

## 2016 Hepatocellular Carcinoma: Global view

# Hepatocellular carcinoma mouse models: Hepatitis B virus-associated hepatocarcinogenesis and haploinsufficient tumor suppressor genes

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## Abstract

The multifactorial and multistage pathogenesis of hepatocellular carcinoma (HCC) has fascinated a wide spectrum of scientists for decades. While a number of major risk factors have been identified, their mechanistic roles in hepatocarcinogenesis still need to be elucidated. Many tumor suppressor genes (TSGs) have been identified as being involved in HCC. These TSGs can be classified into two groups depending on the situation with respect to allelic mutation/loss in the tumors: the recessive TSGs with two required mutated alleles and the haploinsufficient TSGs with one required mutated allele. Hepatitis B virus (HBV) is one of the most important risk factors associated with HCC. Although mice cannot be infected with HBV due to the narrow host range of HBV and the lack of a proper receptor, one advantage of mouse models for HBV/HCC research is the numerous and powerful

genetic tools that help investigate the phenotypic effects of viral proteins and allow the dissection of the dose-dependent action of TSGs. Here, we mainly focus on the application of mouse models in relation to HBV-associated HCC and on TSGs that act either in a recessive or in a haploinsufficient manner. Discoveries obtained using mouse models will have a great impact on HCC translational medicine.

**Key words:** Hepatocellular carcinoma; Mouse models; Hepatitis B virus; Haploinsufficiency; Tumor suppressor genes

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**Core tip:** Hepatitis B virus (HBV) viral products, in particular the oncogenic HBV X protein, and mutations of tumor suppressor genes (TSGs) are the driving force of hepatocellular carcinoma (HCC). Inactivation of a recessive TSG requires mutations in both alleles and fits the “two-hit” model. However, haploinsufficiency occurs when one allele is insufficient to confer the full functionality of a TSG; the gene’s effect can be partial or complete depending on tissue type, genetic modifiers/background, and environmental factors. Mouse models play a pivotal role in demonstrating the oncogenic effects of viral products and in establishing the dose-dependency and quantitative differences when analyzing a TSG involved in HCC.

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## INTRODUCTION

Liver cancer is a member of the top ten most common cancers in both men and women<sup>[1]</sup>. The most common type of primary liver cancer in adults is hepatocellular carcinoma (HCC), which is derived from hepatocytes and accounts for 70% to 80% of cases<sup>[2,3]</sup>. Liver cancer has a high mortality and poor prognosis because patients usually are diagnosed with the disease at a well-advanced stage, making liver resection or transplantation unavailable as a therapeutic option. In addition, the recurrence rate of HCC is high in patients who have received liver resection surgery, resulting in a poor cure rate and low long-term survival<sup>[1,4]</sup>. At present, liver cancer is still a worldwide health issue and remains an unsolved medical problem.

The major risk factors for HCC include: (1) hepatitis virus infection, for example hepatitis B virus (HBV) and hepatitis C virus (HCV); (2) aflatoxin B1 (AFB1) exposure; (3) alcoholic cirrhosis; and (4) metabolic

factors such as obesity and diabetes<sup>[5-8]</sup>. Furthermore, in HBV-associated HCC, HBV replication and the genotype of the HBV *per se* are also risk factors. A higher serum level of HBV DNA is correlated with the future incidence of HCC in patients<sup>[9]</sup>. In a manner similar to the HBV infection rate, the distribution of HBV genotypes also shows significant geographical differences. For example, genotypes B and C are predominant in Asia, while genotypes A and D are predominant in Africa, Europe and India. In addition, in Asia, genotype C has been shown to be correlated with advanced liver disease, cirrhosis and HCC more than genotype B<sup>[1]</sup>. Moreover, there is a highly significant association between patient gender, HBV infection and HCC. Males have a higher HBV infection rate and a higher HCC incidence. The greater susceptibility of males in terms of HCC incidence may be attributed to the tumor-promoting effects of androgens and/or the gender-specific metabolism of carcinogens<sup>[10]</sup>. AFB1, which is produced by fungi and often contaminates maize and peanuts, is a well-recognized carcinogen of the liver. A metabolic intermediate of AFB1 is able to bind to and damage DNA, leading in HCC to a loss of function of various tumor suppressor genes such as *p53*<sup>[10]</sup>. Notably, areas with a high prevalence of chronic HBV infection are usually places having a high risk of AFB1 exposure. This suggests that AFB1 is a promoting factor for HBV-associated HCC and that these two risk factors may have a synergistic effect on hepatocarcinogenesis. Another risk factor related to diet is chronic alcoholism, which leads to fatty liver, fibrosis, advanced liver disease and eventually to HCC. Additionally, smoking has been recognized as a risk factor for HBV-associated HCC development. Furthermore, genetic polymorphisms present in the host are also HCC risk factors. For example, studies have revealed that a loss-of-function deletion of glutathione S-transferase seems to increase the risk of HCC<sup>[1,11]</sup>. Finally, there are synergistic effects between the different risk factors for HCC. For example, co-transfection with HCV and HBV has an additive effect on the HCC incidence, as does hepatitis virus infection plus AFB1 exposure or hepatitis virus infection plus chronic alcoholism.

Genetically modified mice, including transgenic, knock-out and knock-in mice, are able to provide animal models that help to elucidate the molecular mechanisms related to pathogenesis and carcinogenesis in the liver; furthermore, they also help us to evaluate potential chemopreventive agents and new therapeutic targets under physiological conditions. Although mice cannot be infected with HBV due to the narrow host range of HBV and the lack of a proper receptor or other factors for HBV, one of the advantages of using mouse models is the numerous and powerful resources that genetically modified mice make available. In this review, we mainly focus on the application of genetically modified mouse models to obtain a better understanding of HBV-associated carcinogenesis, the potential involvement

of tumor suppressor genes in HCC development, and the potential implications of these findings in mice to translational medicine.

## HBV AND HCC DEVELOPMENT

HBV is one of the most important risk factors for HCC<sup>[1,3,4]</sup>. Clinically, chronic infection with HBV is highly associated with the incidence of HCC and with mortality. Additionally, the geographical distribution of liver cancer seems to be highly correlated with the prevalence of HBV in a population<sup>[1]</sup>. Epidemiological studies have identified areas with a low prevalence of HBV infection, such as in North America, Western Europe and Northern Europe; areas with an intermediate prevalence of HBV infection, such as Eastern and Southern Europe; and areas with the highest prevalence of HBV infection such as East and Southeast Asia and sub-Saharan Africa<sup>[1,3]</sup>. Additionally, the epidemiological results show that in the high HBV prevalence areas there are notable differences in the mean age of diagnosis of HCC between men and women, with men showing a lower mean age of diagnosis, and in the incidence of HCC among men than women, with men showing a higher incidence<sup>[1]</sup>. The age at which infection occurs, as well as environmental and dietary factors, all seem to be related to HCC incidence<sup>[1,3]</sup>. Infection with HBV is divided into two types, acute and chronic. Most adults with an acute infection of HBV (90% of cases) spontaneously recover and develop protective immunity<sup>[12-14]</sup>. HBV chronic infection in the remaining 10% of adults can be subdivided into four phases; these are the immune tolerance phase, the immune clearance [hepatitis Be antigen (HBeAg)-positive chronic hepatitis] phase, the inactive (carrier) phase and the reactivation (HBeAg-negative chronic hepatitis) phase; all of these phases may not all be seen in a given individual patient<sup>[15]</sup>. Up to 30% of HBV carriers develop chronic hepatitis, fibrosis, cirrhosis and eventually HCC<sup>[16,17]</sup>. In an area with high HBV prevalence, chronic HBV infection often occurs during birth (mother-child transmission). Therefore, an early onset of HBV infection and long-term chronic infection-induced advance liver diseases are likely to be the main factors contributing to the high mortality and high incidence of HCC in these regions<sup>[1]</sup>. Taiwan, which is located in an HBV high-prevalence region, was a pioneer of national HBV vaccination in the 1980s. A recent study has revealed that chronic disease mortality, HCC incidence and HCC mortality have significantly declined in both males and females who have received HBV immunization<sup>[18]</sup>. Nonetheless, the life-long effects of HBV vaccination will need to be observed for decades to come. Although immunization against HBV is highly efficacious in terms of preventing HBV-related liver disease, it also results in mutations affecting the genomes of HBV in circulation<sup>[19]</sup>. In addition, there is still no effective treatment that is able to eliminate HBV from infected patients<sup>[12]</sup>.

HBV is a hepatocyte-specific enveloped DNA virus that is composed of large, middle and small surface proteins. Inside the envelope of the virus, the genome of HBV is packaged within the core proteins, which is formed into nucleocapsids. The genome of HBV is a 3.2-kb partially double-stranded circular DNA that is reversely transcribed from a 3.2-kb pre-genomic RNA (pgRNA). A receptor (sodium taurocholate co-transporting peptide, NTCP), which is responsible for HBV entry into host cells, was recently identified<sup>[20,21]</sup>. After entering the host cell, the nucleocapsids transport the HBV genome DNA into the nucleus of the infected hepatocytes. The circular HBV DNA is then converted into covalently closed circular DNA (cccDNA), forming a minichromosome inside the nucleus, and these minichromosomes serve as the template for RNA transcription. In some cases, the HBV genome has been found to integrate into the host genome of a patient with chronic HBV infection; however, this is not necessary for viral replication unlike the situation with retroviruses<sup>[14,22]</sup>. The HBV polymerase, which is covalently linked to the 5' end of the negative strand of the genome, uses the 5' end of oligoribonucleotides on the plus strand for reverse transcription. There are four promoters and a shared polyadenylation signal in the genome that are involved in regulating the transcription of HBV proteins. The core promoter is responsible for transcripts of the pgRNA (3.5 kb) and of the pre-core protein, whose transcript is slightly longer than that of the pgRNA. The pre-core protein is able to be secreted as HBeAg after removal of residues at the N-terminus and C-terminus. In addition, in order to serve as a template for reverse transcription, the pgRNA also encodes the core and polymerase proteins. The core protein, which is also known as hepatitis B core antigen, assembles the nucleocapsids of the virus and is responsible for delivering the viral nucleocapsids into the host nucleus. There are two promoters for the surface proteins, which generate 2.4-kb and 2.1-kb transcripts. The 2.4-kb transcript encodes the large surface protein, while the 2.1kb encodes the middle and small surface proteins. These three surface proteins (HBsAg) are synthesized in the rough endoplasmic reticulum (ER) and then transported to the Golgi apparatus for glycosylation; these proteins are then able to be secreted as non-infectious subviral particles. The smallest transcript (0.9 kb) is the template for the X protein. During viral replication, the core protein packages the 3.5-kb pgRNA and polymerase into nucleocapsids. Then, the reverse transcription of pgRNA is carried out inside of the nucleocapsid; this is carried out by the viral polymerase and generates the minus-strand of the HBV DNA first. The minus strand is then used as a template for the synthesis of the plus-strand of HBV DNA. Finally, the nucleocapsids are enveloped by surface proteins during processing on the ER and Golgi apparatus, which is followed by secretion of the virus from the cell<sup>[14,23,24]</sup>.

