an enteric, positive single stranded RNA with the genome size of 7.5kb that shows undergoes little antigenic variations. The genome of HAV is structured as 5´Non-coding region, 3´ Non-coding region, Non structural and Structural proteins (VP1, VP2, VP3 and VP4) (Sanchez *et al*., 2004; Arauz-Ruiz *et al*., 2001; Robertson *et al*. 1992).

HAV targets and replicates in the hepatocytes thereby damaging liver, eliciting immune response and causes liver inflammation. The symptoms are generally obvious in adults while asymptomatic in children (Koff, 1998). To protect against HAV, two types of HAV vaccines i.e. live attenuated and, inactivated vaccines have been designed and commercially available yet this viral infection shows a significant morbidity rate worldwide. It is also suggested that the pre-exposure to HAV vaccine can prove to be more meaningful than after the viral infection takes place. Furthermore, live attenuated HAV is much more effective than the inactivated vaccine(Irving *et al*., 2012; Zhu, Yuan *et al*., 2012).

It is an enteric virus that spreads through fecal-oral route and a major source of transmission is the unhygienic and poor sanitary conditions and represents the most common cause of acute viral infection. In summary, there are three possible ways to reduce the chances of HAV infection i.e. to improve socioeconomic levels, personnel hygiene and availability of the vaccine(Mohebbi *et al*., 2012; Franco *et al*., 2012) .

2.1.2 Hepatitis B Virus

Hepatitis B virus (HBV) was discovered in 1965 as Australian antigen, now named as Hepatitis B surface Antigens (HBsAg) (Blumberg *et al*., 1965). HBV has a small genome size of about 3.2 kb that gets translated into three envelope proteins and a core protein that resides inside the envelope protein. These double shield virions have a diameter of about 42nm (Ranjbar *et al*. 2011).

HBV represents the most important viral hepatitis that branches throughout the world. It is a relaxed circular double stranded DNA virus classified under *hepadnaviridae* family that

infects the hepatocytes and moves from acute to chronic, occult HBV infection (OBI) leading with the worst outcome of developing HCC (Arababadi *et al*., 2010; Arababadi *et al*., 2012).

It is estimated that about 350million people are infected with HBV infections suffering from acute to chronic conditions. About 95% patients infected with acute HBV infection get cured while it leads to chronic infections in neonates. HBV has been classified into seven genotypes i.e. HBV genotype A to G and four serotypes i.e. adw, ayw, adr and ayr (Kao and Chen, 2002). One of the easiest way to identify HBV infection is the enzyme linked immune sorbent assay (ELISA) targeting Hepatitis B surface Antigens (HBs-Ag). However, in certain cases, this test proves to be false negative as HBV DNA is present in patient's serum as well as hepatocytes and this condition is referred to as occult HBV infection (Arababadi *et al*., 2012). The chronic HBV infection can be attributed to immune tolerance, immune clearance, viral host factors and viral integration processes (Kao and Chen, 2002; Arababadi *et al*., 2012).

One of the most important causes of HBV transmission is blood transfusion. Any contact of HBV infected blood or blood borne products with the normal individual at the site of cut or injection is likely to transmit the disease (Arababadi, Nasiri Ahmadabadi *et al*. 2012).

2.1.3 Hepatitis C Virus

Hepatitis C virus (HCV) is a lethal blood borne pathogen targeting hepatocytes. It was discovered in 1989 as non-heptatitis A, non-heptatitis B virus. HCV is classified in the family *Flaviviridae (*Farci *et al*., 2002*)*. The details of HCV, its molecular organization, replication, life cycle and pathogenesis will be discussed below.

2.1.4 Hepatitis D Virus

Hepatitis D virus (HDV) was first isolated in 1977 as a delta antigen (Rizzetto, Canese *et al*. 1977) . HDV is a replication defective single stranded RNA virus, depending on a HBV as a helper virus. It has been observed that superinfection with HDV is an important risk factor for HCC (Rizzetto *et al*., 1977; Sundquist *et al*., 2012). As far as HDV-HBV infection is concerned, it has been observed that HDV can infect an individual along with HBV simultaneously (known as co-infection) or can infect a patient already infected with HBV (known as superinfection) (Abbas *et al*., 2012). The association of HDV with HBV can increase the chances of developing HCC and mortality rate by three folds and two folds respectively (Fattovich *et al*., 2000).

The major source of HDV is the poor hygienic conditions, yet there is some decline in the HDV cases in Pakistan. The data shows that about 16.6% of the males dwelling rural areas, infected with HBV have either co-infection or superinfection of HDV with HBV (Mumtaz *et al*., 2005).

2.1.5 Hepatitis E Virus

Hepatitis E virus (HEV) is an enteric virus that was discovered in 1983 after infecting a human volunteer with the stool extracts of HAV infected patient. The stool of the experimentally infected patient revealed the presence of about 30nm virions, later named as HEV (Balayan, *et al*., 1983). HEV is a single stranded, positive sense RNA, non-enveloped virus classified under the family *Hepaviriade*. This virus has further classified into 4 mammalian, one avian and one rabbit genotype. Out of the mammalian HEV genotypes, type 1 and 2 are pre-dominant in humans (Zhao *et al*., 2009; Lu, Li *et al*. 2006).

HEV represents the most important cause of acute viral hepatitis (Kamar *et al*., 2012). Generally it is believed that only developing countries have the reservoirs of this enteric virus yet the prevalence of this virus in the developed countries needs to re-evaluate the existing notion about HEV. HEV is transmitted through fecal-oral route and diagnosis involves the determination of viral RNA in blood and feces as well as serological tests can be used (Perez-Gracia and Mateos-Lindemann, 2012). In addition to these routes of transmission, zoonosis (via pigs, cats, dogs etc) is also thought to play a role in the spread of this disease yet the mechanism has to be explored (Wedemeyer *et al*., 2012) and (El-Tras *et al*., 2012).

2.2 History of HCV

In the mid of 1970s, the term Non-A, Non-B hepatitis virus (NANB) was coined to describe the hepatocyte specific inflammatory disorder specific to neither HAV nor HBV (Feinstone *et al*., 1975; Alter *et al*.,1975). When a post transfusion analysis of hepatitis was carried out, researchers found that even the screening of blood with hepatitis A and B virus as well as with cytomegalovirus and Epstein bar virus, lead to the occurrence of post-transfusion hepatitis, eliciting the fact that there is a need to unravel another hepatitis like virus to prevent post- transfusion hepatitis like symptoms (Knodell *et al*., 1975). Upon filtrating these NANB particles with the extremely small filters, it was observed that the size of these viruses lies between 30-60nm in diameter, much smaller than HAV and HBV, later the exact size was found to be 42nm (He, Alling *et al*. 1987) and (Ranjbar *et al*., 2011). This virus was first cloned in 1989 and identified as an RNA virus(Farci *et al*., 2002).

Since the discovery of HCV, comprehensive research has been carried out including its cloning, analysis of molecular organization, replication cycle, replicon system and infectious

systems yet further detailed research is going on delighting host-pathogen associations, in-vitro infectious systems and molecular mechanisms involved in HCV induced pathogenesis (Moradpour *et al*., 2007). A schematic representation of milestones in the field of HCV research is shown in the Figure 2.1.

2.3 Model systems for In-vitro HCV replication

HCV is known to be a noxious pathogen from a long time and much research has been focused on the elucidation of its molecular organization, cell-host interactions and various pathologies. Since 1990s, another area of research in the HCV is the development of *In-vitro* HCV replication system that can unravel various hidden pathways for the host-viral interactions as well as drug development (Tariq *et al*., 2012). Different model systems have been designed to favor *In-vitro* HCV replication like

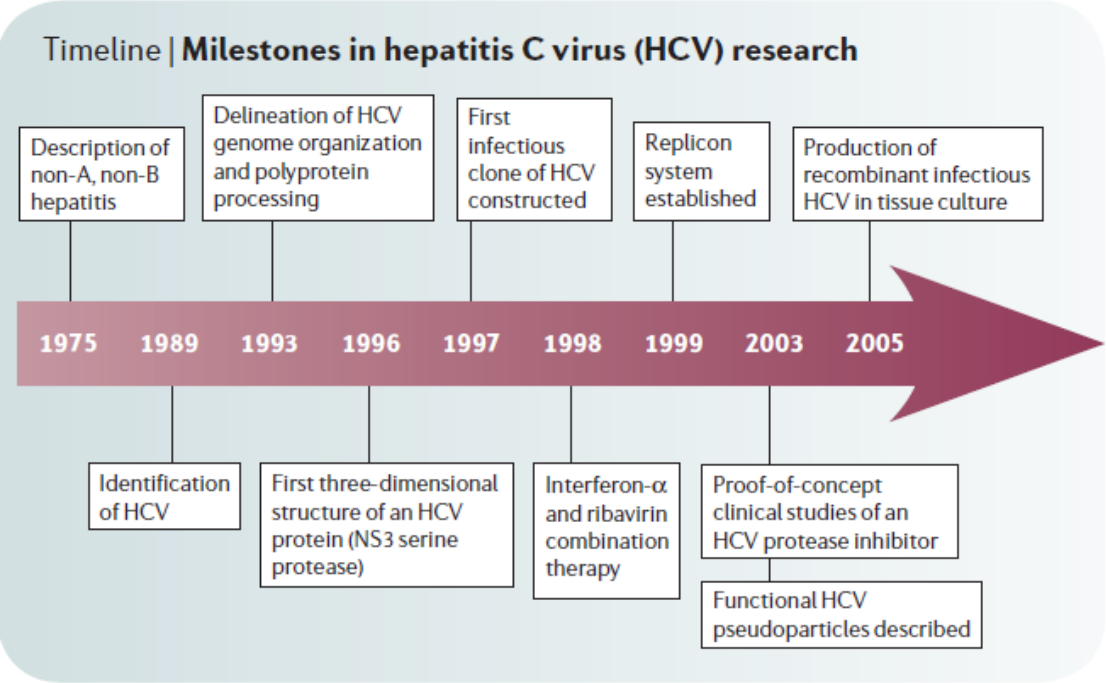


Figure 2.1: Milestones in the HCV research (adapted from: Moradpour *et al*, 2007).

replicon system, cell culture system using patient's sera, stable/transient transfected cell lines etc. Here, we have briefly described some model systems.

2.3.1 Replicon system

Development of the replicon system is one of the hallmarks in the model systems of HCV as it enabled the splitting up of viral components into desired fragments to facilitate biochemical and molecular characterization of the host-viral interactions, paving a way towards drug development. The prototype subgenomic replicon of HCV is bicistronic RNA encoding HCV structural and some part of non-structural proteins while the translation of NS3-5b is mediated by IRES. With the development of this bicistronic RNA, Huh 7 cells were characterized for HCV replication (Moradpour *et al*., 2007). In other studies, it was observed that certain amino acid substitutions can enhance the efficiency of HCV replication with the concomitant decreased production of infectious particles. This indicates the fact that these amino acid substitutions may somehow interfere with the viral packaging, assembly and egress(Blight *et al*., 2000) and (Lohmann *et al*., 2001). The efficiency of HCV infection was greatly improved using sub-lines of Huh 7 like Huh 7.5 cell line as well using chimeric clones like J6 (Wakita *et al*., 2005) and (Pietschmann *et al*., 2006). One of the drawbacks with the HCV replicon system is the colonial formation as there is no cytopathic effect of the cells harboring replicon. Therefore , it becomes difficult to separate them from the cells that are without replicon (Tariq *et al*., 2012).

2.3.2 Cell culture system using patient's sera

Key to the successful HCV replication is the permissive cells that allow the virus to interact with it's recognition receptors and facilitate viral entry followed by the viral replication. To overcome the limitation of replicon system in which the effective infection is a major drawback, HCV positive patient's sera is also used to infect the primary cells and immortalized cells. However, this strategy cannot be used effectively due to the poor reproducibility and replication (Bartenschlager and Lohmann, 2001) and (Sheehy *et al*., 2007).

2.3.3 Transfection

One of the effective means of in-vitro viral replication is transfection based strategy. This methodology allows long term replication; homogeneity among inoculation as well as manipulated viral genome can also be transfected (Bartenschlager and Lohmann, 2001).

2.4 Molecular organization of HCV

HCV is a single stranded RNA virus with a genome size of about 9.6 kb. This much genetic makeup of the virus potentially gets translated into single open reading frame and two flanked regions at the 5´- and 3´- contains Non-coding sequences. The single open reading frame, in turns, gets translated into structural and Non-structural proteins (Farci *et al*., 2002), (Bartenschlager and Lohmann,2000 ; Moradpour *et al*., 2007) as shown in the Figure 2.2

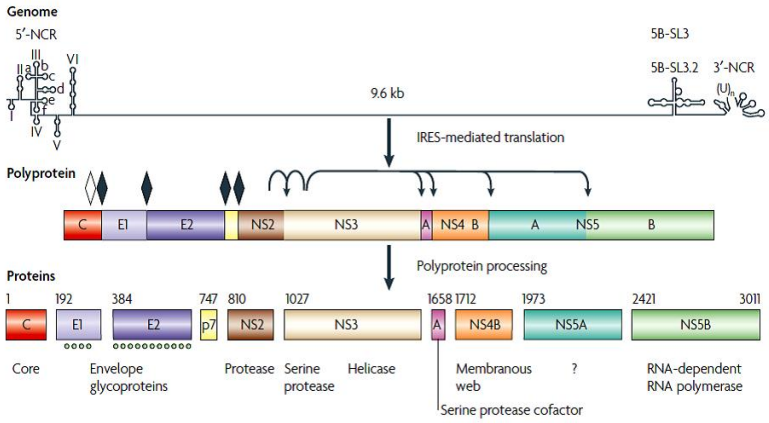


Figure 2.2: Genetic organization and polyprotein processing of HCV. HCV is an RNA virus with the genome size of 9.6kb that gets cleaved into structural proteins (C, E1 and E2) and Non-structural proteins (NS1, NS2, NS3, NS4a, NS4b, NS5a and NS5b). These proteins are hanged between two flanking regions of 5′-UTR and 3′-UTR (adapted from: Moradpour *et al*, 2007).

As mentioned earlier, 5′-NCR has four main domains that have highly conserved sequences in various HCV isolates. Domain II through IV of the 5′-NCR along with the part of core protein (Nucleotide No. 24-40) is involved in the formation of translational machinery of HCV in the form of IRES. Later, this IRES undergoes binary fusion with 40S ribosomal subunit to fully process translation (Otto and Puglisi, 2004).

HCV forms a single polyprotein that gets chopped off into structural and non-structural proteins. These proteins are described in detail below

2.4.1 Structural Proteins

HCV structural proteins include Core (C), Envelope-1 (E1) and Envelope-2 (E2) proteins. Each protein is described in detail below.

2.4.1.1 Core Protein

The first outcome of HCV polyprotein processing is the formation of immature core protein of 191 amino acids that in association with E-1 protein contains certain sequences (signal sequences) that favor further protein processing. Signal peptidases cleaves the signal sequences thereby generating fully mature core protein of 173-179 amino acid sequences (21kDa protein) (McLauchlan *et al*., 2002). HCV core protein has been found to interact with a number of cellular proteins thereby modulating the normal signaling pathways. One of the key functions of C-protein is to establish a capsid around the viral genome and favors its replication (Imran *et al*., 2012). In addition, core protein is associated with a number of pathophysiological conditions and development of hepatocellular carcinoma (Bartenschlager and Lohmann, 2001).

2.4.1.2 Envelope proteins

Ultimate product of HCV polyprotein processing is the formation of two envelope glycoproteins i.e. E1 and E2. These proteins establish a non-covalent interaction and are destined towards endoplasmic reticulum (ER). As far as the signal sequences involved in the envelope proteins docking is concerned, it is observed that the C-terminus of E2 protein (29 amino acid sequences) are critically involved in the retention of envelope proteins towards ER(Cocquerel, Meunier *et al*. 1998). Studies have shown that E2 potentially reduces the antiviral activity of IFN-α by way of interacting with PKR, double-stranded RNA activated protein kinase (Taylor, Shi *et al*. 1999).

2.4.2 Non-structural Proteins

HCV Non-structural (NS) proteins include seven proteins ranging from NS1/ p7, NS2, NS3, NS4A, NS4B, NS5A and NS5B (Bartenschlager and Lohmann, 2001). After the cleavage

of structural proteins from Non-structural proteins, maturation and processing of these Non-structural proteins is done under the influence of three enzymes i.e. protease (NS2/3), helicase (NS3/NS4A) and RNA-dependant RNA polymerase (NS5B). NS3 is a bifunctional protein performing its role as a protease as well as helicase that causes the cleavage at NS3/4A, NS4A/B, NS4B/5A, NS5A/5B. Among Non-structural proteins, NS5A/5B is given more importance because the latter one is the key for the replication of viral RNA while NS5A is involved in oxidative stress and the ongoing metabolic abnormalities (Lohmann *et al*., 1997), (Bartenschlager and Lohmann, 2001) and (Grakoui *et al*., 1993). The role of NS5A in the induction of pathophysiology will be discussed in detail later.

2.5 Replication of HCV

Hepatocytes represent the most important target sites for the HCV. In addition to the hepatocytes, B-lymphocytes and peripheral blood mononuclear cells (PBMC) are also targeting sites for the HCV docking and further pathogenesis (Okuda *et al*., 1999), (Bartenschlager and Lohmann, 2000; Zignego*et al*., 1995). In HCV, viral replication is governed by NS5B that lacks a proof-reading ability. This inability of the virus to undergro proof-reading leads to the formation of quasispecies(Lauer and Walker, 2001). Due to the lack of proof-reading of this viral RNA dependant RNA polymerase (NS5B), HCV undergoes virion production with an error rate of about 10^{-4} that leads to the formation of quasispecies, a major problem towards the detection of viral RNA. Consequently, viral titer appears to be much lower than the actual viral titer in the host cells (Ogata *et al*., 1991 Okamoto *et al*., 1992).

In order to discuss the viral replication, it is necessary to understand the virion structure. Although, the complete structure of HCV virion is still under consideration, yet the electronic

microscopic studies depit HCV as small virion with a diameter of 40-70 nm. As far as the virion structure of HCV is concerned, three of its proteins i.e. Core, E1 and E2 proteins depict the virion structure. Out of these proteins, E2 has been found to be exclusively involved in anchoring to the host cell derived lipid bilayer that encapsulates the virion and promotes the viral trafficking towards the cellular environment. These viral particles have been shown to circulate in the host cell in close proximity with low density lipoproteins (LDL), very low density lipoproteins (VLDL), certain immunoglobulin as well as free virions. This leads to the low viral buoyant density (Moradpour *et al*., 2007).

The complete phenomenon of HCV replication can be summarized in following steps.

i. Viral entry and uncoating
ii. Translation of the viral proteins
iii. Polyprotein processing
iv. Viral replication complex
v. Assembly and egress of virions

2.5.1 Viral entry and uncoating

Hepatocytes are considered to be the best known target sites for HCV replication. Nevertheless, there are some other host cells for HCV replication i.e. dendritic cells, B-cells and some other cells. HCV RNA as well as its Non-structural proteins have been found in the liver of human beings and chimpanzees only, revealing that hepatocytes are the potent target sites for viral targeting. Specialized receptors like low-density lipoprotein receptor (LDLR), CD-81 (a tetraspanin protein found on the cell membrane), scavenger receptor class B type-1 (SR-B1) and Claudin (Bartenschlager and Lohmann, 2000; Moradpour *et al*., 2007; Agnello *et al*., 1999;

Scarselli *et al*., 2002; Evans *et al*., 2007). The dynamics of HCV replication can be correlated to its virion production, quasispecies formation and viral clearance. It is estimated that HCV produces about 50 viral particles per hepatocyte per day (Neumann *et al*., 1998) .

Low density lipoproteins and very low density lipoproteins have specialized receptors like low density lipoprotein receptor (LDLR) for their trafficking inside the cell. Because of the strong interaction between HCV and these lipoproteins, LDLR represent an important class of target receptors for their subsequent endocytosis followed by uncoating and viral replication as shown in Figure 2.3 Apart from LDLR, CD-81 and SR-B1 have been found to critically control viral entry.

HCV enters the host cell by way of clathrin mediated endocytosis with low pH membrane fusion. This endosomal membrane fusion with low pH is a feature of other flaviviruses and *alphaviruses*. The reason for this low endosomal membrane fusion is the activation of internal fusion peptides of the envelope proteins that helps in the further viral replication (Blanchard *et al*., 2006; Koutsoudakis *et al*., 2006).

2.5.2 Translation of HCV proteins

In HCV, the viral translation is under the direct influence of internal ribosomal entry site (IRES) that dictates the translation of proteins on the viral RNA. It is a short stretch of 330 nucleotides located at the 5´- NCR and facilitates translation different from that of other canonical forms of mRNAs (Wang *et al*., 1993). IRES establish a binary complex with the ribosomal unit and eventually form an 80S ribosomal subunit complex by the association of 48S and 60S ribosomal subunit through GTP-dependant manner. This model of HCV translation

provides an insight into the functional aspects of different IRES domains in initiating translation (Otto and Puglisi, 2004).

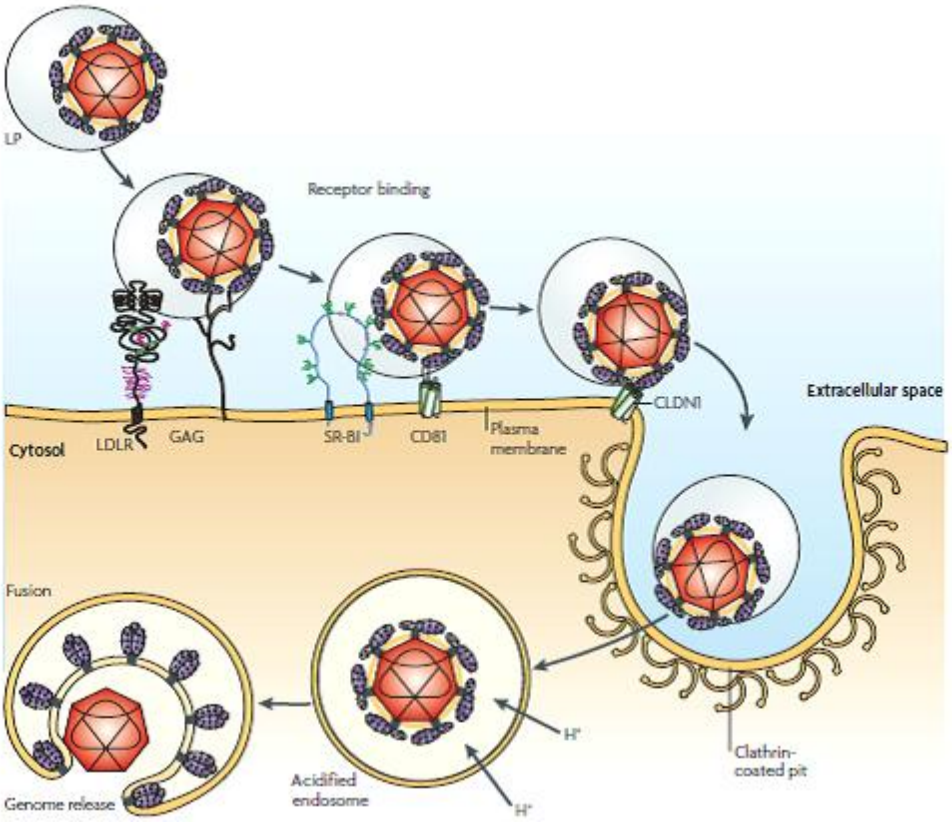


Figure 2.3: Proposed model for Hepatitis C Virus entry. HCV is shown to interact with Low density lipoproteins and very low density lipoproteins which are recognized by specialized receptors like CD81, LDLR, glycosaminoglycans (GAG), Scavenger receptor- class B type-1 (SR-B1) and Claudin 1. Endocytosis of the viral particles is mediated by Clathrin followed by acidification of endosomes that cause rupturing of the endosome and egress of virions.

