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Steatosis, liver injury, and hepatocarcinogenesis in hepatitis C viral infection

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In addition to the link with development of hepatocellular carcinoma (HCC), hepatitis C virus (HCV) infection is associated with several hepatic and extrahepatic manifestations. A role of hepatic steatosis in the pathogenesis of chronic hepatitis C has been shown, implying hepatitis C as a metabolic disease. Furthermore, recent epidemiological studies have suggested a linkage between insulin resistance and chronic HCV infection. In addition to the data indicating the presence of lipid metabolism disturbance and insulin resistance in the cohort of chronic hepatitis C patients, we found evidence showing the association between these two conditions and HCV infection using mice transgenic for the HCV core gene. These mice develop HCC late in life after the phase of hepatic steatosis and insulin resistance. The nonappearance of both steatosis and HCC in HCV core gene transgenic mice that are null for the proteasome activator 28γ implies a close relationship between lipid metabolism disturbance and hepatocarcinogenesis. Also, the core protein is shown to bind with retinoid X receptor (RXR)-α, resulting in the upregulation of some lipid metabolism enzymes, including cellular retinol binding protein II and acyl-CoA oxidase. In addition, the persistent activation of peroxisome proliferator activated receptor (PPAR)-α has recently been found in the liver of HCV core gene transgenic mice, yielding dramatic changes in lipid metabolism and hepatocyte proliferation, including HCC development. These results would provide a clue for further understanding of the role of lipid metabolism in pathogenesis of HCV infection, including liver injury and hepatocarcinogenesis.

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Introduction

Worldwide, approximately 170 million people are persistently infected with hepatitis C virus (HCV), which induces a spectrum of chronic liver diseases from chronic hepatitis to cirrhosis and, eventually, to hepatocellular carcinoma (HCC).[1] HCV has been given increasing attention because of its wide and deep penetration in the community, tied with a very high incidence of HCC in persistent HCV infection. Once liver cirrhosis is established in hosts persistently infected with HCV, HCC develops at a yearly rate of approximately 7%,[2] resulting in the development of HCC in nearly 90% of HCV-associated cirrhotic patients in 15 years. In addition, the outstanding features in the mode of hepatocarcinogenesis in HCV infection, i.e., development of HCC in a multicentric fashion and at a very high incidence, are not common in other malignancies except for hereditary cancers such as familial polyposis of the colon. Knowledge of the mechanism underlying HCC development in persistent HCV infection, therefore, is imminently required for the prevention of HCC.

In addition to the link with development of HCC, HCV infection is associated with several hepatic and extrahepatic manifestations.[3] A role of hepatic steatosis in the pathogenesis of chronic hepatitis C has been shown, implicating hepatitis C as a metabolic disease.[4] Moreover, recent epidemiological studies have suggested a linkage between insulin resistance and chronic HCV infection.[5] In addition to the epidemiological data indicating the presence of lipid metabolism disturbance and insulin resistance in the cohort of chronic hepatitis C patients, detailed analyses on the relationship between

metabolic disorders and chronic hepatitis C have revealed evidence showing a close association between the progression of liver fibrosis and metabolic abnormalities in HCV infection.[6] However, it is unclear yet whether a causative relationship exists between these medical conditions. Moreover, it is unclear whether such metabolic disorders contribute to hepatocarcinogenesis in HCV infection.

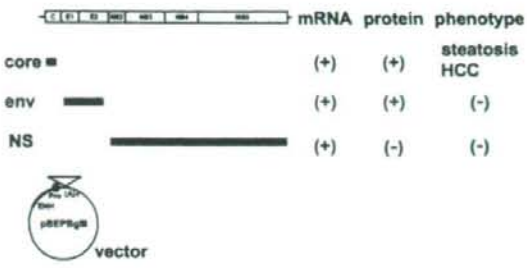

Fig. 1. Transgenic mouse lines carrying the hepatitis C virus (HCV) genome. Three different kinds of transgenic mouse lines, carrying the *core* gene, envelope genes, or nonstructural genes of HCV, respectively, were established under the control of the same regulatory elements. Among these mouse strains, only the transgenic mice carrying the HCV core gene develop hepatocellular carcinoma (HCC) after an early phase with hepatic steatosis in two independent lineages. The mice transgenic for the envelope genes or nonstructural genes do not develop HCC. *HCC*, hepatocellular carcinoma; *env*, envelope genes; *NS*, nonstructural genes

Possible roles of HCV in hepatocarcinogenesis

The mechanism underlying hepatocarcinogenesis in HCV infection is not yet fully understood, despite the fact that nearly 80% of patients with HCC in Japan are persistently infected with HCV.[1,7,8] HCV infection is also common in patients with HCC in other countries, albeit to a lesser extent. These lines of evidence prompted us to seek to determine the role of HCV in hepatocarcinogenesis. Inflammation induced by HCV should be considered, of course, in a study on the hepatocarcinogenesis in hepatitis viral infection: necrosis of hepatocytes caused by chronic inflammation followed by regeneration enhances genetic aberrations in host cells, the accumulation of which culminates in HCC. This theory presupposes an indirect involvement of hepatitis viruses in HCC via hepatic inflammation. However, this context leaves us with a serious question: can inflammation alone result in the development of HCC in such a high incidence (90% in 15 years) or multicentric nature in HCV infection?

The other role of HCV would have to be weighed against an extremely rare occurrence of HCC in patients with autoimmune hepatitis in which severe inflammation in the liver persists indefinitely, even after the development of cirrhosis. This background and reasoning lead to a possible activity of viral proteins for inducing neoplasia. This possibility has been evaluated by introducing genes of HCV into hepatocytes in culture with little success. One of the difficulties in using cultured cells is the carcinogenic capacity of HCV, if any, which would be weak and would take a long time to manifest. Actually, it takes 30–40 years for HCC to develop in individuals infected with HCV. On the basis of these points of view, we started to investigate carcinogenesis in chronic hepatitis C, in vivo, by transgenic mouse technology.

HCV core protein has an in vivo oncogenic activity as revealed by animal studies

Transgenic mouse lines carrying the HCV genome were engineered by introducing the genes from the cDNA of the HCV genome of genotype 1b.[9,10] Established are three different kinds of transgenic mouse lines, which carry the core gene, envelope genes, or nonstructural genes, respectively, under the same transcriptional regulatory element. Among these mouse lines, only the transgenic mice carrying the core gene developed HCC in two independent lineages.[10] The envelope gene transgenic mice do not develop HCC, despite high expression levels of both E1 and E2 proteins,[11,12] and the transgenic mice carrying the entire nonstructural genes have developed no HCC (Fig. 1).

The core gene transgenic mice express the core protein of an expected size, and the level of the core protein in the liver is similar to that in chronic hepatitis C patients. Early in life, these mice develop hepatic steatosis, which is one of the histological characteristics of chronic hepatitis C, along with lymphoid follicle formation and bile duct damage.[13] Thus, the core gene transgenic mouse model reproduces well the features of chronic hepatitis C. Of note, no pictures of significant inflammation are observed in the liver of this animal model. Late in life, these transgenic mice develop HCC. Notably, the development of steatosis and HCC has been reproduced by other HCV transgenic mouse lines, which harbor the entire HCV genome or structural genes including the core gene.[14–16] These outcomes indicate that the core protein, per se, of HCV has an oncogenic potential when expressed in vivo.

Oxidative stress overproduction and intracellular signaling pathway activation are the major pathways in the core-induced liver pathology

It is difficult to elucidate the mechanism underlying the development of HCC, even for our simple model in which only the core protein is expressed in otherwise normal liver. There is a notable feature in the localization of the core protein in hepatocytes; while the core protein predominantly exists in the cytoplasm associated with lipid droplets, it is also present in the mitochondria and nuclei.[10,17] On the basis of this finding, the pathways related to these two organelles, the mitochondria and nuclei, were thoroughly investigated.

One effect of the core protein is an increased production of oxidative stress in the liver. We would like to draw particular attention to the fact that the production of oxidative stress is increased in our transgenic mouse model in the absence of inflammation in the liver. This finding reflects a state of overproduction of reactive oxygen species (ROS) in the liver,[18] or predisposition to it, which is staged by the HCV core protein without any intervening inflammation.[19,20] The overproduction of oxidative stress results in the generation of deletions in mitochondrial and nuclear DNA, an indicator of genetic damage. In addition, analysis of antioxidant system revealed that some antioxidative molecules are not increased despite the overproduction of ROS in the liver of core gene transgenic mice: hemeoxygenase-1 and glutathione peroxidase are not augmented whereas catalase and glutathione S-transferase levels are increased and enhanced by iron overloading (Moriya et al., manuscript in preparation). These results suggest that HCV core protein not only induces overproduction of ROS but also attenuates some of the antioxidant systems, which may explain the mechanism underlying the production of a strong oxidative stress in HCV infection compared to other forms of hepatitis.

