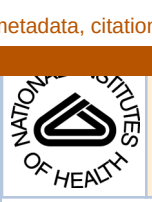

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Virus-specific Mechanisms of Carcinogenesis in Hepatitis C Virus Associated Liver Cancer

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Abstract

The development of hepatocellular carcinoma (HCC) in persons who are persistently infected with hepatitis C virus (HCV) is a growing problem worldwide. Current antiviral therapies are not effective in many patients with chronic hepatitis C, and a greater understanding of the factors leading to progression to HCC will be necessary to design novel approaches to prevention of HCV-associated HCC. The lack of a small animal model of chronic HCV infection has hampered understanding of these factors. Since HCV is an RNA virus with little potential for integration of its genetic material into the host genome, the mechanisms underlying HCV promotion of cancer are likely to differ from other models of viral carcinogenesis. In patients persistently infected with HCV, chronic inflammation resulting from immune responses against infected hepatocytes is associated with progressive fibrosis and cirrhosis. Cirrhosis is an important risk factor for HCC independent of HCV infection, and a majority of HCV-associated HCC arises in the setting of cirrhosis. However, a significant minority arises in the absence of cirrhosis, indicating that cirrhosis is not a prerequisite for cancer. Other lines of evidence suggest that direct, virus-specific mechanisms may be involved. Transgenic mice expressing HCV proteins develop cancer in the absence of inflammation or immune recognition of the transgene. In vitro studies have revealed multiple interactions of HCV-encoded proteins with cell cycle regulators and tumor suppressor proteins, raising the possibility that HCV can disrupt control of cellular proliferation, or impair the cell's response to DNA damage. A combination of virus-specific, host genetic, environmental, and immune-related factors are likely to determine the progression to HCC in patients who are chronically infected with HCV. Here, we summarize current knowledge of the virus-specific mechanisms that may contribute to HCV-associated HCC.

Keywords

hepatitis C virus; hepatocellular carcinoma; cirrhosis; tumor suppressor

Introduction

Liver cancer is the 3rd leading cause of cancer deaths worldwide. While incidence rates are stable or declining for many types of cancers, they have increased substantially for hepatocellular carcinoma (HCC) in recent years in both Japan and the U.S. In Japan, the age-adjusted annual death rate due to primary liver cancer rose from ~10/100,000 persons in 1975 to a peak of 27.5 in 2002, attributable in large part to increases in the prevalence of

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hepatitis C virus (HCV) infection that occurred decades earlier, in the aftermath of World War II (Tanaka et al 2002, Umemura et al 2009, Yoshizawa 2002). HCV infection now accounts for approximately 70% of cases of liver cancer in Japan. The incidence of liver cancer has also undergone a remarkable increase in the U.S., where extensive spread of HCV occurred approximately 30 years later (Tanaka et al 2002). Between 2001-2006, the incidence of microscopically-confirmed HCC increased at an average annual percentage rate of 9.1% among persons aged 50-59 years (Centers for Disease Control and Prevention, 2010). Increased rates of HCC are particularly striking among African American and Hispanic males, reflecting racial disparities in the prevalence of chronic HCV infection, now the leading cause of HCC within the U.S. (Altekruse et al 2009, Armstrong et al 2006). While there are good statistics for the U.S. and Japan, it is difficult to estimate the global burden of HCV-associated liver cancer. In one analysis, HCV infection accounted for 155,000 liver cancer deaths in 2002 (Perz and Alter 2006), but such a figure is likely to significantly underestimate the numbers of HCV-associated HCC deaths today.

Despite advances in therapy for HCC, 1-year survival rates remain less than 50% in the U.S. (Altekruse et al 2009). Even in Japan, where early diagnosis and potentially curative interventions are common, recurrence is frequent and the long-term prognosis poor (Masuzaki et al 2008). Antiviral therapies may prevent progression to HCC in HCV-infected persons, but current interferon-based standard-of-care therapies eliminate the virus in only half of those treated (McHutchison and Fried 2003). Moreover, the preventive benefits of interferon therapy are limited in the absence of virus eradication (Lok et al 2009). Newer, direct-acting antiviral agents are on the horizon and will significantly improve treatment outcomes, but rapid selection of resistant viruses will mandate continued reliance on interferon as the foundation of therapy, at least for the near term future (Shimakami et al 2009). Importantly, only a small proportion of infected persons are likely to have access to these therapies in most countries. The population of persons within the U.S. who have been infected with HCV for more than 2 decades and who are at highest risk of HCC is thus likely to continue to grow over the next 20 years, fueling continued increases in HCC incidence (Davis et al 2010). Worldwide, 130-170 million people worldwide are chronically infected with HCV (Lavanchy 2009).

Direct vs. indirect carcinogenic mechanisms

Most persons who are infected with HCV fail to clear the virus, and become persistently infected and thus at long-term risk for progressive hepatic fibrosis, cirrhosis, or HCC (reviewed in Lemon et al 2007). HCV is a positive-strand RNA virus that replicates in the cytoplasm and has little potential for integration of its genome into cellular DNA. The presence of cirrhosis and chronic hepatic inflammation with associated oxidative stress and accompanying potential for cellular DNA damage are unquestionably important contributing causes to HCV-associated HCC (Bartsch and Nair 2004, Nakamoto et al 1998, Okuda et al 2002). However, there are several lines of evidence supporting a direct role for HCV in cancer promotion. First, although direct comparisons are limited, the incidence of HCC appears to be greater in HCV-associated cirrhosis (7-13% over 5 years) (Degos et al 2000, Lok et al 2009) than in cirrhosis resulting from autoimmune hepatitis (Teufel et al 2009, Yeoman et al 2008). Moreover, there is an increasing recognition that HCV-associated HCC may arise in the absence of cirrhosis, albeit at a much lower rate than in patients with cirrhosis (Bralet et al 2000, Lok et al 2009, Yeh et al 2010). The occurrence of HCC in some lineages of transgenic mice also provides compelling evidence that HCV protein expression may be directly oncogenic (Lerat et al 2002, Moriya et al 1998).

An important question is whether cancer arises in an infected hepatocyte, or in uninfected bystander hepatocytes that are present in far greater numbers in the HCV-infected liver.

Estimates based on observed genome copy numbers in the livers of HCV-infected chimpanzees suggest that less than 30 percent of hepatocytes contain replicating virus (Bigger et al 2004). This is consistent with the 2-20% of cells in which we observed HCV antigen expression by two-photon microscopy of frozen sections of nonmalignant liver tissue (Liang et al 2009). The proportion of hepatocytes expressing the Ki67 proliferation marker is increased in advanced hepatitis C (Farinati et al 1996, Koskinas et al 2005), and a case-control study suggests that patients with a high proportion of Ki67-positive hepatocytes are at increased risk for HCC (Dutta et al 1998). However, it is not known whether the proliferating hepatocytes are infected with HCV, or alternatively, whether they are uninfected cells proliferating in response to the loss of infected cells due to virus-induced apoptosis or immunologically-mediated cell death. Such questions are difficult to answer due to limitations in experimental systems and the technical difficulties inherent in detecting the low abundance of HCV antigen expressed by infected cells in human liver (Liang et al 2009).