2.5.3 Polyprotein processing

HCV genome gets translated into single polyprotein precursor that eventually undergoes proteolytic cleavage to generate three Structural and seven Non-structural proteins as shown in

the Figure 2.2. As far as the processing of structural proteins and p7 is concerned, ER signal peptidases cleave them from the immature polyprotein and help them to establish nucleocapsids. While the Non-structural proteins get processed by two proteins i.e. protease (NS3/4A) (Lohmann *et al*., 1997; Moradpour *et al*., 2007).

2.5.4 Viral replication complex

One of the hallmarks of all RNA viruses is the formation of a membrane associated network consisting of Viral RNAs, proteins and altered membrane proteins. It is thought the viruses may alter the host membranes to facilitate their replication, physical support to the viruses, compartmentalization of the viral products and evasion from the host defense mechanism. However, the alteration of the membranes (that can be ER, Mitochondria, Golgi complex etc) greatly depend upon the viruses (Moradpour *et al*., 2007; Lyle *et al*., 2002; Schwartz *et al*., 2002). In order to explain the phenomenon of viral RNA replication, the term "membranous web" was coined. It is thought that this web is derived from ER membrane. Membranous web is a complex network of viral RNA and proteins associating with the host membranes. Now days, much research is focused on the formation of membranous web and its association with the host factors. In cell culture based system, it has been found that there is a correlation between HCV RNA replication and lipid metabolism, revealing that membrane fluidity is essential for the web formation and subsequent RNA replication (Wang *et al*., 2003), (Kapadia and Chisari, 2005).

2.5.5 Assembly and egress of virions

So far, little is known about the late phases of viral replication i.e. how the virions assemble themselves followed by the egress. In order to unravel this mystery, virus like particle

particles (VLPs) have been synthesized. Two approaches have been employed to study VLPs of HCV. According to the first approach, these virions are about 30-45 nm as non-enveloped and enveloped viruses respectively (Mizuno *et al*., 1995). According to the second approach where baculoviruses have been employed to express a part of HCV genome containing 5´-NCR and Core-E2 region in the insect cell line, the results revealed that VLPs selectively encapsulates viral RNA. Although the latter approach is the most acceptable one, yet virion production is low. Another disadvantage of this system is that VLPs remain in the vesicles and can't be secreted out of the cells (Baumert, Ito *et al*. 1998). Based on these studies it is thought that HCV virions bud-off into the ER membrane or its derived membranes and get secreted into the cell through secretary pathway (Andre *et al*., 2005).

2.6 Serological course of HCV infection

HCV is a blood-borne pathogen that targets hepatocytes and replicates there at a higher rate. On an average, about 10% of the hepatocytes are infected with HCV. There are about 2 x 10^{11} hepatocytes and taken together it can be estimated that about 50 virions are produced per hepatocyte per day (Neumann *et al*., 1998). This rapid replication rate of HCV virions is the basis of its great genetic diversity with 6 main genotypes and more than 90 subtypes. Among this much diversity of HCV, the genotype 1 is the most prevalent one in United States (about 75%), genotype 2 in Japan and genotype 3 are most prevalent HCV genotype in Pakistan. Acute HCV is characterized by the elevation in serum alanine transferase level (ALT) within 1-2 weeks of exposure to HCV. It is estimated that 55-85% of the patients are vulnerable to naturally clear HCV and moves towards chronic HCV phase which gets transformed into a number of pathological conditions like steatosis, fibrosis, insulin resistance and hepatocellular carcinoma. In addition to these conditions, the extrahepatic manifestations of HCV include

cryoglobulinemia, sicca syndrome and glomerulonephritis (Hoofnagle, 2002; Zein *et al*., 1996, McOmish *et al*., 1994; Nousbaum *et al*., 1995).

In acute HCV patients, the viral RNA can be detected after 1-2 weeks of exposure to HCV. However, the clinical symptoms of HCV become prominent within 3-12 weeks of viral infection. In the mean while, the serum ALT gets about 10 folds increase level than the normal level. It is also observed that the HCV RNA level increases rapidly in the first few weeks and reaches a level of 10^5 to 10^7 IU/ml before the rapid increase in serum ALT level (Farci *et al*. 1991; Thimme *et al*., 2001). The acute phase of HCV can be prolonged, but it is rarely transformed into fulminant hepatitis (Farci *et al*., 1996).

About 75-85% of the HCV infected patients with acute phase gets transformed into chronic phase. The chronic infection often takes place after about 6months post-exposure of HCV and varies with various factors like race, gender and immune system. There is less possibility for the development of jaundice like symptoms in chronic HCV patients than that of patients suffering from the acute phase yet there is no clear finding that can predict the chronicity of infection (Farci *et al*., 1996; Alter and Seeff, 2000 and Takaki *et al*., 2000). During the course of progression from acute to chronic infection, there is continuous fluctuation in serum RNA and ALT level while in some studies a "stuttering phase" has been observed in which the viral RNA level becomes undetectable while serum ALT level remains constant (Thimme *et al*., 2001) and (Major *et al*., 1999). Once the chronic infection is established, the spontaneous loss of HCV RNA is unlikely and treatment becomes inevitable (Yokosuka *et al*., 1999).

During the course of chronic infection, the common symptoms of this disease include pain in the upper right quadrant of liver, nausea and poor appetite. Furthermore, the disease

progression does not correlate with the ALT level (Conry-Cantilena *et al*., 1996; Hoofnagle, 1997).

Multiple factors like gender, age, race and immune status have been linked with the chronicity of HCV infection. These factors have been discussed in detail below.

2.6.1 Age and chronicity of HCV infection

Unlike Hepatitis B virus (HBV), the chronicity rate of HCV infection is far more in children than that of adults particularly children getting HCV infection through maternal route or children having chronic leukemia that need to undergo regular blood transfusion (Bellentani and Tiribelli 2001), (Sasaki *et al*., 1997; Strickland *et al*., 2000).

2.6.2 Race and chronicity of HCV infection

As far as the chronicity of HCV infection is concerned, it is observed that ethnic and racial differences do exist. Among Americans infected with HCV, North Americans have a higher chronicity rate than that of Hispanic whites yet this mystery needs to be resolved (Villano, *et al*., 1999; Seeff *et al*., 2001).

2.6.3 Gender and chronicity of HCV infection

The retrospective study has revealed that young women have low rates of HCV infection as compared to the aged women. As far as comparison of chronicity of HCV infection in male and female is concerned, the cross sectional studies have not reported any differences (Bellentani and Tiribelli, 2001;Kenny-Walsh, 1999).

2.6.4 Immune system and chronicity of HCV infection

Likewise other diseases, the status of the immune system determines the chronicity of HCV infection. It has been observed that the patients infected with human immunodeficiency virus (HIV) has high HCV chronicity rate than that of patients without HIV infection (Thomas *et al*., 2000). Similarly, in another study the chronicity of HCV infection was related between the hypogammaglobulinaemic patients, an immune disorder, and immune-competent patients. The results indicated the fact that effective treatment of these patients can be done, yet the recovery efficiency is much higher in immune-competent patients than the immune stressed patients (Christie *et al*., 1997).

2.7 Mode of transmission

HCV is a blood borne pathogen and is strongly associated with the repeated exchange of blood or blood products as in the case of drug users, prisoners, patients undergoing regular dialysis as well as health care workers. Among the risk factors, the most important factor includes the intravenous drug usage (IVDU) (about 71.4%) while the other risk factors include blood transfusions, surgical treatments and hemophilia (Roman *et al*., 2008).

Furthermore, HCV can also be transmitted through nosocomial route. In some of the hospitals the dialysis and hematological wards do not follow strict hygienic conditions and HCV infections are transmitted. Based on the characteristic features of HCV, its nosocomial line of transmission is still a mystery and needs to be studied in detail (Alter, 1997; Amarapurkar, 2000). Furthermore, the gynecological wards treating HCV positive women have been documented with increased risk of HCV cases in the later patients. Therefore, strict hygienic

conditions in the gynecological wards are critically essential to control the nosocomial transmission of HCV infection (Massari *et al*., 2001).

Maternal route of HCV transmission has been documented in some cases yet it varies from 5-12%, depending upon geographical distribution (Kelly and Skidmore, 2002). The possible source of transmission is utero or the mothers who had undergone blood transfusion in the past (Resti *et al*., 1998; Thomas *et al*., 1998; Murakami *et al*., 2012). As the maternal HCV antibodies retain up to 9-10 months, so there is no need of undergoing HCV diagnosis for this period of time while the baby should be followed up for the next 18 months (Kelly and Skidmore, 2002).

In the past it was thought that saliva establishes a good platform for the HCV transmission but later this was rectified as saliva can only act as the source of HCV, but not as a potent route of transmission (Couzigou *et al*., 1993). Similarly, another study revealed that household contact with the patients infected with HCV does not transmit HCV infection (Sagnelli *et al*., 1997).

Another important source of HCV transmission is the sexual contact between an HCV infected patient and a normal one. Among this route of HCV transmission, prostitutes (Nakashima *et al*. , 1992), heterosexual partners infected with HCV and HIV as well as bisexual men (Eyster *et al*. , 1991; Alter, 2011), although the rate of HCV transmission is much less in the latter case than that of HBV with the same route of transmission (Osmond *et al*. , 1993).

2.8 Diagnostic methods

The diagnosis of HCV is a straight forward method that involves the serological analysis of viral RNA. It can be diagnosed using enzyme linked immunosorbant assay (ELISA) as well as qualitative and quantitative polymerase chain reaction (PCR) based methods (Farhana *et al*. 2009). In addition to these conventional diagnostic methods, genotype specific PCR based methods are employed that can be used for the selection of best possible treatment depending upon HCV genotype (Heathcote, Shiffman *et al*. 2000; Idrees 2008).

2.9 Possible treatment

HCV infection progresses through acute towards chronic infection. Some of the HCV infected patients with acute phase have the natural tendency to clear viral RNA because of the strong immune response. However, 55-85% of these patients move toward chronic phase which can be naturally cleared in rare cases. As HCV infection is not merely the infection on hepatocytes rather it involves downregulation of the immune response as well, therefore, the patients are required to undergo treatment (Hoofnagle 2002; Bowen and Walker 2005; Rehermann and Nascimbeni 2005).

Currently, treatment of HCV involves the combination of interferon alpha and ribavirin. The interferon alpha is a cytokine that plays a vital role in the innate immune response and mediates cellular signaling that ultimately leads to the upregulation of interferon stimulated genes (IFGs) and helps in the viral clearance (Sen, 2001; Feld and Hoofnagle, 2005). However, the best known treatment for HCV is the combination of pegylated interferon and ribavirin (Hoofnagle and Seeff, 2006).

Ribavirin is a nucleoside that has a broad range antiviral activity. Its complete mechanism of action is not yet known, but it is thought it may function by the depletion of intracellular Guanosine triphosphate (GTP), essential for viral RNA synthesis as well as reduces viral replication (Crotty *et al*., 2000; Lau, Tam *et al*., 2002; Feld and Hoofnagle, 2005).

2.10 Implication for drug development

HCV life cycle is quite complicated mechanism involving various host-viral factors. Although each of these critical steps can be targeted as viral inhibitors, yet two of the HCV proteins i.e. NS3/4A protease and NS5B (RdRp) are specifically targeted as antiviral agents. Among these inhibitors, serine proteases are potentially important antiviral agents that not only inhibit viral translation but also reverts the host immune system to combat the disease (Lamarre, Anderson *et al*., 2003; De Francesco and Migliaccio, 2005; Moradpour *et al*., 2007). Furthermore, other viral proteins like NS2/3 protease, NS5A and 5´-NCR are been being explored to be used as antiviral drugs against HCV. However, the major problem with the drug development against HCV is the rapid mutations leading towards the formation of multiple quasispecies that hinder the effective viral clearance likewise HIV infection (Trozzi, *et al*. 2003; Tong *et al*., 2006).

2.11 HCV infection and associated pathogenesis

HCV infection is not merely the infection of the hepatocytes rather it alters the homeostasis and immune response of the body in such a way that it triggers a number of metabolic disorders including oxidative stress, endoplasmic reticulum (ER) stress, insulin resistance, steatosis, fibrosis and eventually turns into HCC, if remain uncured (Bureau *et al*., 2001; Bugianesi *et al*., 2005; Choi and Ou, 2006; Parvaiz *et al*., 2011; Presser *et al*. 2011).

Among HCV induced pathogenesis, one of the hallmarks of disease is the development of insulin resistance, a step towards type 2 diabetes mellitus (T2DM). Recent investigations revealed that insulin resistance is not merely a simple disorder rather involves changes at various metabolic levels including oxidative stress, endoplasmic stress, gluconeogenesis and lipogenesis (Parvaiz *et al*., 2011; Arao *et al*., 2003; Romero-Gomez, 2006; Sesti, 2006). Each of these metabolic stresses and abnormalities has been discussed in detail below.

2.11.1 Oxidative stress

In the living cells, there are low levels of free radicals that are not meant to destruct the cell physiology. However, certain diseases have been associated with the elevation of oxidative stress. Likewise, HCV infection is characterized with the elevated oxidative stress by way of ROS and NOS, eventually favoring severe metabolic and pathological conditions like obesity, insulin resistance, fibrosis, cirrhosis and HCC (Choi and Ou, 2006; Vincent and Taylor, 2006).

Antioxidants form the first line of defense against oxidative stress. These oxidants may be enzymatic or non-enzymatic nature. The non-enzymatic oxidants include certain vitamins (like Vitamin C and E) while enzymatic oxidants include Superoxide dismutase (SODs) (Yu, 1994; Sies, 1997; Abdalla *et al*., 2005).

Cellular kinases like MAPK (mitogen activated protein kinase), JNK (c-Jun N-terminal kinase), ERK (extracellular signal regulated kinase) etc are the key enzymes that get activated in response to cellular stress (that may be due to biological, chemical or viral induced stress) and increases the expression of antioxidants (Cohen, 1997; Erhardt *et al*., 2002). SODs forms the key group of antioxidants including MnSOD, CuSOD, etc . In the earlier phase of infection, there is an increased level of these antioxidants. However, there is a concomitant decreased expression of

these antioxidants with the advancement of the disease progression likewise tumor cells have diminished antioxidant activity (Yeh *et al*., 1998; St Clair *et al*., 2002).

HCV is known to induce multiple metabolic disorders, eventually progresses towards HCC. Although the complete mechanism of disease progression is still under consideration, yet oxidative stress is being given much importance because of its key role in the development and progression into lethal pathologies (Choi and Ou, 2006). The phenomenon of oxidative stress has been linked with a number of biological markers and transcription factors (Banerjee *et al*., 2008; (Choi *et al*., 2004; Mahmood *et al*., 2004). Studies have shown that HCV Core (Structural protein) as well as NS3 and NS5A (Non-structural proteins) are involved in the induction of ROS using different possible mechanisms (Gong *et al*., 2001; Okuda *et al*., 2002; Abdalla *et al*., 2005; Korenaga *et al*., 2005; Tardif *et al*., 2005).

Recent studies revealed that HCV core protein is associated with the upregulation of mitochondrial GSH and reduced NADPH concentration in mitochondria (Korenaga *et al*., 2005). Hepatoma cells expressing HCV Core protein shows elevated levels of oxidative stress by way of increasing oxidation of thioredoxin and decreased expression of reduced Glutathione (GSH) (Abdalla *et al*., 2005). Furthermore, HCV Core protein has been found to localize on the mitochondrial membrane, modulates its permeability and facilitates mitochondrial Ca^{2+} uptake, thereby deregulation the mitochondrial functions and disrupts the complex I of electron transport chain, localized in the mitochondrial membrane (Moriya *et al*., 2001; Naas *et al*., 2005). Under such conditions, cells tend to produce Nitric oxide (NO) synthase that triggers synthesis of nitric oxide, which, in turns damage host cells by a number of ways like DNA damage, cell death and furtherROS production (Lim *et al*., 1995; Korenaga *et al*., 2005)

Oxidative stress can be elevated based on the reactive oxygen species (ROS) as well as reactive nitrogen species (RNS) which, in turns, depends upon the expression of NADPH oxidase 2 (Nox 2) protein. NS3 has been strongly linked with upregulation of Nox-2 that elevates the oxidative stress level and damage the liver cells as well as immune cells so that HCV can potentially replicate (Forman *et al*., 2004; Thoren *et al*., 2004). Nox-2 is also known to generate ROS level and prevents the GSH export. During HCV infection, liver is known to undergo oxidative stress by a number of mechanisms, and one of the ways is the upregulation of Nox-2 which in turns prevent the hepatic export of GSH and leads to potent oxidative stress. In some of the studies, TNF-α and cytochrome p450 is found to be upregulated in HCV induced oxidative stress (Choi *et al*., 2004; Gochee *et al*. 2003).

In addition to the Core and NS3, oxidative stress is significantly increased with the HCV NS5A expression in hepatocytes leading towards the perturbations of the cell redox status (Tardif *et al*., 2005; Gong *et al*., 2001). Cells are known to undergo stress with the alteration in cellular kinases. This oxidative stress eventually turns on the antioxidants like MnSOD. Studies have shown that the hepatoma cells expressing HCV NS5A can induce oxidative stress eventually leading towards increased protein expression of MnSOD by way of activating cellular kinases like p38 and JNK via AP-1 (Activated protein -1) that is linked to the increased oxidative stress response (Tardif *et al*., 2005; (Qadri, Iwahashi *et al*. 2004). Some other studies revealed that NS5A can also induce oxidative stress by way of Ca^{2+} uptake from the ER membrane, induces ER stress and potentially increases mitochondrial mediated oxidative stress (Waris *et al*., 2005; Gong *et al*., 2001).

2.11.2 Endoplasmic Reticulum stress

During viral replication, HCV non-structural proteins establishes a web on the ER membrane that facilitates the protein interaction as well as viral propagation in the ER of hepatocytes (Mottola *et al*., 2002). One of the most important signaling pathway for the antiviral activity (IFN-α) is the Jak-STAT pathway. In the previous studies, it has been shown that HCV proteins interact with the Jak-STAT pathway, thereby decreasing the antiviral activity for its further replication and pathogenic mechanisms like ER stress (Heim *et al*., 1999; Gong *et al*., 2001; Blindenbacher *et al*., 2003; Gale and Foy, 2005). Cells can greatly sensitize perturbations in its signaling pathways and undergo some changes to combat with the unusual conditions. Similarly, upon sensitizing ER stress, cells initiates an innate adaptive response known as untranslated protein response (UPR) (Harding *et al*., 2002). As a result of UPR, certain molecular chaperones like GRP78 and GRP94 gets upregulated that prevents further translation by the increased folding of ER. The ER copes with this HCV induced stress by way of transcriptional activation of IRE1α and IRE1β that differentially splice XBP-1 and trans-activates ER stressed genes/molecular chaperones (Lee *et al*., 2003; Yoshida *et al*., 2003). In addition to the UPR, ER can undergo another mechanism known ER-associated degradation (ERAD) that degrades the proteins that have been translated improperly in a proteosome dependant manner (Meusser *et al*., 2005).

Translation and subsequent docking of the proteins into specialized compartments for further processing are the complicated mechanisms that need to be strictly regulated by the host cells. During the course of translation and compartmentalization, there is modulated phosphorylation of the eukaryotic initiation factor-2α (eIF2 α). When the cells sensitize ER stress, there is an increased expression of PERK, an ER resident protein, that mediates

phosphorylation of eIF2 α at serine 51 and blocks translation by preventing association of 40S and 60S ribosomal subunits (Shi *et al*., 1998; Harding *et al*., 1999; Rutkowski and Kaufman, 2004).

ER stress is believed to be the initial outcomes of viral induced stress eventually leading towards lethal metabolic disorders like insulin resistance and T2DM. The initial steps involved in the insulin biosynthesis take place in the ER. Likewise other cells, β-cells have their own ER that can modulate their folding capacity in response to the cellular stress. Studies have shown that mutations in the PERK or its effectors like eIF2α are likely to block β-cell development and promotes hepatic gluconeogenesis (Harding and Ron, 2002). There are two schools of thoughts regarding ER stress and mitochondrial dysfunction and their disease progression. According to the first school of thought, development of ER stress triggers the elevation of Ca^{2+} that induces mitochondrial dysfunction and leads towards disease progression (Gong *et al*., 2001. According to the first school of thought, mitochondrial dysfunction triggers ER stress and modulates the signaling pathways like elevation of p38 MAPK and related transcription factors that increase the key gluconeogenic gene PEPCK for the development of insulin resistance (Lim *et al*., 2009).

Furthermore, studies have shown that ER stress is associated with the transcriptional activation of CCAAT/enhancer binding protein-β (C/EBP β) as well as increased phosphorylation of pCREB eventually promotes the transcriptional activation of PEPCK (phosphoenol pyruvate carboxykinase),a key gluconeogenic gene that leads towards the development of T2DM (Choudhuri *et al*., 2011).

As known already, HCV proteins form a network of ribonucleoprotein complex on the ER membrane and induce metabolic disturbances. Among HCV proteins (structural and non-

structural proteins), NS5A is strongly associated with this membranous web that establishes ribonucleoprotein complex and induces ER stress. ER is believed to be an important reservoir of calcium (Ca^{2+}) that releases Ca^{2+} from its cytosolic domain during HCV induced stress. These calcium ions are held up by the mitochondria and leads towards ROS generation (Murphy *et al*., 1996; Berridge *et al*., 1998; Gong *et al*., 2001). Furthermore, ER stress induced by NS5A is involved in the activation of transcription factors NF-kB and STAT-3, thereby modulating the downstream signaling pathways and contribute towards the development of chronic liver diseases eventually leading towards Hepatocellular carcinoma (Waris *et al*., 2002).

During HCV replication, core protein is localized on the ER membrane and facilitates the virion production and egress that disrupts the host cell homeostasis. Consequently, cells get sensitize and respond to this stress by the activating ER based response known as UPR. As a result of this ER stress, cells become prone towards the development of chronic metabolic disorders including insulin resistance, fibrosis and hepatocellular carcinoma (Nakatani *et al*., 2005; Sheikh *et al*., 2008).

2.11.3 Insulin resistance mechanisms

Insulin resistance is referred to as the condition in which vital tissues of the body like liver, fats and muscles become resistant towards the insulin action, leading towards impaired glucose tolerance and concomitant development of type 2 diabetes mellitus (Saltiel and Kahn 2001). Insulin resistance is a complicated metabolic disorder that is linked with a number of phenomenons like ectopic lipid accumulation, activation of UPR as well as innate immune pathways. Ultimately, these pathways get converge into gluconeogenesis and lipogenesis leading towards insulin resistance and concomitant development of T2DM (Choudhury *et al*., 2011; Samuel and Shulman, 2012).

