REVIEW

Animal Models of Hepatocellular Carcinoma: Current Applications in Clinical Research

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Abstract: In the last decade, relevant advances have occurred in the treatment of hepatocellular carcinoma (HCC), with novel drugs entering the clinical practice, among which tyrosine kinase inhibitors (TKIs) such as lenvatinib, cabozantinib and regorafenib, and immune checkpoint inhibitors (ICPIs) either alone or in combination with VEGF inhibitors. Clinical trials have driven the introduction of such novel molecules into the clinics but, at present, no biomarker drives the choice of first-line options, which relies only upon clinical and imaging assessment. Remarkably, clinical and imaging-based evaluations do not consider the huge heterogeneity of HCC and do not allow to realize the potential of personalized treatments. Preclinical research still does not inform the design of clinical trials, even though many animal models mimicking specific subgroups of HCC are available and might provide relevant information. Although animal models directly informing the clinical practice, such as patients-derived xenografts, are not used to help the choice of treatment in advanced HCC, however, the preclinical research can count on a wide range of valuable models. Here we will review some HCC models which might turn informative for specific questions in defined patient subgroups, and we will describe recent preclinical studies for the mechanistic evaluation of immunotherapy-based treatment approaches. To this aim, we will mainly focus on two issues: (i) HCC models informative on NAFLD-NASH HCC and (ii) HCC models helping to elucidate mechanisms underneath immunotherapy. We have chosen these two settings since they represent, respectively, the most rapidly arising cause of chronic liver disease (CLD) and HCC in western countries and the most promising therapeutic option for advanced HCC.

Keywords: HCC, NASH, animal models, treatments, immunotherapy, TKIs

Introduction

Hepatocellular carcinoma (HCC) is a highly heterogeneous cancer, often accompanied by an underlying chronic liver disease (CLD) and comorbidities that make it difficult to reproduce faithfully this human disease in the experimental setting. HCC is rarely biopsied because its diagnosis mostly relies upon imaging techniques. This hampers tissue availability and large-scale molecular studies on patient subgroups. The heterogeneity of HCC needs to be considered in the preclinical setting allowing animal models to address specific questions, such as the discovery of molecular mechanisms driving cancer development and progression, or the identification of biomarkers, molecular targets, and drug effects in defined subgroups.

Regarding HCC-associated risk factors, metabolic syndrome is on the rise in developed countries. In the liver, it is associated with NASH (non-alcoholic steatohepatitis) or NALFD (non-alcoholic fatty liver disease). NASH represents the more severe form of NAFLD and is characterized by fat accumulation in hepatocytes with ballooning, lobular inflammation and eventual perisinusoidal fibrosis which may progress to cirrhosis and then to HCC.[1] Since liver cancer arises on an inflammatory background, a faithful preclinical model should induce tumorigenesis within an underlying chronic liver disease, especially in the assessment of immune-modulating treatments. Models encompassing not only NASH or NALFD but also obesity and insulin resistance were nicely reviewed.[2,3] These models rely upon diet, genetic manipulation, or a combination of the two. As for humans, most NASH-bearing animals do not develop HCC and are not suitable for cancer research. Thus, models more

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susceptible to HCC were set up to focus on molecular characterization and treatment intervention. A recently identified feature of NASH-HCC is represented by its reduced response to immunotherapy,[4,5] which still remains to be fully clarified.

Since NASH is the fastest increasing cause of HCC and immunotherapy is the most promising strategy for advanced HCC, understanding drivers of NASH-HCC and identifying mechanisms underneath its reduced response to immunotherapy as well as novel strategies to overcome immune exhaustion represent crucial issues for the management of HCC. Due to insufficient tissue availability, animal models designed for these specific purposes are the main tool to answer these questions. For these reasons, here we will focus on two main topics: (i) NASH-HCC animal models associated with dysmetabolic conditions and (ii) HCC models for the evaluation of immunotherapy. Specifically, we will firstly describe the main characteristics of diet-derived and genetically modified NASH-HCC models. Subsequently, we will report the chances to hit lipid metabolism and to screen immune-based treatment combinations in HCC animals with or without NASH to develop more effective therapeutic approaches in well-defined HCC patient subgroups.

Models and Mechanisms of NASH-Associated HCC

NASH is characterized by hepatocellular injury, with hepatocyte ballooning, lobular inflammation, and varying degrees of fibrosis. In humans, NASH is associated with insulin resistance, atherogenic dyslipidemia, hyperinsulinemia and hypertension. Hypotheses have been proposed aiming at explaining how fatty acids and their metabolites promote NASH. Fatty acid-mediated lipotoxicity triggers inflammation and fibrosis, resulting in disruption of hepatic cytoarchitecture, exhaustion of hepatocyte regenerative responses, finally progressing toward cirrhosis and eventually HCC. Several lipotoxins (eg, free cholesterol) have been identified in NASH and their hepatic accumulation is linked to insulin resistance and to activation of innate immunity to recruit cellular inflammatory responses.[6] There is evidence for concurrent immune imbalance in NASH. Although the immune signaling pathways involved are not completely understood, activation of hepatic resident Kupffer cells and neutrophils, in addition to the recruitment of other innate immune cells, is a significant effector of parenchymal inflammation in NASH.[7,8] An ideal drug candidate for NASH should revert underlying metabolic derangements and reduce key clinical endpoints such as inflammation, steatosis, liver cell injury, fibrosis, insulin resistance and obesity.

HCC models recapitulating NASH can be obtained either by administration of specific diets and toxins, or by using genetically modified mouse strains or by combinations of both. Here we will briefly describe some diet and genetic-based NAFLD/NASH models, trying to focus on the mechanisms leading to HCC development, improving the knowledge on molecular evolution of this disease as the first step to design novel therapeutic approaches.

Diet-Based Models of HCC

The high-fat diet (HFD) provides 45% to 75% of the calories as saturated fat, the majority being lard, and it results in hepatic steatosis, obesity, insulin resistance, increased leptin, dysregulated lipid metabolism, and, in the long run, liver inflammation and fibrosis.[9] The western diet (WD), which includes saturated fatty acids, cholesterol, and sucrose, is associated with an objective liver injury as reviewed by Hintze et al,[10] while the fast-food diet recapitulates the high fat diet changes, with fibrosis and hepatocyte ballooning developing much earlier.[11] Despite the high fidelity of both the last two models to the human condition of fibrosing NASH, these diet-induced experimental protocols do not trigger HCC development and are therefore not suitable for the study of cancer progression in a background of predisposing CLD.

