Pre-clinical and clinical investigations of metabolic zonation in liver diseases: The potential of microphysiology systems

Alejandro Soto-Gutierrez1, Albert Gough2,3, Lawrence A Vernetti2,3, DL Taylor2,3,4 and Satdarshan P Monga1,5

1Department of Pathology, University of Pittsburgh, Pittsburgh, PA 15260, USA; 2Drug Discovery Institute, University of Pittsburgh, Pittsburgh, PA 15260, USA; 3Department of Computational & Systems Biology, University of Pittsburgh, Pittsburgh, PA 15260, USA; 4Cancer Institute, University of Pittsburgh, Pittsburgh PA 15232, USA; 5Department of Medicine, Pittsburgh, University of Pittsburgh, PA 15260, USA

Corresponding authors: DL Taylor. Email: dltaylor@pitt.edu; Satdarshan P Monga. Email: smonga@pitt.edu

Impact statement
Liver zonation of oxygen tension along the liver sinusoids has been identified as a critical liver microenvironment that impacts specific liver functions such as intermediary metabolism of amino acids, lipids, and carbohydrates, detoxification of xenobiotics and as sites for initiation of liver diseases. To date, most information on the role of zonation in liver disease including, non-alcoholic fatty liver disease (NAFLD), non-alcoholic steatohepatitis (NASH), cirrhosis, and hepatocellular carcinoma (HCC) have been obtained from animal models. It is now possible to complement animal studies with human liver, microphysiology systems (MPS) containing induced pluripotent stem cells engineered to create disease models where it is also possible to control the in vitro liver oxygen microenvironment to define the role of zonation on the mechanism(s) of disease progression. The field now has the tools to investigate human liver disease progression, diagnosis, and therapeutic development.

Abstract
The establishment of metabolic zonation within a hepatic lobule ascribes specific functions to hepatocytes based on unique, location-dependent gene expression patterns. Recently, there have been significant developments in the field of metabolic liver zonation. A little over a decade ago, the role of β-catenin signaling was identified as a key regulator of gene expression and function in pericentral hepatocytes. Since then, additional molecules have been identified that regulate the pattern of Wnt/β-catenin signaling within a lobule and determine gene expression and function in other hepatic zones. Currently, the molecular basis of metabolic zonation in the liver appears to be a ‘push and pull’ between signaling pathways. Such compartmentalization not only provides an efficient assembly line for hepatocyte functions but also can account for restricting the initial hepatic damage and pathology from some hepatotoxic drugs to specific zones, possibly enabling effective regeneration and restitution responses from unaffected cells. Careful analysis and experimentation have also revealed that many pathological conditions in the liver lobule are spatially heterogeneous. We will review current research efforts that have focused on examination of the role and regulation of such mechanisms of hepatocyte adaptation and repair. We will discuss how the pathological organ-specific microenvironment affects cell signaling and metabolic liver zonation, especially in steatosis, viral hepatitis, and hepatocellular carcinoma. We will discuss how the use of new human microphysiological platforms will lead to a better understanding of liver disease progression, diagnosis, and therapies. In conclusion, we aim to provide insights into the role and regulation of metabolic zonation and function using traditional and innovative approaches.

Keywords: Liver zonation, liver diseases, induced pluripotent stem cells, microfluidics, hepatocytes, microphysiology systems

