Somato Publications ISSN: 2643-2609

Annals of Gastroenterology and Digestive Disorders

Review Article

Review: Modeling in Situ Liver Cancer

Shyama Sasikumar[2#], Matthew Brovold[1#], Rohan Shirwaiker[3], Falguni Pati[2], and Shay Soker[1*]

[1]Wake Forest Institute for Regenerative Medicine, Wake Forest Baptist Medical Center, Medical Center Boulevard, Winston-Salem, North Carolina 27157, USA

[2]Department of Biomedical Engineering, Indian Institute of Technology, Hyderabad, Sangareddy, 502285, Telangana, India

[3]Department of Industrial and Systems Engineering, North Carolina State University, Raleigh, NC 27695, USA

[#]Equal Contribution

*Address for Correspondence: Shay Soker, Wake Forest Institute for Regenerative Medicine, Wake Forest Baptist Medical Center, Medical Center Boulevard, Winston-Salem, North Carolina 27157, USA, Tel: 336-713-7295; E-Mail: ssoker@wakehealth.edu

Received: 28 July 2020; Accepted: 23 October 2020; Published: 24 October 2020

Citation of this article: Sasikumar, S., Brovold, M., Shirwaikar, R., Pati, F., Soker, S. (2020) Review: Modeling in Situ Liver Cancer. Ann Gastroenterol Dig Dis, 3(1): 27-46.

Abstract

Hepatocellular carcinoma (HCC) is the most common form of liver malignancy and the one of the leading causes of deathworld-wide and within the United States. The poor prognosis associated with HCC is due to resistance to chemotherapy and high recurrence rates accompanying other treatment strategies. Traditional models fail to replicate the tumor microenvironment (TME) as well as the complex interactions between the malignant cells and their stroma. *In vitro* hepatic models are essential instruments for understanding the molecular mechanisms of hepatocellular carcinoma while additionally benefiting drug discovery and development. By mimicking tumor stroma interactions present in the native tumor microenvironment,along with the incorporation of relevant cell types and underlying pathologies, has opened new avenues of research for pharmaceutical and clinical applications. These models also enable the development of personalized treatment strategies that addresses the heterogeneity observed in hepatocellular carcinomas. This review focuses on the *in vitro* models that are currently available and their prospects for elucidating the interactions occurring in the tumor microenvironment and chemotherapy.

Keywords: hepatocellular carcinoma, tumor microenvironment, *in vitro* HCC modeling, personalized medicine, liver cancer

Introduction

Hepatocellular carcinoma (HCC) is a major cause of death worldwideand is responsible for approximately 780,000 deaths per year [1,2]. In the United States alone approximately 30,000 deaths are estimated to occur in 2020, which hasbeen on the rise forthe last several decades. Chronic liver diseases and cirrhosis are the major risk factors for the development of HCC, with excessive alcohol intake and viral hepatitis being the lead factors[3,4]. Chronic medical conditions such as obesity and diabetes mellitus can also increase the risk of HCC [5,6]. Gender is a factor, males are more susceptible than females by a ratio of 2:1 – 4:1 [7]. Additionally, persons exposed to aflatoxin [8] or those who suffer from metabolic disorders such as hemochromatosis [9], Wilson's disease [10], tyrosinemia [11], α-1 antitrypsin disease [12], glycogen-storage disease types I and II, and porphyrias [13] are also at increased risk.

HCC develops and progresses by several means one of which is the dysregulation of critical cellular pathways such as cell cycle and apoptosis where mutations arise in proteins involved in the regulation of cell cycle [14]. Hence, most of the advances in systemic treatment of HCC involve targeting the proteins such as cyclin dependent kinases (CDKs) and growth factors [15] such as tyrosine kinase inhibitors (sorafenib [16], regorafenib [17], cabozantinib [18]) and angiogenesis inhibitors (lenvatinib[19]). Apart from chemotherapy, orthotopic liver transplantation (OLT) is the common treatment for HCC [20]. Liver resection (LR) is another treatment strategy considered for HCC, which eases the pressure on the demand for donor organs. However, the development of HCC is tightly linked to underlying chronic liver conditions, such as cirrhosis, where the option of resection can cause a higher occurrence risk [21]. Interventional therapies are generally palliative, and, in combination with repetitive treatments, are used to facilitate the resection or OLT. However, the treatment of HCC is complex as the numerous underlying conditions associated with the disease limits therapeutic possibilities. Therefore, individual factors must be accounted for, including the tumor biologyitself,-to make an appropriate patient evaluation leading to an effective treatment strategy [22].

Liver fibrosis is a risk factor for the development of HCC, which is an underlying condition in approximately 90% of patients diagnosed with HCC. When hepatocytes, the parenchymal cell of the liver, are repeatedly injured produce reactive oxygen species (ROS) and other growth factors that mediate fibrosis and induces hepatic stellate cell activation (HSCs) [23]. Activation is a key cellular event that occurs within HSCs that induces a large phenotypic shift. This leads to the formation of myofibroblasts that are capable of producing large amounts of extracellular matrix (ECM) molecules and pro-inflammatory molecules, both being central to fibrosis [24]. As HCC progresses, the tumor becomes immersed in a complex web of interactions consisting of signaling factors, cells, and structural components collectively referred to as the tumor microenvironment (TME) (Figure 1). The dynamic interactions within the TME highly influence the progression and outcomes of HCC. This review outlines some pertinent HCC background and briefly describes several models developed for HCC from traditional modeling systems (animal and standard tissue culture plate) to advanced modeling techniques (3D cell culture, bioprinting, microfluidics) and an assessment for future development of HCC modeling *in vitro*.

The cellular components of HCC tumor microenvironment

Several different cells comprise the cellular stromal compartment of the TME. Constituent cell types include the tumor infiltrating lymphocytes (TILs), primary immune cells, mainly composed of $CD4^+$ T_H cells, also known as Tregs, that normally account for the host's primary anti-tumor response [25]. However, within the TME, they can induce immune tolerance to the neoplastic cells. Myeloid-derived suppressor cells (MDSC) play a role in T-cell regulation and are also involved in immune suppression within the TME [26]. Tumor associated macrophages (TAM), mainly derived from Kupffer cells within the liver, produce an immuno-inhibitory environment effectively reducing antigen presentation and diminishing the hosts ability for immuno-reduction of the tumor via T-cells. TAMs also produce a pro-inflammatory environment by the secretion of IL-6, which is found in abundance in patients

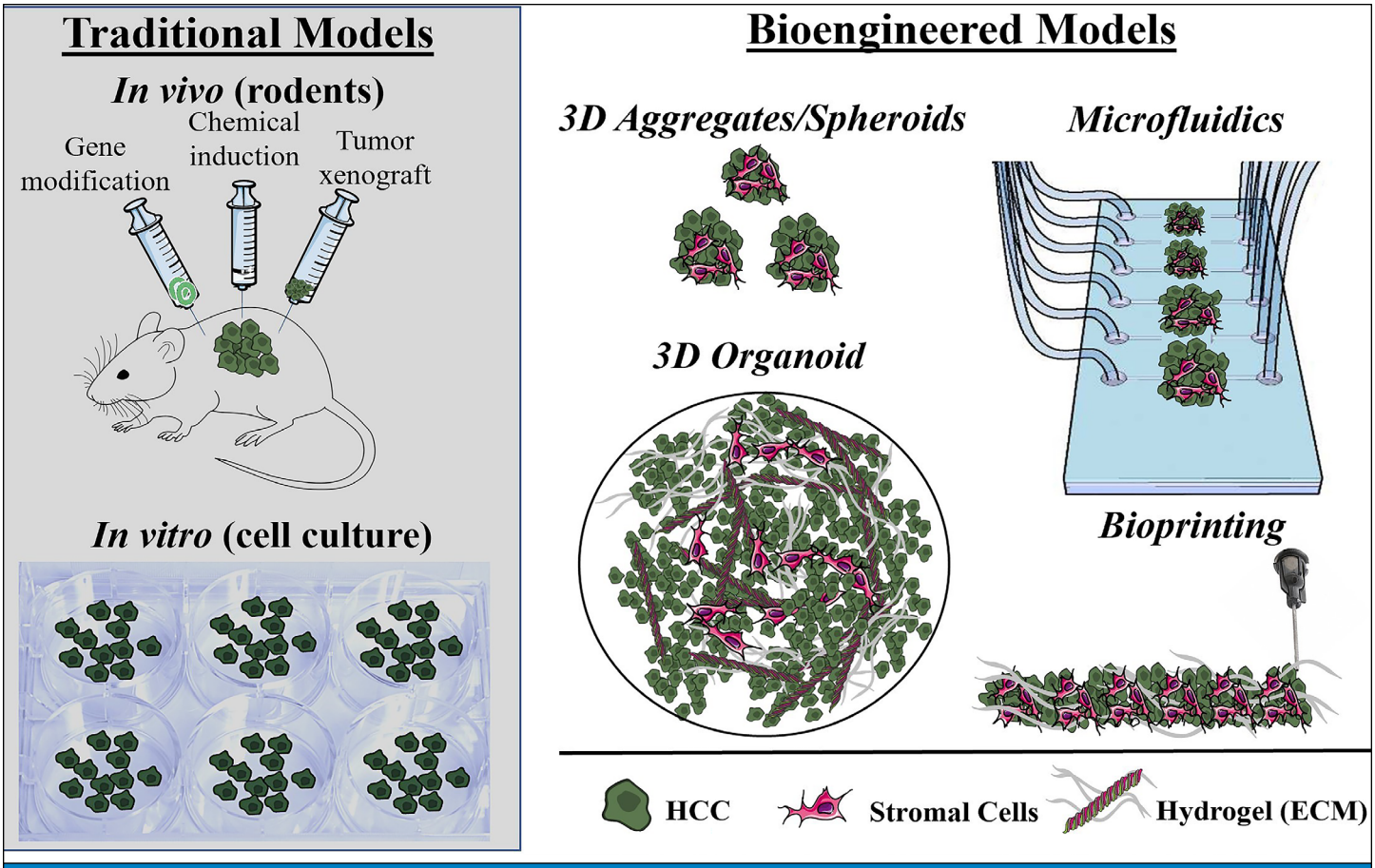

Figure 1: HCC models

Traditional models consist of in vivo rodent models and in vitro cell culture models. New bioengineering technologies support the development of advanced models including 3D cell aggregates, spheroids and organoids enabled by microfluidics and bioprinting.

with poor prognoses [27]. Angiogenic endothelial cells, derived from sinusoid endothelial cells (LSEC), are induced into proliferation and sprouting by HCC that produces platelet derived growth factor (PDGF) in response to hypoxia within the core of the rapidly growing tumor. Cancer associated fibroblasts (CAF), mainly derived from HSCs, play a major role in the physical makeup of the TME. Upon transformation to their activated/myofibroblastic state, HSCs are also the primary cellular agent of liver fibrosis.

