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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

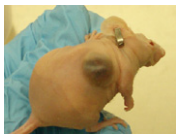
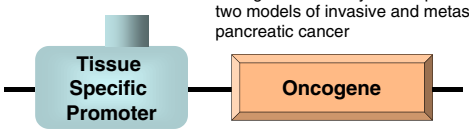
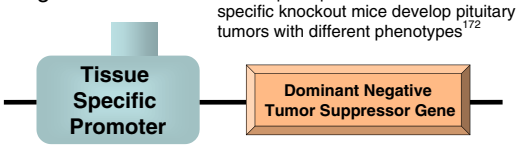
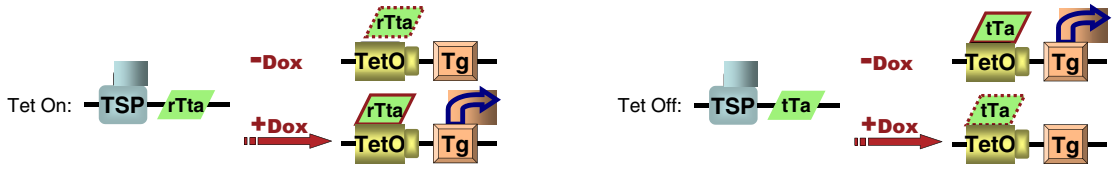
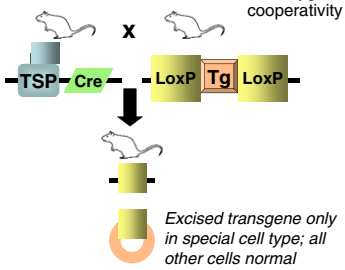
The demonstration that concentrated cancer cells grown *in vitro* could form tumors when implanted subcutaneously into an immunocompromised mouse was first established in 1969 [5]. This xenograft model has since demonstrated several advantages that explain its persistence as the mainstay of pre-clinical 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[16]	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[169]; $Trp53^{R172H}$ 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[166]	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 *re*combination) 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	BgIII-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 (Δ*N90-CTNNB1*), and dominant-negative TCF-1 (*DNHNF1*), by hydrodynamic transfection into livers	Combination *MET* and Δ*N90-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-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

containing either only one maternal allele or two alleles. Imprinting usually blocks IGF-2 expression from the maternal allele in liver. However, IGF-2 re-expression occurred in all 4 of these models, and was chronologically associated with late stages of progression toward HCC [113].

Epidermal growth factor (EGF) is a potent mitogen to hepatocytes. Unlike in other malignancies, the EGF receptor is rarely mutated in HCC, and several reports suggest an EGF-mediated autocrine growth stimulation of hepatoma cells [114]. This was further supported by the accelerated liver tumor formation after constitutive over-expression of a secretable form of EGF (IgEGF). All double-transgenic mice with liver-specific IgEGF over-expression in cooperation with AAT-myc died by 4.4 months from HCC, whereas only 44% of ATT-myc mice had developed HCC by age 14 months [115].

3.3.3.5. Wnt/β-catenin pathway. A key pathway implicated in hepatic tumorigenesis is the canonical Wnt pathway, in which β-catenin acts as a co-activator of the TCF/LEF family of transcription factors and regulates the expression of several genes related to cell proliferation and apoptosis. The Wnt/β-catenin signaling pathway normally functions in cellular differentiation, proliferation, and apoptosis, and has a fundamental role in embryogenesis. Liver development in xenopus, zebrafish, and mouse embryogenesis has been shown to be dependent on functional Wnt signaling [116,117].

There is general agreement that Wnt signaling is upregulated in a subset of HCCs [118]. Mutations of genes encoding several components of the Wnt pathway have been described, including β-catenin (19–44%), AXIN1 and AXIN2 (5–14% and 3–10%) [119–123]. The mutations of β-catenin identified in HCC are located in exon 3 of the CTNNB1 gene, the phosphorylation site for GSK3α/β. In addition, immunohistological studies have demonstrated abnormal cytoplasmic and nuclear accumulation of β-catenin in 17–40% of human HCCs [124,125]. In addition to accumulated mutations, stimulation of proliferation in liver cancer cell lines transfected with Hepatitis C core viral protein is at least partially mediated by upregulation of Wnt-1 protein expression [126]. This correlation between HCV and the Wnt pathway needs to be verified by *in vivo* studies.

