

# Hepatocellular Carcinoma Mechanisms Associated with Chronic HCV Infection and the Impact of Direct-Acting Antiviral Treatment

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Srikanta Dash<sup>1-3</sup>  
Yucel Aydin<sup>1</sup>  
Kyle E Widmer<sup>2</sup>  
Leela Nayak<sup>2</sup>

<sup>1</sup>Department of Pathology and Laboratory Medicine, Tulane University Health Sciences Center, New Orleans, LA 70112, USA; <sup>2</sup>Southeast Louisiana Veterans Health Care System, New Orleans, LA 70119, USA; <sup>3</sup>Department of Medicine, Division of Gastroenterology, Tulane University Health Sciences Center, New Orleans, LA 70112, USA

**Abstract:** Hepatitis C virus (HCV) infection is the major risk factor for liver cirrhosis and hepatocellular carcinoma (HCC). The mechanisms of HCC initiation, growth, and metastasis appear to be highly complex due to the decade-long interactions between the virus, immune system, and overlapping bystander effects of host metabolic liver disease. The lack of a readily accessible animal model system for HCV is a significant obstacle to understand the mechanisms of viral carcinogenesis. Traditionally, the primary prevention strategy of HCC has been to eliminate infection by antiviral therapy. The success of virus elimination by antiviral treatment is determined by the SVR when the HCV is no longer detectable in serum. Interferon-alpha (IFN- $\alpha$ ) and its analogs, pegylated IFN- $\alpha$  (PEG-IFN- $\alpha$ ) alone with ribavirin (RBV), have been the primary antiviral treatment of HCV for many years with a low cure rate. The cloning and sequencing of HCV have allowed the development of cell culture models, which accelerated antiviral drug discovery. It resulted in the selection of highly effective direct-acting antiviral (DAA)-based combination therapy that now offers incredible success in curing HCV infection in more than 95% of all patients, including those with cirrhosis. However, several emerging recent publications claim that patients who have liver cirrhosis at the time of DAAs treatment face the risk of HCC occurrence and recurrence after viral cure. This remains a substantial challenge while addressing the long-term benefit of antiviral medicine. The host-related mechanisms that drive the risk of HCC in the absence of the virus are unknown. This review describes the multifaceted mechanisms that create a tumorigenic environment during chronic HCV infection. In addition to the potential oncogenic programming that drives HCC after viral clearance by DAAs, the current status of a biomarker development for early prediction of cirrhosis regression and HCC detection post viral treatment is discussed. Since DAAs treatment does not provide full protection against reinfection or viral transmission to other individuals, the recent studies for a vaccine development are also reviewed.

**Keywords:** hepatitis C virus, HCV, hepatocellular carcinoma, HCC, interferon, IFN, direct-acting antiviral, DAA, endoplasmic reticulum stress, ER stress, autophagy

## Introduction

Primary liver cancer includes hepatoblastoma (liver cancer among children), angiosarcoma (cancer derived from blood vessels of the liver), cholangiocarcinoma (cancer derived from bile duct), and HCC (cancer derived from hepatocytes). Among those, HCC remains the most common form. Although a high incidence of HCC has been reported in developing countries, the prevalence of HCC has grown tremendously over the last three decades in Western countries as well.<sup>1</sup>

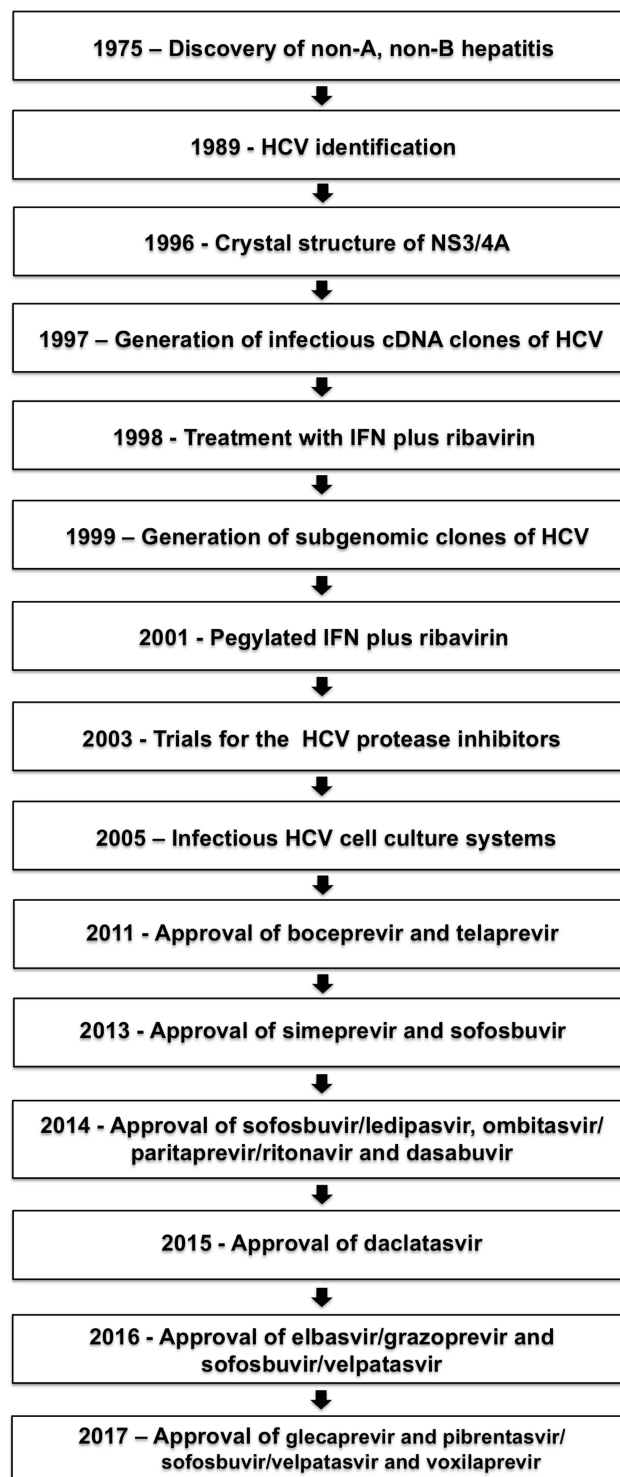
Correspondence: Srikanta Dash  
Department of Pathology and Laboratory Medicine, Tulane University Health Sciences Center, 1430 Tulane Avenue, New Orleans, LA 70112, USA  
Tel +1 504-988-2519  
Fax +1 504-988-7389  
Email sdash@tulane.edu

Currently, HCC is the sixth most common cancer that occurs three times more in males than in females.<sup>2</sup> Approximately 800,000 individuals are diagnosed with HCC worldwide causing more than 700,000 deaths per year.<sup>3</sup> In the US, deaths due to HCC and cholangiocarcinoma are rising more rapidly than deaths from any other type of cancer.<sup>4</sup> The risk factor predominantly contributing to the rise in HCC includes the high rate of HCV infection, followed by increasing rates of alcohol abuse, obesity, NAFLD, and uncontrolled type II diabetes. Autoimmune liver diseases, hemochromatosis, tyrosinemia, glycogen storage diseases, and alpha-1 antitrypsin deficiency can predispose to risk of liver cirrhosis and HCC. Among all these risk factors, HCV alone has about 5 to 20 fold higher risk for HCC development.<sup>5–10</sup>

HCV is a blood-borne pathogen that infects the liver exclusively. Most of the individuals infected with HCV fail to clear the infection naturally, leading to a stage of life-long chronic infection. The long-lasting liver inflammation due to HCV causes the onset of advanced liver diseases, such as liver fibrosis, cirrhosis, and HCC resulting in death. Approximately 71 million people are currently infected with HCV, of which only 20–30% of those develop liver cirrhosis, and 1–4% of cirrhotic patients develop HCC per year.<sup>11</sup> In most cases, HCC develops on the background of cirrhosis.<sup>12–18</sup> Approximately 15% of patients who developed HCC had no cirrhosis supporting the hypothesis that HCV infection can also induce HCC directly.<sup>19</sup> HCC can also develop in non-cirrhotic liver related to NAFLD and HBV infection.<sup>20</sup>

IFN- $\alpha$  or PEG-IFN- $\alpha$  plus RBV combination antiviral therapy has been used as the standard-of-care for patients with chronic HCV infection for many years. This treatment eliminates the virus in only a little more than half of all patients, and many liver cirrhosis patients were unable to clear the infection with this regimen.<sup>21,22</sup> The low responsiveness to IFN/RBV therapy for chronic HCV infection found to be associated with specific IL-28B genotypes.<sup>23</sup> Because of the high association with liver cancer, HCV-related liver research remained as one of the rapidly evolving areas in Hepatology since its discovery in 1989. The development of highly effective antiviral drugs to eliminate chronic HCV infection continued to be the primary focus in many academic laboratories and pharmaceutical industries. Drug discovery efforts of HCV have been hampered for a long time due to the ongoing technical difficulties in the establishment of cell

culture models. There was swift progress in many areas of basic and translational research after the cloning and



**Figure 1** Progress in translational research that leads to significant breakthroughs in DAA-based antiviral drug development resulting in HCV cure. Reprinted from *Semin Cell Dev Biol*, Dash S, Aydin Y, Wu T. Integrated stress response in hepatitis C promotes Nrf2-related chaperone-mediated autophagy: a novel mechanism for host-microbe survival and HCC development in liver cirrhosis. Copyright (2019), with permission from Elsevier.<sup>106</sup>

sequencing of the HCV genome and development of infectious chimpanzee clones (Figure 1). Although HCV is known to replicate well in human hepatocytes, researchers have struggled for a long time to grow the virus in the laboratory using different hepatoma cell lines. It took more than ten years to develop a cell culture model for HCV minigenome (the replicon model), which was later followed by the development of an infectious cell culture system using a unique virus strain derived from a Japanese patient with fulminant hepatitis. This virus strain with a highly adapted Huh-7.5-based liver cell line allowed us to study the replication and assembly of the whole HCV genome.<sup>24</sup> The availability of replicon and infectious cell culture models accelerated the discovery of newer DAAs. Approval of IFN-free DAAs targeting the NS3/4A protease, NS5A, and NS5B polymerase have now rapidly changed the therapeutic landscape for curing HCV infection at a very high rate.<sup>25,26</sup> In the future, HCV cure by DAAs is expected to reduce the incidence of chronic liver disease, liver cirrhosis, HCC, which result in decreased liver-related mortality.<sup>27,28</sup>

Recently, emerging clinical studies with DAAs in patients with liver cirrhosis created a heated debate about the risk of HCC occurrence and recurrence after viral cure. Some studies support that HCV cure by DAAs benefits patients as the liver inflammation decreased, fibrosis reversed, as well as a decreasing incidence of liver transplantation and liver-related extrahepatic complications.<sup>28–39</sup> However, some recent emerging publications from the US, Europe, and Asia indicate that DAA-induced HCV clearance in patients with liver cirrhosis decreases but does not eliminate the risk of HCC occurrence or recurrence.<sup>40–45</sup> In light of this new development, the overall objective of this article is to review relevant pieces of literature about the long-term benefits and risks of HCC development post viral cure by DAAs as compared to previously used IFN-based antiviral therapy. This review has also discussed potential overlapping viral-induced and host-related mechanisms implicated in HCC risk after viral cure by DAAs.