In the absence of inflammation, thus, the core protein induces oxidative stress overproduction, which may, at least in part, contribute to hepatocarcinogenesis in HCV infection. If inflammation were added to the liver with the HCV core protein, the production of oxidative stress would be escalated to an extent that can no longer be scavenged by a physiological antagonistic system. This idea suggests that the inflammation in chronic HCV infection would have a characteristic difference in its quality from those of other types of hepatitis, such as autoimmune hepatitis. The basis for the overproduction of oxidative stress may be ascribed to the mitochondrial dysfunction.[10,19] The dysfunction of the electron transfer system of the mitochondrion is suggested in association with the presence of the HCV core protein.[21]

Other pathways in hepatocarcinogenesis would be the alteration of the expression of cellular genes and modulation of intracellular signaling pathways. For example, tumor necrosis factor (TNF)-α and interleukin-1β have been found to be transcriptionally activated.[22] The mitogen-activated protein kinase (MAPK) cascade is also activated in the liver of the core gene transgenic mouse model. The MAPK pathway, which consists of three routes, c-Jun N-terminal kinase (JNK), p38, and extracellular signal-regulated kinase (ERK), is involved in numerous cellular events including cell proliferation. In the liver of the core gene transgenic mouse model before HCC development, only the JNK route is activated. Downstream of JNK activation, transcription factor activating protein (AP)-1 activation is markedly enhanced.[20,21] At far downstream, both the mRNA and protein levels of cyclin D1 and CDK4 are increased. Thus, the HCV core protein modulates the intracellular signaling pathways and gives an advantage for cell proliferation to the hepatocytes. Interestingly, we found recently that a protein interacting with the core protein, proteasome activator 28γ (PA28γ), is indispensable for the core protein to exert its function for the development of steatosis, insulin resistance, and HCC.[23,24]

Lipid metabolism and HCV infection

Steatosis is frequently observed in chronic hepatitis C patients and is significantly associated with increased fibrosis and progression rate of fibrosis of the liver.[6] A comprehensive analysis of gene expression in the liver of core gene transgenic mice, in which steatosis develops from early in life, revealed that a number of genes related to lipid metabolism are significantly upregulated or downregulated (Table 1).

The composition of fatty acids that are accumulated in the liver of core gene transgenic mice is different from that in fatty liver resulting from simple obesity. Carbon-18 monounsaturated fatty acids (C18:1) such as oleic or vaccenic acids are significantly increased; this is also the case in the comparison of liver tissues from hepatitis C patients and patients with simple fatty liver due to obesity.[20] The mechanism of steatogenesis in hepatitis C was investigated using this mouse model. There are at least three pathways for the development of steatosis. One is the frequent presence of insulin resistance in hepatitis C patients as well as in the core gene transgenic mice, which occurs through the inhibition of tyrosine phosphorylation of insulin receptor substrate (IRS)-1.[25] Insulin resistance increases the peripheral release and hepatic uptake of fatty acids, resulting in an accumulation of lipid in the liver. The second pathway is the suppression of the activity of

Table 1. Cellular genes differentially expressed in hepatitis C virus (HCV) core transgenic mouse liver

	Upregulated	Downregulated
Lipid metabolism	NPC1 Catalase Very long chain acyl-CoA dehydrogenase Carboxylesterase selenoprotein P Carbonic anhydrase Adipose differentiation-related protein Bilirubin/phenol family UDP glucuronosyltransferase	Stearoyl-CoA desaturase Sterol-carrier protein X Alpha-enolase carnitine acetyltransferase Gal beta 1,4(3) GlcNAc alpha 2,3-sialyltransferase Very long chain acyl-CoA synthetase Liver transferrin 4-Hydroxyphenylpyruvate dioxygenase LAF1 transketolase *s*-Adenosylmethionine synthetase Apolipoprotein A-II
Transcription and cell proliferation	Int-6 GCN5L1 *H. sapiens* 8.2k-Da differentiation factor USF1 Initiation factor eIF-4AI Human elongation factor-1-delta Sui1	Human guanine nucleotide regulatory protein Alpha-fetoprotein Retinol binding protein
Inflammation	Alpha-1 protease inhibitor 3 Hemopexin	Alpha-2-macroglobulin LMW prekininogen Complement component C3 AHSG(alpha 2 HS-glycoprotein) homologue
Others	Microvascular endothelial differentiation gene 1 Diazepam-binding inhibitor Argininosuccinate synthetase Skeletal muscle alpha-tropomyosin Ampd3 gene DNA-binding protein	Vitronectin Epithelin 1 and 2 Murinoglobulin

microsomal triglyceride transfer protein (MTP) by HCV core protein[26]; this inhibits the secretion of very low density protein (VLDL) from the liver, yielding an increase of triglycerides in the liver. The last pathway involves sterol regulatory element-binding protein (SREBP)-1c, which regulates the production of triglycerides and phospholipids. In HCV core gene transgenic mice, SREBP-1c is activated, whereas neither SREBP-2 nor SREBP-1a is upregulated.[27]

In relation to lipid metabolism, the core protein has also been found to interact with retinoid X receptor (RXR)-α.[28] RXR-α is one of the nuclear receptors, which forms a homodimer or heterodimers with other nuclear receptors, including PPAR (peroxisome proliferator-activated receptor)-α, and plays a pivotal role in the regulation of the expression of genes relating to lipid metabolism, cell differentiation, and proliferation. In fact, the core protein of HCV activates genes that have an RXR-α-responsive element as well as those with a PPAR-α-responsive element, both in mice and in cultured cells.[28] Based on these results, we, then, examined the expression and function of PPAR-α in the liver of core gene transgenic mice.

PPAR-α activation in HCV-associated hepatocarcinogenesis

PPAR-α, one of the PPAR genes, plays a central role as a heterodimer with RXR-α in regulating fatty acid transport and catabolism. It is also known as a molecular target for lipid-lowering fibrate drugs.[29] On the other hand, prolonged administration of PPAR-α agonists causes HCC in rodents. Currently, there is little evidence that the low-affinity fibrate ligands are associated with human cancers, but it is possible that chronic activation of high-affinity ligands could be carcinogenic in humans.[29]

The level of PPAR-α protein was increased in the liver of core gene transgenic mice as early as 9 months of age. PPAR-α protein is accumulated with age in the nuclei of hepatocytes together with cyclin D1 protein. However, the level of PPAR-α mRNA was not increased at any age. By pulse-chase experiment, the stability of nuclear PPAR-α was increased in the presence of the core protein. In line with the increase of PPAR-α protein, target genes of PPAR-α were activated in the liver of core gene transgenic mice; these genes include

cyclin D1, cyclin-dependent kinase (CDK)-4, acy-CoA oxidase, and peroxisome thiolase.[30] However, in general, the activation of PPAR-α leads to improvement but not aggravation of steatosis. Then, what is the function of PPAR-α activation that is observed in the core gene transgenic mice?

To clarify the role of PPAR-α activation in pathogenesis of steatosis and HCC, we mated a core gene transgenic mouse with a PPAR-α knockout (KO) mouse and studied the phenotype. PPAR-α KO mice have reduced expression of target genes of PPAR-α, and have mild steatosis in the liver, as expected.[31] It was unanticipated, however, that steatosis was absent in PPAR-α-null or -heterozygous core gene transgenic mice but present in PPAR-α-intact core gene transgenic mice at the age of 9 or 24 months.[30] 8-Hydroxy deoxyguanosine (8-OHdG) and peroxylipids, both of which are markers for oxidative stress, were decreased in PPAR-α KO core gene transgenic mice. Mitochondrial dysfunction in the core gene transgenic mice, which contributes to overproduction of oxidative stress,[19] was also improved in PPAR-α KO core gene transgenic mice.

Finally, PPAR-α KO core gene transgenic mice did not develop HCC at the age of 24 months, whereas about one-third of PPAR-α-intact core gene transgenic mice did. It should be noted that core gene transgenic mice that are heterozygous for the PPAR-α gene also did not develop HCC.[32] When clofibrate, a peroxisome proliferator, was administered for 24 months to PPAR-α-heterozygous mice, either with or without the core gene, HCC developed in a higher rate in the core gene (+) mice with greater PPAR-α activation. It should be noted that steatosis was present only in core gene (+) PPAR-α-heterozygous mice. In summary, steatosis and HCC developed in PPAR-α-intact but not in PPAR-α-heterozygous or PPAR-α-null core gene transgenic mice, indicating that not the presence but the persistent activation of PPAR-α would be important in hepatocarcinogenesis by HCV core protein. In general, PPAR-α acts to ameliorate steatosis, but with the presence of mitochondrial dysfunction, which is also provoked by the core protein, the core-activated PPAR-α may exacerbate steatosis. Persistent activation of PPAR-α with "strong" ligands such as the core protein of HCV could be carcinogenic in humans, although the low-affinity fibrate ligands are not likely associated with human cancers.

