

Table 2
Genetically engineered models of hepatocellular carcinoma

Gene	Type of mutation or tissue promoter/construct	Phenotype (+/- and -/-)	Chemically induced/metastasis	References
<i>Viral models</i>				
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-BgIII 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 \times 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]
<i>Cell cycle models</i>				
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 α 1 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 TGF α	c-myc over-expression under albumin enhancer/promoter; TGF α over-expression under metallothionein 1 promoter	4 mos: 70% dysplastic nodules; 18% HCC [90]	Zinc in H ₂ O accelerated nodule formation by 6–8 weeks	[90,182]

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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 I promoter [184]; under $\alpha 1$ 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]
<i>Telomere dysfunction models</i>				
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]
<i>Pathway specific models</i>				
<i>Wnt/β-catenin</i>				
Activating mutation in β -catenin: truncated NH ₂ terminal transgenic	EAB/9K/ Δ N131 β -catenin construct under control of liver-specific enhancer of aldolase 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 ^{loxex3} knockout and fatty acid binding protein Fabp1-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 ^{loxex3} 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 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 ^{loxex3} knockout and H-ras (Tg ^{loxstopAHH-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 ^{loxex14} 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)

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<i>PI3K/Akt pathway</i> 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]
<i>Insulin growth factor pathway</i> 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 TGF α transgenic	TGF α 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) TGF α \times IGF2 ^{wt/wt} and (4) TGF α + IGF2 ^{wt/wt} ; 100% HCC at 18 months	None reported. Zinc in drinking water starting at age 10 months	[188]
<i>Epidermal growth factor pathway</i> 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]
<i>Ras signaling</i> H-ras	Mutant c-H-ras over-expression under albumin promoter	Hepatomegaly, lung tumors [74]	None reported	[74]
<i>HGF/c-Met and TGF-α</i> 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 TGF α 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 TGF α transgenic mice: 33% (3/9 mice) HCC in HGF/ TGF α versus 60% (6/10) in TGF α 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]

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Met and β -catenin	Transposable vectors containing wild-type human <i>MET</i> , constitutively-activated mutated form of β -catenin ($\Delta N90$ - <i>CTNNT1</i>), and dominant-negative TCF-1 (<i>DNHNF1</i>), by hydrodynamic transfection into livers	Combination <i>MET</i> and $\Delta N90$ - <i>CTNNT1</i> : 74% HCC within 1 month (no adenomas); combination <i>MET</i> and <i>DNHNF1</i> : 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 (<i>c-met^{fl/fl}</i>) and Cre driven by albumin promoter (<i>AlbCre^{f/f}</i>)	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	<i>N</i> -nitrosodiethylamine	[94]
TGF α	TGF α 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 ₂ O 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 step-wise 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 potentially 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 Thorgerirsson 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 which were 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 hyperplastic 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, TGF α 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 GSK3 α/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 (*Camb^{loxex3}*) 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 GSK3 β 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 CCl₄ 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 (CCl₄) administered in drinking water, in inhaled gases, or by intraperitoneal injection. The reactive metabolite trichloromethyl radical is produced during the oxidative metabolism of CCl₄ by cytochrome p450, and causes liver damage by eliciting production of reactive oxygen intermediates and by per-

oxidative degradation of membrane phospholipids [138]. Compounds like phenobarbital, ethanol, and acetone induce microsomal cytochrome p450 and therefore potentiate the hepatotoxicity of CCl₄, as does hypoxia; therefore, hepatocellular injury and necrosis are predominantly seen in the centrilobular 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 (DEN)	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-Acetylaminofluorene (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 method 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- β 1) 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- β 1-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 (TBR2), 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- β 1 under CRP promoter, 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 Tamoxifen-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-6 ^{-/-})	Hepatocyte necrosis and compensatory proliferation both decreased in IL-6 ^{-/-} mice	<10% HCC in IL-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-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-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-6^{-/-} versus WT female mice. Estrogens inhibit IL-6 promoter activity by decreasing activity of the transcription factors NF- κ B and C/EBP β , a process dependent on IKK β 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-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 knock-

out 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].

