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REVIEW ARTICLE

Experimental mouse models for hepatocellular carcinoma research

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Summary

Every year almost 500,000 new patients are diagnosed with hepatocellular carcinoma (HCC), a primary malignancy of the liver that is associated with a poor prognosis. Numerous experimental models have been developed to define the pathogenesis of HCC and to test novel drug candidates. This review analyses several mouse models useful for HCC research and points out their advantages and weaknesses. Chemically induced HCC mice models mimic the injury-fibrosis-malignancy cycle by administration of a genotoxic compound alone or, if necessary, followed by a promoting agent. Xenograft models develop HCC by implanting hepatoma cell lines in mice, either ectopically or orthotopically; these models are suitable for drug screening, although extrapolation should be considered with caution as multiple cell lines must always be used. The hollow fibre assay offers a solution for limiting the number of test animals in xenograft research because of the ability for implanting multiple cell lines in one mouse. There is also a broad range of genetically modified mice engineered to investigate the pathophysiology of HCC. Transgenic mice expressing viral genes, oncogenes and/or growth factors allow the identification of pathways involved in hepatocarcinogenesis.

Keywords
Hepatocellular carcinoma, mouse models, hepatology, xenograft, hollow fibre assay, transgenic mice

Hepatocellular carcinoma (HCC) is the most common primary malignancy of the liver. Worldwide, approximately 500,000 new patients are diagnosed with HCC each year, often associated with poor prognosis (Motola-Kuba *et al.* 2006). It is the fifth most frequent neoplasm and ranks third on the list of most lethal cancers (Parkin *et al.* 2001). The highest incidence of HCC is found in Asia and sub-Saharan Africa. Most cases of HCC develop in a background of chronic liver damage such as cirrhosis or hepatitis (Tsukuma *et al.* 1993).

Hepatocarcinogenesis is a multistep process involving different genetic alterations that ultimately lead to malignant transformation of the hepatocytes (Farber 1984). Several rodent models have been used in defining the pathogenesis of HCC and have contributed to the current knowledge of HCC. Because of the physiologic and genetic similarities between rodents and humans, the short lifespan and the breeding capacity, rodents are often used for cancer research. Many chemically induced experiments have been conducted on rats (*Rattus norvegicus*), but mice

(*Mus musculus*) are also a favourite model for cancer because of the availability of gene targeting methods and the possibility of xenograft implantation. Woodchuck or groundhog (*Marmota monax*) is often used for studies concerning hepatitis B infection (HBV) induced HCC (Tennant *et al.* 2004; Cullen *et al.* 2008; Lu *et al.* 2008). The woodchuck hepatitis causes liver inflammation, injury and repair process similar to those in HBV-patients (Gudima *et al.* 2008).

This review focuses only on the available mouse models for HCC research, because of the existence of a broad range of (i) chemically induced models, (ii) xenograft models and (iii) genetically modified models, in mice. The advantages and disadvantages of the three types of experimental mice models will be discussed in this review.

Chemically induced models

Several chemical reagents induce tumour formation when administered in sufficient high doses and time span (Table 1). There are two types of carcinogenic compounds: (i) genotoxic compounds which are characterized by their capacity to induce structural DNA changes and (ii) promoting compounds which lack direct genotoxic capability, but enhance tumour formation after initiation by a hepatotoxic compound (Pitot & Dragan 1991). Treatment with a tumour-promoting agent facilitates the clonal expansion of the preneoplastic cells.

The advantage of chemically induced models is the similarity with the injury-fibrosis-malignancy cycle seen in humans. This makes them the favourite models for HCC research.

Table 1 Summary of chemically induced HCC-models

	Time to develop tumours	% of mice with HCC	Metastasis	Remarks	References
DEN					
Single administration	45–104 weeks (dose dependent)	80–100% in male	/	Poorly reproducible	Chen *et al.* (1993); Frey *et al.* (2000); Hacker *et al.* (1991); Park *et al.* (2008); Teoh *et al.* (2008); Zimmers *et al.* (2008)
Short-term administration	40–60 weeks	100% in male	/		Shiota *et al.* (1999)
Long-term administration	20–35 weeks	100% in male 30% in female	Yes	Very agressive tumours (H-ras mutations)	Finnberg *et al.* (2004)
+ PB	20–40 weeks		/	B-catenin activation	Goldsworthy and Fransson-Steen (2002); Klaunig *et al.* (1988); Weghorst and Klaunig (1989)
+ PH	4–8 weeks (in rats)		/		Farber *et al.* (1977); Klinman and Erslev (1963)
Peroxisome proliferators	50–100 weeks (dose dependent)	Depends on strain, dose and PP-agent	Yes	PP tumorigenicity in humans not known	Hays *et al.* (2005); Reddy *et al.* (1976); Takashima *et al.* (2008)
Aflatoxine	Early HCC in 52 weeks high grade HCC 92–110 weeks	Considerable interstrain differences: DBA/2J: 90% C57BL/N: 25–66%	Yes	Suitable model for AFB-induced HCC in humans	McGlynn *et al.* (2003); Ghebranious and Sell (1998)
CCl4	104 weeks	50–94% (dose dependent) in male and female	Yes		Confer and Stenger (1966); Farazi *et al.* (2006); Weisburger (1977)
Choline deficient diet	50–52 weeks	100%		Steatohepatitis	de Lima *et al.* (2008); Knight *et al.* (2000); Liquori *et al.* (2009)
Thioacetamide	50–80 weeks	70–100%			Palacios *et al.* (2008)

Diethylnitrosamine

N-nitrosodiethylamine (DEN) is often used as a carcinogenic reagent. The target organ in which DEN induces malignant tumours is species specific. Mice mainly develop not only liver tumours, but also gastrointestinal (Binato *et al.* 2008), skin, respiratory (Wang *et al.* 1992) and haematopoietic tumours (Gray *et al.* 1991). The carcinogenic capacity of DEN is situated in its capability of alkylating DNA structures. In the first step, DEN is hydroxylated to α-hydroxylnitrosamine (Verna *et al.* 1996). This bioactivation step is oxygen- and NADPH-dependent and is mediated by cytochrome P450, an enzyme which has its highest activity in the centrilobular hepatocytes. After cleavage of acetaldehyde, an electrophilic ethyldiazonium ion is formed. This ethyldiazonium ion causes DNA damage by reacting with nucleophiles such as DNA-bases. DEN works in a dose-dependent manner (Williams *et al.* 2000); a single low initiation dose does not lead to the formation of neoplasms, administration of a high dose induces HCC after a period of latency. Several DNA-repair mechanisms, such as non-homologous end joining, recombinational repair, base excision repair and many more, prevent the mutagenity of DNA-adducts when DEN is administered in a single low dose (Pegg 1990; Teoh *et al.* 2008).

Furthermore, oxidative stress caused by DEN can contribute to hepatocarcinogenesis (Kolaja & Klaunig 1997; Qi *et al.* 2008). Reactive oxygen species (ROS) generated by the P450-dependent enzymatic system might induce oxidative stress by the formation of hydrogen peroxide and superoxide anions. Production of ROS is known to cause DNA, protein and lipid damage; therefore, oxidative stress can play an important role in carcinogenesis (Kawanishi *et al.* 2002; Valko *et al.* 2006).

Mouse tumours induced by DEN harbour activating mutations in the *H-ras* proto-oncogene (Chen *et al.* 1993; Stahl *et al.* 2005). These mutations are rarely seen in humans and are correlated with metastasis and poor prognosis (Tada *et al.* 1990; Wang *et al.* 2001). However, Ras-activation that does not involve mutations of the ras-oncogene itself is considered a relative frequent event in hepatocarcinogenesis (Calvisi *et al.* 2006). Genetically, the DEN-model proves to be a good representation of HCC associated with poor prognosis (Lee *et al.* 2004).

