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Thinking Out Loud

"Inside-Out" or "Outside-In": Choosing the Right Model of Hepatocellular Cancer

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The incidence of hepatocellular cancer (HCC) is gradually rising. HCC occurs as a sequela to various chronic liver diseases and ensuing cirrhosis. There have been many therapies approved for unresectable HCC in the last 5 years, including immune checkpoint inhibitors, and the overall response rates have improved. However, there are many cases that do not respond, and personalized medicine is lacking, making HCC an unmet clinical need. Generation of appropriate animal models have been key to our understanding of HCC. Based on the overall concept of hepatocarcinogenesis, two major categories of animal models are discussed herein that can be useful to address specific questions. One category is described as the "outside-in" model of HCC and is based on the premise that it takes decades of hepatocyte injury, death, wound healing, and regeneration to eventually lead to DNA damage and mutations in a hepatocyte, which initiates tumorigenesis. Several animal models have been generated, which attempt to recapitulate this complex tissue damage and cellular interplay through genetics, diets, and toxins. The second category is the "inside-out" model of HCC, where clinically relevant genes can be coexpressed in a small subset of hepatocytes to yield a tumor, which matches HCC subsets in gene expression. This model has been made possible in part by the widely available molecular characterization of HCC, and in part by modalities like sleeping beauty transposon/transposase, Crispr/Cas9, and hydrodynamic tail vein injection. These two categories of HCC have distinct pros and cons, which are discussed in this Thinking Out Loud article.

Key words: Hepatocellular cancer (HCC); Animal models; "Outside-in" model; "Inside-out" model

Chronic liver diseases due to a variety of etiologies including viral hepatitis, nonalcoholic fatty liver disease, alcoholic liver disease, and others remain a major cause of morbidity and mortality worldwide. In fact, around 2 million patients succumb every year due to some form of liver disease[1]. A major cause of liver-related mortality is cirrhosis, which is the end result of progressive liver fibrosis due to any underlying etiology. In 2017, cirrhosis caused more than 1.32 million deaths in females and 883,000 in males globally[2]. Currently, cirrhosis is the 11th leading cause of death worldwide[1]. Another major sequela of chronic liver diseases and cirrhosis is hepatocellular cancer (HCC). Almost 90% of HCCs develop in the background of ongoing chronic liver injury, advanced fibrosis, or cirrhosis[3]. The rare development of a de novo HCC in a healthy liver is mostly due to malignant transformation of benign liver tumor such as hepatocellular adenoma[4]. As the incidence of chronic liver diseases increases albeit due to distinct etiologies such a viral hepatitis in Asia, and nonalcoholic fatty liver disease (NAFLD) and alcoholic liver disease (ALD) in the US and Europe, the incidence of HCC has also increased in tandem[1]. Worldwide, for both sexes together, HCC is the sixth most commonly diagnosed cancer and fourth leading cause of death related to cancers[5]. It

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occurs two to three times more in men and thus is the fifth commonest malignancy in men and the ninth commonest cancer in women. In the US, while it only constitutes 2.4% of all tumors, its incidence has been increasing gradually for the last 3 decades[6]. Also, the 5-year survival rate of patients with liver tumors is less than 20%. In fact, around 32,000 patients died due to liver tumors in 2019 in the US. While the advent of immunotherapy and approval of additional agents for medical management of unresectable HCC have improved the overall prognosis, the treatments are suboptimal and nonpersonalized, despite the knowledge of key drivers of hepatocarcinogenesis[7]. Thus, HCC remains a major unmet clinical need requiring additional understanding through generation of better animal models representing human HCC subsets.

