Hepatitis C Virus-Induced Hepatocellular Carcinoma: Cancer Stem Cell and Gene Therapy

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Abstract
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Herein, we have shown that long-term HCV expression results in the acquisition of cancer stem-like cells (CSCs) traits in liver derived cell lines. These traits include enhanced expression of putative stem cell markers DCAMKL-1, Lgr5, CD133 and c-Myc. We also showed that HCV replication is severely impaired by siRNA-led depletion of the microtubule filaments (MTFs)-associated DCAMKL-1. The cholesterol-lowering drug, fluvastatin, reduces DCAMKL-1 RNA and affects its protein localization on the microtubule filaments resulting in marked reduction in the viral RNA and protein abundance. The mouse xenografts, liver biopsy and tissue microarrays unveiled overexpression of DCAMKL-1 during chronic HCV infection and cirrhosis, and in the HCV-replicon expressing cells. We further demonstrate that HCV results in excessive expression of a-fetoprotein, cytokeratin-19, c-Myc, and c-Src, and activation of b-catenin pathway in the absence of Wnt ligand. The result presented here implicates a novel cellular internal ribosome entry site (IRES) element in the c-Src mRNA responsible for the overexpression of c-Src proto-oncogene under stressed conditions such as HCV infection.

These results presented here collectively suggest that HCV exhibits ability to induce reprogramming and/or retrodifferentiation of the host cells and further revealed a novel HCV-(DCAMKL-1)-MTF-CSCs axis that might be responsible for the HCV RNA abundance in the infected cells and HCV-induced hepatocarcinogenesis. The putative stem cell marker, DCAMKL-1, represents a novel cellular target for combating HCV and liver cancer. The concept of a `virus-induced stem cell traits' can also be extrapolated to study diseases caused by other RNA viruses.

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Chapter One: Hepatitis C Virus (HCV): a silent epidemic

1. My exploration in the world of tiny microbes

Viruses are fascinating organic macromolecular complexes that exist as obligate parasites and exhibit high degree of adaptability in their hosts. Although these infectious agents have caused enormous loss to humans and non-humans equally for centuries, they also have taught us about the secrets of life, death and diseases. In fact, the viruses have shaped various life forms on our planet by shuffling and redistributing genes among organisms. Relatively easy manipulation of their genome offers opportunity for the investigators to engineer novel means for combating multitude of diseases including cancer.

Hepatitis C virus (HCV) is one of the highly ‘evolved’ viruses that have clearly challenged modern human intelligence by posing major health problems worldwide including my country (Egypt) in terms of both mortality and morbidity. Recent estimates have shown astounding healthcare spending in USA; rising from $627 million to $6.9 billion (a 10-fold increase) for HCV monoinfection and $63 million to $655 million for HCV-HIV co-infection during last decade. These reasons made me to believe that my medical background combined with extensive research may contribute to alleviate human sufferings due to HCV infection. In this quest, I joined Dr. Naushad Ali’s laboratory who had published numerous papers on molecular mechanism of translation and replication of
HCV. Here, I began working on how HCV survives in human liver for decades without integrating into the host genome, and how its long-term persistence induces cirrhosis and liver cancer (hepatocellular carcinoma). To begin with, I will put my efforts in this chapter to update existing knowledge on the HCV life-cycle, and the advances made in the field of diagnosis, prevention and treatment for hepatitis C. The next 3 chapters (Chapters 2-4) are dedicated to describe my research efforts, important findings during my thesis work, and their implications in designing new treatment strategies against HCV and liver cancer.

2. Historical Perspective of Hepatitis

In the late 1960’s, two hepatitis viruses, hepatitis A virus (HAV) and hepatitis B virus (HBV) that caused infectious jaundice, were known and were distinguished on the basis of transmission route and reactivity of patient’s serum to their respective antigens (Fig 1a). During early 1970’s, transfusion recipients were being screened with the concept that hepatitis B would be the culprit for transfusion-associated hepatitis, but neither hepatitis A nor B viruses were found to be responsible. Another hepatitis agent, hepatitis D virus (HDV) was although identified, it was found to be a defective virus that is always associated and dependent on HBV infection (Manns 2000).

Extensive epidemiological studies from around the world found a history of chronic HBV persistence (over 6 months) in 5% of the infected individual. Intriguingly, over 50% patients with blood transfusion-associated hepatitis exhibited chronic diseases. These observations led to the search for non-A non-B hepatitis agent in these cases. Using molecular biological approach during late 1989, a new RNA virus, named hepatitis
C virus (HCV) was discovered at Chiron Corporation (USA) as a causative agent for the parenterally transmitted NANB hepatitis cases (Choo et al 1992). With the development of RT-PCR and ELISA tests, it was quickly apparent that over 80% of HCV infected individuals develop chronic hepatitis (Guobuaite et al 2008). Follow-up over 20 years and perspective studies showed that many infected persons developed progressive liver fibrosis, often extending to cirrhosis and/or liver cancer. The long-term studies on natural history has been proven to be challenging as the disease onset is often subclinical and progression is extremely slow (Farci et al 1991). Due to these reasons, hepatitis C is also termed as a 'silent killer’ diseases.

The enterically transmitted NANB hepatitis was later found to be caused by a calicivirus-like agent termed hepatitis E virus (HEV). Similar to HAV, HEV usually causes self-limiting acute hepatitis during sporadic infection or outbreaks in the developing countries. However, higher mortality rate (~20%) among HEV patients due to fulminant hepatitis has been reported for pregnant women than other hepatitis viruses (Jilani et al 2007). Recent studies also indicate that HEV exposure to pregnant women is extremely high (85%) in the Nile Delta region of Egypt (Stoszek et al 2006). It is interesting to note that human HEV strains are close relatives of swine and other animal HEV strains. The zoonotic HEV transmission has been reported in Japan and USA due to raw meat consumption, it is likely that animal HEV will adapt to infect humans in future (Renou et al 2007).
3. Epidemiology

HCV belongs to the family Flaviviridae and is the only member of the Hepacivirus genus. The virus is transmitted through direct contact with the blood or body fluids of infected individuals. (Lavanchy 2011, Martins et al 2011).

Hepatitis C is recognized by the World Health Organization (WHO) as a global health problem (Fig. 1B). An estimated 3% of the world population (approximately 170 million) and 4 million in USA alone have been infected with hepatitis C. In countries like the United Kingdom, Scandinavia (0.01% to 0.1%), Americas, Western Europe, Australia, and South Africa (0.2% to 0.5%) the prevalence of HCV infection is low. Meanwhile, Intermediate prevalence is recorded in Eastern Europe, Mediterranean, Middle East, and India. Other countries with intermediate prevalence include Brazil, Eastern Europe, parts of Africa, and Asia. Egypt has a high prevalence of HCV infection (17% to 26%) besides Hubei, Mongolia, and Pakistan. Among injection drug users (IDUs), HCV and HIV coinfection is a major problem. HCV is acquired relatively soon after the intravenous drug use. It is estimated that 50% to 90% of IDUs with HIV also contract HCV infection. As a result, liver disease from HCV has become the leading cause of death in the U.S. in persons coinfected with HIV (Gyarmathy and Racz 2011).

4. HCV in Egypt: a case of unintended medical malpractices

One of the highest prevalence rates (10-13%) of HCV infection in the world is found in Egypt (Fig 1.1B). In certain areas such as delta-Nile region, it may rise up to 40% (Fallahian and Najafi 2011). The epidemic appears to have been initiated during
vigorous public-health campaigns using intravenous tartar emetic to eradicate a parasitic infection, schistosomiasis, during 1950s through 1982 (Frank et al 2000). Before 1986, intravenous tartar emetic was the main schistosomiasis treatment used in Egypt. At that time, glass syringes and needles were inadequately sterilized by boiling due to time restraints and limited resources. As a result, these treatment campaigns improved schistosomiasis-related morbidity but created hepatitis C disease at epidemic proportion in Egypt. Consequently, a high incidence of hepatic morbidity and mortality from the late complications of HCV infection has emerged, such as chronic hepatitis, cirrhosis and hepatocellular carcinoma (HCC) (El-Zayadi et al 2007, Strickland et al 2002). While HCV has six major genotypes worldwide (Simmonds et al 2005), the most predominant in Egypt is genotype 4 representing over 90% of cases, with 4a as the dominant subtype.

5. Natural History of HCV

Exposure to infected blood is the main transmission route of HCV including Blood transfusion especially that was done before 1992 and intravenous drug use. High-risk sexual activity, organ transplantation from an infected donor, occupational exposure, hemodialysis, and birth to an infected mother, household exposure and intranasal cocaine use are other risk factors. According to the Centers for Disease Control and Prevention (CDC), HCV transmission through transfusion of contaminated blood products is now approximately 1 in 500,000 to 2,000,000 transfusions due to the mandatory implementation of HCV screening. High-risk drug (60%) and sexual behaviors (20%) were the most common risk factors for acute HCV infection in the U.S. from 1991-1995. 10% of infections were attributed to other modes of transmission. In 10% of persons
with HCV infection no recognized source of infection can be identified, despite the fact that most persons in this category are associated with low socioeconomic level (Mellor et al. 1995).

a. Acute hepatitis

As the majority of acutely infected cases are asymptomatic, acute hepatitis C infection is frequently missed clinically; 70% to 80% of the blood transfusion documented cases patients were asymptomatic (Chen and Morgan 2006). 20% to 30% of adults with acute HCV infection may show clinical symptoms. The incubation period ranges from 3 to 12 weeks after first exposure. Symptoms includes malaise, anorexia, jaundice and weakness (Czepiel et al. 2008). Serum alanine aminotransferase (ALT) levels, start to rise 2 to 8 weeks after exposure due to hepatocyte necrosis, and can reach levels up to 10-folds the upper normal limits (Vince 2005). The HCV RNA can be detected in the serum 1 to 2 weeks after exposure. In some cases (20%), acute hepatitis C can be self-limited with symptoms lasting several weeks then subside along with ALT and HCV RNA levels decline. Acute HCV infection severe enough to cause fulminant liver failure is rare. The patient becomes positive for anti-HCV antibodies with the onset of symptoms 1 to 3 months after exposure and is detected by enzyme immunoassay. Up to 30% of patients also show false negative results for anti-HCV at the onset of their symptoms, making HCV antibody an unreliable test in the diagnosis of acute infection. Besides, anti-HCV antibody titers can be low or even undetectable in immunodeficient patients.
b. Chronic hepatitis

In Chronic hepatitis C, the viral RNA persists in the blood for at least 6 months after onset of acute infection. Approximately 75%-85% of infected patients do not clear the virus after 6 months and chronic hepatitis develops. Factors affecting the rate of chronic HCV infection includes the age at time of infection, gender, ethnicity, and jaundice severity during acute infection (Fiel 2010).

c. Extrahepatic manifestations

A number of other extrahepatic manifestations has also been demonstrated with hepatitis C that include the development of fatty liver (steatosis), autoimmune phenomena, peripheral neuropathy, sialadenitis, uveitis, corneal ulceration, and polyarteritis nodosa (Jacobson et al 2010).

Deposition of circulating immune complexes in small and medium blood vessels results in the development of a condition known as Essential Mixed Cryoglobulinemia (EMC). EMC presents with rash, arthralgias, and weakness. It is also reviewed that hepatitis C can be found in 95% of all patients with EMC (Jacobson et al 2010). Investigators have concluded that hepatitis C may have a causative role in EMC as Anti-HCV antibodies can be detected in the vessel walls of skin biopsy specimens taken from patients with EMC and chronic vasculitis. Furthermore, Interferon (IFN) therapy has been shown to lead to symptomatic improvement of both rash and joint pains. However, symptoms almost reappear upon cessation of therapy (Pietrogrande et al 2011). Described in several reports, the increased incidence of Non-Hodgkin B-cell lymphoma occurs in patients with hepatitis C.
Figure 1.1 Viral hepatitis and worldwide impact. A. Types of hepatitis viruses and clinical outcomes of the infections. The arrows with red color show clinical outcomes of HCV infection. B. Prevalence and distribution of HCV worldwide. C. Proposed mechanism of attachment/entry of the virus to host cells.
A positive association between HCV and NHL was first described along with cryoglobulinemia using bone marrow biopsy (Marcucci and Mele 2011). Glomerulonephritis is also one of the extrahepatic manifestations associated with hepatitis C infection. Patients are noted to have significant nephrotic range proteinuria and most cases of glomerulonephritis are also associated with cryoglobulinemia. Membranoproliferative glomerulonephritis is the most common histologic lesion found in those patients (Fabrizi et al 2010). Hepatitis C has also been associated with endocrine disorders including an increased incidence of antithyroid antibodies and diabetes mellitus. Recently the association has been uncovered between hepatitis C and diabetes mellitus, and it was demonstrated that among individuals older than 40 years, patients with hepatitis C infection were 3 times or more likely to have type 2 diabetes mellitus than those without hepatitis C infection (Maheshwari and Thuluvath 2011). Also several dermatologic disorders have been described in association with hepatitis C. These include porphyria cutanea tarda (PCT) and lichen planus.

d. **Hepatocellular Carcinoma (HCC)**

Hepatocellular carcinoma (HCC) is the fifth most common malignancy in the world, accounting for approximately one million deaths with an increasing trend of new incidences annually (Farazi and DePinho 2006) HCC incidence and mortality are increasing, while mortality from other cirrhosis-related complications appears to be declining. HCC is also the most prevalent form of primary liver cancer that is histologically and etiologically distinct from other forms of primary liver cancer. High mortality associated with this disease is mainly attributed to the failure to diagnose HCC
patients at an early stage and a lack of effective therapies for patients with advanced stage HCC. In the US and Europe, the rise in incidence is mainly due to the current HCV epidemic and is expected to continue to increase over the next two decades (Marrero and Pelletier 2006).

HCC has a multifactorial etiology, with affecting disease course and prognosis. In most patients (Approximately 70%–90%) with HCC, cirrhosis is a common feature which develops after long periods of chronic liver disease that leads to increased fibrous tissue and destruction of liver cells, eventually allowing tumor development. The risk factors for developing HCC include HBV and HCV infection with an incidence of 54.4% and 31.1% of liver cancer cases globally, respectively, NAFLD, nonalcoholic steatohepatitis (NASH), alcoholic liver disease, intake of aflatoxin-contaminated food, diabetes, obesity, and some hereditary conditions such as hemochromatosis, and some metabolic disorders. The presence of multiple components appears to further drive the hepatocarcinogenic process (Yang and Roberts 2010b).

Cirrhosis due to HBV and HCV infection is the major risk factor for developing HCC. Cirrhosis facilitates the development of cancerous nodules (Caillot et al 2009). Since liver cirrhosis has a central role in the morbidity and mortality associated with HCC, establishing the presence and severity of cirrhosis is essential in all patients to assess prognosis and proceed with the treatment.

There are several oncogenic pathways involved in HCC like phosphoinositol-3-kinase/Akt, myc, Wnt/β-catenin, c-Met, and hedgehog (El-Serag and Rudolph 2007,
Monga 2009). Some of these pathways are developmental pathways, indicating that some HCCs may arise from liver stem cells. Presence of liver cirrhosis and the resultant growth inhibition can activate and transform liver stem cells, and according to the work done by (Lee et al, 2006) there is an evidence suggesting that a stem-cell gene-expression signature exists in a subset of human HCCs (Lee et al 2006c).

e. HCV as a major risk factor for the development of Hepatocellular Carcinoma

The main reasons for the increasing incidence of HCC in the developed world are chronic HCV infection and Non-Alcoholic fatty liver disease (NAFLD) (Regimbeau et al 2004). Chronic Infection with hepatitis C virus (HCV) is considered to be a prominent epigenetic factor for the development of HCC in most patients. Until now, Direct evidence for an HCV carcinogenic role is lacking. In the work done by (Velosa et al, 2011), they showed that in compensated HCV-related cirrhosis, sustained virological response (SVR) due to Interferon alpha treatment markedly reduces the risk of HCC and improves patients' survival (Velosa et al 2011).

In contrast to HBV infection in which, HCC can occur in the absence of cirrhosis due to viral integration into host DNA, HCV will not be incorporated into the cellular genome. It induces the development of cancer cells, which proliferate out of control through different mechanisms including the effect of some proteins of the virus which may induce the oncogenic process. Furthermore, It has been also proposed that the virus acquires its oncogenic effect via oxidative stress, and by activation of different oncogenic
signal pathways. The studies done by (Fishman et al, 2009)(Fishman et al 2009) and (Hu et al, 2009)(Hu et al 2009) showed that Differences were found in HCV core gene sequences between HCC patients and patients with early-stage liver disease with and without cirrhosis. Other factors may have a role in the development of liver carcinoma besides the virus determined factors, like the host reaction, the deficient tumor cell eliminating capacity may also be a factor (Szalay 2010).

Due to the fact that most HCV-related HCC occurs on top of severe fibrosis or cirrhosis it is also proposed that the mechanism of carcinogenesis is possibly indirect, in such way that tissue damage, regeneration and repair are required, rather than a direct viral oncogenic effect or the viral induced inflammatory response (Nash et al 2010). In a study done by marshal et al 2005, It was shown that hepatocytes from patients with chronic HCV infection are arrested in G1 and can undergo replicative senescence, predisposing to malignancy(Marshall et al 2005).

Since chronic HCV infection is a dominant risk factor for the development of HCC, understanding the molecular basis of HCV-induced CSC-like properties in hepatic cells will advance our ability to prevent and treat HCC. In adult liver, hepatic stem/progenitor cells (HSPCs) predominantly reside in bile duct (canal of Herring). These cells are quiescent with a low proliferating rate and are activated only when the mature epithelial cells of the liver are continuously damaged or lost. In severe acute or chronic injury, proliferation and self-renewal of HSPCs are significantly enhanced. Although HCV is a noncytopathic virus, the infection causes loss of hepatocytes and significant reduction in regenerative capacity of the liver that ultimately results in
cirrhosis. These observations clearly indicate that during long-term HCV infectious process, the normal function of liver stem/progenitor cells are lost or impaired.

Telomere length and chromosomal stability in proliferating cells such as hepatocytes are maintained in part by telomerase. Shortening of telomere is therefore thought to reduce the regenerative capacity of organs during aging and chronic disease limiting proliferation. In the study done by Wiemann et al, 2002 (Wiemann et al 2002), it was shown that of in a cirrhotic liver, telomeres are significantly shorter than in a noncirrhotic one, and this shortening is correlating with fibrosis progression. Therefore, telomere dysfunction and alterations in the micro- and macro environment that stimulate cellular proliferation has been proved to affect development of HCC. Therefore, reduction in hepatocyte proliferation is thought to enhance cancer formation in cirrhotic livers. This observation was further supported by an experiment in a rat model in which hepatocyte proliferation was inhibited by chemicals and carcinogen-induced liver tumor formation was accelerated (van Gijssel et al 1997). Cirrhosis also activates stellate cells leading to an increase in the production of growth factors, cytokines, and oxidative stress products (Bataller and Brenner 2005) which all have been shown to affect hepatocyte proliferation and so could have a role in tumor formation (El-Serag and Rudolph 2007). Activation of Akt signaling In liver cirrhosis can promote tumor formation by suppressing transforming growth factor (TGF)-β–induced apoptosis. Activation of this pathway has also been linked to activation of β-catenin signaling, further supporting the hepatocarcinogenic process (El-Serag and Rudolph 2007).
In addition to all mentioned factors that contributes to the development of HCC, the molecular alterations occurring in checkpoints that control DNA damage like the \textit{p53} tumor suppressor gene and inactivation of the p27 cell cycle regulator have been described, which may have a role in promoting tumor formation in the cirrhotic liver (Matsuda 2008).

6. HCV Genotypes and quasispecies

The high spontaneous mutational rate that is characteristic of many RNA viruses results in a considerable heterogeneity throughout the HCV genome. The 5′ and 3′ UTRs are the most highly conserved regions of the genome; and the most variable are the envelope regions (E1 and E2). Genetic heterogeneity has been classified under two headings: genotype and quasispecies. Genotype represents the genetic heterogeneity of the virus between patients, and Quasispecies represent the genetic heterogeneity of the HCV population within an individual patient (Klenerman et al 2000).

Hepatitis C virus has highly related but meanwhile distinct six genotypes, and various subtypes with different geographic distribution and complex nomenclature. Since The C, NS 3 and NS 4 domains are the most highly conserved regions of the genome, these proteins are used as capture antigens for broadly reactive tests for antibodies to HCV (Zuckerman et al 2001). HCV genotyping system based on PCR of the core region with genotype specific PCR primers for determination of HCV genotypes 1a, 1b, 2a, 2b, 3a, 3b, 4, 5a, and 6a were developed (Ohno et al 1997).
The second component of HCV genetic heterogeneity are the quasispecies. Quasispecies are heterogeneous sequences of the HCV genome within a single infected person that result from mutations during viral replication. The rate of nucleotide changes varies significantly among the different genomic regions, with an overall mutation rate of 0.144% to 0.192% nucleotide changes per year. The highest proportion of mutations has been found in the E1 and E2 regions at both the nucleotide (1.2%–3.4%) and the amino acid (1.4%–2.7%) levels, particularly in the hypervariable region (HVR) at the amino terminal end of E2 (Farci et al 2000). Even though this region represents only a minor part of the E2/NS1 region, it accounts for approximately 50% of the nucleotide changes and 60% of the amino acid substitutions within the envelope region. Recent studies have shown that the rate of mutations within the HVR may be accelerated by interferon therapy (Enomoto et al 1995). The quasispecies nature of HCV may be one of the mechanisms by which the virus escapes immune responses. Furthermore, strain-specific viral factors, not distinguishable on the basis of genotype, may play a significant role in disease severity, thereby potentially explaining different courses of disease.

7. Diagnosis of HCV

There are two types of tests used in the diagnosis HCV infection. Those include serologic assays that detect hepatitis C virus specific antibodies (anti-HCV) in patients serum and molecular assays that detect viral RNA.
a. Serological tests

Screening using HCV Enzyme immunoassay (EIA) to detect HCV antibodies is routinely done for all blood donors or persons at risk of contracting the disease. The two enzyme immunoassays (EIAs) approved by the U.S. Food and Drug Administration (FDA) for clinical use, are Abbott HCV EIA 2.0 (Abbott Laboratories, Abbott Park, IL) and ORTHO HCV Version 3.0 ELISA (Ortho-Clinical Diagnostics, Raritan, NJ). Another enhanced chemiluminescence immunoassay (CIA) is VITROS Anti-HCV assay, (Ortho-Clinical Diagnostics, Raritan, NJ). IF The EIA test for HCV antibody is positive, additional testing with recombinant immunoblot assay (RIBA) is done to confirm the diagnosis (Ghany et al 2009, Puoti et al 2009).

b. Molecular tests

Several commercial assays either qualitative or quantitative are available for the detection of HCV RNA. Because HCV antibodies takes several weeks to develop, EIA results could come falsely negative in recently infected individuals. PCR can detect HCV RNA in patient’s serum within 7 to 21 days after exposure and can be used to confirm the diagnosis and also to monitor the patient’s treatment response. Recently, because of the highly sensitive real time polymerase chain reaction (PCR)-based assays and transcription-mediated amplification (TMA) assays that can detect as low as 10-50 IU/mL, monitoring patient’s response during therapy became easier.