Paracrine signaling by Kupffer cells and hepatocytes also play an important role in the process of liver fibrosis. Kupffer cells produce PDGF which is involved in the activation of HSCs [28]. Transforming growth factor beta (TGF-β) is a potent stimulator of fibrosis and isproduced by multiple cell types within the liver, such as hepatocytes, Kupffer cells and endothelial cells. TGF- β directly activatesHSCs, which become highly fibrogenic, also effectively inhibits further degradation of the ECM [29]. Chronic inflammation,as well as the presence of underlying disease conditions,can lead to the production of ROS, causing oxidative stress that can generate mutations within the DNA, contributing to carcinogenesis [30]. Mutations have been found within the telomerase, Wnt signaling pathway, mTOR signaling, p^{53} and Ras signaling [31]. Chronic inflammation also alters the blood flow as the hypoxic hepatocytes produce reactive nitrogen species (RNS) [32]. Hypoxia induces the production of growth factors like vascular endothelial growth factor (VEGF) leading to angiogenesis and sprouting of new blood vessels further facilitating tumor growth [33]. Moreover, the capability of hepatocytes to regenerate predisposes them to malignant transformation [34]. Collectively, the aberrant signaling of these molecules initiates cellular transformation of liver cells into HCC. As HCC progresses, these same cellular and

environmental components makeup the TME.

A large majority of patients with HCC have fibrotic/cirrhotic livers, as such further elucidation of such crosstalk is greatly needed. HSCs induce and influence HCC, that reciprocally induce HSC's into a more pro-fibrotic state further physically altering the TME-via ECM remodeling. ECM modifications such as increased collagen crosslinking, unchecked collagen synthesis, and excess stiffness promote tumorigenesis by activating integrin signaling [35], enabling enhanced survival and proliferation of tumor cells [36]. Similarly, CAF's transformed from HSCs also produce a litany of growth factors, cytokines, and chemokines. Growth factors such as hepatocyte growth factor (HGF) secreted by CAF's can increase tumor cell proliferation, or TGF-β, which when synthesized and secreted at high levels, promotes cancer progression in later stages. SDF-1 and multiple species of CCL/CXCL secreted by CAFs promotes immune cell infiltration as well as promoting tumor progression and angiogenesis [37]. CAFs secrete various growth factors such asVEGF, matrix metalloproteases (MMPs), interferon γ (IFN-γ), interleukin 6 (IL-6) and tumor necrosis factor (TNF) and are known to suppress anti-tumor immunity in HCC [38-40]. Overall, a better understanding of the interplay of all the cell types within the TME will help in the identification of new therapeutic targets for HCC. Ultimately, the incorporation of multiple cell types, along with the ECM component present in the diseased liver, will allow tumor models to mimic the *in vivo* tumor microenvironment more effectively.

HCC Modeling

To study the molecular mechanism of the pathogenesis several HCC models have been developed with various degrees of complexity in order to mimic different aspects of the tumor such as cell migration, intravasation, invasion [41], matrix remodeling [42] and angiogenesis [43]. The ideal *in vitro* model of HCC would incorporate all the different aspects the underlying disease condition including the liver microenvironment, all cell types involved, and fibrosis. This review briefly describes several recently developed models for HCC from traditional modeling systems (animal and standard tissue culture plate) to advanced modeling techniques (3D cell culture, bioprinting, microfluidics) and an assessment for future development of HCC modeling *in vitro*.

Multiple *in vivo* and in *vitro* models are currently available to study the molecular mechanisms and markersfound within HCC, some of which are also useful as drug screening and discovery platforms. *In vitro* systems have helped make advances in understanding the mechanisms of cancer. Most insights into tumor cells, the TME, and drug screening to date have come from *in vitro* models. However, the use of traditional modeling techniques, such as standard 2D tissue plate culture, has often led to confounding results when compared to that of *in situ* HCC found within the highly dynamic native microenvironment. Animal models overcome some aspects of modeling that cannot be achieved in traditional *in vitro* models of HCC. *In vivo* models mainly rely on small rodents as a platform. Such model systems include all of the relevant cell types (save the xenotransplant models) as found in humans, and contain the similar hepatic microenvironmental structures; however, these animal models have downsides in terms of species specific genetic differences, cost, low throughput and clinical translatability that make *in vivo* models a challenge to successfully use as platforms for further discovery.

In vivo models

Small rodent models, used for studying the pathogenesis of HCC, have expanded the current understanding of HCC. Rodent models are known for their ease of breeding, short life span, which is why traditionally rodents have been commonly used as models for studying HCC. HCC has been induced chemically in several different species of rodents, but mainly in rats. Mouse models can be induced chemically,but also have been used for gene targeting and xenograft implantation. Multiple compounds have been used successfully to induce HCC and are listed in Table 1 [44]. Chemically induced models show the injury-fibrosis-malignancy cycle as seen in humans. Diethylnitrosamine (DEN) induced HCC models show similar gene expression patterns seen in human HCC patients with poor prognosis. Although chemically induced carcinogenesis has simulated the cellular alterations and histopathological patterns, there are genetic and physiologic differences between rodents and humans which may have led to erroneous conclusions.

Genetically engineered mouse (GEM) models [45] allow the study of HCC initiation and progression, within the context of oncogenes, in the presence or absence of carcinogenic agents [46]. There

Table 1: Induction agents of in vitro HCC models

Compound	Mechanism of toxicity	Mode of administration	Time for tumor development	Metastasis	Characteristics	References
DEN	Genotoxic, alkylates DNA	Oral, intra peritoneal	45–104 weeks	-	Gender based differences in tumor induction with more tumor formation in males, human HCC features like fibrosis and cirrhosis are not observed	[89-91]
Peroxisome proliferators	Activates the peroxisome proliferator activated receptor a (PPARα), which regulates the expression of various genes that are involved in cell proliferation and apoptosis, induces mutations in the DNA, due to increased intracellular concentration of H2O2	Through diet	50–100 weeks	Yes	Well-defined HCC with a trabecular pattern, inter species differences in PPARa biology	[92-97]
Aflatoxin B1	Genotoxic, metabolized by liver microsomes into exo-8,9-epoxide, an intermediate that binds specifically to guanine residues in the DNA	Bolus injection	Early HCC in 52 weeks, high grade HCC 92–110 weeks	Yes	Genetic alterations in mice differed from the genetic alterations found in humans	[98, 99]
Carbon tetrachloride (CCl4)	Carcinogenic, metabolized to trichloromethyl radicals by cytochrome P450, it causes lipid peroxidation and membrane damage	Gavage, intra peritoneal	104 weeks	Yes	Maintain inflammatory and fibrogenic characters similar to human HCC	[100, 101]
Choline deficient diet (CDD)	Leads to oxidative damage to DNA and chromosomal instability as a result of the reduction in the hepatic antioxidant mechanisms		50–52 weeks	-	Associated with steatohepatitis	[101, 102]
Thioacetamide (TAA)	Carcinogenic, leads to hepatic oxidative stress and damage to the liver	With drinking water, intra peritoneal	50–80 weeks	-	Causes fat deposition, necrosis, and inflammatory cell aggregates in centrilobular area	[103, 104]

are different categories of models such as those which express viral genes, or overexpress oncogenes, growth factors, and ones that create a TME, or those that mimic the injury-fibrosis-HCC cycle (Table 2). The overexpression of oncogenes or knock out of tumor suppressor genes does not mimic the initiation and progression of HCC, especially in patients with chronic infection of hepatitis virus.

To overcome some of the limitations of drug and genetically induced models, tumor xenograft models were developed by injecting human cancer cells or biopsy materials into immune deficient mice (athymic nude) mice or severe combined immune deficient (SCID) mice. There are two different xenograft models: ectopic models where the tumor cells are injected subcutaneously and the orthotopic model where the tumor cells are injected intrahepatically. Orthotopic implantation can also be done in a fibrotic liver, induced by thioacetamide (TAA) or carbon tetrachloride (CCl_4). The tumors in such models have been shown to form a significantly larger mass and are more prone to metastasize and form satellite nodules [47]. This provides a more realistic model to study drug efficiency in fibrotic livers. However, the impaired immune system does not allow for the study of immune evasion seen in HCC and the human cells might not respond to the factors produced by the animal stroma or vice versa.