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Introduction
The liver is responsible for the synthesis of serum proteins; intermediary metabolism of carbohydrates, amino acids, and lipids, as well as the removal of xenobiotic compounds by xenobiotic metabolism. The liver portal vein receives all of the circulation exiting from the small intestine and a majority of the circulation from the large intestine, spleen, and pancreas. The “strategic” position of the liver relative to the intestinal nutrient supply, and the unique gene- and protein-expression patterns of hepatocytes allow it to act as a master metabolic organ. However, hepatocyte functions (e.g. gene expression profiles and biochemical activities) vary based on the physical location within the hepatic lobule.1–3 In the adult liver, the hepatocyte is structurally and functionally polarized and has three separate membrane domains: sinusoidal (basal), lateral (or interhepatocytic), and canalicular (apical). Sinusoids carry the
blood flow from the portal vein and hepatic artery to the central vein and are lined with endothelial cells, Kupffer cells (resident macrophages), and stellate cells (Figure 1). Bile is secreted from the apical side of the hepatocytes and moves towards the bile ducts in the portal triad, traversing biliary canaliculi which are lined initially by the villi-rich apical surface of the hepatocytes, and then by the transitional biliary epithelial cells in the periportal region (Figure 1).

Functionally, the liver sinusoid is divided into three zones, based upon oxygen tension. Zone 1 rings the portal tracts, where the oxygenated blood from hepatic arteries enters and mixes in the sinusoid with blood from the portal vein. Zone 3 is located around the central vein, where oxygenation is much lower, and zone 2 is located between zone 1 and zone 3. The lobular architecture in the adult liver provides a spatial separation and gradation, which exposes only limited numbers of hepatocytes to the same nutrients, toxins, and oxygen but also allows gradation for different metabolic functions including fatty acid metabolism, reactions that produce NADH, gluconeogenesis, and glycolysis. Thus, hepatocytes exhibit specific functions that are centered on their location along the porto-central axis. A pivotal 2017 report using single-cell transcriptome analysis of fixed in vivo mouse liver found 50% of the liver genes are zonated, including genes that are most abundantly expressed in zone 2, an often over-looked zone in research. This report suggests that many more activities and functions of the liver than are currently known may have spatial preferences that are likely to impact liver disease.

Wnt/β-catenin signaling has emerged as a fundamental regulator of liver function and development, including liver zonation. The Wnt/β-catenin pathway is most active around the central vein (pericentral area) and repressed around the portal triad (periportal area). In pericentral hepatocytes, β-catenin regulates the expression of several genes. Hepatic tissues of mice with liver-specific knockout
of β-catenin (Figure 2) do not express glutamine synthetase (GS), cytochrome p450 (Cyp2e1 or Cyp1a2), and many other targets. Several mechanisms that regulate the Wnt pathway in different zones of the liver have been discussed; for instance, the tumor suppressor gene product adenomatous polyposis coli (APC), a negative regulator of Wnt signaling, is expressed basally at higher levels in the periportal hepatocytes. The Ras/MAPK/Erk pathway has also been described as counterbalancing Wnt signaling to favor periportal gene programming. Hepatocyte nuclear factor-4α (HNF4α) has been proposed to play a function in driving liver zonation by modulating zonal expression of genes, acting through cross-talk with the Wnt pathway. HNF4α is believed to regulate gene expression in periportal hepatocytes, as loss of HNF4α from hepatocytes increases the expression of pericentral genes by periportal hepatocytes (similar to mice with a liver-specific knockout of APC).

The activation of β-catenin in pericentral hepatocytes is a function of Wnt signaling. Similar to the liver-specific β-catenin knockout mice, mice lacking Wnt co-receptors LDL-related proteins 5 and 6 (LRP5/6) also show complete lack of expression of pericentral genes, as well as GS (Figure 2(b)). The source and identity of Wnt proteins that regulate pericentral gene expression are not completely known; however, a recent study implicates Wnt2 and Wnt9b from an endothelial cell source in directing β-catenin activation in pericentral hepatocytes. More recently, it was reported that a cell surface signaling element involving R-spondin (RSPO) ligands, the leucine-rich repeat-containing G protein-coupled receptors 4/5 (LGR4/5), the zinc and
ring finger 3 (ZNRF3) and its homologue, ring finger 43 (RNF43) transmembrane proteins spatiotemporally regulate the Wnt/β-catenin signaling gradient in the liver, assisting with the establishment of metabolic zonation. Indeed, RSPO-LGR4/5 signaling are known to synergize with Wnt to amplify β-catenin but are inconsequential on their own.