Thorgeirsson 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/Tgfr 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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METABOLISM, CANCER AND GENETICS

Molecular basis for the synergy between alcohol and hepatitis C virus in hepatocarcinogenesis

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Introduction

Hepatitis C virus (HCV) infects approximately 170 million people persistently worldwide, and induces a spectrum of chronic liver diseases, from chronic hepatitis, cirrhosis to hepatocellular carcinoma (HCC).¹ HCV has been given increasing attention because of its wide and deep penetration in the community, coupled with a very high incidence of HCC in persistent HCV infection. It impacts the medical, sociological and economic domains of society. Once liver cirrhosis is established in hosts infected with HCV, HCC develops at a yearly rate of 5–7%,² resulting in the development of HCC in nearly 90% of HCV-associated cirrhosis patients in 15 years. In addition, the outstanding features in the mode of hepatocarcinogenesis in HCV infection (i.e. development of HCC in a multicentric fashion and a very high incidence), are not common in other

Abstract

Overwhelming lines of epidemiological evidence have indicated that persistent infection with hepatitis C virus (HCV) is a major risk for the development of hepatocellular carcinoma (HCC). In addition, heavy alcohol use has been linked with earlier progression to HCC in chronic hepatitis C patients. However, in the pathogenesis of HCV-associated HCC, it still remains controversial as to whether the virus plays a direct or an indirect role, and as to how alcohol operates in the acceleration of HCC development. Several studies using transgenic mouse models, in which the core protein of HCV has an oncogenic potential, indicate that HCV is directly involved in hepatocarcinogenesis, although other factors such as continuous inflammation or environmental factors seem also to play a role. The downstream events of the HCV core protein expression in the transgenic mouse HCC model are segregated into two pathways. One is the augmented production of oxidative stress in the absence of inflammation along with the attenuation of some scavenging systems in the putative preneoplastic stage with steatosis in the liver. The other pathway is the alteration in cellular gene expression and intracellular signaling, including the mitogen-activated protein kinase cascade. The combination of these pathways would explain the unusually high incidence and multicentric nature of HCC development in HCV infection. In addition, alcohol feeding in this animal model further activated the two pathways synergistically with HCV, leading to an earlier development of HCC. Such a synergy would reveal the molecular basis for the acceleration of HCC development by alcohol in HCV infection.

malignancies except for hereditary cancers such as familial polyposis of the colon. Knowledge of the mechanism underlying HCC development in persistent HCV infection is therefore imminently required for the prevention of HCC.

However, alcohol has been known as an accelerating factor in the development of HCC in persistent HCV infection.^{3–5} The pattern of the risk for HCC due to alcohol intake shows a continuous dose-effect curve without a definite threshold, although most studies have found that HCC risk increased only for alcohol consumption above 40–60 g of ethanol per day. Some evidence supports a positive interaction of alcohol intake, probably with HCV infection and possibly with HBV infection.¹ Synergistic interactions on the additive model were observed between heavy alcohol consumption and chronic hepatitis virus infection and diabetes mellitus.⁶ However, it is unclear how alcohol causes the acceleration of HCC development in HCV infection.

How does HCV contribute to hepatocarcinogenesis?

How HCV is involved in hepatocarcinogenesis is not yet clear, despite the fact that nearly 80% of patients with HCC in Japan are persistently infected with HCV.^{1,6,7} HCV infection is also common in patients with HCC in other countries, albeit to a lesser extent. These lines of evidence force us to determine the role of HCV in hepatocarcinogenesis. Inflammation induced by HCV should be considered in a study on hepatocarcinogenesis in hepatitis viral infection: necrosis of hepatocytes due to chronic inflammation followed by regeneration enhances genetic aberrations in host cells, the accumulation of which culminates in HCC. This theory presupposes an indirect involvement of hepatitis viruses in HCC via hepatic inflammation. However, this context leaves us with a serious question: can inflammation alone result in the development of HCC in such a high incidence or is there a multicentric nature in HCV infection?