A related question is whether the malignant cells in established HCC are productively infected with HCV. The presence of viral RNA in HCC tissue is not a reliable marker of infection in cancer cells, because potentially high numbers of virions circulate in the blood and thus may be expected to be present in any vascular tissue. Importantly, most established human hepatoma cell lines do not support the replication of HCV in cell culture. Such a lack of permissiveness for HCV replication does not exclude a direct role for the virus in the events leading to cancer, however, as the capacity of transformed hepatocytes to support viral replication could be lost due to reduced expression of cellular co-factors required for replication of HCV. As explained below, microRNA 122 (miR-122) is one such co-factor, and it is often expressed at low levels in HCC. These observations do, however, indicate that the malignant phenotype is unlikely to be driven directly by continued viral protein expression, as in the case of papillomaviruses. Rather, they point to a cancer-promoting effect of HCV infection.

While progression to HCC is variable among patients with chronic hepatitis C, suggesting that cancer arises due to a complex interplay between host, viral and environmental factors, a wealth of studies suggest that epigenetic changes in HCV-infected hepatocytes may underlie the development of HCC. Here we summarize the evidence that HCV infection leads to critical stresses on hepatocellular homeostasis and altered cellular response pathways that promote the development of cancer above and beyond the nonspecific pro-carcinogenic effects of cirrhosis and general hepatic inflammation. Such a hypothesis suggests that cancer arises in infected cells and not in uninfected bystander hepatocytes, and is consistent with the exceptionally high rate of HCC observed in HCV-related cirrhosis.

The HCV lifecycle

The HCV genome consists of a single-stranded, positive-sense RNA, approximately 9.6 kb in length with untranslated RNA segments (UTRs) at both ends and a single large open reading frame encoding a 327 kD polyprotein that is processed into 10 mature viral proteins (Fig. 1) (for a review, see Lemon et al 2007). Most if not all of these viral proteins are multifunctional. Indeed, the membrane-bound replicase complexes in which viral RNA is synthesized contain less than 5% of the total complement of nonstructural proteins expressed in cells (Quinkert et al 2005). In addition to their basic functions in supporting viral genome amplification and the production of new virions, viral proteins interact with host proteins in ways that facilitate genome amplification, antagonize host immune responses, or otherwise alter the cellular environment to favor virus replication. Replication of the virus is also dependent upon at least one cellular microRNA, miR-122, as discussed in greater detail below. Many specific details are lacking, but viral RNA replication appears to

be an exclusively cytoplasmic process. This suggests that the mechanisms by which HCV infection leads to cancer are likely to differ substantially from other models of viral carcinogenesis. Nonetheless, HCV replication is associated with altered abundance or localization of some typically nuclear proteins, including the retinoblastoma protein, Rb, and DDX5 (p68) (Goh et al 2004, Munakata et al 2007).

Approaches to the study of HCV pathogenesis

Model systems

HCV is an hepatotropic virus and it replicates primarily if not exclusively within hepatocytes. Some studies have suggested that there may be low level replication in lymphocytes, and this has been well documented in cell culture (Shimizu et al 1998). However, it is controversial whether this occurs in infected persons. There appear to be multiple blocks to replication in peripheral blood mononuclear cells (Marukian et al 2008). Other than humans, the only animal species that is fully permissive for HCV infection is the chimpanzee, *Pan troglodytes*. HCV infection of the chimpanzee recapitulates most if not all features of hepatitis C in humans. Although relatively small numbers of these primates have been infected with HCV since the discovery of the virus, at least one chimpanzee has developed HCC in the setting of chronic infection (Lanford et al 2011). This animal had multiple tumors, but no cirrhosis and only minimal portal fibrosis. The chimpanzee represents the only animal species in which viral pathogenesis can be accurately modeled, but its availability at present for such studies is extremely limited.

There are no robust small animal models of HCV infection. A chimeric mouse model has been developed in which human hepatocytes are transplanted into *SCID/Alb-uPA* mice (Mercer et al 2001). In this system, liver-specific expression of the urokinase plasminogen activator is toxic to the murine hepatocytes, allowing transplanted human hepatocytes to repopulate the mouse liver. The human hepatocytes resident in the resulting chimeric liver can be infected with HCV and this system has proven useful in studying certain aspects of HCV replication. However, since the mice are immunodeficient, it is not possible to study most host immune responses to the virus in these mice. In addition, this system is technically challenging, the mice are not long-lived and they do not develop cancer. As a surrogate for a small animal model of infection, a considerable number of transgenic mouse lines have been developed that express various HCV proteins. These are discussed separately below.

Given the absence of a small animal that is permissive for replication of the virus, it is not surprising that the influence of HCV protein expression on cellular homeostasis has been studied mainly using in vitro systems. These include transient expression studies, the use of cell lines containing HCV RNA replicons (*i.e.* cells that contain autonomously replicating, selectable subgenomic or genome-length viral RNA but do not produce infectious virus) (Lohmann et al 1999) (Fig. 1) and cell culture-infectious virus systems (Lindenbach and Rice 2005, Wakita et al 2005, Yi et al 2006). Aside from the fact that all of these systems are in vitro, each has its limitations. HCV proteins may be expressed by replicons at high levels that are not physiologically relevant, and most replicon cell lines have been generated from Huh-7 cells which express a mutant p53 (Bressac et al 1990). Moreover, the techniques used to select such cell lines are likely to bias cell growth and survival properties. More recently developed systems allowing the propagation of cell culture-produced HCV ("HCVcc") provide the most authentic in vitro system for studying the effects of HCV on cellular homeostasis, but only two viral strains replicate well enough to study. Furthermore, most cell lines that are known to be permissive for HCVcc replication are derived from Huh-7 hepatoma cells. Despite these limitations, the cell culture-infectious virus systems have allowed significant advances in understanding the role of HCV proteins in viral replication and pathogenesis.

HCV transgenic mice and HCC

Transgenic mouse models of HCV pathogenesis typically use liver-specific promoters to drive expression of either individual or multiple HCV proteins. Although they lack viral RNA replication, these transgenic mice provide insights concerning the potential contribution of individual HCV proteins to liver disease and carcinogenesis. Table 1 summarizes HCV transgenic mouse lineages for which the phenotype has been determined up until at least 12 months of age. Of these, only some lineages appear to be at risk for HCC. The propensity of HCV transgenic mice to develop cancer varies with the mouse genetic background, with C57BL/6 mice appearing to be most susceptible (Klopstock et al 2009). In contrast, HCC has not been described in FVB mice expressing similar HCV transgenes. The promoter used to drive expression of the HCV transgene, the nature of the transgene, as well as the abundance of the HCV protein expressed also are likely to contribute to the presence or absence of a cancer phenotype.