HCV core protein causes "fatty acid spiral"

Figure 2 illustrates our current hypothesis for the role of lipid metabolism in HCV-associated hepatocarcinogenesis. Immune-mediated inflammation should also play a pivotal role in hepatocarcinogenesis in HCV infection. However, in HCV infection, the core protein induces steatosis through the aforementioned pathways, leading to "fatty acid spiral" in the presence of the mitochondrial complex 1 dysfunction and PPAR-α activation, both of which are caused by the core protein. These intracellular alterations would contribute to hepatocarcinogenesis by inducing oxidative stress overproduction and cell-growth signal activation. In such a sense, the core protein of HCV is not a classical-type oncoprotein, but rather seems to contribute to hepatocarcinogenesis by modulating intracellular metabolism and signaling.

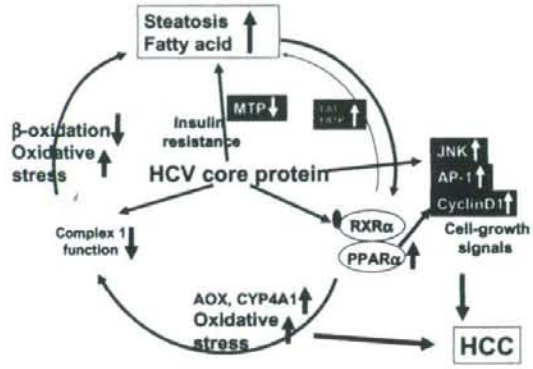

Fig. 2. "Fatty acid spiral" by HCV core protein. In HCV infection, the core protein induces steatosis via several pathways, leading to "fatty acid spiral" in the presence of the mitochondrial complex 1 dysfunction and PPAR-α activation, both of which are also caused by the core protein. These intracellular alterations would contribute to hepatocarcinogenesis by inducing oxidative stress overproduction and cell-growth signal activation. In such a sense, the core protein of HCV is not a classical type oncoprotein, but rather seems to contribute to hepatocarcinogenesis by modulating intracellular metabolism and signaling. *HCV*, hepatitis C virus; *HCC*, hepatocellular carcinoma; *ROS*, reactive oxygen species; *JNK*, c-Jun N-terminal kinase; *ERK*, extracellular signal-regulated kinase; *AP-1*, activating protein-1; *RXR-α*, retinoid X receptor-α; *PPAR-α*, peroxisome proliferator activated receptor-α; *AOX*, acyl-CoA oxidase; *CYP*, cytochrome P450; *MTP*, microsomal triglyceride transfer protein; *FAT*, fatty acid translocase; fatty acid transport protein

The HCV protein may allow some steps in multistep hepatocarcinogenesis to be skipped

The results of our studies on transgenic mice have indicated a carcinogenic potential of the HCV core protein in vivo; thus, HCV would be directly involved in hepatocarcinogenesis. In research studies of carcinogenesis, the theory outlined by Kinzler and Vogelstein[33] has gained wide popularity. They have proposed that the

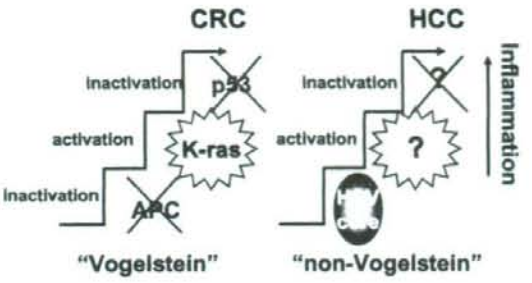

Fig. 3. Mechanism of HCV-associated hepatocarcinogenesis. Multiple steps are required in the induction of all cancers; it would be mandatory for hepatocarcinogenesis that genetic mutations accumulate in hepatocytes. However, in HCV infection, some of these steps may be skipped in the development of HCC in the presence of the core protein. The overall effects achieved by the expression of the core protein would be the induction of HCC, even in the absence of a complete set of genetic aberrations required for carcinogenesis. By considering such a "non-Vogelstein-type" process for the induction of HCC, a plausible explanation may be given for many unusual events happening in HCV carriers

development of colorectal cancer is induced by the accumulation of a complete set of cellular gene mutations. They have deduced that mutations in the APC gene for inactivation, those in K-*ras* for activation, and those in the p53 gene for inactivation accumulate, which cooperate toward the development of colorectal cancer.[33] Their theory has been extended to the carcinogenesis of other cancers as well, called "Vogelstein-type" carcinogenesis (Fig. 3).

On the basis of the results we obtained for the induction of HCC by the HCV core protein, we would like to introduce a different mechanism for hepatocarcinogenesis in HCV infection. We do allow multistages in the induction of all cancers; it would be mandatory for hepatocarcinogenesis that many mutations accumulate in hepatocytes. Some of these steps, however, may be skipped in the development of HCC in HCV infection to which the core protein would contribute (see Fig. 3). The overall effect achieved by the expression of the viral protein would be the induction of HCC, even in the absence of a complete set of genetic aberrations required for carcinogenesis.

By considering such a "non-Vogelstein-type" process for the induction of HCC, a plausible explanation may be given for many unusual events happening in HCV carriers.[34] Now it does not seem so difficult as before to determine why HCC develops in persistent HCV infection at an outstandingly high incidence. Our theory may also give an account of the nonmetastatic and multicentric de novo occurrence characteristics of HCC, which would be the result of persistent HCV infection.

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Review

Experimental models of hepatocellular carcinoma☆

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Hepatocellular carcinoma (HCC) is a common and deadly cancer whose pathogenesis is incompletely understood. Comparative genomic studies from human HCC samples have classified HCCs into different molecular subgroups; yet, the unifying feature of this tumor is its propensity to arise upon a background of inflammation and fibrosis. This review seeks to analyze the available experimental models in HCC research and to correlate data from human populations with them in order to consolidate our efforts to date, as it is increasingly clear that different models will be required to mimic different subclasses of the neoplasm. These models will be instrumental in the evaluation of compounds targeting specific molecular pathways in future preclinical studies.

Keywords: **Liver cancer; Hepatocellular carcinoma; Mouse models; Genetically engineered mice; Cirrhosis**

1. Introduction

Hepatocellular carcinoma is one of the world's deadliest cancers, ranking third among all cancer-related mortalities. Most cases occur in Asia and sub-Saharan Africa, where viral hepatitis is endemic. The incidence is rising in the West, likely due to the increase in patients infected with hepatitis C during the latter half of the last century [1]. The liver, unique in its capacity for regeneration following injury, also gives rise to this malignancy commonly associated with the inflammatory state of advanced fibrosis, or cirrhosis. Potentially curative therapies can be offered to approximately 30% of patients, but are complicated by a high rate of recurrence [2].

Encouraging progress has been made in understanding the molecular pathogenesis of cancer [1,2]. The discoveries of the signal transduction pathways, cascades of protein–protein interactions transmitting information from the cell surface to the nucleus, and of their link to tumor biology, are particularly impressive.

Several key mouse models have been instrumental in defining the pathogenesis of HCC by introducing genetic

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Abbreviations: HCC, hepatocellular carcinoma; HCV, hepatitis C virus; TKR, tyrosine kinase receptor; HBV, hepatitis B virus; TSG, tumor suppressor gene; TSP, tissue specific promoter; Tg, transgene.

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alterations into one or more aetiologic pathways that can be targeted exclusively to the liver. Moreover, these programmed manipulations can be introduced systematically, not only in this specific organ but also at defined times during development, growth and aging of the liver.

Nonetheless, substantial challenges persist in modeling liver diseases whose natural history requires a chronic inflammatory milieu. For example, infectious (hepatitis C virus), toxic (alcohol), metabolic (non-alcoholic steatohepatitis), or congenital (hemachromatosis) diseases share inflammation and fibrosis as precursors to cancer, yet none is easily mimicked in animals. There are few rodent models of HCC arising spontaneously within a background of regenerative nodules and cirrhosis, and most depend on the administration of hepatotoxic and/or carcinogenic agents to recreate the injury–fibrosis–malignancy cycle seen in chronic human liver diseases.

Comparative genomic studies in human HCC samples have begun to identify molecular subgroups with characteristic mutations, gene expression profiles and chromosomal gains and losses [3]. Moreover, since there is no single dominant molecular pathology underlying all HCCs, it is increasingly clear that different models will be required to mimic different subclasses of the neoplasm. These models will be instrumental as pre-clinical tools to evaluate compounds targeting specific molecular pathways.

With these challenges in mind, the objective of this review is to assemble and evaluate the available models of both cirrhosis and HCC, to provide a blueprint for understanding the pathogenesis of HCC and for optimizing preclinical models for drug testing.

2. Experimental models in cancer research

Although many experiments focusing on liver physiology have been conducted in rats due to their propensity for the development of fibrosis, the laboratory mouse (*Mus musculus*) is considered among the best model systems for cancer because of the availability of gene targeting methods, as well as the animal's size and breeding capacity, its lifespan of 3 years, and its physiologic and molecular similarities to human biology [4]. Significant advances have been made in modeling cancer genetics in mice, along a spectrum that ranges from simple xenograft models to more complex, genetically modified mice. Examples of each of the following are illustrated in Table 1.