In certain situations when patients can not produce anti-HCV antibody, HCV RNA testing is required especially in immunosuppressed or immunoincompetent patients
who underwent organ transplant, are on dialysis, are taking corticosteroids, or have agammaglobulinemia. Similarly, the anti-HCV may represent a false-positive reaction due to previous HCV infection, or another liver condition like alcoholism, autoimmunity or iron overload (Hemochromatosis). HCV RNA testing in these situations has a confirmative role (Scott and Gretch 2007). Because the patient response to anti-HCV therapies available differ by and is directly related to the viral genotype, genotyping Assays are employed to determine the appropriate therapeutic approach and required duration of therapy (Scott and Gretch 2007).

c. Liver biopsy

It is crucial to grade the liver disease in patients with hepatitis C to help guide treatment. The role of serum aminotransferase levels, HCV viral load, and hepatitis C genotype is negligible in predicting underlying liver histology. Therefore, it is recommended in the initial evaluation of all hepatitis C patients that liver biopsy to be performed to provide Information that can help exclude other causes of liver disease, determine the rate of progression of disease, and help in monitoring therapies if the patient is experiencing significant adverse effects. Usually persons infected with genotypes 2 and 3 respond well to standard therapy, and treatment can be started without liver biopsy in those patients. The extent of necroinflammatory activity, fibrosis or the presence of cirrhosis are important determinants whether treatment can be effective or not, and also gives an idea about malignant transformation possibility in patients with advanced cirrhosis (Kleiner 2005).
8. Molecular Biology of HCV

The viral structural proteins are encoded by the N-terminal part of the ORF, whereas the nonstructural proteins are coded for by the remaining portion of the ORF (fig.1.2a). Sequence motif-conserved RNA protease-helicase and RNA-dependant RNA polymerase (RdRp) are found at similar locations in the polyproteins of all of the Flaviviridae. In addition, all Flaviviridae share similar polyprotein hydropathic profile, with flaviviruses and hepaciviruses being closer to each other than to pestiviruses. The ORF is flanked in 5′ and 3′ by untranslated regions (UTR) of 95–555 and 114–624 nt in length, respectively, which play an important role in polyprotein translation and RNA replication (Brass et al 2006, Lindenbach and Rice 2005).

The virion contains a positive strand RNA genome encoding a single polyprotein of 3010 aa. The polyprotein is cleaved by cellular and viral proteases into structural (core, and envelope glycoproteins E1 and E2) and nonstructural proteins (p7, NS2, NS3, NS4A, NS4B, NS5A and NS5B) (Fig.1.2b). Translation of the polyprotein is mediated by a unique internal ribosome entry site (IRES) that consists of almost the entire 5’ noncoding region and a few nt of the downstream of the initiator AUG codon.

a. Attachment and entry of HCV into cells

The primary target for HCV replication is the liver hepatocytes. HCV E2 attach with high affinity to CD81 external loop (Fig.1.1C). The low density lipoprotein receptor (LDLR) and scavenger receptor class B type I (SR-BI) have been proposed as components of a putative HCV receptor complex. CD81 is a member of the tetraspanin
protein family that interacts with EWI-2; A cleavage product of which was recently reported to inhibit HCV entry by reducing the association of E1–E2 with CD81 (Schuster and Baumert 2009). This cleavage product was not found in hepatocytes, leading to the suggestion that its expression may explain their inability to support HCV entry in various non-permissive cell types. CD81 can also associate with Claudin-1 in a cholesterol-dependent manner forming complexes that is essential for HCV entry (Meertens et al 2008). Recent Studies supported the idea that lipoproteins play an important role for viral cell entry (Regeard et al 2008). In addition association to high density lipoprotein (HDL) enhanced SR-BI guided cell entry which may be due to protection of viral particles from neutralizing antibodies (Helle and Dubuisson 2008, von Hahn and Rice 2008).

CD81, Claudin-1 and SR-BI expression in normal and HCV-infected human livers is limited to the basolateral–sinusoidal hepatocellular surface (Jia et al 2008) that also contains CD81–Claudin-1 complexes. In addition, CD81 is expressed mainly at the basolateral surface and is excluded from tight junctions with CD81–Claudin-1 complexes formed at the same surface. Concluded from that, HCV enters the liver through the sinusoidal blood, to come into direct contact with the basally expressed receptor complexes (Reynolds et al 2008). Following attachment The virion envelope fuses with cellular membranes delivering the nucleocapsid to the cytoplasm. After entry of the viral genome, translation occurs in the cytoplasm producing a precursor polyprotein, which is then cleaved by both cellular and viral proteases into structural and nonstructural proteins. The virus forms a replication complex which is associated with cellular
Figure 1.2. Genomic organization of HCV and polyprotein processing scheme. **A.** The 5’NCR, a single, open reading frame that encodes 3010 aa precursor polypeptide and its processing by cellular and viral protease into individual proteins are shown. ARF, alternate reading frame protein. **B.** Structure of the 5’NCR. Four structural domains (I-IV), polypyrimidine (Py) motifs, multiple AUGs and pseudoknot structure (PK) are shown. SI and SII are stems of the PK. Translation of the ORF starts at AUG342. **C.** Base pairing of miR-122 at two sites within Domain I of the ‘NCR are shown (adapted from You et al. 2011. Proc. Nat. Acad. Sci. USA 108:3101-3102).
membranes. Replication occurs in the cytoplasm by the synthesis of full-length negative-strand RNA intermediates that act as a template for viral RNA synthesis. Assembly of Progeny virions follows by formation of cytoplasmic vesicles that buds through intracellular membranes and Finally, mature virions are released extracellularly by exocytosis (Stamatakis et al 2008).

**b. Molecular mechanism of HCV protein synthesis: a unique internal ribosome entry site/segment (IRES)**

Hepatitis C virus (HCV) polyprotein translation is mediated by an internal ribosome entry site (IRES) (Wang et al 1995). A 330 nt, located in the 5′ untranslated region (UTR) of the genome, controls translation by a mechanism distinct from other canonical mRNAs. In eukaryotic mRNA The 5′ 7meG-cap structure first assembles with eukaryotic initiation factor 4F (eIF4F). Subsequently, a 43S ribosomal complex, composed of the 40S subunit, eIF3, ternary complex (eIF2·Met-tRNA$_{Met}$·GTP), and other factors, binds to eIF4F at the 5′ end and then scans the 5′ UTR of the mRNA in an ATP-dependent manner until the AUG is positioned the in the ribosomal start site to form the 48S complex. Subsequent GTP-dependent release of initiation factors and 60S subunit joining , followed by first peptide bond formation, complete initiation of translation (Otto and Puglisi 2004).

HCV IRES-mediated translation initiation is independent of the 5′ cap structure or ATP-dependent scanning and requires only a subset of the canonical initiation factors. The HCV IRES direct internal initiation by recruiting translation initiation complexes
directly to the AUG start codon without scanning. Pestova et al., 1998 demonstrated by Reconstitution of IRES initiation that the IRES assembles on purified 40S subunits devoid of initiation factors (Pestova et al 1998). This IRES-40S binary complex is formed near the AUG start codon. The AUG docks in the ribosomal start site after addition of only eIF3 and ternary complex yielding a 48S complex without eIF4F during the first step of translation. (Pisarev et al 2005) The HCV sequence and secondary structure are highly conserved among viral isolates. Based upon phylogenetic comparison to the related pestiviruses and GB virus-B, the predicted IRES secondary structure was constructed using chemical and enzymatic probing and mutagenesis (Zhao and Wimmer 2001). The IRES motifs structures of the HCV, pestivirus, and GB virus-B are similar with regions of conserved sequences. Some sequence differences exists, but the main structural and functional elements are conserved in all HCV-like IRESs, with the same mechanism of initiation.

The HCV 5′NTR has four structural domains, designated I to IV. The most complex is domain III is, having a pseudoknot, multiple helices, and hairpin domains IIIa–IIIf. Domains IIIa–IIIc and IIIe–IIIf form four-way helical junctions, folding of which is dependent on physiological concentrations of divalent metal ions (Melcher et al, 2007)(Melcher et al 2003). This secondary structure and atomic details of IRES architecture was confirmed by computed three-dimensional structures of IRES domains (Kieft et al 2002, Lukavsky 2009).

The Role of these different IRES domains functions during initiation is not fully understood but likely involves regulation of initiation complex assembly. The recruitment
of the translation initiation complexes is mediated by HCV IRES Domain III with its basal portion binding with high affinity to the 40S subunit (Kieft et al. 2002), and also contacting ribosomal proteins involved in the binding and positioning of mRNA and tRNA (Otto et al. 2002). The apical portion of domain III (IIIb) interacts with eIF3 involved in mediating ternary complex stability and subunit assembly (reviewed in Merrick 2000). Domain II interacts with ribosomal protein S5 protruding into the ribosomal start site, leading to a conformational change in the 40S subunit, suggesting roles in both mRNA and tRNA regulation (Fletcher et al. 2002). Domain IV controls Proper positioning of the initiation codon.

Several factors influence the Activity of the HCV IRES. Most important is the X-tail at the 3' end of the HCV genome that appears to enhance IRES-dependent translation by an unknown mechanism (Ito and Lai 1997). Some cellular factors have been demonstrated to bind to the HCV IRES stimulating translation. These cellular factors include polypyrimidine- tract-binding protein (PTB) (Ali and Siddiqui 1995), the La antigen (Ali and Siddiqui 1997), and heterogeneous nuclear ribonucleoprotein L (Hahm et al. 1998).

c. 3' UTR

The 3' UTR contains approximately 225 nt organized in three regions including a variable region of approximately 30–40 nt, a long poly(U)-poly(U/UC) tract, and a highly conserved 3'-terminal stretch of 98 nt (3' X region) that includes three stem-loop structures SL1, SL2 and SL3. The 3' UTR interacts with the NS5B RdRp and with two of
the four stable stem-loop structures located at the 3’ end of the NS5B-coding sequence. The 3’ X region and the 52 upstream nt of the poly(U/C) tract were found to be essential for RNA replication, whereas the remaining sequence of the 3’ UTR appears to enhance viral replication (Tellinghuisen et al 2007).

d. Viral proteins and their functions

The HCV core protein forms the viral capsid and is composed of an N-terminal RNA-binding domain I (DI), a hydrophobic central domain (DII) that supports the association with lipid droplets (LDs), and the C-terminal domain that serves as signal sequence for E1 (Boulant et al 2006). In addition two variant forms of core protein have been described (Eng et al 2009). These core variants are not required for viral replication, but it is unknown yet if they contribute to pathogenesis. The envelope proteins (E1 and E2) are heavily glycosylated type I transmembrane proteins with E1 maturation requiring the coexpression of E2. E1 and E2 form noncovalently linked heterodimers forming a receptor on the surface of the HCV particle (Lindenbach and Rice 2005). The p7 protein act as an ion channel known as viroporin and is required for HCV assembly and release. The NS2 protein does not have a direct role in viral replication, but is required for the production of infectious virus particles (Tellinghuisen et al 2007).

HCV genome encodes four viral enzymes; NS2-3 autoprotease and NS3-4A serine protease, NS3 helicase and NS5B RNA-dependent RNA polymerase, all of which are essential for HCV replication and infectivity (Chevaliez and Pawlotsky 2006).
Inhibiting the activity of NS3 serine protease, stalls the replication of HCV. Therefore, the NS3 serine protease has become an interesting target for viral therapeutics. NS3 is a multifunctional protein, with an N-terminal serine protease domain and a C-terminal RNA helicase/NTPase domain. It cleaves the viral polyprotein at four junctions and its activity is essential for the generation of components of the viral RNA replication complex and helps virion assembly. The serine protease domain is a member of the chymotrypsin serine protease family. NS4A is a small (54-amino-acid) protein that anchors NS3 to cellular membranes through an N-terminal hydrophobic peptide. The serine protease domain requires coordination of Zn2+ by three cysteine residues distal from the active site for Proper folding. In addition, the NS3-4A protease activity has been implicated in blocking the host cell's ability to mount an innate antiviral response (Shi and Lai 2006).

The NS4B protein is a membrane protein with a complex structure. Overexpression of this protein leads to membrane alterations, indicating that it is probably required for the formation of the membrane-associated viral replication complex (Brass et al, 2006). (Brass et al 2006) NS4B was also described to bind to viral RNA and compounds enhancing RNA replication (Glenn 2006). NS5A is another key factor for RNA replication and assembly expressed in a hyperphosphorylated form, which seems to be important for production of infectious virions (Tellinghuisen et al 2007). Hyperphosphorylation of NS5A is suggested to reduce its interaction with the human vesicle-associated membrane protein-associated protein A ( hVAP-A) which acts as a vesicle sorting protein, directing nonstructural proteins to lipid rafts (Poenisch and
Bartenschlager 2010). Several cellular proteins have been described to bind to NS5A. Cyclophilin A (CypA) is one of those proteins, which has become an important target for antiviral therapy.

**e. Mechanism of HCV Replication**

The mature nonstructural proteins and a few cellular proteins forms a membrane associated replicase complex and initiate replication of the genome at the 3’ end for synthesis of negative strand RNA, which in turn, serves as a template for producing more positive strand genome(Tellinghuisen et al 2007). These dynamic processes cause extensive rearrangements in the intracellular membrane structures and alterations in signaling pathways in infected cells forming a membrane-associated replication complex(Lindenbach and Rice 2005). The formation of such a complex is a feature typical of plus-strand RNA viruses like poliovirus or Flaviviruses(Brass et al 2006) and it permits the production of viral proteins and RNA in an isolated compartment. As NS4B is a major component of the viral replication complex and has an RNA-binding activity, it facilitates attachment of the viral positive strand RNA. After NS5B, the RNA-dependent RNA polymerase is recruited by Cyclophilin A, to the viral replication complex, Replication replication results in negative strand intermediate which is further used to make the positive strand progeny that incorporates into new virus particles. The viral helicase unwind newly transcribed positive strand form its template negative strand and this positive sense strand becomes available for translation, transcription or packaging in new virions (O'Leary and Davis 2010).
f. Evasion of immune system by HCV

Viral persistence is attributed to HCV ability to evade the adaptive and innate components of the host's immune system (Fig.1.3). IFN is a major effector of innate antiviral immunity and is naturally produced in response to viral infection when viral pathogen-associated molecular patterns (PAMPs) are recognized as nonself and are bound by cellular pathogen recognition receptors (PRRs), including Toll-like receptors (TLRs) and the RIG-I-like receptors (RLRs). Within hepatocytes, RIG-I is a major PRR of HCV infection wherein PAMP interactions serve to trigger intracellular signaling cascades in the infected hepatocyte to drive IFN production and the expression of interferon-stimulated genes (ISGs). ISGs function to limit virus replication, modulate the immune system, and to suppress virus spread (Guobuzaitė et al 2008). The hepatic innate immune response is triggered when the infected cell recognizes a pathogen-associated molecular patterns or PAMP within the virus as nonself through the actions of cellular pathogen recognition receptors or PRRs. PRRs include Toll-like receptors (TLRs), melanoma differentiation-associated gene 5 (MDA5) and the RIG-I-like receptors (RLRs). The RLRs have a C-terminal RNA helicase domain that has RNA-binding activity. Of this family, only RIG-I and MDA5 have N-terminal tandem caspase activation and recruitment domains (CARDs) that are necessary for RIG-I function and bind long stable double-stranded RNA ligands different from RIG-I ligands. RIG-I is a main PRR in HCV infection (Liu and Gale 2010). The PAMP/PRR interaction triggers signaling cascades that induce the expression of antiviral effector genes in the infected hepatocytes driving IFN production which in turn stimulates the expression of
interferon-stimulated genes (ISGs) both within the infected cell and non-infected cells that respond either locally or systemically to the secreted IFN. ISGs are responsible for inhibiting virus replication, modulating the immune system, and preventing virus spread. During HCV infection, RIG-I and TLRs signaling pathways promote Interferon regulatory factor- (IRF-) 3 and nuclear factor-kappa B (NF-κB) nuclear translocation and transactivation functions through its CARDs signaling domain interaction with the downstream molecule, mitochondria-associated MAVS (also called IPS-1, VISA or CARDIF) (Loo et al 2008) (Liu and Gale 2010).

The HCV NS3 is a multifunctional helicase/protease protein. It harbors N-terminus protease and C-terminus NTPase/RNA helicase domains. The protein plays a remarkable role in the evasion of host innate immunity in addition to its critical roles in polyprotein processing and replication. HCV NS3/4A efficiently cleaves two pathogen associated molecular pattern (PAMP) receptors, RIG-I and IPS-1 (Tasaka et al 2007). In addition, Replication of HCV RNA causes strong ER stress (Waris et al 2005). Among the HCV proteins, core, E1 and NS3, NS5A are shown to be potent inducer of reactive oxygen species (ROS); their expression lead to DNA damage and induction of STAT3 and NFκB (Waris et al 2005);(Waris et al 2002a).

9. Treatment of the HCV infection

Although there are many drugs and HCV protein inhibitors in the pipeline, pegylated interferon with ribavirin is the only option for effective treatment of a few of
Figure 1.3. Current model for HCV-resistance and immune invasion
the HCV strains. However, more than half of the patients do not show sustained viral clearance. With these drugs (Pereira and Jacobson 2009, Vermehren and Sarrazin 2011). Many investigators were able to developed vaccines against HCV proteins but these vaccines have been shown to be ineffective against preventing the viral infection.

The primary goal of therapy in the hepatitis C patient is to achieve a sustained viral response, which is defined as undetectable HCV RNA 6 months after termination of antiviral therapy. Second goal is improvement in histologic findings and quality of life and the prevention of HCC. Patients with persistently abnormal liver enzyme test results, detectable HCV RNA, and an abnormal liver biopsy result are candidates for antiviral therapy. Several regimens have been licensed in the United States for the treatment of chronic hepatitis C. These regimens include IFN-alfa-2a, IFN-alfa-2b, IFN-alfa-con (consensus)-1, pegylated IFN-alfa-2b, combination IFN-alfa-2b and ribavirin, and combination pegylated IFN-alfa-2b and ribavirin. Pegylated IFN-alfa-2a alone and in combination with ribavirin is currently awaiting approval by the US Food and Drug Administration.

The current FDA-approved standard of care (SOC) for HCV infection consists of weekly injections of pegylated interferon-α along with twice-daily oral ribavirin (Peg-IFN/RBV regimen). The sustained viral response (SVR) with this SOC depends on strain of the virus as well as host attributes such as genetic variations. For example, patients infected with genotype 1, the SVR rate is generally <50% with 48 weeks SOC treatment whereas for the HCV genotype 2 and 3, it is between 70-80% (Vermehren and Sarrazin 2011).
A number of drugs that include the specifically targeted antiviral therapies for hepatitis C (STAT-Cs), and cellular targets against HCV (CTACs) are at various stages of clinical trials or have been stopped because of their adverse effects, multiple drug resistance or ineffectiveness in the patients (Kieffer et al 2010b). The most successful among STAT-Cs include drugs against NS3 protease/helicase (telaprevir, boceprevir), NS5B polymerase (nucleoside and non-nucleoside analogues) and NS5A protein (BMS-790052).

The use of telaprevir or boceprevir, in combination with Peg-IFN/RBV regimen was reported to increase the SVR rate by 20% in shorter duration (within 24 weeks) as compared the SOC alone (48 weeks) in patients infected with HCV genotype 1. Similarly, impressive potency of NS5A inhibitor (BMS-790052) has been shown in clinical trial (Gao et al 2010).

With all these successful stories and enthusiasm, cure for the HCV infection has been elusive not only because of adverse effects on patients but also due to emergence of drug-resistance (Fridell et al 2010, Kieffer et al 2010b, Liu et al 2009, Pockros 2010). Anemia (up to 37% with telaprevir and up to 56% with boceprevir), skin problems for telaprevir (rash and pruritus) and dysgeusia for boceprevir in addition to the influenza-like symptoms, cytopenias and depression caused by the SOC are major hindrance in successful treatment.

The viral variants with reduced susceptibility to STAT-Cs occur naturally at low levels due to error-prone HCV replication mechanism, and can be selected in patients not
responding to STAT-C/SOC treatment. Susceptibility of HCV against these drugs diminishes due to gradual incorporation of mutations in the virus that have been demonstrated during in vitro and in vivo testing. These drugs have huge potential for misuse due to prescribing physician’s poor understanding of the therapeutic populations, inadequate viral-assay testing, poor side-effect management, and lack of monitoring for antiviral resistance (Thompson and McHutchison 2009, Toyoda et al 2010).

Statins are well tolerated by patients with HCV and may be added in standard therapy as a cellular target for anti HCV (CTAC) therapy. More recently, Himmelbach et al. (Himmelsbach et al 2009) demonstrated that Sorafenib, an FDA and EU approved drug for the treatment of HCC also inhibits HCV replication in cell culture. Sorafenib has been shown as a potent inhibitor of c-Raf that interacts with the HCV NS5A. The CTAC inhibitors (e.g. NIM811, Debio 025) for the HCV protein-interacting partners such as cyclophilins A and B are also in clinical trials. However, due to adaptive mutations in the HCV proteins, the drug-resistance is inevitable as reported in a number of studies (Yang et al 2008).

HCV induced stem cell-like features may contribute to the long-term persistence of HCV and HCV-induced aggressive HCC. Therefore, new CTAC inhibitors whose potencies are not compromised by the HCV adaptation and drug cocktail with diverse mechanisms of action are needed to combat HCV infection. Such multi-dimensional approach is likely to be effective against both the HCV and the HCV-led initiation of liver carcinogenesis.
10. Models used for investigation of HCV infectious processes

Humans and Chimpanzees are the only known natural host of HCV. However, over three decades, investigators have tried to develop various models to study HCV infection and replication process. The most important ones that contributed in understanding the viral infection are as described below:

a. HCV replicons

The HCV replicons were constructed from cDNA isolated from the liver of subjects chronically infected with a genotype 1b strain and consisted of the HCV 5’ NTR and the genes coding for the capsid protein fused in-frame with the selectable marker gene, neomycin phosphotransferase (Neo), which upon expression confers resistance to the cytotoxic drug G418 and ending with HCV 3’ NTR HCV 3’ NTR. (Figure.1.4) The IRES element from encephalomyocarditis virus (EMCV), drives translation of the HCV non-structural proteins (Blight et al, 2006) (Blight and Norgard 2006). Huh-7 cells transfected with transcripts synthesized in vitro and selection with G418 enabled isolation of a low number of surviving cell colonies.