## The Risk of HCC Occurrence and Recurrence After HCV Cure

Chronic HCV infection is the major risk factor for HCC. A natural course of HCV based on observational model predicts that 60% of infected individuals will develop cirrhosis and 14.4% will develop HCC and remaining 37% will die of other complications related to HCV

infection.<sup>46</sup> Many earlier publications concluded that the IFN-based antiviral therapy prevented HCC risk and reduces all causes of mortality among patients with chronic HCV infection, including patients with liver cirrhosis.<sup>46–54</sup> The overall trends of HCC occurrence and recurrence after HCV clearance by IFN-based antiviral therapy were verified through meta-analyses.<sup>55–57</sup> Morgan et al analyzed 30 observational studies of compromising 31,528 chronic HCV patients in which 10,853 patients achieved SVR (34.4%) by IFN-based antiviral therapy, and 1742 patients developed HCC, suggesting viral cure by IFN does not eliminate the risk of HCC. When they adjusted HCC incidence per year according to the follow-up period and viral clearance, they found that HCV-cured patients develop HCC at a rate of 0.33%/year.<sup>55</sup> Rutledge et al performed similar analysis from 31 studies involving 71,443 HCV patients treated with IFN-based regimens showing that SVR rate with IFN was 45.9%. When they adjusted HCC incidence per year in the SVR population, it was 0.7%.<sup>56</sup> Waziry et al examined HCC occurrence and recurrence in patients treated with IFN-based treatment using an extensive electronic database. Their analysis revealed that the overall risk of HCC development is 1.13%/year. This study also reported the HCC recurrence rate was 9.2%/year when SVR was achieved.<sup>57</sup> Amalgamation of these data is that the risk of HCC occurrence and recurrence was decreased but not eliminated by IFN-based antiviral therapy. The risk and benefit of HCV clearance by DAA therapy have not been fully established since the follow-up timing of DAA-treated patients is not long enough. However, there have been three significant developments on addressing the HCC risk after viral cure since the approval of the DAAs. These results have generated considerable debates among researchers. First, a number of investigators found that, DAA-based antiviral therapy reduced the incidence of HCC development in chronic HCV patients with pre-existing cirrhosis, but did not eradicate the risk, suggesting these patients need ongoing surveillance for HCC after viral clearance.<sup>58–67</sup> Second, some investigators have reported that there is a high chance of HCC recurrence in cirrhotic patients who received DAAs.<sup>68–73</sup> Third, low SVR rate was seen with DAAs in patients with active HCC associated to HCV<sup>74–80</sup> suggests that the DAA-based HCV therapy should be carried out after HCC treatment. There are now considerable debates and disagreements among researchers all over the world on these observations. We will discuss selected publications

addressing the HCC risk after HCV cure by DAAs in patients with liver cirrhosis.

Some studies claimed that the risk of HCC is not decreased after HCV cure by DAA therapy.<sup>58–62</sup> These investigators demonstrated that the annual incidence of HCC development after viral treatment with DAAs is 3–5%, which is much higher than the risk observed with IFN-based therapy. In contrast, some other studies have tried to verify these observations and showed that HCV cure does reduce the risk but does not eliminate the risk of HCC development.<sup>63–67</sup> These studies found that the annual incidence of HCC occurrence was higher in DAA-induced HCV cure as compared to IFN-induced HCV cure (2.9% vs 1.14%). On the other hand, one large study demonstrated that DAA-induced HCV clearance is associated with reduced HCC risk, similar to IFN-induced HCV clearance among veteran patients.<sup>68</sup> Interestingly, a few reports expanded the debates claiming that HCV cure makes the liver cancer grow faster. Mainly, HCV infected patients with HCC who had a complete response to hepatic resection or local ablation subsequently developed high rates of HCC when they received DAAs.<sup>69–71</sup> These results are consistent with two small European studies that have shown a high percentage of HCC recurrence (27.6% and 28.8%) among patients who first received treatment for HCC then received DAA within six months.<sup>59,72</sup> Another study by Cabibbo et al reported a relatively high rate of HCC recurrence among patients who received DAAs after curative HCC therapy with occurrence rates of 12%, 26.6%, and 29.1% in 6-, 12- and 18 months.<sup>73</sup> Moreover, a report from the US shows that 5 out of 18 patients (28%) who were transplanted for HCV-related HCC show unusually high rates of recurrent HCC within six months post DAA therapy. In contrast, only 9.5% recurrence of HCC was seen in those who did not receive HCV treatment.<sup>74</sup> However these unexpected findings were not verified by other publications, which reported no such risk for HCC recurrence after viral cure by DAAs therapy.<sup>75–77</sup>

## HCC Risk After HCV Cure Through Meta-Analysis

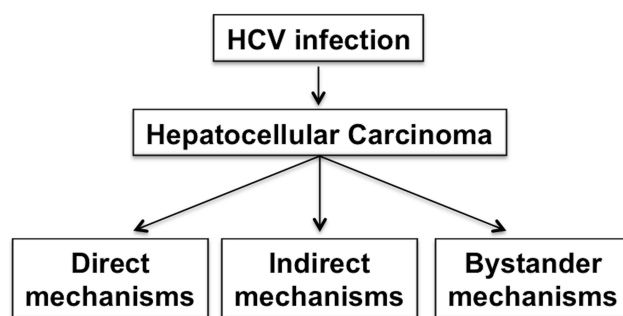
The risk of HCC occurrence and recurrence after HCV cure by DAAs has been determined through meta-analyses. These reports found that the risk of HCC occurrence and recurrence is almost similar between DAAs and IFN-therapy. However, the data agree with the fact that the risk of HCC occurrence persists after HCV treatment.<sup>55–57</sup>

To determine the incidence of HCC occurrence after HCV treatment with DAAs, Rutledge et al performed a more rigorous data analysis of 44 studies with a large number of patients (n=91,249). It is found that the risk of HCC development was 3.57%/year in patients who achieved SVR by DAAs and 9.83%/year in patients who did not achieve SVR.<sup>56</sup> The study of Huang et al involving 61,334 patients who received DAAs from 16 sites showed that the risk of HCC development in SVR-achieved and SVR non-achieved groups was 3.5%/year and 9.1%/year respectively.<sup>81</sup> Waziry et al analysis presented evidence that the overall risk for HCC occurrence is 2.96%/year and recurrence 12.16%/year when HCV infection is cleared by DAA-based therapy.<sup>57</sup> A meta-analysis using 16 studies of IFN-based treatment of 1043 patients and 33 studies of DAA-based treatment of 4876 patients determined the risk of HCC recurrence. The recurrence rate of HCC was 16.7%/year in the DAA-based treatment group and 14.3%/year in IFN-based treatment group.<sup>56</sup> In addition, Huang et al analysis revealed that the HCC recurrence rate is 17.4%/year following DAA-induced viral clearance.<sup>81</sup> A comprehensive review by Singal et al summarized data of the studies that reported the risk of HCC occurrence and recurrence after DAAs treatment.<sup>82</sup> These findings suggest that the risk of HCC occurrence and recurrence remains after viral cure by DAAs treatment. Therefore, most of the authors recommend that patients with advanced liver fibrosis (F3-4) need continuous surveillance for early HCC detection after HCV eradication by DAAs.

## HCC Mechanisms Associated with Chronic HCV Infection

Hepatitis C virus infects hepatocytes, the major cell types that constitute the liver. Only 25% of individuals acutely infected with HCV can eliminate the virus naturally while the rest develop a persistent infection, and chronic liver disease, including liver fibrosis, cirrhosis, and HCC.<sup>83</sup> Chronic HCV infection causes breakdown of immune tolerance leading to a prolonged inflammatory reaction in the liver. A wide range of non-viral agents induces cellular stress and liver injury leading to sterile chronic inflammation. During the early stage of injury, the functional and morphogenic alterations in the liver are reversible if the damage-causing agents or stress factors are removed. The persistence of chronic injury can cause irreversible harm, where the liver undergoes changes from physiological adaptation to

pathological adaptation. Liver cirrhosis is a pathological adaptation to cellular stress causing structural and functional changes in the liver parenchyma to escape from injury. The nature and severity of cellular stress determine whether cellular adaptation to virus infection is reversible or irreversible. Over the years, studies using transgenic mice and cell models revealed that HCV viral proteins altered multiple cell signaling pathways implicated in cell survival, proliferation, migration, and transformation. Many of these cell-signaling pathways overlap among hepatic steatosis, alcoholic liver disease, insulin resistance, and oxidative stress, which cause inflammation, cell death, and liver cirrhosis. The evolution of HCC from cirrhosis is a pathological adaptive response to ISR. Only 1–3% of patients with chronic HCV infection develop HCC after 30 years and HCV does not infect HCC tumor cells as compared to surrounding non-tumorous hepatocytes, supporting the conclusion that HCV is not directly oncogenic. It is possible that HCV infection creates a tumorigenic environment that promote cellular transformation of uninfected hepatocytes through a bystander mechanism as seen in the case of colorectal cancer associated with *Fusobacterium nucleatum* and gastric cancer related to *Helicobacter pylori*. Based on these evidences, we propose that HCC mechanisms associated to HCV infection can be direct virus-induced cellular programming, indirect host-related inflammatory response, and an overlapping host metabolic bystander effect (Figure 2). In the following sections, we discuss the molecular basis of hepatic pathological adaptation to the virus-associated stress response, indirect stress related to inflammation, and metabolic stress response of host that contributes to the persistent fibrosis and HCC. In the end, we discuss possible overlapping synergy mechanisms between viral-induced oncogenic and host-related pathways due to concomitant liver diseases



**Figure 2** Hepatocellular carcinoma mechanisms related to chronic HCV infection are the combination of virus-mediated (direct), host-mediated (indirect), and host-related bystander effects.

that contribute to the persistent HCC risk after DAAs treatment independent of the virus.

## Direct Oncogenic Mechanisms

### HCV Replication in Hepatocytes

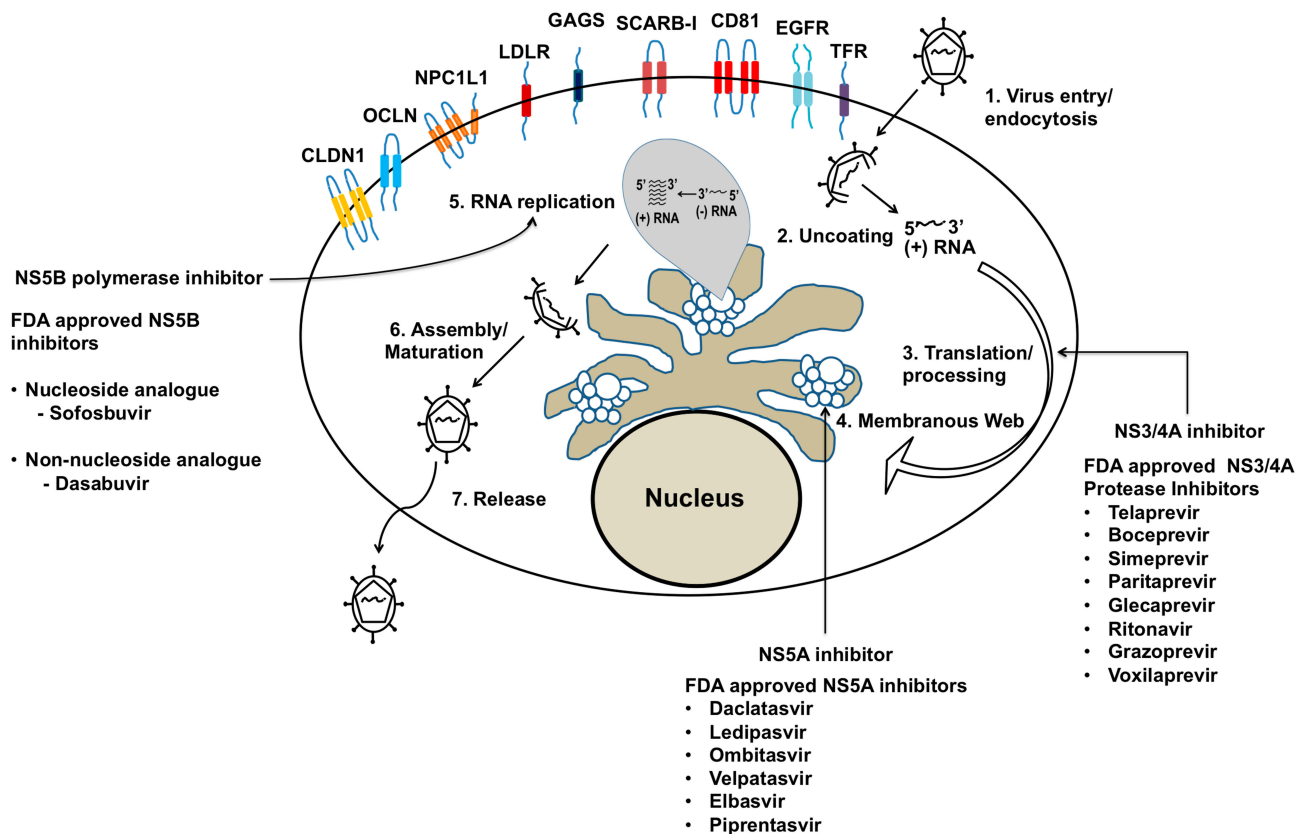
#### Generates an Integrated Stress Response

HCV is a small, enveloped, positive-stranded RNA virus belonging to the *Flaviviridae* family. The complete enveloped HCV particles are icosahedral with a diameter of 56–65 nm, and an embedded viral core around 45nm in size.<sup>84,85</sup>