Gougelet et al. reported that the interaction between β-catenin and T-cell factor-4 (TCF-4) directs specific transcriptional networks that have profound effects on glutamine, lipid, drug, and bile acid metabolism. It is also pertinent to note that while the role of Wnt/β-catenin signaling is of essence in various aspects of hepatic physiology such as in liver development, regeneration, metabolic zonation, and regulating cellular metabolism, its aberrant activation is evident in a significant subset of liver tumors including hepatocellular cancer (HCC), hepatic adenomas (HCA), and hepatoblastoma. In fact, mutations in the hepatocellular cancer (HCC), hepatic adenomas (HCA), and hepatoblastoma. In fact, mutations in the β-catenin gene are present in around 25–40% of all HCC cases. These tumors can be detected by diffuse staining for GS that indicates β-catenin activation (Figure 2(c)).

Herein, we will discuss how several liver diseases have zonal preferences, while others disrupt zonation, which may contribute to disease progression. A great deal of the current knowledge in the field of liver zonation has been produced from experimental animal models. However, emerging studies using human liver microphysiology platforms are expected to provide information on the role and regulation of liver zonation in both normal human physiology and disease.

**Metabolic zonation, NAFLD, and HCC**

Non-alcoholic fatty liver disease (NAFLD) is prevalent in about 30% of Americans, especially in the growing number of individuals who are overweight or obese (currently >60%). An all-encompassing term, NAFLD includes a continuum of aberrations from steatosis, steatohepatitis, hepatocyte ballooning and degeneration, progressive fibrosis, and cirrhosis to HCC. NAFLD is categorized into non-alcoholic fatty liver (NAFL) and non-alcoholic steatohepatitis (NASH). NAFL implies the presence of hepatic steatosis without significant inflammation, while NASH represents hepatic steatosis along with notable hepatic inflammation. While the disease can be histologically indistinguishable from alcoholic hepatitis, the diagnosis of NAFLD is made in the absence of excessive and chronic alcohol intake. NAFLD involves the progression from simple liver steatosis, a largely benign condition, to the more severe NASH, that is characterized by hepatocyte injury (cell ballooning and death) and inflammatory infiltration, often resulting in hepatic fibrosis, liver failure, or cirrhosis (Figure 3). Because of its rising rate of incidence in the US, NAFLD may account for a third of future HCC cases. Intriguingly, NASH may progress to HCC without development of advanced fibrosis and cirrhosis, but the mechanisms remain obscure. NASH is currently the fourth most common indication leading to liver transplantation in the US. However, as the proportion of liver transplant recipients with NASH appears to be increasing, in the next 20 years, NASH may surpass hepatitis C virus infection as the leading indication for liver transplantation.

Metabolic zonation may play a critical role in pathological states, as cells differentially express zone-specific genes, and the exposure of cells to various metabolic insults or toxins is zone dependent. For instance, NAFLD is the result of a combination of abnormalities in hepatocytes that affect both glucose and fat metabolism. As a consequence, perturbations in the metabolic zonation might be both cause and effect in the disease pathogenesis.