## GENETICALLY MODIFIED MOUSE MODELS USED TO STUDY HBV

The host range of HBV is highly limited and is restricted to humans and higher primates<sup>[23]</sup>. Early studies of hepatitis virus were carried out using woodchuck hepatitis virus (WHV), duck HBV (DHBV) and ground squirrel hepatitis virus (GSHV)<sup>[23]</sup>. However, the outbred properties of the hosts of WHV and DHBV limited our understanding of the specific molecular mechanisms involved in host-virus interaction and carcinogenesis. Previous studies revealed that the transgenic mouse carrying the HBV genome is not an ideal animal model to investigate the mechanisms of HBV infection. First, the viruses produced by the transgenic liver cannot infect and re-enter their own hepatocytes in mice. Second, the cccDNA, which is the template for viral transcription in humans, was undetectable in the livers of HBV transgenic mice. Third, the transgenic mice have immune tolerance to HBV therefore cannot develop hepatitis. Currently the chimpanzee and tupaia are the only animal models that can be infected by HBV. However, their use is limited by ethics, large size, and low feasibility as genetic manipulation model systems as well as by a very high cost; this compromises their utility in research and development<sup>[25]</sup>. The problem of establishing a mouse model which can be infected by HBV may be overcome by introducing the receptor of HBV and its critical domain for host-specificity of infection into the hepatocytes of mice by transgenic technology<sup>[20,21]</sup>.

Although the mouse cannot be naturally infected by HBV, transgenic mice carrying the HBV genome have helped scientists to gain insights into the molecular mechanism of viral replication and assembly, and to serve as animal models for evaluation of anti-HBV therapy. First, the viral particles generated in transgenic mouse liver are highly similar to those in human patients. In addition, viruses purified from the blood of HBV transgenic mice can infect human fetal hepatocytes *in vitro*<sup>[26]</sup>. This indicated that the molecular mechanism regulating the synthesis of HBV transcripts and proteins, as well as the viral package and viral secretion, probably is shared in human and mouse hepatocytes. Accordingly, these transgenic mouse models may serve as an *in vivo* platform of animal models to evaluate therapeutic agents against viral replication by nucleoside analog or small interfering RNA<sup>[25]</sup>. Second, the HBV particles can be generated in transgenic liver but cannot re-enter mouse hepatocytes; this suggests mechanistically that mouse hepatocytes lack a proper HBV receptor for viral internalization. Third, the replication and expression of the HBV genome is not cytopathic or toxic to the host cells; there was no sign of hepatocarcinogenesis at a late stage of mouse life<sup>[26]</sup>. Notably, the results obtained from HBV transgenic mice were consistent

with those from infection experiments of HBV in chimpanzee<sup>[14,27]</sup>. Thus, it seems that transgenic mice carrying the HBV genome are capable of recapitulating several aspects of the post-infected hepatocytes in natural hosts such as the human and chimpanzee.

There have been three possible mechanisms proposed for HBV-mediated HCC. These are: (1) that a viral protein *per se* is oncogenic; (2) that there is an infection-promoted immune response and this triggers a long-term process of carcinogenesis in liver; and (3) that the integration of HBV DNA affects the integrity of the host genome<sup>[10]</sup>. Researchers have tried to use small animals such as the mouse, rather than primates with a longer lifespan, in order to establish platforms for the study *in vivo* of mechanisms related to liver carcinogenesis. In 1985, two transgenic mouse lines expressing the HBV surface proteins were independently established by two different groups using genetically modified transgenic technology<sup>[28,29]</sup>. Later, transgenic mice carrying the core gene, the X gene and even the whole genome of the HBV were created. These transgenic mouse models have helped enhance our knowledge of HBV-related biology and liver pathogenesis (Table 1).

### Mouse models for the HBV surface protein

In patients with HBV chronic infection, the surface protein is the dominant viral product and can be considered to be an onco-protein<sup>[28]</sup>. Aiming to mimic the human HBV carrier status, transgenic mice expressing the surface protein were the earliest HBV-related transgenic mice to be created<sup>[28,29]</sup>. One early and important observation was that the accumulation of filamentous surface protein in endoplasmic reticulum leads to hepatotoxicity and carcinogenesis<sup>[22,30-32]</sup>. The pathogenic effect of HBV surface proteins on the mouse liver are relevant to human HCC development in HBV carriers because phenomena such as ground glass cell formation are found<sup>[22,31,33,34]</sup>. The sexual dimorphism found to affect HCC incidence in humans can also be observed in transgenic mice<sup>[22,35-37]</sup>. Another research group generated HBsAg and HBV X protein (HBx) transgenic mice by the knock-in technique in order to reduce the effects of the random integration events that occur with conventional microinjection, and this model system also concluded that the HBV surface protein is oncogenic<sup>[37]</sup>. HBV vaccine is effective at decreasing HCC prevalence; however, mutated forms of the HBVs in circulation have emerged rapidly, particularly in the PreS/S region<sup>[38]</sup>. Clinically, patients harboring the PreS mutation are more susceptible to liver cirrhosis and HCC<sup>[39]</sup>. Efforts were later made to create a transgenic mouse that would help our understanding of the role of the mutated surface protein in pathology. These studies showed that the unfolded protein response (ER stress) is a major factor in HBV surface protein-induced carcinogenesis<sup>[33,40]</sup>.



**Table 1** Hepatitis B virus-related transgenic mice

Transgene	Promoter	Expression	Pathology	Ref.
PreS/S/X	Endogenous	Surface proteins	Until 6 mo: no obvious pathology	[28]
PreS/S/X	Endogenous	Surface proteins	Not determined	[29]
(two TGs)	and mouse metallothionein I			
PreS/S/X	Mouse metallothionein I and Albumin	Surface proteins	Ground glass 7-9 mo: adenoma 12 mo: HCC 18 mo: HCC (100%)	[22,30-32,34]
Genome (2X)	Endogenous	Surface proteins	Not determined	[241]
PreS/S (Knock-in)	Mouse p21	Surface proteins	15-24 mo: HCC [53% (♂KI/+), 72% (♂KI/KI), 0% (♀KI/+ and KI/KI)]	[37]
PreS/S	Endogenous	Surface proteins	HCC	[33,40]
X	Human $\alpha$ -1-antitrypsin	HBx protein	Focal necrosis, hyperplasia nodule (Authors claimed that X is not tumorigenic)	[42]
X	Endogenous	HBx protein	4 mo: altered hepatocyte in multifocal area 10 mo: tumor nodule 16 mo: tumor (80%) 24 mo: HCC (84%)	[43,44]
X	Endogenous	HBx protein	6 mo: neoplastic nodules (66.6%) 15-18 mo: small neoplastic nodule (100%) and HCC (75%)	[41]
X (Knock-in)	Mouse p21	HBx protein	15-24 mo: HCC [60%-64% (♂KI/+ and KI/KI), 43%-46% (♀KI/+ and KI/KI)]	[37]
X	Mouse albumin	HBx protein	12 mo: dysplasia nodule (100%) 16 mo: HCC (80%) 20 mo: HCC (90%-100%)	[45-47]
Precore/core	Mouse metallothionein I	Precore protein	Not determined	[56]
Precore/core	Endogenous	Core protein	12 mo: no hepatitis or HCC	[13,57]
Precore/core	Endogenous	Precore and core proteins	Not determined	[58]
(two TGs)				
Genome (2X)	Endogenous	Replicative intermediates	10 mo: no obvious pathology	[60]
Genome (1.2X)	Endogenous	Replicative virus	12 mo: no hepatitis or HCC 24 mo: no obvious pathology	[59,61]
Genome (1.3X)	Endogenous	Replicative intermediates	Until 12 mo: no obvious pathology	[62]

PreS/S: Coding region of hepatitis B virus (HBV) large, middle and small surface proteins; X: Coding region of HBV X protein; Precore/core: Coding region of HBV pre-core/core proteins; KI: Knock-in allele; TG: Transgenic mice; Genome (2X): Indicates 2 copies of the complete 3.2-kilobase HBV genome.

### Mouse models for the HBx

It has long been suggested that HBx plays a role in hepatocarcinogenesis because infection with DHBV, which lacks the X gene, does not result in the development of HCC, while infection with WHV and GSHV, which do have the X gene, does result in host HCC<sup>[41]</sup>. In addition, the X gene is highly conserved between different HBV subtypes<sup>[42]</sup>. Interestingly, the X transcript is the most abundant mRNA compared to other HBV transcripts in some clinical specimens of human HCC<sup>[41]</sup>. Importantly, several independently generated HBx transgenic mouse models have demonstrated that HBx alone is able to induce malignant transformation of hepatocytes<sup>[37,41-47]</sup> (Table 1).

The functions of the HBx protein have been extensively studied, particularly in cell culture systems, and are known to involve multiple cellular events, including transactivation of transcription factors, cell cycle progression, several signaling transduction pathways, mitochondrial homeostasis, cell death, DNA instability, glucose metabolism and lipid metabolism<sup>[4,10]</sup>. It is possible, using transgenic mice, to test *in vivo* whether the above HBx-mediated cellular events indeed are involved in hepatocarcinogenesis<sup>[4]</sup>. In addition,

these models allow the physiological interacting partners of HBx to be evaluated. Other than mouse, various other model organisms have been used for this type of *in vivo* studies, for example *C. elegans*<sup>[48]</sup>. These studies have shown that the HBx protein binds to CED-9, which is a homolog of members of the anti-apoptotic Bcl-2 protein family; this binding triggers cell death in *C. elegans* and in a human hepG2 cell line<sup>[48,49]</sup>. Interestingly, these studies also discovered that when HBx is mutated, it is unable to induce CED-9-regulated cell death. Further investigation of its role in carcinogenesis using mouse models will help our understanding of the molecular mechanism of HBx-mediated HCC.

Previously, we have generated four lines of HBx transgenic mice, namely A105, A106, A110 and A112, using the C57BL/6 background<sup>[46]</sup>. All of the HBx transgenic lines generated in our laboratory spontaneously develop HCC at 13 to 16 mo of age. At the patho-histological level, the HCC developed in the HBx transgenic mice exhibits a well-differentiated morphology involving the trabecular pattern. Fibrosis, bizarre nuclei, cytoplasmic lipid droplets and hyaline globules can be observed in the HBx-induced HCC

samples, which is similar to the situation observed in human HCC samples<sup>[46]</sup>. Furthermore, a gender disparity for HCC in the HBx transgenic mice has also been observed<sup>[37,43,46]</sup>. The HBx transgenic mice thus provide an animal model for mechanistic studies<sup>[45,50-53]</sup> and for the evaluation under physiological conditions of new chemopreventive agents and new therapeutic agents for HCC<sup>[47,54,55]</sup>.