Many improvements to the replicon system allowing the establishment of transient replication assays for other HCV genotypes eg. 1a, 1b, and 2a have been made. The identification of adaptive mutations that enhance HCV (huh 7.5) led to the development of replication competent full-length genomes. More recently, the HCV subgenomic replicons cell tropism has been expanded to non-hepatoma cell lines and mouse hepatocytes. Recently, the identification of genotype 2a-derived replicons
efficiently replicating in cell culture needless of adaptive mutations has enabled the development of systems supporting the complete virus life cycle (Ashfaq et al 2011).

b. Chimpanzee

As the only animal model available, the chimpanzee has been commonly used to study mechanisms of acute and chronic HCV infection and these studies have greatly contributed to the current understanding of HCV infection, including immunity and pathogenesis (Bukh 2004). However, there are many drawbacks in the chimpanzee model that limit its use. First of all, HCV-infected chimpanzees rarely develop chronic liver disease to the same extent seen in HCV-infected humans (Hiraga et al 2007), making the chimpanzee not an ideal model for studying the mechanisms of HCV pathogenesis. Moreover, chimpanzees are expensive, difficult to handle, and need special housing and care in appropriate non-human primate research facilities. These limitations of the chimpanzee model have stimulated progress toward developing alternative animal models for HCV research.

c. Mouse models

Due to its limited permissiveness in animals, HCV was only studied in humans or in chimpanzees. Recently, a new chimeric mouse model was developed to be permissive for HBV and HCV infection. The new mouse model, uPA+/−-SCID represent mice with a transgene-induced liver disease. Mice are transplanted with primary human hepatocytes early after birth. These human hepatocytes were able to integrate in the parenchyma and progressively repopulate the diseased mouse liver with all their normal metabolic
functions preserved. The successfully transplanted mice were then infected with HCV (Barth et al 2008). This model will allow in vivo expression of HCV proteins and provide an important tool for understanding virus–host interactions, and pathogenesis of HCV infection as many of these mouse models exhibit aspects of HCV-related liver disease. They also will help studies to evaluate the safety and efficacy of new antiviral compounds against HCV.

d. **JFH1 infectious clone**

Studying many aspects of the hepatitis C virus (HCV) life cycle could not be reproduced in cell culture. This obstacle has slowed research and hampered progress on the development of effective antivirals and vaccines against HCV. Recently, Robust production of infectious HCV in cell culture has been achieved using a unique genotype 2a strain HCV genome derived from the blood of a Japanese patient with fulminant hepatitis C (JFH-1) (Berke and Moradpour 2005, Cai et al 2005, Wakita et al 2005). Moreover, the virus particles generated from the JFH-1 clone(fig.1.4) proved to be infectious in vivo both in chimpanzees and in chimeric mice containing transplanted human liver populations (Lindenbach et al 2006, Wakita et al 2005). By introducing multiple adaptive mutations this infectious HCV genotype 1 could also be produced and maintained in cell culture and The full-length JFH-1 RNA was transfected into Huh7 cells. Subsequently, viral RNA efficiently replicated in transfected cells producing nearly $10^{5}$ infectious units per milliliter within 48 hours and viral particles were secreted and was able to infect naive Huh7 cells (Zeisel and Baumert 2006).
11. Hypotheses and Aims

While maintaining their phenotypes by self-renewal process, the cancer stem-like cells (CSCs) function as seed elements of the solid tumors including hepatocellular carcinoma. Because HCV is a dominant risk factor for the HCC development and HCCs contain cells with stem cell gene-expression signatures (discussed above), we hypothesize that long-term infection of HCV causes phenotypic changes. These changes might be due to altered expression of oncogenes and cancer stem cell markers. Keeping in view of the above-described problems, my thesis work was focused on three major specific aims:

i. To investigate if HCV-expressing cells undergo phenotypic changes and exhibit enhanced expression of oncogenes (e.g. c-Src) and putative cancer stem cell markers

ii. To investigate possible mechanism of the HCV-induced hepatocarcinogenesis

iii. To develop novel means for targeting marker (s) that control abundance of the HCV genome
Figure 1.4. JFH1 RNA represents genotype 2, isolated from a Japanese patient with severe acute hepatitis (Wakita et al 2005) and is currently being used as infectious model. The second model is the subgenomic replicon system in which the sequence from core through NS2 is replaced with neomycin resistance gene followed by encephalomyocarditis virus (EMCV) internal ribosome entry site (IRES). The HCV IRES translates Neomycin phosphotransferase (Neo') gene that confers resistance to G418 drug in tissue culture. The EMCV IRES translates NS3-NS5B that are required for the viral replication. The replicon expresses NS5A-GFP chimeric protein. Using this model, we have studied mechanism of viral replication, and function of viral and cellular proteins on the HCV life-cycle. proteins’ Huh-7 or Huh7.5 cells transfected with the replicon synthesized in vitro and selection with G418 enabled isolation of a replicon expressing cells.
12. Conclusion

HCV is one of the most important human pathogens. Its global presence and severity of the liver diseases have proven to be detrimental. Likewise, its economic burden is huge. The research activities in academic institutions and pharmaceutical laboratories have been focused for years on the development of novel therapeutic agents for hepatitis C. The HCV field has taken a giant leap during last ten years by advancing HCV culture methods, unraveling molecular mechanism of HCV entry and infectious processes, and by developing new classes of HCV inhibitors. However, these developments are not enough to fully understand HCV pathogenesis. Furthermore, owing to its quasispecies nature due to its high mutation rate, viral resistance is a major issue for vaccine and drug development. Till now, current treatment approaches are not curative. While HCV on its own is a huge medical problem, the association between HCV infection and hepatocellular carcinoma is an added insult to the injury.

The work presented in this thesis provides a novel concept of HCV virology by delineating relationship between HCV infectious process and development of cancer stem cell-like traits in the host cells. Using RNA interference for targeting a putative stem cell marker (DCAMKL-1), which is known to be involved in colon and pancreatic cancer, this work has set a precedent that delivery of DCAMKL-1 antagonist by gene therapy technology or by small molecule inhibitors will help in reducing the risk of HCV-induced liver carcinogenesis.
Chapter Two: Hepatitis C Virus-Induced Cancer Stem Cell-like Signatures

1. Introduction

Hepatocellular carcinoma (HCC) is the fifth most common malignancy worldwide, accounting for approximately one million deaths annually (Farazi and DePinho 2006, Parkin 2001, Yang and Roberts 2010a). The high mortality associated with HCC is attributed to the failure of early stage diagnosis and lack of effective treatment for patients with advanced stage HCC (Farazi and DePinho 2006, Woo et al 2010, Wu et al 1996). Chronic infection with hepatitis C virus (HCV) is considered to be a prominent risk factor for the development of HCC (Bartosch et al 2009, Levrero 2006, Wurmbach et al 2007). More than 170 million people (>4 million in USA alone) are infected and HCV-related liver disease is increasing globally. Although a strong relationship between HCV-induced chronic liver disease and development of HCC is widely accepted, the molecular mechanism of HCV-induced hepatocarcinogenesis is not clearly understood.

HCV is a positive strand RNA virus classified as a hepacivirus of the family Flaviviridae (Moradpour et al 2007, Poenisch and Bartenschlager 2010). Among the 6 genotypes, 12 subtypes and various quasispecies (Mellor et al 1995), 1a and 1b are the most prevalent strains in the US and are least responsive to the currently available

HCV-induced molecular alterations in infected cells significantly contribute to HCC development and progression. These alterations may include: i) loss of tumor suppressor proteins; ii) activation of oncoproteins such as c-Myc; iii) activation and secretion of cytokines such as transforming growth factor TGF-β; and iv) alterations in the Wnt/β-catenin signaling leading to nuclear accumulation of β-catenin, which are found in 33 to 67% of HCC cases (Bartosch et al 2009, Park et al 2009). Activation of β-
catenin is essential for liver development because deletion of the protein in mice results in fetal death due to impaired liver cell proliferation and increased apoptosis (Thompson and Monga 2007). The activated β-catenin has been demonstrated in cells located at the tumor’s invasive front in colon carcinomas (Zulehner et al 2010). The Wnt/β-catenin signaling pathway is also important for tumor progression because it modulates differentiation and maintenance of stem cells (Alison 2005, Lee et al 2006a, Zulehner et al 2010).

CSCs display several key characteristics of normal tissue stem cells such as self-renewal and unlimited proliferative and differentiation capacity. They also possess the intrinsic ability to reproduce all aspects of the parent tumor after metastasis (Alison 2005). Thus, the “hierarchical model of cancer” considers CSCs as tumor “seed elements”, which are responsible for cancer initiation, re-initiation after metastasis and local recurrence after therapy (Marquardt et al 2010, Shi et al 2008). Histopathology study of chronic liver disease and experiments in mice support the existence of CSCs in HCC (Mishra et al 2009, Shi et al 2008). The origin of CSCs and factors affecting their fate are still being debated. Recent studies indicate that induction of “stemness” in normal tissues or abnormal self-renewal, differentiation and proliferation of stem/progenitor cells might be key elements in the generation of CSCs (Lowry et al 2008, Mishra et al 2009).

Multiple transcription factors and signaling pathways, including Wnt/β catenin, Notch and Hedgehog, may be involved in the induction and maintenance of “stemness”
in fully differentiated cells (Thompson and Monga 2007, Yin et al 2010). Induction of pluripotency/multipotency in adult somatic cells can be carried out by simultaneous expression of several transcription factors (a set of Oct4, Sox2, Klf4 and c-Myc or Oct4, Sox2, Lin28 and Nanog) in recipient adult cells using various delivery vehicles such as viruses, transposons, and bacterial plasmids (Aoi et al 2008, Kaji et al 2009, Park et al 2008). Successful generation of pluripotent stem cells (iPSCs) has been accomplished by manipulating culture conditions and with limited number of pluripotency factors (Kim et al 2009). The most widely accepted evidence for pluripotency/multipotency is the formation of spheroids and teratoma, and unlimited self-renewal of the reprogrammed cells.

Because HCV replicates in fetal hepatocytes and the presence of CSCs have been demonstrated in HCC (Alison 2005, Lazaro et al 2007, Mishra et al 2009), it was essential to investigate whether HCV could induce cancer stem cell-like properties in liver-derived cells. This study shows that continued HCV expression is linked to the overexpression of a putative stem cell marker DCAMKL-1 that is used by the replicon for its efficient replication. Using various cell lines and a tumor xenograft model, the study further demonstrate that majority of HCV replicon-expressing cells acquire characteristic of hepatic progenitor cells, and form a distinct tumor phenotype than the parent cells lacking the replicon. The HCV-related tumors possess CSCs with higher invasive and metastatic potential than their normal counterparts (negative for HCV).
2. Materials and Methods

2.1. Antibodies

Antibodies used in this study were purchased as indicated and used in accordance with the manufacturer’s suggestion: anti-DCAMKL-1, CD133, SOX2, LGR5, CK19, human serum albumin, α-fetoprotein and HCV NS5B polymerase were purchased from Abcam; anti-α-tubulin, Lin28B, c-Src, and actin (Cell Signaling); anti-c-Myc and anti-Oct3/4 (Santa Cruz Biotechnologies); anti-Lin28 (Protein Tech Group), and anti-Klf4 (Abgent).

2.2. Cell culture and transfection assay

The FCA4 cells are derived from Huh7 hepatoma cell line and replication of a subgenomic HCV replicon in this cell line has been characterized previously (Ali et al 2002, Guo et al 2001). The GS5 cells are derived from Huh7.5 cell line [interferon cured-Huh7 cells negative for the HCV replicon (Blight et al 2002)] and express HCV subgenomic replicon that encodes NS5A-GFP (Nelson and Tang 2006). These cell lines were cultured in Dulbecco’s Modified Eagle’s Medium (DMEM) supplemented with 1x Pen/Strep, 1x non-essential amino acids and 10% fetal bovine serum (all from Invitrogen), and maintained at 37°C and 5% CO₂. To evaluate effects of microtubule disruption on HCV replication, Huh7.5 and GS5 cells (each ~ 1x10⁶) were treated with vinblastine (30 μM) or DMSO (vehicle) for 3, 6, 9, 12 and 24 h. Total lysates of cells
were prepared according to the protocol described for Pierce Cell Lysis Kit for Western blot (M-PER).

The siRNA that targets nt 308-327 (5’GGGAGTGAGAACAATCTAC3’) of both AS and AL isoforms of DCAMKL-1 mRNAs (siDCAMKL-1) and scrambled siRNA (SCR) were purchased from Ambion. Incremental amounts of the siDCAMKL-1 or SCR RNAs (0, 20, 50, 100 nM) were transfected into GS5 cells using SiPORT NeoFX transfection agent (Applied Biosystems) according to the manufacturer’s protocol. Similar amounts of transfection reagent alone were used in control transfection. The cells were harvested 48 hr post-transfection to analyze RNA and protein levels.

2.3. **Florescence-activated cell sorting (FACS)**

The DCAMKL-1 antibodies were conjugated with Dylight 549 (Thermo Fisher) using Pierce kit. The conjugated DCAMKL-1 antibodies were purified and allowed to bind cell surface DCAMKL-1 of Huh7.5 or GS5 cells for 30 minutes at RT as described (May et al 2008, May et al 2010). The cells were washed twice with autoMACS rinsing buffer (Miltenyi Biotec), resuspended in the same buffer and sorted using Influx-V Cell Sorter (Cytopeia, Seattle). The instrument was calibrated with unstained cells. The DCAMKL-1-positive and negative cells were collected separately in DMEM and grown as described above. For spheroid assay, 100 DCAMKL⁺ or DCAMKL⁻ cells in 2X DMEM media (50 ul) were mixed with matrigel (50 ul) on ice-bath, plated in non-adherent/ultralow attachment 96-well plates (BD Biosciences, CA). Fifty microliter media was added on the top of the gel 2 hr later and the cells were grown as described
above. Two weeks later, 100 ul DMEM were added in each well. The spheroids were evaluated and photographed under inverted light microscope after 4-5 weeks.

2.4. Real-time reverse transcription-PCR analyses

Total RNAs were isolated from culture cells or tumor xenografts using RNeasy isolation kit (Qiagen). The RNA samples were subjected to reverse transcription with Superscript II and random hexanucleotide primers (Invitrogen). In the subsequent step, the cDNAs were used as templates to perform real-time PCR by SYBR chemistry method (SYBR® Green I; Molecular Probes). The target (HCV and DCAMKL-1) and control (actin) RNAs were amplified using Jumpstart Taq DNA polymerase (Sigma) and the following primers:

Actin: 5’GGTGATCCACATCTGCTGGAA-3’ (forward)

and 5’ATCATTGCTCCTCCTCAGGG3’ (reverse);

DCAMKL-1: 5’AGTCTTCCGATTCCGAGTTGAG3’ (forward);

and 5’CAGCAACCAGGAATGTATTGGA3’ (reverse);

HCV: 5’CGGGAGAGCCATAGTGG3’ (forward)

and 5’AGTACCACAAGGCTTCTCG3’ (reverse).

The crossing threshold values assessed by the real-time PCR were evaluated for the transcripts and normalized with β-actin mRNA. The mRNA levels were expressed as fold change relative to control with ± SEM value.
2.5. Tumor Xenografts in mouse model

Athymic nude Balb/c mice were purchased from Jackson Laboratory and housed in pathogen-free conditions. The animals were treated in accordance with guidelines of the American Association for Accreditation of Laboratory Animal Care and the Policy on Human Care and Use of Laboratory Animals. The studies reported here were pre-approved and supervised by the Institutional Animal Care and Use Committee (IACUC). Huh7.5 or GS5 cells \(5 \times 10^6\) were washed with PBS three times, resuspended in the same buffer, and injected subcutaneously into the dorsal flanks of 4–6 week old mice (3 mice for each injection). Tumors were measured with calipers and the volumes were calculated using formula: \(0.5 \times \text{length} \times \text{width}^2\). The tumors were resected 4-6 weeks after cell transplantation and the portions of the tumors were preserved in 5% formalin for immunohistochemistry and stored at \(-80^0\) C in RNase inhibitors for real-time PCR or directly frozen for Western Blot analyses. The control animals were treated similarly but injected with same volume of the buffer.

2.6. Immunohistochemistry

Heat-induced epitope retrieval was performed on 4 µm formalin-fixed paraffin-embedded sections using a pressurized Decloaking Chamber (Biocare Medical) in citrate buffer (pH6.0) at 99°C for 18 min. Slides were sequentially treated with 3% hydrogen peroxide, normal serum and BSA at room temperature for 20 min. After incubation with primary antibodies and washing with PBS, the slides were incubated in peroxidase-
conjugated EnVision™+polymer detection kit (DAKO). Slides were developed with diaminobenzidine (Sigma).

2.7. Immunofluorescence and confocal microscopy

Cells grown on glass cover-slips (VWR) were rinsed briefly in phosphate-buffered saline (PBS), fixed in 4% paraformaldehyde in PBS pH 7.4 for 20 min at room temperature, washed twice with ice cold PBS and permeabilized in ice-cold acetone. Cells were incubated with blocking buffer (10% serum, 0.01 % Triton X-100, in PBS, pH 7.4) for 1 hour, washed with PBS and treated with primary antibodies in PBS-T containing 1% BSA for 1-2 hr at room temperature or overnight at 4°C. After thorough washing with PBS-T, cover-slips were incubated in appropriate AlexaFluor conjugated secondary antibodies. The nuclei were counterstained with DAPI (0.1-1 µg/ml PBS). The cover-slips were mounted on microscope slides in ProLong Gold antifade reagent (Invitrogen) for detection of Immunofluorescence using Nikon 80i or Leica TCS NT (for confocal microscopy).

2.8. DCAMKL-1 staining in human liver tissue array

The liver tissue arrays were purchased from US Biomax (Rockville, MD). The array contains 30 normal and cancer adjacent normal liver tissues, 10 cases of inflammation with HBV, 9 samples of cirrhosis with HBV, and 31 samples of cirrhosis without HBV infection. The arrays were subjected to immunohistochemical staining as
described above using anti-DCAMKL-1 antibodies (ab31704). The intensity of DCAMKL-1 were evaluated and plotted on the arbitrary scale of 0-3.

2.9. Induction of human iPS cells

The minicircle producer plasmid pMC.BESPX and the E.coli strain ZYCY10P3S2T were obtained from Dr. Zhi-Ying Chen, Stanford University School of Medicine. The ZYCY10P3S2T strain was transformed with pMC.BESPX and grown in 5 ml LB containing Kanamycin (50 μg/ml) at 37°C with shaking at 250 rpm. An overnight culture were grown by combining 100-ul of the previous culture with 400 ml of terrific broth (TB) containing Kanamycin (50 μg/ml). On day 2 after 16-18 hours (OD600 reading between 4-5), the minicircle induction mix (400 ml LB, 16 ml 1N NaoH and 400 ul 20% L-arabinose) was combined with the overnight culture and incubated at 32 °C with shaking at 250 rpm for 5 hours. Bacteria were then pelleted and minicircle isolated using Qiagen kit according to manufacturer’s protocol with some modifications (double volumes of P1, P2, and P3 buffers were used. After purification, just the reprogramming cassette is obtained and bacterial elements were removed.

3. Results

Hepatoma cells harboring HCV replicon express multitude of stem cell-related proteins and exhibit cancer stem cell-like features. Normal adult hepatocytes are fully differentiated cells and lack expression of stem cell markers and pluripotency factors (Mishra et al 2009). However, these cells can be induced to proliferate rapidly after
partial hepatectomy and can also be reprogrammed into pluripotent cells (Aoi et al 2008). In this study we investigated the impact of HCV on partial reprogramming or retrodifferentiation of GS5 cells that are derived from a hepatoma cell line (Huh7.5). These cells harbor a Geneticin (G418)-selectable subgenomic HCV-1b replicon that encodes HCV nonstructural proteins NS3 through NS5B, and a functionally active NS5A-green fluorescent protein (GFP) chimera (Moradpour et al 2004, Nelson and Tang 2006). The cells partially mimic chronic HCV infection in tissue culture conditions due to sustained expression of the replicon in the presence of G418. The intensity of GFP in these cells can be used as a surrogate marker for the efficiency of the HCV RNA replication (Nelson and Tang 2006).

By indirect immunofluorescence microscopy, extensive expression of several putative stem cell/pluripotency markers (CD133, DCAMKL-1, Lgr5, Sox2, Oct4, Lin28) and c-Myc oncogene was observed in the GS5 cells that also demonstrated high level of HCV replicon expression as evidenced by the presence of intense NS5A-GFP (see Fig. 2.1 and Appendix 1). Eccentric staining of DCAMKL-1 was noted in the area where punctate localization of the HCV replication complexes (RCs) was more intense. Previous studies have shown that HCV replication complexes associate with microtubules through NS5A and NS3 proteins and their saltatory movements are fully dependent on the intact microtubule filaments (Lai et al 2008, Wolk et al 2008). Since DCAMKL-1 catalyzes polymerization of tubulins into microtubules (Kim et al 2003, Lin et al 2000), next to be investigated was the possible role(s) of DCAMKL-1 in HCV replication. The Fig. 2.2A shows relative localization of the RCs (marked by punctate
GFP) and DCAMKL-1 in the context of microtubules in GS5 cells. The cells with high HCV expression (indicated by double arrow) showed higher DCAMKL-1 abundance. Both DCAMKL-1 and the HCV RCs were concentrated in the regions showing intense staining for microtubule filaments. In contrast, cells without high HCV expression showed much lower expression and localized staining of DCAMKL-1 (single arrow). These observations suggest a relationship between HCV replicon expression and DCAMKL-1 levels in a subset of GS5 cell line.

3.1. DCAMKL-1-positive GS5 and Huh7.5 cells exhibit cancer stem cell-like properties

Recent studies from this laboratory (May et al 2008, May et al 2009, May et al 2010) showed that DCAMKL-1-positive cells can be isolated from colon and pancreatic cancer cell lines using FACS method (May et al 2009), and that isolated DCAMKL-1-positive cells formed spheroids in matrigel (BD Biosciences) and displayed CSCs properties. This established method was used in this study to isolate DCAMKL-1 and HCV NS5A-GFP double positive cells (HCV⁺DCAMKL-1⁺) from GS5 and DCAMKL-1⁺ cells from Huh7.5 cell line. The FACS data presented in Fig. 2.2B (left panel) revealed relatively higher percentage of DCAMKL-1⁺ cells in GS5 pool (1.42%) than that of the Huh7.5 control (0.36%). Both cell-types positive for DCAMKL-1 formed spheroids in matrigel (with approximately 5-10% efficiency) in 3-4 weeks (middle panel). GS5-spheroids demonstrated higher HCV NS5A-GFP expression in the budding areas than the spheroid body (right panel). The DCAMKL-1 negative pool of these cell lines failed to
Figure 2.1. Indirect immunofluorescence microscopy for visualization of cells coexpressing the HCV replicon (indicated by NS5A-GFP, green) and putative stem/progenitor cell markers or c-Myc (red) in GS5 culture. Blue, nuclear stain. Also see appendix 1.
form spheroids under similar conditions. These results indicate that a subpopulation of GS5 cells co-expressing high levels of HCV replicons and DCAMKL-1 may exhibit CSCs features.