Although the zone-specific histological distribution of steatosis in the liver has been documented, its significance and basis are not completely understood. Steatosis is typically localized in the pericentral region, but it may also occur in the periportal area. This heterogeneity can be observed across the whole organ. A recent study correlated the severity and zonal localization of steatosis with NASH. The histological findings from more than 500 liver biopsies with definite NAFLD showed that increasing severity of steatosis was positively associated with lobular inflammation, fibrosis in zone 3, and steatohepatitis, but was unrelated to hepatocyte ballooning, presence of Mallory bodies, or advanced fibrosis. Moreover, around central veins, where oxygenation is poor, steatosis was more often associated with ballooning, Mallory bodies, and advanced fibrosis. Another study evaluated the association of the zonation-dependent distribution of phospholipids throughout the liver and the occurrence of a pro-inflammatory phenotype. In normal conditions, lipid mobilization and storage in hepatocytes is a dynamic and highly regulated process; however, it is possible that changes in the distribution, storage, and metabolism of specific lipids are directly related to dysfunctional metabolic cycles in specific zones in the lobule. First, a review of normal hepatic protein expression in the Human Protein Atlas identified manifest zonation of enzymes involved in lipid utilization and storage, especially those aiding phosphatidylcholine metabolism. Then, the analysis of human biopsies from livers with simple steatosis and NASH revealed a progressive decrease in the zonal distribution of these phosphatidylcholine biosynthetic enzymes. Further analysis using liquid chromatography mass spectrometry showed that zonation of specific phosphatidylcholines are either lost or preserved in connection with the severity of NAFLD. Thus, loss of lipid zonation appears to be strongly linked to the dysfunction of enzymes related to phosphatidylcholine biosynthesis, suggesting that changes in the microenvironment are related to enzymatic differences and potential alterations in function.

The molecular basis of changes in hepatic metabolic zonation in NASH has recently been linked to Wnt signaling. It is well known that β-catenin regulates gluconeogenesis and hepatic insulin signaling by interacting with the transcription factor Forkhead box protein O1. This interaction has been shown to regulate the transcriptional activation of the genes encoding glucose-6-phosphatase and phosphoenolpyruvate carboxykinase (PEPCK), two critical enzymes in hepatic gluconeogenesis.

The association between insulin resistance and NAFLD is well known. Normally, insulin promotes glycolysis and
suppresses gluconeogenesis thereby reducing glucose levels.\(^{30,31}\) At the same time, insulin also induces lipogenesis.\(^{32}\) However, in insulin resistance, it loses its ability to decrease glucose production while continuing to stimulate lipogenesis.\(^{33}\) Lipogenic effects of insulin and of hyperinsulinemia, which is a characteristic of insulin resistance, play an important role in the development of steatosis. Hepatic lipids can also contribute to the development of insulin resistance.\(^{34}\) In fact, steatosis can occur without preceding insulin resistance and some studies have challenged the idea that steatosis leads to insulin resistance.\(^{34}\) Steatosis can lead to increased fatty acid β-oxidation, which can also contribute to insulin resistance.\(^{35}\) Studies have shown that increased β-oxidation that occurs in high fat diet (HFD) fed animals can result in accumulation of incompletely oxidized fatty acid intermediates, and decreased glycolysis, for example through the inhibition of PDH activity. Also, there is accumulation of citrate, which inhibits phosphofructokinase-1, leading to increased glucose 6-phosphate, which in turn suppresses hexokinase. Inhibition of pyruvate dehydrogenase can preserve the intracellular levels of pyruvate and lactate for use in gluconeogenesis. Based on these observations, treatment strategies that were actually devised to increase fatty acid oxidation to reduce fat accumulation, may be detrimental to insulin resistance and worsen NASH. On the other hand, since fatty acid oxidation appears to contribute to the pathogenesis of NAFLD in part through effects on glycolysis and gluconeogenesis, therapeutic inhibitors of carnitine palmitoyltransferase I (CPT-1), the rate limiting step in fatty acid β-oxidation may be useful and is being developed.\(^{36,37}\)

Another recent study carefully assessed the involvement of β-catenin signaling in lipid metabolism in hepatocytes.\(^{38}\) Hepatocyte-specific β-catenin knockout (KO) mice and hepatocyte-specific stable β-catenin expressing transgenic (TG) mice were fed HFDs. While chow-fed TG and KO mice had normal liver histology, in TG mice the obesogenic HFD led to notable pericentral steatosis with periportal sparing. KO mice fed the same diet showed increased pan-lobular inflammation and hepatocyte apoptosis. Moreover, TG mice developed diet-induced obesity and systemic insulin resistance, while KO mice were resistant. Intriguingly, β-catenin did not directly affect hepatic insulin signaling; however, hepatic expression of both key glycolytic and lipogenic genes was higher in HFD-fed TG mice and lower in KO mice.