2.1. Xenograft models

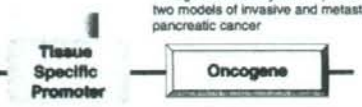
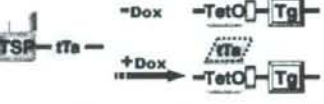
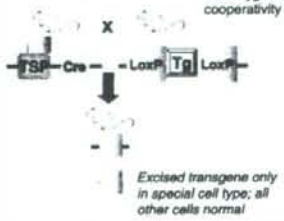
The demonstration that concentrated cancer cells grown *in vitro* could form tumors when implanted subcutaneously into an immunocompromised mouse was first established in 1969 [5]. This xenograft model has since demonstrated several advantages that explain its persistence as the mainstay of pre-clinical studies of anti-neoplastic drugs *in vivo*: the tumors are rapidly and easily induced, and their subcutaneous location enables direct measurement of tumor growth. More recently, however, several critical differences between xenograft- and patient-derived specimens have become apparent, as discussed below. In addition, cancer is now appreciated as a complex disease dependent upon the interaction between transformed cells harboring oncogenic mutations, referred to as the 'cell autonomous compartment', and their surrounding tumor environment, the 'non-cell autonomous constituents' made up of normal cells, stromal cells, and immune cells [4], features that are not part of the xenograft approach.

Mouse models of cancer were first introduced over 60 years ago. Shortly after its inception in 1955, the Developmental Therapeutics Program at the National Cancer Institute (NCI) adopted the use of three transplanted rodent models of sarcoma, carcinoma, and leukemia, for the purposes of selecting agents for clinical use in cancer patients. Thousands of molecules were tested in mice bearing murine leukemias during the first decades of modern cancer drug development, circa 1945–1969 [6]. This tumor panel was later expanded to include human tumor xenografts, with the intention to study drug activity against solid tumors [7]. In 1990, the NCI focused on the development of *in vitro* assays in 60 different cell lines in order to screen pharmaceutical agents for their potency and their selective activity against either a particular disease category or specific cell line [8,9], the most promising of which were to be subsequently evaluated in the nude mouse xenograft model.

The validity of xenografts as a predictive indicator of probable clinical activity is limited, with the most success seen in cytotoxic agents. A retrospective analysis performed by the NCI for 39 compounds in which both xenograft testing and Phase II clinical data were available showing that less than 50% of agents with activity in more than one-third of xenografts showed clinical activity ($p = 0.04$) [6]. The same study demonstrated that activity in a particular histology in a tumor model did not closely correlate with activity in the same human cancer histology [10], with the exception of non-small cell lung and ovarian cancer [11].

There are several variables inherent to the xenograft experiments which may impact on the divergent outcomes compared to human disease, including growth properties and size at initiation of treatment of xenograft tumor, ectopic versus orthotopic location of tumor, local versus metastatic disease [12], tolerance for high doses of chemotherapeutic agents in mice [13],

Table 1
Available mouse models in cancer research

	Technical Method	Advanced Mouse Models of Cancer	Current Models in HCC	Future Prospects: Wish List for HCC
Xenograft	Xenograft 	COLON, BREAST, PROSTATE: Surgical orthotopic implantation: intact fragments of human cancer, including tumors taken directly from the patient, transplanted into the corresponding organ of immunodeficient rodents[18]	Orthotopic xenograft model in which hepatoma 129 cells originating from C3H mice are injected into fibrotic livers of mice pretreated with TAA and EtOH[45]	Mouse HCC cell line derived from GEM tumor with specific molecular pathway dysregulated, with immunofluorescent marker, injected into fibrotic liver of immune-competent mice
Transgenic GEM	Constitutive Transgenic 	PANCREATIC: $Kras^{G12V}$ and chronic pancreatitis[186]; $Trp53^{R172H}$ and $Kras^{G12D}$ double transgenic driven by insulin promoter[170]; two models of invasive and metastatic pancreatic cancer	Mouse C-myc/Human E2F-1 overexpression driven by albumin promoter[171]; HCC at 6-8 months	Double transgenic overexpressing profibrotic gene combined with liver-specific oncogene
	Dominant Negative Transgenic 	PITUITARY: Rb and p27Kip1 Cdk inhibitor tissue specific knockout mice develop pituitary tumors with different phenotypes[172]	Mdr-2 knockout mice are unable to secrete phospholipids into bile, and develop cholangitis and HCC at 6-12 months[188]	Double transgenic liver-specific E-cadherin knockout and β-catenin overexpression
	Inducible Transgenic 	MELANOMA: Double transgenic combining Tet-induced overexpression of mutated $Hras^{V12G}$ and Ink4a knockout[173]	Tet-inducible Met expression under albumin promoter: 60% HCC at 12 months; tumors regressed when transgene (Tg) was inactivated[91] 	Tet-induced, liver-specific overexpression of known oncogene in fibrotic mice
Endogenous GEM	Conditional Gene Targeting 	PROSTATE: Double transgenic Cre-mediated $PTEN^{-/-}$ homozygous loss and $p19Arf^{+/-}$: cooperativity in cancer development[174]	Cre-mediated liver specific PTEN-/- knockout: 66% HCC at 8 months[108]	Cre-mediated, liver-specific knockout of known tumor suppressor gene in fibrotic mice

and variability in selected endpoints. These variables can be minimized if given due consideration in the design of preclinical cancer drug experiments. However, the greatest discrepancies between success of cancer therapies in xenograft models and in human clinical trials are likely due to critical differences in both the tumor cells and their microenvironment. Natural tumor progression is a micro-evolutionary process during which increasingly aggressive clones, generated through genetic instability, emerge from an initially monoclonal lesion. Autochthonous tumors, those that evolve *in situ* from normal cells, tend to have a diminished genetic heterogeneity compared to tumor xenografts, although selective pressures of cell culture or tissue explantation can cause a rapid expansion of a certain clonal constituent of polyclonal tumors [14,15].

One solution to this disparity between cancer cell lines and human tumors is surgical orthotopic implantation, in which intact fragments of human cancer taken directly from the patient are transplanted into the corresponding organ of immunodeficient rodents, as reviewed by Hoffman [16]. This technique has been applied to breast, lung, and prostate cancer among others.

Additional advances have been made in the xenograft model through the addition of mesenchymal stem cells to weakly metastatic cancer cell lines, which enhances the ability of the cell lines to form tumors and to metastasize [17]. Wu et al. were able to isolate a side population (SP) from 29 sarcomas which preferentially formed tumors when grafted into immunodeficient mice; only cells from tumors that developed from the SP cells had the ability to initiate tumor formation upon serial transplantation [18].

Our deepening appreciation of the non-cell autonomous constituents of the tumor microenvironment, including the stroma and immune cells relevant to liver pathology in particular, provides further evidence that the xenograft model is more appropriately termed *animal culture*, as suggested by Tuveson and Frese [4].

2.2. Genetically engineered mouse models (GEM)

The most sophisticated animal models of human cancer are those that have been genetically engineered to mimic the pathophysiological and molecular features of human malignancies [4]. Such models enable the investigation of a range of discrete molecular stages that occur during tumor progression both within tumor cells and within their microenvironment; additionally, mice harboring multiple mutations provide information regarding pathway cooperativity and dependency *in vivo* [19].

Despite these strengths, there are a number of important limitations in mouse models of cancer, such as variation in basic cellular processes, as well as in telomere length and telomerase expression [20,21]. It is also well documented that identical genetic lesions can produce different pathologies in mice than in humans [22]. GEM can be categorized as either transgenic or endogenous models.

2.3. Transgenic models

Transgenic mice are those that are engineered to express either oncogenes or dominant-negative tumor suppressor genes in a non-physiologic manner due to ectopic promoter and enhancer elements [4,19]. Microinjection of recombinant DNA directly into the pronucleus of a fertilized mouse egg is the classic method for generating transgenic mice [23], but transgenic mice can also be produced through gene targeting ("knock-in") and lentiviral transduction in embryonic stem cells.

Constitutive expression of cellular and viral oncogenes and germline disruptions of tumor suppressor genes were the first approaches used to create strains of cancer-prone mice [19,24]. The cDNA constructs can contain promoter elements designed to restrict tissue tropism, so although the effect of the oncogenic gain will be constitutive, its expression can be limited to specific tissues by the use of tissue-specific promoters [19], for example the albumin promoter in liver transgenic models.