Hepatocytes are the only cell type in the human liver that support the entire life cycle of HCV through multiple steps: attachment and entry, viral protein synthesis, virus replication, virus assembly, and release.<sup>86,87</sup> The infection cycle involves the attachment and entry of HCV particles into hepatocytes through the interaction of its two viral envelope proteins (E1 and E2) with the cell surface receptors. Multiple host cell-surface proteins are involved in the attachment and entry of the HCV particles into hepatocytes.<sup>88,89</sup> During the entry process, the fusion of the HCV viral envelope with the host endosomal membrane results in pH-dependent uncoating, releasing the genomic RNA into the cytoplasm. The HCV RNA genome makes viral proteins for its reproduction in a specific region of the ER called the rough ER. The positive-strand HCV RNA genome binds directly to ribosomes through an Internal ribosome entry site present in its 5' untranslated region. Translation of the HCV genome leads to the production of a single large polyprotein of 3000 amino acids. This protein is subsequently cleaved by cellular and viral proteases into the structural proteins (core and envelope proteins E1 and E2), and non-structural proteins (P7, NS2, NS3, NS4A, NS4B, NS5A, and NS5B).<sup>90,91</sup> The viral proteins assemble in the ER membrane vesicles to reproduce more genomic HCV RNA and progeny viral RNA. The non-structural proteins are needed for genome replication.<sup>90</sup> Multiple rounds of viral replication lead to increased accumulation of positive-strand RNA, replicative intermediates (negative-strand RNA), and viral proteins in the ER. Sustained virus replication results in the proliferation and remodeling of the ER membranes into a structure referred to as the membranous web. Structural proteins remodel the ER membranes to recruit more protein components needed for virus replication, assembly, and release.<sup>92</sup> It is not clear how the replication and assembly processes occur one after the other, but they appear to occur in lipid droplets accumulated in the ER vesicles. Intrahepatic HCV replication stimulates lipid metabolism, leading to the accumulation of lipid droplets in multilayer membrane vesicles, which facilitates virus

assembly and maturation. Virus particle assembly and release occur in the membranous web, a link to very low-density lipoprotein synthesis and secretion. HCV RNA replication is catalyzed by the viral NS5B, an RNA-dependent RNA polymerase, via the negative-strand RNA. The protease and helicase domains of NS3 play essential roles during the viral genome replication. The NS5A protein is required for the formation of the membranous web that supports viral replication and assembly. Studies have shown that NS5A inhibitors prevent the formation of membranous web structures in the ER by decreasing the complex formation with phosphatidylinositol 4 kinase III alpha.<sup>93</sup> Several viral proteins and host cellular factors essential for HCV replication could be promising targets for antiviral therapy. Among these, the most successful viral marks used in DAAs treatment are the HCV NS3 protease, NS5A, and the NS5B viral RNA polymerase (Figure 3). A recent review describes the mechanism of how different DAAs inhibit HCV replication.<sup>94</sup> Although the causal

relationship between chronic HCV infection and the development of HCC has been established, the exact HCV cancer mechanism is unknown.<sup>95,96</sup> Hepatocytes are specialized cells in the liver with an elaborate ER membrane network. The ER membrane network transports proteins and lipids to lysosomes, mitochondria, secretory vesicles, the Golgi apparatus, endosome, and the cell membrane. The ER plays an essential role in maintaining hepatocyte function in the liver. The proteins synthesized in the ER must be correctly folded and undergo post-translational modifications such as glycosylation, disulfide bridge formation, and oligomerization. All of these processes take place in the ER. The ER stress response can develop due to alterations in protein synthesis, degradation or folding during viral infection. Due to these reasons, the stress response in the ER can activate pathways that trigger cell injury, inflammation associated with chronic liver disease, liver cirrhosis, and HCC related to both viral and non-viral causes.<sup>97</sup>



**Figure 3** Chronic HCV replication in liver cells induces a multifaceted stress response. The infection is initiated by the attachment and entry of the virus particle through several cell surface receptors. The HCV RNA binds to the ribosome and translates a single large polypeptide, which is processed into structural and non-structural proteins. Accumulation of viral proteins induces proliferation of ER-membranes and formation of membranous web structure. HCV replication in the membranous web produces many new genomic positive-strand and negative-strand RNA. Genomic HCV positive-strand RNA packages into complete infectious virus particles that release through the secretory pathway. Arrows show the emerging new DAAs targeting the viral protein and HCV replication cycle.

**Note:** Adapted from Chapter 8, Figure 2 of *Viral Polymerases: Structures, Functions and Roles as Antiviral Drug Targets*, Dash S, Aydin Y, Stephens CM. In: Gupta SP, editor. *Hepatitis C Virus NS5B RNA-Dependent RNA Polymerase Inhibitor: An Integral Part of HCV Antiviral Therapy*, 211-235, copyright (2019), with permission from Elsevier.<sup>383</sup>

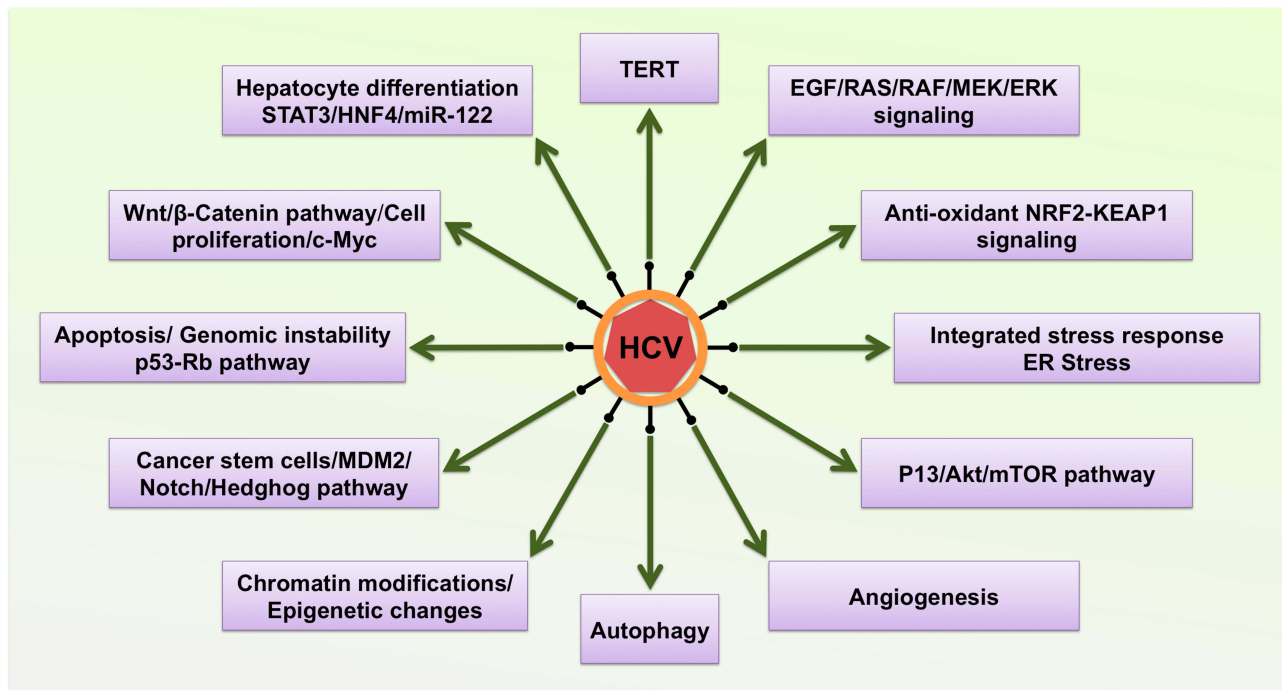
## Hepatocytes in the Liver Undergo Pathological Adaptation to HCV Microbial Stress

The increase in cellular energy demand during chronic virus infection imposes all types of caloric restrictions, and low levels of ATP, amino acids, and sugar, leading to increased ER stress.<sup>98</sup> Autophagy is activated when the ATP and amino acid levels are depleted during infection. Low intracellular sugars will cause a defect in protein glycosylation and alter ER and Golgi function leading to the stress response. Low oxygen supply (hypoxia) during viral replication creates oxidative stress that generates ROS, which can cause ER stress. Chronic HCV infection induces lipid metabolism in the liver, leading to the accumulation of fat in hepatocytes and hepatic steatosis. To study the complex lipid metabolic remodeling in infected cells, Hofmann et al determined the lipid composition of whole cells and subcellular fractions of HCV-infected culture using a bioinformatics approach. They showed that HCV infection accumulates membrane lipids, especially cholesterol and phospholipids, with a higher abundance of phosphatidylcholines and triglycerides with longer fatty acyl chains. The accumulation of longer fatty acids and cholesterol in the infected cells could inhibit the ubiquitin-dependent protein degradation pathway; therefore, increasing ER stress.<sup>99</sup> Persistent virus replication increases cellular DNA damage response, the frequency of DNA repair, transcriptional fidelity, and genomic instability, all of which create additional cellular stress. If the viral-induced stress becomes persistent and severe, that results in irreversible injury or death of infected cells. Hepatocytes experience different levels of cellular stress during virus infection that activates various cell death cascades (apoptotic, necrotic, or autophagic) during chronic liver disease. The release of DAMP from lysed necrotic cells can produce ROS and RNS, which are known to cause lipid peroxidation.<sup>100</sup> This process can activate the unfolded protein response and NF- $\kappa$ B pathway, which is the hallmark of an inflammatory response implicated in the development of liver fibrosis and HCC during chronic HCV infection.<sup>101</sup> In some individuals, combinations of multiple non-viral insults may contribute to liver disease progression. One of the host-related factors responsible for the progression of liver disease is the degree of hepatocellular injury. Aminotransferases are a group of enzymes that synthesize and break down amino acids and convert them into energy storage molecules. Increased ALT and AST levels in the blood are a direct indication of a liver injury. Prior studies found that 1–14% of cells in the HCV-

infected liver showed Ki-67 labeling, suggesting that the hepatic parenchymal mass is maintained through continuous hepatocyte regeneration and self-replication.<sup>102,103</sup> The physiological adaptations at the level of cellular metabolic activity and cellular function, which often occur during chronic infection, are reversible. However, pathological adaptation due to severe microbial stress can result in an increase or decrease in cellular appearance and cell proliferation. Due to these reasons, hepatocyte proliferation rates drop significantly during the stage of liver cirrhosis due to pathological adaptation to stress.<sup>104,105</sup> Infected hepatocytes can manage the stress response through the induction of ISR that promotes the transcription of numerous genes for cell survival.<sup>106</sup> The significant reduction of hepatocyte proliferation in liver cirrhosis could activate stem cell compartment through epigenetic programming.<sup>107–109</sup> The stress signal reprograms to switch cell death to cell proliferation, and it will abet the emergence of malignancies like HCC. In a previously published series, we showed that chronic HCV infection induces ER stress and the expression of ER stress marker remains high in patients with liver cirrhosis.<sup>110</sup> Using the HCV cell culture model, we showed that excessive ER stress activates NRF2-mediated autophagy switching to promote cell survival.<sup>111</sup> The excessive stress contributes to HCC development in liver cirrhosis. HCC grown in the cirrhotic liver undergoes autophagy switching from a protective state characterized by high macroautophagy with low CMA to an HCC-promoting state characterized by low macroautophagy with high CMA.<sup>112,113</sup> Our data are also consistent with previous reports suggesting that persistent activation of NRF2 through the accumulation of p62 is involved in HCC development.<sup>114,115</sup> In the following section, we review how pathological adaptation to HCV-induced stress results in cellular oncogenic programming implicated in HCC development.

## Hepatic Adaptation to HCV Microbial Stress Induces Oncogenic Cell Programming

An integrated analysis of whole-exome sequencing of HCV-related HCC tumors demonstrated many mutations in cancer driver genes associated with malignant transformations.<sup>116–125</sup> The exome sequencing study found that tumor suppressor genes are downregulated due to promoter hypermethylation, and genomic instability in HCC due to the gains and loss in chromosomes was found in more than 80% of HCC associated with chronic HCV infection.<sup>119–125</sup> The most significant



**Figure 4** Hepatic adaptive response to chronic HCV infection activates hepatocyte cell survival programming. HCV structural and non-structural proteins activate multiple cellular pathways.

cellular pathways that are altered in HCV-induced HCC are TERT,  $\beta$ -catenin, p53, Rb, chromatin remodeling/epigenetic modifications, hepatocyte differentiation, PI3K-mTOR pathway, and NRF2-keck-like ECH-associated protein (KEAP), cancer stem cells, angiogenesis and RTKs (Figure 4). In the following section, we discuss the molecular mechanism by which HCV regulates these cancer pathways.