Methionine and Choline-Deficient Diet

Features of severe NASH such as inflammation, hepatocyte ballooning, apoptosis, autophagy deregulation and fibrosis can be better mimicked in mice fed by methionine- and choline-deficient diet (MCD) which induces steatohepatitis by restricting essential nutrients, required for hepatic lipid metabolism, obtaining representative models for investigating the pathobiological mechanisms that cause human NAFLD progression to NASH.[12] These nutrient-deficient diets hamper phospholipid synthesis, lipoprotein secretion, induce oxidative and endoplasmic reticulum (ER) stress[13] and impair mitochondrial respiratory function.[14] The main drawback of these options is the absence of insulin resistance and obesity. Thus, these diets do not recapitulate the features of NASH associated with metabolic syndrome. Indeed, MCD induces fat and lean mass loss, without fasting hyperglycemia or insulin resistance, and is associated with decreased circulating lipid levels. In addition, while recapitulating many pathophysiological markers of NASH, MCD does not trigger IL-6 overexpression in the liver, which

represents a key hallmark of human NASH[15] and does not drive HCC development. Interestingly, by associating long-term choline-deficient diet to high-fat diet, weight loss does not occur and progression to HCC can be observed in nearly 25% of the mice.[16] Interestingly, this mouse model recapitulated critical features of human metabolic syndrome, NASH, and HCC. The activation of intrahepatic CD8 (+) T cells, natural killer T (NKT) cells, and inflammatory cytokines, responsible for hepatocyte damage, together with NF-κB signaling also facilitated NASH-to-HCC transition in this preclinical model.

American Fast-Food Diet

A more representative preclinical tool for the study of NASH-HCC is the ALIOS (American Lifestyle-Induced Obesity Syndrome) model that is generated by feeding animals with a diet similar in composition to an American fast-food diet. In addition, drinking water was provided as gel-water in dishes on the cage floor and cage racks were removed to discourage physical activity promoting a sedentary behavior. The combination of trans fats and high-fructose corn syrup in the diet of sedentary mice induced histological features of NASH in the context of a metabolic profile, better mirroring the human disease. As a further confirm, increased levels of plasma metabolic-associated hormones, such as insulin, resistin, and leptin, reflected the dysregulation observed in NASH patients.[17] Expression of acetyl-CoA carboxylase 1 and fatty acid synthase, both coding for key regulators of lipogenesis, was increased in ALIOS mice at 6 and 12 months compared with control mice, representing increased lipid turnover (increased lipogenesis and β-oxidation) in livers of ALIOS mice. At 12 months, ALIOS mice developed histological features of NASH with severe steatosis and lobular inflammatory cells characterized by a mixed population of neutrophils and lymphocytes. Increased mRNA expression of proinflammatory cytokines (TNF, CCL2, and CCL3) as well as macrophage markers (CD68, CD40, and F4/80) and Kupffer cell infiltration were observed. Comparing the transcriptome from ALIOS mice with publicly available RNA-Seq data from biopsies of patients with NASH, a significant overlap of genes associated with NAFLD and NASH came to light.[18] Fibrosis was histologically observed in ALIOS mice with a pattern similar to that observed in human NASH. Macroscopically visible HCC nodules developed in 60% of the ALIOS mice at 12 months with many features of early human HCC, including the loss of biliary structures, disruption or loss of reticulin fibers, nuclear accumulation of β-catenin and aberrant expression of glutamine synthetase and α-fetoprotein.[19] In the absence of genetic mutations or toxins, high-fat/fructose diet and sedentary lifestyle are sufficient for the induction of NASH and hepatocarcinogenesis in wild-type mice, establishing ALIOS as a novel model for the study of driver genes and treatments in HCC developed on the background of NASH.

Diet and Toxin Combination

Since HCC is rare and late in most of the dietary-based models, hepatocarcinogenesis was triggered by administering toxins or by adding them to NAFLD-inducing diets. Among these toxins, the administration of low dose of streptozotocin after birth (STAM model) destroys pancreatic insulin-producing β cells, resulting in type 1 diabetes. The association of an HFD feeding determines progressively increasing liver changes with steatosis, followed by NASH, fibrosis, development of adenomas and then HCC at approximately 16 weeks.[20] While the rapidity and histopathological similarities of liver changes with respect to human disease are points of strength of the STAM model, yet the lack of obesity, insulin resistance, and type 2 diabetes differentiate the two conditions, limiting its representativeness. In addition, it is not possible to rule out a direct genotoxic role of streptozotocin. In the same context, the association of diethylnitrosamine (DEN) with the HFD induces HCC development on a background of NASH[21]; however the role of DEN is likely to be relevant in the carcinogenic process and this may represent a substantial difference with the human counterpart. Similarly, by adding weekly dosing of carbon tetrachloride (CCl$_4$) to the WD,[22] HCC development can be obtained at 24 weeks but, again, the induction of genotoxic damage by CCl$_4$ raises questions about whether this model can be considered representative of the human disease.

Many other models were obtained by administering NASH-inducing diets to genetically manipulated mouse strains, as reviewed in,[23] each one with its point of strength and limits. In our opinion, the choice of the appropriate model should rely not only upon the representativity with respect to the human disease but also upon the hypothesis and the aims of the studies, and the results should be confirmed in more than one model. Finally, it should be outlined that there is no perfect dietary model inducing NAFLD that faithfully reflects the human disease (Table 1). Remarkably, even though toxins might be considered far from the common dietary intake, yet we have to recognize that daily alcohol consumption,

Table 1 Animal Models of NASH-HCC Induced by Different Diets and Toxins

Diet	Toxic Agent	Metabolic Syndrome	HCC Development	References
HFD	No	No	No	9
Western diet	No	No	No	12
MCD	No	Yes	No	12; 13; 14
HFD+MCD	No	Yes	Yes (25%)	16
American fast-food diet (ALIOS model)	No	Yes	Yes	17; 18; 19
HFD	Streptozoticin	Diabetes Type I	Yes	20
HFD	DEN	Yes	Yes	21
Western diet	CCl4	No	Yes	22

Abbreviations: HFD, high-fat diet; MCD, methionine- and choline-deficient diet; DEN, diethyl nitrosamine; CCl$_4$, carbon tetrachloride.

tobacco smoke, exposure to genotoxic agents, even at very low amounts,[24] usually occur in the everyday life, and might contribute to liver damage. Thus, chronic administration of very low amounts of toxins to animal models might not be so far away from real life.

