The Role of Liver Zonation in Physiology, Regeneration, and Disease

Regeant Panday, BA[1] Chase P. Monckton, BSc[1] Salman R. Khetani, PhD[1]

[1] Department of Biomedical Engineering, University of Illinois at Chicago, Chicago, Illinois

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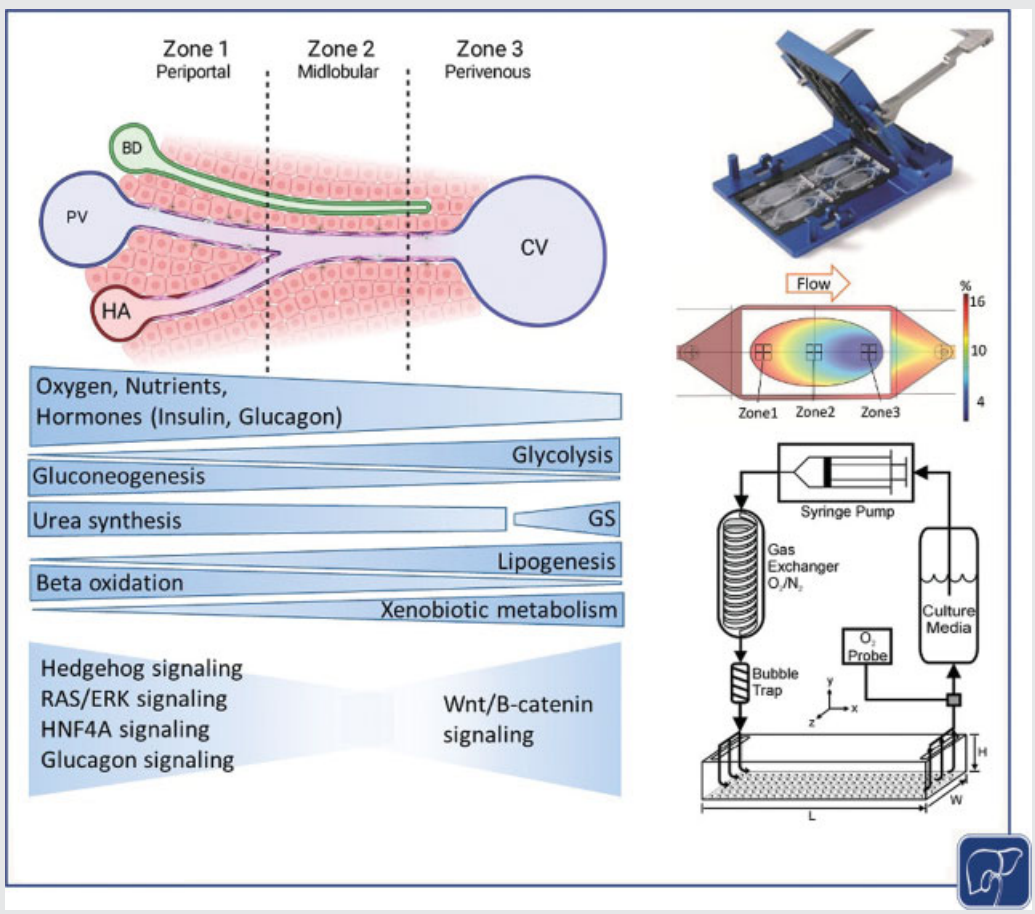
Address for correspondence Salman R. Khetani, PhD, Department of Biomedical Engineering, University of Illinois at Chicago, 851 S Morgan St, 218 SEO, Chicago, IL 60607 (e-mail: skhetani@uic.edu).

Abstract



As blood flows from the portal triad to the central vein, cell-mediated depletion establishes gradients of soluble factors such as oxygen, nutrients, and hormones, which act through molecular pathways (e.g., Wnt/β-catenin, hedgehog) to spatially regulate hepatocyte functions along the sinusoid. Such "zonation" can lead to the compartmentalized initiation of several liver diseases, including alcoholic/non-alcoholic fatty liver diseases, chemical/drug-induced toxicity, and hepatocellular carcinoma, and can also modulate liver regeneration. Transgenic rodent models provide valuable information on the key molecular regulators of zonation, while in vitro models allow for

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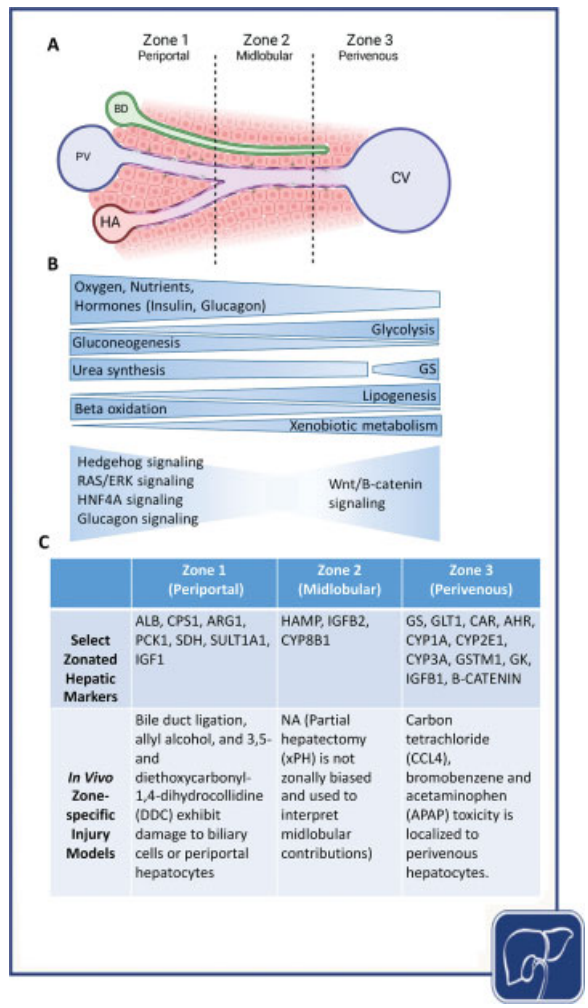
subjecting cells to precisely controlled factor gradients and elucidating species–specific differences in zonation. Here, we discuss the latest advances in both in vivo and in vitro models of liver zonation and pending questions to be addressed moving forward. Ultimately, obtaining a deeper understanding of zonation can lead to the development of more effective therapeutics for liver diseases, microphysiological systems, and scalable cell-based therapies.