In addition, KO mice showed defective hepatic fatty acid oxidation. Since β-catenin interacts with hypoxia-inducible factor 1-α (HIF1α), the authors investigated HIF1α and found higher levels in HFD-fed TG mice and lower levels
in KO mice. Hepatic HIF1α is an important oxygen-sensitive transcriptional regulator of glycolysis. Intriguingly, KO mice showed attenuated pericentral hypoxia, suggesting a disruption of the normal sinusoidal oxygen gradient. In combination, these observations indicate that both Wnt/β-catenin and HIF1α signaling have an impact, and are impacted by carbohydrate and lipid metabolism. Hence, their interactions in this dynamic process of metabolic zonation and homeostasis may have a critical role in diet-induced fatty liver and obesity.

Studies have now begun to show that Wnt/β-catenin activation is apparent in NASH-associated HCC as well. In a subanalysis of our published work, we observed that 32 of the 249 HCC cases in a French cohort had evidence of metabolic disease with or without other comorbidities. Of these, 13 cases had mutations in the catenin β1 gene (CTNNB1) (~40%), which suggests some preferential activation of the Wnt pathway in HCC in a NASH background, requiring a comprehensive study. It should also be noted that recent studies have shown that mutations in CTNNB1, along with activating mutations in telomerase reverse transcriptase promoter, are highest in frequency in HCC associated with alcoholic liver disease. Similarly, mice injected with a chemical carcinogen initially followed by chronic alcohol exposure led to the development of HCC through activation of the Wnt signaling pathway. Thus, whether CTNNB1 mutations in HCC are specific to any particular pathology remains a difficult question to answer; however, a subset of all these etiologies do have a predilection for Wnt pathway activation that eventually leads to HCC due to yet undiscovered mechanisms. A more in-depth study will be of critical significance to elucidate the complex interplay of signaling mechanisms that play a role in injury, repair, and carcinogenesis.

Overall, while zonation and Wnt signaling are key in lipid and carbohydrate metabolism, regulation of inflammation, injury and eventually tumorigenesis, the processes by which the pathways and microenvironment interact to modulate each other remain elusive. More in-depth studies especially in human in vitro models will be critical, especially since NASH is an ongoing therapeutic challenge (see below).

### Metabolic zonation changes, hepatitis C virus infection, and HCC

Hepatitis C virus (HCV) is one of the major etiologies of chronic hepatitis and progressive liver fibrosis that lead to development of lethal complications, most commonly, cirrhosis and HCC. HCC is also the second leading cause of cancer deaths worldwide. Interestingly, it is one of only three cancers that are increasing in incidence in the Western world and the only and most rapidly increasing cause of cancer-related deaths in the US. It is estimated that more than 1 million individuals in the US will develop HCV-related liver cirrhosis and/or HCC by 2020. Recently developed direct-acting antivirals for HCV effectively cure HCV infection, but the high costs limit their widespread use around the world. Further, patients who have already progressed to advanced fibrosis and cirrhosis may still be at high risk to develop HCC, and HCC risk remains high for decades even after effective antiviral therapies. Overall, HCV-related HCC is predicted to increase until 2030 despite the improved viral cure. Thus, an improved understanding of the pathophysiology of HCV-induced HCC and more physiological liver disease models are required to prevent cancer development.