Most transgenic mouse lines express one or more of the HCV structural proteins. Two transgenic lineages with high-level, liver-specific expression of the core protein developed hepatic steatosis with progression to adenomas and HCC (Moriya et al 1998). No hepatic inflammation was observed in these mice, suggesting a direct role for the core protein in carcinogenesis. However, the level of transgene expression was very high in these mice, and probably not reflective of the expression levels in most infected human livers. A separate study demonstrated the development of cancer in mice transgenic for both core as well as the two envelope proteins, E1 and E2 (Naas et al 2005). Although the promoter driving transgene expression in these mice was not liver-specific, transgene mRNA levels were highest in the liver. Steatosis was also observed in the livers of these mice, with the degree of steatosis increasing with age. In older mice, hepatocellular adenomas and carcinomas were observed, as well as tumors of lymphoid origin (Naas et al 2005).

In contrast to these mice, the FL-N/35 lineage expresses the entire HCV polyprotein under the control of the albumin promoter (Lerat et al 2002). These mice have liver-specific expression of the entire HCV polyprotein at very low abundance and thus may represent a more physiologically relevant model of HCV-associated HCC. HCV proteins are detectable in these mice only using very sensitive immunohistochemical methods (Keasler et al 2006), and the expression level is likely to be closer to what it is in infected human tissues. Steatosis was observed in these animals, but no inflammation, and liver cancer developed in older male animals at a relatively low but statistically significant rate (Lerat et al 2002). Interestingly, a companion transgenic mouse line, which expressed a greater abundance of only the structural proteins, core, E1, E2, and p7, did not have a significantly increased incidence of cancer compared to non-transgenic animals, suggesting a possible role for the non-structural proteins in carcinogenesis (Lerat et al 2002). However, it is not possible to exclude differences in the transgene integration sites underlying this difference. There are few other reports of transgenic mice that express the nonstructural proteins of HCV, but mice transgenic for the NS5A protein also develop significant steatosis and HCC (Wang et al 2009a).

In addition to possible direct carcinogenic effects of HCV protein expression, studies with transgenic mice have demonstrated that HCV protein expression may enhance susceptibility to various nonchemical carcinogens. For example, the incidence of liver cancer in FL-N/35 polyprotein-expressing mice, is increased by iron overloading (Furutani et al 2006), co-expression of the hepatitis B virus X protein (Keasler et al 2006), or intestinal colonization with *Helicobacter hepaticus* (Fox et al 2010). Alcohol promoted the development of HCC in NS5A transgenic mice that normally do not develop cancer (Machida et al 2009b, Majumder et al 2003). The synergistic effects of alcohol and NS5A expression on the rate of tumor formation were related to induction of TLR4 expression and a downstream mediator, Nanog.

These findings are particularly interesting because alcohol ingestion increases the risk of HCC in patients with chronic hepatitis C. In another series of experiments, a transgenic mouse line expressing HCV core under control of the serum amyloid P component promoter developed cancer only after repeated carbon tetrachloride-induced liver injury (Kato et al 2003). In this case, tumor development was dependent upon both repeated liver injury and core protein expression. In contrast, transgenic expression of the viral structural proteins resulted in an increase in tumor size, but not frequency, following exposure to diethylnitrosamine (Kamegaya et al 2005).

In summary, cancer phenotypes in HCV transgenic mice suggest roles for both structural and non-structural HCV proteins in hepatocellular carcinogenesis. The lack of detectable inflammation in transgenic mice that develop cancer supports a direct role for HCV proteins in carcinogenesis, while other evidence suggests that HCV protein expression may have broader, co-carcinogenic effects. The variation evident in the phenotypes reported for HCV transgenic mice is disconcerting, however. No single viral protein has been shown to consistently cause liver cancer when expressed at a low abundance comparable to that present in most patients with HCV-related liver disease.

General consequences of HCV infection

Inflammation, fibrosis and cirrhosis

Persistent HCV infections are typically associated with inflammatory and wound healing responses within the liver. Activation of the NF-κB pathway plays a central role in these inflammatory responses and may be important in carcinogenesis (reviewed in Sun and Karin 2008). Chronic inflammation related to HCV infection drives fibrogenesis, with increased deposition of extracellular matrix proteins leading to fibrotic scarring and ultimately cirrhosis. Activation of hepatic stellate cells (HSCs) is known to be important in this process and may be cytokine-driven, but the specific mechanisms by which HCV infection induces HSC activation are not well defined. Oxidative stress and apoptosis of infected hepatocytes are likely to be contributory factors (Brenner 2009). Other studies suggest a pro-fibrotic role for specific viral proteins (Bataller et al 2004, Mazzocca et al 2005) while yet others point to immune responses as an important trigger (Baroni et al 1999).

The sensing of HCV infection by pathogen recognition receptors of the innate immune system likely contributes to these processes. Viral double-stranded RNA replication intermediates produced in infected hepatocytes are sensed by retinoic acid-inducible gene I (RIG-I) and Toll-like receptor 3 (TLR3), leading to activation of interferon regulatory factor 3 (IRF-3) and NF-κB (Saito et al 2008, Wang et al 2009b). Although HCV has evolved redundant mechanisms to counter these responses (reviewed in Lemon 2010), many patients as well as chimpanzees with persistent HCV infection show marked transcriptional up-regulation of interferon-stimulated genes. This may reflect the sensing of infected hepatocytes by plasmacytoid dendritic cells through a TLR7-dependent pathway (Takahashi et al 2010). In addition to these sensing mechanisms, other studies suggest that HCV-induced endoplasmic reticulum stress (Waris et al 2002) or simply the presence of HCV-encoded proteins may activate NF-κB signaling and the expression of pro-inflammatory cytokines (Dolganiuc et al 2004, Sato et al 2006, Waris et al 2003). Core and NS3 stimulate IL-1 receptor-associated kinase (IRAK) activity in multiple cell types, along with phosphorylation of p38 and activation of extracellular regulated kinase (ERK) and c-jun N-terminal kinase (JNK) kinases, via a TLR2-dependent pathway (Dolganiuc et al 2004) .

We have recently demonstrated that there is a patchy distribution of HCV infection within the human liver, with infected cells generally appearing in clusters consistent with cell-to-cell spread of virus (Liang et al 2009) (Fig. 2). Such cells do not always appear to be in close

proximity to inflammatory cells, and interferons and other soluble mediators may largely control spread through the liver. Nonetheless, virus-specific CD8+ cytotoxic T-cells are present, and play a key role in controlling the infection (reviewed in Bowen and Walker 2005). Cytotoxic T cells and virus-induced inflammatory responses are likely to result in repeated cycles of hepatocyte destruction and regeneration. This pattern of chronic inflammation and increased hepatocellular proliferation provides an environment that is highly conducive to the development of cancer, and is likely to be common to the development of HCC due to many other causes. The link between chronic inflammation and HCC is well established from transgenic models of hepatitis B (Nakamoto et al 1998), and it seems certain that this link extends to HCV-associated cancer as well. However, several complementary lines of evidence suggest that HCV also plays a direct role in development of HCC.