Hepatocytes along the liver sinusoid exhibit a spatial distribution of functions, a phenomenon termed "zonation." The liver is a central metabolic organ, and thus, zonation is thought to be optimal for carrying out a multitude of functions in parallel, ranging from maintaining glucose homeostasis to xenobiotic metabolism. A complex interplay between the gradients of multiple soluble factors (e.g., oxygen, hormones, nutritional stimuli) induced via cell-mediated factor depletion and molecular pathways (e.g., Wnt/β-catenin, hedgehog) contributes to the differential regulation of approximately 50% of liver genes along the sinusoid.[1] This complexity has presented challenges in deciphering the major regulators of zonation and its perturbations. Zonation also has implications in the initiation and progression of liver diseases such as non-alcoholic fatty liver disease (NAFLD), alcohol liver disease (ALD), hepatitis B and C viral infections (HBV and HCV), hepatocellular carcinoma (HCC), and chemical/drug-induced toxicity. Furthermore, of key interest is the identification of hepatocyte subpopulations within a zonated lobule which are responsible for repopulating lost or damaged tissue during the process of liver regeneration.

Several in vivo rodent models have contributed to our current understanding of the compartmentalization of hepatocyte functions along the liver sinusoid in both physiology and disease. Furthermore, several in vitro models, such as specialized static plates and microfluidic devices, have been employed to investigate liver zonation by subjecting isolated cells to controlled levels and gradients of oxygen, nutritional factors, hormones, and drugs in higher throughput culture formats than possible with live animal studies. Here, we discuss the latest findings on the molecular regulators of zonation, the known roles of zonation in liver diseases and regeneration, and both in vivo and in vitro model systems that have been employed in the investigations of zonation. Lastly, we discuss advances that will need to be made to further our understanding of this critical feature of the liver toward designing better therapeutics for liver diseases, microphysiological systems, and cell-based therapies for patients suffering from end-stage liver failure.

Structural Anatomy and Physiologic Gradients in the Liver

Liver lobules are hexagonal in shape and serve as the functional units of the liver. They have a central vein (CV) in the middle of the hexagon and located radially in the hexagonal corners are the portal triads, which consist of a portal vein (PV), a hepatic arteriole (HA), and a bile duct (►**Fig. 1A**). Hepatocytes are organized in cords along the liver



	Zone 1 (Periportal)	Zone 2 (Midlobular)	Zone 3 (Perivenous)
Select Zonated Hepatic Markers	ALB, CPS1, ARG1, PCK1, SDH, SULT1A1, IGF1	HAMP, IGFB2, CYP8B1	GS, GLT1, CAR, AHR, CYP1A, CYP2E1, CYP3A, GSTM1, GK, IGFB1, B-CATENIN
***In Vivo* Zone-specific Injury Models**	Bile duct ligation, allyl alcohol, and 3,5- and diethoxycarbonyl-1,4-dihydrocollidine (DDC) exhibit damage to biliary cells or periportal hepatocytes	NA (Partial hepatectomy (xPH) is not zonally biased and used to interpret midlobular contributions)	Carbon tetrachloride (CCL4), bromobenzene and acetaminophen (APAP) toxicity is localized to perivenous hepatocytes.

Fig. 1 Liver zonation. (**A**) Schematic of liver sinusoid segregated to three zones: zone 1, the periportal region; zone 2, the midlobular region; zone 3, the perivenous or pericentral region. Created using Biorender.com. (**B**) PV brings in partially deoxygenated, nutrient and hormone rich blood, whereas HA brings freshly oxygenated blood. Cellular uptake and consumption deplete these soluble factors as the blood flows toward the CV, thereby creating a continuous gradient along the portal-central axis. Key metabolic pathways involved in glucose metabolism, nitrogen metabolism, fatty acid metabolism, and xenobiotic metabolism are distributed in a zonated manner. Complex crosstalk between several signaling pathways regulates zonated expression of approximately 50% genes within the liver lobule. (**C**) Hepatic markers that are enriched in each zone are listed. These markers are used to characterize zonated phenotypes in various in vivo and in vitro models and to assess perturbation in zonated phenotypes during disease and injury. Zonated toxicity can be induced in an animal or in vitro cultures using zone-specific hepatotoxicant chemicals and drugs. BD, bile duct; CV, central vein; HA, hepatic artery; PV, portal vein.

lobule extending from PV to CV. Based on this architecture and the heterogeneity of hepatocytes, the liver lobule can be functionally divided roughly into three zones: zone 1 or the periportal zone is the region by the portal triad, zone 2 or mid-lobular is the intermediate zone between the portal triad and the CV, and zone 3 or perivenous/pericentral zone is by the CV. Hepatocytes along the lobule show functional compartmentalization that enables a multitude of functions to be performed in parallel. This compartmentalization also helps limit acute injuries from different toxicants (e.g., chemicals) to a specific zone.

Gradients of several different soluble factors exist along the liver sinusoid. Liver derives its blood from two afferent vessels, PV and HA (►**Fig. 1A**). The PV brings partially deoxygenated, nutrient rich blood from the gut and supplies approximately 75% of the blood to the liver, whereas the HA brings freshly oxygenated blood from the heart. As the blood flows through the liver acinus, nutrients, hormones, and oxygen are consumed by adjacent hepatocytes, forming a continuous gradient (►**Fig. 1A**). The O_2 tension at the periportal region is approximately 60 to 65 mm Hg, dropping to approximately 30 to 35 mm Hg in the perivenous region.[2] Since O_2 is a known regulator of carbohydrate metabolism, hypoxia-inducible factors (HIFs), and reactive oxygen species (ROS), it is considered to be a key regulator of liver zonation. In addition to O_2 tensions, pancreatic hormones, such as insulin and glucagon, which counteract each other's actions, play important roles in establishing metabolic zonation of the liver[3]; the zone-specific expression of metabolic enzymes affects how hepatocytes respond to these hormones along the length of the sinusoid.

Molecular Mechanisms of Zonation

Wnt/β-Catenin Signaling

Wnt/β-catenin signaling in the liver is upregulated in zone 3 (►**Fig. 1B**) and has been extensively studied as a master regulator of zonated functions. Wnt proteins (19 isoforms in total) act on Frizzled receptors and effectively release β-catenin from a binding complex such that it can translocate to the nucleus.[4] β-catenin works with T-cell factors (TCFs), a family of transcription factors that regulate gene expression (e.g., Axin2, Cyclin-D), to promote a zone 3 phenotype in hepatocytes. Knockout mouse models have elucidated the key roles of proteins in the Wnt/β-catenin signaling pathway and their roles in zonation. For example, the tumor suppressing gene, adenomatous polyposis coli (APC), is highly present in the periportal region and was hypothesized to inhibit Wnt/β-catenin signaling. Deletion of the APC gene resulted in the upregulation of Wnt/β-catenin signaling in the periportal region and dysregulated ammonia metabolism.[5] Interestingly, loss of c-Myc, a downstream target of β-catenin that is a mediator of the Wnt pathway, did not affect the zonation of ammonia-metabolizing enzymes suggesting that liver zonation is maintained independent of c-Myc.[6] A genetic knockout mouse model of Lrp5 and Lrp6 (co-receptors of Wnt) was employed to completely disrupt Wnt signaling in hepatocytes and cholangiocytes using albumin-Cre, which led to the loss of a perivenous phenotype, thereby demonstrating Wnt as a primary regulator of β-catenin.[7]