The time needed after a single DEN-injection to develop HCC does not only depend on the administered dose, but also on sex, age and strain of mice (Rao & Vesselinovitch 1973). The younger the mice, the faster HCC will occur because of the high hepatocyte proliferation rates of juvenile animals (Vesselinovitch & Mihailovich 1983). After a long-term repetitive administration of DEN, HCC develops in 100% of the male mice and in 30% of females. The gender-related difference in HCC incidence is due to the inhibitory effect of estrogens and the stimulating effect of androgens on hepatocarcinogenesis (Nakatani *et al.* 2001). The methylation status of certain genes that contribute to the susceptibility to hepatocarcinogens might be one of the genetic factors that differs between tumour sensitive and tumour resistant strains (Buchmann *et al.* 1991). Tumour sensitive mice, such as C3H/HE-strain are characterized by hypermethylation in promoter regions of tumour suppressor genes after hepatocarcinogen exposure, a high degree of methylation inhibits the DNA-transcription (Bird & Wolffe 1999).

A dose-dependent formation of carcinomas after a single injection (5–90 mg/kg) of DEN in 15-day-old mice is observed after 45–104 weeks (Chen *et al.* 1993; Park *et al.* 2008). When B6C3F1 mice are exposed to a single dose of 5.0 mg/kg DEN, it takes approximately 64 weeks to develop HCC (Hacker *et al.* 1991). HCC occurs either within existing adenomas, which are visible after 24 weeks, or within the hepatocellular parenchyma. Metastasis to the lungs happens quite fast after the development of HCC (Vesselinovitch *et al.* 1978). The time and percentage of tumour development differ between strains, for example sv129 (Park *et al.* 2008; Teoh *et al.* 2008) (20 mg/kg DEN, 80–100% HCC after 30–55 weeks), C3H/HE (Frey *et al.* 2000) (90 mg/kg DEN, 100% HCC after 45–75 weeks), C57BL6/J (Zimmers *et al.* 2008) (5 mg/kg DEN, 84% HCC after 40–70 weeks). When adult mice are subjected to a short time weekly administration of DEN, it leads to a higher tumour incidence in a shorter time span. Six weeks of intraperitoneal injections (3 weeks with 75 mg/kg followed by 3 weeks with 100 mg/kg) lead to 100% tumour incidence after 52 weeks and the first tumours begin to occur after 30 weeks in male mice (Shiota *et al.* 1999). Long-term, weekly administration of 35 mg/kg DEN to mice leads to HCC after 20–35 weeks (Finnberg *et al.* 2004).

A two-stage model in which the initiation by a genotoxic compound is followed by a promotion phase is often used for inducing HCC. DEN can be used as an initiator and phenobarbital (PB) as a promoting agent. Several mechanisms might be responsible for the tumour promoting effect of PB. First, PB can increase the expression of cytochrome P450 a 100-fold, leading to an enhanced effect of DEN (Waxman & Azaroff 1992). Second, the increased cytochrome P450 activity induces oxidative stress (Imaoka *et al.* 2004). Third, PB can cause hypermethylation in promoter regions of tumour suppressor genes, therefore, enhancing hepatocarcinogenesis (Watson & Goodman 2002). Fourth, PB also interferes with the intracellular communication and might influence cell proliferation. Promotion with PB leads

to tumours that exhibit mutations in the β-catenin proto-oncogene (Aydinlik *et al.* 2001; Loeppen *et al.* 2002). Mutant nuclear β-catenin expression is associated with non-invasive tumours and a significantly higher 5-year survival rate in humans (Hsu *et al.* 2000; Mao *et al.* 2001).

The effects of PB promotion on DEN-initiated mice also vary considerably depending upon strain, sex and age of the mice. While the tumour promoting activity of PB is generally accepted a tumour inhibiting effect is seen when juvenile (15-days-old) $B6C3F_1$ mice are treated with DEN followed by a long-term (36 weeks) exposure to PB (Diwan *et al.* 1984; Lee *et al.* 1998). The inhibitory effect is not seen in juvenile female $B6C3F_1$ mice (Weghorst & Klaunig 1989) or other mice strains, such as Balb/c (Klaunig *et al.* 1988) and C3H (Goldsworthy & Fransson-Steen 2002), initiated with DEN promoted with PB.

Timing of initiation with DEN is a critical determinant for the paradoxical effect of PB (Lee *et al.* 1998). DEN initiation alone leads to the formation of lesions that express the Bcl-2 gene (Bcl-2^+), regardless of the moment of initiation. When adult male $B6C3F_1$ mice are initiated with DEN between 6 and 10 weeks of age followed by exposure to PB in drinking water for 36 weeks, PB serves as a tumour promoting agent. Ninety per cent of the tumours consists of eosinophilic lesions that lack the Bcl-2 expression (Bcl-2^-), indicating that PB offers a selective growth advantage for the latter. When juvenile male $B6C3F_1$ mice are initiated with DEN at 15 days of age and submitted to long-term PB exposure, PB serves as a tumour inhibiting agent. Only 4% of the lesions are Bcl-2^-, even after PB promotion (which normally suppresses the proliferation of Bcl-2^+ tumour cells and promotes the proliferation of Bcl-2^- cells). The decrease in the number of tumours is explained by the lack of Bcl-2^- lesions in juvenile mice and the growth-inhibiting effect of PB on the Bcl-2^+ lesions. Juvenile livers are less differentiated because hepatocytes actively proliferate in juvenile mice (Kanamura *et al.* 1990). These qualitative differences between infant and adult hepatocytes might be the reason for the age-dependent initiation properties.

There is also a gender-related difference in the response to PB promotion (Weghorst & Klaunig 1989). Female mice initiated with DEN at 15 days of age followed by long-term exposure to 0.05% PB show an increase of carcinomas compared with the DEN-only group, instead of the decrease that is seen in male mice.

When male BALB/c mice are initiated with DEN, a long-term treatment of PB promotes the development of liver tumours, regardless of the age of the mice during DEN administration. Genetic differences between strains of mice are likely to contribute to the variational response to PB (Goldsworthy & Fransson-Steen 2002). Because PB induces an increase in methylation of GC-rich region, genetic differences in the methylation status might be one of the causes of the differential response to PB (Counts & Goodman 1995; Watson & Goodman 2002).

Another two-step hepatocarcinogenesis model is known as the Solt-Farber protocol (Farber *et al.* 1977). In this model, initiation by a hepatocarcinogenic compound is followed by a partial hepatectomy (PH). Partial hepatectomy induces hepatic cell proliferation of the liver, leading to a fast expansion of the initiated cells (Klinman & Erslev 1963). There is a fast occurrence of altered hepatic foci and visible nodules after PH. Unfortunately, PH is a difficult procedure in mice associated with high mortality and has been conducted mostly in rats.