Germline tumor suppressor cell mutant mice were initially developed to parallel human inherited cancer predisposition syndromes. However, although many of these heterozygous mice were tumor-prone and demonstrated loss of the wild-type allele in their tumors, few of them developed the clinical features of the cognate human syndrome. For example, loss of the retinoblastoma gene product Rb in humans leads to retinoblastomas, osteosarcomas, and small cell lung cancer; whereas Rb heterozygote mice develop thyroid and pituitary tumors but no retinoblastomas [25]. Rb heterozygotes are able to compensate for loss of Rb, a finding that highlights the existence of shared and predictable cellular process within both species [20,26]. So, although identical genetic lesions may not perfectly recapitulate the human disease in mice, there is no doubt that these genetically engineered mice are valuable tools for understanding the underlying biological mechanisms of tumorigenesis [22]. Their ability to recapitulate the genetic features of amplified proto-oncogenes, such as c-myc [27], has contributed greatly to our understanding of cancer biology.

There are, however, additional weaknesses of these models that have spurred the development of more advanced methods. For example, because the genes affected may be vital to normal development, overexpression or ablation may lead to embryonic lethality or infertility [24]. Promoter fragments typically represent the minimal sequence required for tissue-specific expression and do not necessarily allow the same control conferred by endogenous regulatory elements [28]; for example, a typical transgene would not include all transcription factor and microRNA binding sites [4,29]. And, although the DNA fragments are thought to associate by homologous recombination before integration and in most cases insert at a single chromosome site [23], there is little control over site of integration and copy number [22]. This can result in pronounced variability of expression patterns, as the exogenous gene can affect genes near its insertion site or can be affected by endogenous control elements [22,30–32]. Also, although conventional mouse mutants may be useful for modeling familial forms of cancer, they do not mimic sporadic tumorigenesis because the

initiating mutation is present in all cells of the body, including those that constitute the tumor microenvironment [33].

2.4. Inducible systems of oncogene expression

Bujard and colleagues developed a strategy for temporally controlled and reversible transgene expression, using a tetracycline (tet-) inducible system [34]. These drug- or ligand-inducible systems involve the use of a chimeric transcriptional activator that reversibly activates a target gene in response to the administration of the inducing agent.

The *Escherichia coli* tetracycline resistance operon has been applied widely to generate cell lines and murine models with tightly regulated gene expression in response to tetracycline [35]. The tet transactivator functions either as a constitutive repressor that is inducibly inhibited by ligand to allow expression from the *tet* operon (tTA), or it acts as an inducible activator of the *tet* operon upon ligand addition (rtTA) [19]. This system has been particularly useful to study the concept of oncogene addiction; nearly all oncogenes tested thus far seem to be required not only for tumor initiation, but also for tumor maintenance [33].

2.5. Endogenous GEM: knock-out models

Endogenous GEM are those that lose the expression of tumor suppressor genes (TSG) or that express dominant-negative tumor suppressor genes or oncogenes from their native promoters [4]. The original 'knockout' mouse model entailed disruption of an allele in endogenous embryonic stem cells using a targeting vector. Biallelic disruption of TSG often results in embryonic lethality, but heterozygous mice can be used to determine the tumorigenic potential of the genes, such as the retinoblastoma tumor suppressor gene (Rb) [25]. These germline mutations are present throughout the mouse and are constitutively expressed, unlike the sporadic mutations occurring in human tumors that are surrounded by normal tissue.

2.6. Endogenous GEM: conditional gene targeting

As reviewed by Maddison et al. [22], model systems have now been developed which allow both spatial and temporal control of gene expression. These are predominantly dependent on the creation of bi-transgenic mice: those carrying a tissue-specific, inducible transactivator gene are crossed to mice carrying the allele of interest which has been engineered to be controlled by the transactivator. Offspring that carry both transgenic elements are treated with the inducer to express the transactivator gene in a specific tissue, which then acts on the desired allele. This system requires the exogenous delivery of the cre gene (usually by an adeno- or retrovirus), and the induction is irreversible.

Conditional inactivation of tumor suppressor genes relies on the ability of a viral or prokaryotic site-specific recombinase to recognize a pair of target DNA sequences and catalyze recombination at these sites, which results in either deletion or inversion of the intervening DNA sequence [19]. A commonly used tool is the Cre-Lox system, wherein Cre (*C*auses *r*ecombination) recombinase, isolated from bacteriophage P1, catalyses site-specific recombination between defined 34 bp Lox P sites (*Lo*cus χ of crossover *P*1) [36,37]. If gene χ is placed between two Lox P sites and then exposed to Cre, it will be excised, or 'floxed out'. An alternative system to Cre-Lox uses the FLP recombinase, which recognizes the 48 bp Frt site [38]. Transgenic mice that express recombinase from a specific promoter are bred to mice carrying conditional tumor suppressor gene mutations, so that the TSG can be bi-allelically inactivated to allow the generation of organ- and cell-lineage-specific tumors models [19].

Conditional activation of oncogenes is created by the insertion of a LoxP flanked transcriptional silencing element between the promoter and the mutant oncogene-encoding sequence. Conditional oncogenes are constructed using classic transgenic technology, but expression of the oncogene is only activated by the recombinase-mediated removal of the transcriptional silencer. This allows for tissue-specific oncogene expression [39].

This second generation of GEM, which more faithfully recapitulates sporadic tumor formation by the induction of somatic mutations in a time- and tissue-specific fashion, has provided great insight into the contribution of genes in the initiation, progression, and treatment of cancer. We will now discuss how each of these systems has been used to further our understanding of liver cancer.

3. Experimental models of hepatocellular cancer

Hepatocellular carcinoma universally arises upon a background of inflammation and fibrosis. Creation of animal models of HCC presents a particular experimental challenge because of the difficulty in modeling chronic inflammation without using carcinogens to induce liver injury, and because of the heterogeneity of molecular pathways that are dysregulated during this transition from cirrhosis to cancer.

HCC is preceded in both rodents and humans by the development of premalignant lesions including foci of altered hepatocytes and dysplastic nodules, which exhibit a higher risk of malignant evolution than normal cells [40,41]. Various genetic alterations and exposures to chemical carcinogens have been studied in animals

in order to recapitulate the phenotypic, biological, and molecular events that occur during this transformation.

3.1. Xenograft models of HCC

In a recent attempt to characterize primary human xenografts in liver cancer, seven different primary HCC cell lines were injected into SCID mice. The mice were then treated with common chemotherapeutic agents such as cisplatin and gefitinib. There were significant differences in tumor growth inhibition between xenografts, which reinforced the concern for high internal variability of this model in human cancer. Interestingly, the study concluded that most of the chemotherapeutic agents currently used in the treatment of HCC have little or no anti-neoplastic activity in these models [42].

Ma et al. have examined HCC cells expressing CD133 [43], which exhibit stem cell properties and are chemoresistant: purified CD133(+) HCC cells isolated from human HCC cell lines and harvested from xenograft mouse models survived chemotherapy in increased proportions relative to most tumor cells which lack the CD133 phenotype [44]. The inclusion of stem cell-enriched HCC cell lines will likely enhance future preclinical studies in HCC therapeutics (see Table 1).

A group of investigators at the University Hospital Bonn created an orthotopic xenograft model in which hepatoma 129 cells originating from C3H mice were injected into fibrotic livers of mice pretreated with thioacetamide by intraperitoneal injection and alcohol per oral [45]. They found that tumors in fibrotic livers grew significantly larger and more rapidly than those in normal livers, and were able to metastasize and form satellite nodules. Gene expression analysis revealed greater intratumoral expression of vascular endothelial growth factor (VEGF) and its receptor (VEGFR), and of MMP-2 and MMP-9 in the fibrotic liver tumors. This useful model provides a unique tool for testing drug efficacy in orthotopic hepatoma xenograft within the context of liver fibrosis.

3.2. Viral models of HCC

Infection causing latent or chronic viral hepatitis is the most common aetiology of HCC, comprising 80% of cases worldwide. Hepatitis B virus (HBV) is endemic in China, Southeast Asia, and sub-Saharan Africa; there, vertical transmission of the virus results in high rates of HCC. Hepatitis C (HCV) viral infection is more prevalent in the United States and Europe than either HBV or HIV [46]. The woodchuck hepatitis virus (WHV) induces a liver inflammation, injury and repair process in woodchucks similar to those of HBV-positive patients and has therefore proven to be a useful model of the disease.

3.2.1. Hepatitis B virus

HBV is a DNA virus that causes acute and chronic hepatocyte injury, inflammation, and HCC. During prolonged infection, viral DNA sequences integrate into the host cell genome, where they and the flanking cellular sequences are commonly rearranged [47], a phenomenon that can activate an adjacent cellular oncogene. In addition, viral infection can induce hepatocyte injury mediated by the antiviral cellular immune response and, to a lesser extent, by direct injury to the cells. Although most cases of HBV-associated HCC arise in a background of inflammation and fibrosis, the virus is notorious for also causing HCC in the absence of cirrhosis, most likely by integrating into the host chromosome and thereby promoting transcriptional transactivation of mitogenic factors.