β-catenin is thought to be responsible for the localization of drug metabolizing enzymes in perivenous (zone 3) hepatocytes. For example, in vivo liver-specific knockout of β-catenin led to a complete loss of CYP1A2 and CYP2E1 expression, along with the loss of glutamine synthesis (GS).[8] Another study associated β-catenin activation with the elevated expression of CYP-related mRNAs, which was found to be indirectly regulated by β-catenin via it acting on different signaling pathways and nuclear receptors.[9] Similarly, β-catenin is also involved in the regulation of glutathione S-transferases and other phase II enzymes, possibly indirectly through the upregulation of the constitutive androstane receptor and interaction with the retinoid X receptor-dependent transcription.[10] Overall, β-catenin signaling has a dual role within the perivenous region: it upregulates zone 3-specific functions while downregulating zone 1-specific functions (e.g., ammonia detoxification, which is primarily performed by periportal hepatocytes).

R-spondins (RSPO) can facilitate Wnt/β-catenin signaling by binding to leucine-rich repeat-containing G-protein coupled receptor 5 (LGR5) receptors and preventing membrane clearance of Wnt receptors. RSPO3 is secreted from CV endothelial cells and its expression is restricted to the perivenous region. Deletion of Rspo3 in the late stages of development interfered with the establishment of zonation but did not impact Wnt expression level.[11] Additionally, ectopic expression of Rspo1 (functional analog) promoted a zone 3-like phenotype in zone 2 hepatocytes. More recently, a study established the RSPO-LGR4/5-ZNRF3/RNF43 pathway as a spatiotemporal rheostat of the hepatic Wnt/β-catenin activity gradient and metabolic zonation, whereby RSPO1 injection or Znrf3/Rnf43 deletion expanded Wnt/β-catenin expression to the periportal region.[12]

Hedgehog Signaling

Hedgehog (Hh) signaling supports embryogenesis and development; however, Hh ligands are lowly expressed in adult hepatocytes and their role in establishing and/or maintaining liver zonation is poorly understood. Hh ligands, such as sonic hedgehog (Shh), Indian hedgehog (Ihh), and Desert hedgehog (Dhh), bind to Ptch1/2 receptors to relieve patched-mediated suppression of Smoothened (Smo).[13] Activated Smo leads to the stabilization and nuclear translocation of GLI transcription factors. Hh signaling is higher in the periportal region (►**Fig. 1B**), and thus, has been implicated in regulating zone 1-like phenotypes in hepatocytes.

A study using Smo-KO mice identified two key roles of Hh signaling in maintaining zonation: (a) affecting the Wnt/β-catenin pathway by downregulating its target gene, Ihh, and (b) controlling the insulin-like growth factor (IGF) axis, where IGF-1 expression and IGF-1 serum levels decreased in Smo-KO mice whereas IGF-binding protein (IGFBP-1) mRNA levels were upregulated.[14] Specifically, these changes were found to be mediated by the GLI3 transcription factor. The regulation of the IGF-axis is particularly important in zonation because IGFBP-2 and IGF-1 are expressed in the

periportal regions of rat livers (similarly to phosphoenolpyruvate carboxykinase [PEPCK]), whereas IGFBP-1 expression is higher in the perivenous region.[15] These findings highlight the role of Hh signaling in maintaining glucose homeostasis.

While Hh signaling affects local pathways in the periportal region, less is known about interactions with Wnt/β-catenin. Using in vitro and in vivo data from Apc^{homo} (carrying a homozygous floxed exon 14 in the *Apc* allele leading to reduced APC levels) and Smo-KO mice, mathematical modeling was performed to understand the interactions between Wnt and Hh signaling pathways, such that Wnt signaling dominates the perivenous region and Hh signaling dominates the periportal region and communicate primarily by mutual repression.[16] It was believed that Ihh was a major modulator of Hh signaling in the liver, but Ihh is more predominant in the perivenous region (given that it is a Wnt target gene); however, Shh is established as the controller of Hh signaling in liver. Hh inhibition led to the periportal dominance of metabolic functions and Wnt activation led to the extension of zone 3, such that mid-lobular cells assumed perivenous-like phenotypes. Currently, no model is available to replicate Hh signaling in vitro, and thus, there is an important need to further understand its implications in zonation.

Hypoxia-Inducible Factors

HIFs, a family of O_2-sensitive heterodimeric transcription factors, regulate gene expression in response to O_2 availability. In a low O_2 environment, the α subunit (HIF-1α, HIF-2α, and HIF-3α) translocates to the nucleus, interacts with the β subunit (aryl hydrocarbon receptor nuclear translocator or ARNT), and acts on target genes containing hypoxia responsive elements (HREs).[2] Thus, higher activation of HIFs is observed within hepatocytes in the perivenous region that has lower O_2 tensions. Due to their perivenous localization, HIFs have been investigated for their role in interacting with zone 3-specific signaling pathways (i.e., Wnt/β-catenin).

While HIF-2α/IRS2 (insulin receptor substrate 2) preferentially enhanced insulin signaling, thereby suppressing gluconeogenesis, HIF-1α promoted glycolysis, thus further corroborating the roles of HIFs in maintaining zonal glucose metabolism.[17] In an in vitro culture of rat hepatocytes, hypoxia promoted the induction of IGFBP-1, which was found to be regulated through ROS and HIF-2 and -3, further suggesting the important roles of HIFs in glucose homeostasis.[18] Additionally, vascular endothelial cell factor inhibition in normal or diabetic db/db mice led to vascular regression causing hepatic hypoxia that sensitized liver insulin signaling through HIF-2α stabilization.

The interactions between hypoxia/HIFs and β-catenin signaling have not yet been directly shown for liver zonation, but there is evidence of such interactions in other organ systems. For instance, studies in colorectal tissue suggest HIF-1α to be a negative regulator of tumor suppressor APC, and thus has implications in the regulation of β-catenin.[19,20] Depletion of HIF1-α resulted in increased APC expression at the mRNA and protein levels, and conversely, depletion of APC resulted in increased HIF-1α. HIF-1α binding to the HRE present in the promoter region of APC was found to be the mechanism behind this regulation. In embryonic stem cells, hypoxia increased β-catenin signaling and deletion of *hif-1a* and *arnt* reduced the expression of Wnt/β-catenin target genes.[21] Nonetheless, further studies are needed to elucidate the interactions between HIFs and β-catenin signaling specifically in liver zonation.