Peroxisome proliferators

Peroxisome proliferators (PPs) induce hepatomegaly, peroxisome proliferation in hepatocytes and induction of several hepatic enzymes. The development of HCC in mice fed a diet containing a PP was first reported in 1976 (Reddy *et al.* 1976). Currently it is widely accepted that all PPs are capable of inducing HCC after a latency period as a response to a long-term repetitive exposure to these xenobiotics. Methyl clofenapate (Lefevre *et al.* 1994), ciprofibrate (Ledda-Columbano *et al.* 2003; Calfee-Mason *et al.* 2008), fenofibrate (Nishimura *et al.* 2007), clofibrate (Nagini & Nagarajan 1988; Keller *et al.* 1990) and Wy-16,643 (Iida *et al.* 2003) are some examples of peroxisome proliferators that induce liver tumours in rodents (Rao & Reddy 1996). PPs activate the peroxisome proliferator activated receptor α (PPARα) (Hasmall *et al.* 2000). PPARα is a receptor protein that regulates the expression of several genes, including those involved in cell proliferation and apoptosis (Roberts *et al.* 1995; Zhao *et al.* 2007). It is essential for regulating lipid homeostasis and is known to mediate hepatocarcinogenesis (Peters *et al.* 1997; Hays *et al.* 2005). Most of the hepatic tumours induced by PPs are well-defined HCCs characterized by a trabecular histological pattern. In 20 to 40% of the cases metastasis occurs. One of the mechanisms of the carcinogenic effect of PPs is the induction of gene mutations due to an increased intracellular H_2O_2 concentration, because PPs induce transcriptional activation of peroxisomal oxidases, the foremost source of ROS in the liver (Yeldandi *et al.* 2000). In addition, PP inhibits apoptosis of hepatocytes and stimulates hepatocyte growth; exposure to PP, therefore, leads to hyperplasia and hepatomegaly (Ma *et al.* 1997). The dual ability of PPs to induce both cellular proliferation and oxidative stress results in cell transformation and cancer.

When sv129 mice were given a diet containing 0.01 or 0.05% di (2-ethylhexyl) phthalate (DEHP) for 100 weeks, all mice developed hepatic tumours (Takashima *et al.* 2008). After 52 weeks treatment with 0.5% benzafibrate in the diet, the average liver weight is approximately four times higher than in controls (Hays *et al.* 2005). All mice have grossly visible lesions which imply the presence of adenomas or carcinomas. The same exposure does not induce HCC in PPARα knock out mice, neither does a 1-year dietary supply of benzafibrate lead to hepatic carcinomas or adenomas in C57BL/N-mice, even though preneoplastic foci are found in 75% of the mice (Hays *et al.* 2005).

It is not known if a long-term exposure to PPs known to be carcinogenic for rodents represents a hazard to humans. When PPARα humanized mice are treated with the experimental PP Wy 16,643 on a long-term basis, there seems to be no hepatocarcinogenic effect (Cheung *et al.* 2004; Morimura *et al.* 2006). Extrapolation to the human model should be considered with caution because the PP induced hepatocarcinogenesis might be a species-specific process and PP models do not have much in common with human HCCs from the genetic point of view.

Aflatoxin B_1

The hepatotoxin aflatoxin B_1 (AFB), produced by certain fungi of the *Aspergillus* genus such as *Asparagillus flavum*, is known to be a hepatic carcinogen. These fungi thrive in corn, rice and peanuts stored in moist conditions. In China and Western Africa, the combined high prevalence of AFB and HBV contributes to the high rates of HCC in these regions (Autrup & Wakhisi 1988; Wang & Liu 2007). Experimental models involving AFB administration allow research of the mechanisms involved in AFB-induced hepatocarcinogenesis. AFB is metabolized by the liver microsomal system to the exo-8,9-epoxide intermediate that binds selectively to guanine residues in cellular DNA (Gallagher *et al.* 1994). Cytochrome P450 is essential in this process (Ramsdell *et al.* 1991). Exo-8,9-epoxide binds selectively to guanine, transforming it to thymine, hence causing DNA mutations. AFB induces chromosomal aberrations, sister chromatide exchange, chromosomal strand breaks, DNA-adducts, micronuclei and uncontrolled DNA synthesis (Wang & Groopman 1999).

When 7-day-old mice are exposed to 6 mg/kg body weight of AFB, in a bolus injection, HCC is developed after 52 weeks (McGlynn *et al.* 2003). In 90% of the DBA/2J mice (susceptible strain for HCC) HCC occurs after AFB exposure, while only 25% of the C57BL/N mice (relatively resistant strain for HCC) develop HCC after AFB exposure. The difference in susceptibility is a result of differences in several AFB detoxification loci. Ghebranious *et al.* injected C57BL/N mice with 10 mg/kg of AFB and had tumours in 66% of the male mice after 52 weeks, while high grade HCC was not developed until 92–110 weeks of age (Ghebranious & Sell 1998).

Carbon tetrachloride

Carbon tetrachloride (CCl_4) is one of the most potent hepatotoxins (Weisburger 1977). The hepatotoxicity of CCl_4 involves two phases (Avasarala *et al.* 2006). First, CCl_4 is metabolized by cytochrome P450 to form trichloromethyl radicals (Sheweita *et al.* 2001). These radicals are highly energetic and cause lipid peroxidation and membrane damage. Second, the Kupffer cells induce an inflammatory response, which leads to the secretion of cytokines, chemokines and other proinflammatory factors. These factors not only have a direct cytotoxic effect, but also attract and activate monocytes, neutrophils and lymphocytes which contribute to the tissue damage. The repeated cycle of injury, inflammation and repair leads to fibrosis and eventually HCC. Weekly injections of CCl_4 accompanied with alcohol administration through drinking water lead to HCC after 104 weeks (Confer & Stenger 1966; Weisburger 1977; Farazi *et al.* 2006).

Choline deficient diet

Mice subjected to a long-term choline deficient diet (CDD) develop tumours after 50–52 weeks (Knight *et al.* 2000). A CDD induces steatosis in all mice, nevertheless fat accumulation can differ considerably between species and is not necessarily strain-dependent (Knight *et al.* 2000; Liquori *et al.* 2009). The proposed mechanism of carcinogenicity by CDD is through the formation of oval cells (Tarsetti *et al.* 1993), a result of oxidative DNA damage and chromosomal instability because of the depletion of hepatic antioxidant mechanisms. It has been assumed that oval cells, either directly or indirectly through the generation of hepatocytes, function as tumour progenitors. CDD can be combined with the administration of a hepatotoxic compound, such as DEN or CCL4, and serves as a good model for steatohepatitis with further development to HCC (de Lima *et al.* 2008).

Thioacetamide

Thioacetamide (TAA) is a hepatotoxin that can be administered either in drinking water (0.02–0.05%) or by intraperitoneal injections. Repeated administration leads to fibrosis

in mice over a period of 10–15 weeks (Palacios *et al.* 2008). The hepatotoxic action is a result of oxidation properties of the compound, leading to hepatic oxidative stress and liver damage.

Xenograft models

In xenograft models, the tumours are formed by injecting human cancer cells from a lab culture in immune deficient mice. Athymic (nude) or severe combined immune deficient (SCID) mice are often used as hosts. Tumour xenografts can be established either by direct implantation of biopsy material or by inoculation of human tumour cell lines. Several kinds of xenograft models can be distinguished (Table 2). First, the ectopic model, in which human tumour cells are injected subcutaneously in the flank of mice. Second, the orthotopic model, where tumour cells are injected intrahepatically into the mice. The orthotopic xenograft model is more suitable for extrapolation to humans and gives information about the metastatic spread of the tumour. The advantage of xenograft mouse models is the short time span needed for the development of tumours and the fact that it is an efficient way to demonstrate proof-of-principle when enough cell lines are used. This technique provides a model that allows investigation of aspects as *in vivo* toxicity, absorption and pharmacokinetics of a compound in a preclinical trial. Tumour phenotypes can vary remarkably between lines; it is therefore important to use different cell lines when using the xenograft model.