### Telomerase Reverse Transcriptase (TERT)

Telomeres are DNA-protein structures located at the end of each chromosome. They are composed of a tandem repeat of six-nucleotide sequence (5'-TTAGGG-3') coated with shelterin proteins. The telomere can reach a length of 15,000 base pairs. Telomeres function by preventing chromosomes from losing base pair sequences and fusing at their ends. When the telomere length becomes too short, the cell dies by apoptosis. Telomere length is maintained by an enzyme called TERT. Telomerase is an enzyme made of protein and RNA subunits that elongates chromosomes by adding TTAGGG sequences to the end of existing chromosomes. Telomeres and telomerase play an essential role in the progression of liver disease and liver cirrhosis. Hepatocyte senescence and development of liver cirrhosis relate to the absence of telomerase activity and shortening of telomeres. Telomerase detected in human cancer cells is found to be 10–20 times more active than

in healthy body cells.<sup>126</sup> Increase telomerase provides a selective growth advantage to tumor cells. Complete HCC genome sequencing found many hot spots activating mutations in the promoter regions of the TERT gene in about 85% of human tumors, including HCC.<sup>127,128</sup> The modifications are present at nucleotides 124 (mostly G>A and rarely G>T) or 146 (G>A) before the ATG start site in the TERT promoter region.<sup>127</sup> These mutations favor the binding of transcription factors to the promoter, causing TERT overexpression and activation. The long telomeres and increased TERT activity are found to be associated with aggressive HCC with poor prognosis.<sup>129</sup> A previous study showed a mutation in the TERT gene among patients with liver cirrhosis of diverse etiologies: alcohol, hepatitis B, and hepatitis C infection as compared to healthy controls.<sup>128</sup> Reduced telomerase activity could result in telomere shortening, chromosomal instability through end-to-end chromosome fusion, and HCC if the p53 and Rb are defective. Recent publications by Zhu et al suggest that HCV core and NS3 proteins can activate TERT expression and reverse transcriptase activity.<sup>130,131</sup>

### The p53-Rb Pathway

The two tumor suppressors, Rb and the p53 are involved in the etiology of many cancers. The p53 protein levels are increased in response to DNA damage, hypoxia, and



oncogene activation. p53 regulates gene expression that leads to cell cycle arrest or apoptosis. The Rb protein prevents cell proliferation by repressing the activation of E2F transcription factors. The interaction between Rb and E2F transcription factors are critical, preventing the S-phase of the cell cycle and mitosis.<sup>132</sup> Many earlier studies have shown that transient expression core, NS3, and NS5A proteins of HCV alter p53 tumor suppressor function without using the infectious HCV replication model. We discuss here some of the studies that examined the impact of HCV infection on the roles of p53 and Rb tumor suppressors. Mitchell et al showed that HCV infection disrupts p53 function through activation of cellular protein kinase R.<sup>133</sup> Stanley Lemon's laboratory demonstrated that HCV infection negatively regulates Rb protein stability mediated by NS5B protein.<sup>134,135</sup> Studies conducted in our laboratory showed that excessive ER stress due to HCV replication degrades p53 in the lysosomes. The degradation of p53 in HCV culture occurs independently of MDM2. We showed that CMA activation by stress degrades p14ARF, another alternative open reading frame protein (ARF), a tumor suppressor that activates p53.<sup>136</sup> The subsequent publication showed that excessive ER stress induces MDM2-mediated Rb degradation.<sup>137</sup> All these results are consistent with human studies that show more than 70% of HCC cases having alterations in the p53-Rb pathway that leads to mitosis, cell cycle progression, and genomic instability.

### The Wnt/ $\beta$ -Catenin/c-Myc Pathway

The Wnt/ $\beta$ -catenin pathway is altered in 66% of HCC, and in 51% of these cases, activation of Wnt/ $\beta$ -catenin occurs due to altered gene expression.<sup>117</sup> The Wnt/ $\beta$ -catenin pathway is activated when a Wnt ligand binds to the frizzled receptor, leading to activation of the transcription factor  $\beta$ -catenin and subsequent activation of pro-survival genes. Wang et al showed that HCV infection could activate the Wnt/ $\beta$ -catenin pathway. The HCV core protein was shown to activate Wnt/ $\beta$ -catenin signaling at multiple steps, including elevated expression of Wnt ligands, and frizzled receptor, and downregulation of the expression of low-density lipoprotein receptor-related protein 5/6. Core protein has been shown to decrease the expression of the Wnt antagonists; *dickkopf* and secreted frizzled-related protein by recruiting DMT1, and HDAC1 to their transcription start sites.<sup>138,139</sup> The NS5A protein activates PI3K/Akt signaling, leading to the inactivation of GSK3 $\beta$  and subsequently reducing the degradation of  $\beta$ -catenin.<sup>140,141</sup> The activation of c-Myc

oncogene through Wnt/ $\beta$ -catenin pathways has been shown to promote HCC in HCV transgenic mice model.<sup>142</sup>

### Receptor Tyrosine Kinases (RTKs)

The RTKs are a large superfamily of cell surface receptors representing for a wide variety of growth factors, including epidermal growth factor, nerve growth factor, PDGF, VEGF, FGF, insulin and the insulin-like growth factors. Among these, EGFR controls the cascade of oncogenic cell signaling involved in cell proliferation that contributes to hepatocarcinogenesis. The EGFR is highly expressed in the adult liver and plays an essential role in hepatocyte proliferation. The EGFR pathway is activated in 60–80% of HCC and correlates with aggressive tumors and patient survival.<sup>143–150</sup> The receptor-mediated endocytosis and lysosomal degradation are the major negative feedback loops for EGFR signaling. We showed that HCV induces impaired autophagy response to inhibit degradation of EGFR at the level of autophagosome-lysosome fusion leading to the activation of downstream RAS/RAF/MEK/ERK signaling.<sup>111</sup> In principle, impaired autophagy due to HCV could potentially stabilize RTK on the cell surface of infected cells by impairing their endocytosis and lysosomal degradation. Other researchers have also shown that EGFR activation favors the HCV entry process through co-internalization of an HCV-CD81-EGFR complex following binding of EGFR ligands to the receptor and subsequent endocytosis.<sup>151</sup> The viral NS5A protein disturbs EGFR trafficking and degradation, therefore, activates EGFR signaling.<sup>152</sup> All these data support that HCV infection activates EGFR signaling, which contributes to the HCV-associated HCC development. The EGFR pathway activation can cross-talk with Wnt/ $\beta$ -catenin since EGFR can phosphorylate  $\beta$ -catenin at residue Tyr654, therefore dissociating from the multi-receptor complex and leading to nuclear entry and gene expression.<sup>153</sup> The EGFR stimulates PI3K/Akt and RAS/RAF/MEK/ERK cascade that can activate  $\beta$ -catenin through GSK3 $\beta$  activity. Wnt/ $\beta$ -catenin signaling also activates FGF signaling implicated in HCC development secondary to chronic HCV infection by inducing expression of FGF18 and FGF20.<sup>154,155</sup>

### PI3K/Akt/mTOR Pathway

The activation of the mTOR pathway is associated with HCC development related to chronic viral infection.<sup>156,157</sup> Immunohistochemical staining revealed that 33 out of 73 (45%) HCC patients showed increased expression of total S6k, which is correlated with mTOR activation and tumor size.<sup>158</sup> In a large cohort of HCC patients, the activation of the

mTOR pathway was associated with tumor differentiation, staging, vascular invasion, and expression of phosphoS6.<sup>159</sup> The mTOR pathway can be activated by growth factors, cytokines, TLR ligands, low cellular energy (ATP/AMP ratio), hypoxia, and DNA damage. The activation of mTOR can confer many growth advantages to cancer stem cells or progenitor cells, such as promoting cell proliferation and resistance to apoptosis induced by various stress signals such as hypoxia and nutrient deficiency.<sup>160</sup> In addition, the mTOR pathway can regulate telomerase activity in HCC since rapamycin significantly decreases telomerase activity at the protein level.<sup>161</sup> NS5A can activate PI3K-mTOR signaling by directly binding to the p85 subunit of PI3K.<sup>162</sup> The mTOR activation by the NS5A protein blocks apoptosis through binding to FKBP38, an immunosuppressant FK506-binding protein.<sup>163</sup>

### Angiogenesis

Angiogenesis, a physiological process that generates new blood vessels from the existing vessels, is linked to HCC development. It has been shown that HCV promotes angiogenesis process during an advanced stage of liver disease.<sup>164,165</sup> In healthy tissues, the angiogenesis process is inhibited by interactions between proangiogenic and antiangiogenic factors.<sup>165</sup> Angiogenesis is activated when tumor tissue requires additional nutrient and oxygen supply.<sup>166</sup> HCC cells secrete proangiogenic factors and activate endothelial cells by VEGF and FGFs. The expression levels of angiogenic growth factors, VEGF-A, angiopoietin-2, and PDGF are elevated in HCC patients.<sup>167,168</sup> HCV infection can trigger hepatic angiogenesis and HCC growth through HIF-1 $\alpha$  and VEGF regulation.<sup>169,170</sup> The VEGF activation can occur through several other cell survival pathways, including PI3K/Akt, ERK1/2, NF- $\kappa$ B, and STAT3, which stabilize HIF-1 $\alpha$ . It has been demonstrated that the core protein of HCV can trigger angiogenesis through a mechanism that involves cross-talk between TGF- $\beta$ 2, VEGF, and CD34 expression.<sup>171</sup>

### Cancer Stem Cell

Pathological adaptive response to microbial stress results in altered gene expression through epigenetic alterations without changing the DNA nucleotide sequences.<sup>172,173</sup> The epigenetic modifications of chromatin are involved in the maintenance of viral latency and reactivation of human viruses such as HIV, cytomegalovirus, Epstein-Barr virus and many others.<sup>174</sup> The potential role of epigenetic modification in cell differentiation, tissue homeostasis, and

regeneration has been recently understood through stem cell research. The pluripotent embryonic stem cells are capable of giving rise to different cell types in embryo that serve as a valuable model to study epigenetic mechanisms involved in tissue development and tissue-specific gene expression.<sup>175</sup> Disruption of epigenetic processes can lead to altered gene expression and malignant transformation.<sup>176</sup> The chromatin that package chromosomes are the macromolecular complex of DNA and histone proteins. The basic functional unit of chromatin includes a nucleosome that contains 147 base pairs of DNA, which wrapped around a histone octamer that contains two of each histone H2A, H2B, H3, and H4. Condensed chromatin called heterochromatin that leads to gene silencing, whereas open chromatin called euchromatin leads to active gene transcription.<sup>177</sup> The epigenetic modifications occur through DNA methylation, histone modifications, nucleosome positioning, and ncRNAs. The interplay between these chromatin modification mechanisms creates an epigenetic landscape of mammalian gene expression. DNA methylation of CpG-rich DNA sequences called CpG island leads to gene silencing. Histone proteins N-terminal tails undergo a variety of post-translational modifications, including methylation, acetylation, ubiquitination, sumoylation, and phosphorylation. Histone modification can lead to either gene activation or repression, depending upon which histone residues are modified. For example, lysine acetylation correlates with transcriptional activation, whereas lysine methylation leads to transcriptional activation or repression depending on which residue is modified and the degree of methylation. The trimethylation of lysine four on histone H3 (H3K4Me3) is enriched at the promoter that is transcriptionally active, whereas trimethylation of H3K9 (H3K9Me3) and H3K27 (H3K27Me3) of the promoter are transcriptionally inactive.<sup>176</sup> For example, embryonic stem cells possess bivalent domains with both active (H3K4Me3), and repressive (H3K27Me3) marks at the promoters of the developmental genes that regulate multiple cell types in a tissue. The activity of such bivalent domains is regulated by two counteracting groups of chromatin proteins called the trithorax group and polycomb group proteins.<sup>178,179</sup> In mammals, the polycomb group proteins are an epigenetic modifier that promotes H3K27 trimethylation and condenses chromatin resulting in gene silencing. This interaction favors the binding of the trithorax group of proteins to the activating H3K4 trimethylation mark that leads to open chromatin and active gene transcription. This bivalency hypothesis has been found to contribute to phenotypic plasticity and initiation of cancer stem cells. This condition allows differentiated cells to lose