While HIF and Hh pathways probably act in a separate manner, interconnections have been demonstrated previously. For instance, hypoxia induced a systemic Hh response in mice and was shown to be preceded by HIF-1α accumulation in vitro; inhibition or ablation of HIF-1α eliminated Hh activation.[22] Furthermore, hypoxia can induce Smo in pancreatic cancer[23] and neuroblastoma cells.[24] However, investigations of the connections between hypoxia/HIFs and Hh signaling in liver zonation are warranted.

Other Pathways

Apart from the major signaling pathways discussed above, several other pathways have been implicated in maintaining zonated phenotypes of either periportal or perivenous hepatocytes. The Ras/Raf/ERK pathway was initially postulated to be activated by blood borne molecules to attenuate perivenous markers in periportal hepatocytes.[25] This was further validated when hepatocytes cultured in increased serum concentrations showed periportal marker expression and suppression of perivenous markers.[26] Similarly, hepatocyte nuclear factor-4 α (HNF4α) and its interaction with β-catenin have been explored as a regulator of zonation. In liver stem cell-derived hepatocytes, activation of β-catenin through the inhibition of glycogen synthase kinase-3 β led to a perivenous-like phenotype, which was caused by a Wnt downstream target, LEF1, binding to the HNF4α promoter and repressing periportal genes.[27] Another study found that while HNF4α is not zonally distributed, it antagonizes β-catenin through Tcf4 binding to promote a periportal phenotype.[28]

Despite Wnt/β-catenin being considered as the gatekeeper of liver metabolic zonation, factors controlling its spatiotemporal regulation are not fully understood. Dicer, an endoribonuclease III type enzyme that is involved in microRNA processing, is essential for the suppression of periportal proteins by Wnt/β-catenin/TCF signaling; however, Dicer's expression was found to be independent of β-catenin/TCF.[29] The loss of Dicer in hepatocyte-specific Dicer1 knockout mouse livers caused periportal proteins to be diffusely expressed throughout the entire lobule, suggesting that microRNAs are involved in the suppression of periportal protein expression. More recently, glucagon released by the pancreas was found to counteract Wnt/β-catenin signaling to regulate the expression of periportal genes.[3] These studies highlight the complex interplay of pathways and hormonal gradients that are involved in regulating zonated phenotype in liver lobules during homeostasis.

Pathophysiology of Zonation

The liver is the largest glandular organ and is the powerhouse of the body for metabolism. As such, it is a common target for several diseases, such as NAFLD, ALD, HBV, HCV, and HCC. Given that 2 million people die per year from liver diseases,[30]

it is pertinent to further understand etiologies and develop therapies to reduce the high morbidity and mortality. Zonation is a dynamic process that can regulate disease phenotypes and progression, and in turn be perturbed by diseases, which can negatively affect metabolic processes in the liver.

NAFLD and ALD

NAFLD is characterized by greater than 5% lipid accumulation (steatosis) relative to liver weight. In late stages, NAFLD can progress to non-alcoholic steatohepatitis (NASH) and is accompanied by complex inflammatory signals, hepatic insulin resistance, and fibrosis.[31] In the normal liver, fatty acid synthesis and lipid accumulation occur predominantly in the perivenous region, whereas fatty acid oxidation occurs in the periportal region. Due to such a zonal distribution of lipid metabolism, both NAFLD and NASH manifest in zone 3, though the dysregulation can extend to zone 1 with unchecked disease progression.[32] Histology performed on human livers with NAFLD confirmed zone-specific lipid accumulation, in that the majority of patient samples (approximately 37%) displayed perivenous dominant steatosis[33]; interestingly, pan-acinar distribution was the second highest (approximately 34%) suggesting inter-individual differences in disease progression. Similarly, the zonal specification of biosynthesis enzymes is lost in the later stages of the disease[34]; for instance, histological analysis showed that phosphatidylethanolamine methyltransferase expression transitioned from a perivenous localization to a panlobular distribution and then to higher expression at sites of inflammation in healthy, steatotic, and NASH patients, respectively.

Mouse models of NAFLD have also been used to elucidate the phenotypic changes in liver zonation and to determine underlying molecular pathways. For instance, zonal specification of phospholipids (i.e., lipid zonation) was progressively lost in mice with NASH; lipid remodeling enzymes (e.g., LPCAT2) were upregulated and may contribute to the changes observed in vivo.[35] Importantly, this study identified species–species differences in zonation, in that phosphatidylcholine [PC(34:1)] was localized in the periportal zone in mice and in the perivenous zone in humans, whereas PC (32:2) had the opposite profile across the species.

Wnt/β-catenin pathway plays an important role in NAFLD progression and has been identified as a potential contributor of the metabolic perturbations in the liver zones. For instance, β-catenin was found to interact with transcription factor, FOXO1, to regulate gluconeogenic enzymes (e.g., G6Pase and PEPCK) and cause diet-induced obesity[36,37]; this occurred specifically via changes to hepatic lipogenesis and mitochondrial oxidative phosphorylation. β-catenin was also found to interact with HIF-1α, linking several physiologic and molecular gradients together in the regulation of lipid metabolism.[37] However, further work is needed to elucidate these complex and multifactorial mediators of zonal losses in diseases such as NAFLD/NASH. Mathematical modeling has attempted to bridge our understanding of these factors and their implications on lipid zonation[38]; this study concluded that the O_2 gradient and fatty acid uptake, but not the gradients of fatty acids along the sinusoid, contributed to the perivenous accumulation of lipids in the early stages of NAFLD (i.e., steatosis).

The Hh signaling pathway, which controls the molecular expression of periportal hepatocytes, is dysregulated in NAFLD. Healthy adult livers express very low levels of Hh ligands due to the secretion of Hh-interacting proteins (Hhips; Hh antagonists) from quiescent hepatic stellate cells (HSCs).[39] However, dramatic increase in Hh ligands localized to fibrotic regions have been reported in NASH patients.[40] Activated HSCs also play a crucial role in NAFLD progression as primary contributors to extracellular matrix (ECM) deposition and remodeling. The transition of quiescent HSCs to an activated state (myofibroblasts) leads to a downregulation of Hhip and an upregulation of GLI2.[39] Additionally, hepatocyte-specific conditional ablation of Smo in transgenic mice led to the upregulation of lipogenic transcription factors (e.g., SREBP1, PPAR, and PNPLA3) and enzymes (e.g., Acaca, Fasn, Elovl6, etc.) in the perivenous zone with the development of steatosis.[41] Collectively, these findings suggest that active Hh signaling plays a key role in the homeostasis of lipid metabolism and perturbed Hh signaling can promote steatosis through the differential regulation of GLI transcription factors.