Human tumour progression is a complicated process in which the interaction of neoplastic cells and the surrounding tumour environment plays an important role. A microevolutionary process takes place in which advantaged tumour cells expand rapidly, a process that is altered when using cell cultures. Therefore, the resemblance between xenograft tumours and human tumours is rather poor (Kelland 2004). Due to the heterogeneity of cell lines, multiple cell lines should be used for drug screening, which often leads to different outcomes depending on the cell line's phenotype. Cell lines are often used for chemotherapeutic drug screening with common chemotherapeutic agents. Significant differences in tumour growth inhibition were noted between cell lines and discrepancies between previous studies were found. For example, while some studies have shown that EGFR-inhibitor inhibits tumour growth and intrahepatic metastasis by approximately 50% (Matsuo *et al.* 2003), this is not seen in either of the seven cell lines that was used by Huynh (Huynh *et al.* 2006). The lack of predictability of results obtained from xenograft mice models has convinced many researchers to use models in which tumours arise in a background that resembles the natural history of HCC.

An interesting setup consists of orthotopic implantations of HCC cells in fibrotic livers (Kornek *et al.* 2008). Fibrosis is established by intraperitoneal injection of thioacetamide or subcutaneous injection of CCL_4 and oral alcohol intake. Tumours in fibrotic livers not only grow significantly larger and more rapidly than those in normal liver, but also have the

Table 2 Summary of xenograft models

	Pro	Contra	Use in research
Ectopic implantation	Fast occurrence of tumours (5–20 weeks) Easy to perform	Considerable differences between cell lines, multiple cell lines need to be tested No direct interaction with liver tissue Weakly extrapolatable to humans Complex tumour–host interactions (metastasis, angiogenesis, etc.) cannot be tested	Proof-of-principle Drug screening
Orthopic implantation	Fast occurrence of tumours Possibility to test in fibrotic livers Complex tumour–host interactions can be tested	Difficult procedure Considerable differences between cell lines, multiple cell lines need to be tested	Proof-of-principle Drug screening Tumour–host interactions
Hollow fibre model	Fast results (1–2 weeks) Ability for multiplexing reduces the amount of test animals needed Minimal effect on animal welfare Retrieval of tumour cells after experiment for subsequent analysis Angiogenesis can be semi-quantified	No direct interaction with liver tissue Complex tumour–host interactions such as metastasis can not be tested Occurrence of neovascularization around fibres influences results on prolonged periods	Proof-of-principle Drug screening

capacity to metastasize and form satellite nodules. This model provides a useful tool for testing drug efficacy in orthotopic xenografts within the context of liver fibrosis, but there is also variable responsiveness to drugs between cell lines.

Metastatic HCC xenograft models are used for the research on the mechanism of metastasis and relapse. By orthotopic implantation of preserved metastatic tumour tissues of 30 surgical specimens, the first highly metastatic model of HCC (LCI-D20) in nude mice was developed (Sun *et al.* 1996). Orthotopic implantation of LCI-D20 leads to highly metastatic HCC that exhibits several characteristics of human tumour behaviour and the tumour tissue histologically resembles human HCC. HBV-DNA has been integrated in the cellular DNA of the LCI-D20 tumour cells. Metastasis to the lungs occurs in 100% of the mice 15 days after orthotopic inoculation.

Genetically comparing these highly metastatic cell lines to less metastatic cell lines, such as LCI-D35, leads to the identification of several genes involved in the invasive potential of tumours (Shao *et al.* 1999).

To limit the number of mice for drug screening, a low-cost, rapid and efficient method was developed, called the hollow fibre assay (HFA) (Hollingshead *et al.* 1995). This assay involves placing cells from tumour cell lines into small semi-permeable tubes (Figure 1). Tumour cells are inoculated into hollow (1 mm internal diameter) polyvinylidene fluoride fibres which are heat-sealed and cut at 2 cm intervals (Decker *et al.* 2004; Suggitt *et al.* 2006). The fibres are cultured for 24–48 h *in vitro*. Multiple fibres can be implanted subcutaneously or intraperitoneally in athymic mice; therefore, using only one mouse to test several cell lines. In comparison with the traditional tumour xenograft model, the HFA has some important advantages. The ability for multiplexing reduces the number of laboratory animals; this combined with the shorter evaluation time and reduced consumption of test agents makes it an economically interesting method (Shnyder *et al.* 2006). Li *et al.* used HFA to test the therapeutic efficacy of tripeptide tyroserleutide (YSL) in five different human HCC-cell lines (Li *et al.* 2008). The mice were implanted with three hollow fibres containing BEL-7402, SMMC-7721 and Hep3B or two hollow fibres with HepG2 and SK-HEP-1, and were treated with YSL. Because of this multiplex setup, only 30 mice were needed instead of 150 mice if the same experiment was conducted in a classic xenograft model. Other advantages are that the HFA has minimal influence on the animal welfare and it allows retrieval of tumour cells uncontaminated by host cells, which can be used for subsequent analysis. Retrieval of the tumour cells leads to the possibility of using several optical imaging methods to quantify the effect of treatment *ex vivo*. Tumour cell lines can be genetically engineered with constitutive bioluminescent (i.e. luciferase) or fluorescent (i.e. green fluorescent protein) reporter vectors, which allows *in vivo* or *ex vivo* optical imaging (Zhang *et al.* 2007). Although HFA is not suitable for studying complex host–tumour interactions (Hollingshead *et al.* 1995), it is possible to study angiogenesis (Phillips *et al.* 1998). Angiogenic activity around the tumour is measured after 6 days and it takes about 1 month to develop an extensive vascular network towards the tumour (Zhang *et al.* 2007). The presence of a vascular supply to the hollow fibres has a significant influence on drug delivery and chemo sensitivity, which should be taken into account when using the HFA over a prolonged period (Phillips *et al.* 1998). Therefore the HFA is only suitable for experiments that acquire a limited time span, which restricts the usefulness for long-term toxicity studies. In addition, it is not possible to assess data regarding the tumour size, an important factor for the evaluation of chemotherapeutic compounds. Only the effect of the drug on a restricted amount of cells can be examined, leading to a reduced inter-cell and cell-host interaction. Furthermore, the small quantity of cells does not resemble the physiology found in HCC. All these factors should be taken into account when using the HFA technique.

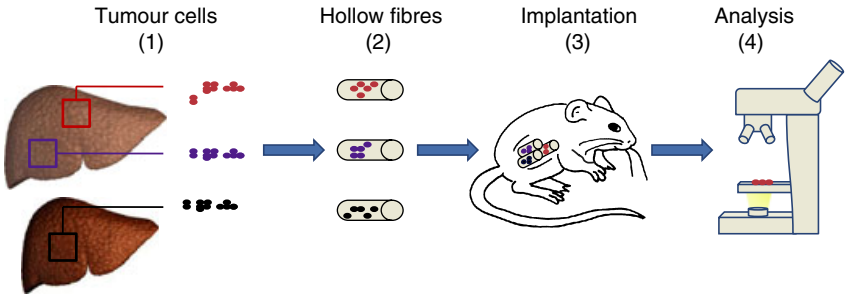

Figure 1 Tumour cells derived from human (or mouse) hepatic tumours (1) are placed in small semi-permeable tubes known as hollow fibres (2). The fibres are cultured for 24-48 hours *in vitro* before subcutaneous or intraperitoneal implantation in nude mice. The cells can be retrieved after the *in vivo* assay and used for subsequent analysis (4).

Genetically modified models

Genetically modified mouse models (GMM) are engineered to mimic pathophysiological and molecular features of HCC (Frese & Tuveson 2007). It is a unique model for assessing the effects of oncogenes either alone or in combination with other oncogenes or carcinogenic agents. GMMs facilitate detailed investigation of carcinogenic pathways and allow the assessment of pathway cooperativity and dependency *in vivo* (Tuveson & Jacks 2002). Tissue-specific expression can be achieved by designing cDNA-constructs that contain promoter elements that are restricted to certain tissues. Although the effect of the mutation is constitutive, its expression is limited by the use of tissue-specific promoters. For hepatic GMMs the albumin promoter is often used. An alternative to the constitutive tissue-specific expression is the induction of specific genes with molecules. This approach allows research towards the role of oncogenes in tumour maintenance and the influence of age on the carcinogenesis.