this bivalency through rigid chromatin structure, whereas the maintenance of active chromatin states favors tumorigenesis, stem cell renewal, and proliferation. Some reviews describe the importance of epigenetic modification on cell fate and cancer initiation.<sup>179–181</sup> The polycomb repressive protein complexes (PRC1 and PRC2) are involved in CSCs to establish dynamic epigenetic changes needed for the embryonic stem cell gene signature.<sup>180–182</sup> These advances have led to the discovery of epigenetic drugs that have been now FDA-approved for the treatment of cancer, and viral infection including DMT inhibitors (azacytidine, decitabine) and HDAC inhibitors (vorinostat, romidepsin, belinostat, panobinostat).<sup>183</sup> A study published by Ali et al shows that HCV replication in cultured cells can induce expression of cancer stem markers including, doublecortin and CaM kinase-like-1, LGR5, CD133, AFP, cytokeratin-19, LIN28, and c-Myc. They showed that curing HCV replication of these cells results in diminished expression of these factors.<sup>184</sup> Another report showed HCV NS5A transgenic mice fed with alcohol developed HCC with stem cell regulator Nanog expression through the TLR4 signaling.<sup>185</sup> Human genome microarray study using a Huh-7.5 cell line stably-replicating HCV sub-genomic RNA revealed the upregulation of cancer stem cell markers: octamer-binding protein 3, SOX2 and suppressor of zeste 12 homolog.<sup>186,187</sup> The authors showed that the expression of enhancer of zeste homolog 2, a member of PRC protein was overexpressed in HCC as compared to the normal liver.<sup>186</sup> Notch signaling is involved in the maintenance of CSCs.<sup>188</sup> Studies found that NS3 protein can activate notch signaling through binding Snf2-related CBP activator protein and enhances hairy enhancer of split-1 promoter activity that leads to increased hairy enhancer of split-1 expression, a transcriptional repressor of cell differentiation, thus providing a mechanism on how HCV infection promotes cancer stemness.<sup>189–191</sup> The Hedgehog pathway is also important for the maintenance of stem cell homeostasis is activated during HCV infection.<sup>192,193</sup> One study found that the Hedgehog pathway plays a role in HCV replication and viral permissiveness.<sup>194</sup> We showed that HCV-induced cellular ER stress could activate MDM2 that degrades Rb tumor suppressor independent of p53.<sup>136</sup> A recent report shows that MDM2 can associate with the PRC2 and enhances stemness through chromatin modification independent of p53.<sup>195</sup> This evidence suggests there are many oncogenic pathways that HCV replication probably induces epigenetic cell programming that induces CSCs leading to HCC.

## Epigenetic Modifications

Epigenetic modifications are frequently seen in human HCC with hypermethylation of tumor suppressor genes, hypomethylation of oncogenes, and methylation of repetitive elements. Stefanska et al reported that approximately 3700 promoters that are hypomethylated in HCC using a combination of methylated DNA immunoprecipitation and hybridization. The demethylated genes are mainly involved in cell growth, cell adhesion and communication, signal transduction, mobility, and invasion, functions that are essential for cancer progression and metastasis.<sup>196</sup> A study by Nishida et al indicates that the number of tumor suppressor genes is hypermethylated in human HCC.<sup>197</sup> Some recent publications have examined genome-wide epigenetic changes during the progression of HCV-associated cirrhosis to HCC, and their findings showed a potential prognostic value of DNA methylation of some specific gene promoters, CpG islands and CpG island shores.<sup>198,199</sup> Alterations in histone modifications also have been observed in HCC development.<sup>200</sup> Increased expression of HDACs that are aberrantly expressed in HCC, which provides a rationale for HCC treatment using a novel pan-HDAC inhibitor (panobinostat).<sup>201</sup> Numerous small RNA and ncRNAs are also involved in the epigenetic mechanisms involved in HCV-induced HCC development. Some of them have been used as biomarkers for early detection of HCC.<sup>202,203</sup> A recent report showed that long ncRNAs are highly expressed in HCC, and they promote liver cancer stem cell growth through epigenetic regulation.<sup>204</sup> Cheng et al study showed that liver-specific miR-122 is epigenetically suppressed by HOTAIR, leading to activation of Cyclin G1 and HCC growth.<sup>205</sup> Recent research from our laboratory shows that hepatic adaptive response to HCV-induced ER stress and oxidative stress can silence miR-122 through induction of the STAT3-HNF4 $\alpha$ -miR-24 inflammatory feedback loop.<sup>206</sup> In this study, we found that miR-122 levels severely depleted among patients who developed cirrhosis. These data suggest that pathological adaptive response to HCV microbial stress epigenetically silence HNF4 $\alpha$  and miR-122.

## Indirect Oncogenic Mechanisms

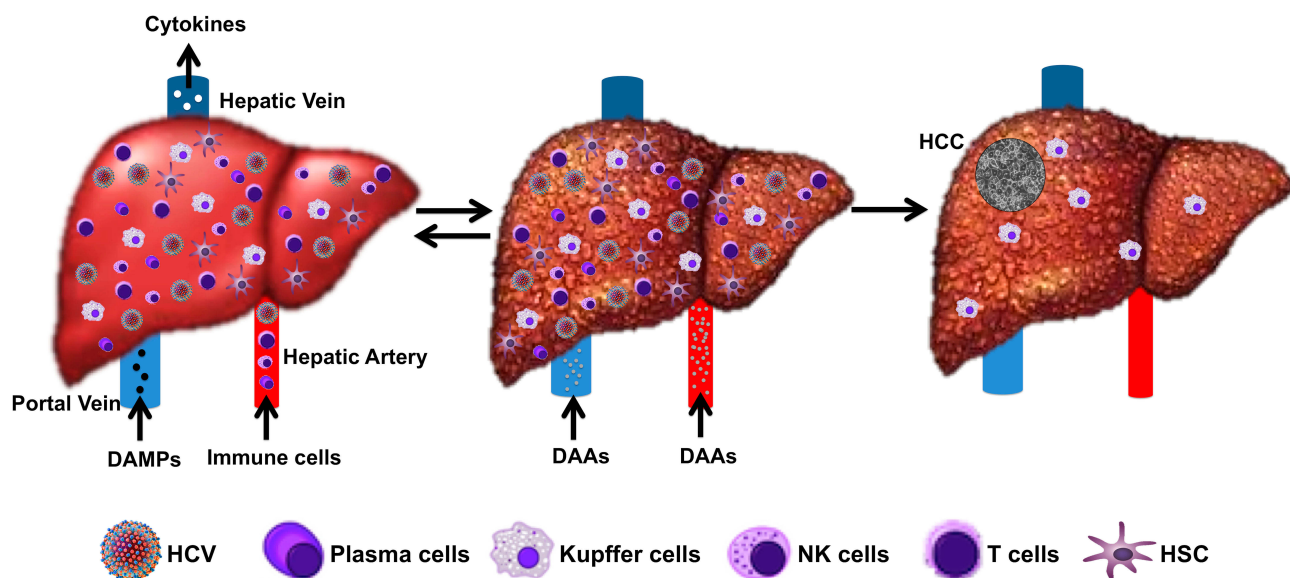
The liver is the most immune-privileged organ in the human body. It filters most of the microbial pathogens that enter through the hepatic artery and portal vein blood. The liver is also regularly exposed to the gut microbiota, toxic metabolites, food allergens derived

from the gastrointestinal organs through the portal vein. A durable protective innate immune response through the activation of immune and non-immune cells eliminates pathogens to prevent liver injury. In this section, we describe basic molecular mechanisms of how persistent HCV replication breakdown immune tolerance in the liver that leads to the chronic infection and liver disease progression. The liver is composed of primary hepatocytes (80%), and the remaining non-parenchymal cells are KCs, stellate cells, sinusoidal endothelial cells that are essential for innate immune surveillance.<sup>207</sup> The liver contains many other cell types that constitute the innate and adaptive immune cells of lymphoid origin, such as T cells, NK cells, NKT cells, MAIT cells, and B cells.<sup>208</sup> The liver houses abundant APCs, such as MDCs, PDCs, KCs, sinusoidal endothelial cells, monocytes/macrophages, neutrophils, stellate cells.<sup>209,210</sup> During HCV infection, a both innate and adaptive immune response is activated through multiple mechanisms. For example, hepatocytes and other antigen-presenting cells quickly sense the conserved PAMP receptors present in HCV (HCV genomic RNA, structural and non-structural proteins) by different pattern recognition receptors such as RIG-1, TLRs. The acute inflammatory response can also be generated by the DAMP released in response to cell death (called sterile agents) during integrated cellular stress and cell damage. The innate immune signaling pathways are amplified

through the production of IFN (type 1 and type III), ISGs, and proinflammatory cytokines to eliminate virus. In the case of HCV, a very few individuals are resolved infection; naturally, the majority of cases disease becomes chronic. It has been demonstrated that several HCV proteins impair the cytotoxic and immunoregulatory activities of immune cells such as APCs, NK cells, CD4 T cells, and CD8 T cells, therefore, overcomes the host innate and adaptive immune response leading to a stage of chronic infection.<sup>211–214</sup> We review different immune cells that involved in innate and adaptive immune response and basic mechanisms of how the rapid expansion of early T cell exhaustion or depletion leads to chronic HCV infection (Figure 5).

### Antigen-Presenting Cells

KCs are the primary tissue-resident macrophages located in the liver sinusoids play an essential role in the intrahepatic innate antiviral response during HCV infection. These cells first encounter microbial pathogens that enter the liver through the natural antiviral program.<sup>215</sup> A number of researchers have shown that HCV core and NS3 proteins can activate KCs via TLR to produce inflammatory cytokines (IFN- $\beta$ , IL-1 $\beta$ , IL-6, TNF- $\alpha$ ) and these inflammatory cytokines suppress HCV replication.<sup>216–220</sup> Numbers and expression of KCs specific markers (CD163 and CD33) were found increased during chronic HCV infection.<sup>221,222</sup>



**Figure 5** Hepatic adaptive response to inflammatory stress progresses to cirrhosis. Blood supply to the liver by both the hepatic artery and the portal vein brings potential pathogens, including HCV. Kupffer cells and sinusoidal endothelial cells recognize HCV-derived pathogen-associated molecular pattern (PAMP). The activation of the innate immune response through Kupffer cells recruit new adaptive immune cells (CD4 and CD8 T cells) and B cells. Sustained inflammation can lead to liver fibrosis, cirrhosis, and HCC. Chronic hepatitis is a physiological adaptation that can be reversible after HCV cure. Pathological adaptation of the inflamed liver can lead to cirrhosis and HCC.

Chronic HCV patients have higher levels of IL-1 $\beta$  as compared to healthy controls. Some researchers showed that the NLRP3/caspase-1 inflammasome activation by hepatic macrophages during HCV infection produces IL-1 $\beta$  leading to proinflammatory cytokine production implicated in the liver disease progression.<sup>223</sup> Although these reports support that HCV generates a strong innate immune response, several studies demonstrated that HCV infection is able to neutralize the proinflammatory innate immune signaling (TLR and RIG-1) in KCs and hepatocytes. Some researchers have shown that HCV is able to neutralize the inflammatory activity of peripheral mononuclear cells and hepatocytes by interfering with TLR and RIG-1 signaling.<sup>224–231</sup> The liver also contains some populations of DCs and macrophages. Liver DCs can be two categories: MDCs and PDCs. The myeloid-derived mononuclear cells (monocytes) are the primary antigen-presenting cells that prime the innate and adaptive immunity during HCV infection. These cells support T cell activation leading to the generation of Th1 response with the production of IL-12 and TNF- $\alpha$ . The PDCs in the liver express PD-L1, a molecule that induces antigen-specific tolerance.<sup>232</sup>