The HBV virus is a circular DNA molecule containing four open reading frames encoding four HB viral proteins: preS/S, preC/C, P and X protein (HBx). The most common viral marker in HCC is the integration of HBV genomic DNA encoding HBx. In 1994, Koike et al. published their description of a transgenic mouse model demonstrating that high levels of HBx expression were sufficient to generate HCC in 84% of male transgenic mice at age 13–24 months [48] (see Table 2). Analysis of proliferation and DNA content in these mice suggested that the continued expression of HBx gene initiated tumor formation by inducing DNA synthesis and placing large numbers of hepatocytes subjective to secondary events for transformation [48]. Yu et al. also confirmed the development of HCC in HBx transgenic mice [49]. Although another research group did not see spontaneous HCC development, those HBx transgenic mice were more susceptible to chemical carcinogenesis than control mice [50]. The reason for this discrepancy is unclear, but the difference in genotype of HBV should be noted: HCC tumors arose in genotype C HBx transgenic mice but not in other genotypes [51].

Chisari et al. described a transgenic model that overproduces the hepatitis B virus large envelope polypeptide and accumulates toxic quantities of hepatitis B surface antigen (HBsAg) [52]. This hepatocellular injury initiates a programmed response within the liver, characterized by inflammation, regenerative hyperplasia, transcriptional deregulation, aneuploidy and eventually HCC. Inappropriate expression of a single structural viral gene was thereby shown to be sufficient to cause malignant transformation. The process of oncogenesis seen in this model also supports the theory that severe, prolonged cellular injury can induce a proliferative response that fosters secondary genetic events that lead to unrestrained growth [47]. However, the level of viral protein expression in this model may well surpass the expression in human infection.

Table 2
Genetically engineered models of hepatocellular carcinoma

Gene	Type of mutation or tissue promoter/construct	Phenotype (+/− and −/−)	Chemically induced/ metastasis	References
Viral models				
Hepatitis B virus large envelope protein	BglII-A fragment of HBV encoding large envelope protein under control of albumin promoter and enhancer	Focal necrosis, inflammation, and subsequent HCC in 72% males	No metastases; rare local invasion	[47,52]
Hepatitis B virus X protein	EcoRI–BglII fragment of HBV including the X gene under its own promoter and enhancer	HCC in 84% after 13–24 months in mice with high HBx expression	Lung metastasis	[48,175,176]
Hepatitis C virus	HCV core-E1–E2 transgenic under albumin promoter and HCV core transgenic under HBV X promoter	No DEN: No HCC in either strain by 21 months. +DEN: 100% HCC at 32 weeks; HCV core-E1–E2 with largest tumors ($p = 0.008$)	DEN injected weekly × 6 weeks	[56]
Hepatitis C virus	HCV core under HBV X promoter; HCV E1–E2 under HBV X promoter	Core transgenics: 32% HCCs in male mice at 16–23 months; E1–E2 transgenics: no HCC. No evidence hepatitis	None reported	[56,59]
Hepatitis C virus	HCV core-E1–E2 transgenic under albumin promoter and the entire HCV transgenic under albumin promoter	HCC in core-E1–E2 transgenic and entire HCV transgenic after 13 months	None reported	[60]
Cell cycle models				
p53 germline knockout and liver-specific viral receptor TVA, injected with PyMT oncogene	p53 germline knockout [177] crossed with mice expressing viral receptor TVA under albumin promoter (Alb-TVA), injected at age 3 days intrahepatically with mouse polyoma virus middle T antigen	HCC in 42% of p53 null mice, in 37% of $p53^{+/-}$, and in 66% of $p53^{+/+}$ mice expressing TVA injected with PyMT at 4 months. No TVA-negative littermates developed HCC	Metastases in p53 null mice (6/16); less in $p53^{+/-}$ (1/14)	[67,177]
Trp53 and INK4a/ARF conditional mutant mice, injected with PyMT oncogene	Albumin Cre mice crossed with Trp53 conditional mutant and INK4a/ARF conditional mutant, injected at age 3 days intrahepatically with mouse polyoma virus middle T antigen	>90% HCC in combined Trp53, INK4a/ARF null mice injected with PyMT compared to single null gene	Metastases in Trp53 null mice (30%) and in combined Trp53, INK4a/ARF mice (63%) at 6 months	[68]
P53 conditional expression	Hepatoblasts transduced with oncogenic ras (Hras V12) and a tet-responsive P53 miRNA design short hairpin RNA	Complete tumor regressions when endogenous p53 reactivated in p53-deficient tumors	None reported	[69]
c-myc	c-myc over-expression under albumin enhancer/promoter [74,90,178,179]; under α1 antitrypsin promoter [180,181]	15 weeks: polyploidy cells, dysplasia >60% [179]; 15 mos: 91% adenomas [74,178]; 54% HCC [178,180,181];	None reported	[74,90,178–181]
c-myc and E2F-1	Mouse c-myc and human E2F-1 over-expression under albumin promoter	6–8 mos: 100% HCC [171,178]	None reported	[90,171,178,179]
c-myc and TGFα	c-myc over-expression under albumin enhancer/promoter; TGFα over-expression under metallothionein 1 promoter	4 mos: 70% dysplastic nodules; 18% HCC [90]	Zinc in H_2O accelerated nodule formation by 6–8 weeks	[90,182]

(*continued on next page*)

Table 2 (*continued*)

Gene	Type of mutation or tissue promoter/construct	Phenotype (+/− and −/−)	Chemically induced/ metastasis	References
SV40 T-antigen conditional and inducible expression	SV40 T-antigen expression under albumin enhancer/ promoter [74]; under major urinary protein enhancer/ promoter [183]; under metallothionein 1 promoter [184]; under α1 antitrypsin promoter [185]; under antithrombin III promoter [186]; tetracycline-inducible expression: mice expressing tTa under albumin promoter crossed with mice expressing T antigen under tTa promoter [75]	3–7 mos: adenomas and HCC [74]; 10–12 weeks: HCC[185]; after 4–6 weeks: 100% HCC [186]	Lung metastases [186]	[74,75,181,183–186]
E2F-1	E2F-1 over-expression under control of albumin enhancer/ promoter	10 mos: 100% adenomas and dysplastic nodules; 12 mos: 33% HCC	None reported	[71,90,179]
Telomere dysfunction models				
mTERT$^{-/-}$ and p53$^{+/-}$ or WT	Germline mTERT and p53 knockout over several generations and CCl_4 liver injury	50 weeks: 100% HCC in p53$^{+/-}$ both generations (G0 and G3/ G4); 44% in wild-type G0 versus 9% HCC in wild-type G3/G4	CCl_4 by IP injection 3×/ week × 4 months	[66]
Pathway specific models				
Wnt/β-catenin				
Activating mutation in β-catenin: truncated NH_2 terminal transgenic	EAB/9K/Δ N131 β-catenin construct under control of liver-specific enhancer of aldorase B gene (expressed throughout embryonic and post-natal development)	Death at 3 weeks from hepatomegaly; no dysplastic foci in liver	N/A	[127]
Activating mutation in β-catenin: exon 3 conditional knockout	Catnb$^{lox(ex3)}$ knockout and fatty acid binding protein Fabpl-cre transgenic	Death at 5 weeks from liver damage/mitochondrial swelling. No dysplastic foci in liver; +intestinal polyps	N/A	[128]
Activating mutation in β-catenin: exon 3 conditional knockout	Catnb$^{lox(ex3)}$ knockout injected with recombinant adenovirus expressing Cre from human CMV promoter	High multiplicity injection (10^9 pfu/mouse): death at 3 weeks with hepatomegaly/ mitochondrial swelling. Low multiplicity injection (10^{7-8} pfu/ mouse): No dysplastic foci in liver >6 mos	N/A	[128]
β-catenin exon 3 knockout and activated H-ras (H-ras^{G12V}) double-transgenic conditional	Catnb$^{lox(ex3)}$ knockout and H-ras (Tg$^{lox(pA)H\text{-}ras^*}$) double-transgenic with recombinant adenovirus expressing Cre from human CMV promoter	Low multiplicity infection (10^8 pfu/mouse): 100% HCC at 6 months	Intrahepatic invasion	[131]
APC knockout liver-specific	Apc$^{\Delta ex14}$ knockout (−/−) injected in tails with recombinant adenovirus expressing Cre (injections infected primarily and massively the liver)	High multiplicity infection (10^9 pfu/mouse): Death within 2 months and hepatomegaly. Lower multiplicity infection (0.5×10^9 pfu/mouse): 67% HCC at 9 months. Apc$^{+/-}$ had no liver abnormalities	None reported	[130]
β-catenin wild-type	β-catenin over-expression under control of albumin enhancer/promoter	Hepatomegaly (15% increased liver/body weight ratio); no dysplastic nodules at 24 months	N/A	[129]

Table 2 (*continued*)