Because of the large number of transgenic mice, only a small selection of representative models for HCC research will be discussed in this review (Table 3).

Transgenic models expressing viral genes

Hepatitis B virus. The main cause of HCC worldwide is the hepatitis B virus (HBV). HBV is an enveloped hepatotrophic DNA virus causing several liver diseases such as acute to chronic hepatitis, cirrhosis and HCC. During prolonged infection, viral DNA sequences integrate into the host genome, causing mutations, chromosomal instability and general genomic rearrangements. The genome of HBV is a circular, partially double-stranded DNA molecule and it is characterized by its four overlapping open reading frames (ORF) that encode for surface (S), core (C), polymerase (P) and X proteins (HBx). There is increasing evidence that the expression of viral genes, in particular genes encoding for HBx, might deregulate the control of cellular growth and viability and sensitize hepatocytes to exogenous and endogenous carcinogens (Wu *et al.* 2001). The oncogenic capacity of HBx is a result of the fact that some of the genes activated by HBx, such as ICAM-1 (Hu *et al.* 1992), c-myc (Terradillos *et al.* 1997) and c-fos (Avantaggiati *et al.* 1993), are important for cell adhesion and proliferation.

Many aspects of HBV-induced hepatocarcinogenesis have been analysed by generating transgenic mice expressing complete fragments of the HBV genome, under control of either the HBV promoter, or constitutive (mouse albumin) or inducible (mouse metallothionein) liver-specific promoters. The first HBV-related transgenic mice model was produced in 1985 by two independent research groups (Babinet *et al.* 1985; Chisari *et al.* 1985).

Most of the HBV-related transgenic animals express the HBx genes, which are associated with altered hepatocellular functions and HCC development (Burk *et al.* 1988; Araki *et al.* 1989; Xiong *et al.* 2003). The livers of HBx transgenic mice exhibit megalocytosis, nuclear pleomorphism, hyperchromatism and a slightly increased nuclear/cytoplasmic ratio after approximately 15 weeks (Lakhtakia *et al.* 2003; Koo *et al.* 2005). The birthrate of the HBx transgenic mice is a bit lower than that of other transgenic mice, indicating an interaction with the prenatal development of mice (Xiong *et al.* 2003). The progression to HCC takes approximately 52–104 weeks (Takada *et al.* 1995; Koo *et al.* 2005). Transgenic lineages with lower HBx copy number showed lower tumour incidence comparable with that of wild-type mice (Koike *et al.* 1994), while other experiments did not encounter any tumours in HBx transgenic mice (Lee *et al.* 1990; Zheng *et al.* 2007). HBx transgenic mice seem to be more sensitive for HCC development after a single DEN-injection when compared with their non-transgenic counterparts (Sell *et al.* 1991; Zhu *et al.* 2004; Zheng *et al.* 2007).

Hepatitis C virus. Viral Hepatitis C (HCV) infection is an important risk factor for HCC (Gerber 1993; ElRefaie *et al.* 1996). Chronic HCV can cause cirrhosis and increases the risk of HCC by approximately 1–3%. The HCV genome is a RNA molecule of approximately 9500 nucleotides. It contains an ORF that counts 9000 nucleotides and encodes for a large glycoprotein that is prone to several post-translational modifications, leading to the final viral proteins and enzymes. A number of HCV proteins, such as core (Feng *et al.* 2007), NS3 (Deng *et al.* 2006) and NS5A (Lan *et al.* 2002) play an important role in the hepatocarcinogenesis. The proteins encoded by the HCV genome, especially core, interrupt the intracellular signal transduction pathways. Unlike HBV, the RNA genome of HCV does not integrate into the host chromosome. Therefore, HCV-related hepatocarcinogenesis is not likely to involve insertional mutagenesis. Numerous transgenic mouse models are made expressing different HCV proteins have been developed and differences occur in the development of tumours. When the complete viral protein is expressed, steatosis and HCC occur in 15% of the mice after approximately 90–100 weeks (Lerat *et al.* 2002). Other researchers did not encounter the tumour phenotype, indicating that genetic background of mice and/or oncogene expression level play an important role in the outcome of transgenic mice models (Kawamura *et al.* 1997).

The core protein interferes with the lipid metabolism by activating PPARα, leading to lipid accumulation in the

Table 3 Summary of transgenic models

	Promotor	Time to develop tumours	% of mice with HCC	Research	References
Viral genes					
HBx	X-gene promotor, mouse albumin or metallothionein	52–104 weeks +DEN: 30 weeks	70–85 85	Hepato-carcinogenesis in a background of hepatitis B or C	Araki *et al.* (1989); Burk *et al.* (1988); Koo *et al.* (2005); Lakhtakia *et al.* (2003); Sell *et al.* (1991); Takada *et al.* (1995); Xiong *et al.* (2003); Zheng *et al.* (2007)
HCV	Albumin	90–100 weeks	15		Lerat *et al.* (2002)
HCV core	HBV	80–105 weeks	32		Moriya *et al.* (1998); Tanaka *et al.* (2008)
HCV core E1-E2	Albumin	+DEN: 32 weeks	100		Kamegaya *et al.* (2005)
	HBV	60 weeks	23		Naas *et al.* (2005)
Oncogenes					
c-myc	Albumin	65–90 weeks	55	Investigation of carcinogenic pathways	Thorgeirsson and SantoniRugiu (1996)
c-myc	WHV	36 weeks	100		Merle *et al.* (2005)
c-myc + E2F1	Albumin	26–35 weeks	100	Cooperativity of oncogenes	Conner *et al.* (2003)
β-catenin + H-ras	Cre-Lox	8 weeks (early HCC) 26 weeks (high grade HCC)	100		Harada *et al.* (2004)
Growth factors					
TGF-α	Metallothionein 1	+Zinc: 40–70 weeks	50	Investigation of carcinogenic pathways	Jhappan *et al.* (1990)
ELF (TGF signalling)	Knock out	58 weeks	40–70	Cooperativity of growth factors with oncogenes	Baek *et al.* (2008)
TGF-α + c-myc	Albumin	40 weeks	100		Murakami *et al.* (1993); Ohgaki *et al.* (1996); Thorgeirsson and SantoniRugiu (1996)
	Albumin	+ Zinc: 16 weeks	100	Influence of growth factors on sensitivity for hepatocarcinogens	
EGF	Albumin	24–36 weeks	100		Borlak *et al.* (2005); Tonjes *et al.* (1995)
EGF + c-myc	Albumin	12–18 weeks	100		Tonjes *et al.* (1995);
FGF19	No liver-specific expression	52 weeks	50		Nicholes *et al.* (2002)
SV40 T-antigen	Albumin, α1 antitrypsin, serum amyloid P component or antithrombin III	4–12 weeks	100	Fast tumour occurrence due to uncontrolled DNA replication	Araki *et al.* (1991); Cullen *et al.* (1993); Sepulveda *et al.* (1989)
	Cre-Lox	20 weeks	100		Lou *et al.* (2005)
Creating tumour environment					
AAT	α1 antitrypsin	52–90 weeks	100	Effect of AAT-deficiency on the liver	Geller *et al.* (1994)
PTEN	Albumin/Cre	40–44 weeks	66 (male) 30 (female)	Effect of steatohepatitis	Watanabe *et al.* (2007)
PDGF TGF-β1	Albumin	52 weeks +AAT: >18 weeks	100		Borkham-Kamphorst *et al.* (2007);
GMNT		40 weeks	100		Campbell *et al.* (2005); Czochra *et al.* (2006); Thieringer *et al.* (2008); Schnur *et al.* (2004) Martinez-Chantar *et al.* (2008)