Several components of the microenvironment appear to play essential roles in the HCV life cycle and immune evasion and have been identified as candidate targets for antiviral therapy. However, the disease pathogenesis that ultimately causes or facilitates HCC is still unclear. Experimental studies to date have suggested models of viral carcinogenesis unique to HCV. Cumulative information shows that HCV transmits signals and modulates hepatocyte gene expression following engagement with cellular receptors. Additionally, viral proteins have been involved in disrupting signal transduction pathways that affect cell survival, proliferation, and transformation. For instance, viral proteins, as well as viral RNA structural motifs, have been suggested to initiate lipogenic signaling. This is known to lead to hepatic steatosis and fibrosis, a frequent complication of chronic HCV. Also, it has been observed that transgenic animals with expression of the HCV in the liver develop a range of liver metabolic disorders, including changes of lipid metabolism and micro-/macrovesicular steatosis. Males are particularly prone to HCC in this scenario.

In a recent study, Moreau et al. studied the HCV-based mechanisms that allow the systemic loss of hepatic metabolic zonation in transgenic mice with hepatocyte-targeted expression of HCV proteins (FL-N/35 model) as well as needle biopsies from patients with hepatitis C. To demonstrate alterations of hepatic metabolic zonation, the authors quantified steatosis in livers of FL-N/35 mice and found that lipid accumulation occurred in a zonated pattern, with lipid-filled hepatocytes occupying only a couple of rows of cells in the midzone of the hepatic lobule. In accordance, the expression of enzymes involved in lipogenesis, such as fatty acid synthase and those necessary for further steps of fatty acid metabolism, such as acyl-CoA synthetase long-chain family member 3 and stearoyl-CoA desaturase 1, was increased. Intriguingly, the expression of GS extended from only pericentral hepatocytes in normal mice all the way to the midzone. Moreover, these alterations in metabolic zonation were validated in 50 human liver HCV-infected biopsies. Interestingly, these changes in HCV-infected human liver biopsies, as well as FL-N/35 mice, appeared to be driven by systemic signaling via the Wnt/β-catenin pathway. The authors found that messenger RNAs of several elements of the Wnt signaling pathway and its target genes were greatly increased in transgenic animal livers. Human liver biopsies revealed a significant increase of Wnt4, Tcf7l2, and nuclear β-catenin. However, there were not major differences in the expression of genes targeted by the canonical Wnt/β-catenin. This is not surprising as Wnt targets show contextual specificity and exact signatures of the pathway under different pathologies are truly unclear. These studies suggest that perturbed
metabolic zonation seems to precede steatosis in early stages of human disease associated with HCV infection.

Several pathologies in the liver are associated with alterations in metabolic zonation during their evolution. For example, cirrhosis has been reported to either totally remove GS expression, suggesting a switch to the peripherally phenotype or, paradoxically, also leading to strong GS expression (a target of Wnt/β-catenin signaling) throughout the lobule, thus suggesting acquisition of centrilobular features by all cells. These studies have also identified increased expression of WNT13, WNT5a, and/or β-catenin. The above observations really suggest the existence of heterogeneity within a hepatic lobule as a major mechanism of hepatocyte survival and expansion during chronic hepatic pathology, where a zonal subgroup of hepatocytes demonstrates clear advantages over other subgroups. Whether this is purely a function of zonation or existence of polyploidy and aneuploidy, or both, requires further investigation. Nonetheless, whether increased expression of the Wnt pathway is a cause or an effect remains a mystery.

HCC has also been studied extensively and specific molecular signatures of HCC have been identified in cirrhotic vs. non-cirrhotic livers. An interesting observation is that HCCs from non-cirrhotic livers more frequently have activation of WNT signaling to β-catenin than HCCs from cirrhotic livers, which frequently have altered P53 activity. Indeed, clinical studies have also shown HCC in non-cirrhotic livers to have more frequent mutations in CTNNB1. A study was also performed to determine if there was any cooperation between mutations in CTNNB1 and ongoing fibrosis in HCC development. Transgenic mice expressing mutant β-catenin in hepatocytes were administered chronic thiocetamide. No differences in fibrosis of HCC development were observed in these mice as compared with control mice. All of the above data suggest that fibrosis and Wnt pathway activation may be two parallel events in HCC. However, a more direct study is essential to corroborate these findings. Intriguingly, frequent alterations have been documented in WNT signaling via β-catenin in HCV-associated HCCs. New models of human liver cirrhosis and cancer could improve our knowledge of the role and regulation of these pathways and to test new therapeutic strategies.