Although mutations in β-catenin are thought to be tumorigenic in human HCCs, transgenic mouse models over-expressing either a stable mutant form of β-catenin [127,128] or a constitutively activated, non-mutated form of β-catenin exhibit hepatomegaly, but no HCC [127–129]. Surprisingly, although mutations in the tumor suppressor APC are very rarely seen in HCC and patients with germline APC mutations do not typically develop HCC, it has been found that liver-targeted loss of APC in mice can lead to HCC through activation of β-catenin signaling [130].

It seems that a second hit from an additional mutation is required to generate tumors in β-catenin transgenic mice. Simultaneous co-expression of a Wnt-activating β-catenin mutation ($Catnb^{lox(ex3)}$) and mutation in H-ras introduced by adenovirus-mediated Cre expression resulted in HCC in 100% of the double-transgenic progeny [131]. The interplay between the growth factor signaling pathways and the Wnt/β-catenin pathway was amply illustrated in the simultaneous over-expression of HGF and β-catenin knockout mouse model generated by Monga and colleagues [96]: the proliferative effects of HGF over-expression were mediated by β-catenin stabilization, and were negated in β-catenin null mice.

3.3.4. Other HCC models

3.3.4.1. Fibroblast growth factor in muscle. While most mouse models of HCC express growth factors and oncogenes under liver-specific promoters, liver-specific expression is not a requirement for development of HCC. For example, a transgenic model over-expressing fibroblast growth factor 19 (FGF19) in skeletal muscle develops HCC in 53% of mice by age 10–12 months [132]. Interestingly, unlike the vast majority of both human tumors and murine models, these tumors are more common in female progeny. Hepatocellular proliferation was significantly increased in these mice and in non-transgenic mice injected with FGF19 protein. Furthermore, immunostaining for β-catenin revealed nuclear staining in 4/4 female mouse tumors, and subsequent sequencing of the GSK3β phosphorylation site of β-catenin revealed mutations in 16%, which implicates activation of the Wnt/β-catenin signaling pathway as a potential mechanism for hepatocellular transformation in this model.

3.3.4.2. Urokinase-type plasminogen activator. Not all genetically modified models of HCC arise from predicted oncogene over-expression, tumor suppressor loss, or liver injury. In a transgenic model over-expressing the urokinase-type plasminogen activator (*uPA*) transgene under the albumin promoter, for example, most mice died from liver hemorrhage within 4 days of birth; in the two transgenic lineages developed from surviving founder mice, there was a surprising 100% incidence of HCC at 8–20 months of age. Moreover, the surviving mice regained normal clotting function, and their livers were repopulated by clonal, regenerative nodules that no longer expressed the transgene. Tumor progenitor cells were found to contain transgene-deleting chromosomal rearrangements which likely extended into flanking DNA. Therefore, the initiating event in this HCC model was likely extensive DNA rearrangements occurring during rapid regeneration [133].

3.4. Chemically-induced fibrosis and hepatocarcinogenesis

Cirrhosis is a major cause of mortality as both a precursor to malignancy and a cause for liver failure. As a disease with clear environmental non-hereditary components to its aetiology, liver fibrosis and cancer is well suited for modeling using chemical induction. Experimental models of liver disease can be categorized as cholestatic, nutritional, alcoholic, immunological, and toxic, and have been reviewed elsewhere [134].

Briefly, several hepatotoxic agents have been used both in the induction of generalized liver disease and HCC (see Table 3). Chemical models of hepatocarcinogenesis often involve initiation by a carcinogen followed by a growth stimulus promoter to induce clonal expansion of initiated cells, such as partial hepatectomy (Solt–Farber method [135]) or phenobarbital [136]. Alternatively, rodents are subjected to repeated administration of carcinogens such as DEN, DMN, or CCl_4 over a prolonged period [136]. Most initiated cells accrue damage and ultimately undergo apoptosis, but the small number that respond to promoters evolve into dysplastic foci and later to dysplastic nodules. These foci and nodules can disappear following the removal of promoters in a process termed remodeling, which typically involves apoptosis of the preneoplastic cells [41]. Nodules which have acquired the capacity for autonomous growth progress to neoplastic nodules and HCC, an irreversible process involving the accumulation of genomic damage [137].

The most commonly employed model for liver disease is carbon tetrachloride (CCl_4) administered in drinking water, in inhaled gases, or by intraperitoneal injection. The reactive metabolite trichloromethyl radical is produced during the oxidative metabolism of CCl_4 by cytochrome p450, and causes liver damage by eliciting production of reactive oxygen intermediates and by peroxidative degradation of membrane phospholipids [138]. Compounds like phenobarbital, ethanol, and acetone induce microsomal cytochrome p450 and therefore potentiate the hepatotoxicity of CCl_4, as does hypoxia; therefore, hepatocellular injury and necrosis are predominantly seen in the centrolobular zone where the oxygen tension is low [134,138].