Gene	Type of mutation or tissue promoter/construct	Phenotype (+/− and −/−)	Chemically induced/ metastasis	References
PI3K/Akt pathway				
PTEN$^{-/-}$	Albumin cre/PTEN$^{lox/lox}$	Steatohepatitis; Adenomas at 44 weeks and 66% HCC at 78 weeks [108]; HCC in 66% of males at 44 weeks and in 83% of males and 50% of females at 78 weeks [109]	Lung metastases	[108,109]
Insulin growth factor pathway				
IGF2 transgenic	IGF2 over-expression under control of urinary protein promoter	HCC in <10% at 18–24 months; also lymphomas, sarcomas, and thyroid carcinomas	None reported	[187]
IGF2 knockout and TGFα transgenic	TGFα over-expression under metallothionein 1 promoter [86] crossed with IGF2 heterozygous knockout mice (paternal null allele; maternal wild-type, normally imprinted)	(1) IGF2$^{wt/wt}$; no HCC; 4% adenoma; (2) IGF2$^{+/-}$: dwarves, normal liver phenotype; (3) TGFα × IGF2$^{wt/wt}$ and (4) TGFα + IGF2$^{wt/-}$: 100% HCC at 18 months	None reported. Zinc in drinking water starting at age 10 months	[188]
Epidermal growth factor pathway				
EGF transgenic	Double-transgenic of the liver construct Alb-DS4 that encodes autocrine growth factor IgEGF crossed with AAT-myc mice	EGF transgenic (Alb-DS4): mortality from HCC by age 7.1 months; EGF/myc double-transgenic: accelerated mortality to 4.4 months		[115]
Ras signaling				
H-ras	Mutant c-H-ras over-expression under albumin promoter	Hepatomegaly, lung tumors [74]	None reported	[74]
HGF/c-Met and TGF-α				
HGF transgenic	Mouse HGF expression driven by metallothionein promoter [95]; by albumin promoter[189]	Hepatomegaly; >17 months: adenomas and rare HCCs [95]; rapid recovery after partial hepatectomy, no dysplasia [189]	Most animals not given zinc because transgene expression adequate	[95,189]
HGF over-expression +/− β-catenin conditional knockout	Hydrodynamic injection of plasmid containing HGF under CMV promoter (pCMV-HGF) into wild-type and into AFP-enhancer albumin promoter-Cre floxed β-catenin knockout mice	HGF over-expression: hepatomegaly and increased Wnt/β-catenin signaling; no dysplastic nodules. HGF over-expression in β-catenin knockout: no alterations in liver	N/A	[96]
HGF + c-myc	Double-transgenic mouse c-myc driven by albumin promoter/enhancer and human HGF driven by albumin regulatory elements	Inhibition of hepatocarcinogenesis by HGF in c-myc transgenic mice: 0% HCC in HGF/c-myc versus 60% HCC at 16 months in c-myc single transgenic, even with addition of phenobarbital	Phenobarbital	[97]
HGF + TGF-α	Double-transgenic mouse TGFα over-expression under metallothionein 1 promoter and human HGF driven by albumin promoter	Increased proliferation and c-myc expression in HGF over-expressing mice. Diminished hepatocarcinogenesis by HGF in TGFα transgenic mice: 33% (3/9 mice) HCC in HGF/ TGFα versus 60% (6/10) in TGFα single transgenic	None reported	[98]
Met transgenic	Tetracycline-inducible expressing human Met under liver-specific promoter crossed with mice expressing tetracycline transactivator under liver-specific liver activating protein (MET-TRE/LAP-tTA) [91,99]	12 months: 60% HCC; tumors regressed when transgene was inactivated [91]; by 4 months, adenomas and HCC [99]. +Recurrence of HCC in mice whose original tumors had regressed on Doxycycline	None reported	[91,99]

(*continued on next page*)

Table 2 (*continued*)

Gene	Type of mutation or tissue promoter/construct	Phenotype (+/− and −/−)	Chemically induced/ metastasis	References
Met and β-catenin	Transposable vectors containing wild-type human *MET*, constitutively-activated mutated form of β-catenin (*ΔN90-CTNNB1*), and dominant-negative TCF-1 (*DNHNF1*), by hydrodynamic transfection into livers	Combination *MET* and *ΔN90-CTNNB1*: 74% HCC within 1 month (no adenomas); combination *MET* and *DNHNF1*: 50% hepatic adenomas within 1 month; each one individually, no tumors	Death within 3 months	[99]
c-Met conditional knockout	c-Met conditional liver-specific knockout (MetLivKO) using floxed Met ($c\text{-}met^{fl/fl}$) and Cre driven by albumin promoter ($AlbCre^{-/-}$)	100% HCC in MetLivKO at 6 months versus 44% in control; greater number and size of tumors in MetLivKO; protumorigenic effects of c-Met deficiency reversed by early administration of antioxidants	*N*-nitrosodiethylamine	[94]
TGFα	TGFα over-expression under metallothionein 1 promoter [86,87,90]	10–15 mos: 50% HCC [87];100% HCC [86] +mammary/pancreatic hyperplasia [86,88]	Zinc in H_2O increased tumor formation; no metastases	[86–88]

The transgenic mouse expressing PreS, S and X proteins (Tg (HBV Alb-1) Bri44) described by Chisari et al. [52] has also been studied more extensively for its stepwise accumulation of liver disease. Gene expression in this model generates hepatocyte damage and inflammation early, generating dysplastic nodules by age 9 months, and macroscopic HCC nodules by age 18 months [53].

3.2.2. Hepatitis C virus

Hepatitis C virus (HCV) infects 170 million people worldwide, and the recent increase in HCC in the United States has been attributed to an increase predominantly among patients with chronic HCV infection. HCV does not cause insertional mutagenesis, but rather is thought to produce HCC through the cumulative effects of chronic infection, injury and repair. Most cases of HCC occur after several decades of infection with HCV and in a microenvironment of cirrhosis [54].

Several models have attempted to emulate HCV viral infection in hepatocytes in order to better understand its oncogenicity. Transgenic mice encoding the core, E1 and E2 structural proteins under control of the albumin promoter did not develop hepatic disease [55], although when the same strains were exposed to diethylnitrosamine (DEN), there were significantly larger HCC tumors in core-E1–E2 transgenic mice relative to the core and non-transgenic strains [56]. Koike et al. described two different mouse strains expressing HCV core protein under control of the HBV promoter; these mice developed steatosis after several months [57] and HCC in 32% of animals after 16–23 months [58,59]. The same study found no adenomas or carcinomas in transgenic mice over-expressing HCV envelope genes. Lerat et al. also described the development of HCC in mice transgenic for the entire HCV genome or core-E1–E2 structural genes under the control of albumin promoter [60].

The mechanism by which HCV core protein promotes oncogenesis is unclear. HCV core transgenic mice have been studied for their gene expression patterns, revealing that interleukin-1 (IL-1) and tumor necrosis factor (TNF) are transcriptionally activated in these models. Reactive oxygen species (ROS) are produced in HCV core transgenic mice even in the absence of hepatitis and inflammation [61]. Alcohol can act synergistically to produce ROS in HCV-core protein transgenic mice [62]. Clinically, heavy alcohol use is known to enhance the development of cirrhosis and HCC [60] in patients chronically infected with HCV; thus, production of ROS may be the common instigator.

3.2.3. Woodchuck hepatitis virus

Woodchucks develop cirrhosis and HCC from chronic Woodchuck Hepatitis Virus (WHV) infection. During the course of infection, WHV DNA is stably integrated into the DNA of 1–5% of hepatocytes [63], and causes HCC within the first 2–4 years of life [64]. Over 50% of these HCCs contain integrations of WHV DNA within, or immediately adjacent to, a unique and functional N-myc 2 retroposon, and are associated with increased IGF-2 expression [65].

3.3. Experimental models recapitulating molecular events of hepatocarcinogenesis

3.3.1. Cell cycling pathways: p53, Rb, E2F, SV40 T antigen

Cancer is a disease of the cell cycle in the majority of cases, as most tumors contain defects in cell cycle

machinery. Fundamental to our understanding of cancer biology have been models simulating loss of tumor suppressors p53 and Retinoblastoma (Rb), key regulators of cell cycling and frequent targets of carcinogens. There is a large body of evidence indicating a pivotal role for cell cycle deregulation during hepatocarcinogenesis [41].

Tumor suppressor p53 acts to restrict proliferation in response to DNA damage or deregulation of mitogenic oncogenes, by leading to the induction of various cell cycle checkpoints, to apoptosis, or to cellular senescence. p53 heterozygous mutant mice appear to be susceptible to HCC formation in the context of liver injury, but only in the absence of intact telomerase [66].

Trp53 knockout mice develop larger, more invasive tumors than wild-type mice when mouse polyoma virus middle T antigen (PyMT) viral oncogene is introduced into the liver under an albumin promoter [67]. Liver-specific knockout of Trp53 when combined with liver-specific PyMT expression also results in an invasive, metastatic phenotype. Concomitant loss of Ink4a/Arf tumor suppressor locus accelerates this process [68].