Even though animal models have various advantages in terms of mimicking and developing the HCC in humans, the heterogeneity of each tumor determines the susceptibility to anti-tumor drugs and no single model can be representative of the human condition. Animal models are also not always good predictors of human-relevant drug induced liver injury (DILI) due to significant species-specific differences in drug metabolism pathways.

In vitro models

In vitro models include 2D and 3D culture of either cell linesor patient-derived cells. Additionally, other systems such as cancer-on-chip microfluidic models, and 3D printing/bioprinting approaches have been developed very recently. Standard 2D models have been used for decades, they can utilize human cells grown on tissue culture plates from either cell lines or from primary derived cells. More advanced 3D models employ a large number of approaches where multiple cell types can be incorporated, as well as multiple components of the microenvironment, including all the relevant proteins from the liver ECM, which can be further manipulated. Additionally, microfluidic models also incorporate the effects of fluid dynamics and drug metabolism, found natively within the liver, on cancer cell physiology. Each model represents aspects of the tumor cells and/or the external influences acting upon them. Each model has their own restrictions and limitations due to a number of reasons such as overall throughput, flexibility of design, or complexity of interactions highlighted in Table 3.

Cell lines and 2D culture models of HCC

In vitro models of the human liver are important to understand human drug metabolism and toxicity prior to clinical trials. Such models can also aid in phenotypic drug discovery against liver diseases. Primary human hepatocytes are an important tool in studying HCC. However, because of their limited proliferation and loss of function in culture, their use for research purposes is limited. Alternate sources include hepatic cell lines and hepatocytes derived from stem cells. Most hepatic cell lines are derived from liver tumor tissues or by immortalization of primary hepatocytes (Table 4). Cell lines are well-characterized, robust systems that can be genetically modified and are known for reproducible results. They are low maintenance, cost-effective, high-throughput platforms used for mechanistic analysis because of their unlimited proliferative potential [48]. The liver-derived immortalized cell lines commonly used in use are HepG2, Hep3B, PLc/PRFs, Huh7, and HepaRG. The HepG2 line expresses various liver-specific genes; however, gene expression involved in phase I and phase II metabolism has been shown to differ between passages and the resulting data can be challenging to interpret. The more recent human hepatoma cell line, HepaRG, maintains the expression of various liver-specific functions as along with many cytochrome P450s, phase II enzymes, nuclear receptors, and membrane transporters. They have a stable karyotype and can differentiate into both hepatocytes or cholangiocytes, witha high proliferative capacity, and produces data that is both reproducible and consistent among experiments. Still, the expression of liver-specific functions in HepaRG cells is still much lower on average than that of primary hepatocytes and also they represent a phenotype from a single donor, reducing

Table 2: Induction pathways of GEM models

Gene	Mechanism	Time for tumor development	Characteristics	References
HBV	HBx genealters the regulation of cellgrowth and sensitize the hepatocytes to carcinogens	52–104 weeks	Hepatocellular carcinoma in the background of hepatitis B	[105-107]
HCV	Core protein altersthe lipid metabolism by the activation of PPARα, which causes lipid accumulation in the hepatocytes	90–100 weeks	Causes progressive hepatic steatosis	[108, 109]
Myc	Expression of several genes by recruiting histone acetyltransferases	65–90 weeks	Genetically close to human HCC with good prognosis	[110-112]
β-catenin	Downstream Effector of the Wnt signaling pathway	8 weeks (early HCC) 26 weeks (high grade HCC)	Leads to hepatomegaly, however additional mutations are required to induce hepatocarcinogenesis	[113, 114]
Transforming growth factor-a. (TGF-α)	Hepatotrophic mitogen that is expressed in the hepatocytes during regeneration	40–70 weeks when supplemented with zinc	Tumors genetically comparable to human HCC which are associated with poor prognosis	[110]
Epidermal growth factor (EGF)	Regulates cell growth, proliferation and differentiation	24–36 weeks	Multiple highly malignant hepatic tumors	[115]
Fibroblast growth factor 19 (FGF19)	Higher metabolic rate which leads to a higher ROS production	52 weeks	HCC has a higher incidence in female mice compares to male	[116]
SV40 T antigen	Suppresses the expression of the p53	4–12 weeks	Metastasis to the lungs can happen, rapid tumor progression which differs from humans where they progress gradually	[117]
PTEN	Tumor suppressor gene that regulates PKB/ Akt pathway leading to hyperproliferation, anti-apoptosis and oncogenesis	40–44 weeks	PTEN-deficient mice develop hepaticsteatosis leading to fibrosis and tumors which are similar to human non-alcoholic steatohepatitis (NASH)	[118, 119]

Table 3: Comparison of available HCC models

	Experimental design	Complexity	Gradients	Reproducibility	Throughput	Challenges	References
2D culture	Great flexibility and control	Cell-cell interactions	No	High	High	Loss of cell-ECM interactions, polarity	[53]
3D aggregates	Great flexibility and control	Cell-cell interactions and cell-ECM interactions	Diffusion	High	High	No control over spatial positioning of cells	[52, 120, 121]
Microfluidics	Tailored for a particular application	Cell-cell and cell-ECM interactions	Perfusion	High	Medium	Non-standard culturing methods, Complex designing of the chip	[122]
3D printing/ bioprinting	Tailored for a particular application	Cell-cell and cell-ECM interactions	Perfusion	High	Low	Non-standard culturing methods, complex designing and printing	[123]
Animal models	Complex involving multiple parameters	Cell-cell interactions Cell-ECM interactions Interactions with the surrounding tissue	Vascular supply	Low	Low	Genetic differences, labor-intensive	[124, 125]

their predictive value for the human population.

A panel of 25 HCC cell lines have been used to understand the molecular mechanisms of the disease and also for drug screening purposes [49]. There is ongoing research with cell lines to establish unique characteristics that are not expressed by currently available cell lines such as gemcitabine resistance [50]. However, 2D culture systems have many disadvantages such as the lack of cell-cell and cell-ECM interactions and the lack of gradients (nutrients, oxygen, hormones) that are present in the native liver [51, 52]. HCC modeling done in 2D culture may not accurately recapitulate function and response to external agents as they do *in vivo* is also in part due to the unnatural and rigid substrate on which they are cultured. 3D culture systems were developed as an alternative to overcome the limitations of 2D systems which have led to numerous failures in the translation of results into clinical relevance.

Bioengineered 3D models of HCC

3D culture systems have a wide range of compositions, but mainly fall within those which use cell aggregates without the use of scaffolding for cell growth and those which encapsulate cells within a large variety of scaffolding composed of synthetic or naturally derived proteins. Collectively, these constructs are referred to as spheroids or organoids depending on cellular composition. 3D models without scaffolding have come into the forefront in liver disease research. Such models incorporate single or multiple cell

Table 4: Common cell lines used in HCC models

Cell line	Species	Chromosome number	Characteristics	Population doubling time (PDT) (Hours)
Hep3B	Human	60	Epithelial human hepatocellular carcinoma – Hepatitis B virus integrated into genome, tumorigenic	24
HepG2	Human	50-56	Well differentiated epithelial-like hepatocellular carcinoma, non-tumorigenic	48
SK-HEP1	Human	aneuploid	Epithelial-like human hepatic adenocarcinoma derived from ascitic fluid – Endothelial origin	46
HepaRG	Human hepatic cell line	Trisomic **chromosome** 7 and a translocated **chromosome** from 22 to 12	Forms bile canaliculi, high plasticity and transdifferentiation capacity	30
SNU398	Human	Aneuploid, 62	Human epithelial liver carcinoma, grade IV, anaplastic, tumorigenic	39
Huh7	Human	55-63	Well differentiated epithelial-like hepatocellular carcinoma taken from a primary tumor, Point mutation in p53	24
PLC/ PRF/5	Human		Hepatoma, epithelial, expression of HbsAg antigen	35-40
HepG2/ C3A	Human	55	Hepatocellular carcinoma, epithelial, non-tumorigenic	72