hepatocytes (Tanaka *et al.* 2008). The expression of HCV core leads to progressive hepatic steatosis in several lines of constitutive transgenic mice, followed by HCC after 80–105 weeks in 32% of the male mice (Moriya *et al.* 1998; Tanaka *et al.* 2008). Inducible expression of core protein by tetracycline and Dox administration leads to a peak in steatosis after 2 months but no HCC occurs (Chang *et al.* 2008). Transgenic mice expressing core, E1 and E2 structural proteins developed HCC after 60 weeks in 23% of the male mice (Naas *et al.* 2005). An accelerated tumour progression occurs after DEN injection; after merely 32 weeks HCC is found in 100% of the male mice. HCV proteins and chemical carcinogens such as DEN have a synergistic influence on HCC development, because HCV proteins blocks apoptosis in the hepatocytes, leading to an accelerated expansion of the neoplastic hepatocytes (Kamegaya *et al.* 2005).

Transgenic mice over-expressing oncogenes

An oncogene is a protein encoding gene which participates in the onset and development of cancer. Genetic alterations resulting in the activation or over-expression of oncogenes increase the chance of tumour development.

Myc protein. The Myc protein is a transcription factor that activates the expression of several genes through binding on consensus sequences and recruiting histone acetyltransferases (Figure 2) (Dang & Lewis 1997; Nilsson & Cleveland 2003). When *myc* is mutated or over-expressed, it is associated with a variety of tumours. The cooperation of myc proteins with other oncogenes and growth factors is critical in the evolution of the malignant phenotype. Transgenic mice over-expressing *c-myc* develop liver tumours after a long period of latency (35–90 weeks) (Thorgeirsson & SantoniRugiu 1996; Merle *et al.* 2005). Five to 10% of the tumours show increased β-catenin expression. Co-expression of E2F1/c-Myc accelerates liver cancer development (Conner *et al.* 2003). Myc transgenic mice are genetically close to human HCC of good prognosis (Lee *et al.* 2004).

β-catenin. β-Catenin, a subunit of the cadherin complex, plays an important role in the development and regeneration of the liver (Figure 3). β-Catenin is one of the key downstream effectors of the Wnt-signalling pathway, a network of proteins that is known for its involvement in tumour development (Schwarz *et al.* 2003; Brembeck *et al.* 2006). β-Catenin mutations are considered to be an early event in the hepatocarcinogenesis (Devereux *et al.* 1999; Laurent-Puig & Zucman-Rossi 2006). Approximately 30% of the

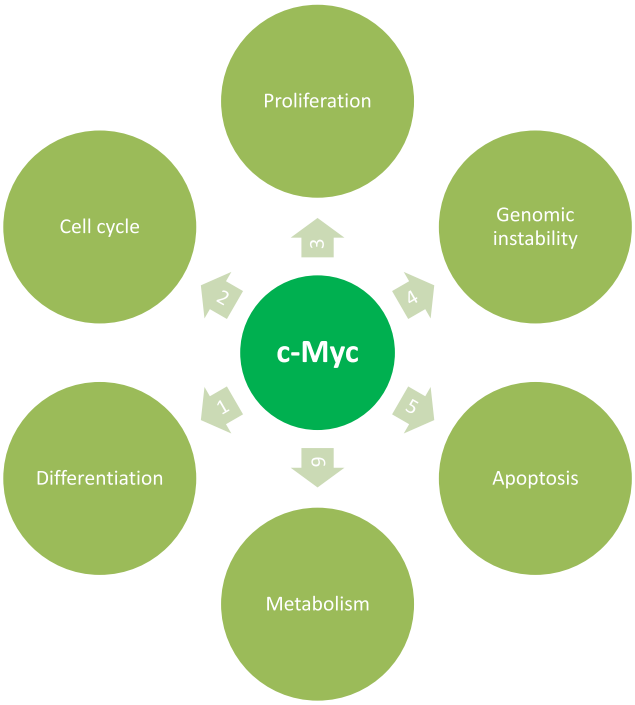

Figure 2 c-myc is one of the key elements in the malignang transformation. By (1) **preventing differentiation** and/or (2) **cell cycle** arrest, deregulated Myc forces cells to remain in a (3) **proliferative** state and causes (4) **genomic instability**. (5) **Apoptosis** is induced by activating death receptor pathways, in which myc has several points of regulation. c-Myc over-expression results in elevated expression of genes involved in regulating the (6) **cellular metabolism**, allowing tumour cells to switch to an anaerobic metabolism when oxygen is depleted).

human hepatic tumours harbour activated β-catenin mutations. A Wnt activating β-catenin mutation alone causes hepatomegaly, but to induce hepatocarcinogenesis additional mutations or epigenetic changes are required (Harada *et al.* 2002). When mutations in both the β-catenin and H-ras genes are introduced by adenovirus-mediated Cre expression, early HCC is found in all the mice killed 8 weeks after induction (Harada *et al.* 2004). High grade HCC was established after approximately 26 weeks.

Transgenic mice models over-expressing growth factors

During HCC an unregulated expression of hepatocyte mitogens occurs, leading to an uncontrolled expansion of the hepatocytes. Over-expression of growth factors that mediate hepatocyte growth induces HCC.

Transforming growth factor-α. Transforming growth factor-α (TGF-α) is a potent hepatotrophic mitogen synthesized

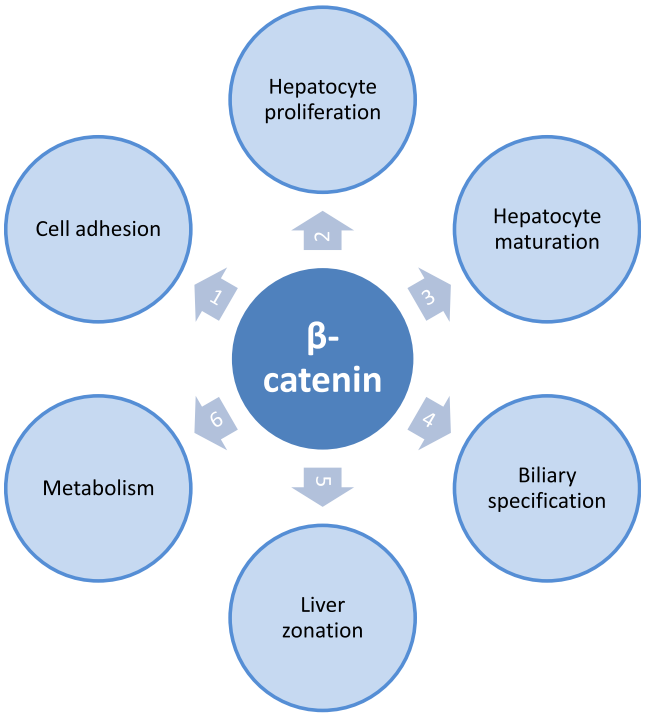

Figure 3 Beta-catenin is an integral componant of the Wnt-signaling pathway, a pathway that is one of the central players in maintaining liver health. The association between beta-catenin and E-cadherin is found in the hepatocyte membrane and has significant implications in (1) **cell-cell adhesion**. Phosphorylation of beta-catenin inhibits the beta-catenin-E-cadherin association, leading to disruption of adherens junctions and loss of intracellular adhesion. The dissociation of this complex induces nuclear translocation of beta-catenin, leading to target gene expression. Nuclear translocation of beta-catenin is associated with **hepatocyte proliferation** during development and after partial hepatectomy. Beta-catenin regulates the expression of genes involved in (3) **hepatocyte maturation** and in (4) **biliary specification**. The interaction between the protein adenomatous polyposis coli and beta-catenin, and the involvement of beta-catenin in the regulation of proteins important in ammonia metabolism, play an important role in the (5) **zonation of the liver**, dividing the liver in several structural and (6) **metabolic** regions.