Genetically Modified Mouse Models of NASH-HCC

The genetic background plays a relevant role in disease development and progression. The heterogeneous phenotypic expression of diseases, when exposure to risk factors is very similar, mostly relies upon the genetic background. So far, we cannot fully define a comprehensive genetic background contributing to HCC development in NAFLD patients, neither we can precisely assess the role of constitutive genetic variants of genes (eg, PNPLA3 or TM6SF2) which are associated with HCC risk. Steatosis-related lipotoxicity and oxidative DNA damage can induce hepatocarcinogenesis in the absence of cirrhosis. Validated models that combine multiple risk factors and fibrosis stage into "HCC risk calculators" are not yet available for patients with NAFLD. Development of such tools would enable risk stratification, identification of high-risk patients even in the absence of cirrhosis, and individualized (risk-based) surveillance strategies.[25]

Similarly, among different mouse strains, some are more prone to HCC development and thus they are commonly used in this kind of research. For example, in the setting of diet inducing NASH/NAFLD, the C57/BL6 mice are more susceptible to both fatty liver changes and inflammation.[26,27] Besides a global and yet uncharacterized genetic background, researchers have developed genetically manipulated models to hit specific mechanisms, which recapitulate the human disease either alone or in combination with other promoting factors. An example of these is the PTEN knockout mouse, which disrupts a crucial pathway involved in metabolism, proliferation, motility, immunity, and carcinogenesis, as detailed below.

PTEN Knockout Mice

PTEN is a tumor suppressor gene frequently mutated in cancer whose manipulation was suggested for the development of preclinical tools in cancer research.[28] The PTEN-null mouse develops steato-hepatitis, insulin hypersensitivity, fibrosis, adenomas and finally HCC.[29] Liver-specific PTEN knockout (KO) in mouse led to severe steatohepatitis and HCC due to PI3K/AKT pathway activation. PTEN is an important regulator of lipogenesis, glucose metabolism, and hepatocyte homeostasis; its deletion in mice mimics metabolic derangements occurring in human NASH where hyperinsulinemia frequently drives hepatic lipid synthesis. Wnt-mediated activation of hepatic stellate cells (HSCs) is responsible for extracellular matrix deposition inducing progressive liver fibrosis in PTEN null livers. In line, PTEN expression negatively correlates with the presence of NASH in human patients.[30] Functional alterations of mitochondria were shown to occur in steatotic hepatocytes supporting progression to NASH.[31] Alterations in mitochondrial function

have a significant role in the generation of ROS, which contributes to the development of NASH as extensively reviewed by Garcia-Ruiz et al[32] Indeed, one mechanism by which ROS exert cellular effects is through the regulation of PTEN that protects against damaging effects caused by high insulin levels, preventing the induction of PI3K/AKT signaling pathway. Increased mitochondrial respiration and ROS production observed in PTEN KO mice might foster malignant transformation of hepatocytes confirming the relevance of the PI3K/AKT pathway in mediating damaging effects of high insulin levels.[33] In line, administration of eicosapentaenoic acid (EPA), which regulates lipid metabolism and inflammation, to PTEN null mice improved liver injury and steatosis; the EPA-treated group showing a less severe chronic hepatic inflammation, decreased ROS production, and reduced HCC development due to inhibition of MAPK activity.[34] Another study showed that combining PTEN and glucose-regulated protein 78 (GRP78) knockdown exacerbates steatohepatitis and induces malignant transformation.[35] An elegant study investigated the role of de novo lipogenesis mediated by sterol regulatory element-binding proteins (SREBPs) in NASH pathogenesis by assessing SREBP inhibition in PTEN KO mice. Interestingly, SREBP inhibition exacerbated liver injury, fibrosis, and carcinogenesis despite markedly reduced hepatic steatosis.[36] Thus, the authors warn against an excessive lipogenesis inhibition for NASH therapy; this finding might have relevant clinical implications for NASH prevention and treatment.

The immune system gives an important contribution to the pathogenesis of NASH-HCC representing the inflammatory component that triggers the progression of this disease. In this regard, Miura et al investigated the role of Toll-like receptor (TLR) signaling in PTEN-deficient mice by generating double-mutant mice containing the contemporaneous deletion of PTEN together with TLR4 or TLR2.[37] The authors reported tumor growth suppression and reduced hepatic inflammation in TLR4 but not TLR2 deficiency in the PTEN KO model. Of note, liver resident macrophages isolated from PTEN-deficient mice produced elevated levels of proinflammatory cytokines IL-6 and TNFα in response to lipopolysaccharide (LPS), activating cancer progenitor cells (eg, oval cells) that play an active role in liver cancer development as we previously reviewed.[38] Thus, immune cell populations create an inflammatory microenvironment that heavily contribute to liver injury and to the development of NASH-related HCC in this preclinical model. In agreement, Vidotto et al have recently focused on the emerging role of PTEN loss in the evasion of the immune response by sustaining immunosuppressive environments in different human tumors.[39] Loss of a PTEN allele was identified in 20–30% of the HCC patients, whereas cDNA microarray analysis indicated that PTEN expression was decreased in a considerable number of HCCs.[40] This might in part explain the lower responsiveness to immunotherapy in patients with NASH-associated HCC, suggesting that a liver-specific PTEN KO mouse model might turn useful to assess the role of the tumor microenvironment (TME) in the immune escape of this disease. Finally, even though this model does not present peripheral insulin resistance and hyperglycemia, PTEN null mice might help to identify crucial downstream pathways and therapeutic targets.

PCSK9 Knockout Mice

The enzyme 3-hydroxy-3-methylglutaryl-coenzyme A reductase (HMGCR) is an endoplasmic reticulum enzyme, which catalyzes the conversion of HMG-coenzyme A to mevalonate, is the rate-limiting enzyme in the mevalonate pathway and it has been identified as a determinant of plasma cholesterol levels.[41] Inhibitors of HMGCR, statins, are potent cholesterol lowering agents that have been widely used to prevent the occurrence of atherosclerotic diseases. Recently, a role of statins in preventing HCC development has been suggested, possibly linked to their pleiotropic effects on improving endothelial function, decreasing vascular inflammation and oxidative stress. Although conflicting findings are reported in humans, preclinical studies provide strong evidence for a favorable effect of statins in HCC as reviewed by Lonardo and Loria.[42] Accordingly, the use of pravastatin prolonged the survival of advanced HCC patients although mechanisms leading to growth inhibition are poorly investigated.[43]