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

Transgenic mouse studies revealed an *in vivo* oncogenic activity of HCV core protein

Transgenic mouse lines with parts of the HCV genome were engineered by introducing the genes from cDNA of the HCV genome of genotype 1b.^{8,9} Three different transgenic mouse lines were established, which carry the core gene, envelope genes or non-structural genes (Fig. 1), respectively, under the same transcriptional control element. Among these mouse lines, only the transgenic mice carrying the core gene develop HCC in two independent lineages.⁹ The envelope gene transgenic mice do not develop HCC, despite high expression levels of both E1 and E2 proteins.^{10,11} The transgenic mice carrying the entire non-structural genes have not developed HCC.

The transgenic mice carrying the core gene express the core protein of an expected size, and the intrahepatic level of the core protein is similar to that in the liver of chronic hepatitis C patients. Early in life, these mice develop hepatic steatosis, which is one of the histological characteristics of chronic hepatitis C, along with lymphoid follicle formation and bile duct damage.¹² Thus, the core gene transgenic mouse model well reproduces the feature of chronic hepatitis C. Of note, any pictures of significant inflammation are not observed in the liver of this animal model. Late in life, these transgenic mice develop HCC. Notably, the development of steatosis and HCC has been reproduced by other HCV transgenic mouse lines, which harbor the entire HCV genome or structural

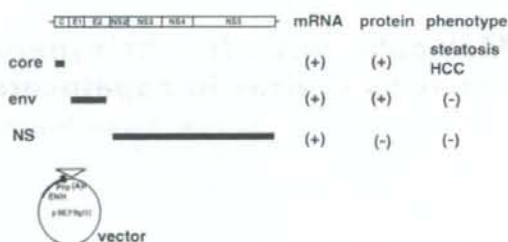


Figure 1 Transgenic mouse lines carrying the hepatitis C virus (HCV) genome. Three different types of transgenic mouse lines, carrying the core gene, envelope genes or non-structural genes of HCV, respectively, were established under the control of the same regulatory elements. Among these mouse strains,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

genes including the core gene.^{13–15} These outcomes indicate that the core protein per se of HCV has an oncogenic potential when expressed *in vivo*.

Oxidative stress overproduction and MAPK activation as consequences to the core protein expression in the liver

It is difficult to determine the mechanism of carcinogenesis even for our simple model in which only the core protein is expressed in otherwise normal liver tissues. There is a notable feature in the localization of the core protein in hepatocytes: while the core protein predominantly exists in the cytoplasm associated with lipid droplets, it is also present in the mitochondria and nuclei.^{9,16} On the basis of this finding, the pathways related to these two organelles, the mitochondria and nuclei, were meticulously analyzed.

One activity of the core protein is an increased production of oxidative stress in the liver. We would like to draw particular attention to the fact that the production of oxidative stress is increased in our transgenic mouse model in the absence of inflammation in the liver (hepatitis). This reflects a state of overproduction of reactive oxygen species (ROS) in the liver, or predisposition to it, which is staged by the HCV core protein without any intervening inflammation.^{17,18} The overproduction of oxidative stress results in the generation of deletions in the mitochondrial DNA, an indicator of genetic damage. In addition, analysis of the anti-oxidant system revealed that some anti-oxidative molecules are not increased despite the overproduction of ROS in the liver of core gene transgenic mice; hemoxygenase-1 and glutathione peroxidase are not augmented whereas catalase and glutathione *S*-transferase levels are increased and enhanced by iron overloading (S Shinzawa *et al.*, unpubl. data, 2007). These results suggest that HCV core protein not only induces overproduction of ROS but also attenuates some of the anti-oxidant system, which may explain the mechanism underlying