3.2. Tumors initiated by GS5 cells are distinct from the Huh7.5-derived tumors in athymic nude mice

Assuming that very high NS5A-GFP expressing cells also have high DCAMKL-1 expression level (Fig. 2.2A), we used FACS to isolate the top 26% of total GS5 population with the highest green florescence intensities (Fig. 2.3A, indicated by R4). These cells were then expanded in tissue culture and injected into the dorsal flanks of the nude mice. In parallel, similar numbers of Huh7.5 cells were injected into the control mice. Since both cell lines contain DCAMKL-1+ cells to varying extents, the transplanted cells formed tumors with 50-60% efficiency over a period of 3-4 weeks. An example of GS5 tumor in one of the mice is shown in Fig. 2.3B (indicated by an arrow). The resected tumors (example shown in Fig. 2.3C) were phenotypically characterized and analyzed for the expression of stem cell/pluripotency markers by immunohistochemistry. A significant number of DCAMKL-1-positive cells were localized in certain areas of GS5-tumors in which most cells exhibited high level of activated c-Src expression, indicating that these tumors are highly aggressive and possess metastatic potential (Fig. 3E). Only rare c-Src and DCAMKL-1 expressing cells were observed for the Huh7.5-tumors (Fig. 2.3D) whereas neither was detected in control mouse liver preparations (Fig. 3F).
Figure 2.2. Correlation among DCAMKL-1, HCV and CSCs traits in the replicon-expressing cells. (A) Confocal microscopy for localization of HCV NS5A-GFP (green), DCAMKL-1 (red) and microtubules (magenta) in GS5 cells as indicated. The field shows two cells as an example; single and double arrows indicate low and high HCV replicon-expressing cells respectively as indicated by the relative GFP intensities. (B) Isolation of DCAMKL-1-positive cells from Huh7.5 and GS5 cells grown in tissue culture by FACS method (left panel), and Spheroid assay (right panel) using isolated DCAMKL-1+ (Huh7.5) or GFP+DCAMKL-1+ (GS5) cells. One hundred cells were plated with culture media containing 50% matrigel in a 96-well plate. The cells were grown in standard culture conditions and spheroids were photographed after four weeks (a-d).
Figure 2.3. Characteristics of tumor xenografts developed by GS5 and Huh7.5 cells. (A) The highest GFP-expressing cells (R4 gated, 26% of total) were isolated by FACS method, expanded in tissue culture and 5 million cells were injected into the dorsal flank of a nude SCID mice (in duplicate). In control animals, similar numbers of Huh7.5 cells were transplanted. (B) An example of GS5 tumor within animal (indicated with an arrow). (C) Resected tumor from the animal. The tumors developed by Huh7.5 (not shown) had varying size but usually smaller than that of GS5-tumors (not shown). Co-staining of DCAMKL-1 and active c-Src in the tumor tissues of Huh7.5 (D) and GS5 (E) by immunohistochemical method. Purple, DCAMKL-1; Brown, activate c-Src [pTyr418 c-Src]. (F) Control, section of the mouse (B) liver stained as in (D, E) showing that both antibodies were not reactive to the animal liver cells.
2.3F). This in vivo observation complements our preliminary finding that activated c-Src is highly expressed in GS5 cells grown in laboratory cell cultures. We next compared expression of a number of stem cell/pluripotency markers in the xenograft tumor tissues and culture cells which were derived from both the cell lines. The total cell lysates (20-30 ug) were subjected to Western blot analysis using specific antibodies against an array of stem cell-associated markers.

As shown in Fig. 2.4A, the levels of DCAMKL-1, LGR5 and CD133 were considerably enhanced in GS5 cells as compared to the control cells (Huh7.5). However, the levels of pluripotency factors (Oct4, Sox2 and Lin28) were similar in both cell-types. Unlike cultured cells, DCAMKL-1, LGR5 and CD133 expression and that of pluripotency factors (Lin28, Sox2, Oct4 and c-Myc) in both tumors were similar. During GS5 cell transplantation, the animals did not receive the replicon selection marker (G418 drug) due to its high cytotoxicity. As a result, GS5-tumors lost the replicon expression over a 3-4 week period as indicated by the absence of NS5B band in the Western blot (Fig. 2.4B, control markers). This is consistent with our observation that withdrawal of G418 from GS5 culture gradually diminishes the HCV expression (see Fig. 2.7, left panel). These data suggest that despite the gradual HCV decline over a few weeks, initiated tumors continued to grow in the animals.

Previous reports indicated that immature hepatic progenitor cells such as oval cells and cholangiocytes in liver express CK19 protein (Aoi et al 2008, Banaudha et al 2010). The protein is also considered a marker for aggressive HCC subtypes. The CK19
expression in HCC (with or without HCV) correlates with the lower patient survival and higher rates of tumor recurrence (May et al 2010, Mellor et al 1995). Striking differences in CK19 expression were observed between the GS5 and Huh7.5 xenografts by immunohistochemistry (Fig. 2.5). Higher percentages of GS5-tumor cells expressed CK19 (Fig. 2.5B) whereas rare Huh7.5-tumor cells expressed this protein (Fig. 2.5A). This difference was confirmed by Western blot analysis using replicon-expressing two cell lines. The total lysates prepared from cells grown in tissue culture revealed that both GS5 (Fig. 2.5C, left panel) and FCA4 (Fig. 2.5D) cells expressed much higher levels of CK19 than their parent cell-types (Huh7.5 and Huh7 cells respectively). This trend was clearly reflected in the respective tumor xenografts derived from GS5 and Huh7.5 cells.

To induce pluripotency in Human hepatoma cell line Huh7, the minicircle vector, p2phiC31-LGNSO was transfected into Huh7 cells. In the minicircle DNA, the reprogramming cassette is driven by a CMV promoter leading to the expression of LIN28, GFP, NANONG, SOX2 and OCT4. The reprogramming genes coding regions in the cassette are linked by 2A protease sequence, so after transfection the expressed proteins undergo self cleavage by peptide 2A. In parallel, Huh7 cells were also transfected with BM45 plasmid harboring HCV replicon, controls were empty vector DNA transfected Huh7 and lipofectamine only. Total lysates analyzed on SDS-PAGE showed that both LSNGO and BM45 transfected cells (Fig. 2.6C, lanes 3 and 5 respectively) expressed higher levels of CK19 than their parent untransfected Huh7 cells, mock or empty vector DNA transfected Huh7 cells (Fig. 2.6C lanes 1, 2, 4 respectively).
Figure 2.4. Relative expression of putative stem cell markers and induced pluripotency factors in the culture cells (A) and tumor tissues (B) as indicated. Western blot analysis was carried out with 20 µg of total lysates of the cells (left panel) and their respective tumors (right panel). Protein bands of stem cell markers (DCAMKL-1, LGR5, and CD133) and induced-pluripotency (iPSCs) factors (Oct4, Sox2, c-Myc, Lin28) were detected by the specific antibodies as indicated. The viral NS5B polymerase and actin were used as controls. (C) Enhanced expression of DCAMKL-1 in another HCV replicon-expressing cell line (FCA4) as compared to the parent cells (Huh7).
Figure 2.5. Unique features of the GS5-tumors. Enhanced expression of CK19 in GS5-derived tumors (B) as compare to that of Huh7.5-derived tumors (A). Immunohistochemical staining (A, B) for CK19 (brown); Blue, nuclear stain. (C) Western blot of total lysates (30 ug) of tumor xenografts (right panel) or culture cells (left panel) of both cell lines probed for CK19. The upper band in the doublet is considered as full-length Ck19. Actin, loading control. (D) Western blot for CK19 expression in FCA4 cells that express a subgenomic HCV-1b replicon (indicated by the presence of NS5B) as that of GS5 but lacks GFP.
Lin28 was much more expressed by LSNGO transfected cells (Fig. 2.6 D lane 3) compared to control and mock cells (lanes 1 and 2 respectively).

A similar strategy was employed to detect the hepatoblast marker, α-fetoprotein (AFP), in xenograft tumors and in tissue culture cells. Western blot analyses reiterated immunohistochemical findings that GS5-tumor or GS5 culture cells expressed AFP at a much higher level than Huh7.5 controls (Figs 2.7 A, B, and C). Lysates of GS5 tumors showed much higher relative expression pattern by Western blot than observed by immunostaining because AFP is secreted extracellularly (Fig. 2.7B vs. 7C, right panel). These observations suggest that GS5 cells although were originated from Huh7.5 cells, they maintain a distinct cellular phenotype in culture conditions as well as in tumor xenografts.

We also observed a number of CK19+AFP+ and CK19+AFP− progenitor-like cells in the GS5 tumors (Appendix 2a), whereas these cells were rarely seen in the Huh7.5-tumors (Appendix 2b). The most likely inference of these observations could be that high level of chronic HCV replication results in retrodifferentiation of cells leading to acquisition of hepatic stem/progenitor-like characteristics.

3.4. HCV replication influences activation of β-catenin

Previous studies have shown that c-Myc and CK19 expression is affected by β-catenin expression (Thompson and Monga 2007, Zulehner et al 2010). The high level of c-Myc and CK19 expression observed in GS5 cells (Figs.2.4A and 5C) prompted
Figure 2.6. HCV replicon and pluripotency factors induce CK19 overexpression in Huh7 cells. (A) Schematic of the reprogramming cassette-expression plasmid. The CMV promoter-driven synthesis of a single mRNA results in the translation of a polyprotein that is self-cleaved by 2A peptide sequence into individual Lin28, GFP, Nanog, Sox2 and Oct4 proteins. (B) Schematic of preparation of ‘Minicircle’ from the vector DNA (A) for transfection into Huh7 cells. (C) Western blot after transfection of HCV subgenomic replicon (lane 5), Minicircle (LGNSO, lane 3), and empty vector DNA (lane 4). Lane 1, untransfected; lane 2, transfection with lipofectamine alone. (D) Western blot showing much higher expression of Lin28 in LNGSO transfected cells (lane 3) than untransfected (lane 1) or Lipofectamine alone transfected Huh7 cells (lane 2). (E) Western blot for two different HCV replicon-expressing cell lines (GS5 and FCA4) in the presence (plus) or absence (minus) of G418 selection marker. The specific bands of CK19, NS5B and actin are as indicated.
Figure 2.7. Expression pattern of hepatoblast marker in the tumor xenografts and parent culture cells. Huh7.5-tumor (A) and GS5-tumor (B) were subjected to immunohistochemistry for staining of a-fetoprotein (AFP, brown). Blue, nuclear stain. (C) AFP expression in the total lysates (30 ug) of tumor xenografts (right panel) or culture cells (left panel) was detected by Western blot. Actin, loading control.
us to investigate the status of β-catenin in response to the HCV replication. The GS5 cells cultured in the presence of G418 support continued high-level replication of the HCV RNA whereas withdrawal of G418 leads to decline in replicon expression as evidenced by decreased NS5B level in the cytoplasm (Fig. 2.8A, left panel). The NS5B reduction was accompanied by decline in both total and active nuclear β-catenin (right panel). These results clearly suggest that continued HCV replication is required to maintain activation of the β-catenin pathway in the absence of external Wnt ligands. We also observed the presence of active β-catenin in tumors as expected (Fig. 2.8B). However, there were no significant differences between the levels of active and total β-catenin in both tumor types, in consistence with the c-Myc expression profile for both tumors and cultured cells (Figs. 2.4A and 2.4B).

### 3.5. DCAMKL-1 is required for the HCV replication

HCV replication complexes associate with microtubule filaments (MTFs) and use the MTF-tracks for their movement and transport (Lai et al 2008, Roohvand et al 2009, Wolk et al 2008). Since DCAMKL-1 protein is known to bind, stabilize and polymerize tubulins into MTFs (Lin et al 2000), we examined the effect of inhibiting DCAMKL-1 expression on HCV replication. The GS5 cells were transfected with scrambled siRNA (SCR, control) or siRNA against DCAMKL-1 (siDCAMKL-1). This siRNA targets nt 308-327 (the predicted site) in the DCAMKL-1 mRNAs encoding both the AL (long form) and AS isoforms [short form, ref. (Engels et al 2004)].
As shown in Fig. 2.9A, siDCAMKL-1 transfection in GS5 cells resulted in the inhibition (average 75% at 100 nM) of DCAMKL-1 mRNA level whereas the same concentration of the SCR had minimal effect. The decrease in mRNA was accompanied by disappearance of the DCAMKL-1 protein in total lysates, suggesting that the siRNA had effectively inhibited DCAMKL-1 expression (Fig. 2.9B, lanes 3, 4). The DCAMKL-1-depletion in these cells also resulted in significant reduction (70-80%) of both the HCV RNA and NS5B polymerase levels (Fig. 2.9A and 9B, lanes 3, 4). This effect appears to be specific as the level of another pluripotency marker, Lin28B (Guo et al 2006), was unaffected by the siDCAMKL-1 treatment (Fig. 2.9B).

The immunosuppressive drug cyclosporine A (CsA) is known to inhibit cyclophilins (CypA and CypB), which interact with crucial components of the HCV RCs such as NS5A and NS5B (Watashi et al 2005, Yang et al 2008). CsA downregulates HCV replication by inhibiting activities of the RCs (Watashi et al 2005, Yang et al 2008). More often, however, CsA-treatment results in drug-resistance (Liu et al 2009, Puyang et al 2010). The CsA treatment of GS5 cells effectively inhibited HCV replication (Fig. 2.9C) and diminished level of NS5B in the GS5 lysates (Fig. 2.9D, lanes 3-5). Interestingly, CsA treatment resulted in a moderate increase in the DCAMKL-1 protein (Fig. 2.9D, lanes 3-5) without increasing its transcription level (Fig. 2.9C). It has been reported that CsA activates AKT and suppresses PTEN; both events culminate in the activation of mTOR complexes (Han et al 2010).
**Figure 2.8.** Activation of b-catenin in response to the HCV replication. (A) The cytoplasmic and nuclear lysates of GS5 cells cultured for 2 weeks in the presence (plus) or absence (minus) of G418 selection marker were subjected to Western blot using specific antibodies as indicated. The unphosphorylated form of β-catenin is shown as active protein. (B) Total lysates prepared from tumor xenografts were analyzed by Western blot for total and active b-catenin.
The activation of AKT/mTOR signaling favors enhanced cap-dependent protein synthesis and cell survival. These observations imply that even though CsA successfully inhibits HCV replication during early phase of treatment, enhanced DCAMKL-1 level may eventually contribute to CsA-resistance. In fact, we noticed a certain level of NS5B was not affected by the sequential increase in CsA concentrations (1 through 6 ug/ml, Fig. 2.9D, lanes 3-5).

3.6. HCV incites resistance to MTFs disruption

The anti-neoplastic drug, vinblastine, binds α-tubulin and inhibits microtubule assembly by preventing addition of new tubulin subunits (Rendine et al 2010). We evaluated the effect of vinblastine on Huh7.5 and GS5 cells at different time-points. As shown in Fig. 2.10 B (lower panel), vinblastine treatment caused clear disruption of MTFs architecture and showed bundling of MTFs within 7 hr in Huh7.5 cells. Most GS5 cells showed similar bundling pattern and inhibition of NS5A-GFP expression. However, the bundling of MTFs was less evident in cells showing high NS5A-GFP presence (Fig. 2.10 A, indicated with an arrow). Longer treatment and further kinetic analysis suggested that nearly all the GS5 cells eventually died within a week. During MTF bundling, we further noticed that DCAMKL-1 staining in GS5 cells was weak and the protein appears to be dislodged from the MTF bundles. Control cells treated with DMSO alone did not show MTF disruption. These results reiterate the study overall findings that survival of HCV requires DCAMKL-1 and its intact substrates (MTFs).
Figure 2.9. DCAMKL-1 is required for the HCV replication. (A) siRNA against DCAMKL-1 (siDCAMKL-1, 50 nM and 100 nM) or scrambled siRNA (SCR, 100 nM) were transfected into GS5 cells in triplicate. Total RNAs were extracted and subjected to real-time RT-PCR. The levels of HCV and DCAMKL-1 RNAs were considered one in untransfected GS5 cells (Cont.). Actin mRNA in each sample was used as internal control for PCR. (B) Western blot analysis of siRNAs transfected (lanes 2-4) and untransfected (lane 1) samples as indicated. (C) Real-time PCR was performed for detection of HCV and DCAMKL-1 RNA levels after treatment with CsA (3 ug/ml). Control, DMSO vehicle. (D) Effect of CsA on the levels of HCV NS5B polymerase and DCAMKL-1. The GS5 cells were treated with 1, 3, 6 ug/ml CsA (lanes 3-5). Lanes 1 and 2 represent untreated and DMSO (vehicle) treated samples respectively. Actin, internal loading control. Each experiment was repeated three times to confirm the results.
4. Discussion

We have used a proven replicon-based system to study long-term effects of HCV-1 on cells, as a chronic infectious model for HCV-1 is not available. We investigated HCV-induced phenotypic alterations in liver-derived GS5 cells that express a subgenomic replicon encoding NS3 through NS5B of the HCV genome. The C-terminus GFP tagged NS5A (NS5A-GFP) expressed from the replicon is efficiently incorporated into active replication complexes (RCs) and is distributed in a punctuate pattern in the cytoplasm with higher concentrations localized in the perinuclear regions [Figs. 2.1 and 2.2 (Gosert et al 2003, Moradpour et al 2007, Poenisch and Bartenschlager 2010)]. GFP intensity correlates with the level of HCV replication in these cells. We demonstrated that a small subpopulation of intensely bright GFP+ cells contain RCs in the area highly enriched in DCAMKL-1 and microtubule filaments (MTFs).

In some cases, co-localization of RCs, DCAMKL-1 and MTFs was observed by confocal microscopy. These observations and subsequent analyses presented in Figs. 2.2, 2.3 and 2.8 support the existence of an HCV-(DCAMKL-1)-MTFs-CSCs axis in GS5 cells. Using both in vitro and in vivo models, our data provided evidence that this collaboration promotes HCV replication and HCV-induced hepatocarcinogenesis. We further found that HCV replication results in the induction of putative stem cells/CSCs markers and activation of β-catenin, which are required for the maintenance and self-renewal of
Figure 2.10. Effects of vinblastin on microtubule filaments and HCV expression. Huh7.5 (B) or GS5 (A) cells were treated with vinblastin (30 uM) for 7 hrs and subjected to indirect immunoflorescence microscopy using antibodies against α-tubulin. Arrow, a vinblastin-resistant cell in GS5 population.
stem/progenitor cells (Lee et al 2009, Zhang et al 2008, Zulehner et al 2010). The stem/progenitor cells in an adult organ such as liver are considered to be: i) ‘privileged cells’ in their niches; ii) relatively resistant to apoptosis, and iii) possess longer life-span than fully differentiated cells. Thus, HCV appears to employ a unique strategy to ensure its long-term survival in the infected cells simply by directing the cells to exhibit stem cell-like properties.

Our studies suggest a novel role of DCAMKL-1 in the HCV replication. Higher expression of DCAMKL-1 in hepatic cells, and its association with MTFs, led to higher levels of HCV replication suggesting a certain advantage for virus replication. MTFs represent dynamic, polarized polymers of α- and β-tubulin heterodimers that undergo phasic polymerization and depolymerization required for cellular transport and cell division (Roohvand et al 2009). DCAMKL-1, by virtue of its MTFs polymerizing and stabilizing activity may provide MT-dependent transport and fast, saltatory movements of RCs over long distances (Fukasawa 2010, Gale and Foy 2005). By promoting RC movement, DCAMKL-1 is likely to increase HCV replication efficiency. It may also compensate for architectural distortion created by HCV-induced membranous web-like structures in the cells. Roohwand et al. (Roohvand et al 2009) demonstrated that MTFs dynamic polymerization/depolymerization also affects post-fusion steps of the HCV life cycle, and that HCV core protein interacts with tubulin thereby enhancing microtubule polymerization. The effects of viral proteins on DCAMKL-1 activities and the DCAMKL1 interaction with viral components are yet to be investigated.
Previous studies done by this lab revealed that DCAMKL-1 level is increased significantly in pancreatic and colon cancers and its knockdown causes marked reduction in tumor size in a xenograft model (May et al 2009, Roohvand et al 2009). In this study, we found evidence of higher DCAMKL-1 expression in human patients with liver cirrhosis than the normal liver tissues (Appendix. B). The cause of DCAMKL-1 overexpression in tumors and its effects on CSCs is not known yet. Our studies presented here provide HCV as one causative agent for the overexpression of DCAMKL-1. The DCAMKL-1\(^+\) cells from both cell lines (GS5 and Huh7.5 cells) behave like CSCs in matrigel further reiterating the previous findings. Therefore, HCV-led induction of DCAMKL1 in conjunction with enhanced expression of CD133, Lgr5, c-Myc and \(\beta\)-catenin may directly force the infected cells on the path of developing into CSCs. However, not all patients living with HCV develop HCC. Studies have shown that HCV NS5A inhibits apoptosis in infected cells by inhibiting oxidative stress-induced p38 MAPK activation and \(K^+\) efflux (Raychoudhuri et al 2010). Similarly, NS3/4A has been shown to promote hepatocyte survival and liver regeneration by enhancing NF\(\kappa\)B activities and hepatoprotective TNF\(\alpha\) (Rendine et al 2010). Thus, other factors may also play important role during HCV-induced hepatocarcinogenesis.

Similar to many solid tumor cell lines, the hepatoma cells used in these studies are expected to contain a population of CSCs because of the expression of many putative stem cell and pluripotency markers (Nakanishi et al 2010). As a result, both GS5 and Huh7.5 cells were able to form solid tumors in athymic nude mice. We also observed that both tumors expressed human albumin suggesting that the tumors were derived from the
transplanted cells and contain mature human hepatocytes. According to the ‘hierarchical model’ of cancer, the original phenotypes of CSCs represented in culture pool must also be maintained in their respective tumors due to self-renewal. We validated this assumption by comparing expression of hepatoblast and hepatic progenitor markers in culture cells and the tumor xenografts. Each tumor, indeed, reflected the original phenotype of the parent culture cells. For example, GS5 cells and its tumor xenografts exhibited much higher expression of CK19 and AFP than that of Huh7.5 controls by Western blot. The GS5-tumors also showed a very high population of CK19\(^+\)AFP\(^-\) (hepatic stem cells), CK19\(^+\)AFP\(^+\) [hepatoblast and transit amplifying cells, ref. (Zhang et al 2008)]. These HCV-induced stem-like features were clearly absent in Huh7.5 culture cells and its tumor xenografts. Higher expression of CK19 in an another HCV replicon expressing cell line (FCA4) than the parent cell line (Huh7) suggests that these properties are not limited to GS5 cells. The detection of CK19\(^+\) oval cells in HCV-positive liver cirrhosis (Sun et al 2006) and co-expression of CK19 and AFP in hepatic progenitor cells (Zhao et al 2009) further support our observations. The HCV-induced distinct traits in culture cells were subsequently maintained in the GS5-tumor tissues even though the viral gene expression was diminished during later stage of the tumor development.

Tsamandas et al. (Tsamandas et al 2006) demonstrated that hepatic progenitor cells (HPC) are frequently present in liver tissues of hepatitis C patients and their number tends to increase as the disease advances to cirrhosis which is a known risk factor for the HCC initiation. It was further demonstrated that a large group of patients that do not respond to the standard HCV treatment show higher expression of CK19 and AFP than
the responders and these proteins can be used as reliable markers for detecting the HPCs in these patients. Using extensive gene expression profiling of HCC tissues, other investigators independently reached to the conclusion that HCC originating from HPC or HPC-like cells express high level of CK19, AFP and certain stem cell signatures (Lee et al 2006b, Woo et al 2010). While supporting these observations, our in vitro and in vivo analyses further provide mechanistic insights that high expression and/or coexpression of CK19 and AFP is, indeed, caused by HCV-induced retrodifferentiation of hepatic cells leading to acquisition of HPC-like phenotype. These characteristics were universally detected in different cell lines and tumor model used here (GS5 and FCA4 cell lines, G418-withdrawl assay, and tumor xenografts). Our studies also revealed that multiple DCAMKL-1 positive cells were embedded in the areas of active c-Src expression in GS5 tumors (Fig. 2.3E). It is known that c-Src expression and activities are enhanced under varying stress conditions that favor tumor growth and metastasis (Allam and Ali 2010). In these aspects, the GS5-tumor traits are reminiscent of HCC with stem cell features (Ding et al 2004, Lee et al 2006b). Thus, it is conceivable that HCV-induced reprogramming of liver cells may contribute to both survival of the virus and hepatocarcinogenesis.