Dimethylnitrosamine (DMN) is a carcinogenic agent which causes liver injury by covalent binding and methylation of nucleic acids and proteins in hepatocytes [139]. Animals administered DMN either per oral or by intraperitoneal injection develop cirrhosis within 3–4 weeks, and can continue to have stable or progressive disease for several months after discontinuation of the agent [140].

Diethylnitrosamine (DEN) induces pericentral foci of small dysplastic hepatocytes and acts by ethylating nucleophilic sites in DNA [141,142], causing cirrhosis and multifocal HCC within 18 weeks [89,143]. Frequent β-catenin mutations have been found in HCCs induced by DENA in mice [144], and when combined with a methyl-deficient diet, DEN administration generates p53 mutation or rearrangement in rats [145].

Thioacetamide (TAA) in drinking water (0.03%) or by intraperitoneal injection induces fibrosis in rats and mice over a period of 2–3 months, which may be secondary to the oxidant properties of TAA and the induction of hepatic oxidative stress [134,146,147]. Acute liver injury and subsequent fibrosis can be created by administration of D-galactosamine (GalN), a hepatotoxin that induces liver damage by depleting uridine nucleotides and therefore diminishing RNA and protein synthesis [148].

Cholestatic cirrhosis has been induced by extrahepatic bile duct ligation (BDL) in rats, rabbits, dogs, and monkeys. Histologically, the BDL model is characterized by infiltration of connective tissue in the portal

Table 3
Toxic models of liver fibrosis and HCC

Diet or chemical	Mechanism of action	Phenotype	References
Choline-deficient and ethionine (CDE) diet	Oxidative DNA damage, DNA strand breaks and chromosomal instability [41]	30–35 weeks: 100% HCC	[135,190–192]
Ciprofibrate	Synthetic peroxisome proliferators, non-genotoxic carcinogen	60 weeks: 100% HCC [193]	[90,193,194]
Diethylnitrosamine (DENA)	Genotoxic hepatocarcinogen	100% HCC in males, 30% in females. Extensive chromosomal damage	[90,168,194,195]
Thioacetamide (TAA)	Metabolites induce oxidative stress	100% HCC	[134,146]
2-Acetylaminoflouren (2-AAF)	Genotoxic	Used primarily as promoter in initiation/promotion protocols	[194,196]
Phenobarbital	Non-genotoxic	Used as promoter in initiation/promotion protocols; increases HCC by 500%. Can inhibit tumor formation in mice given DEN. Associated with β-catenin activation [197]	[197,198]

zone and proliferation of bile duct epithelial cells and hepatocytes. This methods allows rapid four-week induction of cirrhosis, and the mortality is high [134].

Choline-, methionine-deficient diets administered over 3–12 week periods induce cirrhosis and HCC in rats and mice, even when followed by an adequate diet [41]. Injury in these diets is most likely attributable to depletion of hepatic antioxidant mechanisms, such as reduced glutathione, which leads to oxidative DNA damage, inflammation and fibrosis [41,149]. Histologic changes seen in rodents fed this diet include periportal fatty liver, focal hepatocyte necrosis, oval cell proliferation, infrequent cirrhosis [150] and HCC [151]. The variation in animal susceptibility to choline deficiency is a disadvantage to this model [134].

3.5. Models of liver fibrosis and HCC: creating a tumor environment

The tumor microenvironment is emerging as a fundamental determinant of oncogenesis and metastasis. The liver presents an ideal organ in which to study the interaction between tumors and their microenvironment, as hepatocellular carcinoma (HCC) develops in a background of liver fibrosis in about 90% of cases. While the notion that the tumor microenvironment may help instigate tumor formation is gaining acceptance, the manner in which this occurs remains a mystery. In addition to the traditional toxic method of inducing fibrosis in rodents, there are numerous transgenic models that have been designed to recapitulate the phenotype of chronic inflammation leading to fibrosis and HCC seen in humans (see Table 4).