Alcohol is a leading cause of morbidity and mortality worldwide, with ALD being the major cause of alcohol-related mortality.[42,43] As with NAFLD, ALD can progress from simple steatosis to alcohol-associated steatohepatitis with inflammation, hepatocyte ballooning, and pericellular fibrosis.[44] Ultimately, fibrosis can lead to cirrhosis and HCC. Alcohol (ethanol) metabolism is mediated by alcohol dehydrogenase (ADH) that converts ethanol to a highly toxic aldehyde, which is then converted to acetate by acetaldehyde dehydrogenase that produces high levels of NADH. The CYP2E1 enzyme is responsible for the metabolism of approximately 20% of consumed ethanol and can be upregulated with ethanol consumption.[45] Since cytochrome P450 enzymes (CYP450) and ADH are expressed at higher levels in the perivenous region,[46] ALD typically originates in this region though it progresses toward the periportal region with disease progression.[47,48] CYP2E1 is also regulated by Wnt/β-catenin through the LPR6 receptor[7,49]; LPR6-mediated upregulation of CYP2E1 was also associated with an upregulation in ROS, which may lead to the dysregulation of lipid zonation.

HBV and HCV

Both HCV and HBV infections cause dysregulation in the Wnt/β-catenin signaling pathway. Some studies have suggested that hepatocytes in the perivenous region are more susceptible to HCV infection; specifically, perivenous-like cells showed up to 40% more HCV transduction than periportal-like cells in vitro,[50] while hypoxia, which occurs more in the perivenous region, led to enhanced HCV replication in hepatoma cell lines and in correlative studies in liver biopsies from HCV-infected patients.[51] HCV infection can also alter the expression of lipogenic enzymes from the periportal to midlobular region, possibly due to an increase in β-catenin activation mediated by HCV viral proteins.[52,53] Similar to the

zonal perturbations observed in metabolic diseases, zonated features are lost during HCV infection; for example, GS distribution is greatly dysregulated and shows a twofold increase in percent area in histological sections.[52] Interestingly, there is a growing evidence to support that the mutation in catenin β 1 (CTNNB1; gene encoding β-catenin) due to HCV viral proteins suppresses tumor-suppressing APC, leading to the dysregulation of DNA repair which aids in the development of HCC.[53] Approximately 27% of HCV-related HCC is associated with the CTNNB1 mutation as compared with approximately 12% observed in HBV-associated HCC and 21.2% in total non-viral-associated HCC. Furthermore, as compared with colorectal cancer, where CTNNB1 mutations are in the Thr41 and Ser45 residues, a higher frequency of HCV-related HCCs show CTNNB1 mutations in the Asp32 and Ser37 residues. Mutations in Asp32 and Ser37 resulted in higher β-catenin signaling than mutations in Thr41 and Ser45, which could help explain the higher propensity of CTNNB1 mutation in HCV-associated HCCs.

HBV biosynthesis predominantly occurs in perivenous hepatocytes and colocalizes with β-catenin signaling pathway.[54] In a liver-specific β-catenin null HBV transgenic mouse, the zonal biosynthesis of HBV was lost and led to a homogeneous distribution of viral biosynthesis and reduction in viral replication, suggesting a regulatory role of β-catenin in HBV replication.[55] Similarly, in HepG2 cells, HBV infection induced Src kinase-dependent β-catenin signaling and caused the disassembly of adherens junctions which are associated with an epithelial-to-mesenchymal transition observed in HCC.[56]

HCC

Diverse metabolic and viral hepatic etiologies lead to HCC, thus making it biologically and molecularly heterogeneous, which presents major challenges in treatments.[57] Global gene expression analysis of 1,113 HCC samples revealed that well-differentiated HCCs showed preservation of the zonated phenotype.[57] HCCs that maintained a periportal-like phenotype showed dysregulation of eight genes related to amino acid catabolism, lipid and glucose metabolism, and urea cycle. However, perivenous-type HCCs predominantly carried a mutation in CTNNB1 that led to the upregulation of β-catenin target genes. Aberrations in Wnt/β-catenin signaling pathway are detected in over 50% of HCC cases.[58,59] Liver-specific deletion of APC in mice promoted HCCs which was linked to the stabilization and nuclear translocation of β-catenin.[60]

NAFLD-driven steatotic HCCs are common in obese patients[61]; however, the molecular pathways that associate metabolic dysregulation to the onset of tumorigenesis are not well understood. While Hh signaling is below detection level in the healthy liver, metabolic dysregulation such as NAFLD is known to upregulate Hh pathway in the injured liver.[62] A recent study associated hepatocyte-secreted Ihh to the activation of HSCs via Myc and TGFβ2 that led to increases in the secretion of pro-tumorigenic Wnt5a from activated HSCs.[63] Thus, therapeutics targeting the Hh pathway can be an important strategy to combat NAFLD-driven HCC.

The contribution of the β-catenin mutation in HCC progression has also been associated with HCV infection, which upregulates β-catenin via HCV core proteins.[53,64] In both HCV and HBV, hypermethylation of APC was observed and was responsible for aberrant Wnt activation; however, CTNNB1 mutation was comparatively lower in HBV infected samples than HCV ones.[59] Thus, Wnt/β-catenin pathway can be important target for therapeutics in HCC treatment.

Drug Toxicity

Many drugs bioactivated by CYP450 enzymes cause localized hepatotoxicity in the perivenous region due to higher CYP450 expression.[65] Acetaminophen (*N*-acetyl-para-aminophenol or APAP) is a widely studied zonal toxin that induces hepatic necrosis in the perivenous region. APAP hepatotoxicity accounts for approximately 48% of acute liver failures with approximately 29% of patients requiring liver transplantation.[66] While the majority of APAP is transformed into non-toxic glucuronide and sulfate, approximately 5 to 8% of APAP is metabolized by CYP2E1 and CYP3A4 to generate the reactive metabolite, *N*-acetyl-p-benzoquinone-imine (NAPQI). At a toxic dose of APAP, excess NAPQI leads to the depletion of glutathione and reacts with mitochondrial proteins to cause ATP depletion and necrosis.[67,68] APAP toxicity is further exacerbated due to ethanol-mediated induction of CYP2E1 in the perivenous region.[69] A recent study utilized computational methods to incorporate all three metabolic pathways of APAP (i.e., sulfation, glucuronidation, and oxidation) to simulate the spatial distribution of APAP-induced toxicity and found that zonal differences affected glutathione-mediated detoxification and localization of hepatotoxicity.[68] Such computational methods could serve as a platform to study the effects of other metabolites that are zonally distributed. Another hepatotoxin, bromobenzene, also causes necrosis in perivenous hepatocytes due to the depletion of glutathione followed by bromobenzene-induced protein degradation.[70,71] A transcriptomics and proteomics study in rats reported significant alteration of 24 proteins following bromobenzene treatment and perturbation in enzymes involved in glutathione synthesis.[72] In contrast, allyl alcohol (AA) causes periportal toxicity and the mechanism was linked to higher and efficient uptake of AA in the periportal region and subsequent ADH-mediated conversion of AA to the toxic aldehyde, acrolein.[73]