On the other side, statins raise levels of proprotein convertase subtilisin/kexin type 9 (PCSK9), a circulating protein secreted by the liver that binds to hepatic low-density lipoprotein receptor (LDLR) and induces its internalization and degradation. Regarding HCC, PCSK9 increases plasma LDL-cholesterol (LDL-c) resulting in hypercholesterolemia that hampers sorafenib-induced cell killing and promotes HCC cell survival. In fact, LDL-c binds to sorafenib reducing the fraction of drug available for uptake by cancer cells.[44] Additionally, increased levels of pERK were detected in LDL-c exposed/sorafenib-treated cells as compared to sorafenib treatment alone, implying that extracellular LDL-c abrogates the inhibitory activity of sorafenib acting on pro-oncogenic signal transduction. The PCSK9 KO model helps to dissect

these mechanisms aiming to increase sorafenib efficacy. PCSK9 KO mice feeding a high-fat/high-cholesterol diet exhibited higher levels of cholesterol than their WT counterparts and developed significantly more hepatic inflammation, injury and fibrosis.[45] However, the current landscape of studies examining the role of PCSK9 in human and rodent HCCs is still controversial. It was shown that high expression of PCSK9 in HCC is related to microvascular invasion and large tumor size and is an independent risk factor for both disease-free survival and overall survival in patients who underwent curative resection.[46] On the contrary, other studies reported decreased PCSK9 expression in HCC implying that liver tumors can modulate their local and adjacent microenvironment, thus enabling energy supply to fuel tumor growth.[47] A brilliant study demonstrated that deleting PCSK9 gene in rodent cancer cells substantially affects their growth in vivo and, notably, improves the efficacy of anti-programmed cell death protein 1 (PD1) therapy, opening the way towards combined immune checkpoint-based strategies. Mechanisms of action seem independent of the cholesterol regulation, rather relying on the recycling of MHC I molecules for degradation to lysosomes.[48] On a different wavelength, a preclinical study reported that injection of DEN early in life in PCSK9 KO mice leads to significant NASH-HCC development, confirming that PCSK9 deletion predisposes to fibrosis, steatohepatitis and hepatic carcinogenesis when associated with a high cholesterol diet, also showing features of insulin resistance.[49] These studies provide evidence that cholesterol rather than steatosis plays a role in NASH-HCC progression raising the question whether patients on long-term treatment with anti-PSCK9 monoclonal antibodies are at increased risk of steatosis, steatohepatitis or even liver cancer. These findings have interesting clinical implications: modulation of PCSK9 could potentially be therapeutically exploited with the newly developed antibodies or targeted pharmaceuticals, thus challenging metabolism and immuno-phenotype of cancer cells. However, a deeper understanding of specific settings and patient subgroups in which PSCK9 targeting might be beneficial as an antitumor approach in HCC is still needed.

In the light of the central role of lipid accumulation for NAFLD-to-NASH HCC progression, below we explored the therapeutic potential of affecting lipogenesis in different NASH preclinical models.

Hitting Fatty Acid Synthesis for HCC Treatment

Going deeper into mechanisms sustaining NASH and acting as HCC drivers and potential therapeutic targets, Wang and coworkers[50] showed the therapeutic advantage obtained by combining fatty acid synthase (FASN) inactivation with conventional systemic treatments for HCC in preclinical animal models. Specifically, these authors tested several oncogene-driven murine HCC models obtained by hydrodynamic tail vein injection. Since aberrant lipid biosynthesis is required for cancer cell proliferation, energy production and membrane formation, inhibition of fatty acid synthesis was tested as a possible tool to improve cancer treatment (Figure 1). In HCC models sustained by PTEN deletion coupled with c-MET overexpression as well as by AKT and NRAS activation, FASN inhibition led to an antiproliferative effect. Even further, by combining FASN inhibition with the first and second lines TKIs, sorafenib and cabozantinib, a synergistic antiproliferative effect was obtained, in part due to mTOR pathway inhibition. Similar findings were identified by inhibiting FASN in c-MYC amplified/activated HCC models. Thus, FASN inhibition, and especially its association with TKIs might be suggested as a possible therapeutic strategy for FASN-overexpressing HCCs, among which AKT/mTOR and c-MYC activated tumors that are presumably those activating the lipogenesis cascade. Indeed, AKT induces hepatic steatosis, which is suppressed by FASN inhibition. From a molecular point of view, FASN expression is induced by the activation of mTOR and c-Met pathways.[51,52] Consequently, FASN inhibition might play an anticancer role in those cancers triggered by the above-mentioned oncogenic drivers that represent a subgroup of aggressive tumors.[53] Interestingly, FASN inhibitor TVB3664 does not improve the antitumor effects of cabozantinib in FASN-independent models, such as β-catenin mutated mice, confirming the need for a preliminary molecular classification of HCC patients when combined FASN-TKI treatments are considered. Interestingly, FASN inhibition significantly reduces NASH features not only in mouse models but also in patients with NASH, as shown in a recent phase 2 clinical trial.[54] Finally, sgPTEN/c-Met mice showed no tumor growth inhibition with either anti-programmed death ligand 1 (PD-L1) antibody or combined TVB3664/anti-PD-L1 treatment, suggesting the limited efficacy of FASN inhibition and immunotherapy in this oncogene-driven HCC model. Since immunotherapy is the standard of care in advanced HCCs but not all patients respond to it, below we describe innovative preclinical studies assessing immune-mediated treatment combinations in "ad hoc" animal models for the evaluation of the tumor/microenvironment crosstalk.
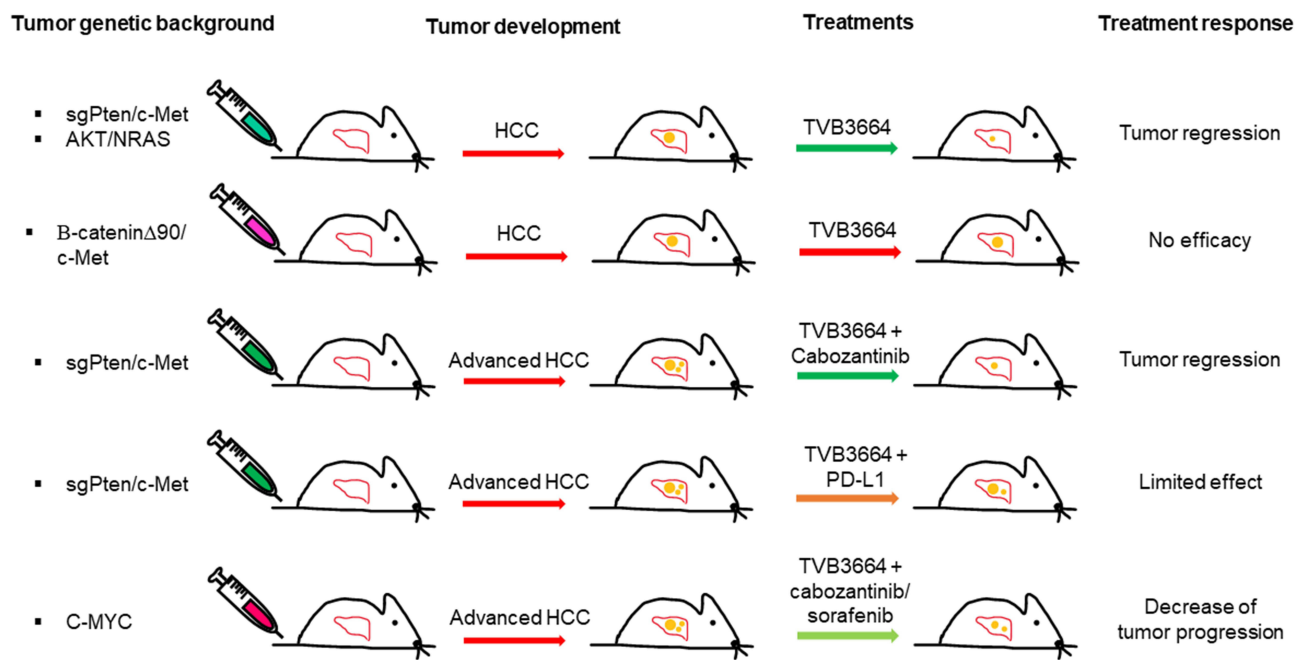
Figure 1 Schematic picture of treatment efficacy of FASN inhibitor (TVB3664) alone or in combination with tyrosine kinase (sorafenib and cabozantinib) and immune checkpoint inhibitors (PD-L1 antibodies). The different genetic backgrounds of tumors were obtained by hydrodynamic tail vein injections in C57BL/6J mice.