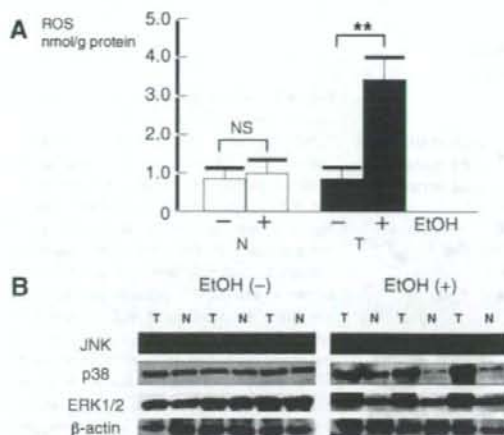


Figure 2 Alcohol administration enhances oxidative stress production and mitogen-activated protein kinase (MAPK) pathway activation in a synergistic fashion with hepatitis C virus (HCV) core protein. Administration of 5% alcohol for 3 weeks provoked an induction of reactive oxygen species (ROS) in HCV core gene transgenic mice, whereas it induced only a marginal increase in control mice, showing a synergy between the HCV core protein and ethanol in inducing ROS. Only the c-Jun N-terminal kinase (JNK) pathway is activated in the core gene transgenic mice before hepatocellular carcinoma (HCC) development, but feeding 5% alcohol for 3 weeks activated the other two pathways, p38 and ERK1/2, which was not observed in control mice. Thus, combining the effect of ethanol to that of the core protein resulted in the activation of all the MAPK pathways, among which only JNK was activated by the action of HCV core protein only in the absence of ethanol. ERK, extracellular signal-regulated kinase; EtOH, ethanol; N, non-transgenic control mouse; NS, statistically not significant; T, transgenic mouse. ** $P < 0.01$.

ing the production of a strong oxidative stress in HCV infection compared to other forms of hepatitis.

Thus, the core protein induces oxidative stress overproduction in the absence of inflammation, which may, at least in part, contribute to hepatocarcinogenesis in HCV infection. If inflammation were added to the liver with the HCV core protein, the production of oxidative stress would be escalated to an extent that cannot be scavenged any longer by a physiological antagonistic system. This indicates that the inflammation in chronic HCV infection would have a characteristic difference from those of other types of hepatitis, such as autoimmune hepatitis. The basis for the overproduction of oxidative stress may be ascribed to mitochondrial dysfunction.^{16,17} The dysfunction of the electron transfer system of the mitochondrion is suggested in association with the presence of the HCV core protein.¹⁸ Hepatic steatosis in hepatitis C, which is also attributed to the action of the core protein,⁹ may work as fuel for oxidative stress overproduction.^{18,20,21}

Other possible pathways would be the alteration of the expression of cellular genes, interacting with cellular proteins, and modulation of intracellular signaling pathways. For example,

tumor necrosis factor (TNF)- α and interleukin-1 β have been found transcriptionally activated.²² The core protein has also been found to interact with some cellular proteins, such as retinoid X receptor (RXR)- α , that play pivotal roles in cell proliferation and lipid metabolism.²³ The mitogen-activated protein kinase (MAPK) cascade is also activated in the liver of the core gene transgenic mouse model. The MAPK pathway, which consists of three routes, c-Jun N-terminal kinase (JNK), p38 and extracellular signal-regulated kinase (ERK), is involved in numerous cellular events including cell proliferation. In the liver of the core gene transgenic mouse model prior to HCC development, only the JNK route is activated. Downstream of the JNK activation, transcription factor activating protein (AP)-1 activation is markedly enhanced.^{22,24} Far downstream, both the mRNA and protein levels of cyclin D1 and CDK4 are increased. Thus, the HCV core protein modulates the intracellular signaling pathways and confers an advantage for cell proliferation to hepatocytes. Interestingly, we found recently that a protein interacting with the core protein, proteasome activator 28 γ (PA28 γ), is indispensable for the core protein to exert its function for the development of steatosis, insulin resistance and HCC.^{25,26}

Such an effect of the core protein on the MAPK pathway, in combination with that on oxidative stress, may explain the extremely high incidence of HCC development in chronic hepatitis C.