Zonation in Liver Regeneration

The liver possesses an incredible capacity to regenerate fully after extreme injury (e.g., removal of approximately 90% of tissues mass) prompting speculation that a subpopulation of liver progenitor-like cells exist. A highly proliferative cell population has been identified in rodents, termed oval cells, though they arise only after severe injury[74]; however, the presence of a progenitor-like hepatocyte remains controversial in human studies. Given the varying functional responsibilities of hepatocytes along the liver sinusoid, investigators have postulated zonation to play a key role in regeneration. Several lineage tracing techniques or knockout models and injury

models have been employed to elucidate a zonal population of self-renewing and regenerative liver cells.

Partial hepatectomy (xPH) in rodents is a well-established model that does not cause zonal damage and allows for the unbiased assessment of zone-specific responses; additionally, this injury model lacks inflammation and necrosis and is accompanied by hypertension and the transient modulation of signaling pathways, some of which are implicated in the regulation of zonation (e.g., Wnt/β-catenin signaling).[75] Cre-labeled mice have been used to elucidate hepatocyte subpopulations responsible for regeneration. For instance, Mfds2+ (a periportal marker) hepatocytes labeled with the Red Fluorescent Protein had a 1.66-fold increase in area after xPH as compared with sham controls.[76] Axin2+ hepatocytes, located around the CV and co-expressing GS, had a twofold higher EdU uptake during homeostasis, suggesting an active role in self-renewal.[77] Hepatocytes expressing high telomerase activity were also found to contribute to self-renewal, though they were distributed throughout the liver lobule.[78] Thus, several subpopulations have been demonstrated to play a role in natural turnover and injury-induced regeneration. However, it is important to note that this method of tracing cells for proliferative responses is inherently biased. For example, deletion of Axin2 did not alter regeneration after xPH, and instead, Axin2 expression was upregulated in hepatocytes in the periportal and midlobular (zone 2) regions (►**Fig. 2A**).[79] Since Axin2 is linked to proliferation and is upregulated after injury, it is likely that strict zonal compartmentalization is lost after severe injury events, and this allows all hepatocytes along the liver sinusoid to upregulate key genes/proteins required for proliferation and regeneration.

β-catenin is immediately upregulated after xPH and contributes to the increased expression of cyclin-D1; β-catenin liver knockout mice experience a delay in the initiation of liver regeneration (peak S-phase occurs at 72 hours as