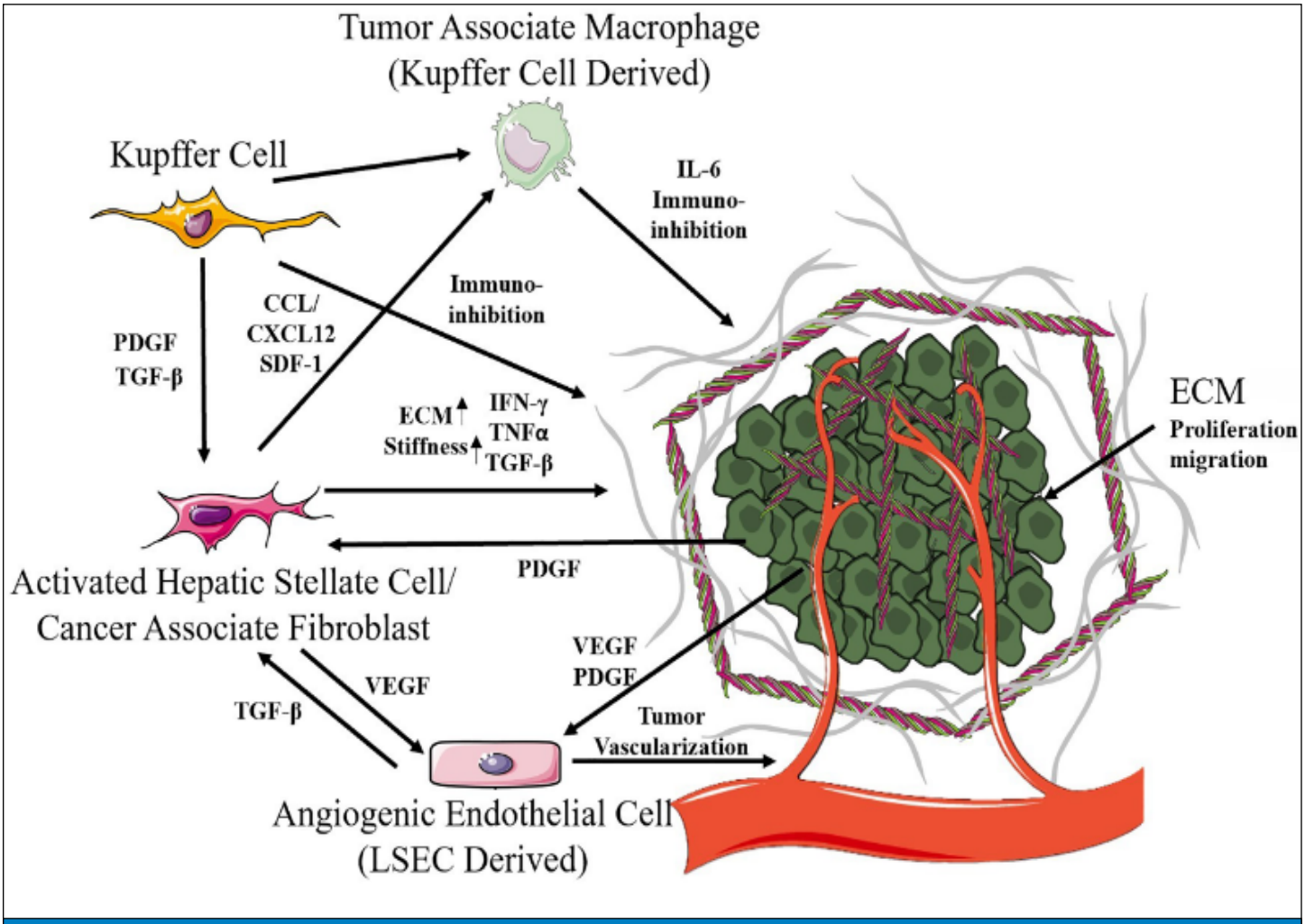

Figure 2: Cellular interactions within the tumor microenvironment

The tumor microenvironment (TME) is composed of a multitude of cell types, signaling factors, extracellular matrix (ECM) components and vasculature. Illustrated are examples of important cell-cell and cell-ECM interactions that drive tumor progression through a dynamic, multivariant process.

types, that include cancer cells lines and patient derived tumor cells [53]. Recently, different studies of Huh7/LX2 and Huh7/HUVECs spheroids were developed to demonstrate the importance of incorporating multiple cell lines in establishing an *in vivo* microenvironment and epithelial-to-mesenchymal (EMT) phenotype. The spheroids showed higher production of collagen I and growth factors such as TGF-β1 and connective tissue growth factor (CTGF) [54] and activation of angiogenic and EMT pathways respectively [55]. Both multicellular spheroids had differential response and a higher resistance to drugs when compared to 2D monolayers. Patient-derived multicellular tumor spheroids (MCTS) generated using patient-derived HCC cells along with WI38, LX2 and HUVECs showed different responses when treated with drugs such as sorafenib, cisplatin and 5-fluorouracil when compared to monolayer cultures [56]. While the aforementioned systems can consider some complex cell-cell interactions, they do not account for the cell-ECM interactions that can influence metabolic functionality. Such systems do not form the more complex structures found in most organs. Aggregate systems generally only contain a small number of cells due to constraints of nutrient diffusion, but can be rapidly manufactured and are well suited for high throughput systems. Several recent studies of HCC utilizing cell aggregates have yielded some promising results [57]. On the other hand, i*n situ* cells exist in a highly complex environment

where dynamic and continual interactions with other cells and the ECM exist. Researchers have increasingly turned to cell cultures of single and multicellular 3D constructs constituted of cells that are encapsulated within various hydrogels comprised of such ECM related proteins such as collagen, fibrin, and hyaluronic acid [58]. In some cases, whole organs can be decellularized, and then the remnant ECM lyophilized, which can then be reconstituted to form a hydrogel composed of largely the same proteins found natively [59]. 3D liver organoids replicating hepatobiliary organogenesis and further forming more differentiated hepatocytes and bile duct structures was demonstrated by using fetal-liver progenitor cells on liver decellularized scaffolds [60].

Tumors themselves are comprised of a tortuous organization of cellular and noncellular components both healthy and cancerous. The ECM is composed mainly of collagens, laminins, proteoglycans and fibronectin creates a complex network of chemical and mechanical signaling essential for appropriate tissue function and cellular behavior [61,62]. The unregulated ECM remodeling, found in fibrosis and cancer, can have deleterious effects on tissue functionality [63]. The use of hydrogels to encapsulate HCC can facilitate various aspects of the TME including the physical cues found within the ECM and the inclusion of stromal cells [64]. Stromal cells, when encapsulated within a hydrogel, can reconfigure certain features of the ECM found within the TME, which would be useful to study individual ECM components involved in cancer progression. Currently, this is a challenge within the field as there are complex relationships between many of the proteins which makeup the ECM.

Encapsulated models more closely mimic these aspects of *in vivo* environmental conditions. Such models can account for mechanical features of the cellular microenvironment. Microenvironmental mechanical features such as stiffness, which can be tuned chemically or induced on a cellular level. In one such study, a two-step crosslinking mechanism was developed using thioacrylate and thioalkyne polymerization to tailor hydrogels of gelatin and hyaluronic acid to desired stiffness [65]. Additionally, encapsulated models can also easily incorporate such beneficial proteins as growth factors to increase cell function, proliferation, or differentiation. Bioprinting of encapsulated cells takes the microenvironmental replication one step further in that the protein substrate and cells can be arranged in such a way that more closely replicates the *in situ* spatial distribution of these components such as in the reproduction of the liver lobule [66]. Benefits aside, organoid models are overall lower throughput than spheroid models and with increasing complexity can confound results. However, in efforts to create more truly organotypic, model systems incorporation of an appropriate substrate, and appropriate spatial distribution is pushing the field forward.

Patient Derived Organoid (PDO) technology has ensured an alternative to pre-clinical drug testing as well as for personalized medicine to modulate the treatment as they are uniquely identical to each patient's genetic makeup. Different types of patient derived models have been produced, which have demonstrated their efficient application in various fields such as disease modeling, pathogenesis, drug screening and regenerative medicine [67]. Initially, long-term cultures of HCC organoids were generated from needle biopsies of patients. These models were able to maintain the tumor specific markers and the heterogeneity of the tumor. They were then treated with sorafenib to test the sensitivities of the tumors towards the drug, providing a tool for tailored therapies [68]. Patient-derived 3D models were used to explore the role of cotreatment with sorafenib and Hedgehog inhibitors. The cells within the 3D construct maintained the tumor morphology and responded to the drug treatments. CD44 positive cells were resistant to sorafenib, however they responded to hedgehog inhibitors [69]. To study initiation of liver cancer, reprogrammed human hepatocytes (hiHeps) organoids along with inactivation of p53 and Rb were established which have the architecture of the liver. HCC were developed by over expression of c-Myc. It also showed that RAS activation induces intra-hepatic cholangiocarcinoma. This model is capable of mimicking of cancer initiation and can be used to characterize cellular and molecular changes as well as to develop to preventive measures [70]. Additionally, 3D HCC models were developed from hepatitis B virus (HBV) infected patients as a platform for modeling HBV and the associated tumorigenesis. The models derived from HBV infected patients showed aberrant early cancer gene signature.Whereas models derived from healthy human donors showed the development of HBV when

treated with HBV infected patient serum or recombinant virus. Moreover, treatment with tenofovir was found to block HBV replication, highlighting the potential of the model as platform for HBV drug screening [71]. Broutier et al., developed a primary liver cancer (PLC) organoids from HCC, cholangiocarcinoma (CC) and a mixed population of HCC and CC. The organoids retained the histological architecture and the gene expression profile of the original tumors. Xenografts studies showed that the tumorigenic potential and metastatic properties of the cancer was retained. This study demonstrates the potential of organoid systems for the understanding of liver cancer biology and for their use in the development of personalized medicine [72].

Although PDOs are regarded as a promising approach for personalized medicine, there are still many limitations associated with them. This includes the replication of the inter and intra tumoral heterogeneity observed between the tumors. They cannot replicate the complex tumor stroma interactions as they lack blood vessels, CAFs, immune cells and other relevant cell types [72]. Moreover, the microenvironment also contributes to an important part of the response of cells to the drugs. Currently used hydrogels such as Matrigel is problematicdue to lack of controlled modifications and undefined growth factors present. Ideally, a hydrogel best reflecting the microenvironment into which an HCC PDO could be cultured would be an acellular liver ECM derived from patient with fibrosis or cirrhosis of the liver which would more closely replicate the relevant protein composition found within the diseased tissue. Combining organoid technology with more relevant matrices, thus more accurately replicating the tumor microenvironment can lead to better understanding of the role of ECM in the process of carcinogenesis [73].