Finally, our studies provide evidence that active β-catenin accumulates in the nucleus in response to HCV expression which may explain higher-level c-Myc expression in GS5 than the controls [also see ref. (Milward et al 2010)]. Active β-catenin was also detected in the tumor xenografts. Using human HCC tissue array and murine liver tumor model, Zulehner et al. (Zulehner et al 2010) demonstrated significant
correlation between CK19 expression and that of nuclear β-catenin. It has been further shown that lack of nuclear β-catenin causes cells to differentiate into mature hepatocytes whereas its presence induced retrodifferentiation into immature hepatocyte progenitors. Therefore, our data, in conjunction with published reports, support the concept of HCV-induced reprogramming of the liver-derived cells. Since the replichon used here lacks NS2 and C, E1 and E2 structural proteins, the impact of these proteins on the ‘cellular renovation and reprogramming’ by the virus could not be investigated. A long-term, chronic HCV infection model that produces high titer HCV particles such as improved version of JFH1 strain-based cell culture (Banaudha et al 2010, Wakita et al 2005) in normal human hepatocytes will likely to provide additional clues on these aspects.

Although both Huh7.5 and GS5 or Huh7 and FCA4 cells originated from the same cell line and propagated under similar culture conditions, the long-term HCV expression (reminiscent to chronic infection) resulted in stable phenotypic changes in GS5 cells. These acquired alterations were reproduced in the tumor xenografts of GS5, which conforms the hierarchical model of tumor initiation by CSCs. Thus, it is highly likely that GS5 pool contains CSCs with features of hepatic stem cells or hepatic/biliary precursors because of retrodifferentiation and acquisition of stem cell/CSCs and oncogenic traits (see model proposed in Fig. 2.11). Consequently, the HCV infection per se including HCV-induced HCCs poses increased threat of resistance to anti-viral and anti-cancer regimens, recurrence of the infection and/or tumors, and high mortality of patients (Frank et al 2010, Kieffer et al 2010a). The Huh7.5 cells, on the other hand, retained their HCC phenotype both in culture and in tumor xenografts as it lacks the
HCV-induced alterations. In conclusion, our data provides DCAMKL-1 as a novel cellular target for combating HCV replication and/or hepatic CSCs. To inhibit, DCAMKL-1 expression, siDCAMKL-1 (Fig. 2.9) can be delivered to the target cells using gene therapy approach by constructing an expression vector for the siRNA. Alternatively, this laboratory has tried delivery of the siDCAMKL-1 using nontoxic biodegradable nanoparticles (NPs) made of poly(lactide-co-glycolide) [PLGA]. The successful delivery of siDCAMKL-1 NPs at the site of tumors in mouse xenograft model led to the reduction in liver and pancreatic tumor size (Suraben et. al 2009). In addition, our studies further argue comprehensive evaluations of anti-HCV drugs in the setting of chronic/long-term infection for their efficacies.

5. Chapter summary

Hepatitis C virus (HCV) infection is a prominent risk factor for the development of hepatocellular carcinoma (HCC). Similar to most solid tumors, HCCs are believed to contain poorly differentiated cancer stem-like cells (CSCs) that initiate tumorigenesis and confer resistance to chemotherapy. This dissertation studies demonstrate that expression of HCV subgenomic replicon in cultured cells results in acquisition of CSCs traits. These traits include enhanced expression of DCAMKL-1, Lgr5, CD133 and c-Myc in conjunction with long-term maintenance of pluripotency factors. The studies also show that HCV replication is severely impaired by siRNA-led depletion of the microtubule-associated putative stem cell marker, DCAMKL-1. The DCAMKL-1-positive cells isolated by fluorescence activated cell-sorting (FACS) form spheroids in matrigel.
work further demonstrates that HCV replicon-expressing cells initiate tumors in mice. The resulting tumors exhibit characteristics of HCCs that are originated from hepatic progenitor-like cells and are phenotypically distinct from the tumors initiated by parent cells lacking the replicon. This phenotype expresses high-levels of α-fetoprotein, cytokeratin-19 and pluripotency markers. HCV-induced activation of β-catenin, c-Myc, and c-Src indicates the aggressive nature of the tumor. These results collectively suggest that HCV exhibits intrinsic ability to induce retrodifferentiation and CSC signatures, and DCAMKL-1 represents a novel cellular target for combating HCV and the virus-induced hepatocarcinogenesis.
Figure 2.11. Proposed retrodifferentiation model for the HCV-induced liver carcinogenesis. Solid arrows, pathways of normal hepatic stem cell differentiation; arrows with broken lines, possible source of Huh7.5- and GS5-derived tumors. Majority of Huh7.5 culture/tumor cells lack hepatic/biliary precursor markers although these cells contain stem/progenitor cell traits. Therefore, they represent intermediate or differentiated hepatocytes. On the other hand, GS5 culture/tumor cells contain stem cell/progenitor cell markers, and high population of CK19^+AFP^- (hepatic stem cells) and CK19^+AFP^+ (hepatoblast, transit amplifying cells or hepatic/biliary precursors). The expression level of CK19 and AFP in these cells are remarkably higher than those of Huh7.5. These acquired distinctions are likely attributed to retrodifferentiation of the parent Huh7.5 cells into GS5 phenotype that was induced by sustained expression of the HCV replicon. HPC, hepatic progenitor cell. (B) Probable consequences of the HCV-induced HPC traits.
Chapter Three: Targeting DCAMKL-1 with FDA-approved drugs for treatment of HCV infection

1. Introduction

A number of drugs that include the specifically targeted antiviral therapies for hepatitis C (STAT-Cs), and cellular targets against HCV (CTACs) are at various stages of clinical trials or have been stopped because of their adverse effects, multiple drug resistance or ineffectiveness in the patients (Kieffer et al 2010b). The most successful among STAT-Cs include drugs against NS3 protease/helicase (telaprevir, boceprevir), NS5B polymerase (nucleoside and non-nucleoside analogues) and NS5A protein (BMS-790052). The use of telaprevir or boceprevir, in combination with Peg-IFN/RBV regimen was reported to increase the SVR rate by 20% in shorter duration (within 24 weeks) as compared the SOC alone (48 weeks) in patients infected with HCV genotype 1. Similarly, impressive potency of NS5A inhibitor (BMS-790052) has been shown in clinical trial (Gao et al 2010). With all these successful stories and enthusiasm, cure for the HCV infection has been elusive not only because of adverse effects on patients but also due to emergence of drug-resistance (Fridell et al 2010, Kieffer et al 2010b, Liu et al 2009, Pockros 2010). Anemia (up to 37% with telaprevir and up to 56% with boceprevir), skin problems for telaprevir (rash and pruritis) and dysgeusia for boceprevir in addition to the influenza-like symptoms, cytopenias and depression are additional hindrance for a
successful treatment. The viral variants with reduced susceptibility to STAT-Cs occur naturally at low levels due to error-prone HCV replication mechanism, and can be selected in patients not responding to STAT-C/SOC treatment. Susceptibility of HCV against these drugs diminishes due to gradual incorporation of mutations in the virus that have been demonstrated during \textit{in vitro} and \textit{in vivo} testing. These drugs have huge potential for misuse due to prescribing physician’s poor understanding of the therapeutic populations, inadequate viral-assay testing, poor side-effect management, and lack of monitoring for antiviral resistance.

Clinical trial by our group (Bader et al 2008) and subsequent studies by others (Harrison et al 2010, Milazzo and Antinori 2010, Milazzo et al 2010) suggest that certain statins (fluvastatin, simvastatin, lovastatin, and mevastatin) but not pravastatin show anti-HCV activities both in experimental models and in patients with hepatitis C. It has been demonstrated that the FDA-approved doses of fluvastatin are well tolerated by patients with HCV and the drug can provide aid standard of care therapy (Delang et al 2009). Statins are inhibitors of 3-hydroxyl-3-methylglutaryl coenzyme A (HMG CoA) reductase, which is a rate-limiting enzyme in cholesterol biosynthesis that catalyzes the conversion of HMG-CoA to mevalonic acid (Fig. 3.1). These drugs are used for the treatment of hypercholesterolemia and have been reported to exhibit antiviral activities against a number of viruses (Gilbert et al 2005). The precise mechanism of the anti-HCV activity of statins has not yet been defined. Recent studies suggest that the anti-HCV activity of statins may result from inhibition of geranylgeranylation of cellular proteins rather than the inhibition of cholesterol synthesis. Geranylgeranylation is a
posttranslational modification that covalently attaches geranylgeranyl to various cellular proteins to facilitate their membrane association. These geranylgeranyl groups are isoprenoids synthesized in the cholesterol biosynthesis pathway. The HCV genome does not code a geranylgeranyl protein suggesting that the replication of the HCV needs the host cell geranylgeranyl pyrophosphate synthesis. More recently, FBL2 has been reported to be a host target for geranylgeranylation. Geranylgeranylation of FBL2 appears to be critical for HCV replication because the association between FBL2 and NS5A, an interaction that is a prerequisite for HCV replication, depends on geranylgeranylation of FBL2 (Kapadia and Chisari 2005, Wang et al 2005, Ye et al 2003).

Himmelbach et al. (Himmelsbach et al 2009) demonstrated that Sorafenib, an FDA and EU approved drug for the treatment of HCC also inhibits HCV replication in cell culture. Sorafenib has been shown as a potent inhibitor of c-Raf that interacts with the HCV NS5A. The CTAC inhibitors (e.g. NIM811, Debio 025) for the HCV protein-interacting partners such as cyclophilins A and B are also in clinical trials. However, due to adaptive mutations in the HCV proteins, the drug-resistance is inevitable as reported in a number of studies (Yang et al 2008). Our studies suggest that HCV induced stem cell-like features may contribute to the long-term persistence of HCV and HCV-induced aggressive HCC. Therefore, new CTAC inhibitors whose potencies are not compromised by the HCV adaptation and drug cocktail with diverse mechanisms of action are needed to combat HCV infection. Such multi-dimensional approach as proposed here is likely to be effective against both the HCV and the HCV-led initiation of liver carcinogenesis.
Figure 3.1. Possible interactions among HCV, miR-122 and cholesterol biosynthesis pathway. The statins block the rate-limiting enzyme, HMGCR. The interaction of viral protein NS5A with geranylgeranylated cellular protein FBL2 and HCV RNA are shown. NS5A also interacts with lipid droplets and membranes (not shown) CoA, coenzyme A; PP, pyrophosphate. Presented with minor modification of Figure 3.1 shown in J. Virol. 2010. 84:666-670.
2. Materials and Methods

Cell Culture, real-time RT-PCR analyses, immunohistochemistry, immunofluorescence and confocal microscopy were done as described in Materials and Methods, Chapter 2.

2.1. Culture of normal human hepatocytes (NHH)

Normal human hepatocytes from a donor (16 yr, female, non-smoker, died due to head trauma) were purchased from BD Biosciences in cryopreserved form (Catalog # 454550). The cells were stored in liquid nitrogen until needed. For confocal and immunoflorescence microscopy, the cells were cultured/maintained in Hepato-STIM Hepatocyte Defined Medium (BD Biosciences) supplemented with Epidermal Growth Factor (10 ng/ml) and 1X antibiotic-antimycotic (Invitrogen) on collagen BD cover slips (22 mm). The cells were incubated at 37°C and 5% CO2, washed after 72 hrs with PBS, fixed with formaldehyde and stained with antibodies as described in earlier chapter. Alternatively, the NHH were also maintained in BD Matrigel 6-well plate in the Hepato-STIM complete media to determine their three-dimensional growth pattern. These NHH were subjected to H&E staining, immunohistochemistry, and staining for DCAMKL-1 and microtubules.
2.2. Cell proliferation assay:

MTT (3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyltetrazolium bromide) was purchased from Biotum, Inc. and used according to manufacturer recommendations. To investigate cellular toxicity of fluvastatin, $2 \times 10^4$ cells were plated into 96-well plates in 100 µl regular complete DMEM media. After 24 h, varying concentrations of fluvastatin were added and the plate kept at 37 C in 5% CO2 for 72 or 94 h. The cells were washed and fresh media (100 µl) and MTT solution (5 mg/ml in PBS) were added to all wells, wrapped the plate in aluminum foil and incubated for 3-4 h at 37 C. Two hundred µl of DMSO was added to each well in order to dissolve the formazan crystals formed as a result of the MTT reaction. Absorbance with an enzyme-linked immunosorbent assay (ELISA) plate reader at a test wavelength of 570 nm and a reference wavelength of 630 nm.

3. Results

3.1. Fluvastatin reduces abundance of the HCV RNA and NS5B polymerase in GS5 cells

Recent studies have shown that statins lower HCV titer in patients (Bader et al 2008). The HCV-specific drugs and/or IFN with certain statins have also been demonstrated to improve efficacy of the treatment (Delang et al 2009, Harrison et al
2010). However, mechanism of action of different statins against HCV is not fully understood.

The HCV subgenomic replicon-expressing GS5 cells were treated with increasing amounts of FLV. Since DMSO is a solvent for FLV, similar amounts of the solvent/vehicle alone were used in the control culture. The cultures without any drug or solvent were considered as positive controls. As shown in Fig. 3.2A, increase in FLV concentration from 0 to 5.0 uM caused marked decrease in the HCV RNA (black bar). Further increase of FLV (10 uM) did not show additional reduction of the HCV RNA. In contrast, DMSO alone did not reduce the HCV RNA level at any of these concentrations (gray bars). The inhibitory effect of FLV on HCV was also confirmed by Western blot analysis. The total lysates prepared from untreated and FLV-treated cells revealed that the expression of NS5B polymerase is significantly reduced at 5 and 10 uM (Fig. 3.2B, upper panel, lanes 5, 6) as compared to the control (lane 1). This reduction was not noticed the DMSO treated lysates (lower panel). These results clearly indicate that FLV considerably inhibits HCV RNA and protein levels.

3.2. Effect of mevalonic acid on FLV-led inhibition of the HCV replicon

Mevalonic acid (Mev) is a product of the enzyme HMG-CoA reductase (HMGR) and is essential substrate for downstream reactions in the cholesterol biosynthesis pathway. Therefore, inhibition of HMGR by FLV can be antagonized and cholesterol biosynthesis pathway can be restored by addition of mevalonic acid in the culture media. Varying amounts of Mev (5, 10, 15 uM) were added to GS5 culture in the presence of
FLV (5uM). The control cultures lacked FLV but were supplemented with DMSO and 
Mev. The HCV RNA levels in these cells were compared with the untreated culture. 
Although, 5 and 10 uM of Mev could not recover the FLV inhibition. However, a modest 
expression level of the HCV RNA was restored at 15 uM of Mev (Fig. 3.3A). The 
DMSO-treated cells alone or with exogenously added Mev did not affect the HCV RNA 
abundance. Next, 5 uM of each FLV, pravastatin (Prav) and rusovasatin (Ruso) were 
added in the absence or presence (15 uM) of Mev. Only FLV showed reduction (Fig. 
3.3B, lane 2) in the NS5B level as compared to the untreated control (lane 1). 
Interestingly, addition of 3-fold molar excess of Mev led to very little recovery of the 
NS5B level (lane 3). Addition of Prav and Ruso to the cell culture in the absence or 
presence of Mev failed to exhibit reduction in the HCV NS5B level under similar 
conditions (lanes 4-7). In similar experiments, we further noticed that the FLV-treated 
GS5 cells displayed marked morphological changes with induced cytoplasmic 
protrusions (Fig. 3.3C-b), which were partially recovered by Mev treatment (Fig. 3.3C- 
d). These changes were not observed for DMSO (Fig. 3.3C-c) or pravastatin treatment 
(not shown). Our MTT cell proliferation assay on these cells including ASPC1 
(pancreatic cancer cell line) and FCA4 (an HCV replicon expressing Huh7 cell lines) 
suggest that the FLV-treated cells are viable during this period (72 hr).
Figure 3.2. Reduction of the HCV RNA and NS5B polymerase abundance by fluvastatin treatment in GS5 cells. (A) Real-time RT-PCR for detection of the HCV RNA in DMSO (control, gray bar) or fluvastatin (FLV) in DMSO (black bar). The cells were treated with different amounts (0-10 uM in media) of FLV. Same amounts (ul) of the solvent (DMSO) were added in the control samples. Total RNAs were extracted from the cells using Qiagen Rneasy Kit for the PCR. (B) Western blot for NS5B in FLV-DMSO (increasing concentrations from lane 2 through 6) and DMSO alone (Cont.) as indicated. Actin, loading control.
3.3. Fluvastatin induces ‘microtubule bundling’ in cancer-related cells but not in normal human hepatocytes

The morphological changes in GS5 cells due to FLV-treatment (Fig. 3.3C) prompted us to investigate the status of microtubule filaments (MTFs) using confocal microscopy. Interestingly, the FLV-treated but not the DMSO-treated GS5 cells exhibited extensive ‘bundling’ of the microtubule filaments (Fig. 3.4A). The induced skeletal change was accompanied by the decrease in NS5A-GFP intensities and displacement of replication complex from the MTF bundles (Fig. 3.4B, lower panel). The MTF bundling effect was observed in Huh7.5 but was less pronounced (Fig. 3.4C, lower panel). In contrast, normal human hepatocytes were completely resistant to the FLV-induced MTF disruption (upper panel).

3.4. Effect of Fluvastatin on DCAMKL1

Because DCAMKL-1 catalyzes polymerization of tubulins into microtubules, we looked into its cellular localization in the FLV-treated and untreated cells. As shown in Fig. 3.5, DCAMKL1 localization in GS5 cells was disturbed and dislodged from microtubules as a result of the bundling effect induced by FLV (lower panel). This bundling and redistribution of DCAMKL1 did not occur in DMSO treated cells (upper panel).

The miR-122 has been shown to facilitate the replication of HCV by binding to the domain I in the HCV 5’UTR at two adjacent sites (Jangra et al 2010, Jopling 2008, Norman and Sarnow 2010).
Figure 3.3. Effect of different statins and mevalonate on the HCV replication, cellular morphology and cell survival within three days of the drug treatment. (A) Relative abundance of the HCV RNA in FLV-treated cells that were supplemented with 5, 10 and 15 μM of mevalonic acid (antagonist of FLV). The untreated culture was used as positive control. (B) Western blot for detection of NS5B expression levels in GS5 cells treated with fluvastatin (FLV, lanes 2, 3), pravastatin (Prav, lanes 4, 5) and rusovastatin (Ruso, lanes 6, 7). Lane 1, untreated control. DMSO, vehicle control. (C) Brightfield photograph of GS5 cells treated and untreated with various drugs as indicated, highlighting morphological sensitivity of the cells by the FLV treatment (b) and its partial reversal by mevalonate (d). a, b; controls. (D) MTT assay for cell proliferation at 72 hrs of the FLV and vehicle treatments. ASPC1, pancreatic cell line; FCA4, hepatoma cell line derived from Huh7 and expresses a subgenomic replicon without GFP, otherwise similar to the GS5 cells.
**Figure 3.4.** Effect of FLV on microtubule filaments (MTFs) in GS5 (A), normal human hepatocytes [(C), upper panel] and Huh7.5 cells [(C), lower panels] shown by confocal microscopy. (B) Higher magnification for vehicle (DMSO alone) treated GS5 cells [(A), upper panel] showing localization of the HCV replication complexes (green), MTFs (red) and their colocalization (yellow).
The miR-122 has also been implicated in cholesterol biosynthesis. For these reasons, we checked if primary miR-122 RNA is affected by the FLV in GS5 cells. As shown in figure 3.6A, DCAMKL-1 RNA (black bar) but not the pri-miR-122 (Fig. 3.6B) level was reduced to significant extent by the FLV treatment. In the control DMSO sample neither DCAMKL-1 nor pri-miR-122 RNA levels were affected. These results clearly suggest that FLV regulates expression of DCAMKL-1 at its RNA level, and the existing DCAMKL-1 localization to the MTFs is severely affected. The net result of FLV effect is reflected by inhibition of the HCV expression in these cells.

3.5. Cirrhotic liver nodules of chronic HCV patients show overexpression of DCAMKL1 and CK19

As a continuing effort to unravel the mechanism of HCC development, we examined liver biopsy samples from chronic HCV patients and liver tissue arrays representing various stages of liver diseases. An example of immunohistochemical staining result is shown in Fig. 3.7. Strikingly, in agreement with the results obtained for our cell culture and xenograft experiments, the biopsies exhibited overexpression of DCAMKL1 and CK19 in the cirrhotic liver nodules which are considered to be risk factor for hepatocarcinogenesis (Fig. 3.7 lower panel). DCAMKL-1 expression was also shown to be increased in cirrhotic liver tissue as shown by our tissue microarray studies (Appendix 3).
Figure 3.5. Immunofluorescence microscopy localization of DCAMKL-1 (red) in relation to MTFs (magenta) and replication complexes (green). The GS5 cells were treated with DMSO or FLV as indicated.
The findings described in this chapter are coherent with those in Chapter 2. Our data further support possible use of the FDA-approved drug, fluvastatin as an adjuvant to the available HCV therapeutics in order to reduce the risk of HCC development. In addition, it is likely that FLV along with DCAMKL-1 antagonist would effectively block both chronic HCV infection as well as HCV-induced HCC.

4. Discussion

We demonstrated inhibitory effects of fluvastatin (FLV) on the abundance of HCV RNA and NS5B polymerase in cell culture. This pleiotropic effect was only partially restored by large excess (3 times molar excess) of the FLV antagonist (mevalonate), which indicates involvement of additional mechanism for the FLV-led inhibition of HCV besides partial contribution of the cholesterol biosynthesis pathway. This notion is also supported by the fact that only fluvastatin but not pravastatin reduces the HCV NS5B level and exerts induced-bundling of microtubule filaments. One of the obvious consequences of the MTF bundling was highly pronounced morphological changes in GS5 cells than that of the parental Huh7.5 cells. Our cell viability assays suggested that these cells were still alive during 3-day of FLV treatment. Interestingly, normal human hepatocytes derived from a healthy donor were completely tolerant to FLV treatment and did not exhibit the MTF bundling. The selective MTF bundling in
Figure 3.6. Effect of fluvastatin on the abundance of DCAMKL-1 and pri-miR-122 RNAs. The GS5 cells were treated with varying amounts of FLV (black bar) or DMSO alone (gray bar) and total RNAs isolated from these cells were subjected to real-time RT-PCR.
cancer cells induced by FLV is likely affecting microtubule dynamics and its associated proteins. In previous chapter, it was demonstrated that HCV induces overexpression of the microtubule-associated putative stem cell marker, DCAMKL-1. Houchen and colleagues from this department (May et al 2008, Sureban et al 2009) have previously shown that both pancreatic and colon cancer cells exhibit high expression of DCAMKL-1 and develop tumors in a mouse xenograft model. However, when DCAMKL-1 expression was inhibited in the tumors, the tumor size shrinks dramatically. These observations prompted us to investigate the status of DCAMKL-1 in FLV-treated cells. We demonstrated that DCAMKL-1 RNA abundance was reduced in these cells and the existing DCAMKL-1 protein was aggregated and dislodged from the microtubule filaments. As a result, the HCV replicon expression was significantly reduced because intact MTFs and DCAMKL-1 (as presented in chapter 2) are required for the HCV replication (Lai et al 2008, Roohvand et al 2009) (Wolk et al 2008).