At the ultrastructural level, we have carried out transmission electron microscope (TEM) examinations and these have revealed that there are alterations in the organelles of the hepatocytes of the HBx transgenic mice (Figure 1). In wild-type mice, each normal hepatocyte typically contains a spherical nucleus with homogeneous euchromatin and heterochromatin. Long and stacked cisternae of rough endoplasmic reticulum are located parallel to the nuclear envelope and the lateral edges of the cell. Numerous mitochondria of varying lengths are present, and these range in shape from spherical mitochondria to dumbbell and rod-like shaped mitochondria. Hepatocellular glycogen is apparent in the electron micrographs as irregular non-membrane bound and rosette structures (Figure 1A and A'). In the HBx transgenic mice, TEM reveals that a dramatic decrease has occurred in the density of cytoplasmic organelles (Figure 1B-D). Many ultrastructural abnormalities of the mitochondria can be detected and these include breakdown and swelling of mitochondrial membranes, as well as an increase in the electron density of the matrix (Figure 1B and B'). In addition, the phagophore isolation membrane (PIm), which is the result of the endoplasmic reticulum engulfing degenerated mitochondria, is now present, which leads to the formation of autophagosomes (Figure 1B and B'). The endoplasmic reticulum is dilated or forms a structure with irregular fragmentation and degeneration (Figure 1C and C'). Part of the degenerated membrane debris of mitochondria and endoplasmic reticulum can be detected as irregular concentric myelin figures (Figure 1D and D'). Furthermore, some hepatocytes of HBx mice have abundant peroxisomes; this is likely to be due to the oxidative stress induced by the HBx protein (Figure 1D and D'). Moreover, the nuclear envelopes of some hepatocytes of HBx transgenic mice have undergone breakdown (Figure 1D and D'; Figure 1C and C'). All of these ultrastructural alterations and organelle degeneration events, particularly the abnormalities in the mitochondria and endoplasmic reticulum, appear at a very early stage (4-8 wk old) and contribute to the progression of carcinogenesis in the livers of HBx transgenic mice.

#### **Mouse models for the HBV precore/core protein and the whole genome**

Previously, several groups have established transgenic mice in order to investigate HBV assembly within the hepatocyte *in vivo* (Table 1). Transgenic mice carrying the precore or core proteins of HBV have no overt

phenotype (no sign of hepatitis and HCC formation) up to 12 mo-old<sup>[13,56-58]</sup>. Transgenic mice carrying the whole HBV genome have also been generated in order to study the HBV life cycle and its interaction with host factors<sup>[26,59-62]</sup> (Table 1). Virus particles that contain HBsAg, HBc/eAg and HBV DNA can be detected in the bloodstream of these transgenic mice. Interestingly, a gender difference in the synthesis of HBeAg and intermediates of the replicative virus can be observed in these mice<sup>[63]</sup>. However, similar to the precore/core transgenic mice, there are no obvious pathological changes detectable in the transgenic liver. Nevertheless, mice carrying the whole HBV genome do provide animal models that are amenable to the study of the HBV life cycle, and they can also help the screening of therapeutic agents against HBV replication.

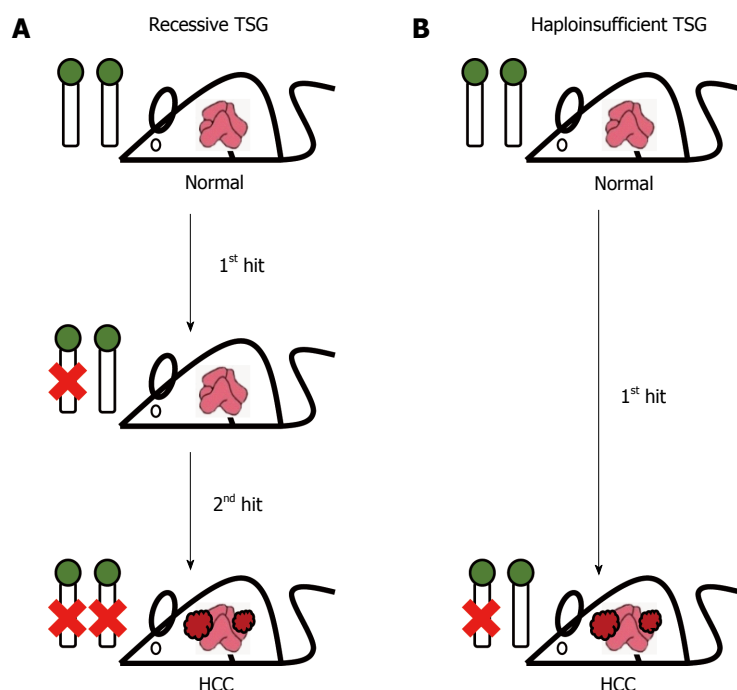
## **CONCLUSION AND PERSPECTIVES IN RELATION TO THE HBV-RELATED MOUSE MODELS**

No single transgenic mouse model is able to cover all aspects of HCC pathogenesis. Currently there are still some barriers when using genetically modified mice, such as their inbred genetic backgrounds versus the complications of human genetic variation, the inability to be infected by HBV, the lack of an immunopathogenesis process, the scarcity of cirrhosis, and the very rare nature of spontaneous metastasis. Accordingly, alternative strategies have been created and applied to mice in order to study HBV biology. For example, hydrodynamic injection of HBV DNA into mice<sup>[25,64]</sup> and humanized mice<sup>[65]</sup>. These models have further extended the feasibility and have helped to create an understanding of virus-host interactions as well as the pathological effects of HBV mutant proteins.

Transgenic mice carrying the HBV genome or expressing various HBV proteins provide valuable models that help to elucidate clinic observations, allowing mechanistic investigations to be performed and helping to test relevant hypotheses. These approaches also help our understanding of HBV replication and the regulation of HBV gene expression, as well as of the inflammation and innate immune response that is induced by HBV proteins under physiological conditions<sup>[13,63,66-68]</sup>. In addition, these previously published transgenic mouse models have been employed as a platform for discovery of therapeutic targets and/or for the identification of preventive chemicals<sup>[4,27,47,54,55,69]</sup>. For example, novel HBV-related microRNAs present in the hepatic and general circulation have been discovered over the past decades and are considered to be potential targets for therapy<sup>[10,70]</sup>. The possible roles of these microRNAs in carcinogenesis and their therapeutic applications can certainly be tested using the available established HBV-



**Figure 1 Ultrastructural alterations in the hepatocytes of the hepatitis B virus X protein transgenic mice revealed by transmission electron microscope.** A: Ultrastructure of the hepatocyte of a wild-type mouse (2-mo old). A': the inset in A was enlarged to provide a better perspective of intact mitochondria (M), rough endoplasmic reticulum (RER), rosette glycogen (Gly) and nucleus (N). B-D: In the HBx transgenic mice (2-mo old), severe ultrastructural alterations can be observed in the hepatocytes. These include nuclear envelope breakdown (NB), a significant decrease in the density of cytoplasmic organelles, abundant peroxisomes (P), the disorganization of rough endoplasmic reticulum [e.g., endoplasmic reticulum fragmentation (ERF), endoplasmic reticulum dilation (ERDil), and/or endoplasmic reticulum degeneration ERD], mitochondria membrane breakdown (MB), swollen mitochondria (SM), the presence of lipid droplets (L), and appearance of myelin figures (MF), which are the membranous debris of mitochondria or ER degeneration. B'-D': Schematic presentation of the ultrastructural alterations in the HBx hepatocytes shown in panel B-D. The inset in B and B' provides a better perspective of a phagophore isolation membrane (Plm) engulfing a degenerated mitochondrion leading to the formation of autophagosomes (AVi).



**Figure 2 Tumor suppressor genes in hepatocellular carcinoma.** A: Recessive tumor suppressor genes (TSGs) need there to be loss-of-function via two hits (mutation or genetic modification) in order to trigger tumor formation. Only when there is homozygous deficiency of a recessive TSG does this lead to hepatocellular carcinoma (HCC) development; B: Haploinsufficient TSGs are functionally insufficient to suppress tumor development when there is loss of only a single allele. Heterozygous deficiency of a haploinsufficient TSG can cause HCC development in the liver even when the second allele remains intact.

related mouse models. The ultimate goal, of course, is still to use transgenic mice to find a cure for patients with HBV infection and HCC. Nevertheless, there is still a great need for a good animal model for HCC and such a model would be expected to meet as many of the following requirements as possible. These are: (1) the faithful reproduction of HCC's progression stages and incidence; (2) a reliable reproduction of the molecular and cellular events during HCC carcinogenesis; (3) the ability to mimic the tumor-host and tumor-metastases interactions; (4) a reproduction of the tumor micro-environment in human patients; and (5) the ability to manipulate in a feasible manner the genome of HBV<sup>[4]</sup>.

Additionally, the HBV virus at present is also evolving in response to environmental changes such as vaccination or drug administration. For example, the emergence of mutated hepatitis B surface protein, which may potentially have vaccine-resistant properties, is a serious issue<sup>[38]</sup>. The generation of new transgenic mouse model systems is one way to dissect the effects of such mutant HBV proteins on liver carcinogenesis. Lately, a newly developed genome-editing tool, the Crips/Cas9 system, has been successfully applied and used to target the HBV DNA and reduce the production of HBV proteins<sup>[71-73]</sup>. Using a hydrodynamics-HBV persistence mouse model, Lin *et al.*<sup>[72]</sup> have demonstrated that the Crips/Cas9 system is able to cleave the intrahepatic HBV genome containing the plasmid and facilitate its clearance in animals. These findings suggest that the genome-editing tool may have potential in the eradication of persistent HBV

infection.

## TUMOR SUPPRESSOR GENES DURING HCC DEVELOPMENT

The identification of tumor suppressor genes (TSGs) is very important to the development of novel therapeutic strategies targeting HCC. Many TSGs have been identified in human HCC tissue samples, using cell culture systems and with mouse models<sup>[74-77]</sup>. These TSGs can be classified into two major groups, the recessive TSGs and the haploinsufficient TSGs (Figure 2). The recessive TSG group follows the Knudson's two-hit hypothesis, in which a deficiency affecting both alleles of a TSG triggers HCC development. For the haploinsufficient TSG group, the loss of a single allele of a TSG is sufficient to promote tumorigenesis, with the second allele remaining intact in the tumors. These latter TSGs are functionally haploinsufficient in the heterozygous condition and it would seem that cellular level of 50% for their gene product is insufficient to suppress tumor formation<sup>[78-80]</sup>. Using mouse models, scientists have been able to delineate the molecular mechanisms underlying the roles of both recessive and haploinsufficient TSGs during the suppression of carcinogenesis in liver. These findings should contribute significantly to the development of novel chemopreventive or therapeutic strategies for HCC. Here, we will review the TSGs in HCC mainly based on the pathways in which they are involved (Table 2).



