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Preclinical Models for Studying NASH-Driven HCC: How Useful Are They?

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Hepatocellular carcinoma (HCC) is one of the most fatal and fastest-growing cancers. Recently, non-alcoholic steatohepatitis (NASH) has been recognized as a major HCC catalyst. However, it is difficult to decipher the molecular mechanisms underlying the pathogenesis of NASH and understand how it progresses to HCC by studying humans. Progress in this field depends on the availability of reliable preclinical models amenable to genetic and functional analyses and exhibiting robust NASH-to-HCC progression. Although numerous mouse models of NASH have been described, many do not faithfully mimic the human disease and few reliably progress to HCC. Here, we review current literature on the molecular etiology of NASH-related HCC and critically evaluate existing mouse models and their suitability for studying this malignancy. We also compare human transcriptomic and histopathological profiles with data from *MUP-uPA* mice, a reliable model of NASH-driven HCC that has been useful for evaluation of HCC-targeting immunotherapies.

Introduction

Hepatocellular carcinoma (HCC) is one of the most common and fatal cancers worldwide (Starley et al., 2010). In developed countries the past three decades have witnessed the incidence of HCC increase 3-fold, becoming the fastest-rising cause of cancer-related deaths (El-Serag and Kanwal, 2014). Initially ascribed to the emergence and spread of hepatitis C virus (HCV), only 50% of the dramatic increase in HCC mortality in developed countries is virus dependent. Up to 50% of newly diagnosed cases of HCC are in ''virus-absent'' patients (El-Serag, 2011), most of whom present with obesity, non-alcoholic fatty liver disease (NAFLD), and, more commonly, non-alcoholic steatohepatitis (NASH), the most severe manifestation of NAFLD (Cohen et al., 2011). It is estimated that at least 30% of the adult population in developed countries has NAFLD (Lazo et al., 2013), with up to 15% of such patients exhibiting some degree of NASH (Cohen et al., 2011). Alarmingly, approximately 25% of NASH patients progress to cirrhosis, a serious premalignant condition that may require liver transplantation or result in liver failure (McCullough, 2004). Although mortality associated with most types of cancer has steadily declined over the last 40 years, both incidence and deaths due to HCC have substantially increased (Ryerson et al., 2016). Our ability to tackle this emerging epidemic has been severely hampered by the poor understanding of NASH. Even less is known about NASH-driven HCC.

NASH-Driven HCC: Etiology and Mechanisms

Initially, NASH was thought to represent just one end of a broad spectrum of clinical and pathological signs associated with NAFLD. Currently, NAFLD is thought to be initiated by simple hepatosteatosis that arises from an imbalance between triglyceride deposition, mainly due to elevated *de novo* lipogenesis (DNL), and removal, either by very low-density lipoprotein-mediated secretion or via fatty acid (FA) oxidation in mitochondria (Cohen et al., 2011). Such an imbalance is often the outcome of a sedentary lifestyle combined with overeating and excessive consumption of fructose-containing processed foods and beverages, but genetic factors (Dongiovanni et al., 2013) and inflammation of unknown origins (Marengo et al., 2016; Tilg and Moschen, 2006) are also of importance. Nevertheless, lipid droplet accumulation alone does not lead to liver damage and inflammation and it was therefore recognized that simple hepatosteatosis, also called ''bland steatosis,'' must be accompanied by a necro-inflammatory response, which is characterized by ballooning hepatocytes, liver damage, inflammatory infiltrates, and fibrosis. This realization gave rise to the so-called two-hit or multiple-hit hypothesis, according to which lipid droplet accumulation is only one of several factors that by itself is insufficient for initiation of NASH pathogenesis. Additional factors include oxidative stress, ER stress, or presence of infectious or commensal organisms that trigger liver inflammation (Day, 2006; Day and James, 1998; Font-Burgada et al., 2016; Tilg and Moschen, 2006). Another important factor that distinguishes bland steatosis from NASH is the accumulation of free non-esterified cholesterol in the latter (Farrell and van Rooyen, 2012; Ioannou, 2016). Free cholesterol and its oxidized derivatives are cytotoxic and can synergize with tumor necrosis factor (TNF), whose synthesis is markedly elevated in NASH (Nakagawa et al., 2014) to cause mitochondrial dysfunction and liver damage (Verbeek et al., 2015). Exactly how NASH progresses to HCC is not entirely clear, and the only reliable mechanistic information comes from studies of mouse models. Like most other drivers of HCC, NASH is associated with chronic liver damage, which triggers so-called compensatory proliferation. The