Humans are heterototrophic in nature that need to feed on various food items for their ultimate survival and propagation. They have evolved anabolic as well as catabolic mechanisms to supplement their dietary requirements. In this regard, secretion and targeted action of insulin establishes a remarkable example. The main purpose of insulin is to consume dietary carbohydrates and convert it into storage molecules like glycogen and lipids, rich energy reservoirs. Therefore, effective secretion and targeted action for insulin is critically essential for the maintenance of homeostasis otherwise insulin deficiency or resistance is likely to favor the development of some lethal diseases like type 1 and 2 diabetes, maturity onset diabetes of the young (MODY) (Samuel and Shulman, 2012; (Boyle *et al*., 2010)).

Insulin resistance has been described in detail below.

2.11.3.1 Insulin receptors

Insulin receptors belong to the sub-family tyrosine kinases including, Insulin growth factor (IGF)-I receptor and insulin receptor-related receptors (IRR). These receptors are the tetrameric proteins with two α- and two β- subunits. These α- and β- subunits are allosteric in nature in which α-subunit inhibits the tyrosine kinase activity of β- subunit (Saltiel and Kahn, 2001). Downstream to these receptors are the signaling molecules like phosphotidyl inositol-3-OH kinase (PI3K), AKT and Fox. Any mutation in these receptors is likely to undergo metabolic disturbance with the resulting outcome of insulin resistance and obesity (Skorokhod *et al.*, 1999).

2.11.3.2 Insulin receptor substrates

Receptor tyrosine kinases of Insulin have at least nine substrates including Gab-1, $p60^{dok}$, Cbl, APS, Shc and Insulin receptor substrates (containing four isoforms i.e. IRS-1, IRS-2, IRS-3 and IRS-4) (White, 1998; Pessin and Saltiel, 2000). All of these isoforms of IRS are highly homologous yet have different effects on the insulin mediated signaling. The results have shown that a defect in IRS-1 induces insulin resistance in peripheral tissues and impairs glucose tolerance (Araki *et al*., 1994; Tamemoto *et al*., 1994). Although, IRS-2 knockout mice also shows insulin resistance in peripheral tissues yet show retarded growth in some tissues like brain. Contrary, IRS-3/4 does not lead to the severity of the disease and their effect is slightly more than that of normal metabolism (Tamemoto *et al*., 1994; Withers *et al*., 1998). In another study, IRS-3 and IRS-4 were found to be the negative regulators of IRS-1 and IRS-2 by suppressing other insulin signaling molecules yet the exact mechanism is still unclear (Tsuruzoe *et al*., 2001).

In addition to the normal tyrosine phosphorylation of Insulin receptor substrates, these signaling molecules may undergo serine phosphorylation at various check points thereby modulating normal insulin mediated signaling pathway and disrupts homeostasis (Hotamisligil *et al*., 1996).These inhibitory phoshphorylations let the insulin receptor substrates to interact with other proteins and lead towards insulin resistance. Kinases that have been implicated in such phoshphorylations include PI3K, AKT, GSK3 (glycogen synthase 3), mTOR mammalian target of rapamycin etc (Kim *et al*., 2001).

Previous studies have shown that insulin receptor substrates can also be deregulated by the catalytic action of a group of enzymes known as protein tyrosine phosphatases (PTPases). These phosphatases cause dephosphorylation of insulin receptors and their respective substrates,

thereby modulating the downstream insulin signaling pathway towards insulin resistance. Among PTPases, PTP1B is important phosphatases whose knockout mice experiments triggered tyrosine phosphorylation and improved insulin sensitivity. Based on this, PTPases can be taken as therapeutic agents against diabetes and obesity (Saltiel and Kahn, 2001).

2.11.3.3 Insulin signaling and regulatory mechanisms

Insulin signaling involves a complex array of cross-talk between different signaling molecules. Upon internalization of insulin, tyrosine phosphorylation of the IRS protein is favored that triggers downstream insulin signaling molecules. After IRS gets tyrosine phosphorylated, PI3K, first target of IRS, gets activated. PI3K plays a key role in the insulin signaling and any dysfunction in the PI3K is likely to deregulate insulin stimulated metabolic functions like glycolysis, gluconeogenesis, glucose transport and lipid synthesis (Myers *et al*., 1992; Shepherd *et al*., 1995) as shown in the Figure 2.4. This lipid moiety, PI3K, contains catalytic as well regulatory subunits that potentially interacts with the other signaling molecules and plays an important role in insulin dependent manner (Kessler *et al*., 2001).

PIP3 is known to regulate three classes of signaling molecules i.e. AGC family of Serine/Threonine kinases, guanine nucleotide-exchange proteins of Rho proteins of GTPases and TEC family of tyrosine kinases (Mackay and Hall, 1998; Peterson and Schreiber, 1999). One of the most important type of AGC proteins is phosphoinositide dependent protein kinase-1 (PDK1) that phosphorylates and activates AKT/PKB, an important insulin signaling molecule (Alessi *et al*., 1997). Under normal conditions, AKT favors phosphorylation and subsequent inactivation of glycogen synthase kinase 3β (GSK-3β) and Forkhead transcription factors (Fox) and favors insulin mediated metabolic activities (Cross *et al*., 1994; Nakae *et al*., 1999). However, studies

using different isoforms of AKT have not come up with a uniformity of insulin mediated actions (Brady *et al*., 1998). It has also been studied that deletion of AKT-2 favors the development of insulin resistance, revealing its role in normal insulin mediated signaling pathways (Brady *et al*., 1998; Cho Mu *et al*., 2001).

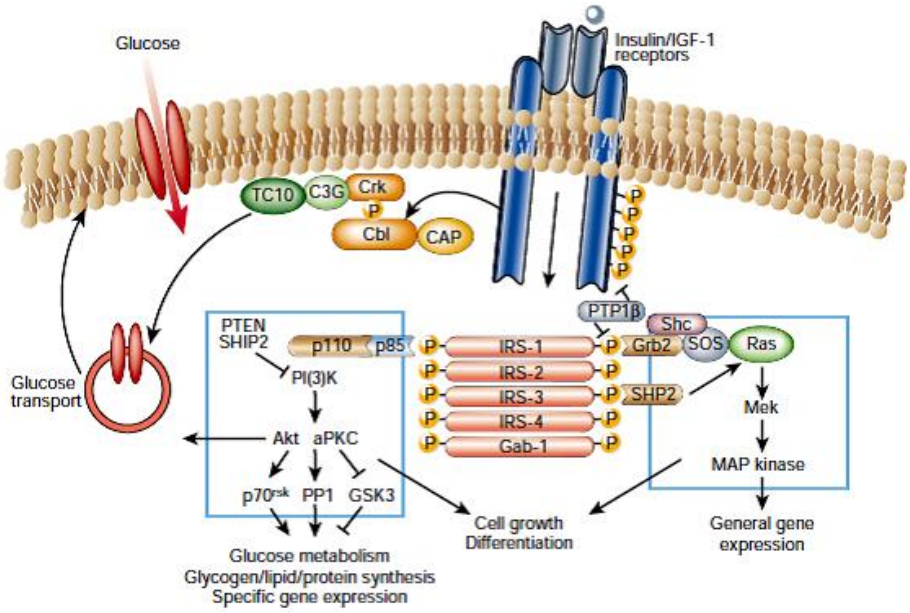


Figure 2.4: Signal transduction in insulin action. Insulin receptor is a tyrosine kinase that undergoes autophophosphorylation upon insulin binding and catalyses the phosphorylation of the downstream insulin signaling molecules like PI3K, AKT, GSK3 etc to favor glucose metabolic pathways including glycogen synthesis, lipid synthesis along with the targeted gene expression. (Adapted from: Saltiel and Kahn 2001))

2.11.3.3.1 Glycogen synthesis

One of the important roles of insulin is to accumulate glucose, perform its transport and conversion into glycogen in a coordinated fashion. Insulin mediates glycogen synthesis by favoring dephosphorylation of glycogen synthase through the coordinated activity of certain kinases like protein phosphatases-1 (PP1) and GSK-3 (Cross *et al*., 1995; Brady *et al*., 1997). In

the insulin signaling pathway, PP1 lies downstream to the PI3K. As a result of activation of PP1, AKT gets phosphorylated that phosphorylates and inactivates GSK-3, thereby favoring glycogen synthesis and tends to maintain normal insulin mediated metabolic pathway as shown in Figure 2.4 (Cross *et al*., 1995).

Downstream to the IRS, there lies a number of signaling molecules that get phosphorylated or dephosphorylated to favor normal metabolic pathways. AKT is an important signaling molecule that gets activated in IRS-dependent pathway. Downstream to the AKT is another kinase i.e. glycogen synthase kinase-3β (GSK-3β).that was originally identified as a key regulator of glycogen synthesis. Under normal conditions, AKT activation triggers the hypophosphorylation of GSK-3β at serine^{9} with its concomitant inactivation and favors glycogen synthesis (Cross *et al*., 1995; Shaw *et al*., 1997; Frame and Cohen, 2001). Furthermore, GSK-3β has been implicated in many biological functions like Wnt signaling pathway, NF-kB signaling as well as in the development of insulin resistance (Frame and Cohen, 2001; (Lee and Kim 2007). Studies have shown that GSK-3β can directly phosphorylate IRS-1 at Ser^{332} while its inhibition can recover normal glucose metabolism, revealing its role in the development of insulin resistance (Eldar-Finkelman and Krebs, 1997; Nikoulina *et al*., 2002; Liberman and Eldar-Finkelman, 2005).

2.11.3.3.2 Gluconeogenesis

The anabolic effect of insulin is the formation of glycogen, rich energy source, that not only depends on the conversion of glucose into glycogen but also the prevention of glycogenolysis i.e. conversion of glycogen into glucose and gluconeogenesis i.e. synthesis of glucose from non-carbohydrate molecules (Bergman and Ader, 2000; Michael *et al*., 2000). One

of the most important factors involved in gluconeogenesis is the formation of free fatty acids generated from visceral fats that are less sensitive against insulin (Bergman, 1997). As a result of their accumulation, there is a net flux of fatty acids from visceral fats to the liver via portal vein where it generates more glucose, favors gluconeogenesis and paves the way towards insulin resistance and T2DM (Bergman, 1997; Saltiel and Kahn, 2001). The metabolic effect of insulin upon glycogen synthesis and prevention of gluconeogenesis is shown in Figure 2.5.

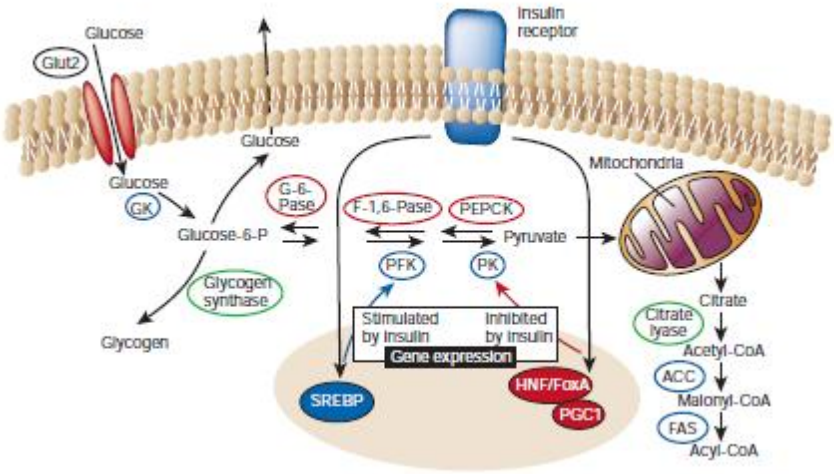


FIGURE 2.5: Regulation of glucose metabolism in hepatocytes. In the hepatocytes, insulin stimulates the glycogen and lipid synthesis to regulate the glucose concentration. Insulin stimulates the expression of glycolytic enzymes (shown in blue color) and inhibits gluconeogenic genes (shown in red color). In this metabolic pathway, a number of genes and transcription factors are involved like PEPCK, G6P, HNF, PGC1, SREBP etc.

2.11.3.3.3 Lipogenesis

Insulin resistance is a complicated phenomenon whereby several metabolic pathways are disturbed and are closely associated with the enhanced lipogenesis with the concomitant

accumulation of specific lipid metabolites like diacylglycerols and ceramides (Samuel and Shulman, 2012).

Lipids and insulin resistance are linked together long time ago. Studies have shown that the exogenous supply of lipids can increase glucose-6-phosphatase (G6P), one of the key gluconeogenic genes (Randle Garland *et al*., 1963; Jucker *et al*., 1997; Cline *et al*., 1999; Dresner *et al*., 1999). In order to investigate whether it has the free circulating plasma lipids or lipids within the insulin responsive tissues, it was found that the intramyocellular triglyceride content is much better indicator of insulin resistance with the concomitant production of diacylglycerols (DAG) (Krssak Petersen *et al*., 1999; Chen *et al*., 2002).

Lipids are not only the important macromolecule but also act as the signaling intermediates (like endotoxins, prostaglandins etc), performing a number of different metabolic functions. Among this much diverse group of molecules, some of these signaling intermediates also act as the second messengers (like diacylglycerol and ceramides), imparting pivotal roles in the signaling pathways. These secondary messengers have been extensively studied in association with pathologies like insulin resistance (Samuel and Shulman, 2012). Such an association between DAG and insulin resistance state has been studied in mice with genetic manipulations of diacylglycerol acyltransferase (DGAT).

There are two isoforms of DGAT i.e. DGAT1 and DGAT2. As far as insulin resistance is concerned, DGAT2 has been found to increase the diacylglycerol content, modulate intracellular signaling and eventually favors insulin resistance (Smith *et al*., 2000; Levin *et al*., 2007). Overexpression of DGAT2 (MCK-DGAT2) in glycolytic fibers increases muscle triglyceride content with a decrease in diacylglycerol content while the transgenic overexpression of hepatic

DGAT2 results in an increase in triglyceride content, diacylglycerol content as well as increase in the ceramides content and is associated with insulin resistance (Jornayvaz *et al*., 2011). Generally, this enzyme is localized on the endoplasmic reticulum however it gets associated with lipid droplets upon ectopic lipid accumulation (Stone *et al*., 2009) and modulates the insulin signaling pathway like serine phosphorylation of IRS-1 is favored, thereby promotes hepatic insulin resistance (Monetti, Levin *et al*. 2007). However, the phenomenon of lipogenesis and insulin resistance can be reduced by the decreasing the hepatic and adipose DGAT2 overexpression (Choi *et al*., 2007).

Further investigation of the lipogenesis and development of insulin resistance revealed that PKCs are also involved in lipid mediated insulin resistance. PKCs are the lipid sensing molecules through a broad range of serine/threonine kinase activity. PKCs are classified as PKC α, β I, β II and γ that requires calcium and diacylglycerol for their activation and subsequent signaling. However, there are some novel types of PKCs like PKC Θ, ε, etc (Anderson and Olefsky 1991; Miyake, Yasui *et al*. 2009). These PKCs have putative phosphorylation sites on the insulin receptors (Coghlan *et al*., 1994). Studies have shown that PKC Θ leads to the serine based phosphorylation of IRS-1 (Ser^{1101}) instead of normal tyrosine phosphorylation, thereby modulating the entire downstream insulin signaling pathway and hampers normal insulin mediated metabolism and favors insulin resistance (Soos *et al*., 2004).

In addition to the DAGs, the tissue accumulation of ceramides is also associated with the development of insulin resistance. Ceramides are known to affect the insulin signaling pathway by targeting AKT2/PKB (Schmitz-Peiffer *et al*., 1999; Stratford *et al*., 2004). AKT is a serine/threonine kinase that plays an important role in the insulin signaling pathway. Despite of the long study, AKT has some controversial roles (Stratford *et al*., 2004). So far there are two

proposed mechanisms regarding ceramides stimulated AKT2 inhibition. First, ceramides trigger the activation of PP2A (protein phosphatases 2A) that leads to the inhibition of AKT2 phosphorylation (Teruel *et al*., 2001). Secondly, ceramides may recruit some novel forms of PKC i.e. PKCz, that can sequester AKT2 from insulin signaling, thereby modulating normal insulin signaling pathway (Powell *et al*., 2003).

2.11.3.3.4 HCV and insulin resistance

HCV is a liver invading pathogen that modulates host cellular machinery, moves from acute to chronic phase and leads towards insulin resistance, a step towards T2DM. Structural and non-structural proteins of HCV are known to induce metabolic disturbances involving multiple pathways like oxidative stress, modulation of the IRS-1phosphorylation, increased gluconeogenesis, lipogenesis etc (Clement *et al*., 2009; Joyce *et al*., 2009; Parvaiz *et al*., 2011).

Insulin resistance is a pre-diabetic phase that is linked up with the chronic HCV infection (about 25%) while other hepatic infections like HBV is less potential to cause insulin resistance (approximately 10%) (Bugianesi *et al*., 2005). Insulin resistance refers to the metabolic disturbance where either adequate amount of insulin is required to bring the homeostasis or abnormalities at the insulin receptor level and its concomitant downstream signaling molecules thereby triggering excessive glucose production (Campbell *et al*., 1988).

One of the leading causes of viral hepatitis is the infection with HCV that infects hepatocytes, modulate cellular signaling and progresses from acute to chronic conditions that eventually comes up with steatosis, fibrosis, insulin resistance, cirrhosis and hepatocellular carcinoma (Choi and Ou, 2006). As far as the disease progression is concerned, studies revealed that it is associated with the increased ROS production and decreased antioxidants (Choi and Ou

2006). In most of the studies, tumor necrosis factor-α (TNF- α) is linked with the disease progression (Sheikh *et al*., 2008; Parvaiz *et al*., 2011; Hotamisligil, 1999). Some studies revealed that HCV infection triggers the upregulation of TNF- α along with the upregulation of suppressor of cytokine signaling-3 (SOCS-3) and favors the development of T2DM (Romero-Gomez, 2006).

HCV is known to induce hepatic as well as extrahepatic manifestations including insulin resistance and development of T2DM. In the course of insulin signaling, glucose transport to the hepatocytes via GLUT2 is critically essential for the glucose homeostasis. However, *in-vitro* studies showed that HCV infection modulates cellular signaling in such a way that glucose homeostasis is imbalanced due to the increased gluconeogenic and decreased glycolytic pathway, leading towards the development of insulin resistance (Shoji *et al*., 2011). In previous studies, HCV cell culture based system revealed increased gluconeogenic gene expression particularly PEPCK (rate limiting enzyme of gluconeogenesis) and G6P. These genes are known to be regulated by FOX01 (forkhead box01) in HCV infected cells (Banerjee, Meyer *et al*. 2010). Some studies have shown that members of the family FOX undergo post-translational modifications to modulate glucose metabolism and favor the development of insulin resistance (Banerjee *et al*., 2008); Banerjee *et al*., 2010; Hsieh *et al*., 2012).

HCV infection and disease progression are tied up with the lipid metabolism of hepatocytes (Herker *et al*., 2010). During the course of HCV infection, ER membrane gets surrounded by lipid droplets because of the accumulation of cholesterol esters and triglycerides (Farese and Walther, 2009). One of the key enzymes regulating triglyceride biosynthesis is DGAT. These enzymes (DGAT1 and 2) are known to localize onto the ER membrane, however DGAT-2 gets localize on to the lipid droplets during cellular uptake of fatty acids (Yen *et al*.,

2008). Herker *et al.* showed that DGAT-1 potentially interacts with the HCV core protein and paves the way for the docking of core protein to lipid droplets to facilitate virion production and disease progression (Herker *et al*., 2010).

Lipid droplet is an important organelle that is meant to store neutral lipids and allows transportation of lipids and proteins between cytoplasm and other organelles. Miyanari *et al*. have shown that these lipid droplets are effectively targeted by HCV particles for the new virion production. Some of the HCV proteins (i.e. core, E1 and E2) have been found to interact with these lipid droplets and recruits replication complex machinery to these lipid droplets for the production of new virions (Miyanari *et al*., 2003; Miyanari *et al*., 2007).

HCV has a genome size of about 9.6kb that gets translated into structural and non-structural proteins. Among this much large number of proteins synthesized by HCV, only few proteins have been found to directly interact with the insulin signaling pathway or induction of the oxidative stress for the concomitant disease progression. A brief description of these HCV proteins with respect to the development of insulin resistance is discussed below.

HCV core protein is an integral component of the HCV nucleocapsid that potentially interacts with the host cells and is known to be involved in the modulation of insulin signaling pathway primarily by targeting phosphorylation status of IRS-1 and IRS-2 with the concomitant increased expression of SOCS-3 (Kawaguchi *et al*., 2004). PI3K is an important downstream insulin signaling molecule that gets activated by p85 subunit. However, studies have shown that HCV core transgenic mice can block normal insulin signaling pathway by blocking p85 subunit and impairs glucose homeostasis (Banerjee *et al*., 2008). Previous studies have shown that c-Jun N-terminal kinase (JNK) and mitogen activated protein kinase (MAPK) are involved in the

disease progression and transfection of hepatocytes with HCV core protein elucidates that ectopic expression of core protein favors the serine phosphorylation of IRS-1 (Ser^{312}) through the activation of JNK. Furthermore, AKT is an important downstream insulin signaling molecule that is found to be involved in various metabolic pathways like cell survival, cell proliferation, glucose metabolism, cancer development etc (Testa and Tsichlis, 2005). Transfection of hepatocytes (HepG2) with core protein resulted in the increased serine phosphorylation of AKT (Ser^{473}) and impairs insulin signaling thereby results in development of insulin resistance (Testa and Tsichlis 2005; Banerjee, Saito *et al*. 2008). Insulin signaling is a complex mechanism involving modulation of gluconeogenic and lipogenic gene expression by modulating the transcriptional and translational activities of Forkhead box transcription factors (FOX01 and FoxA2) (Herker *et al*., 2010; Yabaluri and Bashyam, 2010); Shoji *et al*., 2011; Samuel and Shulman, 2012). HCV infection of the hepatocytes or ectopic expression of HCV core protein showed that serine phosphorylation of FOX01 (Ser^{256}) is inhibited along with the accumulation of FOXA2 in the nucleus that eventually triggers an increased transcriptional expression of G6P, a key gluconeogenic gene, thereby favoring excessive glucose production and leads to insulin resistance condition (Banerjee *et al*., 2010). TNF-α, an antioxidant gene, is an important biomarker for the HCV chronic phase. Previous studies have shown that TNF-α gets upregulated in chronic phase of HCV and is associated with insulin resistance, T2DM, cirrhosis and hepatocellular carcinoma (Knobler and Schattner 2005); Park *et al*., 2012). As far as role of HCV core protein in the induction of insulin resistance is concerned, it has been observed that TNF-α, an antioxidant gene, gets upregulated and aids in the serine phosphorylation of IRS-1 which in turns is associated with insulin resistance. Therefore, TNF-α can be used as an novel bridge

between HCV infection and insulin resistance (Knobler and Schattner, 2005; Banerjee *et al*., 2008; Park *et al*., 2012)).

Among the envelope proteins, E2 protein has been found to modulate normal insulin signaling pathway. Hsieh wt al. have shown that transfection of hepatocytes (Huh 7 cells) with HCV E2 protein resulted in the decreased phosphorylation of IRS-1 with the concomitant impaired AKT serine phosphorylation (Ser^{308}) and decreased serine phosphorylation of GSK3-β (Ser^{9}), leading to decreased glucose uptake and increased gluconeogenesis respectively (Hsieh *et al*., 2012).