**Table 2** Mouse models of tumor suppressor genes in hepatocellular carcinoma

Official gene name (common name)	Mouse genotype	Mouse genetic background	Pathology	TGS type	Functions	Human Chromosome location	Expression in human HCC tumor	Ref.
Autophagy regulator <i>Becn1</i> (Beclin1)	<i>Beclin1</i> <sup>+/-</sup>	Mixed (129Sv/J; C57BL/6J)	16-18 mo: HCC (8%) 18-22 mo: HCC (26%) Promote HBV- mediated HCC	Haplo- insufficiency	Regulation of autophagy	17q21	Down	[85-87]
<i>Atg5</i>	<i>Alb-Cre</i> ; <i>Atg5</i> <sup>flx/flx</sup>	C57BL/6	2 mo: hepatic cell death, proliferation, inflammation, fibrosis and hepatomegaly 12 mo: hepatic adenoma (100%)	Recessive	Control autophagosome formation	6q21	Down	[90,91]
<i>Atg7</i>	<i>Alb-Cre</i> ; <i>Atg7</i> <sup>flx/flx</sup>	C57BL/6	4 mo: fatty liver 12 mo: hepatic adenoma (90.9%)	Recessive	Control autophagosome formation	3p25.3	Up	[92-94]
Cell cycle regulator <i>Klf6</i>	<i>KLF6</i> <sup>+/-</sup>	C57BL/6	Promote DEN-induced HCC	Haplo- insufficiency	Cell cycle regulation	10p15	Down	[99,101]
<i>Plk4</i>	<i>Plk4</i> <sup>+/-</sup>	Mixed (129Sv;CD1)	3 mo: mitotic failure in hepatocyte regeneration 18-24 mo: HCC (30%)	Haplo- insufficiency	Control of cell cycle progression	4q28	Down	[104,105]
<i>Cdkn1a</i> (p21)	<i>p21</i> <sup>+/-</sup>	Mixed (129Sv; C57BL/6)	Promote $\gamma$ -irradiation- induced HCC	Haplo- insufficiency	Inhibits the activity of CDK2 or CDK4	6p21.2	Up	[107,242]
	<i>p21</i> <sup>-/-</sup>	C57BL/6	Promote HCC in Nemo liver-specific KO	Recessive				[108]
<i>Trp53</i> (p53)	<i>p53</i> <sup>+/-</sup>	C57BL/6	Promote HBV- mediated and AFB1- induced HCC	Haplo- insufficiency	DNA damage response transcription factor	17p13.1	Up	[111,243]
	<i>Aflp-Cre</i> ; <i>p53</i> <sup>flx/flx</sup>	C57BL/6	14-20 mo: HCC (90%)	Recessive				[112]
Protein ubiquitination <i>Amfr</i> (Gp78)	<i>Gp78</i> <sup>-/-</sup>	C57BL/6	12 mo: fatty liver, inflammation and HCC (24%)	Recessive	E3 ubiquitin ligase	16q21	Down	[117]
<i>Park2</i> (Parkin)	<i>Parkin</i> <sup>-/-</sup>	Mixed (129/ C57BL6SJL)	11-12 mo: hepatocyte proliferation and hepatomegaly 16-17 mo: HCC (33%) 22-23 mo: HCC (45%)	Recessive	E3 ubiquitin ligase	6q25.2-q27	Down	[118,122]
<i>Trim24</i>	<i>Trim24</i> <sup>-/-</sup>	C57BL/6	2 mo: hepatic cell death and inflammation 4-6 mo: fatty liver, fibrosis and liver nodule 9-21 mo: HCC (55%)	Recessive	E3 ubiquitin ligase	7q32	Up	[119,125]
Genome stability maintenance <i>Anxa7</i> (Annexin 7)	<i>Anx7</i> <sup>+/-</sup>	C57BL/6	10 mo: Hepatomegaly 12 mo: HCC (3.6%)	Haploinsufficiency	Ca <sup>2+</sup> -dependent endocrine secretion	10q22.2	Up	[131,132]
<i>Nbn</i> (Nibrin)	<i>Nbn</i> <sup>+/-</sup>	Mixed (129Sv; C57BL/6)	23 mo: HCC (8.6%)	Haploinsufficiency	DNA repair	8q21	Up	[134,244]
<i>Pinx1</i>	<i>PinX1</i> <sup>+/-</sup>	Mixed (129Sv; C57BL/6)	9-18 mo: HCC (17.7%)	Haploinsufficiency	Telomerase inhibitor	8p23	Down	[136,137]
<i>Sgo1</i> (Shugoshin 1)	<i>Sgo1</i> <sup>+/-</sup>	C57BL/6	4 mo: hepatocyte DNA damage 12 mo: HCC Promote AOM-induced HCC	Haploinsufficiency	Protector of chromosome cohesion and centrosome integrity	3p24.3	Up	[139,140]
Metabolic function <i>Acox1</i> (Acyl-CoA oxidase 1)	<i>Aox</i> <sup>-/-</sup>	Mixed (129/ Ola; C57BL/6)	2-4 mo: fatty liver, inflammation and hepatocyte proliferation 10-15 mo: HCC (100%)	Recessive	Peroxisomal $\beta$ -oxidation	17q25.1	Not determined	[144]

<i>Blmt</i>	<i>Blmt</i> <sup>-/-</sup>	C57BL/6	1 mo: fatty liver 12 mo: HCC (50%)	Recessive	Methionine metabolism	5q14.1	Down	[149,150]
<i>Nr1h4</i> (Farnesoid X receptor)	<i>Exr</i> <sup>-/-</sup>	C57BL/6N	3 mo: hepatic cell death and proliferation 6-9 mo: fatty liver and inflammation 12 mo: fibrosis and HCC (16.1%)	Recessive	Transcriptional regulation of synthesis and transport of bile acids	12q23.1	Down	[155,157]
<i>Gnmt</i> (Glycine N-methyltransferase)	<i>Gnmt</i> <sup>-/-</sup>	Mixed (129Sv; C57BL/6)	3 mo: fatty liver and fibrosis 8 mo: HCC (100%) at 8-mo	Recessive	Methionine metabolism	6p12	Down	[148,151]
<i>Abcb4</i> (Mdr2)	<i>Mdr2</i> <sup>-/-</sup>	129/OlaHsd	3 mo: inflammation and fibrosis 6-mo: HCC	Recessive	Phosphatidylcholine translocase	7q21.1	Down	[156,158]
Hippo signaling pathway								
<i>Stk4</i> (Mst1)	<i>Alb-Cre; Mst1</i> <sup>flx/flx</sup>	Mixed (129Sv; C57BL/6; CD1)	1 mo: hepatocyte proliferation and hepatomegaly 6 mo: HCC (100%)	Recessive	Kinase (negative regulator)	20q11.2	Not determined	[165,166]
<i>Stk3</i> (Mst2)	<i>Alb-Cre; Mst1</i> <sup>flx/flx</sup>	Mixed (129Sv; C57BL/6; CD1)	1 mo: hepatocyte proliferation and hepatomegaly 6 mo: HCC (100%)	Recessive	Kinase (negative regulator)	8q22.2	Not determined	[165,166]
<i>Nf2</i> (Neurofibromin 2)	<i>Alb-Cre; Nf2</i> <sup>flx/flx</sup>	Mixed (FVB/N; C57BL/6)	2 mo: oval cell over-proliferation and hepatomegaly 7 mo: HCC (100%)	Recessive	Negative regulator	22q12.2	Not determined	[168]
Jak/Stat signaling pathway								
<i>Socs1</i>	<i>Socs1</i> <sup>+/-</sup>	C57BL/6	Promote DEN-induced HCC	Haploinsufficiency	Negative regulator	16p13.13	Down	[174]
<i>Socs3</i>	<i>Socs3</i> <sup>+/-</sup>	Mixed (129Sv; C57BL/6)	Promote DEN-induced HCC	Haploinsufficiency	Negative regulator	17q25.3	Down	[176]
<i>Ptpn11</i> (Shp2)	<i>Alb-Cre; Shp2</i> <sup>flx/flx</sup>	C57BL/6	2-3 mo: inflammation and hepatic cell death 8-mo: hyperplasia nodule 12-18 mo: hepatic adenoma (68%) Promote DEN-induced HCC	Recessive	Protein tyrosine phosphatase (negative regulator)	12q24	Down	[179]
<i>Ptpro</i>	<i>Ptpro</i> <sup>-/-</sup>	C57BL/6	Promote DEN-induced HCC	Recessive	Receptor-like protein tyrosine phosphatase (negative regulator)	12p13.3	Down	[181,182]
NF-κB signaling pathway								
<i>Cyld</i> (Cyldromatosis)	<i>Alfp-Cre; Cyld</i> <sup>flx/flx</sup>	C57BL/6 congenic	1-2 mo: hepatic cell death, inflammation and fibrosis 12 mo: HCC	Recessive	Deubiquitinase (negative regulator)	16q12.1	Down	[188,189]
<i>Lgals3</i> (Galectin-3)	<i>Gal3</i> <sup>-/-</sup>	CD1	6 mo: fatty liver and inflammation 15 mo: fibrosis and liver nodule 25 mo: HCC (100%)	Recessive	Regulation of inflammatory responses	14q22.3	Up	[192-194]
<i>Ikbkg</i> (Nemo)	<i>Alfp-Cre; Nemo</i> <sup>flx/flx</sup>	C57BL/6	2 mo: fatty liver, inflammation, hepatic cell death and proliferation 6 mo: dysplastic nodule 12 mo: HCC (100%)	Recessive	Activation of NF-κB	Xq28	Down	[195,196]
<i>Map3k7</i> (Tak1)	<i>Alfp-Cre; Tak1</i> <sup>flx/flx</sup>	Mixed (129/Ola; C57BL/6)	1-2 mo: hepatic cell death, proliferation and fibrosis 4-8 mo: HCC (88%)	Recessive	Serine/threonine protein kinase (Activation of the NF-κB)	6q15	Not determined	[197]
PI3K/ Akt/ mTOR signaling pathway								
<i>Stk11</i> (Lkb1)	<i>Lkb1</i> <sup>+/-</sup>	Mixed (129Sv; C57BL/6)	10-12 mo: Hepatic hyperplasia, HCC (29%) 14 mo: HCC (75%)	Recessive	Serine/threonine kinase (activator of AMPK)	19p13.3	Down	[201,202]