Table 1. Summary of Mouse Models of NASH and HCC

	Obesity	Insulin Resistance	Steatosis	Inflammation/ER Stress	NASH	Fibrosis	p62 Accumulation	HCC
Diet Models								
HFD	✔✔	✔✔	✔✔	✔	×	×	×	×
WD	✔✔	✔✔	✔✔	✔	×	×	×	×
MCD	×	×	✔✔	✔	✔✔	✔✔	?	×
CD	×	×	✔✔	✔	✔✔	✔✔	?	×
HFD/fructose	✔✔	✔✔	✔✔	✔✔	✔✔	✔✔	?	✔✔
CD HFD	✔✔	✔✔	✔✔	✔✔	✔✔	✔	?	✔
Toxins/Diet-Based Models								
STAM	x	✔✔	✔✔	✔✔	✔✔	✔	?	✔✔
HFD/DEN	✔✔	✔✔	✔✔	✔✔	×	×	✔✔	✔✔
CD HFD/DEN	✔✔	✔✔	✔✔	✔✔	×	×	?	✔✔
Genetic/Diet Models								
ob/ob	✔✔	✔✔	✔✔	✔	×	×	?	×
db/db	✔✔	✔✔	✔✔	✔	×	×	?	×
SREBP1c Tg	×	✔✔	✔✔	✔✔	✔✔	✔✔	?	✔
Agouti	✔✔	✔✔	✔✔	✔	×	×	?	×
PTEN ko	×	✔✔	✔✔	✔✔	✔✔	✔✔	?	✔
PPAR-α ko	×	×	×	✔✔	✔✔	✔✔	?	✔
AOX ko	×	×	×	✔✔	✔✔	✔	?	✔
MAT1A ko	×	×	×	✔✔	✔✔	✔	?	✔
MUP-uPA Tg	✔✔	✔✔	✔✔	✔✔	✔✔	✔✔	✔✔	✔✔
DIAMOND	✔✔	✔✔	✔✔	✔✔	✔✔	✔✔	?	✔✔

✔✔, marked expression; ✔, modest expression; ×, no expression; ?, not assessed; HFD, high-fat diet; WD, Western diet; MCD, methionine- and choline-deficient diet; CD, choline-deficient diet; STAM, streptozotocin + HFD-treated mice; DEN, diethylnitrosamine; ob/ob, leptin-deficient mice; db/db, leptin-receptor-deficient mice; SREBP1c Tg, sterol regulatory element-binding protein 1c transgenic mice; Agouti, KK-Ay-a mice; PTEN ko, phosphatase and tensin homolog deleted on chromosome 10 knockout mice; PPAR-α ko, peroxisome proliferator-activated receptor α knockout mice; AOX ko, acetyl-coenzyme A oxidase-deficient mice; MATa1 ko, methionine adenosyl transferase 1A knockout mice; MUP-uPA Tg, mice expressing transiently high amounts of urokinase plasminogen activator in hepatocytes; DIAMOND, isogenic BL6/129 hybrid strain fed an obesogenic WD supplemented by high fructose and sucrose.

same inflammatory cytokine involved in the transition from bland steatosis to NASH, TNF, was found to drive the proliferation of HCC-initiating cells, mainly through activation of nuclear factor κB (NF-κB) signaling (Nakagawa et al., 2014). However, how NASH results in accumulation of oncogenic mutations that lead to HCC initiation is unknown, as the mutational profile and signature of NASH-associated HCC (Shalapour et al., 2017) do not seem to differ from those attached to other HCC etiologies (Schulze et al., 2015).

Role of Cirrhosis and Fibrosis

NASH is a risk factor for both HCC and liver cirrhosis (Adams et al., 2005). Cirrhosis is thought to contribute to the progression from NASH to HCC (Marengo et al., 2016). This is based on a large human clinical study in which less than 1% of patients with NAFLD developed HCC within 8 years after initial diagnosis. However, in NAFLD patients with cirrhosis, the rate of progression was 10% (Yatsuji et al., 2009). It is important to note, however, that patients who develop cirrhosis as a result of NASH have a lower relative risk of developing HCC compared with those with viral- or alcohol-related cirrhosis (Marengo et al., 2016). Although less well documented, there is emerging evidence that HCC can also present in non-cirrhotic NASH patients. From a clinical perspective, it is somewhat dangerous to define