Apoptosis

HCV infection may influence pathways that regulate programmed cell death. Numerous over-expression studies have suggested both pro- and anti-apoptotic functions of individual HCV proteins and have generated a large body of sometimes conflicting data. For example, some reports purport that core protein expression promotes apoptosis (Chou et al 2005, Honda et al 2000, Zhu et al 1998), while others suggest an anti-apoptotic effect of core expression (Lu et al 1999, Ray et al 1998, Sacco et al 2003). In addition, E2 (Lee et al 2005), NS2 (Erdtmann et al 2003), NS3 (Tanaka et al 2006) and NS5A (Gale et al 1999, Lan et al 2002) have all been reported to exert an anti-apoptotic effect when expressed in cultured cells, while other studies suggest that NS3 (Prikhod'ko et al 2004) and NS4A (Nomura-Takigawa et al 2006) may promote apoptosis. Most of these studies have evaluated the effects of transient expression of individual HCV proteins at levels that may be considerably higher than in infected hepatocytes in vivo. Core is a highly basic protein that has been reported to have numerous pleiotropic effects when transiently over-expressed. Moreover, the expression of individual HCV proteins in the absence of the remainder of the polyprotein may lead to different localization or post-translational modifications than expressed as a component of the viral polyprotein. Consequently, the biological significance of these findings is not clear.

On the other hand, apoptosis has been demonstrated in response to HCV infection in more physiologically relevant systems that have become available in recent years. For example, recent reports have been consistent in demonstrating apoptosis in Huh-7 cells infected with HCVcc (Deng et al 2008, Mateu et al 2008, Walters et al 2009). Furthermore, in the chimeric *SCID/Alb-uPA* mouse model, apoptosis was observed in the transplanted human hepatocytes following intrahepatic injection with replication-competent HCV RNA, but not with a replication-defective mutant (Joyce et al 2009). Although HCV may replicate to atypically high levels both in Huh7 cells as well as human hepatocytes within chimeric livers of severely immunocompromised *SCID/Alb-uPA* mice, these studies suggest that HCV infection may be inherently pro-apoptotic.

Steatosis

Hepatic steatosis, characterized by the intracellular accumulation of lipids in hepatocytes, is frequently found in patients with chronic HCV infection (for a review, see Cross et al 2010). Metabolic factors, such as high body mass index and diabetes, influence the development of steatosis in chronic hepatitis C. However, there is a particularly strong association between steatosis and infection with genotype 3 HCV, indicating that steatosis must result, at least in part, from effects on the cell that are virus-specific. Consistent with this, transgenic mice expressing genotype 1 HCV proteins, particularly core, develop steatosis (Lerat et al 2002, Moriya et al 1997). Changes in the expression of lipid metabolism genes also have been

observed in chimeric *SCID/Alb-uPA* mice infected with genotype 1 virus (Joyce et al 2009). Whether directly virus-induced or metabolic in origin, steatosis may contribute to liver cancer through increased oxidative stress or by promoting fibrosis.

Oxidative stress

Both microarray and proteomics studies have demonstrated increased expression of oxidative stress response genes in HCV-associated fibrosis and cirrhosis (Diamond et al 2007, Shackel et al 2002). While no single causative mechanism has been unequivocally identified, increased oxidative stress has the potential to cause DNA damage, potentially leading to the accumulation of mutations, and may also activate HSC thereby promoting fibrosis (Brenner 2009). Furthermore, chronic oxidative stress may lead to activation of cellular signaling pathways that can contribute to cellular transformation (Waris et al 2005).

Oxidative stress has been detected in several models of HCV pathogenesis and is generated, at least in part, by the inflammatory response to chronic infection. However, there may also be a direct effect of HCV proteins, particularly core, on intracellular levels of reactive oxygen species (ROS). Oxidative stress was observed in cultured cells following the conditional expression of core protein under control of a tetracycline-regulated promoter (Okuda et al 2002), and this led to increased expression of cellular antioxidant genes (Li et al 2002). Transgenic mice expressing the core protein also show an increased abundance of both ROS and lipid peroxidation products (Korenaga et al 2005, Moriya et al 2001). When expressed in cultured cells, core protein localizes in part to mitochondria, and this may promote increased ROS production through inhibition of mitochondrial electron transport (Korenaga et al 2005). Other studies suggest that over-expression of NS5A may also cause oxidative stress (Gong et al 2001), but the caveats described above for protein over-expression studies must be considered in interpreting these results.

Epigenetic changes in gene expression accompanying HCV infection

The dependence of HCV upon a number of cellular proteins and even a microRNA for its replication reflects extensive adaptation of the virus to its human host, a process shaped by an extraordinarily high rate of virus production (on the order of 10^{12} new virus particles produced every day over decades of persistent infection in the typical patient) (Neumann et al 1998) and a highly error-prone RNA replicase. In addition to disrupting signal transduction pathways involved in immune responses, these accessory functions of HCV proteins appear to include a number of interactions with tumor suppressor proteins. Such interactions are likely to have evolved because they promote a favorable cellular environment for virus replication and thus survival of the virus. However, they may also have coincidental, pathologic consequences for the host.

Retinoblastoma protein

The retinoblastoma tumor suppressor protein (Rb) is a common target of oncoproteins expressed by DNA tumor viruses, including adenovirus (Whyte et al 1988), simian virus 40 (DeCaprio et al 1988), and human papillomavirus (Dyson et al 1989). The down-regulation of Rb promotes cell cycle entry, activating cellular DNA synthetic pathways required for replication of these viruses. Surprisingly, Rb is also targeted by HCV, an RNA virus, and strongly, negatively regulated by HCV infection in cultured cells (McGivern et al 2009, Munakata et al 2005, Munakata et al 2007). This is due to NS5B, the viral RNA-dependent RNA polymerase, which forms a cytoplasmic complex with Rb (Fig. 3) and recruits to it the E6-associated protein (E6AP). This leads to polyubiquitination of Rb and Rb degradation via the proteasome (Munakata et al 2005, Munakata et al 2007). The end result is activation of E2F-responsive promoters, which would be expected to stimulate entry into the S phase of the cell cycle (Munakata et al 2005). In a fascinating parallel with DNA viruses, the

interaction with Rb is dependent upon an LxCxE motif in NS5B that has homology to pRb-binding domains in DNA virus oncoproteins (Munakata et al 2005).