## Natural Killer Cells (NK Cells)

About 30–50% of total intrahepatic lymphocytes are NK cells in humans. Activated NK cells can kill target cells by releasing perforin and granzyme from cytotoxic granules or by engagement of death receptors on target cells. During acute infection, NK cell activation leads to increased production of IFN- $\gamma$  and cytotoxicity. NK cells secrete TNF- $\alpha$  and IFN- $\gamma$  that inhibit HCV replication as well as cytolytic enzymes that destroy HCV-infected cells.<sup>233</sup> The role of NK cells in HCV infection is supported by an earlier publication showing that gene encoding NK cell receptor KIR2DL3 and its ligand HLA-C1 was associated with spontaneous resolution of infection.<sup>234</sup> The explanation for this genetic association was that the NK cells from HLA-C1 homozygous individuals show increased production of IFN- $\gamma$  and greater degranulation than those from nonhomozygous individuals.<sup>235</sup> The NK cell phenotypes and functional changes were found among patients with chronic HCV infection. Increased expressions of activating NK cell receptors such as NKp30, NKp44, NKp46, and NKG2D were reported to be increased during chronic HCV infection.<sup>236–238</sup> Some researchers published data showing the variation of NK cell effector function, with increased cytotoxicity with reduced production of IFN- $\gamma$  and TNF- $\alpha$ .<sup>237–239</sup> This dichotomy of NK cell function was found to be more pronounced among patients who were receiving IFN- $\alpha$  antiviral

therapy.<sup>240</sup> The mechanistic insight into this observation was found to be due to the preferential activation of STAT1 expression over STAT4 by IFN- $\alpha$  treatment.<sup>241</sup> At present, one report claim that DAAs-induced HCV clearance corrects the altered NK cell phenotypes and reduces STAT1 expression and phosphorylation.<sup>242</sup> Another recent study claims that HCV-induced NK cell functional imprinting is not reversible.<sup>243</sup> NK T cells are another group of innate cells, which comprise 26% of intrahepatic lymphocytes and secrete IFN- $\gamma$ , TNF- $\alpha$ , and IL-2.<sup>244,245</sup> Though its precise role in chronic infection is yet unclear, there are indications that NKT cells may influence the balance of Th1 versus Th2 responses to an HCV infection.<sup>246</sup>

## CD4 and CD8 T Lymphocytes

Adaptive immune response in acute infection is activated when HCV antigen-specific T cells are primed by APCs in the lymphoid organs, where they proliferate and then migrate to the liver to execute their effector functions.<sup>247</sup> HCV-specific CD8 T cells can kill HCV-infected hepatocytes via the perforin/granzyme lytic pathway, but also by secretion of Fas ligand (CD178 or CD95L) and inflammatory cytokines, mainly IFN- $\gamma$  to clear HCV infection. The success of the adaptive immune response to HCV requires proper interactions between CD4 T cells and CD8 T cells. In addition to acute resolving infection, the CD4 T cells also contribute to the maturation of memory CD8 T cells that prevent reinfection.<sup>248,249</sup> However, impairment of CD8 T cells response is associated with chronic HCV infection.<sup>250</sup> The expansion of T-reg, expression of multiple co-stimulatory molecules, impaired proliferation capacity, and cytokine production are some of the mechanisms of impaired CD8 T cell response.<sup>251–255</sup> During the chronic stage of HCV infection, CD4 T cells exhibit different phenotypes with a high expression of inhibitory receptors (TIM3, PD-1, and CTLA-4) decreasing the production of IL-21.<sup>256,257</sup> In chronic HCV infection, PD-1 expression was associated with impaired function of CD8 T cells with studies showing that PD-1 inhibition reactivates CD8 T cells function, proliferation, IFN- $\gamma$  production, and viral clearance.<sup>258–261</sup> Tacke et al study show that HCV infection accumulates CD33+ MDSCs in human peripheral blood that suppresses CD8 T cell response through ROS.<sup>262</sup> Lack of resolution of HCV infection due to insufficient CD8 T cell response during chronic HCV infection relate to the fibrogenic response in the liver.<sup>263</sup>

## B Cell Response

The role of neutralizing antibodies and B cells in the progression of HCV-induced chronic liver disease is unclear. B cells may contribute more towards to humoral immunity and antibody-dependent cellular cytotoxicity of HCV-infected cells in the liver. Increasing evidence suggests that neutralizing antibodies are associated with spontaneous clearance of HCV infection and reinfection.<sup>264–267</sup> HCV accounts for 85–95% of mixed cryoglobulinemia, a common extrahepatic manifestation of HCV. The cryoglobulinemia occurs by HCV antigen-driven B cell clonal proliferation leading to the deposition of circulating immune complexes in small vessels of the skin, nerves, kidney, liver, and joints. About 50% of patients with chronic HCV virus infection have circulating cryoglobulins, only about 10–15% show clinical disease of palpable purpura, arthralgias, neuropathy, and glomerulopathy.<sup>268</sup>

## Pathological Adaption to Chronic Inflammation Leads to Liver Cirrhosis

The immune activation cross-talks with the metabolic pathways of immune and non-immune cells to allocate more nutrients (ATPs, amino acids, sugar, and lipids) therefore creating integrated immune stress response. However, chronic inflammation results in repeated cell death, cell injury, DNA damage leading to liver injury. Inflammatory cells also produce chemokines, metabolites, and growth factors. Production of inflammatory cytokines activates cell signaling, thus creates ISR pathways.<sup>269</sup> Prolonged inflammation and immune activation in the liver lead to pathological adaptation. Due to this mechanism, chronic inflammation promotes liver regeneration, fibrosis, and aberrant accumulation of collagenous connective tissue leading to liver fibrosis. Liver cirrhosis represents the pathological adaptation to a long-lasting inflammation secondary to HCV infection (Figure 5). Chronic inflammation can increase the risk of HCC. The impaired immune regulation due to KCs, NK cells, CD4 T cells, CD8 T cells, CD4 T-reg cells, and inflammasome activation are associated with HCC development. A full discussion of the mechanism of chronic inflammation, liver cirrhosis, and HCC falls outside the scope of this review but can be found elsewhere.<sup>270,271</sup>

## Inflammation and HCC Connection

Inflammation is a critical component of liver cirrhosis and HCC development. It is unclear whether the HCV-induced inflammation prevents HCC development (tumor

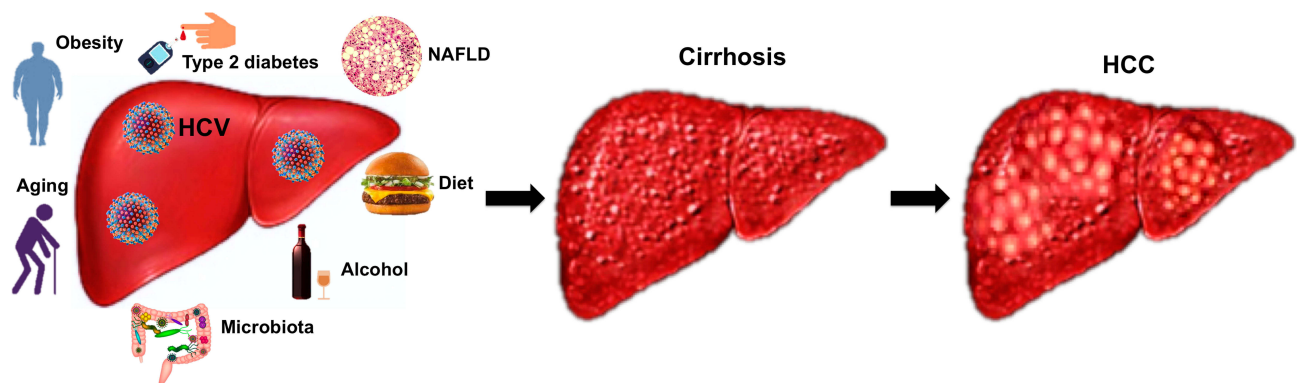
suppressor) or promotes HCC (oncogenic). For example, the presence of life long chronic inflammation with repeated cycles of hepatocyte death and regeneration could be a driver of liver cancer in chronic HCV infection. The inflammatory cells promote HCC by releasing ROS, RNS, lipid peroxidation, and aberrant expression of cytotoxic cytokines. Many inflammatory cytokines released during chronic inflammation such as TNF- $\alpha$ , IL-1 $\beta$ , IL-23, IL-6 are associated with HCC development.<sup>272–274</sup> This hypothesis is supported with the study by Ramzan et al, which showed the presence of high-level liver-infiltrating T and B cells in the cirrhotic liver with HCC as compared to cirrhotic livers without HCC. In this case, the presence of CD8 T cells in the cirrhotic liver promotes HCC development supporting the pro-carcinogenic role of inflammation.<sup>275</sup> On the other hand, lymphocytes infiltrating the liver can be a host response to the virus when the liver failed to clear the virus leading to chronic disease. The sustained accumulation of lymphocytes in the liver can be an unsuccessful host inflammatory response to HCC as well. In this case, the virus-targeted sustained immune activation, and cell death are an anti-tumorigenic response or tumor-suppressive inflammation. The removal of virus-associated immune surveillance by DAAs therapy promotes HCC development, suggesting that HCV-associated inflammation may be tumor suppressive. The presence of low CD8 T cells and a high population of suppressive T-reg cells correlate with HCC recurrence, suggesting that antitumor surveillance role of inflammatory cells inhibited by T-reg cells. In this case, the cirrhotic microenvironment favors recruitment and expansion of committed T-reg cells, thus establishing a state of immune tolerance, ultimately contributing to the evolution of cirrhosis into cancer. A decreased number of innate immunity members, NK and NKT cells, and increased accumulation of T-reg cells supporting the hypothesis that suppressive antitumor immunity correlates with HCC development in chronic HCV infection.<sup>276</sup> The changes in the immune landscape after HCV cure lead to the reactivation of HBV and herpes virus infection, suggesting that HCV induced immune response may also suppress the growth of other microbial contamination.<sup>277,278</sup> The contribution of inflammation in the development of HCC is unclear. Lack of an animal model remains a significant challenge to study the function of different immune components against HCC development related to chronic HCV infection.

## Bystander Oncogenic Mechanisms Overlapping Host-Related Non-Sterile Inflammation

The concomitant liver disease related to non-viral etiologies accelerates HCV-induced liver disease, cirrhosis development, and HCC.<sup>279–287</sup> Obesity is associated with metabolic syndromes such as insulin resistance, type 2 diabetes, and NAFLD. A recent study using more than 900,000 US adults showed that the risk of death related to HCC was 2-fold higher in men with BMI of 30–34.9 and 4.5 times higher with BMI greater than 35.<sup>279</sup> Obesity accelerates liver disease progression and HCC risk among patients after HCV cure.<sup>280</sup> Individuals who have metabolic syndrome with obesity, plus diabetes with HCV contributes to increased risk for HCC development. Diabetes with HCV infection has a 2 to 3-fold increase in HCC risk.<sup>281–284</sup> The researchers also showed that HCC development increased due to the combined effect of alcohol and chronic HCV infection.<sup>285–287</sup> In addition to non-viral agents, co-infection with the hepatitis B virus or human immunodeficiency virus increases the risk of HCC after HCV cure. Patients who have active HBV replication have double risk of HCC development and increased mortality as compared to single virus infection.<sup>288</sup> Likewise, patients with HIV infection have an increased risk of developing liver cirrhosis and HCC.<sup>289–291</sup> The mechanism of chronic inflammation, liver cirrhosis, and HCC development in the non-viral insults are not different than HCV. ER stress regardless of the etiology of liver disease leads to increased inflammation, cell death, tissue-regeneration, fibrosis, and HCC (Figure 6).