<i>Pten</i>	<i>Alb-Cre; Pten<sup>flx/flx</sup></i>	C57BL/6	2-3 mo: fatty liver and hepatomegaly 9-10 mo: inflammation and fibrosis 17-18 mo: HCC (66%)	Recessive	Phospholipid phosphatase (negative regulator)	10q23.3	Down	[204,208]
<i>Raptor</i> (Regulatory associated protein of mTOR, complex 1)	<i>Alb-Cre; Raptor<sup>flx/flx</sup></i>	C57BL/6	2 mo: hepatic cell death, inflammation and fibrosis Promote DEN-induced HCC	Recessive	Activation of mTOR activity	17q25.3	Up	[209,210]
TGF- $\beta$ signaling pathway <i>Sptbn1</i> ( $\beta$ -spectrin)	<i>Elf<sup>-/-</sup></i>	Mixed (129SvEv; Black Swiss)	15 mo: Fatty liver, HCC (40%)	Haploinsufficiency	Propagation of TGF- $\beta$ signal	2p21	Down	[214]
<i>Tgfb1</i> (TGF- $\beta$ 1)	<i>Tgfb1<sup>+/-</sup></i>	C57BL/6NCR	Promote DEN-induced HCC	Haploinsufficiency	Growth factor	19q13.1	Up	[216,245]
<i>Tgfb2</i> (TGF- $\beta$ type II receptor)	<i>Tgfb2<sup>-/-</sup></i>	Mixed (129Sv; C57BL/6)	Promote DEN-induced HCC	Recessive	TGF- $\beta$ receptor	3p22	Down	[217,218]
Wnt signaling pathway <i>Apc</i>	<i>Adenovirus-Cre; Apc<sup>flx/flx</sup></i>	C57BL/6N	9 mo: HCC (67%)	Recessive	Antagonist	5q21	Down	[222,223]
MicroRNA <i>Mir122</i>	<i>Mir122a<sup>-/-</sup></i>	C57BL/6	3 mo: fatty liver, inflammation and fibrosis 11 mo: HCC (75%)	Recessive	Post-transcriptional regulation of gene expression	18q21.31	Down	[227,228]
<i>Mir140</i>	<i>Mir140<sup>-/-</sup></i>	C57BL/6	Promote DEN-induced HCC	Recessive	Post-transcriptional regulation of gene expression	16q22.1	Not determined	[229]
Miscellaneous <i>Ncoa5</i> (Nuclear receptor coactivator 5)	<i>Ncoa5<sup>+/-</sup></i>	Mixed (129Sv; C57BL/6)	6 mo: fatty liver 10 mo: inflammation and fibrosis 10-18 mo: HCC (94%)	Haploinsufficiency	Estrogen receptor coactivator	20q13.12	Down	[231]
<i>Prkar1a</i>	<i>Prkar1a<sup>+/-</sup></i>	Mixed (129Sv/J; C57BL/6)	9-19 mo: HCC (29.4%)	Haploinsufficiency	Regulation of the serine/threonine kinase activity	17q24.2	Up	[233,246]
<i>Ncoa2</i> (Nuclear receptor coactivator 2)	<i>Ncoa2<sup>-/-</sup></i>	Mixed (129Sv/J; C57BL/6)	Promote DEN-induced HCC	Recessive	Transcriptional coactivator	8q13.3	Down	[235]
<i>Nfe2l1</i> (Nuclear factor, erythroid 2-like 1)	<i>Alb-Cre; Nrf1<sup>flx/flx</sup></i>	Mixed (129Sv; C57BL/6)	1-2 mo: fatty liver, necrosis and inflammation 6 mo: fibrosis 12 mo: HCC (100%)	Recessive	Transcription factor	17q21.3	Not determined	[238]

HCC: Hepatocellular carcinoma.

## TSGs AND HCC MOUSE MODELS

### Autophagy regulatory genes

Autophagy is a highly regulated process that degrades damaged cellular organelles and macromolecules allowing recycling of bioenergetic molecules. Many proteins are involved in the autophagy pathway, including the Beclin1 and autophagy-related gene (Atg) proteins. Autophagy plays dual roles in hepatocarcinogenesis; firstly, it acts as a tumor suppressor during the initiation stages of HCC, while exerting a tumor supportive function during the promotion and progression stages. In normal cells, autophagy has a tumor suppressor function that aims to maintain normal metabolism, preserve genetic stability and inhibit inflammation. On

the other hand, autophagy also plays a role in supporting tumor progression by helping cancer cells survive stress-induced cell death<sup>[81,82]</sup>. The tumor suppressor function of autophagy has been demonstrated using mouse models.

**Beclin1:** Beclin1 is one of the major mediators of autophagy; this gene is located on human chromosome 17q21. Mono-allelic deletion has been detected in various human cancers including ovarian, breast and prostate cancers<sup>[83]</sup>. A decrease in the levels of autophagy has been observed in a highly malignant HCC cell line compared with immortalized normal hepatic cells. Furthermore, the expression level of Beclin1 has been found to be decreased in human HCC tissues compared with the adjacent non-tumor



tissues<sup>[84,85]</sup>. Homozygous knockout of *Beclin1* leads to embryonic lethality; this finding demonstrates that *Beclin1* is essential for early embryonic development in mice. Importantly, heterozygous deficiency of *Beclin1* leads to the development of spontaneous HCC and accelerates HBV-induced hepatocarcinogenesis in mice. Moreover, the wild-type allele of *Beclin1* has been found not to be mutated/lost in the HCC tissue of the *Beclin1*<sup>+/-</sup> mice<sup>[86,87]</sup>. These studies demonstrated that *Beclin1* is a haploinsufficient TSG in relation to HCC.

**ATG5 and ATG7:** *Atg5* and *Atg7* are autophagy-related genes (Atg) that promote the elongation of the autophagosome membrane. Homozygous knockout of either *Atg5* or *Atg7* leads to impairment of autophagosome formation and postnatal lethality in mice<sup>[88,89]</sup>. Regarding ATG5, mutation and/or loss of ATG5 expression has been observed in human HCC tissues<sup>[90]</sup>. In mice, hepatocyte-specific knockout of *Atg5* was found to result in hepatomegaly, hepatic cell death, compensatory proliferation of hepatocytes, inflammation and fibrosis, as well as the development of hepatocellular adenomas in mice<sup>[91]</sup>. Regarding ATG7, the expression of the ATG7 protein is increased in human HCC tissue samples compared with their adjacent non-tumor tissue samples, but the mechanism is currently unknown<sup>[92]</sup>. However, mice with a hepatocyte-specific knockout of *Atg7* develop fatty liver and hepatocellular adenoma, indicating that autophagy is involved in lipid metabolism and plays a role in suppressing tumor formation in the liver<sup>[93,94]</sup>.

### Cell cycle regulatory genes

Cell cycle progression is regulated by cyclin-dependent kinases (CDKs) together with several activators (cyclins) and CDKs inhibitors (p21 and p27). In normal tissue, the cell cycle is carefully controlled and regulated in order to maintain cell number homeostasis<sup>[95]</sup>. Abnormal cell cycle progression and sustained cell proliferation is one of the hallmarks of cancer cells<sup>[96]</sup>. Many genes involved in cell cycle control have potential tumor suppressive roles in HCC.

**Kruppel-like factor 6:** Kruppel-like factor 6 (KLF6) is a ubiquitously expressed zinc finger transcription factor that regulates the cell cycle and signal transduction. KLF6 is frequently inactivated by loss of heterozygosity, somatic mutation or promoter methylation in various cancers including prostate, colon and liver cancer<sup>[97,98]</sup>. In mice, heterozygous deficiency of *Klf6* promotes diethylnitrosamine (DEN)-induced hepatocarcinogenesis in *Klf6*<sup>+/-</sup> mice; however, whether the wild-type allele of *Klf6* remained intact in the HCC was not determined during this study<sup>[99]</sup>. Liver-specific knockout of the two alleles of *Klf6* enhanced DEN-induced HCC in mice<sup>[100]</sup>. In humans, expression of KLF6 is decreased in HCV-related HCC tissue samples<sup>[99]</sup>. Down-regulation of KLF6

has also been observed in HBV-related human HCC<sup>[101]</sup>. These studies in humans and mice suggest that *KLF6* may function as a haploinsufficient TSG in HCC.

**Polo-like kinase 4:** Polo-like kinase 4 (PLK4) is a member of the polo-like kinase protein family that plays a critical role in cell cycle progression<sup>[102]</sup>. Homozygous knockout of *Plk4* resulted in embryonic lethality due to a mitotic defect in mice<sup>[103]</sup>. Importantly, heterozygous deficiency of *Plk4* leads to mitotic failure of regenerated hepatocytes and spontaneous HCC development in the heterozygous knockout mice. Furthermore, the wild-type allele of *Plk4* has been shown to remain intact in these HCC tissue samples<sup>[104]</sup>. In humans, the expression level of PLK4 is down-regulated in HCC tissue samples<sup>[105]</sup>. These studies indicate that *PLK4* functions as a haploinsufficient TSG in HCC.

**p21:** The p21 protein is an inhibitor of CDK and is involved in cell cycle control, cell senescence and cell death<sup>[106]</sup>. Heterozygous and homozygous knockout of *p21* promotes  $\gamma$ -irradiation-induced tumor formation in many types of cancers including HCC. The p21 protein has been found to be expressed in malignant tumors from irradiated *p21*<sup>+/-</sup> mice, suggesting that *p21* functions as a haploinsufficient TSG in this situation<sup>[107]</sup>. Moreover, homozygous knockout of *p21* in hepatocytes has been shown to enhance NEMO-mediated hepatocarcinogenesis in mice<sup>[108]</sup>. These results suggested that *p21* is able to function as a recessive or haploinsufficient TSG across various different tissues under a range of different physiological/environmental settings in mice<sup>[107]</sup>.

**p53:** The p53 protein is a transcription factor that regulates DNA damage and stress responses. This protein functions as a tumor suppressor in many types of cancers<sup>[109,110]</sup>. Heterozygous knockout of *p53* has been shown to promote HBV-mediated and AFB1-induced HCC development in mice; furthermore, the wild-type allele of *p53* has been found to remain intact in the HCC tissues of these *p53*<sup>+/-</sup> mice, suggesting that *p53* functions as a haploinsufficient TSG in these circumstances<sup>[111]</sup>. Liver-specific knockout of the two alleles of *p53* leads to spontaneous HCC development in mice. These findings suggest that *p53*, like *p21*, is able to function as either a recessive TSG or a haploinsufficient TSG in different tissues under a range of different physiological/environmental settings in mice<sup>[112]</sup>.

### Protein ubiquitination

Intracellular protein degradation occurs *via* two major pathways; these are the ubiquitin-proteasome system and the lysosomal-mediated proteolysis pathway. The ubiquitin-proteasome system is a multi-step process involving protein labeling by polyubiquitination, which is followed by protein degradation *via* the proteasome.