These findings are unique among RNA viruses and suggest a novel theoretical framework for the origins of liver cancer. In addition to controlling the G1 to S phase transition in the cell cycle, in part through repressive effects on E2F transcription factors (Chellappan et al 1991, Classon and Harlow 2002, Takahashi et al 2000), Rb also regulates DNA damage responses, the mitotic spindle checkpoint, and apoptosis (Classon and Harlow 2002, Hernando et al 2004, Khidr and Chen 2006, Lentini et al 2002). While the virus might benefit from enhanced hepatocellular proliferation (which might favor virus replication based on observations in cell culture), the disruption of Rb-mediated regulatory functions may also restrict DNA damage responses. Such a defect would be particularly important in a liver with ongoing inflammation and oxidative stress. Expression of the core protein has also been suggested recently to uncouple the mitotic spindle checkpoint and induce chromosomal polyploidy in transgenic mice and cultured hepatocytes through defective Rb signaling (Machida et al 2009a). In this case, Rb expression was thought to be restricted at the transcriptional level.

The analogy with the DNA tumor viruses only goes so far, however. While the p16-cyclin D-Rb pathway is frequently disrupted in HCC, including tumors associated with HCV (Azechi et al 2001, Edamoto et al 2003), this does not result from continued virus replication or NS5B expression. If it plays a role, NS5B-induced loss of Rb expression seems likely to be an early event in the development of cancer, occurring while hepatocytes remain sufficiently well differentiated to support virus replication, and prior to the heterogeneous chromosomal mutations that are found commonly in HCC (Thorgeirsson and Grisham 2002).

p53 pathway

Over-expression studies suggest that HCV proteins, including core, may deregulate the p53 pathway. Again, however, the available experimental systems are limited and published reports conflicting with respect to the effects on p53 activity. Some results suggest that core activates p53-dependent gene expression (Lu et al 1999, Otsuka et al 2000) while others show repression (Ray et al 1998). It is possible that this reflects varied levels of expression, with low levels of core expression activating p53 and high levels resulting in repression of p53 activity (Kao et al 2004). Other in vitro evidence suggests that NS3 may interact with p53 (Ishido and Hotta 1998) and repress p53-dependent transcription (Kwun et al 2001). NS3 expression blocks actinomycin D-induced apoptosis; this activity of NS3 was found to be sequence-dependent and correlated with p53 interaction (Deng et al 2006, Tanaka et al 2006). Yet a third HCV protein, NS5A has been reported to interact with p53, resulting in its redistribution to the perinuclear membrane in HepG2 hepatoma cells (Majumder et al 2001). NS5A also interacts with p53 in the rat hepatoma cell line FAO, and inhibits p53-dependent transcription in HCT116 cells (Qadri et al 2002). Anchorage-independent growth of NIH3T3 cells is promoted by NS5A (Ghosh et al 1999). Finally, while ectopic expression of p53 in the p53-null Hep3B cell line induces apoptosis, this effect is blocked by NS5A (Lan et al 2002).

It is possible that HCV infection may also impact p53 function indirectly. As discussed in greater detail below, there is good evidence that core interacts with the cellular RNA helicase DDX3 (Fig. 4), a candidate tumor suppressor protein that regulates activity of the p21(waf1/cip1) promoter (Chao et al 2006). In addition, NS5B interacts with DDX5 (p68), another RNA helicase, resulting in its relocalization from the nucleus to the cytoplasm (Goh et al 2004) (Fig. 5). DDX5 is a transcriptional co-activator of p53 (Bates et al 2005), a function that would be impeded by its relocalization to the cytoplasm. Interactions of HCV

proteins with the p53 pathway may have evolved to prevent stress-induced growth arrest or apoptosis, both of which would be counter to survival of the virus. On the other hand, recent studies have revealed surprising roles for p53 and DDX3 in TLR3 and RIG-I-mediated induction of interferon synthesis (Dharel et al 2008, Oshiumi et al 2010, Schroder et al 2008, Taura et al 2008), and it is possible that such interactions might reflect yet another mechanism by which the virus escapes innate immune defenses.

Many of the immortalized cell lines used to study the impact of HCV protein expression on p53 have defects in the p53 pathway. Huh-7 cells, which are commonly used for propagation of HCV, overexpress a functionally defective p53 mutant (Bressac et al 1990). To date, this has precluded a direct analysis of the effects of viral infection on p53 function in cell culture. Similarly, NIH3T3, COS-7 and HeLa cells all express viral oncoproteins that directly interact with the p53 protein. An additional consideration is that the level of protein over-expression in many of these studies may be much higher than in the HCV-infected liver. Therefore, while these findings may be relevant to the development of HCV-associated HCC, they need to be interpreted with considerable caution.

Wnt/β-catenin

The Wnt pathway is a key signal transduction pathway in animal development. In the canonical Wnt pathway, Wnt ligands bind to their receptor to activate a signal transduction cascade resulting in inhibition of a β-catenin degradation complex. Components of the Wnt pathway are frequently mutated in liver cancer, resulting in β-catenin stabilization. Stabilization of β-catenin allows it to enter the nucleus and interact with proteins that regulate transcription of genes that influence cell survival and proliferation.

NS5A may indirectly regulate the Wnt pathway through its interaction with the p85 regulatory subunit of phosphoinositide 3-kinase (PI3K), which results in activation of the p110 catalytic subunit of PI3K and initiates signaling that activates the downstream kinase, Akt (Street et al 2004). This leads in turn to phosphorylation and inactivation of glycogen synthase kinase-3β(GSK-3β), a key component of the multiprotein complex that normally targets β-catenin for proteasomal degradation (Street et al 2004). Thus, NS5A expression results in the stabilization of β-catenin and increased β-catenin-dependent transcription (Park et al 2009, Street et al 2005). Increases in β-catenin abundance have been observed in cells containing autonomously replicating HCV replicons, and to some extent in cells infected with HCVcc. More recent data, however, suggest that β-catenin may be activated through a direct interaction with the NS5A protein (Milward et al 2010, Park et al 2009). The functional significance of the activation of β-catenin within HCV infected cells is uncertain. While dysregulation of Wnt signaling does not appear to be independently capable of causing malignant transformation of hepatocytes, β-catenin abundance is increased in most HCC and may promote tumor growth (reviewed in Whittaker et al 2010)

ATM and Chk2

The ataxia telangiectasia mutated kinase (ATM) is a tumor suppressor protein that detects double-strand DNA breaks and regulates signal transduction pathways controlling the DNA damage checkpoint. An interaction with the HCV NS3/4A protease results in partial relocalization of ATM from the nucleus to the perinuclear region of the cytoplasm (Ariumi et al 2008, Lai et al 2008). NS3/4A may also interact with another DNA damage sensor, checkpoint kinase 2 (Chk2) (Ariumi et al 2008). Knockdown of either ATM or Chk2 impaired viral RNA replication and reduced virus yields, suggesting that these interactions may have evolved to promote replication. Impaired ATM and Chk2 functions may explain why NS3/4A expression results in abnormal responses to DNA damage following ionizing radiation (Lai et al 2008).