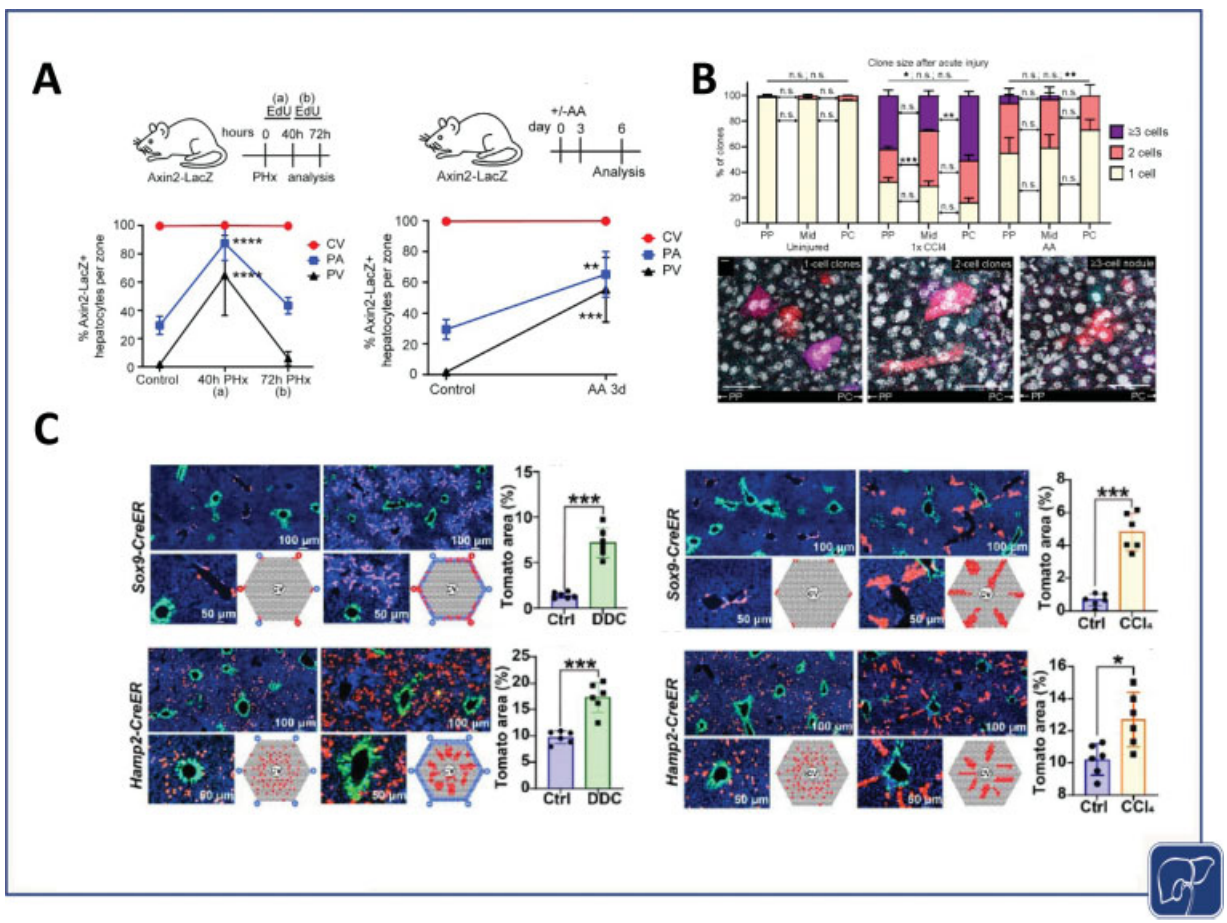


Fig. 2 Assessments of hepatocytes in in vivo models of liver regeneration. (**A**) Axin2-LacZ+ mice were subjected to xPH and assessed using EdU, a marker of proliferation, after 40 or 72 hours. The percent of Axin2-LacZ+ hepatocytes was upregulated in the periportal (PV) and midlobular (PA: parenchyma) regions at 40 hours which correlated with peak in hepatocyte proliferation post-injury (a). Similarly, high doses of allyl alcohol (AA) upregulated Axin2-LacZ+ hepatocytes in the periportal (PV) and midlobular (PA) regions as measured after 3 days of recovery (b) (adapted from Sun et al[79]). (**B**) Livers of R26R$^{rb/wt}$ mice activated with AAV8-TBG-Cre at low doses were subjected to AA (periportal injury) and carbon tetrachloride (CCL4) (perivenous injury) and assessed for the number of clones present in each zonal region 2 weeks post injury. Images demonstrate types of clones (i.e., 1-cell clones [*left*], 2-cell clones [*middle*], or ≥ 3-cell nodules [*right*]) (adapted from Chen et al[83]). (**C**) Lineage-tracing mice were subjected to 0.1% 3,5-diethoxycarbonyl-1,4-dihydrocollidine (DDC) or CCl4 for 2 weeks post tamoxifen injections and were analyzed 6 weeks post injury for glutamine synthetase (GS; *green*) and Tomato (*red*) immunostaining of histological sections. Hamp2-CreEr mice showed a significant increase in tomato area (%) for DDC (*top-left*) and CCl4 (*top-right*). Sox9-CreER mice similarly experienced a significant increase in tomato area (%) for DDC (*bottom-left*) and CCl4 (*bottom-right*) (adapted from Wei et al[84]).

opposed to 40 hours in wild type mice).[80] Similarly, Wnt secretions from Kupffer cells were inhibited and peak S-phase detection in hepatocytes declined by 33%. Similarly, a loss of cyclin-D1 expression was observed after xPH in liver sinusoidal endothelial cells (LSECs) in Wnt-KO mice as compared with controls.[81] Interestingly, β-catenin induction and the increased expression of Wnt proteins (Wnt2 and Wnt9b) were observed across all the zones. This suggests Wnt/β-catenin signaling to be supported by the non-parenchymal cell (NPC) compartment and necessary for normal liver regeneration. Macrophages have been identified to modulate Wnt/β-catenin signaling to induce a compensatory stage during regeneration, such that non-proliferating hepatocytes upregulate key metabolic functions to compensate for the lost or damaged tissue.[82] These studies suggest a critical role of liver NPCs, which have zonated responses, in regulating regeneration.

Regeneration is most prominent after injury; therefore, several injury models have been employed to study the contribution of zonation in regeneration. Most common models are carbon tetrachloride (CCL4) and AA administrations to induce perivenous and periportal damage, respectively. In zone-specific injury, the repopulation of cells appear to come from non-damaged hepatocytes (i.e., perivenous damage using CCL4 will prompt a regenerative response from periportal hepatocytes, likely because they did not experience damage). To assess lineage-traced hepatocytes, Rosa26-Rainbow (R26R) Cre reporter mice can be activated using adeno-associated virus (AAV) capsids and subsequently interrogated for hepatocyte proliferation (e.g., clones) along the liver sinusoid.[83] R26R mice subjected to CCL4 or AA to induce zone-specific injury displayed a significant proliferation of adjacent hepatocytes located farther away from the injury site. However, the percentage of clones in each zone was not significantly different for either injury type (►**Fig. 2B**). Similar trends were confirmed by tracing the proliferation of hepatocytes using CreER mice after 3,5-diethoxycarbonul-1,4-dihydrocollidine (DDC) treatment (preferentially injures biliary cells to cause periportal damage) and CCL4 treatment.[84] During the period of recovery, transient loss of zonal hepatic phenotypes was observed. For instance, CCL4 treatment in R26R-EYFP mice led to a transient loss of zone-3 specific markers (e.g., Gls2, GS, Cyp2e1) that recovered after 6 days[85] and caused an upregulation of key Wnt proteins (e.g., Wnt2, Wnt4, Wnt5a, Wnt9b) for up to 14 days post injury.[86] Therefore, all hepatocytes, regardless of zonal compartmentalization of functions, can contribute to proliferation and tissue repair after injury. The process of modulating hepatic phenotype is likely linked to the transient abolishment of soluble factor gradients in the liver during the turbulent period post injury, though zonation is re-established after recovery (e.g., periportal cells that expand into the perivenous region using lineage tracing will assume zone 3-like characteristics).

Broadly distributed lineage tracing of hepatocytes under homeostasis has been utilized as an in vivo technology to elucidate hepatocytes responsible for self-renewal and the implications of zonation. Labeling efficiency of R26R hepatocytes is known to be increased in the perivenous region,[87] and thus, sustained fluorescent gradients of reporter hepatocytes suggested that the long-term self-renewal during homeostasis was due to equal contributions from hepatocytes without zonal specificity (i.e., rejects the streaming theory).[83] Interestingly, a significant increase in clonal size (>2 cells) was observed in the midlobular region; this study confirmed proliferation to be independent of AAV vector transduction.

Proliferation tracer (ProTracer) technology is an advancement to lineage tracing as it allows for (1) unbiased, (2) non-toxic, (3) single cell type-specific labeling, and (4) spatio-temporal resolution of long-term proliferation of hepatocytes in mouse models. ProTracer was used to demonstrate zonation in hepatocyte proliferation under homeostasis with the greatest recordings in zone 2 > zone 1 > zone 3, which corroborated earlier findings.[88] Recently, Wei et al developed and deployed 13 CreER mouse lines, each representing a labeling technique for zone-specific markers (e.g., zone 1 centric: Arg1.1, Arg1.2, Gls2; zone 3 centric: Cyp1a2, Oat, GS; zone 2 centric: Hamp2, Mup3, Tert), to systematically locate zone-specific contributions of hepatocytes.[84] Positive cells in zone 1 using the Gls2-CreER mice showed a decrease from approximately 60 to 37% over 12 months whereas Cyp1A2- and Oat-CreER expression (zone 3 with portions of zone 2 showing expression) increased proportionally, suggesting that zones 2 and 3 expand and give rise to zone 1 cells under normal conditions. Interestingly, GS-CreER, a more faithful marker of approximately three cell layers around the CV, was unchanged over the 12-month period suggesting the midlobular hepatocytes to be the key contributors to repopulation during homeostasis. To reduce bias from single gene tracing, this finding was further confirmed in Hamp2-CreER lines where an increase from approximately 7.4% to approximately 27.4% in zone 2 expression was observed. These mice were subjected to periportal or perivenous injury and specific subpopulations (e.g., Hamp2 and Sox9) greatly contributed to proliferation after either injury type (►**Fig. 2C**); Sox9-positive cells were also previously implicated as a potential progenitor-like cell.[89] The response of Hamp2-positive cells in the study by Wei et al corroborates findings that midlobular hepatocytes are responsible for regeneration and this transition region is not usually a target for damage which offers it a biological advantage over the other zones.[84]