Microfluidics

Microphysiological systems are changing the way we study human physiology [74]. Microfluidic devices allow for culturing of living cells in continuously perfused chambers to model physiological functions of tissues and organs. The goal is to develop minimal functional units that recapitulate tissue- and organ-level functions. The simplest system is a single, perfused microfluidic chamber containing a single cell type that exhibits functions of one tissue. In more complex designs, two or more microchannels are connected by porous membranes, lined on opposite sides by different cell types, to recreate interfaces between different tissues. These systems can incorporate physical forces, including physiologically relevant levels of fluid shear stress, cyclic strain, and mechanical compression.These systems also permit analysis of organ-specific responses, including recruitment of circulating immune cells, in reaction to drugs, toxins or other environmental perturbations [75]. A metastasis-on-a-chip platform was developed to model an HCC to bone metastasis of HepG2 cells and to analyze the inhibitory effect of thymoquinone (TQ) on the migration of HepG2 cells. A microporous membrane was used in place of the vascular barrier. The HepG2 cells were seen to migrate from the HCC chamber to the hydroxyapatite (HAp) containing bone chamber, which Hap acting as a chemoattractant. The presence of TQ was shown to have an inhibitory effect on the migration of HepG2 cells [76]. Incorporation of patient-derived HCC cells into the device allows for personalized testing of anticancer drugs. Moreover, the incorporation of the tumor sample as such will retain the heterogeneity of the tumor, leading to a better assessment of the response of a tumor to the anticancer drug [77,78].

A vascularized, multi tissue-on-a-chip microenvironment was developed containing both breast tumor and healthy/cancerous liver tissue which allowed for the study of dynamic and spatial distribution of particles. This device allows the determination of vessel permeability, the measurement of drug and nanoparticle transport, and the assessment of the associated efficacy and toxicity to the liver. The study showed that larger particles tend to accumulate in the tumor site when compared to smaller particles because of the leaky vasculature, showing that porosity of the tumor vasculature has to be taken into consideration when selecting drugs and delivery vehicles [79]. A biomimetic tumor-on-a-chip was developed to recreate the TME of the liver using a mix of decellularized liver matrix and GelMA. The inclusion of decellularized matrix increased the viability and function of hepatocytes under perfusion. The chip demonstrated dose-dependent toxicity to sorafenib and acetaminophen [80].

Bioprinting

3D bioprinting techniques allows for the precise control over bio-

material deposition and of cell distribution within the 3D construct. There are a multitude of printing methods inkjet, extrusion-based, immersion-based, digital light processing amongst others and many printable biomaterials (bioinks) of various compositions [81]. The patterning of cells and biomaterials using a bioprinting strategy allows for the reproduction of the tissue microarchitecture present *in vivo*, enabling the simulation of TME on multiple levels. In the context of HCC, bioprinting can assist the development of a more accurate replication of the TME and its influence on tumor cells. The selection of an appropriate bioinks to encapsulate cells is of primary concern. They vary, in terms of complexity, from single proteins to decellularized ECM, each of which elicits different responses in terms of cell proliferation, migration, or adhesion amongst others. Recently, researchers used a combinations gelatin/alginate/fibrin gels were used to encapsulate HepG@ cells [82]. Utilizing this composition investigators were able to test tumor cell sensitivity for drugs such as mitomycin C (MMC), 5-fluorouracil (5-FU) and a combination of both. HepG2 cells showed higher resistance to MMC in 3D while 2D cells showed higher resistance to 5-FU [82]. In another study, HepG2 cells encapsulated in sodium alginate gels were 3D printed by extrusion bioprinting to generate a 3D model for HCC. The 3D construct showed higher expression of tumor related genes such as ALB, AFP, CD133, IL-8, EpCAM, CD24, and TGF-β. Transcriptome analysis revealed a huge difference in gene expression between the 3D and 2D groups. The model also showed a differential drug response to different concentrations of cisplatin, sorafenib and regorafenib [83]. A 3D bioprinted HCC model was developed by Xie et al., using primary HCC cells from patients with gelatin and sodium alginate as bioink. The 3D models maintained a genetic profile similar to the original tumor when compared to their 2D counterparts. With this study they have achieved a simple procedure for establishing tumor models with high speed, an adjustable and rewritable printing process and a model capable of long-term culture [84].

Stiffness is a key physico-mechanical micro environmental parameter that influences cellular physiology. In the context of liver fibrosis and cirrhosis, diseased liver has higher overall stiffness than that of a healthy liver. Utilizing a digital light processing printing technique, Ma et al used photo-crosslinkable bioink containing porcine derived decellularized ECM, which had tunable mechanical properties to enable stiffness manipulation within the constructs [85]. These constructs showed mechanical properties similar to normal and cirrhotic tissue. Interestingly, HepG2 cells encapsulated within higher stiffness constructs showed reduced growth and an upregulation of invasion markers, which is at odds with previous research in a tunable 2D substrate [85]. Further studies on optimization and validation of 3D printing strategies will help in the generation of high-efficiency 3D tumor models to study the molecular mechanisms of HCC pathogenesis and to accurately screen anticancer drugs [86].

The challenges faced by the field of bioprinting largely revolve around the benefits and drawbacks of the printable bioink. One such aspect is the survivability of certain cell types found within the TME within bioinks. Some bioinks have superior printability but lack realistic liver ECM mechanical and biomolecular features. The loss of such cues from the perspective of the physico-mechanical environment greatly dissuades from the clinical applicability of such research. An ideal bioink would have excellent multi-platform printability, contain much of the native ECM proteins as well as an appropriate substrate stiffness, which could be modified by the resident hepatic stellate cells to reorganize the environment to one that more closely resembles fibrosis. Additionally, such a gel would allow for highly localized modifications similar to those found within the aberrant makeup of the tumor stroma.

Future perspectives

The treatment of HCC poses a significant challenge as the current treatment strategies used havea very low survival rate. The poor prognosis associated with HCC can be attributed to chemotherapy resistance and the high recurrence rates associated with resection and other locoregional therapies. Another factor that contributes to the complexity of disease development and progression is the underlying chronic diseases such as fibrosis or cirrhosis that are known to promote cancer initiation and progression. Current liver cancer models that aid in the understanding of the molecular mechanisms of disease progression and the development of treatment strategies have fallen short in several respects.

Traditional modeling techniques have only focused on the cancerous cells themselves; they fail to recreate the TME and replicate the intricacies of the human stroma and signaling. When developing an HCC model, research should consider several important aspects while focusing mainly on the tumor stroma interactions present in the native tumor microenvironment, the different cell types that need to be incorporated, and the ECM components or the altered ECM components in the diseased liver. It would also be ideal if the models could incorporate or mimic the fibrotic/cirrhotic conditions. Such conditions that are present in the liver that can initiate cancer progression and also pose a significant challenge to the current treatment modalities that are available for the treatment of HCC. The addition of patient derived cells, in the form of PDOs, *in vitro* are becoming increasingly useful in terms of personalized anti-cancer therapies. Their predictive power of treatment sensitivity is especially noteworthy and of high utility in the clinical world. As an example, a recent study using PDOs demonstrated 100% sensitivity, 93% specificity, 88% positive predictive value, and 100% negative predictive values when comparing with patient response to chemotherapeutics or targeted therapeutics [87]. Studies such as this have paved the way to further clinical trials and according to clinicatrials.gov, there are currently 31 ongoing clinical trials employing cancer organoids. These models can be used not only for personalized medicine, but also for the identification of cancer sub-populations that are or can become resistant to care. Efforts have been made to gather large collections of such PDOs and to deposit them into living biobanks for further study especially those of rare cancer subtypes with poorly understood biomolecular mechanisms.

The integration of multiple model types may yield the greatest advances yet such as the integration of patient derived tumor cells into bioprinted constructs [88]. If additional research were to be conducted utilizing patient derived cancer cellsand other key cell types within the TME, a liver ECM capable of being remodeled, and also effectively incorporating the influence of fluid dynamics,one could effectively achieve a model recapitulating most aspects of the TME and its dynamics.

Conclusion

HCC still lacks adequate treatment strategies as a result of chemoresistance, recurrence, and an unavailability of models that can be specifically used to study the initiation and progression of cancer. Current HCC models are not able to mimic the heterogeneity of the TME in terms of the cell types, cell-ECM interactions,and theoften-present underlying liver diseases. To make further progress, there is a critical need to generate clinically relevant models that can replicate the native liver environment with all its interacting components within the TME. Apart from incorporating the majority of microenvironmental components, the addition of patient-derived cells would be a step forward in the development of HCC models, allowing for personalized anti-cancer therapies. Incorporation of fluid dynamics into the systems using microfluidic or 3D printing approaches will also help in mimicking the dynamic nature of the *in vivo* microenvironment. Development of a fully functional hepatic model that can mimic the TME of HCC including an underlying fibrotic background would pave the way for the advancement and establishment of new treatment modalities for HCC.

References

1. Rawla, P., Sunkara, T., Muralidharan, P., Raj, JP. (2018) Update in global trends and aetiology of hepatocellular carcinoma. Contemp Oncol (Pozn), 22(3): 141-150.

2. O'Connor, S. (2010) Hepatocellular carcinoma-United States 2001-2006. MMWR Morb Mortal Wkly Rep, 59(17): 517-520.

3. Lyon, F., IARC monographs on the evaluation of carcinogenic risks to humans. Some industrial chemicals, 1994. 60: p. 389-433.

4. Cancer, I.A.f.R.o., IARC monographs on the evaluation of carcinogenic risks to humans. Re-evaluation of some organic chemicals, hydrazine and hydrogen peroxide, 1999. 71.

5. Calle, EE., Rodriguez, C., Walker-Thurmond, K., Thun, MJ. (2003) Overweight obesity and mortality from cancer in a prospectively studied cohort of US adults. N Engl J Med, 348(17): 1625-1638.

6. Gao, C., Fang, L., Zhao, HC., Li, JT., Yao, SK. (2013) Potential

role of diabetes mellitus in the progression of cirrhosis to hepatocellular carcinoma: a cross-sectional case-control study from Chinese patients with HBV infection. Hepatobiliary Pancreat Dis Int, 12(4): 385-393.