Previous studies have shown that HCV NS3 is involved in the activation of oxidative stress as well as ER stress. NS3 is known to favor these stresses primarily by the production of ROS species either directly or indirectly through the upregulation of NOX2 (nicotinamide dinucleotide phosphate oxidase 2) (Bureau *et al*., 2001).These oxidative species are then involved in the immunomodulatory mechanisms, cell deterioration and disease progression (Thoren *et al*., 2004; Kasprzak and Adamek, 2008). It is thought that NS3 dependant oxidative and ER stress may activate NF-kB and favors the development of insulin resistance and Hepatocellular carcimoma yet its direct role in the development of insulin resistance is not established so far (Bureau *et al*., 2001; Sheikh *et al*. 2008).

PKR, RNA dependant kinase, is an essential cellular component that has antiviral activities through the phosphorylation of eIF-2α thereby favoring the antiviral response through the activation of interferon stimulated genes (ISGs) (Clemens and Elia, 1997; Kumar *et al*., 1997).Among the HCV non-structural proteins, NS5A has been found to potentially interact with this cellular kinase, PKR through its interferon stimulated determining regions (ISDR) that

masks the phosphorylation of eIF-2α and favors viral propagation and development of interferon resistance (Waris *et al*., 2002; Sheikh *et al*., 2008). Likewise, Core and NS3 proteins, NS5A is also known to induce oxidative stress by the upregulation of ROS that damage the hepatocytes by a number of mechanisms like upregulation of PP2A (Georgopoulou *et al*., 2006); Gong *et al*., 2001; Christen *et al*., 2007). This enzyme, PP2A, has the potential of modulating normal insulin signaling pathway by the impairment of AKT phosphorylation (Bernsmeier *et al*., 2008). Furthermore, HCV infection of hepatocytes or ectopic expression of NS5A has been found to increase the gluconeogenesis primarily by the upregulation of key gluconeogenic gene, PEPCK, indicating its role in the impairment of the normal insulin signaling pathway yet the exact pathway has to unraveled (Deng *et al*., 2011). Previous studies have also shown that NS5A gets co-localized onto the ER membrane, hampers lipid metabolism, accumulates cholesterols and favors the disease progression like steatosis, insulin resistance and hepatocellular carcinoma (Wang, Lee *et al*. 2009).

MATERIAL AND METHODS

3.1 CELL LINES AND CULTURE CONDITIONS

The cell lines, Huh 7 and Huh 7.5, used in this study were generously provided by Dr. Charles Rice to Dr. Gulam Waris at RFUMS (Department of Microbiology and Immunology, USA). These cell lines were gown in Dulbecco's modified eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100U of penicillin/ml and100µg of streptomycin/ml and cultured at 5℃ in a humidified cell culture incubator containing 5% CO_2. The cultured medium was replaced with the fresh medium after a confluency of 70% was reached.

3.2 HCV CELL CULTURE INFECTION SYSTEM

The genomic RNA of HCV JFH-1 was transcribed and delivered into Huh 7.5 cell line by electroporation or liposome mediated transfection. For electroporation mediated transfection of the Huh 7.5 cells, the cells were suspended in cytomix buffer at 10^7cells/ml. The JFH-1 RNA (8-10µg) was mixed with 0.2ml of cells in a 2mm cuvette and subjected to electroporation using a single pulse of 0.27kV, 140Ω and 960µF (Biorad Gene Pulser system) and then the cells were plated in 100 culture dishes as described previously by Burdette *et al*., 2010. Liposome mediated transfection was performed using Lipofectamine 2000 (Invitrogen). The transfected cells were then plated in DMEM medium supplemented with 10% FBS and passaged for 2-3 days. The corresponding HCV infected cell culture supernatant was determined by quantitative real-time PCR (Applied Biosystems). The HCV infected cell culture supernatants were collected at appropriate times and used to infect the naïve Huh 7.5 cells (m.o.i of 1). These cells were then

subjected to incubation for about 6h at 37°C and 5% CO_2. Multiple aliquots of the HCV infected cell culture supernatants were made in our lab and each aliquot was first subjected to western blot assay using NS3 antibody for the confirmation of HCV infection as previously reported (Burdette *et al*., 2010). The infectious HCV JFH-1 construct was provided by Dr. Takaji Wakita (NIID, Tokyo, Japan) to Dr. Gulam Waris at RFUMS.

3.3 TRANSIENT TRANSFECTION ASSAYS

Cells were cultured and allowed to attain a confluency of about 70% in 60mm petri dishes. The cells were then transfected with the indicated plasmids using lipofectamine 2000 (Invitrogen, CA). Upon attaining confluency of about 70%, cultured cells were washed thrice with phosphate buffer saline (PBS) to remove the cellular debris followed by treating with 100nM insulin for three hours before harvesting the transfected cells.

3.4 RNA EXTRACTION, PURIFICATION, QUANTIFICATION AND REVERSE TRANSCRIPTION

3.4.1 RNA extraction

RNA was extracted from the cultured cells using the following protocol.

i. Aspirated media from culture plates
ii. Added 1 ml Trizol (Invitrogen)
    a. Pipette up and down to wash off and mix followed by transferring the liquid to 1.5 ml tube.
    b. Incubated at room temp 2-3 min
iii. Added 200 µl chloroform

a. Shake it vigorously 15 sec

b. Incubated at room temp 2-3 min

iv. Centrifuged at 12,000 RPM for 15 min at 4^{o}C. The homogenate separated into 2 phases: Lower red is phenol/chloroform while upper colorless layer is aqueous phase.

v. Transferred the aqueous phase to a fresh 1.5 ml tube

vi. Added 500 µl Isopropanol

a. Incubated 5-10 min at room temp

vii. Centrifuged at 12,000 RPM for 10 min at 4^{o}C

a. RNA formed a small white pellet

viii. Washed pellet once in 70% EtOH

a. Resuspended pellet

b. Centrifuged at 12,000 RPM for 10 min at 4^{o}C

ix. Discarded EtOH

x. Air dry pellet

xi. Dissolved in RNAse free H_20 (Roughly 20-100µl depending on size of pellet)

xii. Stored at -80^{o}C

3.4.2 RNA Purification

The extracted RNA was then subjected to purification using following protocol.

i. In the ependorff containing RNA along with RNAase free water, I added 2µl of DNAase1.

ii. Added 200µl of RNAase free water.

iii. 600µl of PCI was added from the bottom of its bottle.

iv. Vorex it for 15-20 sec followed by microfugation for 30 sec.

v. This lead to the formation of two layers in the tube. I took upper liquid portion carefully and added 600ul of PCI. This was again subjected to vortexing, microfugation and took the upper the liquid portion.

vi. Added 300µl of chloroform, vortex it for 10 sec and allowed it to settle down to take the upper the liquid portion. This step was repeated for once and again the upper the liquid portion was obtained.

vii. Now, I added sodium acetate (100µl) and 1ml of chilled absolute alcohol.

viii. Stored at -80°C.

ix. Centrifuged at 13,000rpm for 15min at 4°C.

x. Took the pellet and washed it thrice with 70% alcohol.

xi. Air dry pellet in biosafety cabinet.

xii. After the dry pellet was obtained, I added 50ul of RNAase free water and stored at -80°C.

3.4.3 RNA quantification

The purified RNA was quantified using eppendorf Biophotometer (6131) at absorbance of 260/280. The quality of RNA was checked on gel electrophoresis (1ug of RNA +1µl of 6X DNA loading dye + 1µl of nuclease free water) using 1% agarose gel, stained with ethidium bromide. Using Gel Doc system, the RNA results were photographed. However, the best quality samples of RNA were used for reverse transcription.

Reverse transcription

The selected RNA samples were subjected to cDNA synthesis using following protocol.

i. Extracted cellular RNA (1ug)……. (1-5µl)

ii. Random hexamer …… (1 µl)

iii. 10mM dNTP ……. (1 µl)

iv. DEPC / RNAase free …….. make up the volume up to 10 µl.

v. Incubate the mixture at 65°C for 5min in PCR.

vi. Incubate on ice for 1min.

vii. To each tube add,

   a. 10X RT buffer……. (2µl)

   b. 25mM $MgCl_2$…….. (4µl)

   c. 0.1mM DTT …….. (2µl)

   d. Reverse inhibitor……. (1µl)

   e. Reverse transcriptase …… (1µl)

viii. The RT-PCR conditions were as follows:

   a. 25°C for 10min

   b. 42°C for 2h

   c. 70°C for 15min

ix. The PCR product i.e. cDNA was stored at -20°C for further use.

3.5 PRIMER DESIGNING

Primers (sense and anti-sense) for the target genes were designed using primer3 software (http://bioinformatics.weizmann.ac.il/cgibin/primer/primer3.cgi). Some of those genes (CREB,

DGAT and PPAR-γ) were optimized in ASAB, NUST laboratory while rest of the primers (PEPCK, TNF-α, PGC-1α, MTP and G6P) were obtained from the published data.

The sequences of these primers for the gene specific amplification of the genes from cDNA are listed in Table 3.1.

Table 3.1: List of primer sequences used in the current study

Sr.No.	Primer Name	Primer Sequence: 5'-3' Sequence
1	CREB-F	GATCTTAGTGCCCAGCAACC
2	CREB-R	GACGGACCTCTCTCTTTCGT
3	DGAT-F	CACCATCCAGAACTCCATGA
4	DGAT-R	GTCTCCAAACTGCATGAGCT
5	PPAR-γ-F	GCAGTGGGGATGTCTCATAA
6	PPAR-γ-R	GTCAGCGGGAAGGACTTTAT
7	PEPCK-F	GGCTACAACTTCGGCAAATACC
8	PEPCK-R	GGAAGATCTTGGGCAGTTTGG
9	PGC-α-F	TGTGCAACTCTCTGGAACTG
10	PGC-α-R	TGAGGACTTGCTGACTTGGTG
11	TNF-α-F	AGGCGCTCCCCAAGAAGACA
12	TNF-α-R	TCCTTGGCAAAACTGCACCT
13	MTP-F	GGACTTTTTGGATTTCAAAAGTGAC
14	MTP-R	GGAGAAACGGTCATAATTGTG
15	G6P-F	CATTGACACCACACCCTTTGC
16	G6P-R	CCCTGTACATGCTGGAGTTGAG
17	GAPDH-F	ACCACAGTCCATGCCATCAC
18	GAPDH-R	TCCACCACCCTGTTGCTGTA
19	18S-F	ACATCCAAGGAAGGCAGCAG
20	18S-R	TCGTCACTACCTCCCCGG

3.6 OPTIMIZATION OF PCR AMPLIFICATION

Reaction conditions were optimized for the primers to be used for conventional PCR using 1µg of cDNA concentration. The reaction conditions for different genes are as follows:

i. Optimization of CREB

Nuclease free water……10.5 µl

dNTP (2mM)…….. . 2 µl

Taq buffer……...…… 2 µl

2.5mM $MgCl_2$……. 2 µl

Primer (F)…… 1 µl

Primer (R)…… 1 µl

Taq Polymerase……. 0.5 µl

cDNA …….. 1 µl

Total reaction volume = 20 µl

The PCR tubes with the above mentioned reagents were placed in the thermal cycler. The samples were preheated at 95°C for 5min followed by 35 cycles of the parameters: 94°C for 30sec, **59°C for 40sec** and 72°C for 40sec. Final extension step was done at 72°C for 7min. The resulting PCR product was then subjected to agarose gel electrophoresis using 2% agarose. To visualize the DNA bands, the gel was stained with ethidium bromide and photograph was taken

using gel documentation system. A 50bp ladder (Fermentas) was run in gel as DNA size marker. From this PCR product, a sharp band was obtained with the expected size of about 173bp.

ii. Optimization of DGAT

Nuclease free water…… 10.1 µl

2mM dNTP……..…….. .2 µl

Taq buffer……...…… 2 µl

$MgCl_2$ (3 mM) ……. 2.4 µl

Primer (F)…… 1 µl

Primer (R)…… 1 µl

Taq Polymerase……. 1 µl

cDNA (1µg/µl) …….. 0.5 µl

Total reaction volume = 20 µl

The PCR tubes with the above mentioned reagents were placed in the thermal cycler. The samples were preheated at 95°C for 5min followed by 35 cycles of the parameters: 94°C for 30sec, **54°C for 40sec** and 72°C for 40sec. Final extension step was done at 72°C for 7min. The resulting PCR product was then subjected to agarose gel electrophoresis using 2% agarose. To visualize the DNA bands, the gel was stained with ethidium bromide and photograph was taken using gel documentation system. A 50bp ladder (Fermentas) was run in gel as DNA size marker. From this PCR product, a sharp band was obtained with the expected size of about 166bp.

iii. Optimization of PPAR- γ

Nuclease free water…….. 10.5 µl

2mM dNTP……..……. . 2 µl

Taq buffer……...…… 2 µl

$MgCl_2$ (2.5 mM) ……. 2 µl

Primer (F)…… 1 µl

Primer (R)…… 1 µl

Taq Polymerase……. 0.5 µl

cDNA (1µg/µl) …….. 1 µl

Total reaction volume = 20 µl

The PCR tubes with the above mentioned reagents were placed in the …….. thermal cycler. The samples were preheated at 95°C for 5min followed by 35 cycles of the parameters: 94°C for 30sec, **55°C for 40sec** and 72°C for 40sec. Final extension step was done at 72°C for 7min. The resulting PCR product was then subjected to agarose gel electrophoresis using 2% agarose. To visualize the DNA bands, the gel was stained with ethidium bromide and photograph was taken using gel documentation system. A 50bp ladder (Fermentas) was run in gel as DNA size marker. From this PCR product, a sharp band was obtained with the expected size of about 173bp.

3.7 GEL ELECTROPHORESIS

Gel electrophoresis was performed using agarose gel of different percentage composition depending upon the need of the band separation. The agarose was dissolved in 1X TAE buffer, shaked it well and heated in microwave until the agarose become clear in TAE buffer. In order to photograph the DNA/RNA bands, ethidium bromide was added to the semi-solidified gel.

Poured the gel into the cast and allowed it to settle down. After the gel solidified, the gel comb was removed carefully so that samples can be loaded in the gel wells.

3.8 QUANTITATIVE REAL TIME PCR

Total cellular RNA was extracted from the cultured cells using TRIzol followed by treatment with DNAase using RQ1 RNAase-free DNAase prior to the cDNA production. The cDNA was synthesized from 1µg of total RNA using oligo(dT) primers according to manufacturer's protocol (Applied Biosystems, CA).Using SYBR green master mixture and specific sets of primers, Quantitative RT-PCR was performed. The 18s was used as an internal control. All amplification reactions were performed in 25 µl mix using RT-PCR reagent kit and the template RNA. The amplification reactions were performed under following conditions: **2min at 50°C, 10min at 95°C, 40 cycles of 15sec at 95°C and 1min at 60°C**. Relative transcript levels were calculated using ΔΔCt method as specified by the manufacturer.

Following reagents were added to the real time PCR tubes:

SYB Green Master mixture …….. 12.5 µl

Forward primer …….. 0.75 µl (10 µM)

Reverse primer ……..…… 0.75 µl (10 µM)

cDNA (1µgl)…..……2ul

RNAase free water ……...……. 9 µl

Total reaction volume …… 25 µl

3.9 PROTOCOL FOR HARVESTING CELLS

For protein quantification and further use in SDS-gel electrophoresis and western blot assays, the cultured cells were allowed to attain a confluency of about 70% followed by harvesting. The cells were harvested using the following protocol.

i. Aspirated all the media from the cultured plates.
ii. Rinsed the culture dish with 5X PBS thrice to remove the cellular debris.
iii. Then, I added 1ml of PBS in each culture plate to effectively scarp the cultured cells using a sterilized scraper.
iv. These cells were then transferred to 1.5 ml tube and placed on ice until subjected to centrifugation.
v. Centrifugation was done at 13000 rpm for 10min at 4℃.
vi. Supernatant was aspirated while pellet was stored at -80℃.

3.10 PROTOCOL FOR CELL LYSIS

The harvested cells were then subjected to cell lysis by adding Radio immunoprecipitation assay (RIPA) lysis buffer (70-100 µl) depending upon the pellet. The cellular pellet was pipette out vigorously in lysis buffer to perform cell lysis effectively. Then it was incubated on ice for 30 min followed by centrifugation at 13000 rpm for 10 min. Now, supernatant was obtained and used for protein quantification. The composition of RIPA lysis buffer is: 50mM Tris Base pH 7.5, 150 mM NaCl, 1% NP-40, 0.50% sodium deoxycholate, 0.10% SDS, 1mM orthovanadate, 1mM sodium formate and 10µl/ml of protease inhibitor cocktail).

3.11 PROTEIN ESTIMATION

Each protein sample was quantified by adding 98 µl of 0.1N sodium chloride (NaCl) in a 1.5 ml tube and added 2 µl of the protein sample. Pipette it well and added 1 ml of Bradford's reagent. Then, the sample was incubated at room temperature for 2 min and then subjected to protein quantification using eppendorf Biophotometer. Bovine serum albumin (BSA) was used as a protein standard.

3.12 SDS-Gel Electrophoresis

3.12.1 Preparation of SDS-gels

In order to perform SDS-gel electrophoresis, resolving and stacking gels were prepared. The composition of these gels is given in Table 3.2.

Table 3.2: Composition of resolving and stacking gel for SDS-gel electrophoresis

Resolving gel			
Composition	**7.5%**	**10%**	**12%**
30% Acrylamide/0.8% Bisacrylamide	1.875 ml	2.50 ml	3.00 ml
4x Tris-Cl/SDS pH8.8 (Resolving)	1.875 ml	1.875 ml	1.875 ml
ddH_2O	3.75 ml	3.125 ml	2.625 ml
10% Ammonium Persulfate	25 ul	25 ul	25 ul
TEMED	5 ul	5 ul	5 ul
Stacking gel			
30% Acrylamide/0.8% Bisacrylamide	650 µl		

4x Tris-Cl/SDS pH6.8 (Stacking)	1.250 ml
ddH_2O	3.05 ml
10% Ammonium Persulfate	25 µl
TEMED	6 µl

The mixture was pipette out well and poured into the cassette along with the comb. The gel was poured in a way to minimize bubble formation. After the stacking gel got polymerized (30-45min), comb was removed and protein samples were loaded in each lane.

3.12.2 Running SDS-gel

The SDS-gel was run in following manner:

i. Got the quantified protein samples and transferred the desired volume of the protein into fresh 1.5 ml tubes and labeled each tube.
ii. Added 5 µl of 6x protein loading dye to each tube.
iii. Incubated tubes at 90°C for 5min.
iv. Assembled gel box and fill it up to the mark with 1X SDS buffer, Composition given in Table 3.3.
v. Loaded the protein samples in separate lanes carefully.
vi. 5 µl of the protein marker was used.
vii. The samples were run at 100v until they get through the stacking gel and then it was run up to 150v. It was carefully noted that protein samples should not be eluted into the gel assembly.

Table 3.3: Composition of 5X SDS-buffer

SDS- buffer 5x (4L)	
Tris Base	60.6 g
Glycine	288 g
10% SDS	20 ml
ddH_2O	Make up the volume up to 4L

3.13 WESTERN BLOT ASSAYS

The protein samples that were separated though SDS-gel electrophoresis were then subjected to the western blot by running it at 100v for about 1h in a western blot assembly. Western blot was done in following manner.

i. The SDS-gel containing resolved protein samples were shifted to the transfer buffer (composition shown in Table 3.4)
ii. Furthermore, the desired size of nitrocellulose membrane was cut and immersed in the transfer buffer for 10-15min.
iii. The assembly was filled with cold transfer buffer.
iv. The gel was sandwiched between the nitrocellulose membranes and then western blot was performed at 100v for 1h.
v. Western blot was performed with a continuous shaking of the buffer using a magnet.
vi. After the western blot was run, all of the resolved proteins on the gel transferred on to the membrane and then placed in blocking solution (containing 1X TBST and 5% non-fat dry milk) for 1h.

vii. The blot was then washed with TBST buffer thrice (composition shown in Table 3.4).

viii. The membranes were then probed with suitable primary antibody for 1h at room temperature or overnight at 4°C followed by washing with TBST buffer thrice.

ix. The membranes were then incubated in suitable secondary antibody for 1h at room temperature.

x. The membranes were scanned using Odyssey infrared imaging system (Li-Cor Biosciences, Lincoln, NE).

xi. Cellular actin was used to determine protein loading in each lane.

Table 3.4: Composition of buffers used in western blot

Transfer Buffer (1L)	
Tris Base	3.03 g
Glycine	14.4 g
Methanol	200 ml
ddH_2O	Make up the volume upto 1L
1X TBST (1L)	
1M Tris Base pH 7.9	10 ml (10mM)
5M NaCl	20 ml (100mM)
ddH20	970 ml
Tween-20	1 ml

3.14 LIPID DROPLET STAINING AND IMMUNOFLOURSCENCE

Bodipy staining was used for lipid droplet assay. The protocol used for bodipy staining is as follows:

i. Cells (Mock and infected) were grown on cover slips in a 60 mm culture dish and allowed to attain a confluency of about 70%.
ii. Then, entire media was aspirated followed by the addition of paraformaldehyde (4%) for 20 min.
iii. The cultured cells were then washed with PBS thrice to wash out all the cellular debris.
iv. The cultured cells were then treated with Triton to facilitate permeability. The cells were then blocked for 45min with 5% bovine serum albumin in PBS.
v. Again cells were washed with PBS thrice.
vi. Stained with BODIPY for 30 min. BODIPY is a fluorescent dye that specifically stains lipid droplets in cells.
vii. The cells were again washed thrice with PBS followed by treatment with antifade reagent DAPI (4,6-diamino-2-phenylindole) (Invitrogen) and observed under an Olympus Fluo View FV10i with laser-scanning microscope Olympus Fluo Viewer version Olympus Fluo Version 3.0.

3.15 TRANSFORMATION

In our study, two NS5A expressing plasmids (genotype 1a and 2a) were used. They were kindly provided by Dr. Gulam waris as previously reported (Aleem Siddique *et al.*, 2001 and Hak Hotta *et al.*, 2008). In order to transform these DNA, LB-plates were made and incubated

them at 37°C for 30min. In a 1.5 ml tube, 25 µl of the DH5-α competent cells were added followed by the addition of 1 µl of the DNA to be transformed. Then the tubes were placed on ice for 30 min and then subjected to heat shock for 2 min at 42°C. After this, 1 ml of LB-broth was added to each tube and incubated at 37°C for 1h and centrifuges at 13000rpm for 5 min. After centrifugation was done, supernatant was removed and pellet was resuspended in 100 µl of LB-broth. The resuspended pellet (200 µl) was streaked in each LB-ampicillin resistant plate. These plates were then incubated at 37°C overnight. Then the isolated colonies were picked and grown in 500 ml of LB-broth (containing amplicillin) at 37°C with continuous shaking overnight.