As mentioned, HCC occurs as a consequence of years of chronic liver injury. In fact, the evolution of HCC is a result of sometimes decades of chronic insult and ensuing repair. In most chronic liver diseases, the primary cell afflicted by an injurious agent, be it a virus [hepatitis B (HBV) and hepatitis C (HCV)], toxin like alcohol, or metabolic stressors as in NAFLD, is the hepatocyte. As the hepatocytes are injured, they die only to be replaced by proliferation of neighboring hepatocytes or dedifferentiate and adapt to escape injury, but lose function, which may also trigger proliferation of other hepatocytes, as overall hepatic function is compromised and realized. In addition, a well-differentiated hepatocyte is anti-inflammatory and proactively suppresses immune cell infiltration as has been shown by effect on spontaneous inflammation after conditional deletion of HNF4α or mir-122 from the liver[8,9]. Hepatocyte injury through generation of damaged-associated molecular patterns (DAMPs) or dedifferentiation triggers immune response, partially driven by the specific etiology (alcohol vs. metabolic vs. viral injury) of hepatocyte injury (Fig. 1).

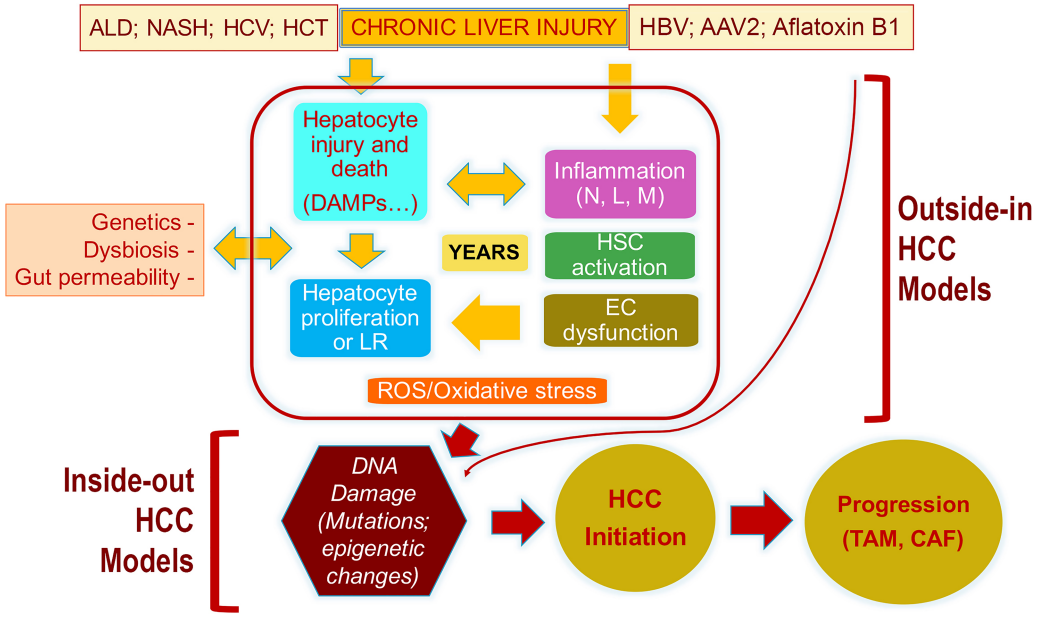

Figure 1. The pathogenesis of HCC and the descriptions of events captured by the "outside-in" and the "inside-out" models of HCC development. Because of any of the noted chronic injuries to the liver (ALD, alcoholic liver disease; NASH, nonalcoholic steatohepatitis; HCV, hepatitis C virus; HCT, hemochromatosis), there is hepatocyte injury, release of DAMPs, inflammation (N, neutrophils; L, lymphocytes; M, macrophages), hepatic stellate cell (HSC) activation, and endothelial cell (EC) dysfunction, all of which result in the presence of reactive oxygen species (ROS) and oxidative stress. Loss of hepatocyte viability or differentiation also induces hepatocyte replication, which in the presence of ROS/oxidative stress is prone to DNA damage and, eventually, mutations or misexpression of genes. Any such alteration that provides significant growth and survival advantage to a hepatocyte is really the initiation of malignant transformation and HCC development. Once HCC develops, it creates its own microenvironment in the form of cancer-associated fibroblasts (CAFs) and tumor-associated macrophages (TAMs), which help in further perpetuation of the tumor. There are models that are more relevant in studying events leading up to the transformation and are referred to as "outside-in" models as these address the microenvironmental changes that are upstream of DNA damage and mutations in the hepatocyte. There are models that utilize expression of sets of genes (normal or mutant) in a subset of normal hepatocytes, which lead to tumor initiation and growth and are referred to as "inside-out" models as the expression of these sets of genes/shRNA is sufficient to induce clinically relevant HCC quite promptly without the need for any previous injury or fibrosis. This suggests that once such DNA aberrations occur in a hepatocyte and the cell is transformed, it does not require any additional cues from the microenvironment to grow and propagate, and the tumor then creates its own microenvironment, which is optimal for its development.