Immunotherapy-Based Approaches in HCC Animal Models

The combination of atezolizumab and bevacizumab shows improved overall survival over sorafenib and it is actually approved for the front line treatment of advanced HCC.[55] In addition, several combinations of immune checkpoint inhibitors (ICIs) and tyrosine kinase inhibitors (TKIs) are being tested in double-blind phase-III trials.[56] Since the composition of TME and immune infiltration are determinant for the success of immunotherapies in oncology, the employment of proper preclinical models is critical for the assessment of novel therapeutic strategies. To study cancer immunotherapy, animal models should recapitulate not only molecular and genetic alterations of the human disease but also the complexity of the immune system, mirroring the pathogenesis of specific subgroups of HCC patients that might benefit from personalized immune-based strategies. Patient-derived xenograft (PDX) mouse models opened the way towards the development of suitable preclinical tools accounting for tumor heterogeneity which is a crucial aspect of human HCCs. Even after several in vivo and in vitro passages, these lines maintained the essential features of the original specimens at the histological, transcriptome, proteomic and genomic levels.[57] Even though very useful for biomarker discovery and for the study of the antitumor effect of TKIs, this animal model, lacking the immune system component, is not suitable for investigating the immunomodulatory effects of ICIs. In this regard, PDX humanized mice (humice) represent an interesting example of preclinical models that can be employed as screening tools for testing immunotherapeutic drugs in HCC. Zhao et al[58] developed a PDX humanized mouse model by injecting hematopoietic stem cells into immunocompromised NOD/SCID gamma mouse (NSG) pups followed by subcutaneous transplantation of HCC-PDX in 8–10 weeks-old humice. Of note, HCC-PDX in NSG mice were significantly smaller than those grown in humice, highlighting the interaction between the tumor and the human immune system that, after tumor infiltration, was educated by cancer cells to develop exhaustion phenotypes. In addition, tumors grown in humice expressed higher levels of immune checkpoint ligands that were further induced by T cell-mediated secretion of INF-γ in the TME. As a final confirm of the reliability of this model for immunotherapy studies, a decrease of tumor size after 4 weeks of pembrolizumab (anti-PD-L1) and ipilimumab (anti-cytotoxic T-lymphocyte antigen 4, CTLA4) treatment was detected in humice but not in PDX mice. Remarkably, this model partially overcomes the lack of TME by reconstituting some immune-related features. However, the liver TME is much more complex showing peculiar liver blood supply, made of

both arterial and venous flow including gut-derived components, and it is therefore hardly reproducible in ectopic sites. This feature can make the difference also when dealing with drug delivery. In this regard, an interesting study by Finkin S and coworkers[59] investigated the role of TME and focused on the role of ectopic lymphoid structures (ELS) in the development of HCC. To establish a suitable animal model, these Authors identified those signaling pathways differently expressed in the liver of patients with high versus low ELS. Among these pathways, the NF-kB was confirmed in different data sets, with relevance of I Kappa B Kinase (IKK)-NF-kB activation. Two genetically distinct mouse models constitutively overexpressing IKK-NF-kB in hepatocytes were developed, which displayed increased macrophage infiltrate, liver damage markers and hepatocyte proliferation associated with ELS. As observed in the human counterpart, ELS from rodent HCCs were composed by T and B lymphocytes, neutrophils, NK cells, T regulatory cells, follicular dendritic cells, and endothelial venules. These models allowed to reveal the growth of neoplastic hepatocytes within ELS suggesting that immune micro-niche with a functional adaptive immune system has a pro-tumorigenic effect. These peculiar immune structures group together different lineages of immune cells generating immune-derived cytokines and growth factors. Specifically, T lymphotoxins within ELS were shown to induce HCC via a paracrine stimulation and were suggested as targets of novel treatments. This elegant study proves the primary relevance of TME through the development of mouse models recapitulating the complex environment of a subgroup of human HCCs, namely the "ELS high". These models also provide an experimental tool to test preventive strategies against HCC development or recurrence.

Comprehensive reviews on mouse models of oncoimmunology in HCC have been conducted.[60,61] Despite programmed cell death protein 1 (PD1) immune checkpoint inhibitors have changed the landscape of cancer medicine and produced encouraging results in HCC,[62,63] not all patients resulted sensitive to anti-PD1 therapies indicating the onset of drug resistance mechanisms. Indeed, clinical responses are observed only in 20% of treated HCC patients. To make this picture even worse, the lack of biomarkers for patient stratification to optimal therapeutic regimens still affects the achievement of personalized medicine in HCC. As a proof of concept, immunotherapy in NASH-HCCs showed an impaired efficacy due to the presence of aberrantly activated CD8+ T cells.[5] In this regard, many efforts have been made to identify more effective treatment combinations. Here, we summarize some of the most recent findings on HCC mouse models that investigated the antitumor immune response to different therapeutic approaches to define deregulated pathways responsible for tumor resistance and escape (Table 2). These preclinical studies provide the rationale for the identification of new target genes and for design of novel treatment combinations for increasing the efficacy of current immunotherapies.