3.16 DNA EXTRACTION, PURIFICATION AND QUANTIFICATION

Using 500 ml of LB culture that was incubated overnight at were subjected to DNA extraction, purification and quantification using the following protocol (QIAGEN Maxi Prep):

i. The LB-culture was pellet down at 6000 x g for 15 min at 4°C.
ii. Homogenously resuspended the bacterial pellet in 10 ml of buffer P1.
iii. Added 10 ml of buffer P2 and mixed it thoroughly by inverting it multiple times and incubated at room temperature for 5 min.
iv. Then, 10 ml of buffer P3 was added to the mixture, mixed it vigorously and incubated on ice for 20 min.
v. The suspension was centrifuged at 2000 x g for 30 min at 4°C. This step was repeated at 2000 x g for 15 min at 4°C.
vi. The plasmid DNA was eluted on QIAGEN-tip.

vii. Then the QIAGEN-tip was washed with 10 ml of buffer QBT and allowed the column to get empty by gravity flow.

viii. To the QIAGEN-tip, I added supernatant from step 5.

ix. The QIAGEN-tip was washed with 2 x 30 ml of buffer QC and allowed the buffer to pass through QIAGEN-tip by gravity flow.

x. Then, the DNA was eluted with 15 ml buffer QF into a clean 50 ml tube.

xi. The DNA was then precipitated by adding 10.5 ml isopropanol and centrifuged at 15000 x g for 30 min at 4°C. The supernatant was removed carefully.

xii. To facilitate effective DNA elusion, the DNA pellet was washed with 5 ml of 70% ethanol at room temperature and centrifuged at 15000 x g for 10 min. the supernatant was discarded carefully.

xiii. The pellet was air dried and re-dissolved in a suitable volume of buffer.

xiv. The eluted DNA was quantified using eppendorf Biophotometer.

xv. The DNA (1µg) was run on 1% agarose gel and the DNA samples with sharp bands were selected for further use in transfection assays.

3.17 ANTIBODIES

In this study, a number of different antibodies were used that were incubated with their host specific secondary antibody and scanned with odyssey Infrared imaging system (Li-Cor Biosciences, Lincoln, NE). Actin was used as an internal control to determine protein loading in each lane. The details of the antibodies being used in this study are listed in Table 3.5.

Table 3.5: List of antibodies

Antibody	Mol. Wt. (kDa)	Dilution	Host	Scientific Company
IRS-1 ($pSer^{307}$)	165-180	1:500	Rabit	Calbiochem
AKT ($pSer^{473}$)	60-65	1:200	Mouse	Calbiochem
Actin	42	1:500	Mouse	Sigma
GSK3 (pSer α^{21} β^{9})	α 51 β 47	1:1000	Rabit	Cell signaling
Fox01($pSer^{256}$)	82	1:1000	Rabit	Cell signaling
Flag NS5A	56	1:1000	Mouse	Sigma
c-Myc	64	1:200	Mouse	Santa Cruze
NS3	70	1:1000	Mouse	Virogen
Actin	42	1:500	Mouse	Sigma

The secondary antibodies (Anti-Mouse and Anti-rabbit) were used that were specific for odyssey Infrared imaging system.

3.18 OTHER REAGENTS

Human recombinant insulin was purchased from Invitrogen. A stock solution of 100 nM insulin was prepared and then its multiple aliquots were made and stored at -20°C. In this study, we used insulin of 100 nM concentration and treated it with mock, infected and transfected cells. For QRT-PCR, SYB green master mixture (Invitrogen) was used. RNAase free water was purchased from Fischer Bioreagents. For transfection based assays, lipofectamine 2000 was purchased from Invitrogen.

3.19 STATISTICAL ANALYSIS

Real time PCR data was analyzed using Graph Pad Prism version 5.0 and 2-tail error bars represent Standard error of mean (SEM) of the data from three individual trials. P-value of <0.05 was regarded as statistically significant and represented as *.

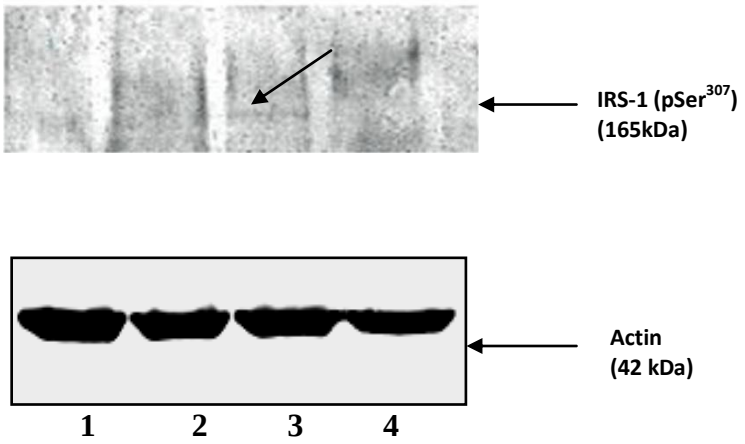
RESULTS

4.1 Insulin signaling in hepatoma cells

In order to examine the insulin signaling pathway, two hepatoma cell lines i.e. Huh 7 and Huh 7.5 cells were used. Cells were treated with insulin (100nM) while untreated cells were taken as control. Using the cellular lysates from treated and control group, western blot was done for p-IRS-1 (Ser^{307}) and -pAKT (Ser^{473}), the key insulin signaling molecules. It was observed that there was no expression of p-IRS-1 (Ser^{307}) in Huh 7 cells treated while a very faint expression of p-IRS-1 (Ser^{307}) was observed in the Huh 7.5 cells only as shown in Figure 4.1. However, a little increased expression of p-AKT (Ser^{473}) was observed in Huh 7 and Huh 7.5 cells treated with insulin as shown in Figure 4.2. To compare protein loading in each lane, cellular actin was used as an internal control.

4.2 Insulin signaling in infected hepatoma cells

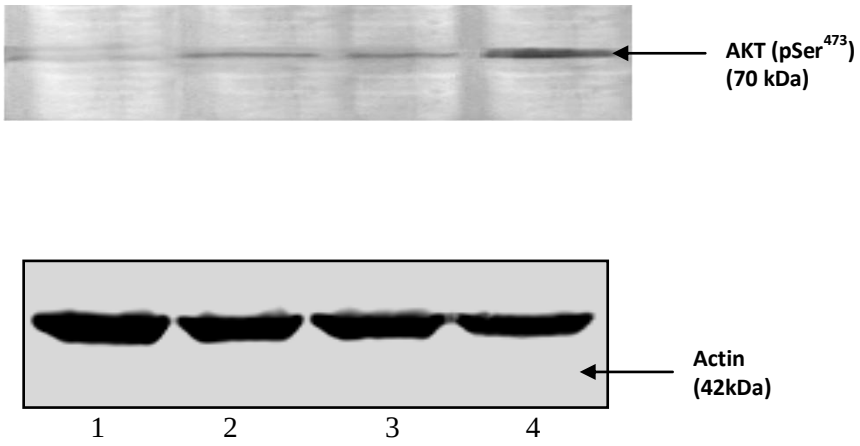
To examine the effect of HCV infection upon insulin signaling pathway, Huh 7 and Huh 7.5 cells were infected with HCV cell culture supernatant and confirmed the HCV infection using western blot assay against HCV NS3 antibody. The results showed sharp bands for HCV NS3 in the HCV infected cells while no NS3 specific band was observed in the mock-HCV infected cells as shown in the Figure 4.3. Furthermore, sharper band for HCV NS3 band was observed in Huh 7.5 infected cells compared to Huh 7 infected cells leading to the conclusion that Huh 7.5 cell line is much susceptible to HCV infection. Cellular actin was used as an internal loading control.



Lanes

Lane-1: Huh 7
Lane-2: Huh 7+ Insulin (100nM)
Lane-3: Huh 7.5
Lane-4: Huh 7.5+ Insulin (100nM)

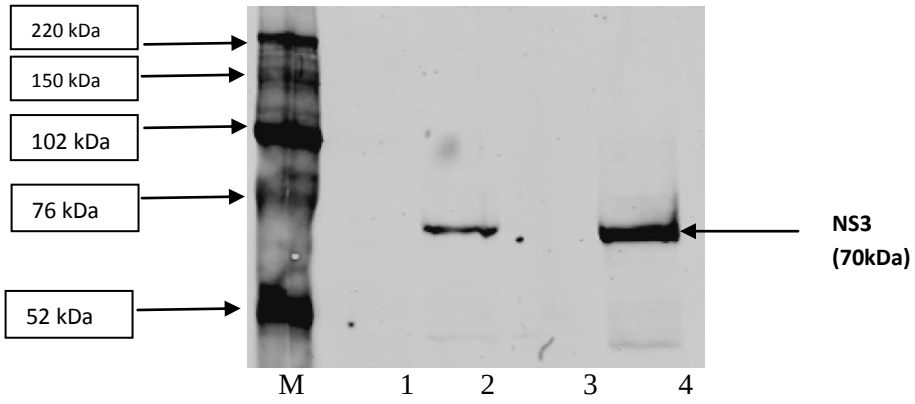
Figure 4.1: Western blot showing pIRS-1 (Ser^{307}) phosphorylation in uninfected hepatocytes. Equal amount of Cellular lysates were made from uninfected Huh 7 and Huh 7.5 cells and were subjected to western blot analysis using anti-p- IRS-1Ser^{307}. Cellular actin was used as an internal control to verify protein loading in each lane.



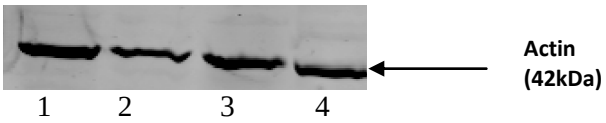
Lanes

Lane-1: Huh 7
Lane-2: Huh 7+ Insulin (100nM)
Lane-3: Huh 7.5
Lane-4: Huh 7.5+ Insulin (100nM)

Figure 4.2: AKT Serine phosphorylation in uninfected hepatocytes. Cellular lysates were made from uninfected Huh 7 and Huh 7.5 cells. Equal amount of cellular lysates were subjected to western blot assay using $pSer^{473}$-AKT. Cellular actin was used as an internal control to verify protein loading in each lane.



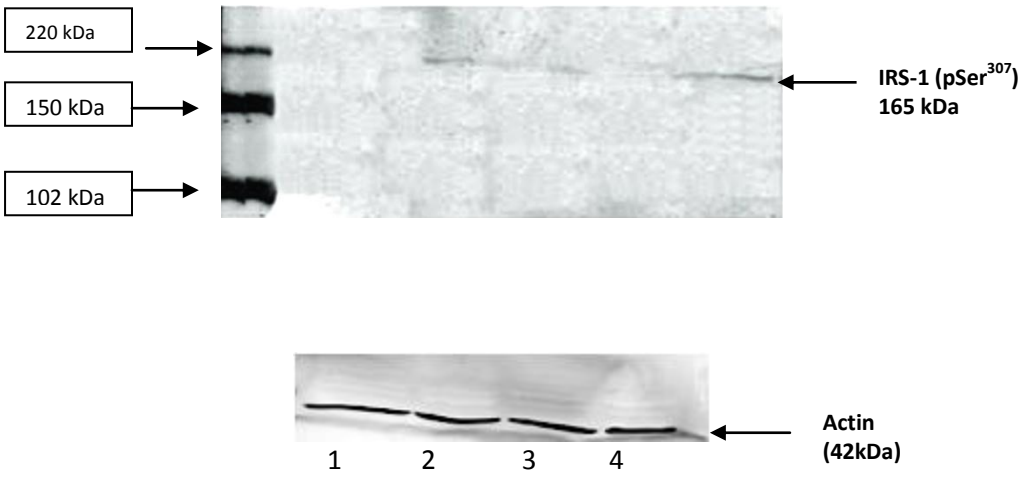
Actin



Lanes

Lane-1: Huh 7
Lane-2: Huh 7+ HCV
Lane-3: Huh 7.5
Lane-4: Huh 7.5 + HCV
Lane-M: Marker

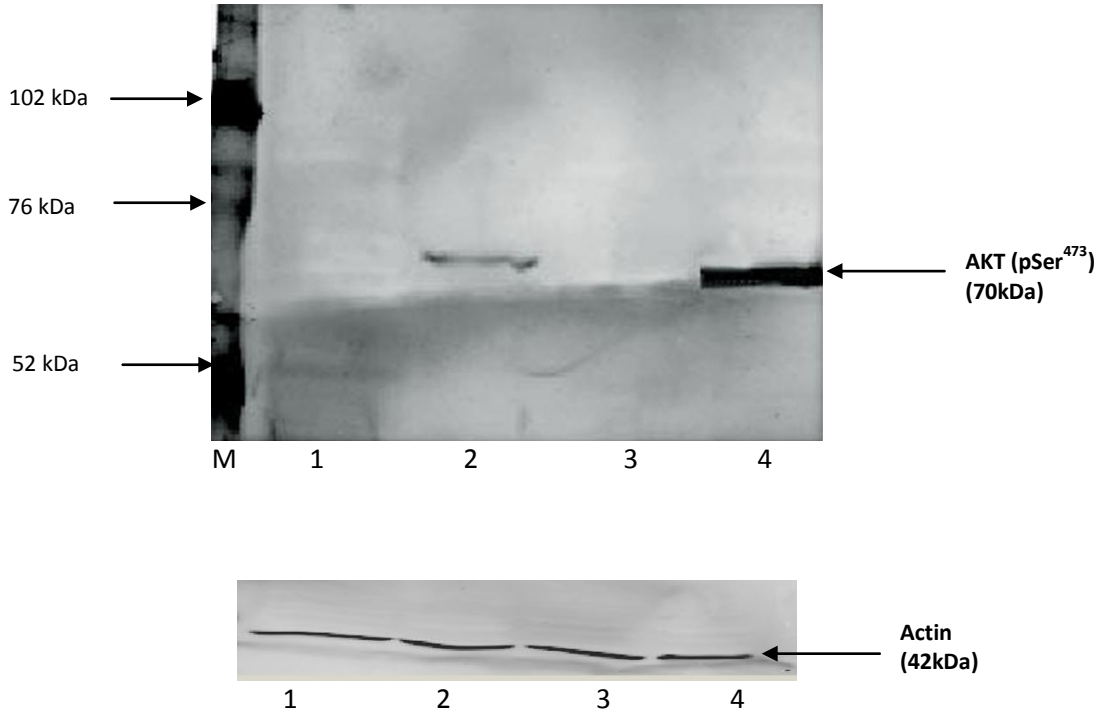
Figure 4.3: Western blot showing HCV infected hepatocyte cell line. Cellular lysates were made from mock and HCV infected Huh 7 and Huh 7.5 cells. Equal amount of cellular lysates were subjected to western blot analysis using antibody against NS3 protein. The sharp band for HCV NS3 represents the level of HCV infection. Cellular actin was used as an internal control to verify protein loading in each lane.



Lanes

Lane-1: Huh 7
Lane-2: Huh 7 + HCV
Lane-3: Huh 7.5
Lane-4: Huh 7.5 + HCV
Lane-M: Marker

Figure 4.4: IRS-1 Serine phosphorylation in HCV infected hepatocytes. Cellular lysates were made from mock and HCV infected Huh 7 and Huh 7.5 cells. Equal amount of cellular lysates were subjected to western blot assay using $pSer^{307}$-IRS-1. Cellular actin was used as an internal control to verify protein loading in each lane.



Lanes:

Lane-1: Huh 7
Lane-2: Huh 7 + HCV
Lane-3: Huh 7.5
Lane-4: Huh 7.5 + HCV

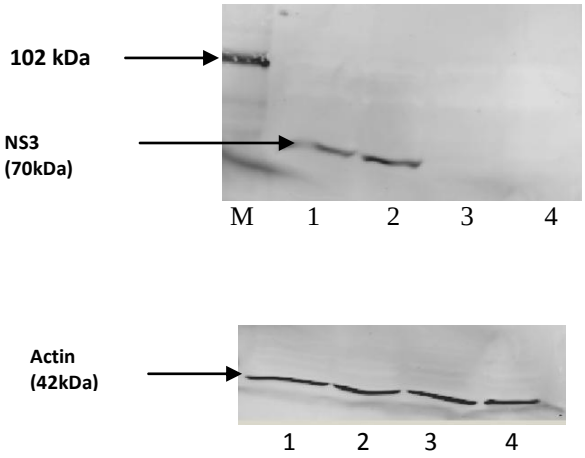
Figure 4.5: Western blot showing AKT Serine phosphorylation in HCV infected hepatocytes. Cellular lysates were made from mock and HCV infected Huh 7 and Huh 7.5 cells. Equal amount of cellular lysates were subjected to western blot assay against p-Akt Ser^{473} antibody.

Using equal amount of cellular lysates, western blot was performed for p-IRS-1 Ser^{307} and p-AKT Ser^{473}. Using the cellular lysates of HCV infected Huh 7 and Huh 7.5 cells, an increased expression of p-IRS-1 Ser^{307} was observed as shown in the Figure 4.4. Furthermore, we observed a significant increased expression of p-AKT Ser^{473} in both of the HCV infected hepatoma cells as shown in Figure 4.5. However, it was observed that the expression of these insulin signaling molecules was much pronounced in HCV infected Huh 7.5 cells as compared to Huh 7 infected cells.

4.3 Effect of Insulin treatment

Based on pronounced expression of the insulin signaling molecules in Huh 7.5 cell line, Huh 7.5 cell line was selected for further experimentation. Furthermore, the effect of exogenous supply of insulin on insulin signaling pathway in HCV infected hepatocytes was observed. Using equal amount of cellular lysates, western blot was performed against p-IRS-1 Ser^{307} and p-AKT Ser^{473} and HCV NS3. Firstly, HCV infection was confirmed in HCV infected hepatocytes using HCV NS3 antibody while the mock-HCV infected cells did not show any NS3 specific band as shown in the Figure 4.6.

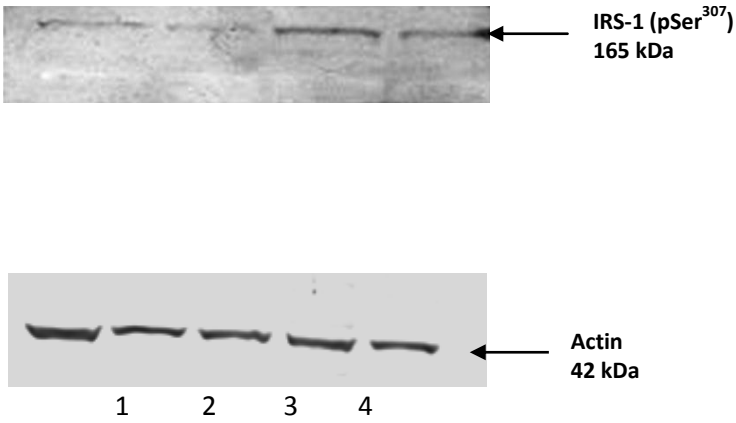
The data indicates that there is an increased serine phosphorylation of $pSer^{307}$ IRS-1 in HCV infected Huh 7.5 cells compared to the mock-HCV infected Huh 7.5 cells Figure 4.7. Contrary, upon the addition of insulin, p-IRS-1 Ser^{307} expression was not significantly decreased revealing the fact that HCV infection favors serine phosphorylation and gets resistant towards the downregulation of serine phosphorylation of IRS-1, a key step towards the modulation of normal insulin signaling pathway.



Lanes

Lane-1: Huh 7.5 + HCV + Insulin (100nM)
Lane-2: Huh 7.5 + HCV
Lane-3: Huh 7.5+ Insulin (100nM)
Lane-4: Huh 7.5
Lane-M: Marker

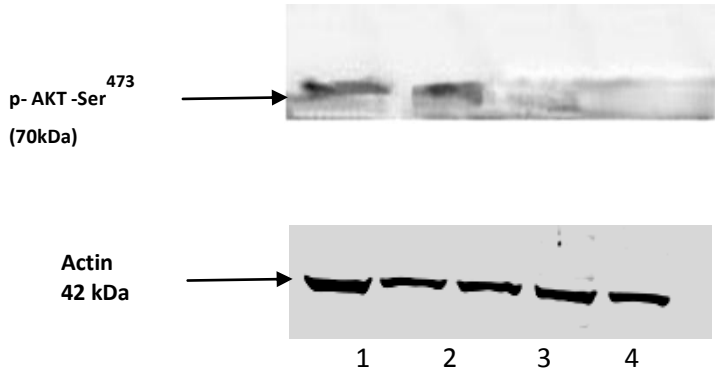
Figure 4.6: Confirmation of HCV infection in Huh 7.5 cells stimulated with insulin. Mock and HCV infected Huh 7.5 cells were treated with insulin (100nM) and equal amount of cellular lysates were subjected to western blot analysis for HCV NS3 to confirm the HCV infection in hepatocytes. Cellular actin was used as an internal control to verify protein loading in each lane.



Lanes:

Lane-1: Huh 7.5
Lane-2: Huh 7.5 + Insulin (100nM)
Lane-3: Huh 7.5 + HCV
Lane-4: Huh 7.5 + HCV + Insulin (100nM)

Figure 4.7: Western blot showing enhanced phosphorylation of pSer^{307} IRS-1 in HCV infected Huh 7.5 cells. Cellular lysates were made from HCV infected and Mock-HCV infected Huh 7.5 cells that were treated or untreated with insulin (100 nM). Equal amount of the cellular lysates were subjected to western blot analysis for the phosphorylation status of pSer^{307} IRS-1. Cellular actin was used as an internal control to verify protein loading in each lane.



Lanes

Lane-1: Huh 7.5 + HCV + Insulin (100nM)
Lane-2: Huh 7.5 + HCV
Lane-3: Huh 7.5+ Insulin (100nM)
Lane-4: Huh 7.5

Figure 4.8: p-Ser^{473} Akt phosphorylation status in HCV infected hepatoma cell line with insulin treatment. Cellular lysates were made from HCV infected and Mock-HCV infected Huh 7.5 cells that were treated or untreated with insulin (100 nM). Equal amount of the cellular lysates were subjected to western blot analysis for the phosphorylation status of pSer4^{73} AKT. Cellular actin was used as an internal control to verify protein loading in each lane.

Furthermore, the effect of insulin upon phosphorylation of AKT (p-AKT^{Ser473}) was investigated. The results indicated that the phosphorylation level of p-AKT Ser^{473} was increased in HCV infected as well as HCV infected cells treated with insulin as shown in the Figure 4.8, indicating the fact that HCV infection modulates AKT phosphorylation and paves a way towards modulation of insulin signaling.

4.4 Effect of HCV NS5A in the modulation of insulin signaling

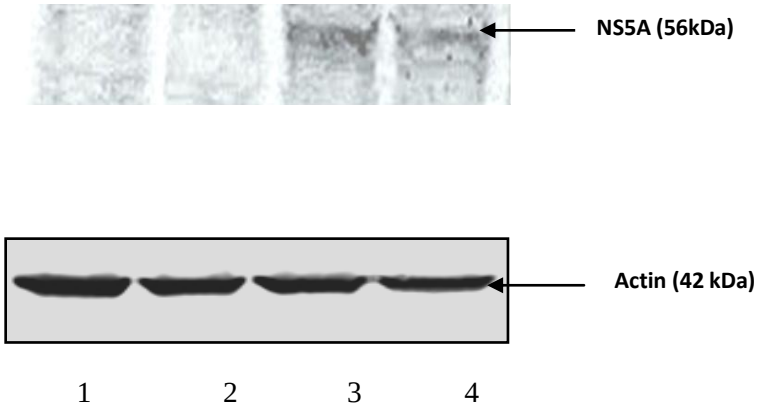
To examine the role of HCV NS5A in the induction of insulin resistance, we transfected Huh 7.5 cells with the indicated HCV NS5A expression plasmids and phosphorylation status of various proteins involved in insulin signaling pathway were examined. Using equal amount of cellular lysates from mock-transfected (with and without insulin treatment) and HCV NS5A transfected (with and without insulin treatment) were subjected to western blot assay against NS5A (Flag and Myc-tagged), p-IRS-1 (Ser^{307}), p-AKT (Ser^{47}), GSK-3 (α-Ser^{21}/β- Ser^{9}) and Fox01 Ser^{256} antibodies.