7. Balogh, J., Victor, D., Asham, EH., Burroughs, SG., Boktour, M., Saharia, A., et al. (2016) Hepatocellular carcinoma: a review. J Hepatocell Carcinoma, 3: 41-53.

8. Liu, Y., Wu, F. (2010) Global burden of aflatoxin-induced hepatocellular carcinoma: a risk assessment. Environ Health Perspect, 118(6): 818-824.

9. Ko, C., Siddaiah, N., Berger, J., Gish, R., Brandhagen, D., Sterling, RK., et al. (2007) Prevalence of hepatic iron overload and association with hepatocellular cancer in end-stage liver disease: results from the national hemochromatosis transplant registry. Liver Int, 27(10): 1394-1401.

10. Xu, R., Hajdu, CH. (2008) Wilson disease and hepatocellular carcinoma. Gastroenterol Hepatol (N Y), 4(6): 438-439.

11. Topic, A., Ljujic, M., Radojkovic, D. (2012) Alpha-1-antitrypsin in pathogenesis of hepatocellular carcinoma. Hepat Mon, 12(10 HCC): e7042.

12. van Ginkel, WG., Pennings, JP., van Spronsen, FJ. (2017) Liver cancer in tyrosinemia type 1. Adv Exp Med Biol, 959: 101-109.

13. Andersson, C., Bjersing, L., Lithner, F. (1996) The epidemiology of hepatocellular carcinoma in patients with acute intermittent porphyria. J Intern Med, 240(4): 195-201.

14. Bisteau, X., Caldez, MJ., Kaldis, P. (2014) The complex relationship between liver cancer and the cell cycle: a story of multiple regulations. Cancers, 6(1): 79-111.

15. Adnane, L., Trail, PA., Taylor, I., Wilhelm, SM. (2006) Sorafenib (BAY 43-9006, Nexavar) a dual-action inhibitor that targets RAF/MEK/ERK pathway in tumor cells and tyrosine kinases VEGFR/PDGFR in tumor vasculature. Methods Enzymol, 407: 597-612.

16. Keating, GM., Santoro, A. (2009) Sorafenib: Sorafenib: a review of its use in advanced hepatocellular carcinoma. Drugs, 69(2): 223-240.

17. Bruix, J., Qin, S., Merle, P., Granito, A., Huang, YH., Bodoky, G., et al. (2017) Regorafenib for patients with hepatocellular carcinoma who progressed on sorafenib treatment (RESORCE): a randomised double-blind placebo-controlled phase 3 trial. Lancet, 389(10064): p. 56-66.

18. Cochin, V., Gross-Goupil, M., Ravaud, A., Godbert, Y., Le Moulec, S. (2017) Cabozantinib: Mechanism of action efficacy and indications. Bulletin du cancer, 104(5): 393-401.

19. Yamamoto, Y., Matsui, J., Matsushima, T., Obaishi, H., Miyazaki, K., Nakamura, K., et al. (2014) Lenvatinib an angiogenesis inhibitor targeting VEGFR/FGFR shows broad antitumor activity in human tumor xenograft models associated with microvessel density and pericyte coverage. Vasc Cell, 6(1): 18.

20. Liver, EAFTSOT. (2018) EASL clinical practice guidelines: management of hepatocellular carcinoma. J Hepatol, 69(1): 182-236.

21. Llovet, JM., Schwartz, M., Mazzaferro, V. (2005) Resection and liver transplantation for hepatocellular carcinoma. Semin Liver Dis, 25(2): 181-200.

22. Zou, W.Y., et al., Tu1489-Improved Treatment Delivery and Overall Survival Associated with Multidisciplinary Tumor Board Evaluation of Patients with Advanced Hepatocellular Carcinoma. Gastroenterology, 2018. 154(6): p. S-1236.

23. Zhou, WC., Zhang, QB., Qiao, L. (2014) Pathogenesis of liver cirrhosis. World J Gastroenterol, 20(23): 7312-7324.

24. Li, JT., Liao, ZX., Ping, J., Xu, D., Wang, H. (2008) Molecular mechanism of hepatic stellate cell activation and antifibrotic therapeutic strategies. J Gastroenterol, 43(6): 419-428.

25. Qin, LX. (2012) Inflammatory immune responses in tu-

mor microenvironment and metastasis of hepatocellular carcinoma. Cancer Microenviron, 5(3): 203-209.

26. Zamarron, BF., Chen, W. (2011) Dual roles of immune cells and their factors in cancer development and progression. Int J Biol Sci, 7(5): 651-658.

27. Hoshida, Y., Villanueva, A., Kobayashi, M., Peix, J., Chiang, DY., Camargo, A., et al. (2008) Gene expression in fixed tissues and outcome in hepatocellular carcinoma. N Engl J Med, 359(19): 1995-2004.

28. Borkham-Kamphorst, E., Herrmann, J., Stoll, D., Treptau, J., Gressner, Am., Weiskirchen, R. (2004) Dominant-negative soluble PDGF-β receptor inhibits hepatic stellate cell activation and attenuates liver fibrosis. Lab Invest, 84(6): 766-777.

29. Liu, X., Hu, H., Yin, JQ. (2006) Therapeutic strategies against TGF-β signaling pathway in hepatic fibrosis. Liver Int, 26(1): 8-22.

30. Frank, A., Seitz, HK., Bartsch, H., Frank, N., Nair, J. (2004) Immunohistochemical detection of 1 N 6-ethenodeoxyadenosine in nuclei of human liver affected by diseases predisposing to hepato-carcinogenesis. Carcinogenesis, 25(6): 1027-1031.

31. Llovet, J., Zucman-Rossi, J., Pikarsky, E., Sangro, B., Schwartz, M., Sherman, M., et al. (2016) Hepatocellular carcinoma. Nat Rev Dis Primers, 2: 16018.

32. Mohammed, NA., Abd El-Aleem, S., Appleton, I., Maklouf, MM., Said, M., McMahon, RFT. (2003) Expression of nitric oxide synthase isoforms in human liver cirrhosis. J Pathol, 200(5): 647-655.

33. Yu, DC., Chen, J., Ding, YT. (2010) Hypoxic and highly angiogenic non-tumor tissues surrounding hepatocellular carcinoma: the 'niche'of endothelial progenitor cells. Int J Mol Sci, 11(8): 2901-2909.

34. Marquardt, JU., Andersen, JB., Thorgeirsson, SS. (2015) Functional and genetic deconstruction of the cellular origin in liver cancer. Nat Rev Cancer, 15(11): 653-667.

35. Schrader, J., Gordon-Walker, TT., Aucott, RL., van Deemter, M., Quaas, A., Walsh, S., et al. (2011) Matrix stiffness modulates proliferation chemotherapeutic response and dormancy in hepatocellular carcinoma cells. Hepatology, 53(4): 1192-1205.

36. Yang, C., Zeisberg, M., Lively, JC., Nyberg, P., Afdhal, N., Kalluri, R. (2003) Integrin α1β1 and α2β1 are the key regulators of hepatocarcinoma cell invasion across the fibrotic matrix microenvironment. Cancer research, 63(23): 8312-8317.

37. Orimo, A., Weinberg, RA. (2006) Stromal fibroblasts in cancer: a novel tumor-promoting cell type. Cell Cycle, 5(15): 1597-1601.

38. Kalluri, R., Zeisberg, M. (2006) Fibroblasts in cancer. Nat Rev Cancer, 6(5): 392-401.

39. Friedman, SL. (2008) Hepatic stellate cells: protean multifunctional and enigmatic cells of the liver. Physiological reviews, 88(1): 125-172.

40. Lu, P., Weaver, VM., Werb, Z. (2012) The extracellular matrix: a dynamic niche in cancer progression. J Cell Biol, 196(4): 395-406.

41. Martin, TA., Ye, L., Sanders, AJ., Lane, J., Jiang, WG. (2013) Cancer invasion and metastasis: molecular and cellular perspective, Madame Curie Bioscience Database [Internet].

42. Théret, N., Musso, O., Turlin, B., Lotrian, L., Bioulac-Sage, P., Campion, JP., et al. (2001) Increased extracellular matrix remodeling is associated with tumor progression in human hepatocellular carcinomas. Hepatology, 34(1): 82-88.

43. Zhu, AX., Duda, DG., Sahani, DV., Jain, RK. (2011) HCC and angiogenesis: possible targets and future directions. Nat Rev Clin Oncol, 8(5): 292-301.

44. Pitot, HC., Dragan, Y. (1991) Facts and theories concerning the mechanisms of carcinogenesis. FASEB J, 5(9): 2280-2286.

45. Frese, KK., Tuveson, DA. (2007) Maximizing mouse cancer models. Nat Rev Cancer, 7(9): 654-658.

46. Tuveson, DA., Jacks, T. (2002) Technologically advanced cancer modeling in mice. Curr Opin Genet Dev, 12(1): 105-110.

47. Kornek, M., Raskopf, E., Tolba, R., Becker, U., Klöckner, M., Sauerbruch, T., et al. (2008) Accelerated orthotopic hepatocellular carcinomas growth is linked to increased expression of pro-angiogenic and prometastatic factors in murine liver fibrosis. Liver Int, 28(4): 509-518.

48. Wilding, JL., Bodmer, WF. (2014) Cancer cell lines for drug discovery and development. Cancer Res, 74(9): 2377-2384.

49. Hirschfield, H., Bian, CB., Higashi, T., Nakagawa, S., Zeleke, TZ., Nair, VD., et al. (2018) In vitro modeling of hepatocellular carcinoma molecular subtypes for anti-cancer drug assessment. Exp Mol Med, 50(1): e419-e419.