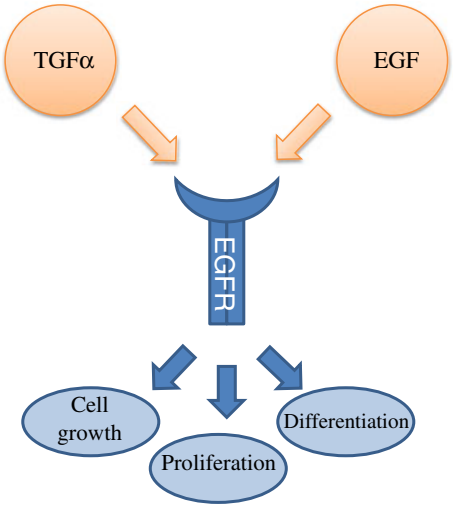

Figure 4 Transforming growth factor alpha (TGF-alpha) and epidermal growth factor (EGF) are important hepatic mitogens that both bind on the epidermal growth factor receptor (EGFR). Upon activation, EGFR undergoes dimerisation which stimulates its intrinsic intracellular protein-tyrosine kinase activity. This leads to the initiation of several signal transduction cascades, leading to cell growth, proliferation and differentiation.

in hepatocytes during regeneration (Figure 4). Transgenic mice over-expressing human TGF-α under the inducible methallothionein 1 promoter develop liver tumours as well as an abnormal development of the pancreas and mammary glands (Jhappan *et al.* 1990). These tumours are genetically similar to human HCC associated with poor prognoses (Lee *et al.* 2004). Zinc administered through drinking water enhances the tumour formation and after 40–70 weeks, HCC occurs in 50% of the mice. Heterozygotic mice with a defect in embryonic liver fodrin ($elf^{+/-}$), a stem cell adaptor protein that plays a pivotal role in TGF-signalling, develop HCC in 40–70% of the mice after 58 weeks (Baek *et al.* 2008). Double transgenic mice carrying both *c-myc* (albumin promoter) and TGF-α (methallothionein promoter) lead to a tremendous acceleration of the neoplastic development compared to the single transgenic mice overexpressing either c-myc or TGF-α (Murakami *et al.* 1993; Ohgaki *et al.* 1996; Thorgeirsson & SantoniRugiu 1996). After approximately 17 weeks, 20% of the mice is diagnosed with HCC that consists of multiple foci of carcinomas and adenomas, with 100% HCC at 40 weeks. The faster occurrence of HCC in the double transgenic model, compared with the parental lines, suggests that the interaction of *c-myc* and TGF-α increases the malignant conversion by the selection and expansion of preneoplastic cells.

Epidermal growth factor. Epidermal growth factor (EGF) is a growth factor that plays an important role in the regulation of cell growth, proliferation and differentiation (Figure 4). Its over-expression is associated with hepatocellular carcinoma. Over-expression of the secreted form of EGF results in multiple highly malignant hepatic tumours after 24–36 weeks (Tonjes *et al.* 1995; Borlak *et al.* 2005). In double transgenic mice expressing EGF and *myc*, tumour development and mortality are accelerated (Tonjes *et al.* 1995).

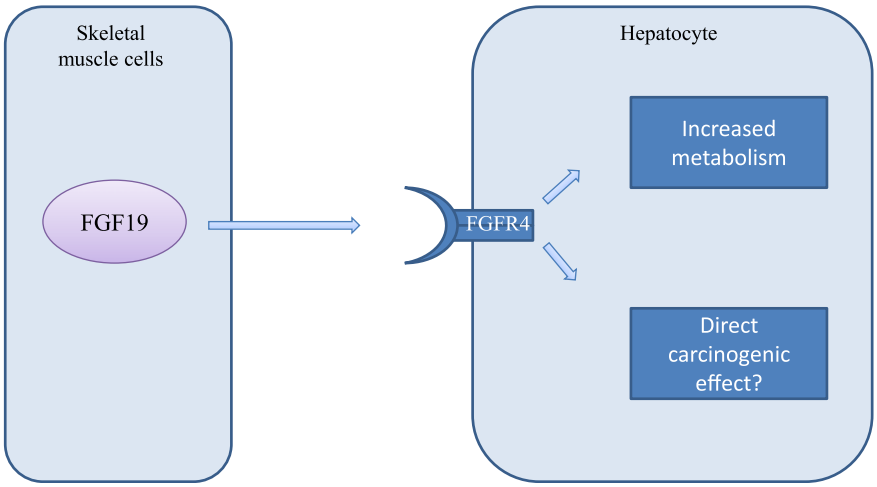

Figure 5 The fibroblas growth factor 19 (FGF19) is a high affinity ligand for the fibroblas growth factor receptor 4 (FGFR4) expressed in hepatocytes. Binding of FGF19 to FGFR4 leads to an increased cellular metabolism, associated with the production of reactive oxygen species (ROS). Wether HCC formation is an indirect effect of altered metabolism or a direct affect of FGF19 is unknown.

Fibroblast growth factor 19. While most transgenic mouse models use tissue-specific promoters to ensure liver-specific expression, there is one model that does not require liver-specific expression for the development of HCC (Figure 5). A transgenic mouse model over-expressing fibroblast growth factor 19 (FGF19) in skeletal muscle leads to the occurrence of HCC in 50% of the mice after approximately 52 weeks (Nicholes *et al.* 2002). In contrast to the other mice models, this model produces a higher HCC incidence in female mice than in male. All tumours in the female mice exhibit an increased β-catenin expression, which is not found in tumours in the male mice. The exact mechanism of the FGF19-induced hepatocarcinogenesis is still unclear, but might be a result of the increased metabolic rate which leads to an increase in ROS production.

Simian vacuolating virus 40. The Simian vacuolating virus 40 (SV40) is a DNA-virus that has the potential to cause tumours. It suppresses the transcriptional properties of the tumour-suppressing p53, which is responsible for initiating apoptosis and cell cycle arrest. When the SV40 T-antigen is brought to expression under a specific or inducible promoter such as albumin (Cullen *et al.* 1993), α1 antitrypsin (Sepulveda *et al.* 1989), serum amyloid P component (Araki *et al.* 1991) or antithrombin III (Dubois *et al.* 1991; Lou *et al.* 2005), liver tumours are found after a short period of latency (4–12 weeks). Metastasis to the lungs can occur. Tumour progression is very rapid in these models and, therefore, differs radically from the development of human tumours which progress more gradually. Transgenic mice expressing regulatory SV40 sequences by activating oncogenic sequences upon Cre-mediated excision develop HCC on a slower pace (20 weeks) (Lou *et al.* 2005).

Creating a tumour environment

These models aim to create a tumour environment and mimic the injury-fibrosis-HCC sequence found in naturally occurring tumours.

Alpha-1 antitrypsin. Alpha-1 antitrypsin (AAT) is a glycoprotein that is produced in the liver. Transgenic mice expressing a human form of transport-impaired AAT represent a good model for studying the effects of AAT deficiency on the liver. AAT deficiency is an autosomal recessive disorder in which a mutation causes the production of AAT that is unable to be transported. This leads to decreased AAT activity in serum and deposition of excessive AAT in the liver. Both heterozygous and homozygous individuals develop cirrhosis and HCC. AAT-deficient mice develop HCC after 52–90 weeks (Geller *et al.* 1994).