HCV is regarded as a lipoviroparticles because lipids play pivotal roles in the organization of HCV particles as well as infectious processes of the virus. Cholesterol esters account for nearly half of the total lipid content of cell culture generated HCV infectious particles. Both HCV NS5A and core proteins are known to associate with lipid droplets (LD) that are cellular lipid storage organelles involved in lipid homeostasis. Chronic hepatitis C virus (HCV) infection affects host lipid metabolism and induces LD accumulation in the liver. In addition, the ‘niche’ of active replication of the HCV RNA
Figure 3.7. CK19 and DCAMKL-1 overexpression in an HCV-positive patient liver detected by immunohistochemistry. Sample 2 represents neoproliferative cells in the cirrhotic liver biopsy taken after 2 years of the first biopsy (Sample 1) from the same patient. Brown stain, DCAMKL-1 and CK19; Blue stain, nucleus.
complexes consists of the viral NS proteins including NS5A and lipid rafts that are enriched in caveolin-2, sphingomyelin and cholesterol. It has been suggested that geranylgeranylation of a cellular protein, F-box/leucine-rich repeat protein 2 (FBL-2) in an HMG-CoA reductase (HMGCR)-dependent manner is required for its interaction with the HCV NS5A protein and its role in HCV RNA replication. In such situation, supplementation of mevalonate would have completely rescued the HCV replication as it functions as a substrate downstream of HMGCR activities. These observations also argue FLV-mediated control of HCV abundance in favor of its selective effects on MTFs-dependence DCAMKL-1. Because the HCV replicon expressed in GS5 cells lacks all of the structural proteins (C, E1 and E2) and assembly of the particle, the observed FLV effect is likely to be directed at the translation and replication steps.

Chronic HCV infection over several months to a few years results in cirrhosis including disruption of normal liver architecture and development of regenerative nodules. We examined multiple nodules from HCV-positive cirrhotic liver. The immunohistochemistry results clearly showed overexpression of both DCAMKL-1 as well as CK19 in these nodules. In many cases, such nodules are considered to be precursors for the development of HCC (Caillot et al 2009). Therefore, FLV inclusion in the treatment of chronic HCV infection will have multiple advantages. First, it will reduce cholesterol biosynthesis and disrupt the excessive accumulation of lipid droplets and lipid-rafts, which are required in the replication, assembly and intracellular transport of HCV. Second, generally well-tolerated fluvastatin may reduce the risk of HCC development due to its effects on DCAMKL-1 and MTFs. It has been shown by other
investigators that the pleiotropic effects of statins act as adjuvant to other HCV targeting drugs, and may sensitize the cells against anti-neoplastic drugs (Demierre et al 2005).

In conclusion, the studies presented in this chapter clearly strengthen this notion and recommends inclusion of fluvastatin with new HCV-specific drugs (e.g. boceprevir) for the treatment of chronic infection and HCC.
Chapter Four: Mechanism of c-Src proto-oncogene expression in HCV expressing cells

1. Introduction

The studies on tumor xenotransplantation (Chapter 2, Fig. 2.3E) suggest that c-Src, a prominent member of the non-receptor tyrosine kinase family, was highly expressed and activated in the area rich in DCAMKL-1. This led us to believe that c-Src expression may be important factor for the HCV-induced carcinogenesis. The level of cellular Src (c-Src) protein, is known to increase in a variety of tumors (Fizazi 2007, Horn et al 2003). However, it is not known whether the enhanced expression is regulated by transcriptional and/or post-transcriptional mechanisms. The c-Src protein promotes cell differentiation, tumor growth, metastasis and angiogenesis (Bjorge et al 2000, Finn 2008). It activates STAT3 which transcriptionally regulates expression of Bcl-XL, c-Myc and cyclin D1 leading to activation of anti-apoptotic and cell-cycle progression pathways (Diaz et al 2006, Garcia et al 2001). It has been shown that activated c-Src-focal adhesion kinase (FAK) complex promotes cell mobility, cell-cycle progression and cell survival. The c-Src activities are also important for promoting VEGF-associated tumor angiogenesis and protease-associated metastasis (Mitra and Schlaepfer 2006).
Post-translational modifications such as phosphorylation and myristoylation are key regulators of the c-Src activities. While non-myristoylated c-Src readily moves to the nucleus in G0 and at the G1/S phase, myristoylation at the N-terminus is required for its membrane attachment and transforming activities (David-Pfeuty et al 1993, Oneyama et al 2008).

The intramolecular interaction between its SH2 domain and phosphorylated Tyr530 (numbered according to NM_198291) residue at the C-terminus induces closed or inactive conformation in the c-Src molecule. Under basal conditions in vivo, 90-95% of Src is found in this state (Zheng et al 2000). The dephosphorylation of Tyr530 by protein tyrosine phosphatase (PTP) and autophosphorylation of Tyr419 by its kinase domain causes induction of an enzymatically active, open conformation (Bjorge et al 2000, Fizazi 2007).

The Src gene is composed of 14 exons (Bonham and Fujita 1993, Bonham et al 2000). Transcription of this gene in hepatoma cells from two different promoters and alternative splicing results in mature transcripts that differ only in the extreme 5’ ends but encode the same 60 kDa c-Src protein (Fig. 4.1A). The c-Src Type-1A mRNA contains a 350 nt long 5’ noncoding or untranslated region (5’NCR or 5’UTR) with multiple AUGs located at nt positions 147, 179, and 351 (Fig 4.1B). However, only AUG351 is used to initiate translation of the c-Src open reading frame (ORF). The Type-1a c-Src transcript contains a 451 nt long 5’NCR and differ with Type-1A only in the first exon (1A or 1a) (Bonham et al 2000). The second and third exons (1B and 1C) are shared in both
transcripts. Hepatoma cells have been shown to express both transcripts (Bonham et al 2000). The regulatory role(s) of these noncoding/untranslated elements during translation of c-Src mRNAs and their role(s) in c-Src overexpression are not known.

Most eukaryotic mRNAs are translated by cap-dependent mechanism where eIF4F complex binds to the 5’ cap structure through its eIF4E subunit (Sonenberg and Hinnebusch 2007). This binding event results in activation of mRNA and assembly of 48S pre-initiation complex. The 48S complex scans mRNA in 5’ to 3’ direction until an appropriate AUG initiation codon is encountered, which is followed by joining of the 60S subunit (Sonenberg and Hinnebusch 2007). Many cellular conditions such as apoptosis, stress, mitosis, heat-shock, hypoxia, infections and nutrient deficiency alter the function of normal translation initiation machinery. This is largely affected by post-translational modifications (e.g. phosphorylation) and/or cleavage of canonical initiation factors (e.g. eIF4B, eIF3, eIF2a and eIF4G family members) (Baird et al 2006, Sonenberg and Hinnebusch 2007, Wek et al 2006). A considerable number of cellular and viral mRNAs have been shown to be translated by cap-independent mechanism due to the presence of an IRES element in the mRNAs (Hellen and Sarnow 2001, Johannes and Berger 1993). Nearly 125 IRES elements have been described in a variety of species ranging from viruses to humans (Baird et al, 2006). The IRES elements have been detected in a number of eukaryotic mRNAs that encode proteins involved in signal transduction pathways, gene expression and development, differentiation, apoptosis, cell-cycle or stress response (Holcik and Sonenberg 2005, Lewis et al 2008, Sonenberg and
Hinnebusch 2007). For example, cellular stress causes dephosphorylation of eIF4E and hypophosphorylation of 4E-BPs, both of which are unfavorable for the assembly of translation pre-initiation complex by the cap-dependent mechanism (Gingras et al 1999, Sonenberg and Hinnebusch 2007). However, under these conditions, Bcl-2, XIAP, eIF4G, VEGF, ODC, PDGF, PITSLRE, c-Myc family members, and a whole host of proteins maintain their presence due to their IRES-controlled translation (Chappell et al 2000, Johannes and Sarnow 1998, Nanbru et al 1997, Semler and Waterman 2008, Spriggs et al 2008).

All of the viral and cellular IRESs initiate translation of a downstream open reading frame (ORF) by cap-independent mechanism in spite of their rich structural diversities (Filbin and Kieft 2009). The distinct structural features allow the IRESs to attract different set of canonical and non-canonical translation factors for their efficient activities and/or regulation. For examples, some of the viral and cellular IRESs require initiation factors such as eIF4G and PABP while others show enhanced activities when these factors are cleaved or their function is inactivated (Holcik and Sonenberg 2005). A few of the IRESs seek support from ITAFs (IRES-specific trans-acting factors) such as hnRNP family members, PTB, La antigen and PCPB for their efficient function (Ali and Siddiqui 1997, Semler and Waterman 2008, Spriggs et al 2008, Wang et al 1995). The IRESs also exhibit variations in the mode of assembly of pre-initiation complex. Poliovirus-like IRESs recruit the 48S pre-initiation complex upstream of the initiation site and require scanning of the complex for the initiator AUG codon, whereas an
extensively studied encaphalomyocarditis virus (EMCV) IRES recruits the pre-initiation complex at the initiation site that includes AUG (Baird et al 2006, Filbin and Kieft 2009). The IRESs of hepatitis C virus (HCV, a hepacivirus), classical swine fever virus (CSFV, a pestivirus), cricket paralysis virus (CrPV, a dicistrovirus) and simian picornavirus type 9 (SPV9) constitute a distinct class because of their ability to directly bind and make multiple contacts with the 40S ribosomal subunit (Filbin and Kieft 2009, Jan and Sarnow 2002, Lancaster et al 2006, Pestova et al 1998). The assembly of productive initiation complexes on these IRESs is energy-efficient and can ignore the need of several critical translation initiation factors (eIFs 4F/4A/4B/1/1A) that are controlled by a variety of external and internal cellular regulators (Robert et al 2006, Spriggs et al 2008). This ‘40S-binding signature’ has not been reported for the known cellular IRESs.

2. Material and Methods

2.1. Plasmid Constructs

RNA was isolated from hepatoma-derived Huh7 cell line using Qiagen RNeasy kit. Nucleotides 1-383 of the Type-1A c-Src mRNA were amplified from the total RNA using high fidelity RT-PCR Kit (Promega) and a pair of primers (Sense: 5’CATAGCAAGCTTGCGGAGCGCCAGGCCGCGTCTG3’; Antisense: 5’GCGCCGTGCTCATGAGGCATCCTTGGGCTTGCTTTGTTGCTACC3’, restriction enzymes sites are underlined). The amplified DNA contains wild type full-length 5’NCR and 33 nt coding region that encodes first 11 aa of the c-Src protein. The
PCR products were digested with HindIII and BspHI for ligation into a vector backbone. The backbone was created by digestion of a previously described plasmid pT7C1-DC29-332 (Wang et al 1993) with HindIII and NcoI and the PCR products were ligated at this site after restriction digestion. The resulting plasmid p5'Src-FLuc contains T7 promoter at the 5’ end of the c-Src sequence which is followed by ORF of firefly luciferase (FLuc) and ends with oligoA tail. The in vitro transcription of the HpaI digested plasmid by T7 RNA polymerase produces an RNA containing the entire 5’NCR followed by coding sequences representing N-terminus 11 aa (MGSNKSKPKDA) of c-Src fused with the FLuc ORF ending with poly(A) tail. The plasmid p5'SrcΔC-FLuc was similarly constructed as described for p5'Src-FLuc except that it lacks 33 nt of the c-Src coding sequence. To construct this plasmid, the c-Src PCR-amplified DNA described above was digested with HindIII and NcoI and cloned at the same site in the vector backbone. In both cases the Kozak sequence context was maintained at the translation site. The sequence of each construct was verified by restriction enzyme digestion and Big Dye DNA sequencing method (Applied Biosystems).

The mutant plasmids p5'SrcΔ1-FLuc, p5'SrcΔ2-FLuc, p5'SrcΔ3-FLuc are derived from the p5'Src-FLuc except that these plasmids have deletions of 19 (nt 216-350), 253 (nt 95-348) and 171 (nt 47-216) bases in the c-Src motif respectively. The plasmids were constructed by appropriate restriction digestion and religation of the open ends. A dual luciferase plasmid construct (p5'Src-RFLuc) was engineered which contains renilla
luciferase (RLuc) gene and stop codon upstream of the c-Src 5’NCR in the original plasmid p5'Src-FLuc.

The plasmid 5’PV-FLuc contains wild type, full-length poliovirus (PV, Mahoney strain) 5’NCR (nt 1-742) cloned in frame with FLuc ORF followed by poly(A) tail. The mutant plasmid 5’PV(D286-605)-FLuc was constructed by restriction enzyme digestion of the 5’PV-FLuc plasmid with BplI and BsaBI followed by filling with T4 DNA polymerase and religation with Quick T4 DNA ligase (New England BioLabs). The resulting plasmid contains a deletion of 319 bp (nt 286-605) in the PV 5’NCR.

2.2. In vitro transcription

The plasmids p5'Src-FLuc, p5'SrcΔ1-FLuc, p5'SrcΔ2-FLuc, p5'SrcΔ3-FLuc and p5'SrcΔC-FLuc were linearized with HpaI and transcribed with T7 RNA polymerase to produce luciferase reporter RNAs. The uncapped RNAs were prepared with Promega’s RiboMax Large Scale RNA production kit. The capped RNAs were synthesized in the presence of the ARCA cap analogue using mMassage mMachine Ultra kit (Ambion) in accordance with the manufacturer’s instructions. The transcribed RNAs were passed through G25 column and purified by extraction with phenol:chloroform:isoamyl alcohol (PCI) followed by water-saturated cold ether. Following precipitation and washing with 70% ethanol, the final preparations were dissolved in RNase-free water and checked for integrity of RNAs by formaldehyde-agarose gel electrophoresis. Concentrations of RNA were determined spectrophotometrically. For preparation of the c-Src NCR probe, 5’Src-
FLuc was linearized with XbaI and transcribed with T7 RNA polymerase in the presence of [α-32P]CTP. The 5’PV(D286-605)-FLuc was similarly digested with XbaI and transcribed for preparation of an inactive IRES control probe. The probes were purified using Qiagen RNeasy purification method. The plasmid pRL-HCV1b encodes upstream Renilla luciferase followed by the HCV IRES (nt 1-357 of the HCV genotype 1b) linked to the second reporter FLuc (Collier et al 1998). The plasmid was linearized with HindIII and transcribed in the presence of cap analogue using T7 RNA polymerase.

2.3. Cell culture and preparation of cell lysates

Huh7 cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 1x Pen/Strep and 10% fetal bovine serum (Invitrogen), and maintained at 37°C and 5% CO2. HeLa S3 cultures were carried out in spinner flask containing Joklik modified minimum essential medium Eagle (Sigma) supplemented with 5% bovine calf serum, 2% fetal clone II (Hyclone), 1x Penicillin/Streptomycin, and incubated at 37°C and 5% CO2.

HeLa translation lysates (S10) and lysates containing initiation factors (IFs) were prepared according to the protocol described by Barton and colleagues (Barton et al 1996). The rabbit reticulocyte lysate nuclease-treated (RRL) was purchased from Promega. The total lysates from cultured Huh7 cells were prepared using M-PER Kit (Pierce) as instructed.
2.4. In vitro translation of RNAs

The in vitro transcribed wild type 5’Src-FLuc and its mutant derivatives were translated in HeLa cell-free system. The standard HeLa cell-free translation mixtures contain 20 ul S10, 10 ul IFs, 5 ul 10X buffer (155 mM HEPES-KOH, pH 7.4, 600 mM potassium acetate, 10 mM ATP and 2.5 mM GTP, 300 mM phosphocreatine, 4 mg/ml creatine phosphokinase), 20 units RNasin, 5 to 10 ug RNA template in a 40 ul final volume. One microliter [\(^{35}\)S]Methionine was added for radio-labeling of the newly synthesized proteins. The translation mixtures were incubated for 1 to 2 hr at 30°C, and the FLuc activity was assayed using 2 ul aliquots. For detection of protein bands, the samples were subjected to SDS-PAGE followed by autoradiography. For detection of the 5’Src-RFLuc RNA expression, a dual luciferase assay protocol (Promega) was employed, and renilla and firefly luciferase activities were simultaneously assayed. Varying amounts of m\(^7\)GDP or m\(^7\)GTP were added in the standard HeLa translation mixtures for inhibition of cap-dependent translation. Unmethylated GDP or GTP served as negative control. Translation of the RNA in RRL was carried out as described in the supplier’s protocol (Promega).

2.5. RNA stability assay

Equal amounts of \(^{32}\)P-labeled wild type or mutant reporter RNAs were incubated in standard HeLa translation reactions and total RNAs were extracted from each sample
using RNeasy Kit (Qiagen). The recovered RNAs were subjected to formaldehyde-agarose gel electrophoresis followed by autoradiography of the dried gel. The bands of 18S or 28S rRNA in each lane were measured by ethidium bromide staining before drying the gels. During transfection experiments, $^{32}$P-labeled reporter RNAs (1-2x10$^6$ dpm) were transfected into Huh7 cells using standard transfection method and total RNAs were isolated. The radioactive full-length RNAs were detected by autoradiography.

**2.6. Transfection of RNA into cells**

Huh7 cells were transfected with in vitro transcribed RNAs using Lipofectamine 2000 reagent (Invitrogen) according to the manufacturer’s protocol. After 3, 5, 8, 24 and 48 hours post-transfection, the cells were harvested and resuspended with lysis buffer (100 mM potassium phosphate pH 6.8, 1 mM DTT, 0.5% Igepal). The samples were then subjected to two freeze-thaw cycles and supernatants were assayed for Luc activities. For fluorescence microscopy, the cells were grown on coverslips (Fisher Scientific) followed by RNA transfection. The cells were fixed with 4% formaldehyde 48 hours post-transfection, permeabilized and stained with anti-firefly luciferase monoclonal antibody (Bionovus). A FITC-labeled secondary conjugate was used to visualize the FLuc distribution in the transfected cells.
2.7. Isolation of 40S ribosomal subunit

HeLa S10 lysate was prepared from HeLa S3 cells grown in a spinner flask as described by Barton and colleagues (Barton et al 1996). The ribosomes were pelleted from S10 lysate by centrifugation in Ti70.1 rotor (Beckman, 45,000 rpm) for 3h at 4 °C and the pellet was resuspended in buffer A (20 mM Tris-HCl, pH 7.5, 2 mM DTT, 50 mM KCl, and 4 mM MgCl₂) at a concentration of 150 U/ml measured at A₂₆₀ as described by Pisarev et al. (Pisarev et al 2007). Puromycin (1 mM) and KCl (0.5 M) were added, stirred at ice-bath for 10 min followed by incubation for 10 min at 37°C. The mixture was then loaded onto a 10%-30% sucrose density gradient and centrifuged for 16 h at 4 °C in a Beckman’s SW28 rotor (22,000 rpm). The peak fractions containing 40S ribosomes (as determined by the presence of only 18S rRNA) were pooled and concentrated in Ultracell-100 K (Millipore). The final preparation was dialyzed in buffer C (20 mM Tris-HCl pH 7.5, 2 mM DTT, 100 mM KCl, 2 mM MgCl₂, 0.25 M sucrose), aliquoted and stored at -80 °C (Pisareva et al 2008).

2.8. Sucrose density gradient analysis

Capped or uncapped ³²P-labeled mRNAs were incubated in standard HeLa translation lysates that were treated with 1mM GMP-PNP for 5 min on ice-bath. The mixtures were then incubated for 15 min at 30°C, layered onto a 10%-30% sucrose gradient in buffer K (20 mM Tris-HCl pH 8.0, 100 mM potassium acetate, 5 mM
magnesium acetate, and 2 mM DTT), and centrifuged for 3 hours at 45,000 rpm and 4 °C in a SW-51 rotor. Fractions (250 ul) were collected from the bottom of gradient and analyzed by scintillation counter. Total RNAs from peak fractions were isolated using Qiagen RNeasy Mini column for analysis of the RNA contents in the fractions.

For 40S-IRES binary interaction assay, the 32P-labeled c-Src IRES or a nonspecific RNA probe derived from 5'PV(D286-605) as scrambled IRES was mixed with purified HeLa 40S subunit in buffer K containing 20 U RNasin in a final volume of 40 ul and incubated for 15 minutes at 30°C. The reaction mixtures were analyzed by sucrose density gradient method as described above. A sample of RNA probe without 40S was used to specify position of the free probes during centrifugation.

3. Results

3.1. Cellular stress induces c-Src overexpression as well as its activation

Our mouse xenograft experiments (Chapter 2, Fig. 2.3E) clearly demonstrated that sporadic DCAMKL-1 expressing cells were embedded in the GS5 tumor areas where c-Src oncoprotein was overexpressed. It has been established by other studies that stress is one of the culprits for inducing tumorogenesis (Alvarez et al 2006, Chang et al 2007, Diaz et al 2006, Horn et al 2003). Since HCV induces oxidative stress and NF-kB (Waris et al 2005), we investigated molecular mechanism of c-Src expression under cellular stress that impairs cap-dependent translation.
Thapsigargin (TG) causes eIF-2 phosphorylation resulting in global translation inhibition and ER stress response due to inhibition of Ca\(^{2+}\)-ATPase activities (Chao et al 1997, Wong et al 1993). A modest increase in the total c-Src protein level was observed in DMSO-TG-treated cell lysates as compared to the untreated (control) or DMSO alone-treated lysates (Fig. 4.1A). The c-Src mRNA level remained unchanged in these cells (Fig. 4.1B). Serum-deprivation of cells also causes suppression of cap-dependent translation due to competitive inhibition of eIF4E binding to the 5’ cap structure by poly(A)-specific ribonuclease (PARN) (Seal et al 2005), and phosphorylation of eIF2a (Wek et al 2006). When Huh7 cells were subjected to serum starvation, the total c-Src protein level was moderately enhanced within 72 hours (Fig. 4.1.C, minus) as compared to the control (plus) although the c-Src mRNA level was not affected (Fig. 4.1.D).