patients at risk of developing HCC by the presence or absence of cirrhosis. It should also be emphasized that while cirrhosis is not a universal feature of NASH-driven HCC, liver fibrosis is a cardinal sign of NASH. Therefore, a faithful mouse model of NASH-driven HCC need not necessarily present with cirrhosis but should exhibit extensive "chicken wire"-like pericellular fibrosis (see Table 1). Nonetheless, the mechanisms by which fibrosis, which usually refers to the deposition of collagen fibers, affects HCC development are obscure. Furthermore, the cells that are responsible for collagen deposition in the liver, hepatic stellate cells (HSCs), are related to activated myofibroblasts or cancer-associated fibroblasts (CAFs), which are known to have broad-spectrum tumor-promoting activities, not all of which are related to collagen deposition (Affo et al., 2017). Activated HSCs and CAFs produce transforming growth factor β as well as many other cytokines and chemokines that recruit follicular helper (T_{FH}) cells and naive B cells into the liver and stimulate conversion of the latter to immunosuppressive immunoglobulin A$^+$ (IgA$^+$) plasma cells, which accumulate in human NASH and promote NASH-to-HCC progression in mice (Shalapour et al., 2017). Activated HSC also produce interleukin-6 (IL-6), another cytokine that is elevated during hepatic steatosis and can support HCC progression (Park et al., 2010).

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Inflammation and Adaptive Immunity in HCC

The concept that obesity leads to a state of low-grade chronic inflammation is now well accepted (Hotamisligil et al., 1993). Work from our group has demonstrated that infiltrating macrophages and resident Kupffer cells produce inflammatory cytokines, such as IL-6 and TNF (Nakagawa et al., 2014; Naugler et al., 2007; Park et al., 2010), which contribute to both NASH and HCC development through c-Jun terminal kinase and IκB kinase signaling (Font-Burgada et al., 2016). However, the role of adaptive immunity in HCC development has, until recently, been unclear. Using mouse models of NASH that progress to HCC, we found that immunosuppressive IgA$^+$ plasma cells that accumulate in fibrotic livers dismantle CD8$^+$ cytotoxic T cell (CTL)-mediated immunosurveillance, thereby allowing early neoplastic lesions to escape immune elimination and progress to full-blown HCC (Shalapour et al., 2017). The immunosuppressive function of the fibrosis-stimulated IgA$^+$ plasma cells most likely depends on expression of programmed death ligand 1 (PD-L1) and IL-10, which can directly induce CTL exhaustion or dysfunctionality. These findings explain why programmed death 1 (PD-1) inhibitors, which reverse CD8$^+$ T cell exhaustion, are highly effective for the treatment of HCC (El-Khoueiry et al., 2017), despite the low mutational load associated with this type of cancer (Alexandrov et al., 2013). A different NAFLD-related mechanism was described by Greten and coworkers (Ma et al., 2016), who attributed the decline in liver-resident CD4$^+$ T cells in high-fat diet (HFD)-fed mice that express a *MYC* transgene (which is not a relevant model of NASH-driven HCC) to the production of linoleic acid by steatotic hepatocytes. Although HFD also leads to a decline in liver-resident CD4$^+$ T cells in *MUP-uPA* mice, it remains to be determined whether this has any effect on anti-HCC immunity (Shalapour et al., 2017).

Genetic Factors that Contribute to NASH-Driven HCC

A limited number of genetic risk factors that predispose individuals to the development of NAFLD have been identified, the most notable of which is a missense mutation in the patatin-like phospholipase domain-containing 3 (*PNPLA3*) gene (Romeo et al., 2008). There are also a limited number of rare variants of other genes, such as nonsense mutations in the apolipoprotein B (*APOB*) gene and the telomerase reverse transcriptase (*TERT*) gene, which influence disease progression (Dongiovanni et al., 2014). It is important to note that deep sequencing of tumors and adjacent non-tumor tissue from NASH-driven HCC and comparison with hepatitis B virus/HCV-driven HCC is of importance, but to date no such studies have been reported. This is of importance considering some of the basic differences in liver metabolism between steatohepatitis and viral hepatitis. For example, hydroxysterols accumulate in NAFLD/NASH-afflicted livers (Li et al., 2017) but not in livers of viral hepatitis patients. Another important player is the liver-specific microRNA 122 (miR-122). Reduced miR-122 expression in HCV-related HCC correlates with increased metastasis and poor prognosis (Filipowicz and Grosshans, 2011), while delivery of miR-122 to a *MYC*-driven mouse HCC markedly reduces tumorigenesis (Hsu et al., 2012). Paradoxically, inhibition of miR-122 in HFD-fed mice with antisense oligonucleotides markedly improved hepatosteatosis and reduced liver damage, prerequisites for NASH development (Esau et al., 2006). While genetic factors play a role in almost every disease, it is important to note that weight-loss surgery

leads to improvement and/or resolution of NASH in approximately 80% of patients (Clark and Diehl, 2003), suggesting that environmental factors may dominate over genetic factors in this disease. However, when it comes to NASH-to-HCC progression, not a single genetic or epidemiological study has led to the identification of factors that specifically affect this transition. Therefore, our knowledge of how NASH progresses to HCC is almost exclusively dependent on preclinical models.