## Bystander HCC mechanisms through non-sterile inflammation:

The activation of oncogenes through point mutation and loss of tumor suppressor was thought to be a dominant mechanism for viral-induced cancer. The tumor suppressor loss and the oncogene activation are not consistent with data generated from the whole-genome sequencing of HCC tumors. This tumor centric-view is changing because HCC still develops in the absence of HCV after DAA treatment. The viable alternative hypothesis is that the cirrhotic micro-environment promotes HCC through bystander mechanisms. The recent development of microbiota studies suggesting that microbe alters the microenvironment that transform the surrounding cells through a bystander mechanism. Studies found that the metabolites derived from gut bacteria-infected cells enter the surrounding cells, induces DNA damage, and chromosomal instability that causes colon cancer.<sup>292–294</sup> This new information has generated considerable interest in understanding whether HCV infection-associated cellular stress can promote cancer by promoting the survival of nearby uninfected cells through a bystander mechanism. A study by Kofahi et al has shown that HCV infection of cultured cells induces apoptosis and pyroptosis in both infected and uninfected bystander cells.<sup>295</sup> The HCV-induced bystander apoptosis occurring in neighboring cells is cell-cell contact-dependent. Intracellular communication between cell-cell occurs through adhesion complexes, including adherens junctions, tight junctions, and gap junctions. Among those, intracellular communication mediated by gap junctions is vital for cell survival in various tissues.<sup>296</sup> This observation suggests that GJIC plays an essential role in



**Figure 6** Sterile inflammation associated with host-related non-viral factors accelerates HCC progression during chronic HCV infection. Multiple host-related factors induce hepatic stress (ER stress) and low-grade inflammation in the liver. The most common causes of hepatic stress and inflammation include metabolic syndrome, type 2 diabetes, NAFLD associated with obesity and high-calorie diet, alcohol, gut microbiota, and autoimmune diseases. The bystander effect of inflammation associated with these non-viral causes can accelerate liver damage, persistent fibrosis, and HCC risk.

tissue homeostasis, and its downregulation causes many human diseases. In the liver, normal hepatocytes express Cx26 and Cx32, forming gap junction at the cell-cell contact areas.<sup>297,298</sup> Altered expressions of these proteins occur in HCC tumors. One study shows that Cx26 expression abolished in HCC,<sup>299</sup> and other studies show that Cx32 remained cytoplasm instead of the plasma membrane.<sup>300</sup> Subsequent research indicates that the cytoplasmic Cx32 is critical for the expansion and self-renewal of cancer stem cells in hepatocellular carcinomas.<sup>301</sup> The cytoplasmic expression of Cx32 is an integral part of cancer stem cells and high-grade malignancy.<sup>302</sup> The radiation therapy is the most widely cited inducer of the bystander effect in cancer cells. Molecules such as ROS, RNS, protein factors, and DNA molecules can also utilize GJIP to spread from damaged cells to the surrounding healthy cells. The exosomal transfer of cargoes is implicated in the bystander cancer mechanism. The number of molecules, including TNF- $\alpha$ , TGF- $\beta$ 1, IL-6, IL-8, and nitric oxide are involved in cell-cell communications.<sup>303,304</sup> Earlier studies showed that the overexpression of Cx32 reduces HCC growth.<sup>305</sup> Cx32 knockout mice show increased susceptibility to chemical hepatocarcinogenesis.<sup>306-308</sup> A similar mechanism triggered by the overlapping metabolic liver disease promoting HCC after viral cure needs to be determined.

## Mechanisms of HCC Development After Viral Cure by DAAs

In this section, we review literature showing the impact of antiviral medicine on the long-term healing of liver inflammation and hepatic parenchymal injuries associated with chronic infection. We discuss some of the recent publications that have examined the benefits of viral cure at the level of cirrhosis regression, virus-induced cell programming, and indirect effect of a component of innate and adaptive response after HCV cured by DAAs.

## Reversal of Cirrhosis After DAAs Treatment

Patients who have not developed cirrhosis at the time of DAAs treatment have a significantly lower risk of developing HCC as compared to those who have established cirrhosis.<sup>309</sup> The status of cirrhosis after viral treatment is an essential predictor of HCC development. Cirrhosis is also a risk factor for increased mortality due to liver failure. The survival of patients with cirrhosis varies considerably based on the stage of the disease. For instance, one-year

mortality for stage 1 fibrosis is 1%, stage 2 is 3%, stage 3 is 20%, and stage 4 is nearly 57%.<sup>310</sup> Not all patients show cirrhosis regression after viral cure by IFN-based antiviral therapy. Previous studies have shown that SVR is associated with cirrhosis regression in nearly 55% of patients. A meta-analysis of a large number of multicenter clinical studies demonstrated that SVR by IFN-based antiviral therapy have a three-fold increased chance of cirrhosis regression that those who did not show SVR.<sup>310-316</sup> Data on cirrhosis regression after HCV cure by DAAs is emerging based on the assessment of liver stiffness assessment by transient elastography.<sup>317-322</sup> The available data suggest that viral cure by DAAs display significant benefits and cirrhosis regression. A study reported by Fehily et al summarizes that the DAA-induced HCV treatment improves clinical outcome and mortality associated with cirrhosis.<sup>323</sup> Tacke et al study examined the baseline risk factors related to the success of DAAs treatment using 4946 chronic HCV patients. They found that obesity, diabetes, cirrhosis, and alcohol consumption are associated with persistent liver enzyme elevation post HCV cure.<sup>324</sup> These data suggest that overlapping liver injury related to non-viral etiologies also contributes to persistent cirrhosis after HCV cure.

## Reversal of Direct Mechanisms After DAAs Treatment

To understand the reason for increased risk of HCC after DAA-based antiviral treatment, the number of investigators started examining the cancer pathways either in liver and tumor samples after viral eradication. As discussed earlier, epigenetic changes can contribute to open chromatin, increase expression of the cancer-specific gene that leads to HCC development. Two studies have used chromatin immunoprecipitation and DNA sequence analysis from chronic HCV patients with or without DAAs treatment.<sup>325,326</sup> Hamdane et al showed that the expression levels of 2 genes: sphingosine kinase 1 and SOX-2, a transcription factor, did not change after HCV cure. The expression levels of these two genes were examined in the samples of patients before and after HCV cure as well as HCV-induced HCC samples. These two genes are important in HCC development since the functional knockout of these two genes has been shown to inhibit HCC growth.<sup>325</sup> A recent publication supports the data showing that SOX gene signatures are associated with the expression of liver cancer dedifferentiation markers.<sup>327</sup> The development of an oncogenic signature



relates to SOX expression. Perez et al also found that epigenetic signature was maintained after viral cure by DAAs, not by IFN treatment, therefore explaining as to why HCC is more frequent in DAA-treated group as compared to IFN. They identified Wnt10A, JUNB, FOSL2, MYCN, TNFAIP3, KLF4, and EDNA1 genes that are not reversed after DAAs. The study identified numerous genes related to cytoskeleton remodeling, endocytosis, virus release, and host cell cycle.<sup>326</sup> Exosomes are major modulators of tumor microenvironment in cirrhosis as they carry numerous miRs implicated in cancer. Santangelo et al examined the impact of DAAs on exosomal miRs in plasma samples of chronic HCV patients. They have looked at miR-122 since it is involved in HCV replication, and miR-122 loss is associated with HCC development. They showed that miR-122-5p, miR-222-3p, miR-146-5p, miR-150-5p, miR-30C-5p, miR-378a-3p, miR-20a-5p were enriched in exosomes derived from HCV-infected cells. They found liver-specific miR-122 levels showed a significant decrease after DAAs therapy. They also found decreased expression of all these miRs mentioned above after DAAs.<sup>328</sup> Koberle et al study also demonstrated that serum miR-122 levels remained low among patients who had SVR after DAAs.<sup>329</sup> Villani et al study tested the hypothesis that DAAs treatment-induced VEGF expression promotes liver cancer angiogenesis. They performed an observational study using 117 cirrhotic patients who have been treated with DAAs to determine whether increased serum VEGF levels correlate with HCC development. They found serum VEGF levels increased after four weeks and remained elevated up to the end of treatment.<sup>330</sup> Fallaci et al verified a similar hypothesis on HCC occurrence and recurrence among cirrhotic patients after DAAs. They showed that the VEGF expression was significantly related to serum angiopoietin-2 levels. They also showed that angiopoietin-2 expression in HCC and cirrhotic tissue before DAAs treatment relates to the risk of HCC recurrence and occurrence.<sup>331</sup> Recently, we reported that HCC developed in the cirrhotic liver when the hepatic adaptive response reprogrammed to switch from pro-death to the pro-survival state through autophagy switching. In addition, our laboratory illustrated that virus-associated ER stress and tumor suppressor loss (p53 and Rb, miR-122 levels) were restored more by IFN-induced HCV clearance than DAAs in cell culture models.<sup>136,137,206</sup> Based on these data, we propose that understanding the impact of viral cure on the resolution of direct hepatic parenchymal injuries and indirect immune restoration may explain as to why specific individuals remain at risk of HCC occurrence and recurrence.

## DAAs Treatment on Innate Immunity

In the following section, we will review data correlating with the impact of HCV cure on the reversal of immune mechanism that could explain the possible reason of HCC recurrence after HCV cure. Meissner et al examined the impact of HCV clearance by DAAs on endogenous intra-hepatic IFNs. They compared the expression level of type I, type II, and Type III IFN and ISGs before and after HCV treatment. The study showed that HCV clearance resulted in decreased expression of type II and type III IFNs, their receptors, and ISG in the liver. Unexpectedly, HCV cure by DAAs did not reduce the appearance of type I IFN signaling, IFNA2 gene expression remained high among those who achieved SVR. The study concluded that activation of type I IFN signaling is important for HCV elimination.<sup>332</sup> A study by Alao et al showed that patients who achieved SVR at 12 weeks after treatment displayed higher ISG expression levels in baseline liver biopsies and a higher frequency of pSTAT1 and TRAIL-expressing, degranulating NK cells in baseline blood samples than those who experienced a virological breakthrough. The downregulation of ISGs was rapid after HCV RNA suppression by DAAs.<sup>333</sup> Another study by Sung et al showed that the expression levels of IFN- $\beta$ , IFN-induced protein 44, and CXCL10 cytokine levels decreased and normalized after DAAs treatment in the peripheral blood mononuclear cells. The conclusion of this study was that DAAs treatment normalizes type I IFN response.<sup>334</sup> Debes et al study measured serum cytokines levels among 13 patients who developed HCC after DAAs. Among 22 different cytokines, nine of them (MIG, IL-22, TRAIL, APRIL, VEGF, IL-3, TWEAK, SCF, and IL-21) presented significantly higher in serum before treatment, which eventually developed HCC.<sup>335</sup> These studies now provide information regarding that DAA-induced HCV clearance does not restore the innate immune surveillance completely. NK cells are an essential component of the innate immune response in HCV infection. People who clear HCV infection naturally show increased expression of the activating receptors (NKp44 and NKp46) and decreased expression of the inhibitory receptors NKG2A associated with strong T cell response.<sup>336</sup> Golden-Mason et al reported that rapid viral clearance by DAAs results in normalization of NK cell function, reduced cytotoxicity and downregulation of cytotoxic NK cell signaling by TRAIL, NKp30, and NKp46.<sup>337</sup> Spaan et al showed downregulation in TRAIL-mediated killing by NK cells during DAAs therapy.<sup>338</sup>

A report by Stevenson et al demonstrated that DAAs therapy normalized NK cell function, and decreased expression of CXCL-10, CXCL-11 levels associated with NK cell activation and function suggesting functional restoration of NK cells after viral cure by DAAs.<sup>339</sup> However, some researchers claim that rapid decrease or normalized immunosurveillance causes early HCC occurrence after DAAs. The activating receptor NKG2D and its ligands play a crucial role in the immune response to HCC. Two studies found that reduced NKG2D and ligand expression in HCC correlates with HCC recurrence and early occurrence.<sup>340,341</sup> MICA, which is one of the ligands for the NKG2D and their interaction, is vital for NK-cell cytotoxic effect for HCC cells. HCC sheds membrane-bound MICA as soluble MICA and downregulates the expression of NKG2D on the NK cell surface, explaining a mechanism of how HCC escape NK-cell mediated immune surveillance.<sup>342,343</sup>

DCs play a critical role in sensing virus infection through pattern recognition receptors. This process coordinates the innate and adaptive immune response to HCV infection. Several reports claim that the impairment of MDC function and production of cytokines (IL-12, TNF- $\alpha$ , IFN- $\alpha$ /IFN- $\beta$ ) in chronic HCV infection.<sup>344-346</sup> Laursen et al examined soluble CD163 released from activated liver macrophages in chronic HCV, and histological activity after DAAs. They found that serum CD163 levels decline rapidly after successful DAAs therapy and are associated with histological inflammatory activity and fibrosis.<sup>347</sup> Kostadinova et al evaluated the serum level of ALT with the plasma level of sCD14, sCD163, autotaxin, and Mac2BP with HCV treatment response to DAAs. They found levels of these immune activation markers declined and normalized after DAAs. AST level was well correlated with sCD163, which is a KC activation marker.<sup>348</sup> MAIT cells are innate-like lymphocytes that are activated in chronic HCV infection due to the synergistic action of IL-18, IL-12, IL-15, and IFN- $\alpha$ /IFN- $\beta$  that trigger granzyme B release. MAIT cell number is significantly decreased and inversely correlates with liver inflammation and fibrosis.<sup>349</sup> One report claims that DAA-induced HCV clearance does not restore MAIT cell defects, which seems different than IFN-based antiviral therapy.<sup>350</sup> Cannizzo et al also found that MAIT cell function is not restored after DAAs in HIV/HCV co-infected patients.<sup>351</sup>