50. Varamo, C., Peraldo-Neia, C., Ostano, P., Basiricò, M., Raggi, C., Bernabei., P., et al. Establishment and characterization of a new intrahepatic cholangiocarcinoma cell line resistant to gemcitabine. Cancers, 11(4): 519.

51. Oudin, MJ., Weaver, VM. (2016) Physical and chemical gradients in the tumor microenvironment regulate tumor cell invasion, migration, and metastasis. Cold Spring Harb Symp Quant Biol, 81: 189-205.

52. Pampaloni, F., Reynaud, EG., Stelzer, EH. (2007) The third dimension bridges the gap between cell culture and live tissue. Nature reviews Molecular cell biology, 8(10): 839-845.

53. Duval, K., Grover, H., Han, LH., Mou, Y., Pegoraro, AF., Fredberg, F., et al. Modeling physiological events in 2D vs 3D cell culture. Physiology, 32(4): 266-277.

54. Khawar, IA., Park, JK., Jung, ES., Lee, MA., Chang, S., Kuh, HJ. (2018) Three dimensional mixed-cell spheroids mimic stroma-mediated chemoresistance and invasive migration in hepatocellular carcinoma. Neoplasia, 20(8): 800-812.

55. Jung, HR., Kang, HM., Ryu, JW., Kim, DS., Noh, KH., Kim, ES., et al. Cell spheroids with enhanced aggressiveness to mimic human liver cancer in vitro and in vivo. Scientific reports, 7(1): 1-14.

56. Song, Y., Kim, JS., Kim, SH., Park, YK., Yu, E., Kim, KH., et al. (2018) Patient-derived multicellular tumor spheroids towards optimized treatment for patients with hepatocellular carcinoma. Journal of Experimental & Clinical Cancer Research, 37(1): 109.

57. Bhatia, SN., Underhill, GH., Zaret, KS., Fox, IJ. (2014) Cell and tissue engineering for liver disease. Sci Transl Med, 6(245): 245sr2-245sr2.

58. Tibbitt, MW., Anseth, KS. (2009) Hydrogels as extracellular matrix mimics for 3D cell culture. Biotechnol Bioeng, 103(4): 655-663.

59. Saldin, LT., Cramer, MC., Velankar, SS., White, LJ., Badylak, SF. (2017) Extracellular matrix hydrogels from decellularized tissues: structure and function. Acta biomaterialia, 49: 1-15.

60. Vyas, D., Baptista, PM., Brovold, M., Moran, E., Brovold, M., Gaston, B., et al. (2018) Self-assembled liver organoids recapitulate hepatobiliary organogenesis in vitro. Hepatology, 67(2): 750-761.

61. Özbek, S., Balasubramanian, PG., Chiquet-Ehrismann, R., Tucker, RP., Adams, JC. (2010) The evolution of extracellular matrix. Mol Biol Cell, 21(24): 4300-4305.

62. Balkwill, F.R., Capasso, M., Hagemann, T. (2012) The tumor microenvironment at a glance. J Cell Sci, 125(Pt 23): 5591-5596

63. Belgodere, J.A., Connor T. King, CT., Bursavich, JB., Burow, ME., Martin, EC., Jung, JP. (2018) Engineering breast cancer microenvironments and 3D bioprinting. Front Bioeng Biotechnol, 6: 66.

64. Beck, J.N., Singh, A., Rothenberg, Ar., Elisseeff, JH., Ewald.,

Aj. (2013) The independent roles of mechanical, structural and adhesion characteristics of 3D hydrogels on the regulation of cancer invasion and dissemination. Biomaterials, 34(37): 9486-9495.

65. Skardal, A., Devarasetty, M., Kang, HW., Mead, I., Bishop, C., Shupe, T., et al. (2015) A hydrogel bioink toolkit for mimicking native tissue biochemical and mechanical properties in bioprinted tissue constructs. Acta biomater, 25: 24-34.

66. Zhang, Y.S., Yue, K., Aleman, J., Moghaddam, KM., Bhakt, Sw., Yang, J., et al. (2017) 3D bioprinting for tissue and organ fabrication. Ann Biomed Eng, 45(1): 148-163.

67. Ashok, A., Choudury, D., Fang, Y., Hunziker., W. (2020) Towards manufacturing of human organoids. Biotechnology Advances, 39: 107460.

68. Nuciforo, S., Fofana, I., Matter, Ms., Blumer, T., Calabrese, D., Boldanove, T., et al. (2018) Organoid models of human liver cancers derived from tumor needle biopsies. Cell Rep, 24(5): 1363-1376.

69. Wang, S., Wang, Y., Xun, X., Zhang, C., Xiang, X., Cheng, Q., et al. (2020) Hedgehog signaling promotes sorafenib resistance in hepatocellular carcinoma patient-derived organoids. Journal of Experimental & Clinical Cancer Research, 39(1): 22.

70. Sun, L., Wang, Y., Cen, J., Ma, X., Cui, L., Qui, Z., et al. (2019) Modelling liver cancer initiation with organoids derived from directly reprogrammed human hepatocytes. Nat Cell Biol, 21(8): 1015-1026.

71. De Crignis, E., Carofiglio, F., Moulos, P., M.A. Verstegan, M., Romal, S., Khalid, MM., et al. (2019) Human liver organoids; a patient-derived primary model for HBV Infection and Related Hepatocellular Carcinoma. bioRxiv, 568147.

72. Broutier, L., Mastrogiovanni, G., Verstegan, MM., Francies, HE., Gavarro, LM., Bradshaw, CR., et al. (2017) Human primary liver cancer–derived organoid cultures for disease modeling and drug screening. Nat Med, 23(12): 1424-1435.

73. Gjorevski, N., Sachs, N., Manfrin, A., Giger, S., Bragina, ME., Moran, Po., et al. (2016) Designer matrices for intestinal stem cell and organoid culture. Nature, 539(7630): 560-564.

74. Truskey, G.A. (2018) Human microphysiological systems and organoids as in vitro models for toxicological studies. Front Public Health, 6: 185.

75. Bhatia, S.N., Ingber, DE. (2014) Microfluidic organs-on-chips. Nat Biotechnol, 32(8): 760-772.

76. Sharifi, F., Celiktas, OY., Kazan, A., Saghazadeh, S., Firoozbakhsh, K., et al. (2020) A hepatocellular carcinoma–bone metastasis-on-a-chip model for studying thymoquinone-loaded anticancer nanoparticles. Bio-Design and Manufacturing, 3: 189–202.

77. Holton, A.B., Sinatra, FL., Kreahling, J., Conway, AJ., Landis, DA., Elitok, S. (2017) Microfluidic biopsy trapping device for the real-time monitoring of tumor microenvironment. PLoS One, 12(1): e0169797.

78. Astolfi, M., Peant, B., Lateef, MA., Rousset, N., Dupont, JK., Carmona, E., et al. (2016) Micro-dissected tumor tissues on chip: an ex vivo method for drug testing and personalized therapy. Lab Chip, 16(2): 312-325.

79. Ozkan, A., Ghousifam, N., Hoopes, PJ., Yankeelov, TE., Rylander, N. (2019) In vitro vascularized liver and tumor tissue microenvironments on a chip for dynamic determination of nanoparticle transport and toxicity. Biotechnology and bioengineering, 116(5): 1201-1219.

80. Lu, S., Cuzzucoli, F., Jiang, J., Liang, LG., Wang, Y., Kong, M., et al. (2018) Development of a biomimetic liver tumor-on-a-chip model based on decellularized liver matrix for toxicity testing. Lab on a Chip, 18(22): 3379-3392.

81. Chameettachal, S., Yeleswarapu, S., Sasikumar, S., Shukla, P., Hibare, P., Bera, Ak., et al. (2019) 3D Bioprinting: Recent Trends and Challenges. Journal of the Indian Insti-

tute of Science, 99: 375–403.

82. Zhou, X., Liu, C., Zhao, X., Wang, X. (2016) A 3D bioprinting liver tumor model for drug screening. World J. Pharm. Pharm. Sci, 5: 196-213.

83. Leijia, S., Yang, H., Wang, Y., Zhang, X., Jin, B., Xie, F., et al. (2020) The application of a 3D bioprinted hepatocellular carcinoma cell model in anti-tumor drug research. Front Oncol, 10: 878.

84. Xie, F., Leijia, S., Pang, Y., Xu, G., Jin, B., Xu, X., et al. (2020) Three-dimensional bio-printing of primary human hepatocellular carcinoma for personalized medicine. Biomaterials, 265: 120416.

85. Ma, X., Yu, C., Wang, P., Xu, W., Wan, X., Lei, CSD., et al. (2018) Rapid 3D bioprinting of decellularized extracellular matrix with regionally varied mechanical properties and biomimetic microarchitecture. Biomaterials, 185: 310-321.

86. Knowlton, S., Onal, S., Yu, CH., Zhao, JJ., Tasoglu, S. (2015) Bioprinting for cancer research. Trends Biotechnol, 33(9): 504-513.

87. Vlachogiannis, G., Hedeyat, S., Vatsiou, A., Jamin, Y., Mateos, JF., Khan, K., et al. (2019) Patient-derived organoids model treatment response of metastatic gastrointestinal cancers. Science, 359(6378): 920-926.

88. Ma, X., Liu, J., Zhu, W., Tang, M., Lawrence, N., Yu, C., et al. (2018) 3D bioprinting of functional tissue models for personalized drug screening and in vitro disease modeling. Adv Drug Deliv Rev, 132: 235-251.