Immune cell infiltration including macrophages can be sustained by continued insult and often cross-talks with stellate cells, leading to their activation and fibrogenesis[10], which can also be triggered directly by hepatocyte injury. Stellate cell activation to myofibroblast occurs as a wound healing process, but chronic and sustained collagen deposition itself can contribute to hepatic dysfunction and eventually to cirrhosis and can also contribute to the environment that is permissive to HCC development[11]. Immune cells and stellate cells can also impact sinusoidal endothelial cell function and can undergo capillarization to further perpetuate immune cell infiltration, stellate cell activation, and hepatic dysfunction[12]. Several cell-extrinsic and cell-intrinsic factors, such as gut dysbiosis[13], gut permeability[14], genetic variants such as *PNPLA3* polymorphisms[15], and alterations in bile acid metabolism[16], can also impact the overall adverse hepatic milieu. Another common feature of chronic liver injury is the ductular reaction composed of hyperproliferating cholangiocytes[17]. There is evidence that this ductular reaction may in part be a transdifferentiation mechanism giving rise to hepatocytes and, thus, contributing to hepatocyte repair[18,19]. However, these reactive ductules can also be proinflammatory and profibrogenic and thus can also contribute to an adverse microenvironment in the liver[17].

Thus, chronic liver diseases are characterized by cycles of hepatocyte injury, immune cell infiltration, stellate cell activation, and endothelial cell dysfunction, which provide a signal to relatively healthy hepatocytes due to innate heterogeneity within these cells owing to zonation and ploidy, to proliferate and allow liver regeneration. Chronic hepatocyte proliferation in an adverse milieu of inflammation and fibrosis, which are associated with oxidative stress (partially dependent on primary etiology) from generation of reactive oxygen species, can lead to DNA damage, errors in DNA repair, and mutations, especially in vulnerable replicating cells[20]. Most of the DNA damage is repaired through a host of mechanisms, and only a rare cell that is unable to repair may undergo senescence. However, DNA repair mechanisms can sometimes be deficient, leading to an error that can eventually give rise to mutations or epigenetic alterations. If such a change, especially a mutation, yields a survival or a proliferative advantage to a hepatocyte within this adverse microenvironment of existing inflammation, oxidative stress, fibrosis, and endothelial cell dysfunction, this cell will outgrow and outcompete other cells and thus mark the beginning of neoplasia. Over time, these cells may gain additional mutational events and will continue to grow and expand and lead to development of HCC. Thus, HCC can very well be defined as an adaptation and survival response of a subset of hepatocytes during chronic wound healing and may very well represent the dark side of the liver's ability and will regenerate to maintain its functional mass. It is relevant to mention that some injuries to the liver can bypass these chronic bouts of injury, regeneration, and fibrosis by directly impacting DNA and inducing mutations that provide cells with replicative and survival advantage as in HBV, adeno-associated virus 2 (AAV2), and aflatoxin exposure.