NASH Pipeline: Hope or Hype?

Currently there are only two options for the treatment of early NASH, one of which is lifestyle modification through diet and exercise. However, adherence to such simple and noninvasive therapy is not overly successful and the benefits of exercise are understudied. The second option, bariatric surgery, is quite effective, but like any major surgery is associated with potential health risks and is also costly. Nonetheless, NASH is now the second most common cause for liver transplantation in the United States and is anticipated to become the leading cause by 2020. The cost and risk of liver transplantation outweighs those associated with all of the aforementioned preventive and early treatment measures. New and effective NASH therapies are therefore an urgent necessity.

Unfortunately, no new drugs have been approved for the prevention or treatment of NASH, although the development of targeted NASH therapies is a highly active field of translational medicine. Given that the NASH drug market is estimated to reach US$40 billion by 2025, it is not surprising that many companies have embarked on this effort. Accordingly, there are more than 35 clinical trials in various phases testing new pharmacological agents in NASH patients, most of which were discussed in detail in a recent review (Lazaridis and Tsochatzis, 2017). Notably, there are four drugs in current phase III clinical trials: the farnesoid X nuclear receptor ligand obeticholic acid or Ocaliva (Intercept Pharmaceuticals), the peroxisome proliferator-activated receptor α/δ (PPAR-α/δ) agonist GFT505 or elafibranor (Genfit), the apoptosis signal-regulating kinase 1 selonsertib (Gilead Sciences), and the dual chemokine receptor 2/5 (CCR2/CCR5) inhibitor cenicriviroc (Allergan). It should be noted, however, that in September 2017 the Food and Drug Administration issued a safety communication warning of a risk for serious liver injury and death from incorrect dosing of Ocaliva in patients with primary biliary cholangitis after 19 such deaths occurred. Elafibranor, selonsertib, and cenicriviroc are currently still in phase III with no results posted. Several other NASH-targeting candidate compounds, such as the pan-caspase inhibitor emricasan (Conatus), were recently found ineffective in proof-of-concept trials, although a few other NASH trials are still ongoing. Another clinical candidate that has failed in reducing NASH-associated high blood pressure, although it was found effective in ameliorating hepatic ballooning, is the galectin-3 ligand GR-MD-02 (Galectin Technologies).

Undoubtedly, in order for successful drug development, the biological understanding of NASH needs dramatic improvement and biomarkers that can be used for patient stratification and evaluation of therapeutic outcomes need to be developed. Our opinion is that any significant progress on these fronts depends on the availability of mouse models that closely mimic human NASH. Since mouse models of NASH have been recently

reviewed (Haczeyni et al., 2018; Hansen et al., 2017; Takakura et al., 2018), we focus the following discussion on preclinical models for studying NASH-driven HCC, the most dangerous outcome of untreated NASH. In addition to replicating the human pathology and showing robust progression to HCC within a reasonable time frame (less than 12 months) and without the use of chemical carcinogens, a suitable preclinical model of NASH-driven HCC should also be responsive to the only effective HCC treatment identified thus far, inhibitors of the PD-L1:PD-1 checkpoint (El-Khoueiry et al., 2017). Most likely, future advances in HCC therapy will be based on different ways for increasing the response rates and efficacy of established anti-PD-L1:PD-1 drugs.