Molecular basis for the synergy between alcohol and HCV infection in hepatocarcinogenesis

As described above, the production of oxidative stress is increased in the liver of aged HCV core gene transgenic mice in the absence of inflammation. In young mice, the increase in oxidative stress is apparently marginal. However, feeding 5% ethanol to mice for 3 weeks induced ROS in the liver of core gene transgenic mice, whereas it induced only a minimal increase in control mice, demonstrating a synergy between the core protein and ethanol in inducing ROS (Fig. 2a).¹⁷ In contrast, only the JNK pathway is activated in the core gene transgenic mice before HCC development, but feeding 5% ethanol for 3 weeks activated the other two MAPK pathways, p38 and ERK1/2 in the core gene transgenic mice, the activation of which is not present in control mice (Fig. 2b). Thus, combining the effect of ethanol to that of the core protein provoked the activation of all the MAPK pathways, affording advantage to cell proliferation.²⁴

In a long-term observation experiment, feeding 2% ethanol to the core gene transgenic mice for 9 months resulted in the acceleration of HCC development (Moriya K *et al.*, unpubl. data, 2007). Screening by the high-throughput immunoblot analysis revealed differential expression of proteins in the liver with or without ethanol feeding; some proteins, the levels of which were either increased or decreased by the effect of the core protein, such as Rho GTPase activating protein (GAP) or caspase-8, are down- or upregulated by the effect of ethanol feeding.

In summary, we postulate that the induction of oxidative stress, together with the activation of MAPK cascade, followed by AP-1 activation and cyclin D1 overexpression, plays a pivotal role in the development of HCC (Fig. 3). Alterations in cellular gene expressions, such as TNF- α or suppressor of cytokine signaling-1, and the

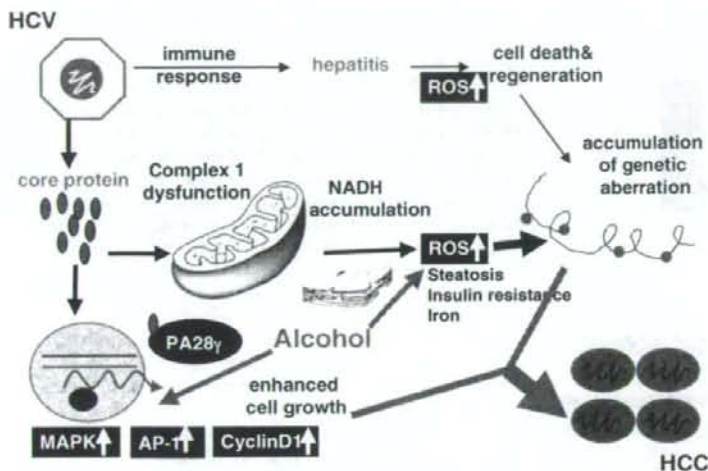


Figure 3 Molecular pathogenesis of hepatocellular carcinoma (HCC) development in hepatitis C virus (HCV) infection in association with alcohol. We postulate that induction of oxidative stress through the dysfunction in the mitochondrial electron transfer system, together with alterations in cellular gene expressions and the intracellular signaling pathways, including the mitogen-activated protein kinase (MAPK) cascade, play a pivotal role in the development of HCC. Alcohol activates both of these pathways and augments the development of HCC in HCV infection. AP-1, activating protein-1; NADH, nicotinamide adenine dinucleotide; PA28 γ , proteasome activator 28 γ ; ROS, reactive oxygen species; SOCS-1, suppressor of cytokine signaling-1; TNF- α , tumor necrosis factor- α .

presence of steatosis and insulin resistance are co-accelerators to hepatocarcinogenesis in HCV infection. Finally, alcohol augments both of these pathways that are activated by the core protein, and further enhance the development of HCC in HCV infection (Fig. 3).

Conflict of interest

No conflict of interest has been declared by the authors.

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