DDX3

Among its many putative interactions, yeast two-hybrid screens and co-immunoprecipitation studies have revealed a now well-documented interaction of the core protein with the DEAD box RNA helicase, DDX3 (Mamiya and Worman 1999, Owsianka and Patel 1999, You et al 1999). This results in a profound change in the cellular distribution of DDX3, from what is normally a diffuse, predominantly cytoplasmic distribution to punctate cytoplasmic foci that co-localize with core protein in HCV-infected cells (Fig 4). DDX3 knock-down studies suggest that it may specifically facilitate HCV replication (Ariumi et al 2007, Randall et al 2007). It is difficult to distinguish virus-specific effects of gene knockdown or over-expression, however, from general pleiotropic effects on the cell that may indirectly influence replication, as shown recently in studies of another cellular helicase, DDX6 (Rck/p54), which also interacts with core (Jangra et al 2010a). At any rate, the ability of DDX3 to modulate HCV replication is independent of its core-binding activity (Angus et al 2010). Like many DEAD-box helicases, DDX3 appears to be multifunctional. As described above, it regulates activity of the p21(waf1/cip1) promoter and represses cap-dependent translation through inhibition of eIF4E (Chao et al 2006, Shih et al 2008), and may have significant tumor suppressor activity (Botlagunta et al 2008, Chang et al 2006). However, DDX3 is also required for efficient induction of interferons in response to virus infections (Oshiumi et al 2010, Schroder et al 2008), and this may be the function underlying its targeting by HCV.

Innate immune signaling

Like many other viruses, HCV has evolved mechanisms to antagonize host innate immune signaling pathways (reviewed in Lemon 2010). These pathways are closely linked to tumor suppressor functions, as alluded to above, and the long term targeting of innate immune signaling pathways by viral proteins during persistent infection may result in the promotion of cell growth and inhibition of apoptosis. The dsRNA-activated protein kinase (PKR) is interferon-inducible and down-regulates translation via phosphorylation of eIF-2α, thereby inhibiting cell growth and promoting apoptosis to restrict virus infection. These properties confer tumor-suppressor activity on PKR (Meurs et al 1993). In vitro data suggest that the NS5A protein can bind to and functionally repress PKR (Gale and Katze 1998, Gale et al 1997), possibly contributing to oncogenesis via this pathway (Gale et al 1999). In addition to the effects of HCV on PKR, the NS3/4A protease targets essential adapter molecules in both the TLR3 and RIG-I signaling pathways, TIR-domain containing inducer of interferon-β (TRIF) and mitochondrial antiviral signaling protein (MAVS, also known as IPS-1), thereby interfering with activation of IRF-3 (Li et al 2005a, Li et al 2005b, Meylan et al 2005, Wang et al 2009b), a transcription factor with strong anti-proliferative properties (Kim et al 2003). These interactions evolved to protect the virus from the antiviral actions of numerous IRF-3 dependent genes, including type I interferons, but may also contribute to the cancer-promoting effects of HCV.

Growth factor signaling

Growth factor signaling pathways play important roles in the initiation and possibly maintenance of HCC (reviewed in Whittaker et al 2010). The transforming growth factor beta (TGF-β) signaling pathway exerts both an anti-proliferative and pro-apoptotic influence, and is important in regulating the expansion of progenitor cells in regenerating liver (Thenappan et al 2010). The HCV core protein has been suggested to interact with Smad3, a transcriptional modulator that is activated through the TGF-β pathway, thereby impairing the tumor suppressive properties of TGF-β (Battaglia et al 2009, Cheng et al 2004, Pavio et al 2005). In addition, NS5A has the capacity to interact with Src homology 3 (SH3) domains that are found in many proteins involved in signal transduction. Such interactions may involve Grb2, an adaptor protein involved in growth factor signaling (Tan

et al 1999), the p85 subunit of PI3K, as described above (Street et al 2004), and some members of the Src tyrosine kinase family (Macdonald et al 2004).

miRNAs and HCV-associated cancer

miRNAs are likely to play important roles in both the causation and maintenance of cancers, including HCC (Coulouarn et al 2009, Pineau et al 2010). Of 940 recognized human miRNAs, miR-122 is uniquely expressed at high abundance in the adult liver, representing approximately 50% of miRNAs and with 50,000 or more copies per cell (Chang et al 2004). It is developmentally regulated and expressed almost exclusively in liver, controlling the expression of numerous hepatocyte-specific genes and promoting hepatocellular differentiation (Chang et al 2004, Elmen et al 2008, Krutzfeldt et al 2005).

HCV replication is critically dependent upon miR-122 (Jangra et al 2010b, Jopling et al 2005). miR-122 binds to two conserved, tandem sites in the HCV 5′UTR that are complementary to its “seed” sequence (Jopling et al 2008). This interaction is required for effective genome amplification, but available data do not suggest that it directly enhances viral RNA synthesis (Jopling et al 2005, Norman and Sarnow 2010). Instead, miR-122 binding promotes cap-independent translation of the viral RNA (Henke et al 2008, Jangra et al 2010b). However, the magnitude of this effect appears insufficient to completely explain the dependence of HCV on miR-122, and thus the requirement for miR-122 as a host factor for HCV replication remains incompletely explained. Importantly, however, HCV replication was potently suppressed in chimpanzees after therapeutic silencing of miR-122 by administration of an antisense locked nucleic acid (LNA) oligonucleotide against miR-122 (Lanford et al 2010). These recent data have fueled enthusiasm for miR-122 antagomirs as potential antiviral agents, making a better understanding of the role of miR-122 in carcinogenesis imperative.

There is some evidence that miR-122 may have tumor suppressor properties. Expression of miR-122 is low or undetectable in the human hepatoma cell lines, Hep3B and HepG2 cells, in which its over-expression inhibits anchorage-independent growth, migration, invasion, and tumor formation in nude mice (Bai et al 2009). IL-1α is one of many genes regulated by miR-122, and polymorphisms in the miR-122 binding site in the IL-1α 3′UTR confer an increased risk for HCC (Gao et al 2009). Cyclin G1 is also regulated by miR-122, influencing the stability of p53 and affecting the growth properties of HCC-derived cells (Fornari et al 2009). Although a number of studies have profiled miRNA expression in HCC, it remains unclear whether miR-122 abundance is altered in HCV-associated HCC. Overall, miR-122 expression is reduced in 50-70% of HCC (Gramantieri et al 2007, Kutay et al 2006, Ura et al 2009). However, it may be increased in a subset of HCC with mutations in β-catenin (Pineau et al 2010), and some but not all studies suggest that miR-122 expression is preserved specifically in HCV-associated cancers (Coulouarn et al 2009, Ura et al 2009, Varnholt et al 2008). This is an important question that deserves further study, as loss of miR-122 would limit the ability of HCV to replicate in HCC cells.