Immunotherapy-Based Approaches in β-Catenin-Activated HCC Animal Models

WNT is a frequently mutated pathway in HCC, with beta β-catenin (CTNNB1) representing one of the most common driver gene in hepatocarcinogenesis. Unfortunately, β-catenin, together with other driver genes identified so far in HCC (eg, TP53), is an undruggable target.[64] Recent studies reported that β-catenin pathway activation is responsible for anti-PD1 resistance in HCC;[65] nevertheless, molecular mechanisms linking genetic alterations to treatment response are still missing. De Galarreta et al developed transgenic mouse models leading to the expression of exogenous immunogenic antigens and investigated molecular networks responsible for tumor escape from T cell-mediated immune surveillance.[66] Specifically, MYC/TP53$^{-/-}$ mice were obtained by hydrodynamic tail vein injections of transposon and CRISPR-Cas9 vectors leading to MYC overexpression and p53 deletion, respectively, directly into the liver of C57BL/6 immunocompetent mice. Different degrees of immunogenicity were obtained with the introduction of modified luciferase genes (MYC-luc) inducing the expression of exogenous antigens that, once recognized by CD8$^+$ T cells, led to tumor formation delay and improved survival. From a molecular point of view tumors that escaped from the immune system showed similarity with the CTNNB1-mutant gene signature of human HCCs and displayed Axin2 overexpression, suggesting that β-catenin activation might impair immune surveillance favoring tumor escape. Mechanistically, a reduced number of dendritic cells (DCs) and antigen-specific CD8$^+$ T cells was detected in mice experiencing tumor escape, whereas their number was restored after CCL5 chemokine overexpression, suggesting that β-catenin is determinant for immune exclusion in HCC. The same mouse model above described was employed by Chiu and coworkers to investigate the antitumor effect of anti-PD1 antibodies alone or in combination with inhibitory molecules against exhaustion markers

expressed on the external surface of CD8^{+} T cells.[67] No growth inhibition of spontaneous liver tumors was reported following anti-PD1 treatment in MYC/TP53^{−/−} mice. A comprehensive mass cytometry analysis (CyTOF) revealed the overexpression of inhibitory molecules including LAG3 (Lymphocyte Activating 3) and TIGIT (T cell Immunoreceptor with Ig and ITIM domains) in PD1+ CD8+ T cells collected from treated mice. PVRL1 (Poliovirus receptor-related protein 1), which stabilizes the TIGIT ligand PVR, is overexpressed in human HCCs with respect to surrounding tissues and correlates with poor survival. Its knockdown in mouse Hepa1-6 cells decreased tumor volume in an orthotopic syngeneic mouse model showing a greater number of TIGIT^{+} infiltrating CD8^{+} T cells. This study demonstrated the combination of TIGIT antagonists with anti-PD1 antibodies as a new therapeutic approach to improve immunotherapy efficacy in HCC and suggested PVRL1 as a biomarker for predicting anti-PD1 response. Another study employed syngeneic orthotopic models for studying the role of natural killer (NK) cells in HCC immune response by engrafting murine H22 cells into the liver of BALB/c mice.[68] Flow cytometric analysis revealed fewer conventional and liver resident NK cells and a decreased production of their effector molecules (eg, INF-γ) in tumors compared to normal livers. Of note, a low number of NKs correlated with decreased overall survival (OS) in HCC patients. In accordance, a higher expression of immune checkpoint inhibitors such as T-cell immunoglobulin and mucin domain-containing protein 3 (Tim-3), TIGIT, PD1, and LAG-3 was detected in NKs cells isolated from human HCCs. Moreover, Tim-3 levels negatively correlated with OS of HCC patients. From a molecular point of view, phosphatidylserine residues activated Tim-3 phosphorylation inhibiting the PI3K/Akt/mTOR pathway that is essential for the NK cell biology, including their development and cytotoxicity. H22 homograft together with CD3^{−}Dx5^{+} NK cells bearing Tim-3 knockdown showed a reduction in tumor growth in vivo; in line, anti-Tim-3 antibodies delayed tumor growth of T-cell–deficient nude mice bearing H22 tumors proving the central role for NK cells in liver tumor engraftment and progression. This preclinical study identified Tim-3 as a promising immunotherapeutic target in HCC, showing the complexity of the tumor milieu and the cooperation of circulating and liver resident immune-derived cell populations.

Combined Immunotherapy-Based Approaches in HCC Animal Models

Due to encouraging clinical findings from a phase Ib study,[69] syngeneic mouse models (Hepa1-6 and Hep53.4) were used to investigate molecular and immunomodulatory mechanisms of lenvatinib in association with anti-PD1 treatment.[70] The rationale behind this study resides on the multi-targeting effects of lenvatinib that, switching off the immunosuppressive and proangiogenic activity of the self-sustaining VEGF pathway, might enhance the antitumor potential and the clinical benefits of anti-PD1 treatments. In subcutaneous and orthotopic syngeneic models, both anti-PD1 and drug combination treatments reduced tumor growth and increased DC cells infiltration in the tumor microenvironment. On the contrary, only tumors from treatment combination showed a contemporaneous increase in T cell infiltration and decrease in regulatory T cells (Treg), leading to the activation of the proinflammatory cell component that is likely responsible for the higher proportion of necrotic areas observed in these tumor tissues compared to monotherapy. Interestingly, tumors from Hepa1-6 syngeneic mice clustered together with human HCCs, proving the suitability of this animal model. A transcriptomic analysis confirmed the activation of proinflammatory pathways (eg, B and T cell, chemokine signaling) in the combination arm compared to placebo. Strikingly, only tumors from the combination group were enriched in the "immune-active" class showing the presence of an adaptive T cell response; on the contrary, the anti-PD1 group showed an immune exhausted profile. Inhibition of the immunomodulatory transforming growth factor (TGFß) signaling together with impairment of the Wnt/β-catenin pathway was observed in tumors from the drug combination arm. Interestingly, signature profile analyses identified ~22% of the human HCC patients characterized by reduced proinflammatory signaling, high Treg levels, and VEGF activation that could potentially benefit from the combination therapy.

To specifically address NASH-HCC, an orthotopic syngeneic model was established by injecting the murine Hep53.4 cell line in a background of liver steatosis induced by feeding mice with a modified high-sugar high-fat diet. In line with clinical findings,[5] anti-PD1 showed no benefit on the tumor growth, immune infiltration and mouse survival in steatotic mice, whereas an antitumor effect was seen in controls. Similar findings were registered in an autochthonous model of NASH obtained by combining diethylnitrosamine (DEN) with the American lifestyle-induced obesity syndrome diet (DEN/ALIOS) showing tumor resistance to anti-PD1 treatment. The authors reported the accumulation of tumor-associated neutrophils (TANs) in several NASH-HCC models both before and after ICIs treatment. An upregulation of ligands (Cxcl1, Cxcl2, Cxcl3, Cxcl5) for