Mouse Models of NASH-Driven HCC
Dietary Intervention Models

The HFD, in which 60% of the caloric value is provided by saturated fats, causes simple hepatic steatosis but practically no liver injury or inflammation in most strains of mice (Nakagawa et al., 2014). A considerably higher degree of hepatic injury is elicited by the so-called Western diet (WD), which contains pork lard, beef tallow, saturated FA, cholesterol, and sucrose. This is particularly apparent in $ApoE^{-/-}$ mice, which are defective in triglyceride export, where NASH-like symptoms and modest fibrosis are observed after WD consumption (Schierwagen et al., 2015). However, no HCC has been reported to date in such mice. More severe NASH-like pathology is produced by feeding mice toxic diets that are deficient in either methionine and choline (MCD) or choline alone (CD) (Machado et al., 2015). Both diets cause rapid weight loss (including loss of both fat mass and lean mass), reduced fasting glycemia, decreased circulating lipidemia, and no insulin resistance or increases in circulating or liver IL-6 (Kammoun et al., 2017). Unfortunately, this phenotype bears little resemblance to the etiology and metabolic features of human NASH. Moreover, neither MCD- nor CD-fed mice develop HCC. The CD model can be improved by combining it with HFD (CD + HFD), resulting in inhibition of weight loss and progression to HCC in about 25% of mice in a manner dependent on NF-κB signaling (Wolf et al., 2014). However, a major drawback of this model is the low penetrance of HCC induction and lack of information regarding factors that differentiate the few mice that progress to HCC from the majority that remains tumor free.

Recently, it has become apparent that elevated fructose consumption is an important contributor to the NAFLD and NASH epidemic, acting primarily by induction of liver ER stress and DNL (Softic et al., 2016). Accordingly, mice maintained on a diet high in *trans* fats and high-fructose corn syrup along with a sedentary lifestyle developed liver inflammation and bridging fibrosis within a year, and 60% of them showed hepatocellular neoplasms (Dowman et al., 2014). Although this dietary manipulation appears very useful as a model, the metabolic and immune mechanisms that drive neoplastic progression in response to fructose remain to be investigated, and the tumors need to be shown to be similar to human HCC.

Toxins Plus Diet-Based Models

A new mouse model of NASH-driven HCC was recently developed based on administration of streptozotocin (STZ), a naturally occurring chemical that is toxic to pancreatic insulin-

producing β cells that is often used in preclinical settings to induce type 1 diabetes. In this so-called STAM model, low-dose STZ is given to mice immediately after birth, followed by HFD feeding at 4 weeks of age. Simple steatosis appears after 5 weeks and NASH after 7 weeks, followed by fibrosis after 9 weeks, adenomas after 12 weeks, and evidence of HCC at 16 weeks (Fujii et al., 2013; Saito et al., 2015). The STAM model has the advantage of being rapid, which may be the main reason for its wide adoption. However, unlike NASH-driven human HCC that almost always develops in the context of obesity, insulin resistance, and type 2 diabetes, STZ-treated mice exhibit type 1 diabetes and are lean. In addition, one cannot rule out an independent carcinogenic effect of STZ, as STAM mice display very mild fibrosis (Shalapour et al., 2017) and STZ is a DNA-damaging alkylating agent (Ridolfi et al., 1991). Thus, this model may not be all that different from the diethylnitrosamine (DEN) + HFD model, which depends on administration of the procarcinogen DEN (Park et al., 2010). As mentioned above, HFD alone does not induce NASH and its combination with DEN does not model NASH-driven HCC. Nonetheless, the DEN + HFD model provided the first demonstration that obesity and simple steatosis increase HCC risk by enhancing local production of IL-6 and TNF. Likewise, the administration of DEN to CD + HFD-fed mice allows for much more efficient HCC induction (Kishida et al., 2016), although it does not model NASH-induced HCC. Another recently developed model has combined WD with weekly dosing of carbon tetrachloride (CCl_4) (Tsuchida et al., 2018). This model has the advantage of rapid disease progression, as the mice develop stage 3 fibrosis by 12 weeks and HCC by 24 weeks. Moreover, the model exhibits hepatic transcriptomic alignment with human NASH. It should be noted, however, that CCl_4 is a potent hepatotoxin that can induce genotoxicity and oxidative DNA damage in rodents (Alkreathy et al., 2014).

Genetics Plus Diet-Based Models

The most popular genetic models of obesity, both leptin (*ob/ob*)-deficient and leptin-receptor (*db/db*)-deficient mice, do not develop NASH or HCC but are more susceptible to HCC induction on DEN administration (Park et al., 2010). However, several other genetically modified mice that are susceptible to NASH development have been described. SREBP-1c transgenic mice, the agouti (*KK-Ay/a*) mouse, and mice with global deficiencies in phosphatase and tensin homolog (PTEN), PPAR-α, acyl-coenzyme A oxidase (AOX), and methionine adenosyltransferase 1A (MAT1A) have all been studied in the context of NASH-driven HCC. As previously reviewed (Takahashi et al., 2012), these models have limitations since they fail to either display obesity (PTEN-, PPAR-α-, AOX-, MAT1A-deficient, and SREBP-1c transgenic mice) or NASH (*KK-Ay/a* mice). Moreover, RNA-sequencing (RNA-seq) analysis had shown that the liver gene expression profile of $Pten^{-/-}$ mice is quite different from other mouse models of NASH (Teufel et al., 2016). Furthermore, $Pten^{-/-}$ mice are insulin hyper-responsive rather than insulin resistant, in marked contrast to patients presenting with NASH.