Phosphatase and tensine homologue. Phosphatase and tensine homolog (PTEN) is a tumour suppressor gene that regulates the serine-threonine kinase protein kinase B (PKB/akt) pathway (Figure 6). PTEN deficiency induces cellular hyperproliferation, anti-apoptosis and oncogenesis (Horie *et al.* 2004). Liver-specific PTEN-deficient mice develop hepatic steatosis, inflammation, fibrosis and tumours that are very similar to human non-alcoholic steatohepatitis (NASH) (Watanabe *et al.* 2007). Liver tumours are present in 66% of male and 30% of female PTEN-deficient mice by 40–44 weeks of age.

Platelet-derived growth factor. Members of the platelet-derived growth factor (PDGF) family are known to play an important role in the embryonic development, cell

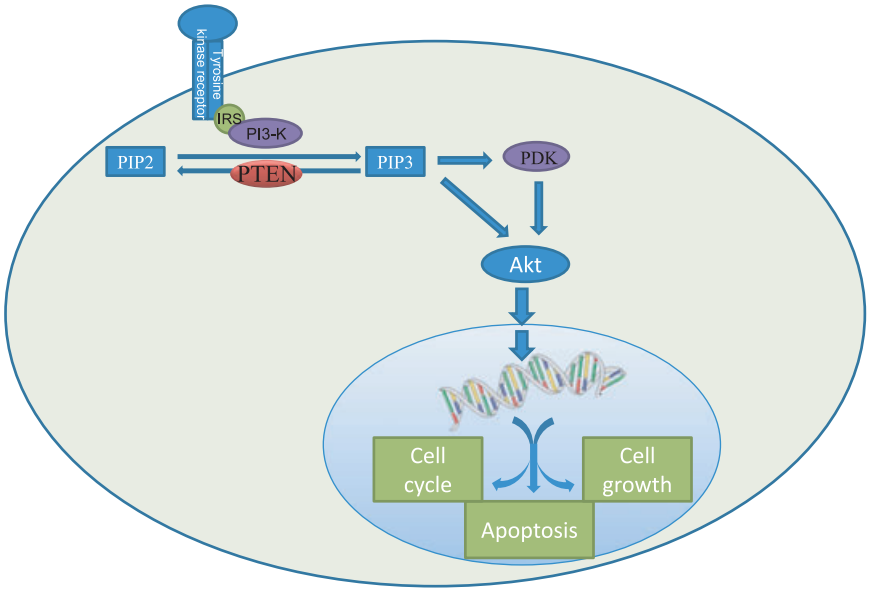

Figure 6 Phosphatase and tensin homolog (PTEN) is a tumour suppressor gene involved in the regulation of the threonine kinase protein kinase B (PKB/akt) pathway, by dephosphorylating phosphatidylinositol (3,4,5)-triphosphate (PIP3). The PKB/akt pathway is involved in the regulation of the cell cycle, apoptosis and cell growth.

proliferations, cell migration and angiogenesis. Furthermore, they have been associated with several diseases, including hepatic fibrosis (Bonner 2004). Liver cirrhosis induced by over-expression of PDGF-A, PDGF-B, PDGF-C or PDGF-D is associated with hepatic stellate cell activation possibly as a result of the induction of profibrotic genes such as transforming growth factor-beta1 (TGF-β1) (Campbell *et al.* 2005; Czochra *et al.* 2006; Borkham-Kamphorst *et al.* 2007; Thieringer *et al.* 2008). Long-term over-expression of the PDGF genes will induce HCC after approximately 52 weeks (Campbell *et al.* 2005).

Transforming growth factor-beta. Transforming growth factor-beta1 is known to be an important factor in the pathogenesis of fibrosis (Williams & Knapton 1996). TGF-β1 enhances the production of several matrix components and decreases their degradation, thereby causing an accumulation of extracellular matrix which is the basic underlying cause of fibrosis (Lechuga *et al.* 2004; Qi *et al.* 2006). Transgenic mice over-expressing TGF-β1 present the most extensive liver fibrosis after approximately 10 weeks; nevertheless, fully developed cirrhosis is not seen, probably because of the high mortality rate in the transgenic strains (Sanderson *et al.* 1995). Repeated administration of AAT can induce cirrhosis after 18 weeks, which would lead to HCC if the experiment would be continued for a prolonged period (Schnur *et al.* 2004).

Glycine N-methyltransferase. Glycine *N*-methyltransferase (GMNT) is the main enzyme responsible for catabolism of hepatic *S*-adenosylmethionine (SAM), leading to the formation of *S*-adenosylhomocysteine (SAH), an inhibitor of methyltransferases (Mato & Lu 2007). By controlling the ratio of SAM/SAH, GMNT contributes to the genetic stability by regulating DNA methylation. GNMT-knock-out mice have SAH levels that are 71 times higher than that in control mice (Liu *et al.* 2007). The transgenic mice start to develop steatosis and liver fibrosis after 3 months, a phenomenon that becomes more prominent after 8 months (Martinez-Chantar *et al.* 2008). At 8 months, 100% of the mice have developed multifocal HCC.

Conclusion

The availability of a wide range of experimental mice models for HCC research has given researchers the opportunity to assess tumour–host interactions, to perform drug screening and to mimic the complex multistep process that leads to the malignant transformation of hepatocytes. When selecting a model for HCC research, one must first understand the limitations and advantages that the specific model possesses. HCC rarely occurs spontaneously in humans, in contrast to certain mouse strains which have a high background incidence of spontaneous hepatic tumour formation (Diwan *et al.* 1986). The same mouse strains are very susceptible to hepatocarcinogens. The first step in constructing an experimental setup is choosing an optimal mouse strain.

Chemically induced models are favourable for research that requires HCC to develop in a natural background of

liver damage. Different hepatocarcinogenic agents are metabolized through diverse metabolic pathways and extrapolation should be considered with caution when it is uncertain if the agents would be metabolized differently in humans. Age of the mice and dose of administration of hepatotoxins might lead to different tumour phenotypes, which might influence experimental outcomes.

Xenograft models offer a fast solution for drug screening and are an easy way for proof-of-principle experiments. Xenograft models are often used for drug screening; nevertheless, it must be noted that the spectacular results often seen in mice models can seldom be repeated in cancer patients. It should also be noted that multiple cell lines have to be used in experiments because of the heterogenity between cell lines. Hollow fibre assays (HFA) offers a model that allows testing of multiple cell lines in one mouse, leading to a tremendous decrease in required lab animals. HFA and ectopic implantation do not allow research about complex tumour–host interactions, but orthotopic implantation makes it possible to investigate the neoplastic hepatocytes in their natural environment.

Genetically modified mouse models are favourable to identify a range of discrete molecular and histological stages during the multistep process of hepatocarcinogenesis. GMMs harbouring multiple mutations allow the investigation of cooperation and dependency between oncogenes, growth factors and viral genes. Use of an inducible promoter offers significant advantages because the mutation can be switched on and off when needed. The biological insight gained from transgenic mice is invaluable. Nevertheless, a mutation in a gene does not always result in the expected phenotype and the phenotypic outcome can be influenced by many environmental and genetic factors. It should also be stated that most models do not demonstrate the advanced neoplastic lesions and that metastasis occurs only seldom. One should also consider the fact that natural tumours consist of a heterogenic group of cells in which multiple mutations have occurred. The interpretation of results from studies using GMMs is not always simple, especially for extrapolation to humans.

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