Firstly, western blot assay was performed for the transfection with both HCV NS5A expressing plasmids and observed NS5A specific band in the NS5A transfected cells as compared to the mock-transfected cells as shown in Figure 4.9 and 4.10. We observed that transfection with HCV NS5A resulted in the significant increased expression of IRS-1 pSer^{307}. While the transfected cells that were treated with insulin showed a decreased expression of IRS-1 pSer^{307} yet exogenous supply of insulin could not abolish serine phosphorylation of IRS-1 in the presence of NS5A as shown in the Figure 4.11 4.12 . Similarly, we observed that HCV NS5A modulates serine phosphorylation of AKT Ser^{473} in the presence as well as absence of insulin,

thereby modulating the downstream insulin signaling pathway as show in the Figure 4.13 and 4.14.

Downstream to the AKT are the signaling molecules that have been implicated in the context of modulation of insulin signaling. One of the important downstream insulin signaling molecules is GSK-3. Therefore, we examined the effect of HCV NS5A upon phosphorylation status of GSK-3 (α/β) and observed that there was a decreased phosphorylation of GSK-3 in the HCV NS5A transfected cells as compared to the mock-transfected cells while addition of insulin triggered an increased serine phosphorylation of GSK-3 as shown in Figure 4.15 and Figure 4.16. To compare protein loading in each lane, cellular actin was used. In addition to the GSK-3, Fox01 is also considered to be an important protein involved in the insulin signaling pathway. Our results showed a decreased phosphorylation of Fox01 (Ser^{256}) in HCV NS5A transfected cells (Figure 4.17 and Figure 4.18) revealing the fact that these changes at the post-translational level of the insulin signaling molecules is triggered by HCV NS5A and may be involved in the modulation of subsequent downstream signaling pathways like gluconeogenesis and lipogenesis.

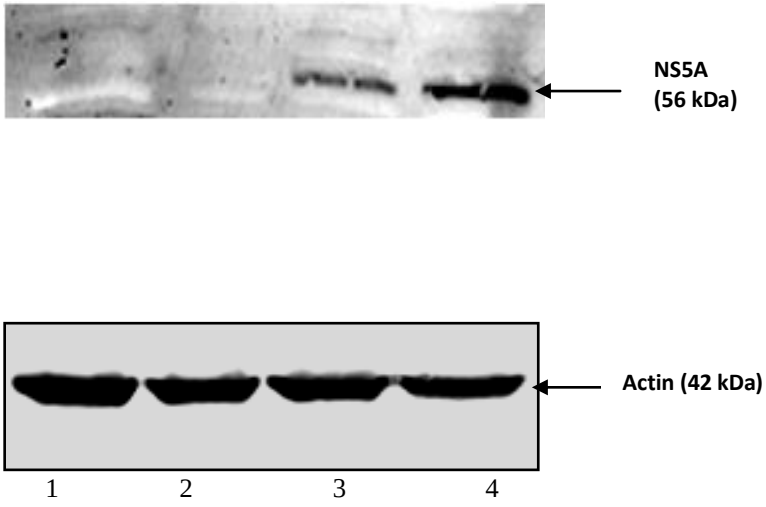
Insulin signaling pathway involves complex metabolic signaling including changes at post-translational level and mRNA levels. In this study, the effect of ectopic expression of HCV NS5A upon modulation of various proteins at phosphorylation level was investigated. Lying downstream to these key insulin signaling molecules are various genes involved in the regulation of key metabolic pathways like gluconeogenesis and lipogenesis. In order to evaluate the effect of HCV and HCV NS5A on the modulation of these metabolic pathways, we did quantitative real-time PCR.



Lanes

Lane-1: Huh 7.5
Lane-2: Huh 7.5 + Insulin (100nM)
Lane-3: Huh 7.5 + NS5A
Lane-4: Huh 7.5 + NS5A + Insulin (100nM)

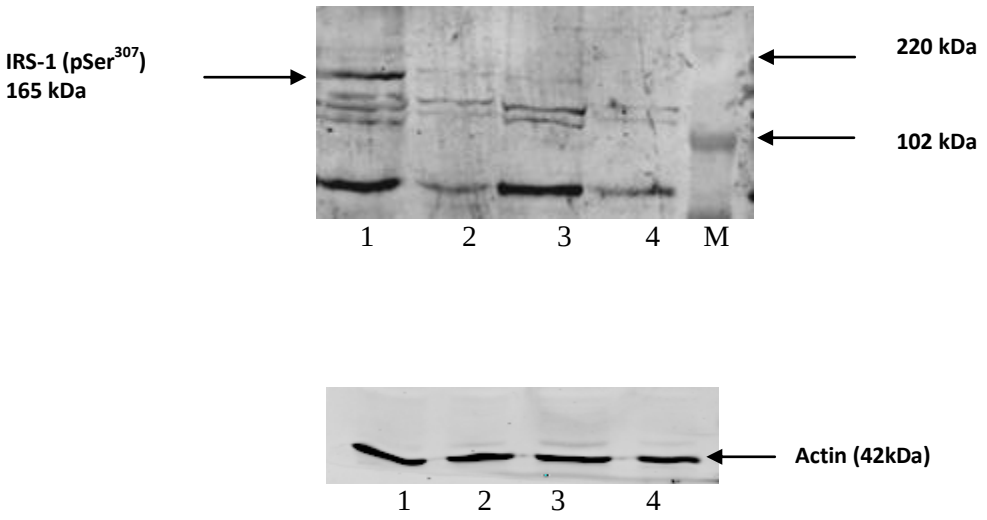
Figure 4.9: Transfection of Huh 7.5 cells with HCV NS5A (genotype 1a). Mock-transfected and HCV NS5A transfected cells were incubated with insulin (100nM) for 3h. Equal amount of cellular lysates were subjected to western blot analysis Flag tagged NS5A. Cellular actin was used as an internal control to verify protein loading in each lane.



Lanes

Lane-1: Huh 7.5
Lane-2: Huh 7.5 + Insulin (100nM)
Lane-3: Huh 7.5 + NS5A
Lane-4: Huh 7.5 + NS5A + Insulin (100nM)

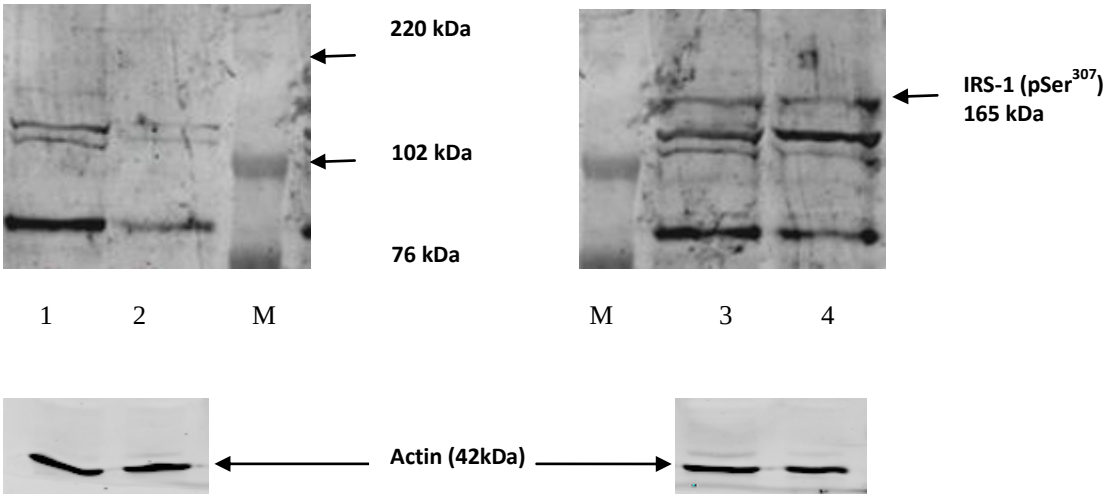
Figure 4.10: Transfection of Huh 7.5 cell line with HCV NS5A (genotype 2a). Untransfected and HCV NS5A transfected cells were incubated with insulin (100nM) for 3h. Equal amount of cellular lysates were subjected to western blot analysis against Myc tagged NS5A. Cellular actin was used as an internal control to verify protein loading in each lane.



Lanes

Lane-1: Huh 7.5 + NS5A (Gen.1a)
Lane-2: Huh 7.5 + NS5A+ Insulin (100nM)
Lane-3: Huh 7.5
Lane-4: Huh 7.5 + Insulin (100nM)

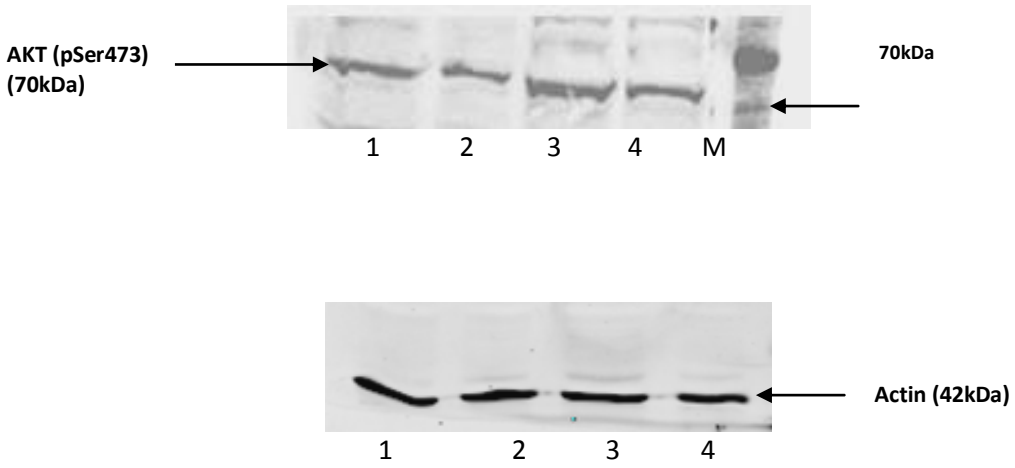
Figure 4.11: p-IRS-1 Ser^{307} phosphorylation level in HCV NS5A (genotype 1a) transfected Huh 7.5 cells. Untransfected and HCV NS5A (Flag tagged) transfected cells were incubated with insulin (100nM) for 3h. Equal amount of cellular lysates were subjected to western blot analysis using anti-p-IRS-1 Ser^{307}. Cellular actin was used as an internal control to verify protein loading in each lane.



Lanes:

Lane-1: Huh 7.5
Lane-2: Huh 7.5 +Insulin (100nM)
Lane-3: Huh 7.5 + NS5A
Lane-4: Huh 7.5 + NS5A + Insulin (100nM)
Lane-M: Marker

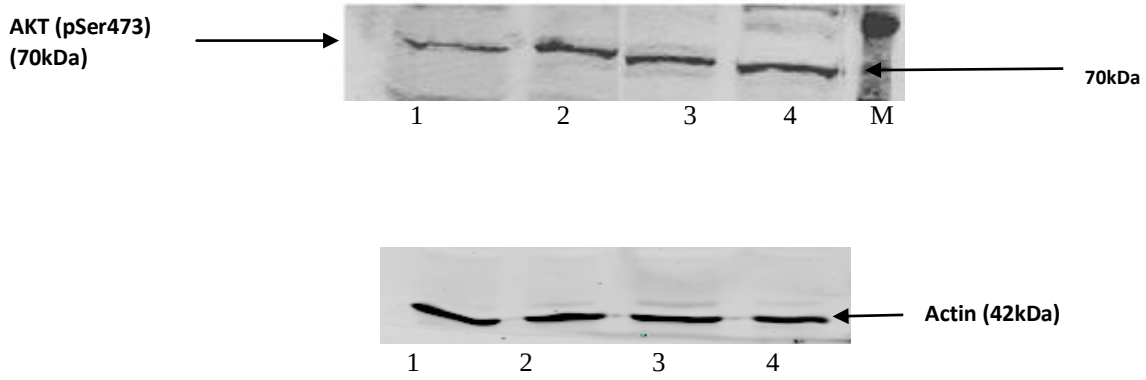
Figure 4.12: HCV NS5A (genotype 2a) favors IRS-1 serine phosphorylation. Untransfected and HCV NS5A (Myc tagged) transfected cells were incubated with insulin (100nM) for 3h. Equal amount of cellular lysates were subjected to western blot analysis against anti-p-IRS-1 (Ser^{307}). Cellular actin was used as an internal control to verify protein loading in each lane.



Lanes:

Lane-1: Huh 7.5
Lane-2: Huh 7.5 + Insulin (100nM)
Lane-3: Huh 7.5 + NS5A
Lane-4: Huh 7.5 + NS5A + Insulin (100nM)
Lane-M: Marker

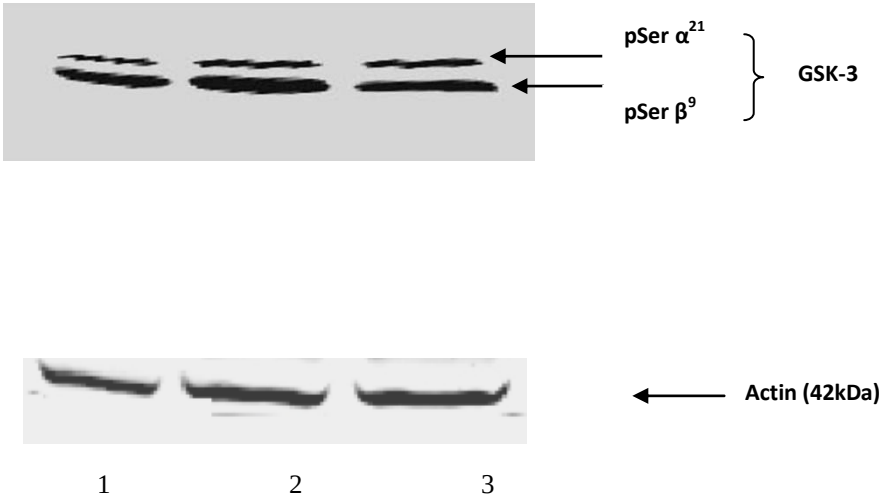
Figure 4.13: Effect of HCV NS5A (genotype 1a) upon AKT^{Ser473} phosphorylation. Untransfected and HCV NS5A (Flag tagged) transfected cells were incubated with insulin (100 nM) for 3h. Equal amount of cellular lysates were subjected to western blot analysis using anti-p-Akt Ser^{473}. Cellular actin was used as an internal control to verify protein loading in each lane.



Lanes:

Lane-1: Huh 7.5
Lane-2: Huh 7.5 + Insulin (100nM)
Lane-3: Huh 7.5 + NS5A
Lane-4: Huh 7.5 + NS5A + Insulin (100nM)
Lane-M: Marker

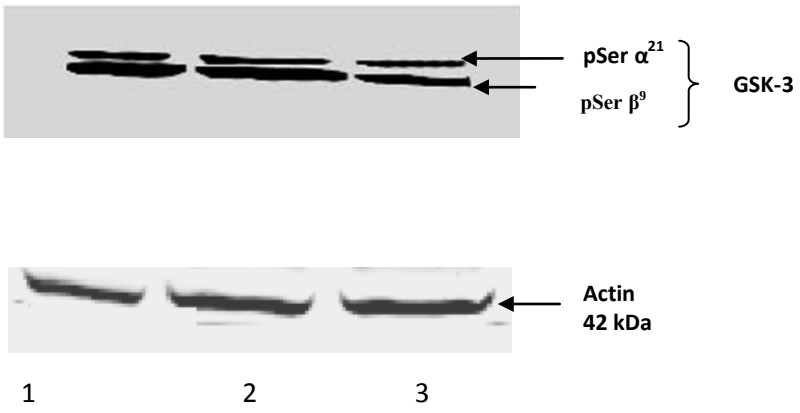
Figure 4.14: Effect of HCV NS5A (genotype 2a) upon AKT^{Ser473} phosphorylation. Untransfected and HCV NS5A (Myc tagged) transfected cells were incubated with insulin (100 nM) for 3h. Equal amount of cellular lysates were subjected to western blot analysis using anti-p-Akt Ser^{473}. Cellular actin was used as an internal control to verify protein loading in each lane.



Lanes

Lane-1: Huh 7.5
Lane-2: Huh 7.5 + Insulin (100nM)
Lane-3: Huh 7.5 + NS5A (genotype 1a)

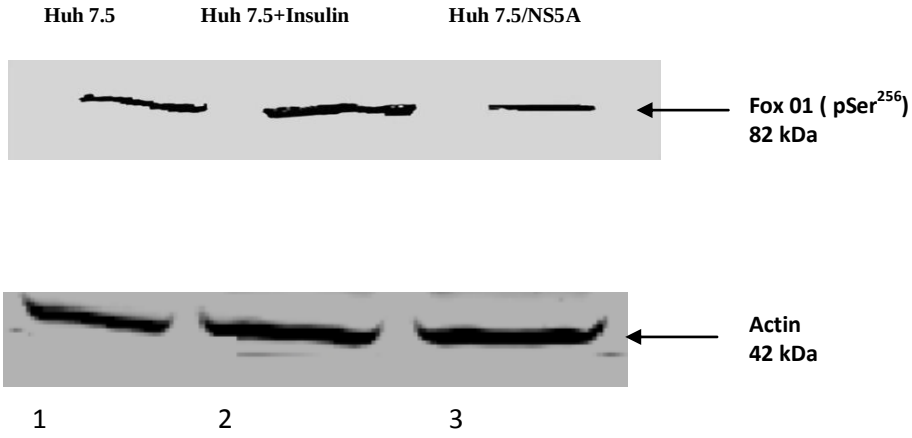
Figure 4.15: HCV NS5A (genotype 1a) decreases the serine phosphorylation of GSK-3. Using the equal amount of cellular lysates from HCV NS5A transfected (flag tagged) and untransfected cells, western blot assay analysis was done against GSK-3 (α/β) antibody. Cellular actin was used as an internal control.



Lanes

Lane-1: Huh 7.5
Lane-2: Huh 7.5 + Insulin (100nM)
Lane-3: Huh 7.5 + NS5A (genotype 2a)

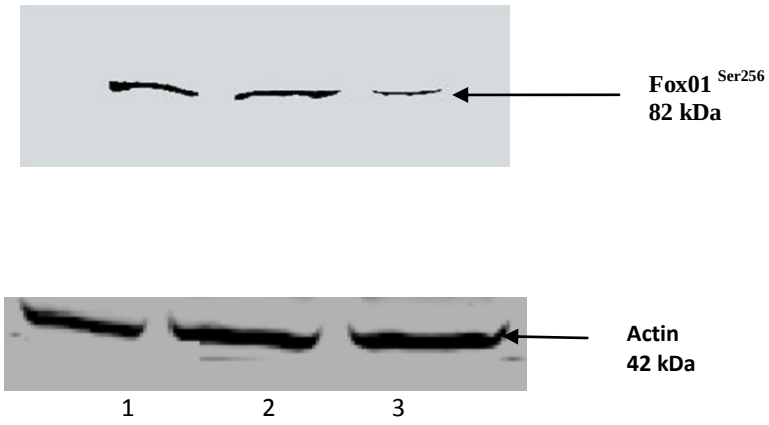
Figure 4.16: Effect of HCV NS5A (genotype 2a) upon serine phosphorylation of GSK-3. Using the equal amount of cellular lysates from HCV NS5A transfected (Myc tagged) and untransfected hepatocytes were subjected to western blot assay using GSK-3 (α/β) antibody. Cellular actin was used as an internal control.



Lanes

Lane-1: Huh 7.5
Lane-2: Huh 7.5 + Insulin (100nM)
Lane-3: Huh 7.5 + NS5A (genotype 1a)

Figure 4.17: HCV NS5A (genotype 1a) decreases the phosphorylation level of FOX01 Ser^{256}. Using the equal amount of cellular lysates from HCV NS5A transfected (flag tagged) and untransfected hepatocytes were subjected to western blot assay using pSer^{256}-Fox-01 antibody. Cellular actin was used as an internal control.



Lanes:

Lane-1: Huh 7.5
Lane-2: Huh 7.5 + Insulin (100nM)
Lane-3: Huh 7.5 + NS5A (genotype 2a)

Figure 4.18: HCV NS5A (genotype 2a) decreases the phosphorylation level of FOX01 Ser^{256}. Using equal amount of cellular lysates from HCV NS5A transfected (Myc tagged) and untransfected hepatocytes western blot analysis was done against pSer^{256}-Fox-01 antibody. Cellular actin was used as an internal control.

4.5 RNA isolation from HCV infected and HCV NS5A transfected cells

Mock infected, HCV infected, mock-transfected and HCV NS5A transfected hepatocytes were cultured at the previously mentioned conditions. After confluency was achieved, cells were harvested for RNA isolation and the resulting RNA was run on 1% agarose gel along as shown in the Figure 4.19.

4.6 Optimization of target genes

4.6.1 cDNA Synthesis

Using the selected RNA samples, cDNA were synthesized. For the optimization of genes, GAPDH was used as an internal control as shown in the gel electrophoresis picture (Figure 4.20).

4.6.2 Optimization of some target genes using conventional PCR

Our targeted markers involved a list of gluconeogenic and lipogenic genes. Using primer 3.0 and BLAT software, we designed primer sequences for all of those genes. However, we could only optimize some genes i.e. CREB, DGAT and PPAR-γ as shown in the Figure 4.21. Rest of the gene specific primer sequences were taken from already published data. In this study, various gluconeogenic (CREB, PEPCK, G6P, PGC-1α) and lipogenic genes (DGAT, PPARγ, MTP) were used. These genes are present downstream to the insulin signaling molecules and are important to regualte metabolic pathways.

4.7 Transcriptional expression of quantitative real-time PCR using HCV infected Huh 7.5 cells

Mock-infected and HCV infected Huh 7.5 cells were cultured at the above mentioned conditions followed by harvesting RNA and used in QRT-PCR.

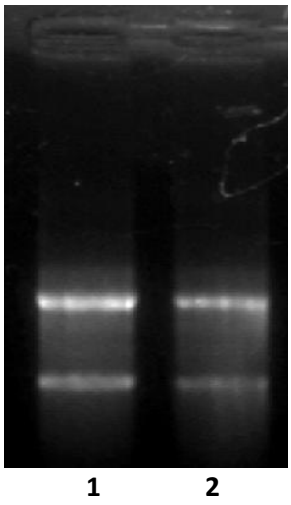


Figure 4.19: RNA isolation from HCV infected hepatocytes. Huh 7.5 cells were cultured under the given conditions of temperature and CO_2 concentration. After attaining a confluency of about 70%, Mock (lane-1) and HCV infected cells (Lane-2) were harvested for RNA isolation to be used for PCR.