Protein ubiquitination is a tightly regulated process that involves three major enzymes, namely E1 ubiquitin-activating enzyme, E2 ubiquitin-conjugating enzyme and E3 ubiquitin ligase<sup>[113,114]</sup>. The E3 ubiquitin ligases act by targeting specific proteins, including gene products from oncogenes and tumor suppressor genes. Abnormal regulation of the E3 ubiquitin ligase is involved in the development of many types of cancer, including HCC<sup>[115,116]</sup>. Many E3 ubiquitin ligases have been demonstrated to function as tumor suppressors of HCC in mice, including Gp78, Parkin and Trim24<sup>[117-119]</sup>. These mouse models have been able to uncover the role of E3 ubiquitin ligases in hepatocarcinogenesis.

**Glycoprotein 78:** Glycoprotein 78 (GP78) is an endoplasmic reticulum (ER) membrane-anchored E3 ubiquitin ligase, which is involved in the ER-associated degradation (ERAD) pathway. The ERAD pathway is a protective mechanism that helps to maintain ER protein homeostasis through degradation of misfolded proteins<sup>[120]</sup>. In mice, homozygous knockout of *Gp78* results in the development of non-alcoholic steatohepatitis (NASH), liver fibrosis and HCC. In humans, GP78 has been shown to be down-regulated in HCC tissue samples compared to adjacent non-tumor liver tissue samples<sup>[117]</sup>. These studies suggest that *Gp78* is a regulator of ER homeostasis in the normal liver, and functions as a recessive TSG in HCC.

**Parkin:** Parkin is an E3 ubiquitin ligase involved in protein turnover, the stress response, mitochondrial homeostasis, metabolism and cell growth. Inactivation of Parkin has been frequently detected in many types of human cancers including HCC<sup>[121]</sup>. In humans, the protein levels of Parkin have been shown to be decreased in HCC tissue samples and HCC cell lines<sup>[122]</sup>. In mice, Parkin deficiency leads to hepatomegaly and HCC *via* an increase in the hepatocyte proliferation and a reduction in hepatic cell death. Additionally, disruption of Parkin results in impaired lipid uptake by hepatocytes when the mice are fed a high fat diet (HFD)<sup>[118,123]</sup>. These studies reveal that Parkin plays an important role in maintaining cell numbers and lipid homeostasis in the liver and functions as a recessive TSG in HCC.

**Tripartite motif 24:** Tripartite motif 24 (TRIM24) is located in the HCC critical region on human chromosome 7q32, suggesting that *TRIM24* may function as a tumor suppressor in HCC. TRIM24 is an E3 ubiquitin ligase, which negatively regulates the level of p53<sup>[124]</sup>. Upregulation of the TRIM24 protein has been observed in human HCC tissue samples compared with their adjacent non-tumor tissue samples<sup>[125]</sup>. Trim24 deficiency has been found to result in the development of fatty liver, hepatic injury and spontaneous HCC in homozygous knockout mice,

which suggests that *Trim24* functions as a recessive TSG in HCC<sup>[119]</sup>. In addition, these studies have also revealed that the expression level of Trim24 is an important factor in the progression of HCC.

### Genes related to genomic stability maintenance

Genomic stability is very important because it allows cells to avoid neoplastic transformation and tumorigenesis. The major mechanisms for maintaining genomic stability are high-fidelity DNA replication, accurate chromosome segregation, faithful DNA repair and cell cycle checkpoint control<sup>[126]</sup>. Genomic instability is a hallmark of most cancers and significantly contributes to cancer initiation and progression<sup>[127,128]</sup>. Many haploinsufficient TSGs in HCC are functionally involved in the maintenance of genomic stability.

**Annexin 7:** Annexin 7 (ANX7) is a member of the annexin family and is a Ca<sup>2+</sup>-dependent phospholipid-binding protein<sup>[129]</sup>. Homozygous knockout of *Anx7* in mice leads to postnatal lethality at embryonic day 10 due to cerebral hemorrhage. Interestingly, heterozygous knockout of *Anx7* results in a Ca<sup>2+</sup>-dependent endocrine secretory defect in mice, indicating that *Anx7* plays an important role in the regulation of endocrine secretion<sup>[130,131]</sup>. In addition, heterozygous deficiency of *Anx7* exhibits a phenotype of hepatomegaly and HCC develops spontaneously in these mice; the wild-type allele of *Anx7* has been found to remain intact in the HCC tissue from these *Anx7*<sup>+/-</sup> mice<sup>[132]</sup>. Genomic instability and downregulation of tumor suppressor genes have been observed in the HCC of *Anx7*<sup>+/-</sup> mice. These findings suggest that *Anx7* helps to maintain genomic stability and functions as a haploinsufficient TSG in HCC.

**Nibrin:** Nibrin (NBN) is involved in the repair of DNA double stranded breaks and has the potential to function as a tumor suppressor gene<sup>[133]</sup>. Homozygous knockout of *Nbn* causes embryonic lethality between embryonic day E3.5 and E7.5 in mice. Importantly, heterozygous deficiency of *Nbn* results in many types of spontaneous tumors including prostate cancer, mammary gland tumors, lymphoma and HCC in mice. Various chromosome aberrations, including fragmentation and end-to-end fusion, have been observed in the mouse embryonic fibroblasts (MEFs) of *Nbn*<sup>+/-</sup> mice. Loss of heterozygosity was found not to occur in the HCC tissues from the *Nbn*<sup>+/-</sup> mice<sup>[134]</sup>. These findings indicate that *Nbn* plays an important role in the maintenance of chromosome stability and would seem to function as a haploinsufficient TSG in HCC.

**PIN2/TRF1-interacting telomerase inhibitor 1:** PIN2/TRF1-interacting telomerase inhibitor 1 (PinX1) is a potent telomerase inhibitor involved in maintaining

telomeres at optimal length and its expression level is frequently down-regulated in the HBV-related HCC<sup>[135,136]</sup>. Heterozygous deficiency of *PinX1* has been found to result in telomere elongation and chromosome instability in MEF. Spontaneous HCC development has been observed in the *PinX1*<sup>+/-</sup> mice. The expression levels of *PinX1* are similar when HCC and its surrounding non-tumor tissues in the *PinX1*<sup>+/-</sup> mice are compared<sup>[137]</sup>. These findings indicate that *PinX1* may function as a haploinsufficient TSG and is an essential factor for chromosome stability.

**Shugoshin 1:** Shugoshin 1 (SGO1) is a guardian of chromosome cohesion that is associated with the fidelity of chromosome segregation during mitosis<sup>[138]</sup>. In mice, heterozygous deficiency of *Sgo1* has been found to lead to persistent hepatocyte DNA damage, to promote azoxymethane (AOM)-induced HCC and to result in the development of spontaneous HCC in *Sgo1*<sup>+/-</sup> mice. Interestingly, the SGO1 protein level has been shown to be higher in HCC tissue samples compared with the adjacent non-tumor tissues from the *Sgo1*<sup>+/-</sup> mice, suggesting that the wild-type allele of *Sgo1* is still present and is likely to be upregulated in HCC tissue<sup>[139]</sup>. In humans, upregulation of SGO1 expression has been observed in the HCC tissue samples compared with their adjacent non-tumor liver tissue samples<sup>[140]</sup>. These findings reveal that *Sgo1* helps maintain the accuracy of chromosome segregation and may function as a haploinsufficient TSG in HCC.

#### Genes related to various metabolic functions of the liver

The liver is an important metabolic organ and plays key roles in glucose, lipid, protein, bile acid and methionine metabolism. Dysregulation of metabolism may cause liver disease, including HCC<sup>[141]</sup>. Abnormal lipid metabolism, including decreased fatty acid oxidation, very low-density lipoprotein (VLDL) secretion or enhanced lipid uptake and lipogenesis, leads to hepatic steatosis, which is one of the risk factors for HCC. Lipid accumulation in hepatocytes results in lipotoxicity, oxidative stress, chronic liver damage and liver regeneration, which all contribute to hepatocarcinogenesis<sup>[142,143]</sup>. The effect of abnormal metabolic functioning, including lipid metabolism, methionine metabolism and bile acid metabolism, on hepatocarcinogenesis had been clearly demonstrated using mouse models.

**The fatty acyl-CoA oxidase:** The fatty acyl-CoA oxidase (AOX) protein, which is involved in lipid metabolism, is the first step enzyme of peroxisomal  $\beta$ -oxidation. AOX deficiency leads to steatohepatitis, oxidative stress, chronic liver damage and liver regeneration, as well as the development of spontaneous HCC in mice. Homozygous knockout of AOX results in the development of HCC due to sustained activation

of peroxisome proliferator-activated receptor alpha (PPAR $\alpha$ ), indicating that abnormal lipid metabolism may contribute to HCC development<sup>[144]</sup>.

**BHMT and GNMT:** Methionine metabolism is very important in maintaining the metabolic homeostasis of liver. One of the key metabolites is S-adenosylmethionine (AdoMet), which is the major methyl donor for methylation of several substrates, including DNA, RNA, histones, and various other small molecules. Abnormal methionine metabolism can cause fatty liver and HCC<sup>[145-147]</sup>. Reduced expression of the main enzymes involved in methionine metabolism, including methionine adenosyltransferase (MAT), glycine methyltransferase (GNMT) and betaine homocysteine (BHMT), have been observed in human HCC<sup>[148,149]</sup>. These findings suggest that impairment of methionine homeostasis may contribute to hepatocarcinogenesis.

BHMT is an enzyme that catalyzes the conversion of homocysteine (Hcy) to methionine. High levels of activity of BHMT have been detected in the livers of humans and mice. Disruption of *Bhmt* perturbs methionine metabolism and results in fatty liver and HCC development in mice. The fatty liver in *Bhmt*-deficient mice has been found to be due to a decrease in hepatic VLDL secretion<sup>[150]</sup>. These studies indicate that BHMT is important for methionine metabolism, the maintenance of liver homeostasis and the suppression of liver cancer.

GNMT is a methyltransferase that contributes to the maintenance of AdoMet homeostasis, which helps to avoid aberrant methylation. Loss of *Gnmt* causes fatty liver and HCC in mice. Aberrant methylation of DNA, which contributed to activation of the Janus kinase (JAK)/signal transducer of activators of transcription (STAT) pathway, has been observed in the livers of *Gnmt*-deficient mice<sup>[151]</sup>. These findings indicate that GNMT suppresses HCC development by maintaining AdoMet levels and allowing normal DNA methylation to occur.

#### Farnesoid X receptor and multidrug resistance

**2:** Bile acids are cholesterol metabolites and largely synthesized by hepatocytes. Hepatocellular bile acid synthesis and transport are highly regulated by canalicular transporter multidrug resistance 2 (MDR2) and nuclear transcriptional regulator farnesoid X receptor (FXR). Bile acids play key roles in the liver, including lipid metabolism, glucose metabolism and liver regeneration. Abnormal bile acid homeostasis may contribute to the development of fatty liver and HCC<sup>[152-154]</sup>. In mice, deficiency in the *Fxr* or *Mdr2* results in impairment of bile acids metabolism, an increase in inflammation, and the development of spontaneous HCC<sup>[155,156]</sup>. In humans, reduced expression levels of FXR and MDR2 have been observed in HCC tissue samples<sup>[157,158]</sup>. These findings suggest that deregulation of bile acid homeostasis may be involved



in hepatocarcinogenesis.