The mutations in hepatocytes are key to the origin of HCC. Gain-of-function (GOF) mutations in *TERT* promoter are the earliest mutations to be mapped through analysis of very early preneoplastic nodules in patients with cirrhosis[21]. Other earlier mutations in HCC include loss-of function (LOF) mutations in *P53* and GOF mutations in *CTNNB1*, the gene encoding for β-catenin[21,22]. Further, these mutations in *TERT, P53*, and *CTNNB1* have also been ascribed as trunk mutations based on their presence in early lesions, their presence in sampling of more than one part of a large tumor, their presence in all nodules in multinodular tumors, and their presence in both primary and metastatic site[22]. As tumors evolve, they gain additional mutations, and the spectra of common mutations in HCC is now well described as mostly limited to around 30–35 genes with varying frequency. The top few leading mutations are those affecting *TERT* promoter, *P53, CTNNB1, ARID1A, ALB, AXIN1, APOB, CDKN2A, EEF1A1, ARID2, RPS6KA3, SMARCA4*, and *NFE2L2,* among others[23,24]. These various mutations have been uniformly observed through whole-exome sequencing (WES) of several independent HCCs in various databases, suggesting their potential relevance in disease initiation and progression. Intriguingly, most studies have been limited to analysis of relatively early stage lesions by virtue of sampling bias since the available materials for WES are often tumors available in explanted or hepatectomized livers. A recent study analyzed HCCs ranging from early to advanced stages for various well-known mutations[25]. This analysis showed persistence of the commonly mutated genes (*TERT* promoter, *TP53*, *CTNNB1*, *ARID1A*, etc.) irrespective of the stage of the disease. Intriguingly, this study identified enrichment of *P53* mutations in a large series of HCCs in their cohort, while mutations in *TERT* promoter or *CTNNB1* were more or less comparably distributed across HCCs at various BCLC stages[25]. Lastly, as now shown in multiple studies, there are some mutations in HCC that are mutually exclusive, while others show significant association and often coexist. A classic example is that of the significant association of *CTNNB1* mutations with *TERT* promoter, *NFE2L2*, *ARID2*, and *RPS6KA3*, while *CTNNB1* mutations never occur with mutations in *AXIN1*[23–25]. All of these clinical observations are highly relevant and should be considered when generating animal models of human HCC.

Chemical carcinogenesis using agents such as diethylnitrosamine (DEN), which leads to DNA adducts, has

been a popular means to study HCC in rodents. Around 70% of DEN-induced HCC in C3H/He mice, however, have been shown to be due to mutations in genes such a Ha-ras and B-raf and hence very dissimilar from clinical mutational spectra[26]. Since HCC in patients is almost always associated with ongoing injury, inflammation, and fibrosis, mouse models have been generated to induce such chronic insult by high doses of repeated DEN or combining DEN with toxicants like alcohol[27], carbon-tetrachloride[28,29], or Western diet[30,31]. Others have combined even three forms of injury or more to create an even optimal environment and accelerate HCC development in rodents[30,32]. Several genetic knockout or transgenic mice can also demonstrate spontaneous injury, fibrosis, and HCC, such as hepatocyte-specific PDGF-CC transgenic mice[33] and hepatocyte-specific miRNA-122 knockout mice[9], or can be combined with insults such as feeding high-fat diet to the major urinary protein-urokinase-type plasminogen activator transgenic or the MUP-uPA mice, to yield chronic injury and HCC development[34]. However, while these "outside-in" models lend themselves well to studying various specific events leading up to tumorigenesis such as mechanisms of hepatocyte injury, induction of immune response, and mechanisms of stellate cell activation and fibrosis, the mutational spectra of the observed tumors are heterogeneous and distinct from what is observed in patients (Table 1). Further complicating these studies, other than chronicity of events, are how mice differ from patients in their immune cell composition and signaling[35], relative resistance, and differences in fibrosis[36], and other modifiers of chronic liver injury such as gut microbiota[37] and bile acid metabolism[16]. Thus, while the "outside-in" models of HCC have distinct advantages in terms of studying the underlying mechanisms of injury involving various cell types that lead to initiation of dysplasia, the molecular underpinnings in the process of hepatocarcinogenesis itself, from initiation to progression, are distinct and disparate from humans. Thus, these models should be cautiously used to study tumor biology, tumor microenvironment, and therapeutics.