Recently, we developed a new model for NASH-driven HCC that does not suffer from most of the limitations listed above. This model is based on feeding HFD to *MUP-uPA* transgenic mice, which express high amounts of urokinase plasminogen

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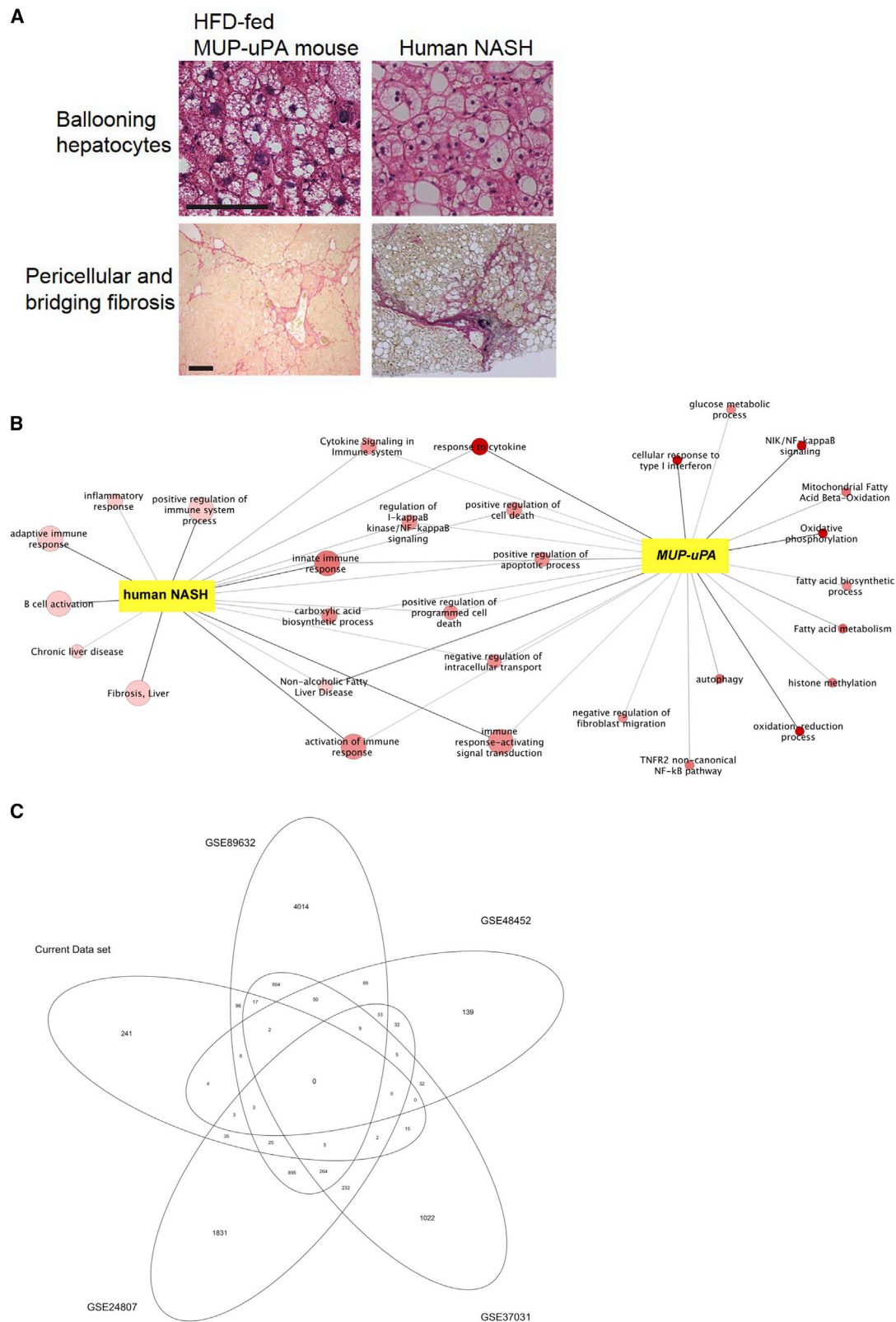
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activator (uPA) specifically in hepatocytes during the first 6 weeks of life (Nakagawa et al., 2014). Excessive uPA expression induces hepatocyte ER stress and transient liver damage, both of which have been implicated in human NASH development (Lebeaupin et al., 2018). Unlike wild-type BL6 mice kept on HFD, all HFD-fed *MUP-uPA* mice develop NASH-like disease with up to 85% of them spontaneously progressing to HCC. This pattern of disease progression allows for biomarker discovery and identification of molecular drivers of HCC progression, since mice can be examined at the onset of NASH and then followed until they separate into those that develop tumors and those that do not. HFD-fed *MUP-uPA* mice show substantial upregulation of collagen gene expression, HSC activation, and upregulation of fibrogenic markers. Classical NASH signs, including ballooning hepatocytes, inflammatory infiltrates, and pericellular and bridging fibrosis indistinguishable from the pattern observed in human NASH, are observed within 4 months of HFD initiation (Nakagawa et al., 2014) (Figure 1A). Both NASH development and HCC progression depend on elevated TNF production, ER stress, and hepatic expression of p62, all of which also participate in the human pathology (Nakagawa et al., 2014; Umemura et al., 2016). Altogether, the HFD-fed *MUP-uPA* model is almost identical to human NASH in its pathological features (Maurel et al., 2014). As mentioned above, HCC progression in *MUP-uPA* mice depends on accumulation of immunosuppressive IgA+ plasma cells, a prominent feature of human NASH (Shalapour et al., 2017). The involvement of immunosuppressive IgA+ plasma cells that express PD-L1 and IL-10 makes the HFD-fed *MUP-uPA* mouse highly suitable for testing various treatments that can further increase the efficacy of PD-L1:PD-1 signaling inhibitors.