### **The Hippo signaling pathway**

Aberrant cellular signaling pathways are critical events in the process of hepatocarcinogenesis. Previous studies have identified many critical signaling pathways involved in HCC development<sup>[159-161]</sup>. Previous studies in mice have revealed that dysregulation of many important signaling pathways contribute to the development of HCC; these included the Hippo pathway, the JAK/STAT pathway, the NF- $\kappa$ B pathway, the PI3K/AKT/mTOR pathway, the TGF $\beta$  pathway and the WNT/ $\beta$ -catenin pathway.

The Hippo signaling pathway is a highly conserved kinase cascade that regulates cell proliferation, cell survival and organ size control. The Hippo pathway can be divided into three major parts, namely the upstream regulators, the Hippo core kinase components, and the downstream target genes. The Hippo pathway is critical for the maintenance of hepatocyte quiescence and the regulation of liver size. Dysregulation of the Hippo pathway has been observed in many types of human cancers, including HCC<sup>[162-164]</sup>. In mouse models, the tumor suppressive role of various Hippo signaling components has been identified, including the core kinases MST1/MST2 and the upstream regulator NF1.

**Mammalian sterile 20-like kinase 1 and 2:** Mammalian sterile 20-like kinase 1 (Mst1) and Mst2 are the components of the Hippo core kinase cascade. The single knockout of *Mst1* or *Mst2* results in no obvious phenotypes in mice, which suggests the functional redundancy of Mst1 and Mst2 during embryonic development and hepatocarcinogenesis. However, hepatocyte-specific knockout of both the *Mst1* and *Mst2* genes causes an increase in hepatocyte proliferation, hepatomegaly and spontaneous HCC development in mice<sup>[165,166]</sup>. These studies demonstrate that Mst1 and Mst2 are required for organ size control and tumor suppression.

**Neurofibromatosis type 2:** Neurofibromatosis type 2 (NF2) is the positive regulator of the Hippo signaling pathway<sup>[167]</sup>. NF2 deficiency leads to hepatic progenitor cell proliferation, hepatomegaly and hepatocarcinogenesis in mice. The tumor promoting phenotypes in the NF2-deficient liver have been shown to be disrupted by loss of the Yes-associated protein (YAP), which is inhibited by the Hippo pathway<sup>[168,169]</sup>. These studies indicate that the tumor suppressor function of NF2 in HCC occurs *via* inhibiting YAP activation.

**JAK/STAT signaling pathway:** JAK/STAT pathway is a plasma membrane to nucleus signaling pathway that regulates cell proliferation, differentiation and cell death. Activation of the JAK/STAT pathway is regulated by various positive (cytokine and tyrosine kinase) and

negative regulators [protein tyrosine phosphatase (PTP) and suppressor of cytokine signaling (SOCS) proteins]. Constitutive activation of the JAK/STAT pathway is associated with tumorigenesis. The SOCS protein is one of the negative regulators of JAK/STAT pathway and its expression is decreased in many types of cancers including HCC<sup>[170-172]</sup>. The role of the negative regulators of the JAK/STAT pathway in hepatocarcinogenesis has been studied and evaluated using mouse models.

**Suppressor of the cytokine signaling-1:** Suppressor of the cytokine signaling-1 (SOCS1) is a negative regulator of JAK-mediated cytokine signaling *via* the protein's direct binding to JAK<sup>[173]</sup>. In humans, a lower level of SOCS1 expression has been observed in HCV-associated HCC samples compared with adjacent non-tumor liver tissue samples<sup>[174]</sup>. In mice, heterozygous deficiency of *Socs1* has been found to increase dimethylnitrosamine (DMN)-induced and diet-induced liver fibrosis; furthermore, heterozygous deficiency has also been found to promote DEN-induced hepatocarcinogenesis in the *Socs1*<sup>+/-</sup> mice. These results suggested that SOCS1 is a haploinsufficient TSG in HCC.

**Suppressor of the cytokine signaling-3:** Suppressor of the cytokine signaling-3 (SOCS3) is a negative regulator for the JAK and interleukin (IL)-6-related signaling pathways<sup>[175]</sup>. In humans, expression of SOCS3 has been shown to be reduced in HCV-mediated HCC samples and this has been found to be accompanied by enhanced activation of STAT3. In mice, heterozygous deficiency of *Socs3* has been found to promote DEN-induced HCC in *Socs3*<sup>+/-</sup> mice<sup>[176]</sup>. These findings indicate that SOCS3 may function as a haploinsufficient TSG in HCC.

**Protein tyrosine phosphatase non-receptor type 11:** Protein tyrosine phosphatase non-receptor type 11 (PTPN11) is a tyrosine phosphatase that dephosphorylates activated STAT3 and attenuates proinflammatory IL-6 signaling<sup>[177,178]</sup>. In humans, a decrease in PTPN11 protein expression has been observed in HCC tissue samples compared with the adjacent non-tumor tissue samples<sup>[179]</sup>. In mice, *Ptpn11* deficiency in hepatocytes has been shown to result in hepatic inflammation, cell death and hepatocellular adenoma formation. In addition, *Ptpn11* deficiency also promotes DEN-induced HCC development in mice. Furthermore, *Stat3* is required for the tumor promoting effect of *Ptpn11* deficiency to occur. Together, these studies indicate that *Ptpn11* functions as a tumor suppressor in HCC by controlling the oncogenic activity of *Stat3*.

**Protein tyrosine phosphatase receptor type O:** Protein tyrosine phosphatase receptor type O (PTPRO) is a receptor type of protein tyrosine phosphatase that

is frequently down-regulated in human HCC tissues through promoter hypermethylation<sup>[180,181]</sup>. Studies have revealed that *Ptpro* deficiency promotes DEN-induced HCC and enhances *Stat3* activity in mice<sup>[181,182]</sup>. These findings indicate that PTPRO suppresses HCC development *via* control of the activity of Stat3.

#### **The nuclear factor- $\kappa$ B signaling pathway**

Nuclear factor- $\kappa$ B (NF- $\kappa$ B) is an important transcriptional regulator of inflammation and is required for survival of hepatocytes in the liver. Upon tumor necrosis factor (TNF) stimulation, the I $\kappa$ B kinase (IKK) complex is recruited and activated by the kinase TAK1, and this then stimulates NF- $\kappa$ B activity by phosphorylating the negative inhibitor I $\kappa$ B protein<sup>[183]</sup>. Various studies have revealed that NF- $\kappa$ B is constitutively activated in many cancers including HCC. This suggests a tumor promoting role for NF- $\kappa$ B signaling<sup>[184]</sup>. However, evidence from different groups has indicated that NF- $\kappa$ B signaling may also have a tumor suppressor role in HCC. It is likely that NF- $\kappa$ B signaling has a dual role and that this role changes at different stages of carcinogenesis<sup>[185,186]</sup>.

**Cylindromatosis:** Cylindromatosis (CYLD) is a deubiquitinase that inhibits the NF- $\kappa$ B pathway *via* deubiquitination of upstream regulatory factors<sup>[187]</sup>. In humans, reduced expression levels of CYLD have been observed in HCC tissue samples compared with the adjacent non-tumor tissue samples<sup>[188]</sup>. In mice, liver-specific disruption of CYLD triggers hepatic cell death, inflammation, fibrosis and spontaneous HCC development. Constitutive hyperactivation of TAK1, a NF- $\kappa$ B upstream regulatory factor, is required for liver pathogenesis in liver-specific CYLD knockout mice<sup>[189]</sup>. These findings show that CYLD suppresses hepatocarcinogenesis *via* control of the NF- $\kappa$ B signaling pathway.

**Galectin-3:** The galectin-3 is a down-stream target gene of NF- $\kappa$ B and plays an important role in inflammation response. Under normal conditions, galectin-3 is expressed in the bile duct epithelial and Kupffer cells, but not in the hepatocytes of livers<sup>[190,191]</sup>. However, expression of galectin-3 has been detected in human HCC cells, indicating that galectin-3 may be involved in HCC development<sup>[192]</sup>. In mice, homozygous knockout of galectin-3 results in fatty liver, inflammation, fibrosis and spontaneous HCC formation<sup>[193,194]</sup>. These studies indicate that galectin-3 plays an important role in hepatocarcinogenesis.

**NF- $\kappa$ B-essential-modulator:** NF- $\kappa$ B-essential-modulator (NEMO) is a regulatory subunit of the IKK complex and is essential for NF- $\kappa$ B activation. Ablation of NEMO blocks NF- $\kappa$ B activation in hepatocytes. In humans, loss of NEMO protein expression has been found in a substantial proportion of HCC tissue samples compared with their adjacent non-tumor tissue samples<sup>[195]</sup>. In mice, disruption of NEMO in

hepatocytes leads to nonalcoholic steatohepatitis (NASH) and the spontaneous development of HCC<sup>[196]</sup>. These findings show that NEMO acts as a tumor suppressor in the liver.

**TGF- $\beta$ -activated kinase 1:** TGF- $\beta$ -activated kinase 1 (TAK1) is a kinase that activates the IKK complex upon TNF stimulation. Ablation of TAK1 in the hepatocytes of mice results in liver damage and the early onset of spontaneous HCC development, which suggests a tumor suppressor function for TAK1 in liver cancer<sup>[197]</sup>.

#### **PI3K/AKT/mTOR signaling pathway**

The PI3K/AKT/mTOR pathway has a critical role in the regulation of cell growth, proliferation and metabolism. The PI3K/AKT/mTOR pathway is frequently activated in diverse cancers including HCC. The pathway is negatively regulated by phosphatase and tensin homolog deleted from chromosome 10 (PTEN) and AMP-activated protein kinase (AMPK). Alterations in PTEN or the AMPK activator LKB1 have been observed in many types of human cancers<sup>[198,199]</sup>. Knock-out mice having loss of *PTEN* and *LKB1* have been generated and used to evaluate the roles of the PI3K/AKT/mTOR signaling pathway in HCC development.

**Liver kinase B1:** Liver kinase B1 (LKB1) is a serine/threonine kinase. LKB1 positively regulates downstream kinases including AMPK, which inhibits mTOR pathway. LKB1 has identified as the disease gene of Peutz-Jegher polyposis and cancer syndrome<sup>[200]</sup>. In addition, down-regulation of LKB1 has been observed in human HCC tissue samples compared with the adjacent non-tumor tissue samples<sup>[201]</sup>. In mice, heterozygous knockout of *Lkb1* leads to increased proliferation of hepatocytes and the development of spontaneous HCC. Loss of heterozygosity of the wild-type allele of *Lkb1* has been detected in the HCC tissues from the heterozygous knockout of *Lkb1* mice, indicating that *Lkb1* acts as a recessive TSG in HCC<sup>[202]</sup>.