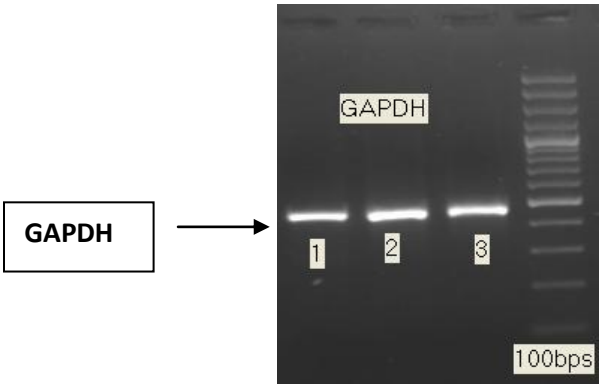


Figure 4.20: cDNA synthesis. Huh 7.5 cells were cultured and harvested for RNA isolation followed by the cDNA synthesis. GAPDH was used as housekeeping gene. The PCR products for GAPDH (Lane 1-3) were run on agarose gel (1%) along with 100bp ladder.

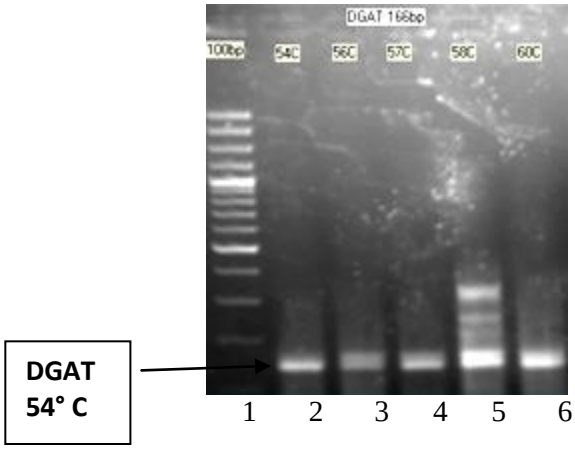


Figure 4.21 (a): Optimization of diacyl glycerol acyltransferase (DGAT). Using the cDNA, the DGATgene was run on agarose gel and optimized at 54°C (Lane-2)**.**

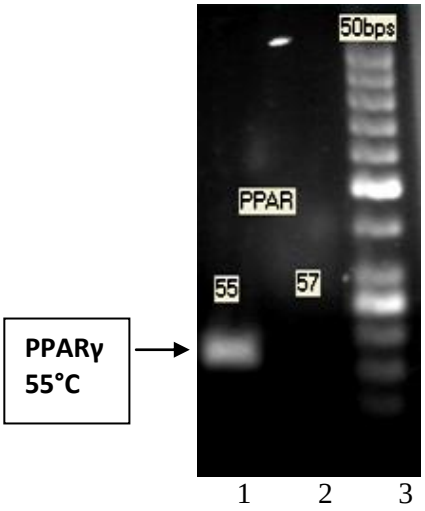


Figure 4.21(b): Optimization of peroxisome proliferator activated receptorγ (PPARγ). Using the cDNA, the gene for PPARγ was run on agarose gel and optimized at 55°C (Lane-1)**.**

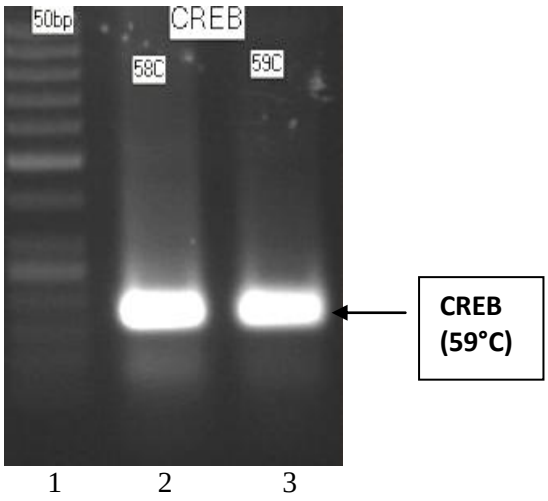


Figure 4.21(c): Optimization of cAMP response element binding protein (CREB). Using the cDNA, the gene for CREB was run on agarose gel and optimized at 59°C (Lane-3)**.**

Total cellular RNA was extracted using trizol method (Invitrogen, CA) and equal amounts of cDNA were subjected to QRT-PCR using an ABI-prism 7500 sequence detector (Applied Biosystems, CA). Amplifications were done in triplicate and 18S was used as an internal control. Amplification reactions were performed in 25 µl mix using QRT-PCR reagent kit and the cDNA. The reaction conditions were: 2min at 50°C, 10min at 95°C, 40 cycles of 15sec at 95°C and 1min at 60°C.

4.7.1 Anti-inflammatory genes

In order to determine the mRNA expression of TNF-α, an anti-inflammatory gene, Huh 7.5 cell line was infected with HCV along with the mock-HCV infected Huh 7.5 cell line as control. Equal amount of cells were plated in 100mm cultured flasks at same time and under same conditions of CO_2 concentration, moisture and temperature. RNA was isolated from the cultured cells and subjected to quantitative real-time PCR that revealed a significant increased expression (p-value of 0.0005) of TNF-α in Huh 7.5-HCV infected cell line compared to the mock-infected cells as shown in the Figure 4.22a.

4.7.2 Gluconeogenic genes

To discern the effect of HCV infection upon gluconeogenesis in hepatoma cell line, we used gluconeogenic markers i.e. PEPCK, PGC-1α and CREB. Using the HCV infected Huh 7.5 cell line, RNA was isolated and qRT-PCR was performed for both of these genes under same conditions as mentioned above. The results showed that PEPCK (p-value of 0.0001) as well as CREB (p-value of 0.0003) increased significantly in Huh 7.5-HCV infected cell line as compared to the control cell line (Figure 4.22b). Similarly there was a significant increased

transcriptional expression of PGC-α (p-value of 0.0001) in HCV infected Huh 7.5 cells compared to the mock infected cells (Figure 4.22 c).

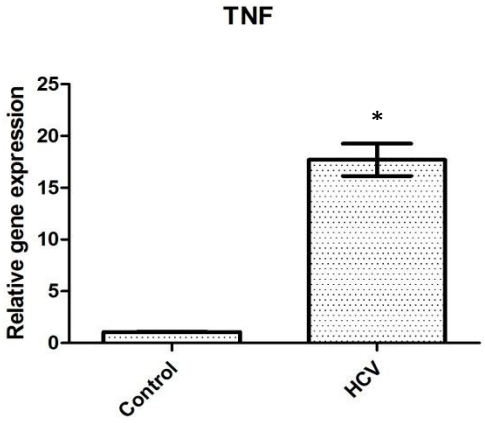


Figure 4.22 (a): Tumor Necrosis factor (TNF)-α. HCV infected Huh 7.5 cells were harvested for RNA isolation and subjected to real time PCR for the transcriptional expression of TNF-α. 18S was used as an internal control. Data represents mean of three independent experiments. Data was analyzed by Graph Pad Prism and p-value less than 0.05 was regarded as statistically significant, represented as *. 2-tail Error bars represent SEM of the data.

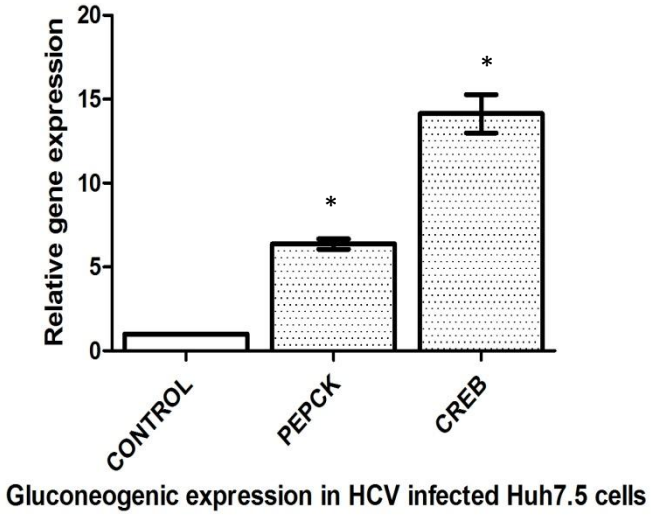


Figure 4.22 (b): Phosphoenol pyruvate carboxy kinase (PEPCK) and cAMP response element binding protein (CREB). HCV infected Huh 7.5 cells were harvested for RNA isolation and subjected to real time PCR for the transcriptional expression of PEPCK and CREB. 18S was used as an internal control. Data represents mean of three independent experiments. Data was analyzed by Graph Pad Prism and p-value less than 0.05 was regarded as statistically significant, represented as *. 2-tail Error bars represent SEM of the data.

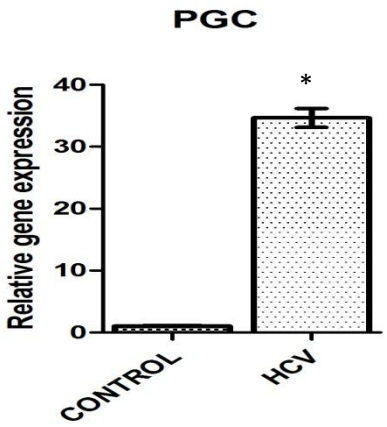


Figure 4.22 (c): Peroxisome proliferator activated receptor γ Co-activator-1α (PGC-1α). HCV infected Huh 7.5 cells were harvested for RNA isolation and subjected to QRT-PCR for the transcriptional expression of PGC-1α. 18S was used as an internal control. Data represents mean of three independent experiments. Data was analyzed by Graph Pad Prism and p-value less than 0.05 was regarded as statistically significant, represented as *. 2-tail Error bars represent SEM of the data.

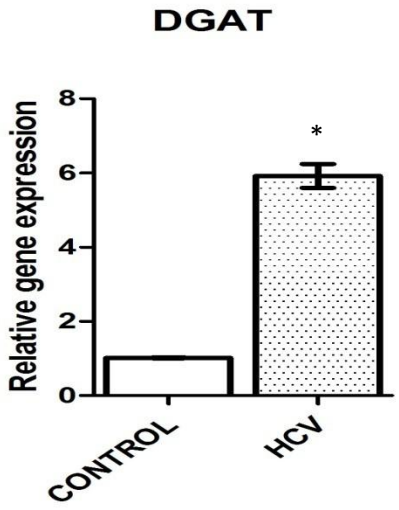


Figure 4.22 (d): Diacylglycerol acyltransferase (DGAT). HCV infected Huh 7.5 cells were harvested for RNA isolation and subjected to QRT- PCR for the transcriptional expression of DGAT. 18S was used as an internal control. Data represents mean of three independent experiments. Data was analyzed by Graph Pad Prism and p-value less than 0.05 was regarded as statistically significant, represented as *. 2-tail Error bars represent SEM of the data.

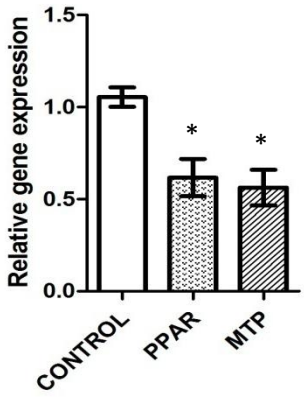


Figure 4.22 (e): Peroxisome proliferator activated receptor (PPAR)- γ and Microsomal triglyceride transfer protein (MTP). HCV infected Huh 7.5 cells were subjected to real time PCR for the transcriptional expression of PPARγ and MTP. 18S was used as an internal control. Data represents mean of three independent experiments. Data was analyzed by Graph Pad Prism and p-value less than 0.05 was regarded as statistically significant, represented as *. 2-tail Error bars represent SEM of the data.

4.7.3 Lipogenic genes

Using the same RNA from Huh 7.5 cells infected with HCV, QRT-PCR was used to determine the mRNA expression level of lipogenic markers. In this study, we used three lipogenic markers i.e. DGAT, PPAR-γ and MTP. The results showed that there was significant increased transcriptional level of DGAT (p-value of 0.0001) in Huh 7.5-HCV infected cell line as compared to the control as shown in the Figure 4.22d. Furthermore, it was observed that there is significant decreased transcriptional expression of PPAR-γ (p-value of 0.0187) in Huh 7.5-HCV infected cell line. Similarly, a significant decreased transcriptional level of MTP (p-value of 0.0109) was observed in HCV infected Huh 7.5 cells as compared to the control as shown in the Figure 4.22e.

4.8 Quantitative real-time PCR using HCV NS5A transfected Huh 7.5 cells

4.8.1 Gluconeogenic genes

Using the confirmed HCV NS5A (genotype 1a and 2a expressing plasmids) transiently transfected Huh 7.5 cell line, RNA was isolated from the cultured cells and subjected to QRT-PCR to evaluate the transcriptional level of gluconeogenic genes in HCV NS5A expressing cell line. In this experiment, key gluconeogenic markers were evaluated i.e. PECK, G6P and PGC-1α. The results showed that there was a significant increased expression of PECK in HCV NS5A (genotype 1a) as well as HCV NS5A (genotype 2a) with the respective p-value of 0.0009 and <0.0001 as shown in the Figure 4.23 a.

Furthermore, the results showed that there is increased transcriptional level of G6P in HCV NS5A (genotype 1a) as well as HCV NS5A (genotype 2a) with the p-value of 0.0001 and <0.0001 respectively (Figure 4.23 b). Similarly a significant increased transcriptional expression

of PGC was observed in NS5A expressing plasmids (genotype 1a and 2a) with the respective p-value of 0.0003 and 0.0001 (Figure 4.23c).

4.8.2 Lipogenic genes

To discern the effect of HCV NS5A upon modulation in lipogenesis, we transfected Huh 7.5 cell line with the indicated HCV NS5A expressing plasmids and RNA was isolated from the cultured cells. Using the NS5A transfected RNA, QRT-PCR was performed for DGAT, a key lipogenic marker. The data revealed that there is significant increased expression of DGAT (p-value of p-value of 0.0004 and 0.0005 in HCV genotype 1a and 2a respectively) as shown in the Figure 4.23d.

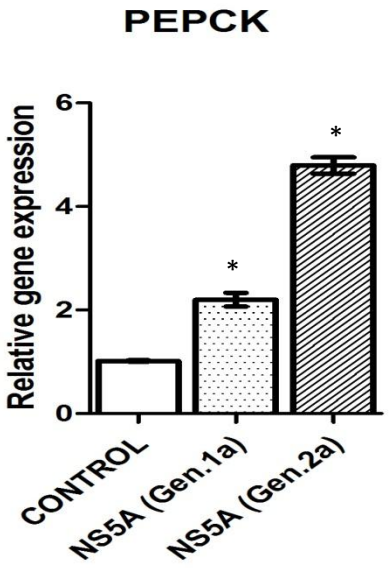


Figure 4.23 (a): Phosphoenol pyruvate carboxy kinase (PEPCK). HCV NS5A favors gluconeogenic gene expression. Huh 7.5 cells were transiently transfected with HCV NS5A and the isolated RNA was subjected to QRT-PCR analysis of the targeted gene i.e. PEPCK. 18S was used as housekeeping gene. Untransfected Huh 7.5 cells were used as control. Data represents mean of three independent experiments. Data was analyzed by Graph Pad Prism and p-value less than 0.05 was regarded as statistically significant, represented as *. 2-tail Error bars represent SEM of the data.

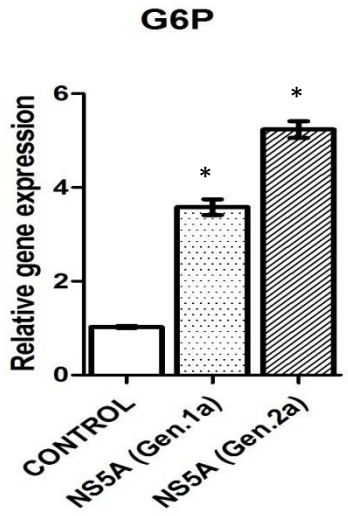


Figure 4.23 (b): Glucose-6-phosphatase (G6P). HCV NS5A favors gluconeogenic gene expression. Huh 7.5 cells transfected with HCV NS5A were subjected to RNA isolation followed by QRT-PCR analysis of the targeted gene i.e. Glucose-6-phosphatase (G6P). 18S was used as housekeeping gene. Untransfected Huh 7.5 cells were used as control. Data represents mean of three independent experiments. Data was analyzed by Graph Pad Prism and p-value less than 0.05 was regarded as statistically significant, represented as *. 2-tail Error bars represent SEM of the data.

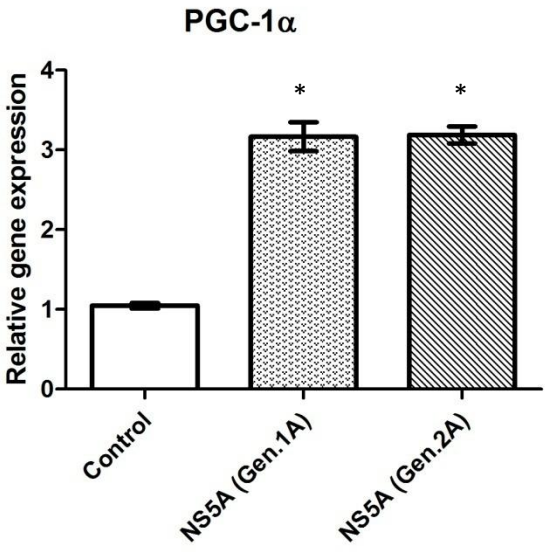


Figure 4.23 (c): Peroxisome Proliferator Activator Receptor γ- Coactivator -1α (PGC-1α). HCV NS5A favors gluconeogenic gene expression. Huh 7.5 cells transfected with HCV NS5A were subjected to RNA isolation followed by real time PCR analysis of the targeted gene i.e. Peroxisome proliferator activator receptor γ- Coactivator (PGC)-1α. 18S was used as housekeeping gene. Untransfected Huh 7.5 cells were used as control. Data represents mean of three independent experiments. Data was analyzed by Graph Pad Prism and p-value less than 0.05 was regarded as statistically significant, represented as *. 2-tail Error bars represent SEM of the data.

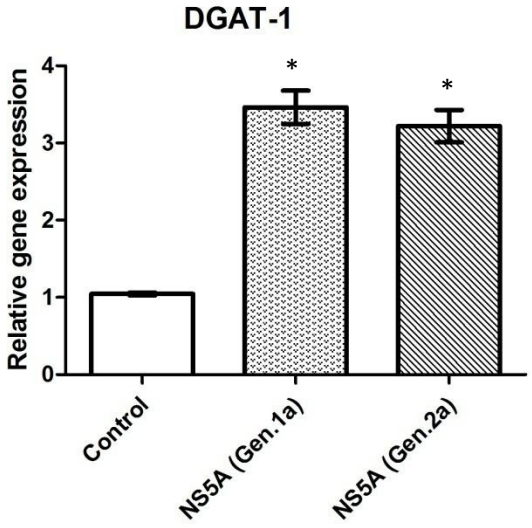


Figure 4.23 (d): Diacylglycerol acyl transferase (DGAT). HCV NS5A favors lipogenic gene expression. Huh 7.5 cells transfected with HCV NS5A were subjected to RNA isolation followed by real time PCR analysis of the targeted gene i.e. Diacylglycerol acyl transferase (DGAT).18S was used as housekeeping gene. Untransfected Huh 7.5 cells were used as control. Data represents mean of three independent experiments. Data was analyzed by Graph Pad Prism and p-value less than 0.05 was regarded as statistically significant, represented as *. 2-tail Error bars represent SEM of the data.

4.8.3 Effect of HCV infection and HCV-NS5A on ectopic lipid accumulation

Our results indicated the effect of HCV infection and NS5A upon modulation of lipogenesis. As the previous data also shows that lipid droplet formation is an important phenomenon towards enhanced lipogenesis, we also investigated the lipid droplet formation with respect to HCV infection and transient transfection of NS5A in the hepatocytes. Our results showed that there is an enhanced lipid droplet formation in the perinuclear region of the HCV-infected hepatocytes as well as Huh 7.5 cells transiently expressing NS5A as shown in the Figure 4.24.

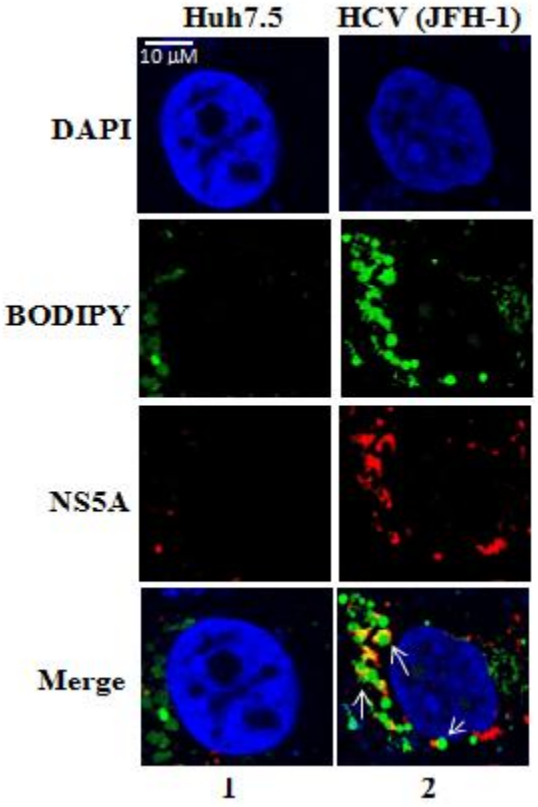


Figure 4.24: Lipid droplet formation. Cells were stained with BODIPY followed by primary and secondary antibodies as described in Materials and Methods. Arrows indicate the formation of lipid droplets around the nucleus. Scale bar, 10 µM.

DISCUSSION

Hepatitis C Virus (HCV) is a blood borne pathogen that targets hepatocytes and moves from acute to chronic phase, favoring multifaceted hepatic and extra-hepatic pathologies like insulin resistance, fibrosis and cirrhosis, eventually turns into hepatocellular carcinoma (HCC). HCV is known to induce metabolic disturbances involving a number of different strategies like oxidative stress, modulation of IRS-1 phosphorylation and increased transcriptional expression of gluconeogenic and lipogenic genes thereby facilitating the development of insulin resistance (Clement, Pascarella *et al*. 2009; Joyce, Walters *et al*. 2009; Parvaiz, Manzoor *et al*. 2011).

HCV shows hepatocyte specific tropism, where it propagates, infects the neighboring hepatocytes and takes control over the host machinery by perturbing normal signaling pathways to favor its replication (Clement, Pascarella *et al*. 2009; Joyce, Walters *et al*. 2009). The previous study indicated that HCV and its certain proteins like core protein and envelope proteins modulate the cellular signaling pathways in such a way that cells become resistant towards the insulin mediated action with the concomitant increased transcriptional expression of gluconeogenic or lipogenic genes (Banerjee, Saito *et al*. 2008); Banerjee, Meyer *et al*. 2010, Shoji, Deng *et al*. 2011, Herker, Harris *et al*. 2010, Farese and Walther 2009). However, there is a great need to unravel the potential role of other proteins and the detailed mechanism to develop potential therapeutic targets against T2DM.

In this study, HCV NS5A was chosen as a target due to its strong role in the promotion of oxidative stress, ER stress, mitochondrial stress and impairment of the homeostasis. It is already known that insulin resistance arises because of metabolic abnormality, therefore, we hypothesized the possible role of HCV NS5A in the induction of insulin resistance. In order to

unravel this possible role of HCV NS5A in the modulation of insulin signaling pathway, we studied key proteins involved in insulin signaling pathway as well targeted various genes lying downstream to the insulin signaling pathway in the context of HCV infection and transfection of hepatocytes with HCV NS5A.