Another recently developed genetically modified mouse that progresses to NASH and HCC is the isogenic B6/129 hybrid strain fed a WD with a high-fructose-sucrose solution (WD-SW diet) (Asgharpour et al., 2016). These mice, nicknamed DIAMOND mice, show zone 3 hepatic steatosis within 8 weeks and steatohepatitis 16–24 weeks after WD-SW initiation, including ballooning hepatocytes and Mallory-Denk bodies (Asgharpour et al., 2016). Of note, 90% of DIAMOND mice develop HCC after 32–52 weeks, which is quite similar to HFD-fed *MUP-uPA* mice. However, the unique genetic background of DIAMOND mice makes it difficult to cross them with other gene-targeted mice, most of which are on a pure BL6 background, which is required for the genetic identification of additional factors and signaling pathways involved in NASH-to-HCC progression. Nonetheless, DIAMOND mice may be suitable for testing immunotherapies and other interventions, as the mechanisms of tumor development in these mice should be similar to those operating in *MUP-uPA* mice. This, however, remains to be demonstrated. Without further comparison it seems that both the *MUP-uPA* + HFD and DIAMOND models are well suited for studying NASH-driven HCC and are far more physio-

logically relevant than the STAM model. Nonetheless, HCC development in either *MUP-uPA* or DIAMOND mice is considerably slower than in STAM mice. Furthermore, the mutational landscape of *MUP-uPA* (and probably also DIAMOND) mice varies from mouse to mouse (Shalapour et al., 2017). Although this is much more similar to human HCC, which is one of the most heterogeneous cancers (Schulze et al., 2015), this heterogeneity may complicate drug development studies.

Role of Microbiota
Diet and other environmental factors modulate the composition and metabolic activity of the gut microbiota, which in turn can affect health. In particular, HFD and obesity induce dysbiosis and increase the representation of inflammation-evoking bacteria in the gut (Hildebrandt et al., 2009; Parks et al., 2013; Shalapour et al., 2017). HFD- or fructose-induced dysbiosis may also enhance energy harvest from ingested food and, together with inflammation, promote energy storage and insulin resistance (Conlon and Bird, 2014). Not surprisingly, NASH in both humans and mice was found to be associated with dysbiosis (Brandl and Schnabl, 2017; Shalapour et al., 2017; Yu and Schwabe, 2017). Dysbiosis can contribute to deterioration of the intestinal epithelial barrier, resulting in translocation of inflammation-provoking bacterial products and metabolites (and even intact bacteria) that reach the liver via the portal circulation to further enhance NAFLD and NASH progression. As the gut microbiota is metabolically active, metabolomic studies have identified metabolomic profiles unique to NASH patients (Brandl and Schnabl, 2017). Some of these metabolites, such as the bile acid deoxycholic acid, may also contribute to NASH-to-HCC progression via yet to be identified mechanisms (Yoshimoto et al., 2013; Yu and Schwabe, 2017). Gut bacteria and their metabolites also influence adaptive immunity. For example, gut bacteria support the development of IgA+ immunosuppressive plasmocytes (Shalapour et al., 2017), and microbial-derived bile acids control a chemokine-dependent accumulation of hepatic NKT cells and anti-tumor immunity in the liver (Ma et al., 2016), indicating a dual role of bacteria in the context of cancer immunity. Further studies are needed to fully comprehend how the gut microbiota affects hepatic metabolism and immunity and to determine whether bacteria play a primary or secondary role in NASH-driven HCC.