Another way to study human HCC in preclinical models is the sleeping beauty transposon/transposase

Table 1. Examples of "Outside-In" and "Inside-Out" Models of Liver Tumors

	Type	Phenotype	Reference(s)
Outside-in models			
DEN + carbon tetrachloride	Chemical carcinogen + injury	Liver inflammation, fibrosis, and hepatocellular carcinoma	27,28
DEN + alcohol	Chemical carcinogen + injury	Liver inflammation, fibrosis, and hepatocellular carcinoma	27
DEN + high-fat diet	Chemical carcinogen + injury	Obesity, liver inflammation, and hepatocellular carcinoma	30,31
DEN + alcohol + carbon tetrachloride	Chemical carcinogen + injury + injury	Liver inflammation, fibrosis, cirrhosis, and hepatocellular carcinoma	32
PDGF-CC transgenic mice	Spontaneous genetic mouse model	Fibrosis, adenomas, and hepatocellular carcinoma	33
Liver-specific miR-122 knockout mice	Spontaneous genetic mouse model	Fatty liver, liver inflammation, and hepatocellular cancer	9
MUP-uPA transgenic mice + high fat diet	Genetic mouse model + injury	Fatty liver, liver inflammation, and hepatocellular cancer	34
Outside-in models			
S127AYap-S45Y/S33Y-β-catenin or S127AYap-Δ90-β-catenin	SBTT-HDTVI	Hepatoblastoma	49,50
Met-S45Y/S33Y-β-catenin or Met-Δ90-β-catenin	SBTT-HDTVI	Hepatocellular cancer	45,47,52
Met-sgAxin1	SBTT-HDTVI	Hepatocellular cancer	47
MYC-lucOS-N90-CTNNB1	SBTT-HDTVI (immunogenic)	Liver inflammation and hepatocellular cancer	41
MYC-lucOS-sgp53	SBTT (immunogenic), and CRISPR based HDTVI	Liver inflammation and hepatocellular cancer	41
myr-AKT-YapS127A	SBTT-HDTVI	Intrahepatic cholangiocarcinoma	53
myr-AKT-NICD	SBTT-HDTVI	Intrahepatic cholangiocarcinoma	51

DEN, diethylnitrosamine; MUP-uPA, urokinase plasminogen activator overexpression under hepatocyte-specific major urinary protein promoter; PDGF-CC, platelet derived growth factor-CC; SBTT-HDTVI, sleeping beauty transposon-transposase and hydrodynamic tail vein injection; NICD, notch intracellular domain.