**PTEN:** PTEN is a phospholipid phosphatase that negatively regulates the highly oncogenic pro-survival PI3K/AKT signaling pathway<sup>[203]</sup>. In humans, expression levels of PTEN are decreased in HCC tissue samples compared with the adjacent non-tumor tissue samples<sup>[204]</sup>. In mice, homozygous knockout of *Pten* results in embryonic lethality. Furthermore, *Pten* heterozygous mice develop tumors in multiple organs including the liver. Loss of the wild-type *Pten* allele has been observed in liver tumors from *Pten*<sup>+/-</sup> mice<sup>[205-207]</sup>. Moreover, *Pten* deficiency in hepatocytes leads to steatohepatitis and HCC formation in mice<sup>[208]</sup>. These studies indicate that *Pten* functions as a recessive TSG in HCC.

**Regulatory-associated protein of mTOR:** Regulatory-associated protein of mTOR (RAPTOR) is one of the associated proteins of mTOR and is required for

mTOR activity. RAPTOR expression has been found to be up-regulated in advanced human HCC tissue samples compared with the adjacent non-tumor tissue samples, suggesting that the RAPTOR may contribute to hepatocarcinogenesis<sup>[209]</sup>. Hepatocyte-specific disruption of RAPTOR has been shown to result in hepatic damage, inflammation, fibrosis and the acceleration of DEN-induced hepatocarcinogenesis in mice. The HCC promoting effect of RAPTOR deficiency may be attributed partly to the hyper-activation of AKT in such livers<sup>[210]</sup>. These loss-of-function studies indicated that a persistent inhibition of mTOR may not be a suitable way to treat HCC.

### **TGF- $\beta$ signaling pathway**

TGF- $\beta$  signaling is involved in many important pathways related to cell growth, including proliferation, motility and cell death. TGF- $\beta$  inhibits hepatocyte proliferation in the liver, suggesting that it has potential tumor suppressive effects in HCC<sup>[211]</sup>. Although TGF- $\beta$  has tumor suppressive functions in benign and early-stage tumors, it can also act as a tumor promoter in advanced-stage cancers. TGF- $\beta$  has dual functions of tumor prevention and tumor promotion at different cancer stages<sup>[212,213]</sup>. Scientists have applied genetically modified mouse models to evaluate the complex roles of the TGF- $\beta$  signaling pathway in HCC development.

**Embryonic liver fodrin:** Embryonic liver fodrin (ELF) is an adaptor protein that mediates access to the receptor and SMAD3 activation in the TGF- $\beta$  signaling pathway. In humans, ELF has been found to be significantly decreased in HCC tissue samples compared with the adjacent non-tumor tissue samples<sup>[214]</sup>. In mice, heterozygous deficiency of *ELF* leads to the development of fatty liver and spontaneous HCC, which is accompanied by enhanced activation of cyclin D1. These findings indicate that *ELF* functions as a haploinsufficient TSG in HCC.

**Transforming growth factor  $\beta$ 1:** Transforming growth factor  $\beta$ 1 (TGF- $\beta$ 1) is a negative factor that inhibits hepatocyte proliferation<sup>[215]</sup>. In mice, heterozygous deficiency of *TGF- $\beta$ 1* increases hepatocyte proliferation and promotes DEN-induced HCC in *TGF- $\beta$ 1*<sup>+/-</sup> mice. Tumors from *TGF- $\beta$ 1*<sup>+/-</sup> mice do not show loss of the wild-type allele of *TGF- $\beta$ 1*<sup>[216]</sup>. These findings indicate that the *TGF- $\beta$ 1* functions as a haploinsufficient TSG in HCC.

**TGF- $\beta$  type II receptor:** TGF- $\beta$  type II receptor (T $\beta$ R-II) is a receptor of TGF- $\beta$  growth factor. In humans, down-regulation of T $\beta$ R-II has been frequently observed in HCC tissue<sup>[217]</sup>. In mice, heterozygous deficiency of *T $\beta$ R-II* results in increased hepatocyte proliferation and promotes DEN-induced HCC carcinogenesis; in addition, a decrease of T $\beta$ R-II expression has been observed in the DEN-induced HCC tissue samples compared to the

non-tumor liver in *T $\beta$ R-II*<sup>+/-</sup> mice suggesting loss of the wild-type allele of *T $\beta$ R-II* in HCC<sup>[218]</sup>. These studies show that *T $\beta$ R-II* functions as a recessive TSG in HCC.

### **The WNT signaling pathway**

The WNT/ $\beta$ -catenin signaling pathway plays critical roles in liver development, growth and metabolism. Moreover, WNT/ $\beta$ -catenin signaling is important for cancer development, including tumor initiation, growth and metastasis. Aberrant activation of WNT/ $\beta$ -catenin signaling has been observed in human HCC, suggesting that this pathway is involved in hepatocarcinogenesis<sup>[219-221]</sup>.

**Adenomatous polyposis coli:** Adenomatous polyposis coli (APC) is the antagonist of WNT signaling and acts by enhancing  $\beta$ -catenin degradation. In humans, the expression level of the APC protein has been found to be decreased in HCC tissue samples compared to the adjacent non-tumor tissue samples<sup>[222]</sup>. In mice, liver-specific disruption of *Apc* causes activation of  $\beta$ -catenin signaling and spontaneous HCC formation<sup>[223]</sup>. These findings demonstrate that APC functions as a tumor suppressor in HCC *via* regulation of the  $\beta$ -catenin signaling.

### **MicroRNAs**

MicroRNAs (miRNAs) are short noncoding RNAs and act as post-transcriptional regulators of gene expression. miRNAs may function as oncogenes or tumor suppressor genes through their targeting of various different genes involved in tumorigenesis. Deregulation of miRNA expression has been observed in human HCC indicating that miRNAs are involved in hepatocarcinogenesis. Importantly, the direct role of miRNA in hepatocarcinogenesis has been demonstrated using mouse models; examples of such miRNAs include miRNA-122 and miRNA-140<sup>[224-226]</sup>.

**miR-122 and miR-140:** miR-122 is a major form of miRNA found in the liver. Downregulation of miR-122 has been observed in human HCC<sup>[227]</sup>. Disruption of miR-122 leads to hepatic steatosis, inflammation, fibrosis and spontaneous HCC in mice. The fatty liver in miR-122-deficient mice is caused by impaired VLDL secretion in the liver<sup>[228]</sup>. These studies demonstrate the metabolic and tumor suppressor function of miR-122 in livers. Another miRNA, miR-140, has also been suggested to act as a tumor suppressor in HCC. miR-140 deficiency results in enhanced NF- $\kappa$ B activity and promotes DEN-induced HCC development, indicating that the miR-140 functions as a tumor suppressor *via* regulation of the NF- $\kappa$ B activity in mice<sup>[229]</sup>.

### **Miscellaneous**

**Nuclear receptor coactivator 5:** Nuclear receptor coactivator 5 (NCOA5) is an estrogen receptor



coactivator that is able to enhance estrogen receptor  $\alpha$  activity in the presence of estradiol<sup>[230]</sup>. In humans, expression of NCOA5 mRNA has been found to be decreased in about 40% of HCC tissue samples compared with their adjacent non-tumor tissue samples<sup>[231]</sup>. In mice, heterozygous deficiency of *Ncoa5* leads to fatty liver, inflammation, fibrosis and spontaneous HCC development. In addition, expression of the NCOA5 protein has been detected in HCC samples from *Ncoa5*<sup>+/-</sup> mice. These findings indicate that *Ncoa5* functions as a haploinsufficient TSG in HCC.

**Regulatory subunit 1a of protein kinase A:** Regulatory subunit 1a of protein kinase A (PRKAR1A) is the major component of type I protein kinase A and regulates protein kinase activity<sup>[232]</sup>. Heterozygous deficiency of *Prkar1a* leads to multiple tumor formation events including HCC. Loss of heterozygosity of the *Prkar1a* allele was not observed in the tumors of *Prkar1a*<sup>+/-</sup> mice, suggesting that *Prkar1a* functions as a haploinsufficient tumor suppressor<sup>[233]</sup>.

**Nuclear receptor coactivator 2:** Nuclear receptor coactivator 2 (NCOA2) is a transcriptional coactivator that regulates fasting hepatic glucose release by controlling the expression of glucose-6-phosphatase, which is the key enzyme of gluconeogenesis<sup>[234]</sup>. Homozygous deficiency of *Ncoa2* promotes DEN-induced hepatocarcinogenesis in mice revealing a tumor suppressor role for *Ncoa2* in liver cancer<sup>[235]</sup>.

**Nuclear factor erythroid 2-like 1:** Nuclear factor erythroid 2-like 1 (NFE2L1; also known as NRF1, LCR-F1 and TCF11) is a transcription factor that controls the redox balance and lipid metabolism<sup>[236,237]</sup>. Hepatocyte-specific disruption of the *Nrf1* gene causes hepatic steatosis, cell death, inflammation, oxidative stress, fibrosis and spontaneous hepatic cancer in mice<sup>[238]</sup>. These findings suggested that *Nrf1* may function as a tumor suppressor in HCC by reducing oxidative stress in liver.

## CONCLUSION

Spontaneous tumor formation largely occurs due to an accumulation of multiple somatic mutations. Discoveries from genetically modified mice are very informative and allow the identification of potential TSG based on clinical findings and characterization of carcinogenic mechanisms. HCC is tremendously heterogeneous at the pathological, clinical, genomic and molecular levels; this may be due to the natural function of liver, which serves as a major organ responsible for metabolism and detoxification of the whole body<sup>[239]</sup>. This situation also indicates the complexity of the signaling pathways active in the liver with multiple signaling pathways and cellular processes intercrossing in hepatocytes in order to regulate and control the cell cycle, metabolism and detoxification.

Inactivation of TSGs might involve a range of different modulations in addition to mutation, deletion and loss-of-heterozygosity; for example promoter silencing by DNA methylation. One of the therapeutic strategies is to take a TSG as a molecular target, for example *p53*. Not only the TSG itself can be a target; its interacting proteins and/or the downstream targets of the TSG are also candidates for suppressing tumor formation. In this regard, HCC mouse models, which recapitulate the pathology and progression of hepatocarcinogenesis in human HCC, provide a useful *in vivo* animal model for therapeutic testing and mechanistic studies under physiological conditions. Several TSGs are currently under evaluation in order to test whether they can serve as a target for treating lung or ovarian cancer<sup>[240]</sup>. Taking into consideration tissue specificity and the possibility of potential off-target problems, it may be useful to identify liver-specific TSGs for the development of precision medicine when tackling HCC.

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