The potential of human liver MPS as experimental models of human liver diseases

Within the past decade, in vitro liver models have evolved from the gold standard of isolated mammalian primary hepatocytes in 2D monolayer plate culture to multicellular, 3D, microphysiology liver systems. The evolution of this process has been extensively reviewed elsewhere. In vitro study of the effect of low oxygen tension on hepatocellular function was initially demonstrated in monolayer cultures of rat hepatocytes maintained in CO2 incubators supporting 4% and 13% oxygen environments. These first studies looked at the effect of oxygen tension on isolated rat hepatocytes and found low oxygen levels increased glycolysis, elevated protein, and activity levels of cytochrome P450 drug metabolizing enzymes and induced zone-dependent compound effects and toxicity.

Liver models that have included zonation produced by microfluidic flow or other means to generate the zonal modulation of hepatocyte functions are presented in Table 1.

Allen and Bhatia first demonstrated the spatial zones with different oxygen tensions by measuring the protein distribution of PEPCK (increased in zone 1) and cytochrome p450 2B (increased in zone 3) in rat hepatocytes in a microfluidic bioreactor. Later, the same authors demonstrated acetaminophen toxicity in the oxygen levels of zone 3 in a co-culture of rat hepatocytes and mouse 3T3-J2 fibroblasts in a perfused bioreactor. Sato et al. reported an in vitro hepatic zonation model in which media oxygen levels of 70.0 mmHg and 31.4 mmHg corresponding to zone1 and zone 3 regions of the liver could be recreated in mouse hepatocytes cultured in a novel MPS device with controllable air/fluid gas exchanger. This study confirmed high levels of PEPCK mRNA in zone 1 and higher levels of glucokinase mRNA in zone 3 hepatocytes which is concordant with in vivo zonation. A commonality for all of the described models is the control of media oxygen over the cell surface to generate the functional effects of zonation. A notable exception to creating zonation through the use of media flow to control oxygen levels was achieved in a microfluidic device that controlled glucose metabolism by creating a gradient of the soluble hormones glucagon and insulin on opposite ends of the MPS device.

However, the heretofore described zonation models used rodent hepatocytes. In addition to the well-documented species, differences in biotransformation of xenobiotics between rodents and humans, differences in glucose regulation at the levels of genes, proteins, and signaling pathways exist between the two species. It is also shown that rodent models reproduce only a limited number of pathogenic events found in NAFLD. The rodent does not adequately replicate the underlying conditions that lead to the prerequisite liver damage necessary for HCC. The absence of an optimal rodent model for HCC has led to the use of the standard subcutaneous tumor models for efficacy testing and these have not been effective in identifying new drugs. Finally, humans are more prone to drug-induced cholestasis from BSEP inhibitors due to an increased production of toxic glycine-conjugated bile acids compared with the production of less toxic taurine-conjugated bile acids found in the rodent. These differences suggest that the use of human cell-based liver models is likely necessary to expand our understanding of human liver diseases.

The first report of human liver cells in a system capable of zonation occurred in 2007 using HepG2 cells in a static plate culture that controlled the oxygen tension by adjusting the media height above the cells. The zonal differential functions of hepatocytes in the equivalent of zone 3 oxygen state were demonstrated in these experiments as the increase in Cyp 1A1 activity and increased glucose consumption. Bavli et al. demonstrated zonation as an increase in glycolysis in zone 3 equivalent oxygen tension in a microfluidic bioreactor built using HepG2/C3A spheroids.
We have created zone 1 and zone 3 microenvironments in our improved, human 3D, microfluidic, multicellular Liver Acinus Microphysiology system (LAMPS) device using computational modeling of oxygen tension confirmed by experimental measurements of oxygen tension. Various zone-specific physiological measurements including acetaminophen toxicity, glycolysis, oxidative phosphorylation, and protein secretions were consistent with measurements in animals (Table 1). In particular, the human-based LAMPS models can be used to exploit the role of canonical Wnt/β-catenin signaling in liver zonation to control zone 3 hepatocyte gene expression to generate human-based models of the normal liver and to study the importance of zonation on liver disease (Figures 1 and 3). Future perspectives