Stellate cell transactivation is a hallmark of hepatic fibrogenesis. Many genetic models of liver fibrosis have focused on the over-expression of TGF-β, a major fibrogenic factor that drives matrix deposition from activated stellate cells [152]. Sanderson et al. generated transgenic mice containing a fusion gene (Alb/TGF-β1) under the control of the regulatory elements of the mouse albumin gene; these mice developed mild fibrosis by 12 weeks, and rarely developed cirrhosis [153]. Similar mild to moderately fibrotic phenotypes have been demonstrated by other investigators [154,155]. When exposed to thioacetamide, TGF-β1-over-expressing transgenic mice develop fibrosis at an accelerated rate [155], and develop HCC more frequently than wild-type mice (9/9 versus 4/10 mice at 9 months) [156].

Intracellular signaling from TGF-β occurs through signaling members TGF-β receptor type II (TBRII), SMAD2, SMAD4, and SMAD adaptor, which are tumor suppressors in gastrointestinal cancers. None of

Table 4
Genetically modified models of liver fibrosis, inflammation, and HCC

Gene	Type of mutation or tissue promotor/construct	Phenotype	Dysplasia or HCC	References
TGF-β	Porcine TGF-β over-expression under albumin promoter	Early death due to extra-intestinal manifestations [153]; mild fibrosis [155,156]	100% HCC in transgenic mice treated with TAA [156]	[153,155,156]
TGF-β inducible transgenic	Fusion CRP/TGF-β1 under CRP promotor, induced by LPS injection	Collagen deposition at age 6 weeks	None reported	[154]
$ELF^{+/-}$ knockout	ELF+/− knockout mice	Steatosis	40% HCC at >15 months	[157,199]
PDGF-B	PDGF-B over-expression using Cre-LoxP under albumin promoter; made Tamoxifene-inducible by breeding with mice expressing Cre under transthyretin receptor promoter	100% liver fibrosis at age 4–6 weeks	None reported	[159]
PDGF-C	Human PDGF-C expression driven by albumin promoter	Fibrosis and steatosis	80% HCC at 12 months	[160]
IL-6 knockout	IL-6 knockout ($IL\text{-}6^{-/-}$)	Hepatocyte necrosis and compensatory proliferation both decreased in $IL\text{-}6^{-/-}$ mice	<10% HCC in $IL\text{-}6^{-/-}$ mice compared to 100% HCC at 8 months in male WT mice; 13% HCC in female WT mice	[162,163]
MyD88 knockout	$MyD88^{-/-}$	Diminished production of IL-6 in $MyD88^{-/-}$ mice	Suppression of DEN-induced HCC: $MyD88^{-/-}$ mice had fewer smaller HCCs than WT mice	[162,163]
Alpha-1-antitrypsin (AAT)	Transgenic mice using AAT Z genomic clones	High copy Z lineage: AAT accumulation in endoplasmic reticulum; hepatitis and HCC	82% HCC at 16-18 months	[164]
Mdr-2	Mdr-2 gene knockout	Early: non-suppurative inflammatory cholangitis	HCC at 6–12 months with +lung metastasis [166]	[165,166]
$Acox1^{-/-}$	Fatty acyl-CoA oxidase null ($AOX^{-/-}$) [167]	Steatohepatitis followed by regeneration	100% HCC at 15 months [167]	[167]

the SMAD mutant models have developed HCC, however. SMAD function is dependent upon adaptor proteins such as embryonic liver fodrin (ELF), a β-spectrin protein. ELF associates with SMAD3, SMAD 4, and the TGF-β receptor complex, and ultimately leads to their translocation to the nucleus. Mishra et al. report that $ELF^{+/-}$ knockout mice develop steatosis and spontaneous HCC. Loss of ELF in these mice results in cell cycle disruption with significant increases in Cdk4, cyclin D1 and pRb hyperphosphorylation [157].

In addition to TGF-β, activated stellate cells produce a number of other profibrotic cytokines such as platelet derived growth factor (PDGF). Induction of PDGF receptor mRNA is one of the earliest events in stellate cell activation, and its over-expression has been linked to fibrosis [158]. Kanzler's group developed a model in which the PDGF-B ligand is inducibly over-expressed in the liver. They found that PDGF-B expression caused hepatic stellate cell activation and collagen deposition [159]. Campbell et al. have described a PDGF-C transgenic model expressing human PDGF-C driven by the albumin promoter. These mice develop fibrosis and steatosis, and 80% develop HCC by 12 months of age [160]. Interestingly, no cirrhosis or regenerating nodules were observed in either of these models.