(SBTT)-mediated stable expression of clinically relevant combination of genes found to be overexpressed or mutated in patients[38]. Delivered to a subset of hepatocytes in vivo in mice by a hydrodynamic tail vein injection (HTVI), this methodology is able to directly address the relevance of specific genes in the carcinogenesis. This "inside-out" model relies on the selection of gene or combinations of genes for expression in 1%–5% of hepatocytes, based on data from Whole Genome Sequencing (WGS) or WES studies performed on HCCs from patients. Several publically available databases including The Cancer Genome Atlas (TCGA)[39], Catalogue of Somatic Mutations in Cancer (COSMIC)[40], and others have curated information on several well-characterized HCC patient cohorts. Other groups have collected and characterized their own HCC cohorts and hence generated their own databases, which may be available through collaborations[24]. However, multiple analyses have now validated the presence of common alterations including mutations and changes in gene expression across multiple cohorts of HCC cases[23,24]. Having such validated data from patients provides a unique and impactful opportunity to generate these "inside-out" models and also allows to test the relevance of such alterations if they are truly an oncogenic and driver, or just secondary, bystander, and passenger events. The premise behind these models is that irrespective of upstream events, if the DNA errors or mutations are the end result of years of damage due to chronic liver injury and are truly the initiator events for HCC, then expression of those aberrations, singly or in combination (based on clinical data), would be sufficient to lead to tumorigenesis even in a normal liver and without the need of any other microenvironmental cues. Indeed, many studies using SB-HTVI have now conclusively demonstrated the feasibility of this "inside-out" model and the lack of requirement of an adverse microenvironment to induce or propagate a tumor, as long as the initiator event of expression of specific DNA aberrations has occurred in a healthy hepatocyte. Once these mutations or misexpression occurs and a tumor is induced, the tumor cells will create their own microenvironment, such as generating cancer-associated fibroblasts or tumor-associated macrophages, which will be conducive to the survival and growth of tumor cells via autocrine, paracrine, or endocrine signals. The disadvantage of this model is the lack of its suitability to study mechanisms of injury leading up to the cancer, but the advantage of this model is its appropriateness to study tumor biology, biomarker discovery, and therapies since these tumors are induced by clinically relevant genes and show high similarity in gene expression to the respective human HCC subsets. Since these models are typically immune naive, more recently, use of artificial antigens in plasmids has even enabled studies of tumor immunology in these SB-HTVI-induced HCC models[41].

Our group along with collaborators have focused on *CTNNB1* mutations as these have been described to be consistently present in early and advanced HCC in around 25%–35% of all HCC cases. Knowing that expression of *CTNNB1* (point mutant or deletion mutant) alone is insufficient to induce HCC in murine models[42–44], and knowing that *CTNNB1* mutations occur frequently with other mutations such as TERT promoter, NFE2L2, and others[23–25], or overexpression of genes such as Met (or downstream Ras) and Myc[41,45,46], we have used SB-HTVI to coexpress mutant CTNNB1 and the "second-hit." Similarly, LOF mutations in *AXIN1,* which are mutually exclusive from *CTNNB1* in their occurrence, have also been coexpressed as shRNA along with Met to yield HCC[47]. All of these models demonstrate a clear cooperation of these abnormal genes in HCC pathogenesis, which significantly resemble subsets of human HCCs at a molecular level. This has also been extended to other liver tumors like hepatoblastoma and intrahepatic cholangiocarcinoma. Since around 80% of all hepatoblastomas in patients showed activation of β-catenin because of mutations or deletions, and yes associated protein-1 (Yap1) due to unidentified reasons[48], coexpression of these two genes in mouse liver using SB-HTVI led to the development of hepatoblastoma[49,50]. Also, coexpression of myristoylated-Akt and notch intracellular domain (NICD) led to the development of intrahepatic cholangiocarcinoma[51]. All of these findings clearly suggest that coexpression of specific clinically observed mutant genes or aberrantly expressed genes in a subset of hepatocytes in a normal adult liver is sufficient to lead to development of liver tumors.

To conclude, the preclinical models of HCC that exist have been highly useful in elucidating the cellular and molecular basis of this dreadful disease. Based on the question that needs to be addressed, an investigator should select the most appropriate model that may help in answering a specific question of interest after weighing the many pros and cons of each model. The "outside-in" models are of clear value in investigating the mechanisms leading up to the cancer and even in devising chemopreventive strategies. However, one must be cognizant of limitations imposed by innate differences between a mouse model and humans. The "inside-out" model is of value in using clinically relevant genes identified in HCCs in patients to address tumor biology specific to the genetic aberrations and to study biomarkers and therapy. This reductionist approach leads to clinically relevant tumors in mice while precluding opportunities to study mechanisms leading up to tumor initiation. Both categories of HCC models thus have unique applications in oncology research.

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