There has been remarkable progress and advances in 3D, microfluidic, multicellular microphysiology human liver models. Several platform technologies have demonstrated zonation, and three have demonstrated zone-specific toxicity. Further investigations are needed to understand how these models can best be used to interrogate the role zonation may have on drug-induced liver injury and in disease initiation and progression.

One example of a model which adds zonation to a human MPS liver designed for toxicology, ADME, and disease modeling is shown in Figure 4. A major component of the platform is the inclusion of a database to manage and track data and provide tools and resources for computational modeling. Integrated platform systems such as this can be used to unravel the unknown molecular interactions between liver disease initiation, progression from simple steatosis to NAFLD, NASH, cirrhosis, and HCC and the three broadly defined zones 1, 2 and 3 compartments of the liver. Furthermore, the liver MPS can be

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**Table 1 Key animal and human liver zonation models**

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<tr>
<td>1st Human hepatocytes, endothelial</td>
<td>path length across cells</td>
<td>↑Oxidative phosphorylation zone 1</td>
<td></td>
</tr>
<tr>
<td>cells, Kupffer-like cells in 3D</td>
<td>O₂-sensitive ruthenium soluble dye</td>
<td>↑Albumin section zone 1</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>↑Urea section 1</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>↑α1-antitrypsin secretion zone 3</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>↑Cyp 2E1 expression zone 3</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>↑Steatosis zone 3</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>↑Acetaminophen toxicity zone 3</td>
<td></td>
</tr>
</tbody>
</table>

PEPCK: Phosphoenolpyruvate carboxykinase; Cyp: Cytochrome P450.
coupled to intestine and fat chips to explore the role of these systems on disease progression. However, a number of important biological, device design, and biomaterial selection factors still need to be addressed before the full potential of human liver biomimetics can be achieved. Critical to success for precision medicine and study of disease progression is the use of patient-specific iPSCs to create all the liver cell types from a single donor. This is necessary to provide consistent sources of cells to test healthy and diseased tissues, while avoiding the use of established cell lines or primary cells from multiple genetically diverse donor sources. In addition, human iPSCs will need to be engineered with gene knock-in and knock-outs to accelerate the disease process, which can be as long as 30 years in some patients. Novel non-invasive methods to monitor cellular health, activation of toxic, therapeutic, or disease pathways processes are needed along with improved in-line media readouts (i.e., media O₂, glucose, lactate, pH). Currently, most 3D, MPS liver models require manual preparation, but 3D bio-printing methods may offer better control of the cell-cell organization and the placement, composition, and thickness of extracellular protein matrices. Finally, the inclusion of metabolic zonation and eventually a bile duct collecting system is also seen as a critical need to realize the full potential of the human, 3D, multicellular, microfluidic, MPS liver, and other organ models needed to improve toxicity assessment, efficacy, and the study of liver diseases.

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DECLARATION OF CONFLICTING INTERESTS

DLT is a consultant for AstraZeneca. SPM is a consultant for Abbvie Pharmaceuticals and Dicerna Pharmaceuticals. He also has corporate research agreements with both companies.

REFERENCES

12. Guadon AM, Song KX, Qu S. Nonalcoholic fatty liver disease: pathogenesis and therapeutics from a mitochondria-centric perspective. Oxid Med Cell Longev 2014;2014:637027
25. Calzadilla Bertol L, Adams LA. The natural course of non-alcoholic fatty liver disease. Int J Mol Sci 2016;17:1