3.2. Characteristics of the computer-generated c-Src RNA structures

The 5’NCRs in the c-Src transcripts have been shown to be relatively longer than those in most cellular mRNAs (Bonham and Fujita 1993). Sequence analysis revealed multiple pyrimidine-rich motifs and two cryptic AUGs with short ORFs at positions 147 and 179 in the 350-nt long Type-1A 5’NCR (Fig. 4.2). Only the AUG located at position 351 is known to serve as the initiator codon in this mRNA. Our nucleotide blast search using BLASTN 2.2.20+ program (Schwartz et al 2000) revealed that the exon 1C and 11 amino acid N-terminus coding sequences are highly conserved in humans, chimpanzees and rhesus monkeys (94%-100% identity) whereas mouse sequences in this region are 76-80% identical to the reference human c-Src mRNA sequences (NM_005417.3 and
NM_198291). Using *M-Fold* program (Zuker 2003), we examined a number of predicted secondary structures representing three segments (nt 1-353, 1-383, 1-410) of the Type-1A c-Src mRNA. A representative structure (dG = -135) for nt 1-383, which was found to be similar to the structure obtained for nt 1-410 segment, is shown in (Fig. 4.3) This Y-shaped secondary structure appears to contain three domains designated as domain I, II and III. We further observed that a large portion of the domains I and II were conserved in the structures predicted for all three c-Src segments. In addition, a high degree of conservation in the apical loops contributed by AACAAGA (nt 360-366), GUGCCA (SL II, nt 289-294) and UAUUUC (SL III, nt 255-260) motifs was also noticed in the predicted structures. The structures in domain III, however, showed less conservation among various structures generated for the three c-Src segments. A 14 nt pyrimidine (Py)-rich motif (nt 330-344) is located 6 nt upstream of the initiator AUG, which conforms Py-tracts found in many viral IRESs. These characteristics and the Y-shaped architectural features are considered as important elements of many viral and cellular IRESs (Le and Maizel 1997). The predicted structure for the c-Src nt 1-353 that represents the entire 5’NCR and AUG codon lacked a significant portion of domain II structure.

3.3. A c-Src mRNA motif supports cap-independent translation of reporter RNAs

Firefly luciferase (FLuc)-based reporter mRNAs were engineered to test if the c-Src 5’NCR supports cap-independent translation (Fig. 4A). Because a 33-nt sequence
Figure 4.1. A, The cytoplasmic lysates from experiments described in section B (above) were subjected to Western blot for the total c-Src protein with monoclonal anti-Src antibody (clone 327, Santa Cruz, upper panel) or anti-actin antibodies (lower panel). B, Northern blot analysis of total RNA extracted from Huh 7 cells described in section B, probed with $^{32}$P-labeled oligonucleotide corresponding to nt 320-350 of the c-Src 5’NCR (upper panel). Lower panel shows 18S rRNA in the same samples. C, Total c-Src level in Huh7 cell lysates cultured for 72 hrs in serum-deprived (indicated as minus) or 10% serum containing (plus) media. Western blot was carried out with anti-Src antibody as described above. D, Northern blot for probing c-Src mRNA (as described above) in total RNA extracted from Huh 7 cells cultured in serum-starved (minus) and serum supplemented (plus) regular media. The 18 S rRNAs in each lane is shown in the lower panel.
Figure 4.2. A, Organization of the c-Src gene. Transcription from two promoters (indicated as P) and alternative splicing result in Type-1α (NM_005417) and Type-1A (NM_198291) mRNAs in liver cells. Both transcripts differ only in the 5’ distal region of the 5’NCR. The sequence of the exons 1B and 1C and open reading frame (ORF) are shared in both transcripts (35). AUGi, initiator AUG codon. B, Schematic of the Type-1A 5’NCR. Two cryptic AUGs at nt 147 and 179, the initiator AUG at nt 351, and a pyrimidine-tract (nt 330-344) located 6 nt upstream of the initiator AUG are shown. The gray lines with arrows on both sides show sequence locations in different putative domains whereas similar arrows with black solid lines indicate position of the conserved stem-loop structures as shown in Fig. 4.2
motif downstream of the initiator AUG in the c-Src mRNA forms conserved stem-loop structure at the translation initiation site (Fig. 4.3), we included the region with the 5’NCR for engineering a parent reporter 5’Src-FLuc RNA. The RNA contains c-Src nt 1-383 (full-length 5’NCR plus 33 nt of the coding region) that is fused in-frame with luciferase ORF and ends with poly(A) tail (Fig. 4.4A). In vitro transcribed capped and uncapped RNAs were translated in rabbit reticulocyte cell-free lysate (Michel et al 2000, Soto Rifo et al 2007) in the presence of [35S]Methionine and the synthesized products were visualized by autoradiography. As expected, the capped 5’Src-FLuc RNA was translated to produce active luciferase protein (Fig. 4.4B, lane 3) than its capped counterpart (lane 3). During the assay, we used a reporter RNA [5’PV(D286-Interestingly, the uncapped 5’Src-FLuc RNA was also translated but with higher efficiency (lane 2) (from nt 286 through 605). Thus, the noncoding region (420 nt) in the mutant PV construct represents nt 1-285 followed by nt 606-746 of the PV 5’NCR. This PV region, although, is known to form stable stem-loop structures, provides stability to the RNA and binds a number of cellular factors (Murray et al 2001), failed to support translation of FLuc in RRL (lane 4). However, synthesis of FLuc was successfully achieved when the same RNA had 5’m7G cap structure (lane 5). These results clearly demonstrate that the c-Src nt 1-383 allows cap-independent translation of downstream ORF and its activity is enhanced when cap function is absent. The translatability of the mRNA constructs was further tested in HeLa cell-free translation lysates which has been widely used for investigating IRES-mediated translation initiation (Barton et al 1996). An uncapped reporter FLuc RNA that contains wild-type, full-length PV 5’NCR (5’PV-
Figure 4.3. Computer-assisted folding of the Type-1A c-Src mRNA sequence. The Zucker’s M-Fold program (version 3.2, 41) was used for prediction of the secondary structures representing various segments of the c-Src mRNA. Only a representative structure of the c-Src nt 1-383 that includes the entire 5’NCR followed by 33 nt coding sequence, is shown here. The initiator AUG at position 351 (arrow), and putative stem-loop (SL) structures, domains and nt positions are indicated.
FLuc, Fig. 4.4C, lane 4) and 5’Src-FLuc (lane 2) were efficiently translated while the mutant 5’PV(D286-605)-FLuc again failed to support synthesis of luciferase (lane 3). Next, we examined the c-Src 5’NCR-promoted translation in the context of a dicistronic mRNA (5’Src-RFLuc). The RNA is similar to the monocistronic 5’Src-FLuc RNA except that it contains renilla luciferase (RLuc) ORF and stop codon upstream of the c-Src sequence (Fig. 4.5A). The in vitro transcribed capped RNA was transfected into hepatoma Huh7 cells for 3 hr and the lysates were subjected to dual luciferase assay. As shown in Fig. 4.5B, both ORFs were translated in these cells. The capped dicistronic RL-HCV1b and RL-Vector RNAs were used as positive and negative controls respectively during the transfection. The RL-HCV-1b is similar to the 5’Src-RFLuc (Fig. 4.5A) except that the translation of downstream FLuc ORF is controlled by the HCV IRES instead of the c-Src IRES. In RL-Vector, the c-Src IRES between upstream RLuc and downstream FLuc ORF is deleted. Both of the RNAs produced results as expected (Appendix 5). In a parallel experiment, total RNAs isolated from the dicistronic 5’Src-RFLuc RNA-transfected cells were subjected to Northern blot analysis using a 32P-labeled oligonucleotide probe that detects 3’ end of FLuc ORF. The result showed that the dicistronic RNA was intact in the transfected cells (Fig. 4.5C, lane 3). The migration of isolated RNA was similar to that of in vitro transcribed dicistronic RNA (lane 2), and was not cleaved into monocistronic form (lane 1). The 5’Src-FLuc RNA encodes a chimeric firefly luciferase with aa 1 to 11 (MGSNKSKPKDA) of the c-Src protein at its N-terminus (Appendix 4A). This c-Src motif has been shown to play important role in
**Figure 4.4.** c-Src 5’NCR-mediated translation in cell-free lysates.  

**A.** Organization of in vitro transcribed uncapped reporter RNAs. The 33 nt coding sequence (dotted box, C), and Kozak sequence are shown at translation initiation site. The solid line represents 5’NCR. An, poly(A) tail.  

**B.** Five ug of uncapped (lanes 2, 4) and capped (lanes 3, 5) of 5’Src-FLuc (lanes 2, 3) or 5’PV(D286-605)-FLuc (lanes 4, 5) RNAs were translated in RRL for 1.5 hr in the presence of [35S]Methionine. The FLuc protein bands were visualized by autoradiography after SDS-PAGE. Two ul of the translation lysates were assayed for enzymatic activity (shown as RLU) of FLuc using D-luciferine substrate. Lane 1, translation without exogenous RNA (control).  

**C.** Translation of uncapped 5’Src-FLuc (lane 2), 5’PV(D286-605)-FLuc (lane 3) and 5’PV-FLuc (lane 4) RNAs in HeLa cell-free lysate as described above. The FLuc activity and the protein bands are shown.
membrane localization and translocation of the protein into the nucleus (David-Pfeuty et al 1993, Resh 1999). Transfection of an uncapped 5′Src-FLuc RNA into Huh7 cells resulted in the synthesis of luciferase protein that was primarily localized in the nucleus and perinuclear membranes (Appendix 4). This observation was in sharp contrast to the diffused cytoplasmic localization of luciferase that was encoded by 5′HCV-FLuc RNA in which translation of FLuc occurs under the control of HCV IRES. The resulting FLuc lacks c-Src aa 1 to 11 motif. These results suggest that the luciferase synthesized from 5′Src-FLuc RNA contains the c-Src protein motif, which is possible when translation is initiated at the authentic AUG351 (also see Fig. 4.6 for translation of 5′SrcD1-FLuc).

3.4. Identification of an IRES element in the c-Src mRNA

We introduced several deletion mutations in c-Src motif of the 5′Src-FLuc RNA to determine its putative IRES function. The mutant 5′SrcDC-FLuc is similar to the wild type 5′Src-FLuc RNA except that it lacks the c-Src coding sequence (nt 354-383, Fig. 4.6A). The mutant 5′Src-D1-FLuc contains a 19 nt deletion (nt 344-362). This deletion resulted into the loss of Kozak sequence and a major portion of SLI structure at the translation initiation site (Figs. 4.3 and 4.6A). As shown in Fig. 4.6B, both of the deletions caused dramatic reduction in the synthesis of FLuc (lanes 4, 7) as compared to the wild type RNA (lane 3). Similarly, the 5′SrcD2-FLuc mutant RNA that contains a large deletion (nt 95-348) upstream of initiator AUG also failed to support efficient synthesis of FLuc (lane 5). Unlike these mutants, a 5′SrcD3-FLuc RNA that maintains nt 1-47 and 216-383 of the c-Src mRNA showed cap-independent translation of FLuc.
Figure 4.5. c-Src 5’NCR-mediated translation in Huh7 cells. A, Schematic of an in vitro transcribed dicistronic reporter mRNA (5’Src-RFLuc). B, The Huh7 cultured cells (80% confluency, 50 mm dish) were transfected with 10 μg capped 5’Src-RFluc for 3 hr, and renilla and firefly luciferase activities were assayed in the cytoplasmic fractions. Each transfection was carried out in triplicate, and the experiment was repeated three times to confirm the results. The cytoplasmic fractions of untransfected cells were used as negative control. C, Northern blot analysis of total RNA isolated from 5’Src-RFLuc-transfected (lane 3) and untransfected (lane 4) Huh7 cells. Lanes 1 and 2 show position of monocistronic (5’Src-FLuc) and dicistronic (5’Src-RFluc) RNAs respectively as RNA markers. The $^{32}$P-labeled oligonucleotide probe was derived from 3’ end of the FLuc ORF. Overexposure of the film during autoradiography (for more than a week) did not show any fragment of the dicistronic mRNA in lane 3.
(lane 6) and was comparable to that of wild type 5′Src-FLuc RNA. The predicted structure of this mutant c-Src motif (not shown) by M-Fold program showed significant similarities in the domains I and II of the wild type structure (Fig. 4.3).

To determine stability of the reporter constructs, we translated 32P-labeled uncapped mutant and wild type RNAs in HeLa cell-free lysates as described above. Total RNAs from each reaction were isolated by RNeasy column method and the input probes were visualized by autoradiography. As shown in Fig. 4.6C (upper panel), the amounts of full-length mutant RNAs recovered (lanes 2-5) were similar or better than that of the wild type 5′Src-FLuc (lane 1). The quantity of 18S rRNA (internal control) in each lane had minor variations (lower panel). This observation suggests that the mutant RNAs were although present in the lysates, yet these RNAs were unable to support translation of FLuc due to absence of essential elements in the c-Src sequence motif. The different band intensities observed for the RNA probes may likely be due to minor differences in stability and/or loss during purification process. A similar observation was also made during transfection of three mutant RNAs (D2, D3 and DC) into Huh7 cells. Although full-length mutant RNA probes were purified from the transfected cells (Fig. 4.6F), only D3 mutant showed efficient synthesis of reporter FLuc (Fig. 4.6E). These results further suggest that a functional IRES that is represented by the c-Src motif in D3 mutant (Domains I and II, Fig.4.3) was capable of directing translation by cap-independent mechanism in cells as well as in the cell-free lysates. Unlike known cellular IRESs, this IRES requires coding region for its optimal function.
We carried out kinetic analysis of translation promoted by the wild type and mutant c-Src motifs in HeLa translation lysates. The time-course experiment presented in Fig. 4.6 D shows that translation of the 5’Src-FLuc RNA exponentially increased with time whereas the mutant 5’SrcD2-FLuc was translated inefficiently at all time-points. During our investigations (Fig. 4.6D and 4.6E), a minor translation was consistently observed for D2 mutant RNA. It is possible that sequence motifs that form domain I and translation site in this RNA might have played a role in the residual translation. These motifs are, however, absent in D1 and DC mutants that are completely incompetent for translation initiation.

3.5. Assembly of 80S initiation complex on the c-Src IRES in HeLa cell-free translation lysates is not affected by inhibition of eIF2

Sucrose density gradient analysis was carried out to study assembly of 80S translation initiation complex on the c-Src IRES. The $^{32}$P-labeled uncapped 5’Src-FLuc RNA or capped FLuc was incubated for 15 min in HeLa cell-free translation lysates containing 1 mM GMP-PNP, a nonhydrolyzable GTP analogue, which causes accumulation of 48S complex and inhibition of 80S formation on a capped mRNA (Pestova et al 1998, Pestova et al 2008). The reaction mixtures were analyzed for ribosome assembly on the mRNAs by 10%-30% sucrose density gradient centrifugation method. The initiation complexes in the gradient fractions were determined by incorporation of the input RNA probe into the ribosomal complexes (Fig. 4.7A). A
single peak (Peak I, solid line with squares) of ribosomal complex containing the input RNA probe, 18S and 28S rRNA was obtained for the 5’Src-FLuc RNA (Fig. 4.7B, lane 4). The result clearly established assembly of 80S complex on the c-Src 5’NCR motif, which was not inhibited by 1 mM GMP-PNP (Fig. 4.7A and 4.7B). In a similar translation reaction, we further reduced GTP and ATP concentrations by omitting the 10X reaction buffer from the translation mixture. This omission caused 10-times increase in the GMP-PNP to GTP ratio during translation. Interestingly, 80S assembly on the 5’Src-FLuc mRNA probe occurred (Peak I, broken line with squares; Fig. 4.7B, lane 5) similar to the standard reaction conditions described above. On the contrary, when a capped FLuc mRNA probe that lacks the c-Src motif was used in a standard translation reaction supplemented with 1 mM GMP-PNP, only 48S complex was obtained as expected (Peak II, solid line with triangles). This conclusion was based on the observation that peak II lacks 28S RNA, showed lower sedimentation than peak I, and contains only input RNA probe and 18S rRNA (Fig. 4.7B, lane 7). In addition, the 48S complex assembly was considerably reduced when cap structure was absent in the FLuc RNA probe (Peak II, broken line with triangles, Fig 4.7A and Fig. 4.7B, lane 6). The GMP-PNP is known to inhibit GTPase function that is required for the assembly of 80S complex on a capped mRNA. Thus, the observed cap-independent 80S assembly on the c-Src IRES is most likely to be independent of eIF2 function.
Figure 4.6. Effect of deletion mutations on the c-Src sequence-controlled translation of reporter RNAs.  

A, The organization of in vitro transcribed uncapped wild type reporter RNA (5’Src-RFluc) and the mutants containing various lengths of deletions (D) in the c-Src sequences are shown; dashed line, extent of deletion. AUG, initiator codon is underlined.  

B, The RNAs were translated for 1.5 hr in HeLa lysates in the presence of [35S]Methionine and the FLuc protein bands were visualized by autoradiography after SDS-PAGE.
Figure 4.6 continued. C, Stability of the reporter RNAs in HeLa translation lysates. The $^{32}\text{P}$-labeled reporter RNAs (~$1\times10^6$ dpm) were added to a standard HeLa translation mixture for 1.5 hr and total RNAs were isolated by Qiagen RNeasy column method. Half of the eluted RNA samples (20 ul) were subjected to formaldehyde-agarose gel electrophoresis. The gel was photographed after ethidium bromide staining for detection of ribosomal RNAs (lower panel), dried and autoradiographed (upper panel). Reporter RNAs are as indicated for each lane. D, Comparison of the kinetics of translation between wild type 5’Src-FLuc and a deletion mutant (5’SrcD2-FLuc). The RNAs were translated in a standard HeLa lysates mixture and FLuc activities were assayed with an aliquot (2 ul) of the reaction at various time-points. Control, translation lysates without exogenous RNA.
Figure 4.6 continued. **E.** Transfection of uncapped monocistronic c-Src mutant RNAs. The Huh7 cells (80% confluent in 60 mm culture plates) were transfected in triplicates with uncapped RNAs as indicated and FLuc activities were assayed 3 hrs post transfection in the total lysates. **F.** Relative stability of mutant reporter RNAs in the transfected cells. The $^{32}$P-labeled mutant RNAs (as indicated) were transfected as above. The total RNAs were isolated and the labeled RNAs were detected by agarose gel electrophoresis followed by autoradiography of the dried gel (upper panel). Lower panel, the same gel showing 18S rRNA in each lane.
In a similar experiment, the assembly of ribosomal complex on a mutant poliovirus 5’NCR containing FLuc (PVD286-605-FLuc) was compared with the 5’Src-FLuc RNA in the presence of 1 mM GMP-PNP (Figs. 4.7C and 4.7D). The uncapped 5’Src-FLuc RNA showed assembly of 80S in a reproducible manner (Peak I, squares with solid line; Fig. 4.7D, lane 3). However, the uncapped PVD286-605-FLuc RNA showed a peak at lower sucrose density (Fig. 4.7C, Peak II, triangles with broken line; Fig 4.7D, lane 4) and the complexes were spread over a wide range of sucrose density, most likely due to varying composition of ribonucleoprotein complexes formed with the mutant PV RNA.

The assembly of 80S on the c-Src IRES was further strengthened by our finding that purified HeLa 40S ribosomal subunit directly interacts with the c-Src IRES (Fig. 4.8A). The purified 40S subunit was mixed with uncapped 32P-labeled IRES fragment of the 5’Src-FLuc and bound complex was separated from the free probe by sucrose density gradient centrifugation. Characterization of the peak fractions revealed the presence of 18S rRNA and the probe in the same peak (inset, lane 1) while the free IRES probe showed a peak at lower sucrose density. A non-specific RNA probe of similar length and nucleotide contents (PVD286-605 5’NCR) failed to form 40S-RNA binary complex in this assay (Fig. 4.8B and inset lane 2). These results together provide evidence that the c-Src mRNA contains an IRES element that directly interacts with the 40S ribosomal subunit and is capable of assembling 80S complex during conditions when eIF-2 function is significantly compromised.
3.5. **c-Src IRES-mediated translation is enhanced when cap-dependent translation is inhibited.**

The eIF4E protein is a key translation initiation factor that binds the 5’ cap structure of an mRNA and initiates assembly of 48S pre-initiation complex (Sonenberg and Hinnebusch 2007).

It has been shown that m\(^7\)GDP inhibits eIF4E function by occupying its cap-binding site. Therefore, cap-dependent translation is efficiently inhibited by the m\(^7\)GDP cap analogue (Kentsis et al 2005). The cap-dependent translation of a FLuc RNA which contains 5’cap and 3’polyA tail at the respective ends of the luciferase ORF but lacks an IRES (5’Cap-FLuc), was inhibited by m\(^7\)GDP in a dose-dependent manner in RRL (Fig. 4.9A). In contrast, the HCV IRES-controlled translation of a reporter FLuc ORF (5’HCV-FLuc) was stimulated until a threshold concentration (10 ug) of m\(^7\)GDP was reached. Above this concentration, both cap- as well as HCV IRES-dependent translations were inhibited. Interestingly, translation of the 5’SrcD3-FLuc RNA (genetic organization shown in Fig. 4.6A) was considerably enhanced in the presence of m\(^7\)GDP as observed for the HCV IRES-mediated translation initiation.
Figure 4.7 Assembly of translation initiation complexes on the c-Src IRES in HeLa cell-free lysates and analysis by sucrose density gradient centrifugation. A, The translation mixtures were incubated with 1 mM GMP-PNP on ice-bath for 5 min and in vitro transcribed $^{32}$P-labeled RNAs: 5’Src-FLuc (squares in solid line) or capped FLuc (lacking IRES, triangles in solid line) or uncapped FLuc (triangles in broken line) were added to the translation reaction and incubated for 15 min at 30°C. The complexes formed in the absence of exogenous ATP and GTP on the 5’Src-Fluc probe is shown as squares in dashed line. The lysates were separated by 10%-30% sucrose density gradient centrifugation and fractions (250 ul) were collected from the bottom of the tube to determine RNA contents. B, Total RNAs from the peak fractions (Peak I or Peak II) were isolated and analyzed by agarose gel electrophoresis. Marker lanes: 1, input 5’Src-FLuc RNA probe; 2, total RNA extracted from HeLa S10 translation lysate showing 18S and 28S rRNAs; 3, 18S rRNA extracted from purified 40S subunit. The total RNAs extracted from Peak I (fractions 5 and 6) for 5’Src-Fluc probe are shown in lanes: 4, standard translation reaction shown as squares in solid line (section A), and 5, translation reaction deficient in exogenous ATP/GTP (squares in broken line shown in A). RNAs isolated from Peak II are shown in lanes 6 (triangle, broken line) and 7 (triangle, solid line).
Figure 4.7 continued: C, Comparison of ribosomal complex formed at wild type c-Src IRES and a mutant PV 5’NCR used as scrambled IRES in the presence of 1 mM GMP-PNP. The $^{32}$P-labeled uncapped 5’Src-Fluc (squares in solid line) or 5’PV(D286-605)-Fluc (scrambled IRES, triangles in dashed line) RNAs were used in sucrose gradient centrifugation analysis as described above. D, The total RNA isolated and resolved by agarose gel electrophoresis is shown. Lanes 1 and 2, rRNA (as markers) isolated from lysates and purified 40S subunit respectively; lanes 3 and 4, RNA isolated from Peak I and Peak II respectively.
Figure 4.8. Direct binding of the c-Src IRES with purified HeLa 40S ribosomal subunit. A, Purified HeLa 40S subunit was mixed with \(^{32}\)P-labeled RNA representing c-Src nt 1-383 (solid line) and subjected to sucrose density gradient centrifugation. A separate sample without 40S was run to locate position of the free probe (broken line) during sedimentation. Peak fractions were analyzed for RNA contents (inset). Inset, lanes: M, 18S rRNA as marker; 1, RNA extracted from 40S plus probe peak; 2, RNA from free probe peak.
Figure 4.8. continued. B. An experiment similar to that described in section A but repeated with a scrambled IRES [5'PV(D286-605)] as indicated. Inset: lane M, 18S rRNA; RNA isolated from c-Src IRES probe (lane 1) and scrambled IRES probe (lane 2).
Next, we examined translation of a capped dicistronic RNA (5'Src-RFLuc, Fig. 4.5A) in RRL in the presence of increasing concentrations of m$^7$GDP. The wild type c-Src IRES-controlled translation of FLuc was initially enhanced in the presence of m$^7$GDP (5-10 ug) as observed for its monocistronic counterpart, whereas cap-dependent translation of the upstream renilla luciferase (RLuc) ORF continued to decline with increasing concentrations of m$^7$GDP (Fig. 4.9B). Although the requirement of inhibitor concentration to inhibit overall translation was a little higher than that of the monocistronic RNAs, the stimulation pattern of the c-Src IRES in the presence of m$^7$GDP was similar for both mono- and dicistronic templates. During several control experiments, we observed that m$^7$GTP cap analogue also causes stimulation of the c-Src IRES in HeLa and RRL cell-free translation systems whereas the unmethylated nucleotides (GTP or GDP) had no effects within the concentration range used in our studies. These results together with those described above (Figs. 4.4, 4.6 and 4.7) established the presence of a functional IRES in the c-Src mRNA that can be activated when cap-function is absent or significantly inhibited and/or eIF-2 activity is inadequate in the translation system.