Interleukin-6 (IL-6) is the cytokine largely responsible for hepatic response to infections and inflammation. IL-6 serum concentrations are increased in patients with HBV and HCV infections and with HCC [161]. Naugler et al. induced liver disease with DEN in IL-6 knockout ($IL\text{-}6^{-/-}$) mice to determine whether gender bias in IL-6 production accounts for the sex difference seen in HCC development in both humans and in rodent models [162]. The carcinogenic effects of DEN were suppressed in $IL\text{-}6^{-/-}$ male mice: <10% developed HCC by 8 months of age, compared to 100% in wild-type male mice. No difference was seen in $IL\text{-}6^{-/-}$ versus WT female mice. Estrogens inhibit IL-6 promoter activity by decreasing activity of the transcription factors NF-κB and C/EBPβ, a process dependent on IKKβ and toll-like receptor (TLR) adaptor Myd-88. In the same study, Myd-88 was found to be required for IL-6 induction by necrotic hepatocyte debris, and Myd-88 knockout ($Myd\text{-}88^{-/-}$) male mice developed fewer and smaller HCCs in response to injury by DEN than did WT male mice. The results of this experiment provide a potential explanation for the gender differences in the incidence of liver cancer, which ranges between 2:1 and 4:1 male to female ratio [163].

Alpha-1-antitrypsin (AAT)-deficient transgenic mice express the transport-impaired Z variant of the human disease. These mice accumulate AAT and form foci of hyperplasia surrounded by inflammatory infiltrates [41], developing hepatitis, adenomas after 12 months, and HCC after 16–20 months [164].

The Mdr-2 gene encodes a protein involved in transport of phosphatidylcholine into the bile. Mdr-2 knockout mice accumulate toxic bile salts in their intrahepatic biliary system, which causes a non-suppurative inflammatory cholangitis and ductular proliferation and eventually nodules and HCC at 6–12 months [165,166]. A similar pathogenesis occurs in acyl-CoA oxidase (AOX) knockout mice, which develop steatohepatitis followed by a complete liver regeneration; this sequence of inflammation followed by proliferation results in the formation of HCCs by the age of 15 months [167].

4. Integrating functional genomics in HCC: from mice to humans

The progression from dysplastic foci to HCC involves the accumulation of genetic changes which can be monitored with cytogenetic studies that show karyotypic alterations in various chromosomes [137]. This type of chromosomal gains and losses are particularly numerous in lesions from rodents subjected to the carcinogen initiator–promoter protocol, or in SV40/T antigen transgenic mice. Various genes involved in hepatocarcinogenesis such as c-H-ras, met, HGF, myc, and p53 are located on rat chromosomes exhibiting frequent aberrations [41].

Thorgeirrson et al. applied a genome-wide microarray analysis to three transgenic mouse models of HCC, and found that although gene expression profiles in tumors derived from the three transgenic lines were highly similar, it was possible to identify oncogene-specific gene expression signatures at an early dysplastic stage of hepatocarcinogenesis [168]. In a related study, gene expression patterns of HCC tumors from seven different mouse models and 91 human HCCs from predefined subclasses were measured to compare the molecular features of mouse and human HCCs [90]. The authors found that gene expression patterns in tumors from Myc, E2f1 and Myc/E2f1 transgenic mice were similar to those of the better survival group of human HCC, whereas the expression patterns in HCCs from Myc/Tgfα transgenic mice and from DEN-treated mice were most similar to those of the poorer survival group of human HCC. Gene expression patterns in HCC from $Acox1^{-/-}$ mice and in ciprofibrate-induced HCCs were least similar to those observed in human HCCs. This study supports the notion that comparison of gene expression between the two species can be used to identify the mouse models of HCC that most closely mimic the tumors in humans.

5. Conclusion

We have described both traditional models of carcinogenesis in which the expression of oncogenes and tumor suppressor genes is genetically altered to produce

HCC, and other models in which tumor formation is dependent on inflammation. The natural history of HCC development in humans, combined with the evidence that genetic mutations alone sometimes do not generate tumors unless initiated by a proinflammatory agent, underscore the need to develop new models in which HCCs develop spontaneously in an environment of fibrosis, in order to best recapitulate the human disease process. In addition, integrative functional genomic studies have suggested that human HCCs can be classified into subgroups based on molecular pathway activation. Comparison of gene expression between mouse models and human HCC may allow us to create mouse models in future which recapitulate the various subgroups, which would make ideal models for preclinical studies.

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We dedicate this work to our friend and colleague Eric Lemmer, M.D., Ph.D., whose presence at its inception was highly inspirational, and whose absence today we still lament.

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