the chemokine receptor CXCR2, predominantly expressed by Ly6G+ neutrophils, was identified in DEN/ALIOS tumors. In human HCCs, neutrophil expression signatures were enriched in human NASH-HCCs compared with other etiologies and CD66b+ neutrophils expressed high levels of CXCR2 in the tumor tissue but not in the surrounding liver. The CXCR2 inhibitor AZD5069 improved the antitumor efficacy of anti-PD1 immunotherapy in both DEN-ALIOS and orthotopic NASH-HCC models by decreasing tumor burden and extending survival. Mechanistically, anti-PD1/CXCR2 inhibition determined TANs reprogramming to an anti-tumor phenotype and allowed reprogrammed TANs to locally proliferate producing granzyme B+ immune clusters physically associated with CD8+ T cells and antigen presenting cells. As the CXCR2 antagonist AZD5069 has been demonstrated to be safe in humans, this preclinical study lays the foundation for testing this novel immunotherapy-based combination in clinical trials in the well-defined NASH-HCC patient subgroup.[71] In this regard, a recent study[72] identified five tumor immune microenvironment (TIME) subtypes by performing a single-cell RNA-sequencing (scRNA-seq) analysis in samples collected from both liver cancer patients and mouse models (TP53KO C57BL/6 mice subjected to hydrodynamic tail vein injection of Myc-90ΔCtnnb1 or Myc-KRas^{G12D} transposon vectors). These authors reported the association of TANs with poor prognosis of the myeloid-cell-enriched group. CCL4+ TANs were responsible for macrophage recruitment and PD-L1-mediated T cell exhaustion; their depletion in vivo slowed down tumor progression confirming their pro-tumorigenic activity. A large degree of similarity was reported between human and mouse TANs, demonstrating the suitability of these animal models for the development of immunotherapies hitting defined immune cell populations. These studies nicely clarify how well-designed preclinical investigations addressing TIME complexity and the molecular events sustaining ICIs resistance in NASH-HCCs might open the way to mechanistically based treatments. Along this line we will hereafter recall some NASH-HCC models which investigation elucidates pathogenetic mechanisms and suggests possible therapeutic solutions.

Immunotherapy-Based Approaches in NASH-HCC Animal Models

Animal models provide a valuable contribution to the understanding of mechanistic events sustaining disease onset and progression and set the basis to design possible therapeutic interventions. As an example, we can cite the investigation of dysmetabolism and immune system interplay by Shalapour and coworkers in different animal models of NASH-HCC.[73] In this very interesting study, the authors assessed the IgA-mediated inhibition of cytotoxic CD8+ lymphocytes in the development and progression of HCC complicating NASH. They investigated multiple NASH-HCC models characterized by low/absent, moderate, high fibrosis to assess the contribution of fibrosis itself to the accumulation of IgA-expressing plasma cells in the liver. Animal models were also evaluated for their representativity of human HCC. By exploring many different models, each one informative for specific characteristics, these authors identified complex mechanisms sustaining CD8+ T lymphocytes suppression and dissected the contribution of specific factors driving the inhibition of anti-cancer immunity in the context of NASH-induced liver fibrosis. TGF beta, LPS, IL33, IL21 and CD40 were shown to induce class switch recombination of IgM expressing cells to IgA expressing cells. These last cells display a regulatory activity and inhibit anticancer immunity by interfering with the activation of cytotoxic CD8+ lymphocytes, thus fostering HCC development. Interestingly, these events occur only in the presence of fibrosis as confirmed by the investigation of a variety of NASH-HCC models with variable fibrosis levels. Indeed, liver cell-bound IgA, liver interstitial IgA and serum IgA are elevated in patients with liver fibrosis as well as in mouse models of NASH-HCC with liver fibrosis, such as the HFD MUP-uPA and the HFD fed STAM model.

In particular, the HFD fed MUP-uPA transgenic mouse of NASH-HCC couples a high fat diet with a genetic predisposition to HCC, based on high expression of urokinase plasminogen activator (uPA) in hepatocytes.[74] On this genetic background, the HFD causes ER stress and liver damage, developing NASH, with ballooning hepatocytes, inflammatory infiltrates, and bridging fibrosis, and subsequently HCC in nearly 85% of the cases. HCC morphology is very similar to the human counterpart and from a mechanistic point of view, high TNF levels and ER stress mimic the human disease. Specifically, the MUP-uPA mouse model developing HCC after 7 months of high-fat diet was shown to contain 50–100 non-recurrent mutations in the same pathways that are mutated in HCC patients. Interestingly, in this model, HCC progression is associated with the recruitment of immunosuppressive IgA+ plasma cells as it occurs in human NASH.[73] Conversely, serum IgA, liver IgA expressing plasma cells are not elevated in other HCC mouse models without fibrosis such as the DEN-induced and the HFD wild-type mouse models. Most liver IgA positive plasma cells also expressed PD-L1 and IL10, whereas IgA knockout

abolished PD-L1 expression in IgA secreting cells. In cases of a beneficial effect of anti-PD-L1 treatment, tumoral lymphocyte accumulation and decreased steatosis were observed, together with decreased liver IgA+ IL10+ cell abundance and increased activated CD8+ T cell infiltration. Interestingly, administration of broad-spectrum antibiotics reduced gut bacteria and attenuated HCC development in all mouse strains by acting on the microbial dysbiosis induced by the HFD. Remarkably, microbiota was in turn modulated by the immune alterations associated with this hypercaloric diet. This study nicely elucidates the use of different mouse models to dissect the relevance of pathologic features and the associated mechanistic events that cause immune suppression in NASH-related HCCs.