## DAAs Treatment and Adaptive Immunity

Cytotoxic T cell response is restored when someone recovers from HCV infection naturally, which is characterized by the

appearance of HCV-specific T cells expressing IL-7 receptor alpha (CD127), a marker of memory T cells and anti-apoptotic molecule Bcl-2.<sup>352</sup> Chronic HCV infection results in T cell exhaustion and the loss of effector T cell function with the eventual disappearance of the majority of HCV-specific CD8 T cells from the blood and liver. Burchill et al examined the composition of CD8 T cells in the peripheral blood from nineteen HCV patients treated with DAAs. They found that HCV clearance leads to a rapid increase in the frequency of HCV-specific CD8 T cells. In contrast, a significant reduction of the PD-1 on HCV-specific T cells was observed, suggesting the partial restoration in the HCV-specific CD8 T cell exhaustion state.<sup>353</sup> Weiland et al reported that TCF1+CD127+PD-1+ HCV specific T cells expressing both exhaustion and memory markers were found in subjects with chronic HCV infection, and those T cell subsets levels were found to remain unchanged during and after DAAs treatment. This subset was increased in one patient who relapsed and differentiated into terminally exhausted HCV-specific CD8 T cells. These cells express higher levels of eomes and produce low IFN- $\gamma$  and TNF- $\alpha$  upon antigen challenge. These CD8 T cells were distinct from memory CD8 T cells (CD127+ PD-1-) that appear during spontaneous resolution.<sup>354</sup> Aregay et al studied whether HCV cure by DAAs could restore the functionality of exhausted HCV-specific CD8 T cell response. Their data showed that the exhausted HCV-specific CD8 T cells remain functionally impaired after HCV cure in all 40 patients treated with DAAs.<sup>355</sup> Vranjkovic et al evaluated the function of circulating CD8 T cells in HCV-related cirrhotic patients. They found a progressive shift in CD8 T cells subset distribution in all cirrhotic patients even after HCV cure. The authors concluded that the severity of liver fibrosis in these patients relates to the presence of hyperfunction CD8 T cells in the liver.<sup>356</sup> All these results suggest DAA-induced HCV cure does not restore the exhausted phenotype of HCV-specific CD8 T cell response.

CD4 helper T cells are essential for mounting appropriate HCV-specific CD8 T cell response and are needed for natural clearance of infection.<sup>357,358</sup> Intrahepatic regulatory CD4 T cells and CD4(+)CD25(+)FoxP3(+) T-reg cells are expanded in the blood and liver of patients with chronic HCV infection, and they are not decreased after viral cure by IFN-based or DAA-based therapy.<sup>359,360</sup> Langhans et al analyzed T-reg cells before and after DAAs at 12 and 24 weeks. They found that DAAs do not normalize the activation status of T-reg cells, and these cells persist for a long period after viral elimination.<sup>361</sup> One recent study showed

that gamma delta T cell dysfunction in chronic HCV infection is not reversed after HCV cure by DAAs, indicating this functional defect may have implications for susceptibility to HCC.<sup>362</sup> Burchill et al showed that DAAs therapy results in (i) the reconstitution of lymphocyte populations in the peripheral blood; (ii) re-differentiation of memory T cells towards an effector phenotype; (iii) a reduction in the expression of some, but not all inhibitory molecules on bulk and viral-specific CD8 T cell populations; and (iv) a reduction in the activation status of circulating NK cells. B cells are the source of humoral anti-HCV antibodies that are important in resolving HCV infection.<sup>353</sup> Although the human immune response is not the sole contributor to HCV resolution, activation of B cell response is associated with B cell-related abnormalities such as mixed cryoglobulins among 40–60% patients and CV in 5–10% patients. Comarmond et al tested the impact of DAAs treatment on HCV-related CV of 27 patients. They reported that about 88.9% of patients show the complete clinical response of CV after DAAs and restored disturbances in peripheral B cell homeostasis.<sup>363</sup> Saadoun et al examined the remission of vasculitis after the DAAs treatment of 41 patients, and they reported 37 patients (90.2%) showed complete clinical response, which was associated with virological response.<sup>364</sup> A study by Chang et al demonstrated that resolution of HCV infection with DAAs leads to a marked reduction in the frequency of T-bet+ B cells. Re-exposure of convalescent (cured) B cells to viremic plasma and recombinant HCV E2 protein led to the re-expression of T-bet. This study posited that the antigen-driven T-bet expression, a critical suppressor of B cell activation in chronic HCV infection, is reversed after DAAs therapy.<sup>365</sup>

## Development of Accurate Biomarker for Cirrhosis Regression and Early HCC Detection

The current DAAs therapy is inducing an incredibly high rate of HCV clearance in most patients, including those with liver cirrhosis. It is expected that the cured patient population will increase in the future, and many of these cured patients will have overlapping non-viral liver diseases. There is substantial evidence showing liver injury persists after HCV cure among cirrhotic patients. These patients remain at risk of persistent cirrhosis and HCC development. The regression of liver fibrosis is a slow process after HCV treatment. Some cirrhotic patients who have underlying liver disease related to alcohol,

type 2 diabetes, and obesity are still at risk of HCC development. Screening for HCC is recommended among patients who have cirrhosis at the time of DAAs treatment. There remains a clinical challenge to develop accurate tests for persistent risk of HCC in the early stratification of these patients. Liver biopsies are no longer performed to diagnose cirrhosis. The assessment of liver cirrhosis is now performed by noninvasive approaches, including liver imaging (hepatic elastography/fibroscan) and serum biomarkers.<sup>366</sup> Two serum fibrosis scores, namely fibrosis 4 (FIB-4) and the AST to platelet ratio index (APRI), have been widely used in staging liver fibrosis.<sup>367–369</sup> The only currently accepted HCC tumor biomarker, AFP is a 70-kDa-serum glycoprotein produced by fetal liver during development. The serum levels of AFP are increased during HCC. AFP is present in three different forms based on the lectin-binding pattern: AFP-L1, AFP-L2 and AFP-L3. Serum levels of AFP-L1 increased during chronic hepatitis and cirrhosis whereas AFP-L3 levels increased during HCC. The AFP generally has suboptimal sensitivity and specificity for HCC surveillance.<sup>370</sup> This protein can be detected in ovarian, pancreatic, stomach and testicular cancers as well. Des- $\gamma$ -carboxyprothrombin is an abnormal form of prothrombin induced by vitamin K absence-II that serum levels are elevated in patients with HCC. In addition to these serum proteins, ultrasound-based imaging is also recommended for HCC screening. Recently, computed tomography scanning and magnetic resonance imaging have shown to have better sensitivity for HCC screening. As discussed in this review, there are many potential targets that can be explored in the development of a serum biomarker for early HCC. Since the current method still lacks sensitivity for early prediction of neoplastic growth in liver, it is necessary to develop a novel biomarker that accurately measures the hepatic adaptive response to virus-associated cellular stress, immune cell activation, and liver injury.

## Development of Protective Vaccine

Although the DAA-based antiviral treatment is inducing a very high rate of viral cure, the number of new HCV infection rates is increasing worldwide. Most of the new infections remain undiagnosed because both acute and chronic HCV infections are primarily asymptomatic. In the US, only 50% of infected people are aware of their HCV status.<sup>371</sup> The risk of reinfection after DAAs treatment remains a major problem especially among intravenous drug users.<sup>372,373</sup> The current DAA-induced viral clearance

does not provide protection for reinfection or reversal of liver disease-related complications. A prophylactic vaccine is needed since DAAs treatment is unaffordable in many developing nations. Protective immunity to HCV reinfection requires expansion of a broad and polyclonal memory of CD4 T and CD8T cell response<sup>374,375</sup> as well as generation of neutralizing antibodies.<sup>376</sup> There are a number of challenges that exist for the development of a multivalent vaccine for HCV, including the extraordinary diversity of the virus, seven genotypes with more than 80 subtypes, viral variants associated with resistance to T cell and antibody response. At present, there are two main vaccine candidates moving for clinical trials. The first vaccine was based on purified recombinant proteins targeting the viral envelope glycoproteins called E1E2 mixed with MF59 adjuvant (an oil-in-water emulsion). The initial study shows that this formulation induced neutralizing antibody responses in chimpanzee.<sup>377,378</sup> Preclinical evaluation of this vaccine in human volunteers showed that it generates a neutralizing antibody response as well as CD4 T cell response against viral envelope proteins E1 and E2.<sup>378,379</sup> A second vaccine was developed based on the use of recombinant replication-defective adenovirus containing the entire non-structural proteins.<sup>380,381</sup> The second generation of this vaccine approach includes heterologous priming first with the Chimpanzee Adenovirus 3 Nonstructural (AdCh3NS), followed by boosting with another Modified Vaccinia Ankara NS (MVA NS) vaccine. This approach appears to be generating a broad and strong T cell response. A recent study in human volunteers shows that optimal priming and boosting of these vaccine generate high frequencies of polyfunctional, elicited broad CD8 T cells response, IFN- $\gamma$  production, memory cell development, and durable maintenance of HCV-specific T cell response.<sup>382</sup> A Phase II clinical trial of this vaccine is underway in a cohort of HCV-infected intravenous drug users (clinicaltrials.gov, NCT01436357). Vaccines are available for hepatitis A and hepatitis B. Availability of vaccine for HCV will be a step forward for global immunization strategies for hepatitis viruses.

## Summary and Conclusions

HCV infection is one of the major causes of cirrhosis and HCC worldwide and is associated with high mortality. It is expected that DAA-based antiviral treatment will reduce the progressive liver disease and liver-related mortality associated with HCV infection. However, the risk of HCC persists after HCV treatment among cirrhotic patients. The population of HCV-cured cirrhotic patients is expected to increase. These patients remain at

risk for liver disease progression due to non-viral risk factors including obesity, alcohol, diabetes, and other autoimmune diseases. Fatty liver is a particular threat to the long-term healing of cirrhotic patients after HCV cure. These patients need to remain under surveillance for liver complications, including HCC. Since cirrhosis regression is a slow process, the long-term benefits of viral cure among cirrhotic patients are uncertain. This review provided molecular basis on how hepatic pathological adaption to viral insults and the immunological mechanisms associated with development of cirrhosis and HCC occurs during chronic HCV infection. The fibrosis regression and restoration of hepatic injury are important for reduction of HCC risk. The development of novel biomarkers to evaluate the extent of immune restoration and reduction of cellular injuries is needed for early prediction of persistent cirrhosis and HCC. The current DAA-induced viral elimination does not provide immune protection for reinfection among intravenous drug users. The prevention of HCV transmission and reinfection through vaccination is an important aspect of post-cure management and a key to the global elimination of HCV infection.

## Abbreviations

AFP, alpha-fetoprotein; ALT, alanine aminotransferase; APC, antigen-presenting cells; AST, aspartate aminotransferase; BMI, body mass index; CMA, chaperone-mediated autophagy; CSC, cancer stem cell; DAA, direct-acting antiviral; DAMP, damage-associated molecular pattern; DMT, DNA methyltransferase; EGFR, epidermal growth factor receptor; ER, endoplasmic reticulum; ERK, extracellular signal-regulated kinase; FGF, fibroblast growth factor; GJIC, gap junctional intracellular communication; GSK3 $\beta$ , glycogen synthase kinase 3 $\beta$ ; HAT, histone acetyl transferase; HBV, hepatitis B virus; HCC, hepatocellular carcinoma; HCV, hepatitis C virus; HDAC, histone deacetylase; HIF-1 $\alpha$ , hypoxia-inducible factor 1 alpha; HNF4 $\alpha$ , hepatocyte nuclear factor 4 alpha; IFN, interferon; IL, interleukin; ISR, integrated stress response; MDM2, mouse double minute 2; MEK, mitogen-activated kinase; miR, microRNA; mTOR, mammalian target of rapamycin; NAFLD, non-alcoholic fatty liver disease; ncRNA, non-coding RNA; NF- $\kappa$ B, nuclear factor kappa-light-chain-enhancer of activated B cells; NRF2, nuclear factor erythroid 2-related factor 2; PAMP, pathogen-associated molecular pattern; PDGF, platelet-derived growth factor; PI3K, phosphoinositide 3-kinase; PRC, polycomb repressive protein complex; RTK, receptor tyrosine kinase; Rb, retinoblastoma protein; RIG-1, retinoic acid-inducible gene-1 receptor; RBV, ribavirin; RNS, reactive nitrogen species; ROS, reactive oxygen species; SOX,

sex determining region Y-box; SVR, sustained virological response; TERT, telomerase reverse transcriptase; TLR, toll-like receptor; TGF, transforming growth factor; VEGF, vascular endothelial growth factor.

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## Disclosure

The authors report no conflicts of interest in this work.

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