Previous studies reveal that chronic sub-clinical inflammation is often associated with insulin sensitivity and precede the development of T2DM. Several inflammatory cytokines like TNF-α, IL-6, SOCS have been linked with the insulin resistance (Pradhan AD *et al*. 2002, Pradhan AD, Manson JE *et al*. 2001, Banerjee, Saito *et al*. 2008, Greenberg *et al*. 2002, Ruan H *et al*.2003). Therefore, we first tried to examine the effect of HCV infection upon oxidative stress. The findings of the current study showed that there is a significant increased mRNA level of TNF-α in HCV infected Huh 7.5 cells compared to the mock-HCV infected cells. This data highlights the fact that hepatocytes are likely to undergo oxidative stress in response to HCV infection.

Insulin resistance is a complex array of metabolic disorder associated with lipid accumulation, unfolded protein response (UPR) and modulation of proteins involved in insulin signaling pathway (Samuel and Shulman 2012). The previous studies indicated that normal insulin signaling begins with the insulin receptor activation via undergoing tyrosine phosphorylation of the IRS, the docking proteins for insulin signaling (Anderson and Olefsky 1991, Li, Soos *et al*. 2004). However, in case of impairment of insulin signaling pathway, serine phosphorylation is known to take place at various residues (Li, Soos *et al*. 2004, Weiping Sun *et al*. 2011, Joo Hyun Lim *et al*. 2009, Banerjee, Saito *et al*. 2008, Banerjee, Meyer *et al*. 2010). In accordance to the previous work, this study suggests that HCV infected hepatocytes undergo impairment of insulin signaling pathway and this modulation is initiated at the upstream insulin

signaling protein molecule i.e. p-Ser^{307} IRS-1. In spite of the exogenous supply of insulin, this serine phosphorylation of IRS-1 was not abolished. This indicates the fact that HCV infection is potentially capable of modulating insulin signaling pathway and can trigger further metabolic abnormalities. To further discern this pathway, two HCV NS5A expression plasmids (as mentioned in the materials and methods) were used. The results indicated an increased serine phosphorylation of p-Ser^{307} IRS-1 in both of the HCV NS5A transfected hepatocytes while the exogenous supply of insulin was unable to abolish serine phosphorylation of IRS-1, suggesting the potential role of HCV NS5A in the impairment of insulin signaling pathway by favoring its serine phosphorylation. IRS-1 is a key insulin signaling molecule that has to regulate the downstream signaling molecules thereby governing the metabolic processes. Akt is an important downstream molecule of this signaling pathway.

Akt, an important biomolecule lying downstream to the tyrosine kinases, is known to be involved in various regulatory mechanisms like homeostasis, cell survival, cell proliferation, metabolism and angiogenesis (Alessi, James *et al*. 1997, Cross, Alessi *et al*. 1994, Deborah A Altomare *et al*. 2005). Previous studies also suggest that any change in functional activity of Akt is likely to undergo pathological conditions including T2DM and oncogenesis (Lewis C. Cantley and Deborah A Altomare *et al*. 2005). The present study also suggests that hepatocytes infected with HCV infection are likely to favor serine phosphorylation of Akt (Ser^{473}). As a comparison, we used two hepatoma cell lines i.e. Huh 7 and Huh 7.5 cell line. The data suggests an increased serine phosphorylation of Akt in both of the HCV infected hepatocytes nevertheless; there was a robust upregulation of pSer^{473} Akt in Huh 7.5 infected cells as compared to the Huh 7 cells infected with HCV. Therefore, Huh 7.5 cell line was selected for further experimentation. Furthermore, the present study also suggested an increased serine phosphorylation of Akt (p-

Akt-Ser^{473}) the presence of exogenous supply of insulin, revealing multiple biological functions of Akt as shown in previous studies (Banerjee, Saito *et al*. 2008). Furthermore, using NS5A transfected Huh 7.5 cells, an increased Akt phosphorylation (p-Ser^{473} Akt) was observed, indicating that HCV NS5A is also involved in the modulation of Akt (p-Ser^{473}) phosphorylation. Therefore, HCV NS5A is an important candidate for modulation of subsequent downstream insulin signaling pathway and may hamper the metabolic pathways. In addition, Fox01 and GSK-3, are two important signaling molecules lying downstream to the insulin signaling pathway and impart their role in homeostasis.

Fox01 and GSK-3β that are known to favor homeostasis by facilitating insulin mediated actions. The distribution and regulatory actions of Fox01 are mediated in Akt dependant manner (Gross *et al*. 2008, Joo Hyun Lim *et al*. 2009). The previous study suggests a decreased phosphorylation of Fox01 Ser^{256}, an important residue located in the basic region of DNA binding domain, in the immortalized human hepatocytes (IHH) infected with HCV (genotype 1a) and is linked with the impairment of insulin signaling (Banerjee, Meyer *et al*. 2010). Previous studies also suggest that GSK-3 that tends to block glycogenolysis and gluconeogenesis and promotes glycogen synthesis (Cross, Alessi *et al*. 1995, Sanhua *et al*. 2010, Frame and Cohen 2001, Nikoulina, Ciaraldi *et al*. 2002, Eldar-Finkelman 2005, Pamela A. Lochhead *et al*. 2001). In this study, we already demonstrated the role of HCV infection upon modulation of upstream insulin signaling molecules. In order to pull down this signaling pathway, hepatocytes were transfected with HCV NS5A expression plasmids, and it was observed that phosphorylation of p-Ser^{256} Fox01 was significantly reduced in the presence of HCV NS5A while an increased serine phosphorylation of Fox01 was observed when insulin was supplied exogenously revealing the fact that HCV NS5A blocks p-Ser^{256} Fox01 phosphorylation in an IRS dependant manner.

Furthermore, this results also suggest that serine phosphorylation of Fox01 (Ser^{256}) was more reduced in case of HCV NS5A genotype1a. Similarly, the present study suggests a decreased phosphorylation of GSK-3 in HCV NS5A expressing hepatocytes. Likewise Fox01, we observed much decreased level of GSK-3 phosphorylation in hepatocytes infected with HCV NS5A of genotype 1a. These results suggested that HCV NS5A interacts and reduces the serine phosphorylation of GSK-3 in such a way that it triggers inhibition of glycogen synthesis and favors gluconeogenesis, thus imparting its role in the development of insulin resistance.

Taken together, this study suggests the molecular mechanism by which ectopic expression of HCV NS5A is modulating upstream insulin signaling molecules and leads towards insulin resistance.

GSK-3 is an important target for insulin mediated actions as it gets inactivated and reduces the downstream gluconeogenic gene expression. Therefore, any molecule that can alter insulin mediated actions is likely to activate GSK-3 activity and favor gluconeogenesis, thereby paving a way towards insulin resistance (Pamela A *et al*. 2001, Cross, Alessi *et al*. 1995). Many genes are reported to be involved in gluconeogenesis, yet the key ones are PEPCK and G6P. Furthermore, any dysfunction in the expression of these gluconeogenic genes are likely to be linked with the pathophysiology of T2DM (O'Brien *et al*. 1990, Sutherland C *et al*. 1996, Foster 1997, Lange 1994, Zhaofan *et al*. 2009, Rui-Hong Wang *et al*. 2011, Pamela A *et al*. 2001 and Nirmala 2010).

PEPCK is known to catalyze the rate limiting step in gluconeogenesis by facilitating the conversion of oxaloacetate back to phosphoenol pyruvate (Hanson 1994). Previous studies revealed that modulated phosphorylation of downstream insulin signaling molecules like Akt

triggers PEPCK transcriptional expression and favors hepatic gluconeogenesis, thereby leading towards insulin resistance (Zhaofan *et al*. 2009, R.R. Banerjee *et al* .2004). Furthermore, studies have also linked ER stress and mitochondrial stress with the development of insulin resistance through the transcriptional activation of PEPCK (Joo Hyun Lim et a.l. 2009, Min-Woo Lee *et al*. 2010). In other studies, metabolic abnormalities like obesity and insulin resistance are associated with the elevated levels of PEPCK involving upregulation of TNF-α, indicating that there is an increased gluconeogenesis followed by the inflammatory response (Amit K. Pandey *et al*. 2009). The present study also indicates a significant increase in the mRNA expression of PEPCK in HCV infected Huh 7.5 cells. Similarly, a significant increased mRNA level of PEPCK in HCV NS5A transfected Huh 7.5 cells were observed, indicating the potential role of this non-structural protein in the upregulation of key gluconeogenic gene.

Insulin is known to know to mediate its functions through inhibiting the transcriptional expression of a number of genes like PEPCK and G6P. However, in case of T2DM, the inhibitory actions of insulin are disfavored thereby favoring enhanced gluconeogenesis (Pamela A *et al*. 2001). G6P catalyzes the conversion of glycogen into glucose, thereby facilitating gluconeogenesis (Van *et al*. 2002). Furthermore, resistin has been found to modulate insulin signaling pathway and favor gluconeogenesis through the transcriptional activation of G6P (Zhaofan *et al*. 2009). HCV core protein has been shown to favor hypophopshorylation of $pSer^{256}$FOX01 and upregulating gluconeogenesis through the elevated transcriptional expression of G6P eventually favoring the development of insulin resistance (Banerjee, Meyer *et al*. 2010). Similarly, these results also suggest a significant increased mRNA level of G6P in HCV NS5A expressing Huh 7.5 cells revealing an increased gluconeogenesis through the upregulation of PEPCK as well as G6P.

cAMP response element binding protein (CREB) is also considered being an important marker for the insulin resistance. CREB is known to be involved in the glucose regulation as its level is increased in certain conditions like fasting and obesity. However, selective knocking out of this potentially decreases blood glucose level and can prevent the chances of developing T2DM (Yiguo Wang *et al*. 2010). The previous studies showed that transcriptional activation of PEPCK is strongly upregulated by a number of transcription factors like CREB (S. Herzig *et al*. 2001). In addition, mice models having diminished CREB activity have also unraveled the fact that CREB is not only involved in the upregulation of gluconeogenesis but also favors fatty liver phenotype through the upregulation of PPARγ. This also prompted us to quantify the mRNA level of CREB in HCV infected hepatocytes. The present study also indicates a significant increased mRNA expression level of CREB in the HCV infected Huh 7.5 cells,revealing that CREB is likely to be involved in gluconeogenesis as reported in previous studies. Furthermore, we also considered lipogenic gene expression in HCV infected cells as the reported data shows a potential role of CREB in the modulation of β-oxidation of fatty acids and favors lipogenesis.

Among the set of gluconeogenic genes, another important gene that mediates the transcriptional expression of various gluconeogenic genes like PECK and G6P is PGC-1α (Herzing *et al*. 2001, J.C. Yoon *et al*. 2001 and P. Puigserver *et al*. 2003). Blood glucose level should be maintained between the hepatic glucose output and uptake by peripheral tissues. This effective blood glucose level is maintained by the transcriptional regulation of gluconeogenic genes. Previous studies indicate an elevated level of PGC-1 in mice during fasting and diminished insulin sensitivity conditions with the concomitant elevated mRNA expression of PEPCK and G6P (J.C. Yoon *et al*. 2001, Chang Liu *et al*. 2010, Rodgers *et al*. 2005 and P. Puigserver *et al*. 2003). The studies have also showed that PGC-1α is an important co-activator

that binds to other transcription factors like HNF-4α in an Akt dependant manner, thereby modulating the transcriptional activity of various gluconeogenic genes (Puigserver *et al*. 2003, Schilling *et al*. 2006, Rhee *et al*. 2006 and Li X *et al*. 2007). The present study also reveals that ectopic expression of HCV NS5A or infection with HCV triggers a significant increased mRNA level of PGC-1α in hepatocytes. Collectively, these findings suggest that HCV NS5A is potentially capable of increasing transcriptional expression of gluconeogenic genes that are the downstream targets of IRS-1 and paves a way towards the development of insulin resistance.

HCV is known to be a causative agent of multiple hepatic diseases (Liang TJ *et al*. 1993). As far as HCV infection and replication are concerned, it has been found that it co-localizes itself onto the ER membrane via Lipid droplet (LD) formation. HCV proteins including core, E1/E2 and Non-structural proteins are recruited towards LD and facilities in the viral assembly (Moradpour D *et al*. 1996; Deleersnyder V. *et al*. 1997). Lipid droplets are considered to be an important organelle that dynamically moves between the host cell membrane and other organelles including ER, thereby facilitating ectopic lipid accumulation as well as virion assembly followed by effective virion production (Yusuke Miyanari *et al*. 2007). The previous study also indicates that LD formation is also associated with the abnormal lipid metabolism that is likely to disrupt homeostasis of the body (Moriya K. *et al*. 1997). The present study also indicates that HCV infection favors enhanced lipid droplet formation across the perinuclear region of hepatocytes as compared to the mock-HCV infected hepatocytes. This enhanced lipid droplet formation is believed to be involved in the abnormal lipid metabolism. Therefore, these results are is in consistence with the previous results revealing that LD formation is associated with the modulated the gene expression involved in key metabolic pathways.

An important event during HCV induced pathogenesis is the viral assembly and its subsequent egress that infects the neighboring hepatocytes and favors disease progression through LD formation (Miyanari *et al*. 2007, Moriya K *et al*, 1997 and Miyanari Y *et al*. 2003). As far as LD formation is concerned, it is associated with a number of abnormal lipid metabolisms (Moriya K. *et al*. 1997). One of the important cellular targets of lipid metabolism is Microsomal triglyceride transfer protein (MTP) that are considered to play a potent role in the reduction of plasma lipids through Apo B (lipoprotein) biosynthesis that helps in the transfer of lipoproteins towards ER membrane (Joby Josekutty *et al*. 2013, Ahmed Bakillah *et al*. 2001, M Mahmood Hussain *et al*. 2012, Irani Khatun *et al*. 2011 and Mahmood Hussain *et al*. 2011). MTP is also reported being involved in the assembly of lipoproteins particles likewise LDs thereby facilitating the number of lipid particles associated with the MTP activity (Mahmood Hussain *et al*. 2012). The previous study has linked selective inhibition of MTP in fed mice with the enhanced triglyceride content and induction of oxidative stress as well as ER stress that are likely to disrupt the cellular machinery, thus impairing homeostasis (Joby Josekutty *et al*. 2013). Furthermore, it is also known that cellular stress like oxidative stress and ER stress are the hallmarks of metabolic abnormalities like insulin resistance that are likely to favor disease progression (Joo Hyun Lim *et al*. 2009, Gong G *et al* 2001). HCV is known to circulate in blood as lipo-viral particle containing Apo-B, viral RNA and proteins. Although, virus make use of lipoproteins for its propagation, yet it is independent of the lipoprotein assembly thereby giving a clue that HCV propagation takes place in the absence of lipo-protein assembly (Mahmood Hussain *et al*. 2012). In another study, it was found that HCV core protein results in the diminished MTP activity without affecting viral protein expression and suggesting its plausible role in metabolic abnormality (Icard V *et al* 2009). There are two schools of thoughts regarding

viral induced MTP inhibition. According to *the first school* of thought, virus decreases the transcriptional activity of MTP while the other works with some undefined mechanism that plays an important role in the decreased MTP activity yet not lowering the viral titer (Mahmood Hussain *et al*. 2012, Mirandola S. *et al*. 2010). In consistence with the previous studies, the present study also indicates a decreased mRNA level of MTP activity in HCV infected Huh 7.5 cells suggesting that HCV infection might be involved in deteriorating VLDL assembly that leads to the enhanced lipid accumulation, thereby favoring lipogenesis and virion egress.

Furthermore, Peroxisome proliferator activated receptors (PPARs) are the class of molecules that are involved in the homeostasis through the metabolism of biomolecules and have also been linked with severe ailments like diabetes, atherosclerosis and cancer. One of the most important types of PPARs is PPARγ that favors insulin sensitization and favors glucose metabolism (Sander Kersten *et al*. 2000 and Sandeep tyagi *et al*. 2011). The previous studies led to the findings that PPARγ agonist can be used as potential antidiabetic drugs. Although, the direct role of PPARγ on insulin mediated actions has not been established yet it is considered to upregulate free fatty acid contents in the cells thereby protecting the host cells from deleterious effects of free fatty acids and prevents gluconeogenesis (Sander Kersten *et al*. 2000, Terauchi Y *et al* 2005, and Ferre P. 2004).Previous studies have shown that mice with diminished CREB activity has been shown to favor upregulation of PPARγ as well as PGC-1, revealing the fact that CREB is not only involved in gluconeogenesis but also controls the lipogenic activity probably through the transcriptional expression of PPARγ. Therefore, it can be inferred that there is a co-ordinated action of various transcription factors and co-activators that provide a metabolic bridge between insulin and counter regulatory mechanisms (Stephan Herzig *et al*. 2003, Herzing *et al*. 2001 and Matsusue K. *et al*. 2003). These results also indicate a significant decreased mRNA

level of PPARγ in HCV infected Huh 7.5 cells, suggesting that PPARγ is a potential target of HCV by which it can knock down fatty acid oxidation and favors the accumulation of lipids, thereby hampering lipid metabolism. This also suggests that HCV dependant inhibition of PPARγ could also play an important role in the enhanced lipid accumulation that can favor LD formation and further disease progression.

Lipids form a diverse group of molecules executing multiple functions in the cell that are essential for homeostasis and any disturbances in their regulation is likely to facilitate metabolic abnormalities like insulin resistance (Samuel and Shulman 2012). One of the most important lipid moiety reported being involved in insulin resistance is Diacylglycerol acyltransferase (DGAT) (Smith, Cases *et al*. 2000; Levin, Monetti *et al*. 2007). Previous studies revealed that overexpression of DGAT in mice results in increased triglyceride content that are potently involved in the modulation of metabolic pathways (Jornayvaz, Birkenfeld *et al*. 2011). Although Triglycerides (TGs) are essential for normal physiological functions, but its overexpression in adipose and non-adipose tissues is likely to favor insulin resistance (Unger *et al*. 2002 and Friedman J. *et al*. 2002). As far as insulin resistance is concerned, DGAT is known to block tyrosine phosphorylation of IRS-1 through novel class of PKC molecules as well as by modulating phosphorylation of the downstream insulin signaling molecules (Samuel and Shulman 2012).Some studies have also suggested the possible role of DGAT overexpression in HCV infection, viral trafficking as well as a suitable therapeutic target against HCV infection (Herker *et al*. 2010, Charles Harris *et al*. 2011). The present study suggests a significant increased expression of DGAT in HCV infected hepatocytes revealing that HCV infection targets the hepatocytes through impairing lipid metabolism of the host machinery thereby facilitating its trafficking between cellular organelles as well as infecting other hepatocytes. In

consensus with the previous research work, our results indicate that HCV infection is potentially capable of hunting lipid metabolism not only for its propagation, but also for the progression of insulin resistance. Furthermore, it was also revealed that there is significant increased mRNA level of DGAT in HCV NS5A expressing hepatocytes suggesting that this non-structural protein is a potential target for the enhanced lipogenesis as well as modulation of the insulin signaling pathway, thereby paving a way towards insulin resistance.

The results of this study suggest that HCV NS5A modulates insulin signaling pathway from the upstream molecule and modulates its subsequent downstream targets as shown in Fig. 5.1. The results suggest that serine phosphorylation of p-IRS-1Ser^{307} is critically involved in the insulin resistance mechanism as its phosphorylation lead to the reduced phosphorylation of GSK-3 that eventually favors gluconeogenesis. Furthermore, HCV NS5A is also found to be involved in the enhanced lipogenesis. Taken together, we discern a mechanism by which HCV NS5A infects hepatocytes and favors the development of insulin resistance through the upregulation of gluconeogenesis and lipogenesis. Therefore, HCV NS5A is a potential candidate for viral induced pathogenesis that should be considered as therapeutic target to prevent chronic liver diseases.

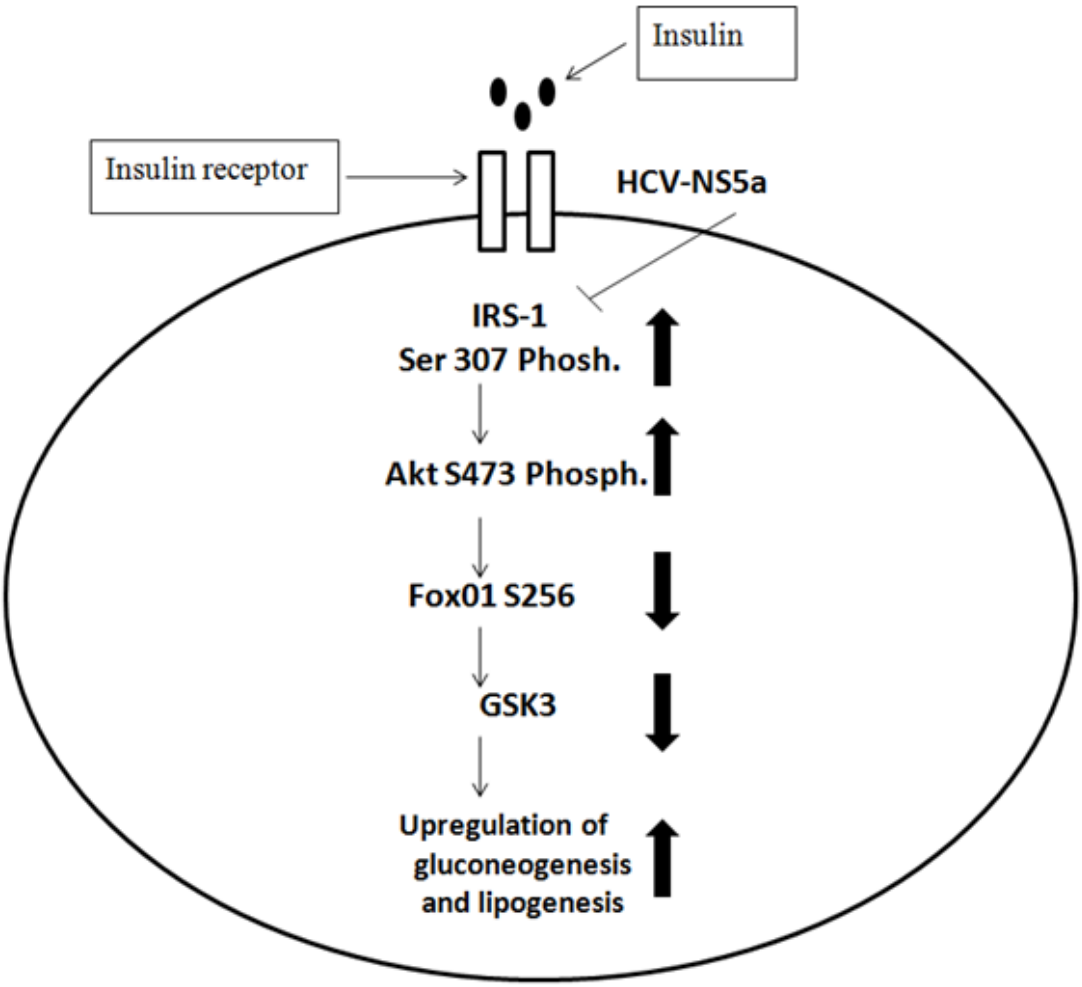


Figure 5.1: Schematic representation of HCV NS5a induced insulin resistance. The (↑) arrows represent the upregulation and (↓) arrows represent downregulation of the target gene/protein.

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