NASH-HCC animal models also help to explore the roles of commonly used drugs. Metformin is the most prescribed drug for the treatment of type 2 diabetes, and it was reported to decrease incidence of HCC in diabetic patients.[75–77] Beside clinical studies considering the HCC chemo-preventing functions of metformin, its therapeutic effects were highlighted as well. Indeed, in vitro and in vivo studies have shown that metformin exerts an antitumor effect by targeting multiple oncogenic pathways such as AMPK/mTOR, ERK1/2 and JNK1/2[78] controlling migration, invasion,[79] tumor growth[80–82] and potentiating the effects of other treatments such as trans-arterial chemo-embolization[83] or sorafenib.[84] Thus, metformin was suggested in the prevention and treatment of HCC and metabolic associated tumors. Due to its anticancer effects, data gained in animal models of NASH-HCC provide interesting suggestion for possible combination approaches with ICIs, as well. In a recent study, Wabitsch et al[85] investigated different strains of syngeneic models of HCC in mice fed by regular diet or MCD diet or choline-deficient L-amino-defined (CDAA) diet or WD, all determining NASH development. Anti-PD1 treatment inhibited orthotopic tumor growth in mice fed by normal diet, while it had no effect or even a trend towards increased tumor size in mice with NASH induced by different approaches. In the case of syngeneic cancer cells implanted subcutaneously, anti-PD-1 treatment was able to reduce their growth and weights in animals fed by regular as well as WD, suggesting that NASH impacts anti-PD-1-mediated immune responses more strongly inside the liver environment. Since CD8+T cells depletion attenuated the effect of PD1 inhibition in mice fed by regular diet, this lymphocyte population was further studied. By using an intravital imaging by 2-photon laser microscopy for in situ imaging of the microenvironmental dynamics, CD8+ T cells were compared in tumors of different HCC mouse models, fed by WD or MCD diet and they displayed a higher motility and speed in mice on normal diet when compared to animals with western diet-induced NASH. Interestingly, while CD8+ T cells infiltrating tumor tissue of animals fed by normal diet showed rapid movements, those infiltrating tumor tissue of animals fed by WD remained resident, without motility and this observation was particularly evident for both HCC and liver infiltrating CD8+ T cells. This decreased motility of intratumoral CD8+ T cells was ascribed to aberrant regulation of T cell metabolism induced by NASH microenvironment. The transcriptional profiling of hepatic CD8+ T cells from NASH-livers revealed alteration of key metabolic pathways. More in detail, a lower expression of four genes, Pck1, Adh4, Fbp1, and Adh1, related to glycolysis, fatty acid oxidation, and mitochondrial respiration turned out to be altered in CD8+ T cells derived from mice with NASH induced by CDAA. Remarkably, NASH was shown to strongly impair mitochondrial depolarization and mass in CD8+ T cells. These experimental findings not only deepen the understanding of HCC development in NASH, but also provide elements to hypothesize therapeutic interventions. Indeed, metformin alters cell energy metabolism and in in CD8+ T cells it induces a metabolic reprogramming[86] and enhances their action against infections.[87] In CD8+ T cells from mice with NASH, metformin increased mitochondrial mass and functional capacity, oxidative consumption, and enhanced CD8+ T-cell mobility in terms of cell speed and track displacement length, ultimately showing an improvement of their metabolic function and enhancing CD8+ T-cell response to anti-PD1. Metformin did not enhance anti-PD-1 effect in mice fed by normal diet, whereas its combination with PD1 inhibition showed anti-tumor effects in mice fed by methionine/choline-deficient and western diets. Finally, in this very informative study, the authors tested the contribution of metformin to anti-PD-L1 plus anti-VEGFR2, a therapeutic association approved for the first line of advanced HCC. Interestingly, metformin restores the efficacy of this treatment in mice with NASH in which anti-PD-L1 plus anti-VEGFR2 association was only marginally effective.

These findings summarize how animal models may help dissect the driver components of a complex disease. In addition, preclinical tools help to focus on the contribution of each single event, on the crucial factors driving therapeutic effects and on phenotypes of resistance to treatments, taking advantage of mechanistic understanding. Findings of such studies have relevant clinical implications, and they should be considered as hypothesis-drivers when designing clinical trials.

Table 2 Animal Models for Immunotherapy-Based Studies in HCC

Mouse Model	Mouse Strain	NASH	Treatment	Antitumor Response	References
Humice	NSG	No	Anti-PD-L1 Anti-CTLA4	Reduced tumor size	58
MYC/Tp53$^{-/-}$ Syngeneic mice (orthotopic)	C57BL/6	No	Anti-PD1 + TIGIT antagonist	Reduced tumor volume, infiltrating C8+ T cells	67
Syngeneic mice (orthotopic, subcutaneous) Knockout mice	BALB/c C57BL/6	No	Anti-Tim-3 antibodies	Reduced tumor growth, NK activation	68
Syngeneic mice (orthotopic, subcutaneous)	C57BL/6J	No	Anti-PD1 + lenvatinib	Reduced tumor growth, infiltrating CD8+ T cells and DC cells, decreased Tregs	70
Syngeneic mice (orthotopic) DEN/ALIOS	C57BL/6	Yes	Anti-PD-1 + CXCR2 inhibitor (AZD5069)	Decreased tumor burden, extended survival, TAN infiltration, Granzyme B clusters	71
MUP uPA + HFD STAM + HFD GEMM	C57BL/6 FVB/ NJ	Yes	Anti-PD-L1	Tumor eradication, lymphocytes infiltration, reduced steatosis	73
Syngeneic mice (orthotopic) + MCD or CDAA or WD	BALB/c C57BL/6 B6 (Cg)-Tyr/J B6.CXCR6-GFP	Yes	Anti-PD1 + metformin or Anti-PD-L1 + anti-VEGFR + metformin	Reduced tumor growth, increased CD8+ T cells motility	85

Abbreviation: GEMM, genetically modified mouse models.

Conclusions

Understanding pathogenic mechanisms allows to identify causative factors, biomarkers and, ultimately, therapeutic targets. The dissection of the steps and components of the pathological processes can be performed by investigating animal models reproducing the key features of diseases that may also allow to test different targeted treatments.

In the case of liver cancer, human tissues are not widely available because clinical guidelines do not recommend biopsy when imaging criteria are satisfied. A further layer of complexity stems from the heterogeneity of liver cancer, in terms of etiology, clinical and molecular features, which are now recognized to have a deep therapeutic impact. This awareness has led to the development of a broad variety of animal models, mimicking risk factors, genetic lesions, and their combinations. We can count on a wide range of animal models of HCC, each one with its own points of weakness and strength. Remarkably, the choice of the most informative model and the investigation of more models allow reliable results and dissection of mechanistic drivers. Inconsistent results might stem from the use of different strains, which however reflect the relevance that the genetic background plays for both animal models and in the human setting, making the choice of the proper model essential for cancer research. In the case of the liver, the hydrodynamic transfection allows the modulation of the genetic background easier than the establishment of transgenic strains and can be used to create models to test specific hypothesis. A further layer of complexity in the case of liver cancer is the concomitance of chronic liver disease. In this regard, we have not reported ectopic models of HCC, except for humice and a study considering both subcutaneous and orthotopic mice.[68] Indeed, most ectopic models lack several pivotal features of human HCC, including the TME. Remarkably, ectopic tumors are devoid of the liver-specific extracellular matrix, the complex liver cell infiltrate, as well as of the peculiar liver vascular supply. All these features can make a relevant difference when drugs and non-conventional treatments, such as nucleic acids, are concerned. Specifically, not only the type of blood supply is relevant for drug delivery but also the hyperactivation of the stromal component that can impair the extent to which tumors are reached by any kind of drug. Cho et al showed how specific oncogenic pathways, such as the yes-associated protein/transcriptional co-activator with PDZ-binding motif (YAP/TAZ) model, can lead to a constitutive

activation of the stromal component impairing the penetration of treatments into the cancer tissue.[88] In fact, besides molecular dissection of mechanisms, animal models are pivotal for therapeutic approaches. Experimental tools include not only models mimicking different risk factors and molecular subgroups but also a wide range of tumor induction techniques, since the liver is highly receptive for any kind of molecules, including nucleic acids and viral vectors. All these opportunities suggest that animal models might also be used to inform the design of clinical trials, providing the rationale to test novel approaches in selected subgroups and resulting in more focused and successful clinical investigations.

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