We treated Huh7 cells with 1 uM TG for different time-points (0.5 to 6 hrs) and monitored the status of eIF2a by Western blot. We observed a considerable increase in the phosphorylation level of eIF2a within 30 min of TG-treatment, which remained elevated for 6 hr (Fig. 4.10A, lanes 2-6, upper panel). The total eIF2a level, on the other hand, was not affected by this treatment (Fig. 4.10A, lower panel). The enhanced eIF2a
phosphorylation may be considered as an indicator for TG-induced cellular stress and reduction in global cap-dependent translation in the treated Huh7 cells. In the subsequent experiments, Huh7 cells were pre-treated with 1 uM TG for 3 hr before transfection with the capped 5’Src-RFLuc or RL-HCV1b RNAs while maintaining 1 uM TG. The RL-HCV1b is similar to the 5’Src-RFLuc except that the translation of FLuc ORF in the RNA is driven by an HCV IRES. The FLuc and RLuc activities were assayed in the cytoplasmic fractions 3 hrs post-transfection. The translation of reporter luciferases in untreated transfected cells (control) were considered as 100% and compared to that of DMSO- or DMSO-TG- treated cells (Fig. 4.10B). Both the cap-dependent and IRES-dependent (HCV or c-Src) translation was not affected by DMSO treatment of the cells. In contrast, the cap-dependent translation of RLuc was dramatically reduced for both the RNAs due to DMSO-TG treatment of the cells. In these cells, however, the c-Src or HCV IRES-controlled translation of FLuc was moderately enhanced. These results together complement our findings that the c-Src level in Huh7 cells is modestly enhanced or maintained to steady level under varying cellular stress conditions that are unfavorable for cap-dependent translation. This effect is likely to occur due to increase in the c-Src IRES activities.

4. Discussion

An overwhelming majority of reports including polysome-profiling data strongly advocate for IRES-dependent translation initiation of a subset of cellular mRNAs during cell division, apoptosis, cellular stress, and viral infections where cap-dependent
translation initiation is compromised (Spriggs et al 2008). Unlike known cellular IRESs, the c-Src IRES demonstrated here exhibits many unique attributes that are analogous to the characteristics of HCV-like IRESs. To identify an IRES function in viral and cellular mRNAs, mono- and di- cistronic RNA-expressing plasmids have been extensively used during transfection studies. This approach, however, has been a subject of criticism due to expression via cryptic promoters, and faulty transcription and splicing of the reporter constructs (Kozak 2007). To avoid spurious results generated by this method, we have used only in vitro transcribed capped and uncapped reporter RNAs for cell-free translation assays, transfection studies, and sucrose density gradient analyses. The transcription reactions were digested with DNase I prior to purification and checked by agarose gel electrophoresis for the absence of DNA contamination in the final RNA preparations. Furthermore, the reporter RNA transcription is under the control of T7 promoter and transcription of the RNA from plasmid DNA contamination is not possible in any of the system used here.

We also demonstrated that wild type and mutant c-Src motif containing reporter RNAs were intact during various translation assays. These measures permitted us to present reliable data for the identification of c-Src IRES.

We established here that the c-Src IRES-controlled translation can be stimulated similar to the HCV IRES when eIF4E function is blocked (Fig. 4.9). The initiation factor eIF4E has been shown to be a negative modulator of the IRES-mediated translation, and
**Figure 4.9.** Stimulation of the c-Src IRES-controlled mRNA translation when eIF4E function is inhibited. A, Capped RNAs: 5’Src-FLuc (triangle), and FLuc (5’Cap-FLuc, circle) or uncapped 5’HCV-FLuc (square) RNAs were translated in triplicate in the presence of increasing amounts of m\textsuperscript{7}GDP in RRL for 1 hr, and one-tenth of each reaction mixture was assayed for FLuc activity. Average FLuc activity of three reactions is shown for each m7GDP concentration. The FLuc activity in samples without m\textsuperscript{7}GDP was considered as 100% translation and compared with those containing the cap analogue (inhibitor). Similar translation reactions were carried out twice to confirm the results. B, Translation of a capped dual luciferase RNA construct (5’Src-RFLuc) in RRL in the presence of increasing amounts of m\textsuperscript{7}GDP as described above. Relative cap-dependent translation of renilla luciferase (RLuc) and c-Src IRES-dependent FLuc synthesis are shown. Each translation mixture was carried out in triplicate. The results were confirmed by three independent experiments.
translation of IRES-containing RNAs is accelerated when eIF4E availability is reduced (Svitkin et al 2005). This is likely attributed to a decrease in eIF4F complex formation that may be accompanied by an increased availability of eIF4G/eIF4A or eIF4A RNA helicase or other initiation factors. Based on these observations, we believe that a direct binding of m7GDP to eIF-4E in our assay may lead to increased availability of translation factors that are required for efficient activities of the HCV or c-Src IRESs.

Our in vitro studies that defined presence of an IRES in the c-Src mRNA were further corroborated by the results of transfection of mono- and dicistronic reporter RNAs into hepatoma-derived cells and induction of cellular stress in the transfected cells. Uncapped reporter RNAs containing nt 1-383 of c-Src mRNA at their 5’ends were efficiently translated in two cell-free translation systems (RRL and HeLa lysates) and in Huh7 cells. Our genetic analysis shows that nt 200-383 of the c-Src mRNA, which harbors initiator AUG (at nt 351), plays pivotal role in promoting cap-independent translation. An extensive analysis of the secondary and/or possible higher-order structures within this region is, however, needed for accurate understanding of its role in loading productive initiation complex. We found that the c-Src IRES promotes assembly of stable 80S complexes in the absence of cap structure and in the presence of 1mM GMP-PNP. Under similar conditions, however, only 48S complex can be trapped on a capped reporter RNA lacking a 5’NCR or contains a scrambled IRES.
Figure 4.10. c-Src IRES-controlled translation is not inhibited during cellular stress. 

A. Phosphorylation of eIF2α by thapsigargin (TG) treatment. The cytoplasmic lysates (40 μg) from Huh7 cells that were treated with 1 μM TG for 0.5, 1, 2, 4 and 6 hrs (lanes 2-6) were subjected to Western blot analysis using anti-[Phospho-eIF2α(Ser51)] antibody (Cell Signaling, upper panel) and anti-eIF2α antibodies (Sigma, lower panel). Lane 1, 0 min treatment.

B. Huh7 cells in triplicate were treated with DMSO alone or 1 μM thapsigargin dissolved in DMSO (DMSO-TG) for 3 hrs followed by transfection with in vitro transcribed capped 5’Src-RFluc RNA (solid black bar) or RL-HCV1b (solid gray bar). The upstream RLuc in RL-HCV1b RNA is translated by cap-dependent mechanism whereas HCV IRES mediates downstream FLuc translation. The cytoplasmic lysates were assayed for FLuc (IRES-dependent translation) and RLuc (cap-dependent translation) activities 3 hours post-transfection. The activities of FLuc and RLuc in untreated (control) samples were considered as 100% and compared with the solvent alone (DMSO) or TG-treated cells.
Furthermore, similar to the HCV-like IRESs, a direct binding of purified HeLa 40S with the c-Src nt 1-383 was detected in the absence of initiation factors. These evidence together strongly support existence of a physiologically relevant IRES element at the 5’ end of c-Src mRNA. The c-Src IRES appears to be functionally similar to the HCV IRES as both IRES elements directly interact with the purified 40S subunit, require coding region for their functions, promote eIF2-independent assembly of 80S complex (Figs. 4.4, 4.6 and 4.7) (Pestova et al 2008, Reynolds et al 1995, Robert et al 2006), and are stimulated when eIF4E or eIF2a function is impaired (Figs. 4.9 and 4.10). Therefore, our studies reported here present several unique attributes of a cellular IRES that have been demonstrated only for HCV-like IRESs.

The sucrose gradient analyses further provided insights into the mechanism of ribosome assembly on the c-Src IRES. The nonhydrolyzable GTP analogue, GMP-PNP, blocks eIF2-dependent initiation pathway at the 48S complex stage (Pestova et al 1998, Pisarev et al 2007). This effect was clearly evident for the cap-dependent translation initiation of the FLuc mRNA in HeLa cell-free lysates in which the 48S complex was trapped by 1mM GMP-PNP treatment (Fig. 4.7). Thus, the GMP-PNP concentration used here during translation initiation assembly was sufficient to block 80S assembly by cap-dependent initiation mechanism in the translation mixture. In sharp contrast, assembly of 80S complex took place on the c-Src IRES in the presence of GMP-PNP or in a reaction mixture containing the analogue but was also deficient in exogenously added ATP and GTP. Generally, 60S subunit joins the 48S complex to form 80S only
after eIF5-induced GTP hydrolysis and dissociation of eIF2.GDP complex (Terenin et al 2008). This step is preceded by ATP-dependent scanning by the 48S complex to locate AUG codon (Sonenberg and Hinnebusch 2007). From the data presented here, c-Src IRES appears to evade both of the critical energy-dependent steps that are needed for the 80S assembly by cap-dependent mechanism. Because the c-Src IRES directly binds 40S subunit (Fig. 4.8) and the structural motifs from flanking regions of the initiator AUG are required for efficient function of the c-Src IRES (Fig. 4.6), it is highly likely that the 48S complex formed at this element may not require energy-dependent scanning for the initiator AUG. This notion is supported by the genetic analysis of the c-Src IRES. A 19 nt-deletion at translation site in 5’Src-D1-FLuc RNA resulted in complete impairment of the IRES function in spite of the presence of upstream AUG147 and AUG179. Recently, the HCV IRES was shown to switch from classical eIF2-dependent initiation to eIF2-independent pathway under cellular stress that favors inactivation of eIF2 due to phosphorylation of its a subunit. This alternative pathway was further shown to require only eIF3 and eIF5B (an analogue of bacterial IF2) for Met-tRNAiMet delivery at the P site. Based on these observations, it was proposed that the 80S assembly on the HCV IRES is analogous to bacterial-like mode of translation initiation (Terenin et al 2008). In this context, the c-Src IRES appears to follow HCV IRES-like mode of translation initiation when the GTPase function of the ternary complex is blocked. This conclusion is further supported by RNA transfection studies in which thapsigargin-led induction of cellular stress in Huh7 cells failed to inhibit the c-Src IRES in spite of increased Ser51 phosphorylation of eIF2a as compared to the normal (unstressed) cells (Fig. 4.10).
The studies presented here demonstrate significant resistance of the c-Src IRES activities to reduced level of ternary complex (TC) and eIF-2a phosphorylation. In contrast to the eIF2-dependent initiation pathway in which the eIF-2 complex delivers Met-tRNAi to 40S subunits in a GTP-dependent manner, the eIF2A has been shown to deliver the Met-tRNAi to 40S subunits by AUG-dependent and GTP-independent mechanism (Robert et al 2006, Zoll et al 2002). In addition, a number of RNA-binding proteins have been shown to stabilize IRES structure and/or promote ribosomal complex assembly (Ali and Siddiqui 1997, Wang et al 1995). A comprehensive analysis is needed to ascertain whether these factors contribute to the reduced TC-dependence of the c-Src IRES.

In the cells, stress and serum-deprivation causes inhibition of PI3K/AKT/mTOR pathway-dependent phosphorylation of eIF4E-BP. The unphosphorylated protein forms a tight complex with eIF4E and prevents its binding to eIF4G and the cap structure (Gingras et al 1999). Similarly, hypophosphorylation of eIF4E that is controlled by Ras-MAPK pathway also reduces its cap-binding ability. Both of these events culminate into suppression of global cap-dependent translation. In addition, phosphorylation of eIF2a by cellular kinases (e.g. PKR, PERK, HR1 and GCN2) in response to various cellular stress and viral infections leads to reduction in the level of ternary complex (eIF2-GTP-Met-tRNAiMet) due to inhibition of guanine nucleotide exchange factor (GEF) activity (Fernandez et al 2002). Our investigations revealed that 80S assembly on the c-Src IRES occurs when function of eIF2 and eIF4E are inhibited. Therefore, it is possible that
the c-Src mRNA can easily escape from tight regulation of both of these translation initiation factors, which may ultimately lead to continued c-Src protein synthesis during adverse conditions (e.g. ER stress and starvation). Enhanced c-Src level has been shown to correlate with its activated state in hepatocellular carcinoma (Ito et al 2001). Activated c-Src is known to induce phosphorylation of 4E-BP1 via PI3K/mTOR and eIF4E via Ras/Raf/ERK pathway, both of which favor cell survival and proliferation (Karni et al 2005, Vojtechova et al 2008). Thus, the c-Src IRES controlled translation provides an important recovery mechanism from translational blockade during cellular stress.

It has been shown that the cap-dependent translation of c-Src mRNA is regulated by elements located in its long 3’NCR through interaction with hnRNP K (Naarmann et al 2008). It would be interesting to investigate if hnRNP K or miRNAs can affect the c-Src IRES-controlled translation through the 3’NCR interactions. Both transcripts of the c-Src gene (Type-1A and Type-1a, Fig.4.1A) contain conserved sequences that constitute most part of the IRES element. However, the extreme 5’ ends in these mRNAs are dissimilar in length and nucleotide composition. It is not known if these sequences play any role in regulating the c-Src translation.

supply of c-Src in the tumor cells. Many of the small molecules that target c-Src activities also inhibit other protein kinases and/or show high degree of cytotoxicity (Ma and Adjei 2009). Adaptation for growth during cellular stress is a hallmark feature of many cancer cells and c-Src has been shown to play very important role during this process (Yamamoto et al 2006). Our report presents c-Src IRES as a new therapeutic target for treatment of cancer. Because the c-Src IRES is located downstream of the cap structure in the mRNA, interference with the IRES structure and/or function will likely result in the inhibition of cap-dependent as well as IRES-dependent c-Src synthesis. This strategy will prevent unabated c-Src supply in the cancer cells, and hence is likely to reduce the chances of cancer cell survival.

5. Chapter Summary

Overexpression and activation of c-Src protein have been linked to the development of a wide variety of cancers. The molecular mechanism(s) of c-Src overexpression in cancer cells is not clear. We report here an internal ribosome entry site (IRES) in the c-Src mRNA that is constituted by both 5' noncoding and coding regions. The inhibition of cap-dependent translation by m\textsuperscript{7}GDP in cell-free translation system or induction of ER stress in hepatoma-derived cells resulted in stimulation of the c-Src IRES activities. Sucrose density gradient analyses revealed formation of a stable binary complex between the c-Src IRES and purified HeLa 40S ribosomal subunit in the absence of initiation factors. We further demonstrate eIF2-independent assembly of 80S initiation complex on the c-Src IRES. These features of the c-Src IRES appear to be
reminiscent to that of hepatitis C virus-like IRESs and translation initiation in prokaryotes. Transfection studies and genetic analysis revealed that the c-Src IRES permitted initiation at the authentic AUG351 which is also used for conventional translation initiation of the c-Src mRNA. Our studies unveiled a novel regulatory mechanism of c-Src synthesis mediated by an IRES element, which exhibits enhanced activity during cellular stress, and is likely to cause c-Src overexpression during oncogenesis and metastasis.
Chapter Five: Summary

Hepatitis C virus (HCV) is a positive strand RNA virus and has been classified as a hepacivirus of the family Flaviviridae. More than 180 million people (>4 million in USA alone) have already been infected and HCV-related liver diseases are on rising trend globally due to addition of 3-4 million new cases per year. The HCV infection causes chronic hepatitis (70%-80%) that may lead to the development of cirrhosis, steatosis and hepatocellular carcinoma (HCC). In spite of aggressive efforts during last 15 years by scientists and pharmaceutical companies, cure for the infection as well as vaccine against the virus remained elusive. The current standard-of-care treatments (e.g. interferon with ribavirin for 24-48 weeks) alone or in combination with the HCV-specific drugs (e.g. telaprevir and boceprevir) have shown partial success. The HCV-related HCC are believed to contain a subpopulation of poorly differentiated cancer stem-like cells (CSCs), which exhibit a strong potential to initiate tumor formation and resistance to anti-neoplastic drugs. Keeping in view of the above described problems, my thesis work was focused on three major specific aims: 1) To investigate if HCV-expressing cells undergo phenotypic changes and exhibit enhanced expression of oncogenes (e.g. c-Src) and putative cancer stem cell markers, 2) To investigate possible mechanism of the HCV-induced hepatocarcinogenesis, 3) To develop novel means for targeting marker (s) that control abundance of the HCV genome.
In these studies, we demonstrate that expression of HCV subgenomic replicon in cultured cells results in acquisition of CSCs traits. These traits include enhanced expression of DCAMKL-1, Lgr5, CD133 and c-Myc in conjunction with long-term maintenance of pluripotency factors. We also show that HCV replication is severely impaired by siRNA-led depletion of the microtubule-associated putative stem cell marker, DCAMKL-1. The DCAMKL-1-positive cells isolated by fluorescence activated cell-sorting (FACS) form spheroids in matrigel. We further demonstrate that HCV replicon-expressing cells initiate tumors in nude mice. The resulting tumors exhibit characteristics of HCCs that are originated from hepatic progenitor-like cells and are phenotypically distinct from the tumors initiated by parent cells lacking the replicon. This phenotype expresses high-levels of α-fetoprotein, cytokeratin-19 and pluripotency markers. HCV-induced activation of β-catenin, c-Myc, and c-Src indicates the aggressive nature of the tumor. These results collectively suggest that HCV exhibits ability to induce reprogramming and/or retrodifferentiation of the host cells.

DCAMKL1 is a putative stem cell/CSC protein that affects ‘dynamic instability’ of microtubule filaments (MTFs), a process required for movement of HCV replication complexes (RCs), cellular transport and survival. Our findings that this protein is overexpressed in HCV replicon model and DCAMKL-1 antagonist leads to specific reduction in the HCV RNA and protein levels are novel. Subsequent studies showed that the cholesterol-lowering drug, fluvastatin (FLV, inhibitor of HMG CoA reductase), induce MTF bundling specifically in the HCV-expressing cells and hepatoma cells but not in the
normal human hepatocytes. However, pravastatin failed to show such effects under similar conditions. The fluvastatin effect on MTFs was accompanied by changes in localization of the RCs and DCAMKL1 with the MTFs. DCAMKL1 RNA level was also reduced to a considerable extent by the FLV treatment.

Because overexpression and activation of c-Src protein have been linked to the development of a wide variety of cancers including HCC, we investigated molecular mechanism(s) of c-Src overexpression in hepatoma cell lines. The studies revealed a novel type of internal ribosome entry site (IRES) in the c-Src mRNA that is constituted by both 5’ noncoding and coding regions. The inhibition of cap-dependent translation by m7GDP in cell-free translation system or induction of ER stress in hepatoma-derived cells resulted in stimulation of the c-Src IRES activities. Sucrose density gradient analyses revealed formation of a stable binary complex between the c-Src IRES and purified HeLa 40S ribosomal subunit in the absence of initiation factors. We further demonstrate eIF2-independent assembly of 80S initiation complex on the c-Src IRES. These features of the c-Src IRES appear to be reminiscent to that of hepatitis C virus-like IRESs and translation initiation in prokaryotes. Transfection studies and genetic analysis revealed that the c-Src IRES permitted initiation at the authentic AUG351 which is also used for conventional translation initiation of the c-Src mRNA. Thus, these results unveiled a novel regulatory mechanism of c-Src synthesis mediated by an IRES element, which exhibits enhanced activity during cellular stress, and is likely to cause c-Src overexpression during HCV-induces liver tumorigenesis.
In conclusion, the studies presented here revealed a novel HCV-(DCAMKL-1)-MTF-CSCs axis that might be responsible for the HCV RNA abundance in the infected cells and HCV-induced hepatocarcinogenesis. The putative stem cell marker, DCAMKL-1, represents a novel cellular target for combating HCV and liver cancer. We showed that its role in the HCV replication could be controlled by a combination of anti-DCAMKL-1 agent with FDA-approved fluvastatin and/or HCV-specific drugs. The concept of a ‘virus-induced stem cell traits’ can also be extrapolated to study diseases caused by other RNA viruses.
References


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Indirect immunofluorescence of stem cell and cancer related markers as indicated
Appendix 2

Expression and co-expression (indicated with arrows) pattern of cytokeratin 19 (purple) and a-fetoprotein (brown) in GS5-derived (A) or Huh7.5-derived (B) tumor xenografts.
Appendix 3

DCAMKL1 staining results of the human liver tissue array. The arrays were processed for immunohistochemical staining. (A) Examples of DCAMKL1 intensities in different liver diseases. (B) Evaluation of DCAMKII intensities in different cell populations using the array on an arbitrary scale of 0-3.
Translation of capped dicistronic RNA controls in Huh7 cells during transfection assay. The Huh7 cultured cells (80% confluency, 50 mm dish) were transfected with 10 ug capped RL-HCV1b or a dual luciferase vector lacking an IRES between upstream RLuc and downstream FLuc ORFs. Renilla and firefly luciferase activities were assayed in the cytoplasmic fractions as described for Fig. 4.4. The cap-dependent Rluc and HCV IRES-dependent FLuc for RL-HCV1b are shown.
Immunofluorescence detection of FLuc expressed by c-Src IRES-controlled translation of 5'Src-FLuc RNA in Huh7 cells.  

A. The amino acid sequence encoded by the coding region in the c-Src IRES is shown. HCV IRES containing FLuc (5'HCV-FLuc) lacks this sequence.  

B. Huh7 cells were transfected with uncapped 5'Src-Fluc or 5'HCV-FLuc RNAs. Indirect immunofluorescence staining for FLuc (green) was carried out with monoclonal FLuc antibody 48 hr post-transfection. Dapi, nuclear staining with bis-benzimide.