Mouse versus Human
In addition to histological and metabolic similarities between mouse models and human NASH-driven HCC, transcriptomic alignment between the species is also informative. We therefore analyzed RNA-seq data from three patients with NASH and three healthy individuals and compared these with publicly available RNA-seq data (GEO: GSE94097) collected from *MUP-uPA* mice kept on either normal chow or HFD (Shalapour et al., 2017). For human studies, all participants provided informed consent to participate in this study. Ethics approval

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Figure 1. Histological and Transcriptomic Alignment between the *MUP-uPA* Mouse Model and Human NASH
(A) Histological comparison of *MUP-uPA* mice and human NASH; data previously published (Nakagawa et al., 2014).
(B) RNA-seq data from human NASH patients and normal individuals (left) and *MUP-uPA* mice fed either HFD or normal chow (right). All terms are enriched with p < 0.05; the color coding represents the p value for enrichments calculated for the *MUP-uPA* + HFD mice, the darker red the more significant, and the size of the nodes represents the p value for enrichments calculated for the human data (all nodes that are only enriched for the human data are light red, and all nodes that are only enriched for *MUP-uPA* are small).
(C) Gene alignment between our current dataset of NASH patients (n = 3) compared with four other publicly available datasets.

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was obtained from Alfred (195/15), Avenue (190), and Cabrini (09-31-08-15) Human Research Ethics Committee. For animal studies, mice were studied in accordance with NIH guidelines and approved by the UCSD Institutional Animal Care and Use Committee under Dr. Karin's Animal Protocol S00218. Figure 1B shows gene ontology pathways that were enriched in both human and mouse NASH, as well as pathways that were exclusive for one species or the other. In the human data, the inflammatory response was more pronounced than in *MUP-uPA* mice, but it should be acknowledged that none of the analyzed patients were drug naive, that only a very small dataset was analyzed, and that human NASH is extremely heterogeneous. In fact, one should note that the human dataset previously described by Teufel et al. (2016) was not unique to NASH and did not make any discrimination between NASH and simple steatosis, as liver samples were obtained from patients undergoing biopsy for suspected NAFLD or NASH. To illustrate the degree of heterogeneity in human datasets, we compared our human NASH RNA-seq data with four other publicly available NASH datasets, whereby only a low degree of transcriptomic alignment was found (Figure 1C). Notwithstanding the limitations to these analyses, we observed some degree of transcriptomic alignment when comparing the *MUP-uPA* model with our human dataset, notably in regulation of the immune system, innate immune response, and the response to cytokine gene sets. These transcriptomic alignments are fully consistent with our recent descriptions of metabolic and signaling similarities between human NASH and the *MUP-uPA* + HFD model (Shalapour et al., 2017).

Conclusions

NASH-driven HCC is a fast-growing, serious, and complex malignancy that requires better understanding and further study before it reaches epidemic proportions. Preclinical models are a cornerstone of further research, especially given the difficulties in obtaining suitable human specimens representing different stages of the disease. The *MUP-uPA* model, while not perfect or rapid, exhibits many similarities to the human disease. We, therefore, suggest its adaptation by researchers exploring those aspects of NASH-driven HCC that are best replicated by this model or by the related DIAMOND mouse. Both models are highly suitable for the development of preventive early treatment and diagnostic procedures. Regardless of the final model to be adapted by the community, one should be cautious of mechanistic, immunological, or drug development studies that are performed with inappropriate models. Despite their so-called convenience, such models should be avoided.

AUTHOR CONTRIBUTIONS

S.S., S.R., G.O., and M.J.W. collected and/or analyzed the data in Figure 1. M.A.F., S.S., and M.K. wrote the manuscript.

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