Despite the unique relationship between HCV and miR-122, few studies have examined how HCV infection influences miR-122 expression. Acute infection of Huh-7 cells with HCVcc was reported recently to cause a significant decline in miR-122 abundance (Liu et al 2010). Many other miRNAs were differentially regulated by HCV infection in this study, however, and it is difficult to predict how these results relate to infection of normal hepatocytes in vivo. miRNA biogenesis is modulated by p53 through an interaction with the Drosha processing complex that involves an association with DDX5 (Suzuki et al 2009). Since various HCV proteins interact with both p53 and DDX5, as described above, it is not surprising to find that HCV infection might alter global miRNA expression profiles. However, the lack of normal p53 function in Huh-7 cells mandates a great deal of caution in

interpreting the results of such studies. There are large gaps in our understanding of how HCV regulates miRNA expression, and the role of miRNAs in development of HCC. Future studies should be directed at addressing these questions.

Summary and conclusions: A model of HCV-associated hepatocarcinogenesis

Although HCV is increasingly associated with HCC in the U.S. and probably many other countries, attempts to understand the underlying pathogenetic mechanisms are limited by the absence of good cellular and animal models of HCV pathogenesis. Those data that are available suggest that HCV-associated HCC is likely to be caused by a combination of environmental, epigenetic and genetic factors. In patients who are chronically infected with HCV, cancer typically develops in the setting of cirrhosis. Repeated cycles of immune-mediated destruction of infected hepatocytes and virus-induced apoptosis together with regeneration of damaged tissue cause disturbances in the normal cellular homeostasis of the liver. Furthermore, inflammatory responses associated with persistent infection cause oxidative stress that can damage chromosomal DNA, leading to heritable changes in the genome. These processes mirror the development of HCC due to many other causes. However, the occurrence of HCV-associated HCC in the absence of cirrhosis, the particularly high rate of HCC in cirrhosis caused by chronic HCV infection, and the development of HCC in HCV transgenic mice in the absence of inflammation or fibrosis, suggest that persistent HCV infection and viral protein expression is likely to have a direct cancer-promoting effect. Numerous interactions between viral proteins and cellular tumor suppressor pathways may act not only to further viral replication, but also to deregulate normal control of the cell cycle and cellular responses to DNA damage. These cancer-promoting actions of HCV may be eliminated largely if not completely by effective antiviral therapy. Antiviral therapies remain limited in efficacy, however, and a better understanding of the effects of HCV infection on these regulatory pathways may suggest new opportunities for preventive measures that may be taken in the absence of virus eradication.

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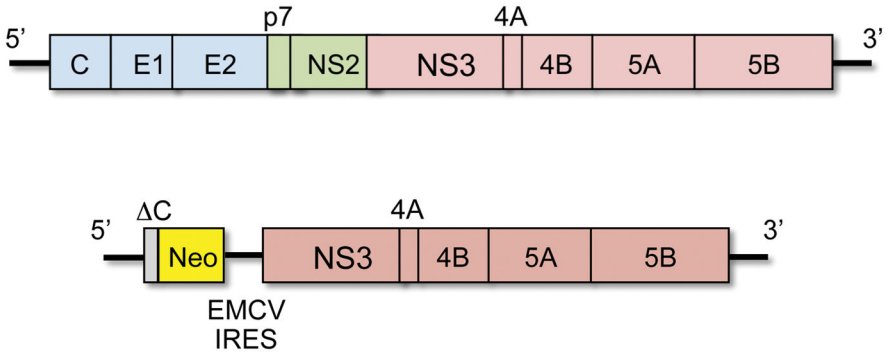

Figure 1.

HCV genome structure. **(top)** Organization of the positive-sense RNA genome of HCV. 5′ and 3′ UTRs contain *cis*-acting elements required for viral protein expression and RNA synthesis, including an internal ribosome entry site (IRES) in the 5′UTR that directs cap-independent translation of the viral polyprotein. The polyprotein (large box) is processed by both host and virus-encoded proteases to produce the individual proteins required for viral genome replication, virus assembly, and production of infectious progeny virus (reviewed in Moradpour et al 2007). The structural proteins (blue) that form the HCV virion include core (nucleocapsid), E1 and E2 (envelope glycoproteins). Non-structural proteins of HCV serve a variety of functions including RNA genome replication, virus assembly and maturation: those from NS3-NS5B (pink) are required for genome replication. NS3 has distinct proteinase and helicase domains, while NS4A is cofactor that intercalates with NS3 and is required for full expression of NS3 protease activity. NS4B is involved in the formation of the membranous web: a cytoplasmic structure associated with the viral RNA replicase. NS5A is an essential co-factor for virus replication and assembly, while NS5B is the viral RNA-dependent RNA polymerase. p7 and NS2 (green) are additional nonstructural proteins that are not required for viral RNA synthesis but contribute to virion assembly and egress. **(bottom)** Organization of a dicistronic, selectable HCV "replicon". The 5′ and 3′UTRs are as in the viral genome. A truncated core-protein sequence is fused to the neomycin phosphotransferase gene, followed downstream by a heterologous picornaviral IRES driving translation of the nonstructural proteins. This RNA is capable of autonomous amplification in permissive cells, but does not generate infectious virus.

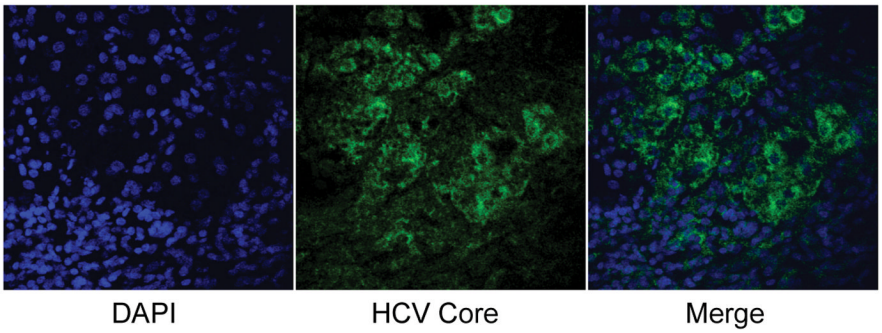

Figure 2.

HCV core antigen visualized in frozen liver tissue from a 50 year old female with genotype 3 HCV infection, cirrhosis, and a serum viral RNA load of 3.3×10^5 IU/ml. Tissue was collected at the time of resection of HCC. Antigen was visualized by multiphoton microscopy following tissue labeling with a monoclonal antibody to core conjugated to a fluorescent quantum dot probe (Liang et al 2009).