89. Verna, L., Whysner, J., Williams, G.M. (1996) N-nitrosodiethylamine mechanistic data and risk assessment: bioactivation, DNA-adduct formation, mutagenicity, and tumor initiation. Pharmacol Ther, 71(1-2): 57-81.

90. Binato, M., Schmidt, MK., Volkweis, BS., Ribeiro, GBS., Edelweiss, MI., Gurski, RR. (2008) Mouse model of diethylnitrosamine-induced gastric cancer. J Surg Res, 148(2): 152-157.

91. Tolba, R., Kraus, T., Liedtke, C., Schwarz, M., Weiskirchen, R. (2015) Diethylnitrosamine (DEN)-induced carcinogenic liver injury in mice. Lab Anim, 49(1_suppl): 59-69.

92. Zhao, W., Iskandar, S., Kooshki, M., Sharpe, JG., Payne, V., Robbins, ME. (2007) Knocking out peroxisome proliferator-activated receptor (PPAR) α inhibits radiation-induced apoptosis in the mouse kidney through activation of NF-κB and increased expression of IAPs. Radiat Res, 167(5): 581-591.

93. Hasmall, S.C., James, MH., Macdonald, N., Gonzalez, FJ., Peters, JM., Roberts, RA. (2000) Suppression of mouse hepatocyte apoptosis by peroxisome proliferators: role of PPARα and TNFα. Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis, 448(2): 193-200.

94. Nishimura, J., Dewa, Y., Okamura, T. (2007) Mechanistic study on fenofibrate-induced hepatocarcinogenesis in a 2-stage hepatocarcinogenesis model of rats: Involvement of oxidative stress. Toxicology Letters, 172: S173.

95. Reddy, JK., Rao, MS., Moody, DE. (1976) Hepatocellular carcinomas in acatalasemic mice treated with nafenopin, a hypolipidemic peroxisome proliferator. Cancer Res, 36(4): 1211-1217.

96. Cheung, C., Akiyama, TE., Ward, JM., Nicol, Cj., Feigenbaum, L., Vinson, C., et al. (2004) Diminished hepatocellular proliferation in mice humanized for the nuclear receptor peroxisome proliferator-activated receptor α. Cancer Res, 64(11): 3849-3854.

97. Takashima, K., Ito, Y., Gonzalez, FJ., Nakajima, T. (2008) Different mechanisms of DEHP-induced hepatocellular adenoma tumorigenesis in wild-type and Pparα-null mice. J Occup Health, 50(2): 169-180.

98. Gallagher, E., Wienkers, LC., Stapleton, PL., Kunze, KL., Eaton, DL. (1994) Role of human microsomal and human complementary DNA-expressed cytochromes P4501A2 and P4503A4 in the bioactivation of aflatoxin B1. Cancer

Res, 54(1): 101-108.

99. Wang, J., Liu, XM. (2007) Contamination of aflatoxins in different kinds of foods in China. Biomed Environ Sci, 20(6): 483-487.

100. Sheweita, S., El-Gabar, MA., Bastawy, M. (2001) Carbon tetrachloride changes the activity of cytochrome P450 system in the liver of male rats: role of antioxidants. Toxicology, 169(2): 83-92.

101. Avasarala, S., Yang, L., Sun, Y., Leung, AWC., Chan, WY., Chan, WY., et al. (2006) A temporal study on the histopathological, biochemical and molecular responses of CCl4-induced hepatotoxicity in Cyp2e1-null mice. Toxicology, 228(2-3): 310-322.

102. Tarsetti, F., Lenzi, R., Salvi, R., Schuler, E., Rijhsinghani, K., Lenzen, R., et al. (1993) Liver carcinogenesis associated with feeding of ethionine in a choline-free diet: Evidence against a role of oval cells in the emergence of hepatocellular carcinoma. Hepatology, 18(3): 596-603.

103. Palacios, R.S., Roderfeld, M., Hemmann, S., Rath, T., Athanasova, S., Tschuschner, A., et al. (2008) Activation of hepatic stellate cells is associated with cytokine expression in thioacetamide-induced hepatic fibrosis in mice. Lab Invest, 88(11): 1192-1203.

104. Köhn-Gaone, J., Dwyer, BJ., Grxelak, CA., Miller, G., Shackel, NA., Ramm, GA., et al. (2016) Divergent inflammatory, fibrogenic, and liver progenitor cell dynamics in two common mouse models of chronic liver injury. Am J Pathol, 186(7): 1762-1774.

105. Lakhtakia, R., Kumar, V., Reddi, H., Mathur, M., Dattagupta, S., Panda, SK. (2003) Hepatocellular carcinoma in a hepatitis B 'x'transgenic mouse model: A sequential pathological evaluation. Journal of gastroenterology and hepatology, 18(1): 80-91.

106. Koo, J.S., Seong, Jk., Park, C., Yu, DY., Kyeong, B., Oh, SH., et al. (2005) Large liver cell dysplasia in hepatitis B virus x transgenic mouse liver and human chronic hepatitis B virus-infected liver. Intervirology, 48(1): 16-22.

107. Wu, C.-G., Salvay, DM., Forgues, M., Valerie, K., Farnsworth, J., Markin, RS., et al. (2001) Distinctive gene expression profiles associated with Hepatitis B virus x protein. Oncogene, 20(28): 3674-3682.

108. Tanaka, N., Moriya, K., Kiyosawa, K., Koike, K., Aouyama, T. (2008) Hepatitis C virus core protein induces spontaneous and persistent activation of peroxisome proliferator-activated receptor α in transgenic mice: Implications for HCV-associated hepatocarcinogenesis. Int J Cancer, 122(1): 124-131.

109. Lerat, H., Honda, M., Beard, MR., Loesch, K., Sun, J., Yang, Y. (2002) Steatosis and liver cancer in transgenic mice expressing the structural and nonstructural proteins of hepatitis C virus. Gastroenterology, 122(2): 352-365.

110. Lee, JS., Chu, Is., Mikaelyan, A., Calvisi, DF., Heo, J., Reddy, JK., et al. (2004) Application of comparative functional genomics to identify best-fit mouse models to study human cancer. Nat Genet, 36(12): 1306-1311.

111. Merle, P., Kim, M., Herrmann, M., Gupte, A., Lefrancios, L., Califano, S., et al. (2005) Oncogenic role of the frizzled-7/β-catenin pathway in hepatocellular carcinoma. J Hepatol, 43(5): 854-862.

112. Nilsson, JA., Cleveland, JL. (2003) Myc pathways provoking cell suicide and cancer. Oncogene, 22(56): 9007-9021.

113. Brembeck, FH., Rosário, M., Birchmeier, W. (2006) Balancing cell adhesion and Wnt signaling, the key role of β-catenin. Curr Opin Genet Dev, 16(1): 51-59.

114. Harada, N., Oshima, H., Katoh, M., Tamai, Y., Oshima, M., Taketo, MM. (2004) Hepatocarcinogenesis in mice with β-catenin and Ha-ras gene mutations. Cancer Res, 64(1): 48-54.

115. Borlak, J., Meier, T., Hlater, R., Spanel, R., Borowski, Ks. (2005) Epidermal growth factor-induced hepatocellular

carcinoma: gene expression profiles in precursor lesions, early stage and solitary tumours. Oncogene, 24(11): 1809-1819.

116. Nicholes, K., Guillet, S., Tomlinson, E., Hillan, K., Wright, B., Frantz, GD., et al. (2002) A mouse model of hepatocellular carcinoma: ectopic expression of fibroblast growth factor 19 in skeletal muscle of transgenic mice. Am J Pathol, 160(6): 2295-2307.

117. Lou, DQ., Molina, T., Bennoun, M., Porteu, A., Briand, P., Joulin, V., et al. (2005) Conditional hepatocarcinogenesis in mice expressing SV 40 early sequences. Cancer letters, 229(1): 107-114.

118. Watanabe, S., Horie, Y., Kataoka, E., Sato, W., Dohmen, T., Ohshima, S., et al. (2007) Non-alcoholic steatohepatitis and hepatocellular carcinoma: lessons from hepatocyte-specific phosphatase and tensin homolog (PTEN)-deficient mice. J Gastroenterol Hepatol, 22(1): S96-S100.

119. Horie, Y., Suzuki, A., Kataoka, E., Sasaki, T., Hamada, K., Sasaki, J., et al. (2004) Hepatocyte-specific Pten deficiency results in steatohepatitis and hepatocellular carcinomas. J Clin Invest, 113(12): 1774-1783.

120. Meng, X., Leslie, P., Zhang, Y., Dong, J. (2014) Stem cells in a three-dimensional scaffold environment. Springerplus, 3(1): 80.

121. Page, H., Flood, P., Reynaud, EG. (2013) Three-dimensional tissue cultures: current trends and beyond. Cell Tissue Res, 2013. 352(1): 123-131.

122. Halldorsson, S., Lucumi, E., Sjoberg, RG., Fleming, RMT. (2015) Advantages and challenges of microfluidic cell culture in polydimethylsiloxane devices. Biosensors and Bioelectronics, 63: 218-231.

123. Gu, Z., Fu, J., Lin, H., He, Y. (2019) Development of 3D bioprinting: from printing methods to biomedical applications. Asian Journal of Pharmaceutical Sciences.

124. Heindryckx, F., Colle, I., Van Vlierberghe, H. (2009) Experimental mouse models for hepatocellular carcinoma research. Int J Exp Pathol, 90(4): 367-386.

125. Zhang, H.E., Henderson, JM., Gorrell, MD. (2019) Animal models for hepatocellular carcinoma. Biochimica et Biophysica Acta (BBA)-Molecular Basis of Disease, 2019. 1865(5): 993-1002.