Lowe and colleagues assessed the extent to which p53 loss is required for maintaining established tumors [69]. To do so, they first transduced hepatoblasts *in vitro* with oncogenic *ras* (*HrasV12*) and a tet-responsive p53 shRNA (miR30 design short hairpin RNA), and then injected the cells into the spleen of nude mice. Next, they used RNA interference (RNAi) to conditionally regulate p53 expression in the nodules that had formed by transduced hepatoblasts seeding in the liver. The authors concluded that p53 loss can be required for the maintenance of aggressive carcinomas, and that the cellular senescence program can act together with the innate immune system to potently limit tumor growth.

The retinoblastoma (Rb) pathway plays its role in cell cycle regulation by guarding and triggering DNA replication and cell cycle division in late G1. Rb binds members of the E2F family, and in doing so represses transcription of E2F regulated genes, which mediate DNA synthesis and cell cycle regulation [70]. After noting upregulation of E2F in liver tumors from their c-myc/TGF-α double-transgenic mice, Conner et al. generated E2F transgenic mice under control of the albumin enhancer/promoter [71]. All of these mice formed adenomas after 10 months, and a minority developed HCC (2/6). When crossed with c-myc transgenic mice, HCC development was accelerated, with 100% tumor formation within 6–8 months. Further investigation of this model revealed activation of the Wnt/β-catenin pathway in a majority of the tumors, as demonstrated by accumulation of nuclear β-catenin; this occurred in the absence of mutations of β-catenin [72].

SV40 (Simian Vacuolating Virus 40) large T antigen (TAg) is an oncoprotein derived from the polyomavirus SV40 which is capable of transforming a variety of cell types. The transforming activity of TAg is due mainly to its perturbation of the retinoblastoma (pRB), p53 and p105 tumor suppressor proteins. This causes the cells to leave G1 phase and enter into S phase, which permits DNA replication of both the cell and the viral genome [73]. In addition, TAg binds to several other cellular factors, including the transcriptional co-activators p300 and CBP, which may contribute to its transforming capacity. SV40 T-antigen expression under the albumin enhancer/promoters provoked the appearance of adenomas and HCC within 3–7 months [74]. A tetracycline-inducible binary transgenic mouse model of SV40 was found to develop hepatic neoplasia in 60% of cases (3/5); no neoplasia was observed in mice with suppression of transgene expression by tetracycline administration [75].

3.3.2. Telomere dysfunction

Telomeres are regions of DNA near the ends of eukaryotic chromosomes that act to prevent loss of genetic information during chromosomal replication. They are synthesized and maintained by telomerase, part of a group of enzymes called TERT (telomerase reverse transcriptases). Because of cell division mechanisms and because telomerase expression is repressed in most human cells (with the exception of stem cells and some leukocytes), telomere length decreases with each cell division. Once telomeres reach a critically short length, they unfold; this uncapping is detected and the cell undergoes senescence (the "Hayflick limit") [76]. Neutralization of p53 or Rb function results in continued telomere attrition, culminating in chromosomal instability and cell death [77]. Low levels of telomerase are associated with aging and tumorigenesis in some tumors such as colorectal cancer [78] but levels are typically increased in HCC [79,80].

Telomere attrition has been documented in hepatocytes from cirrhotic patients [81,82]. It is thought that repeated rounds of hepatocyte injury and regeneration may promote telomere shortening, which would ultimately lead to chromosomal instability (CIN), a common feature of HCC. Indeed a correlation between CIN, telomere shortening, and HCC was demonstrated in a series of 39 patients with HCC by analysis of liver biopsies for ploidy and telomere length [83].

In mice, reduction in telomere length is not observed, probably due to long initial telomere length and active telomerase expression [84]. However, in p53-mutant mice, deficiency of telomerase promotes formation of non-reciprocal translocations and epithelial cancers [85]. The cooperative roles of telomerase-induced chromosomal instability and attenuated p53 function in the liver was illustrated by a study which showed enhanced HCC formation in p53-mutated telomerase knockout mice ($mTERT^{-/-}$). In the setting of intact telomeres, however, p53 mutation had no effect on tumor formation [66].

3.3.3. Growth factor signaling pathways

3.3.3.1. TGF-α and c-myc. Transforming Growth Factor (TGF)-α binds and activates EGFR and is mitogenic toward hepatocytes. In most organs, metallonein-driven over-expression of TGF-α causes epithelial hyperplasia [41]. In liver and breast tissue, the phenotype extends to neoplastic transformation. Tumor incidence is 100% in susceptible mice strains after a substantial latency [86–88]. Gefitinib, an EGFR inhibitor, significantly reduces HCC development in rats with cirrhosis induced by DEN administration [89].

Co-expression of TGF-α and c-myc can occur in HCC. Liver-specific c-myc over-expression induces persistent hepatocyte proliferation and eventual HCC. When c-myc and TGF-α are co-expressed, this process is accelerated [90].

3.3.3.2. Hepatocyte growth factor and c-Met pathway. When stimulated by its ligand, hepatocyte growth factor (HGF) elicits multiple biological responses including proliferation, migration, invasion, and morphogenesis [91]. Over-expression, amplification, and mutation of the *MET* proto-oncogene which encodes protein tyrosine kinase receptor Met have been demonstrated in human HCC samples [92,93]. Nevertheless, experimental mouse models of HCC have revealed that the net outcome of HGF/c-Met activation could be either stimulation or inhibition of hepatocarcinogenesis [94]. Transgenic mice over-expressing HGF driven by the metallothionein promoter developed HCC [95], but when HGF expression was driven by the CMV promoter, mice developed hepatomegaly but not dysplasia [96]. Inhibition of hepatocarcinogenesis by HGF in c-myc transgenic mice was demonstrated by Thorgeirsson et al. in 1996: none of the liver-specific HGF/c-myc over-expressing mice developed HCC and only 30% developed adenomas, versus HCC in 60% of the c-myc single transgenic, even with addition of phenobarbital [97]. Similarly, HGF co-expression inhibited tumor formation in TGF-α transgenic mice [98].

The paradoxical effects of HGF ligand expression are mirrored in Met receptor expression. Bishop and colleagues demonstrated that over-expression of wild-type Met in hepatocytes of transgenic mice leads to the development of HCC [91]. Interestingly, these mice were found to have frequent activating mutations of β-catenin, and it was subsequently discovered that there was a correlation between MET activation and β-catenin mutations in human HCCs. Spurred by these findings, vectors of human MET and β-catenin with activating mutations were hydrodynamically cotransfected: these mice developed larger HCCs with short latency periods, confirming a cooperative relationship between *MET* over-expression and β-catenin mutations [99].

Recently, however, Takami et al. reported that loss of c-Met signaling enhanced rather than suppressed the early stages of chemical hepatocarcinogenesis [94]: C-met conditional knockout (MetLivKO) mice treated with *N*-nitrosodiethylamine developed significantly more and bigger tumors and with a shorter latency compared with control mice. These knockout mice had increased oxidative stress demonstrated signs of was reversed by administration of antioxidant *N*-acetyl-L-cysteine. The authors concluded that intact HGF/c-Met signaling is essential for maintaining normal redox homeostasis in the liver. Further studies will be needed before definitive conclusions can be drawn regarding the role of HGF/c-Met signaling in HCC.

3.3.3.3. PTEN/Akt/mTOR signaling pathway. The serine/threonine kinase Akt (PKB) was first isolated as an oncogene transduced by the acute transforming retrovirus [100,101]. Its role in human cancer was established shortly thereafter by demonstration of its frequent amplification and over-expression in various cancers, including breast and ovarian [102]. Akt acts as a cytoplasmic central regulator of numerous signals related to cell cycling (Cyclin D1), cell survival (Mdm2/p53), cardiovascular homeostasis (eNOS), and cell growth (mTOR), among others [103]. PTEN is a negative regulator of the pathway and its loss activates Akt.

Tissue-specific knockout models of PTEN in pancreas develop tumors with high penetrance [104]. Transgenic animals over-expressing Akt develop a hyperplasic but not malignant phenotype, typically requiring a second hit to generate cancer [105,106]. Notably, mTOR inhibition can reverse these phenotypes, suggesting the presence of an mTOR-dependent survival signal downstream of Akt [107]. Liver-specific deletion of PTEN results in hepatomegaly and steatohepatitis by 10 weeks and HCC in a majority of male mice by 20 months [108,109].

3.3.3.4. IGF and EGF signaling pathway. The insulin growth factor (IGF1 and IGF2) signaling pathways regulate cell growth, differentiation and survival, and play a central role in embryogenesis and regulation of lifespan. IGF-2 possesses both mitogenic and metabolic properties; 16–40% of human HCCs demonstrate over-expression of IGF-2 [110].

The coordinated expression of IGF-2 and its receptor suggests a role for IGF-2R in regulation of extracellular IGF-2 concentration; alterations in the expression of IGF-2R in human tumors suggest it may act as a tumor suppressor gene [111].

Transcriptional activation of IGF2 has been demonstrated in HCCs arising in HBV-associated human samples [112] and in HBV transgenic mice [41]. To investigate whether IGF-2 has a promoter role in a slowly developing HCC model, TGFα transgenic mice were crossed with IGF-2 hemizygous knockout mice