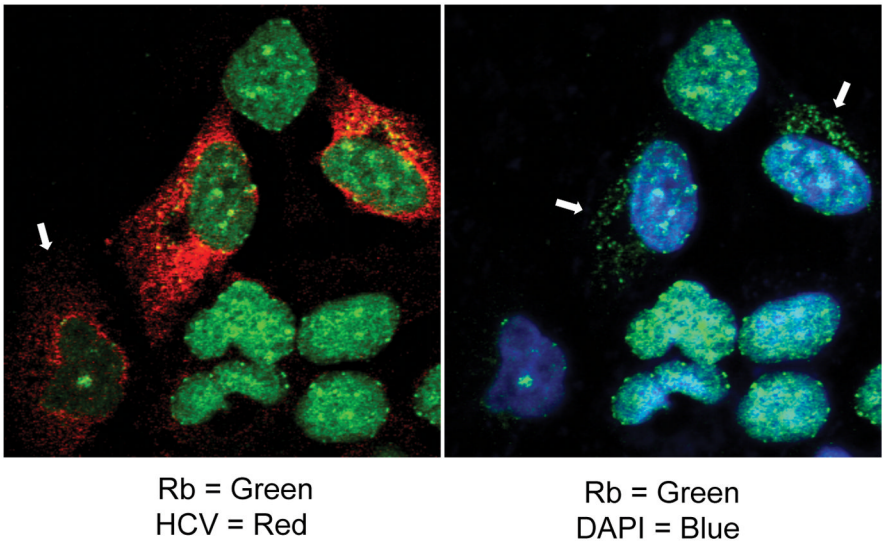

Figure 3.

Laser-scanning confocal microscopy of FT3-7 (Huh7) cells infected with HCVcc (HJ3-5 virus) leads to reductions in nuclear Rb expression (left panel, arrow) and transient accumulation of Rb in the cytoplasm (arrows, right panel). HCV antigens (red) were labeled with polyclonal human anti-HCV antibodies, while Rb was labeled with a murine monoclonal antibody (green) (McGivern et al 2009). Nuclei were counterstained with DAPI.

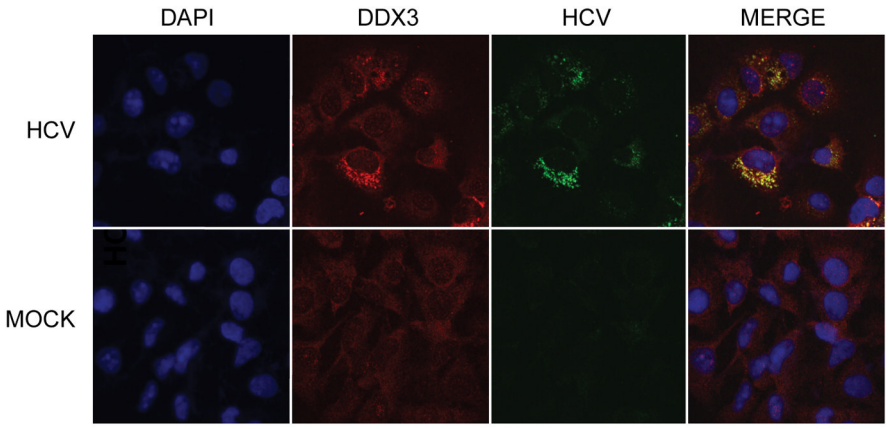

Figure 4.

Confocal microscopic image showing aggregation of DDX3 in association with HCV core protein in the cytoplasm of HCVcc-infected cells. Huh7 cells were infected with HJ3-5 virus 11 days previously. Labeling was with rabbit anti-core (green) and murine anti-DDX3 (red) antibodies (gifts of Dr. Arvind Patel, Medical Research Council Virology Unit, Glasgow). Nuclear counterstain was with DAPI. Mock = mock infected.

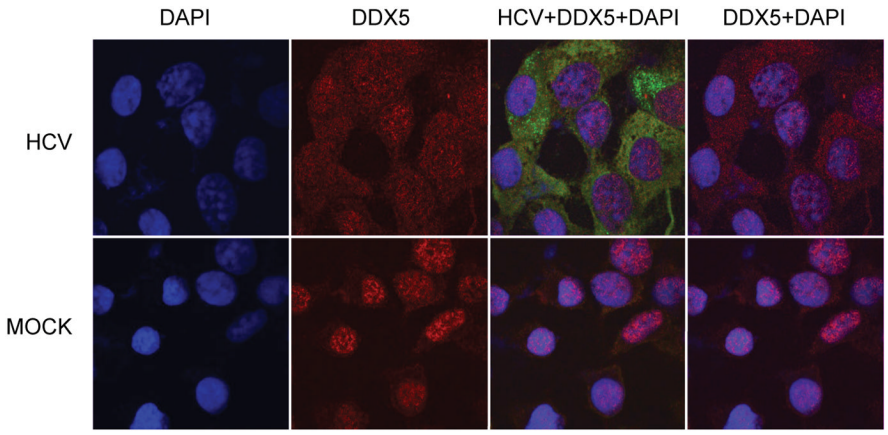

Figure 5.
DDX5 is redirected from the nucleus to the cytoplasm in Huh7 cells infected with HCVcc (JFH1 strain). Cells were labeled with murine anti-DDX5 (red) and human polyclonal anti-HCV (green) antibodies. Nuclei were counterstained with DAPI. Mock = mock infected.

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Table 1

Transgenic mouse lines expressing HCV proteins

HCV transgene	Genotype	Promoter	Genetic background	Pathology	Cancer frequency	Reference
Polyprotein	1b	Albumin	C57BL/6	Steatosis, HCC	5/37	(Lerat et al 2002)
Core	1a	MUP	C57BL/6 × SJL	none	0*	(Pasquinelli et al 1997)
Core	1b	HBV	C57BL/6	Steatosis, HCC	14-31%	(Moriya et al 1998)
Core	1b	HBV	FVB × C57BL/6	none	0*	(Kamegaya et al 2005)
Core-E1-E2	1b	MHC	C57BL/6		0*	(Honda et al 1999)
Core-E1-E2	1b	Albumin	FVB × C57BL/6	none	0*	(Kamegaya et al 2005)
Core-E1-E2	1a	CMV	B6C3F1	Steatosis, variety of tumors of hepatic and non-hepatic origin	17/185	(Naas et al 2005)
Core-E1-E2	1b	MUP and Albumin	FVB	none	0*	(Kawamura et al 1997)
E1-E2	1b	HBV	CD1	None in liver	0*	(Koike et al 1995)
E2 (a.a. 384-715)	1a	MUP	C57BL/6 × SJL	none	0*	(Pasquinelli et al 1997)
Core-E1-E2-p7	1b	Albumin	C57BL/6	Steatosis, HCC	1/42	(Lerat et al 2002)
NS5A	1a	apoE	FVB	none	0/72	(Majumder et al 2003)
NS5A	1a	MUP	FVB	none	0/40	(Majumder et al 2003)
NS5A	1b	HBV	C57BL/6J × CBA/J	Steatosis, tumors	10/163	(Wang et al 2009a)

* total number of mice in study was not stated.