In Vitro Liver Platforms to Model Zonation

Many in vitro liver models have been developed that can functionally stabilize hepatic functions for days to weeks and are advantageous for disease modeling, drug screening, and regenerative medicine.[90] Such models include conventional two-dimensional (2D) monocultures, micropatterned cocultures, self-assembled spheroids, bioprinted tissues, and microfluidic devices. Though throughput is compromised with more complex culture models, the increasing technological complexities allow for higher order control over physiological phenomenon (e.g., establishment of key gradients associated with the regulation of zonation). Here, we review relevant

platforms that aim to replicate zonated features of hepatocytes and identify key areas for improvements.

Oxygen tension is a key gradient established along the liver sinusoid and implicated in regulating zonation. However, conventional well-plate cultures typically subject cells to a single concentration that is based on ambient O_2 in the air (approximately 21%) and diffusion through culture medium. A straight-forward method for modulating O_2 tension onto cell cultures is via specialized incubators capable of regulating the infusion and mixing of three gases (CO_2, N_2, and O_2). Adjusting the height of the cell culture medium, which modulates the oxygen tension at the cell culture surface due to the low solubility of O_2, has been similarly employed to replicate aspects of zonation.[91] For instance, HepG2 cultures supported a 10-fold difference in CYP450 activity between hypoxic and hyperoxic conditions, though differences in activity within the physiologic O_2 range were not discernable. Conversely, using the cell culture platform height relative to the air–liquid medium interface to modulate oxygen tension (between 4 and 15% O_2) has been adapted to a perfusion system to mimic physiologic-like flow over a primary rat hepatocyte (PRH) ECM sandwich culture.[92] Protein analysis supported an upregulation of periportal markers (CPS1 and Arg1) and perivenous markers (GS and CYP3A4) in the high and low platform heights, respectively (►**Fig. 3A**); however, this method relied on raising the position of the cell culture surface within the device to manipulate the medium height, which also led to a concomitant doubling in the magnitude of the fluid shear stress on the cells.

Cell microenvironments that are three-dimensional (3D) are a closer representation of physiologic cell–cell and cell–ECM interactions; generally, 3D liver models support higher functions than 2D conventional monolayers cultures for several weeks in culture,[90] which allows for long-term appraisal of hepatic responses to molecular gradients. HepG2 cells cultured in a thin hydrogel using a paper-based culture platform demonstrated higher hepatic functions at physiologic O_2 as compared with 2D culture.[93] Importantly, gene expression analysis supported upregulation of drug metabolizing genes (e.g., CYP2E1, UGT1A1, AhR) at 3% or 8% O_2 relative to 20% O_2 controls, and the HepG2 were more sensitive to APAP toxicity potentially due to an increase in drug metabolizing capacity (e.g., glucuronidation and sulfation) under perivenous-like oxygen tensions. Spheroid cultures also create soluble factor gradients along the radius of the spheroid due to diffusion limitations, which can be modulated by the size of the spheroids. For instance, spheroids created using C3A hepatoma cells displayed higher CSP1 (a periportal marker) protein at the periphery as compared to the low oxygen region in the core of the spheroid.[94]

Gas-permeable plates created using polydimethylsiloxane (PDMS; a popular biocompatible polymer for cell culture with a high O_2 permeability coefficient) membranes have also been utilized to modulate O_2 tensions at the cell surface using tri-gas incubators[95]; such specialized plates were used to modulate the O_2 consumption rates of PRHs in high oxygen flux (i.e., gas permeable) and low oxygen flux (gas impermeable) conditions to mimic periportal, perivenous, and hypoxic O_2 tensions using tri-gas incubators set to approximately 10, 5, and 2.5%, respectively (►**Fig. 3B**). PRHs in the high oxygen flux conditions maintained a high oxygen consumption rate (OCR), while low flux conditions reduced their OCR with low ambient oxygen. In the periportal-like O_2 range, gluconeogenesis markers were upregulated (e.g., glucose-6-phosphate) which correlated with glucose measured in the media, whereas intracellular localization of perivenous markers (active β-catenin and its downstream target, GS) was observed in perivenous-like O_2 conditions.

In contrast to static cultures, bioreactors allow for the generation of soluble factor gradients over cell cultures. A flat-plate bioreactor made of gas-impermeable polycarbonate was coupled with a perfusion pump and an O_2 exchanger system to create O_2 gradients over 2D monocultures of PRHs.[96] The cell-mediated depletion of O_2 from the inlet (periportal-like, set to 76 mm Hg) to the outlet (perivenous-like, set to 5 mm Hg) regions of the bioreactor established steady-state O_2 gradients (►**Fig. 3C**). Hepatocytes in this bioreactor displayed an in vivo-like zonal distribution of PEPCK at the inlet and CYP2B at the outlet regions. A second iteration of the device expanded the culture period from 24 to 72 hours by functionally stabilizing PRHs via coculture with 3T3-J2 murine embryonic fibroblasts; in this model, upregulation of drug metabolizing enzymes (CYP2B and CYP3A) and increased APAP toxicity were observed in the low O_2 region as compared with the regions with higher O_2, presumably due to the metabolism of APAP into its toxic metabolite via CYP450 enzymes as in vivo.[97] Another laboratory-scale bioartificial liver model cultured with porcine hepatocytes found O_2-independent localization and upregulation of GS suggesting that O_2 alone may not be sufficient to regulate GS expression.[98] The ExoLiver bioreactor platform can similarly create an O_2 gradient through perfusion across the cell culture surface and is amenable to coculture to mimic in vivo-inspired PHH-NPC interactions.[99] Dynamic coculture of hepatocytes and LSECs from freshly isolated human and rat sources supported differential expression of periportal (e.g., Gls2, Aqp1) and perivenous (e.g., Glul, Oat) markers at the inlet (periportal-like) and outlet (perivenous-like) regions, respectively. The LiverChip platform contains multiple bioreactors in a single perfusion system and can support 3D hepatocyte spheroidal cultures tethered to ECM-coated pores.[100] The platform generates O_2 gradient from the inlet to the outlet, supports long-term hepatic functions, and can be coupled with NPCs, such as Kupffer cells, to investigate immune-mediated liver responses; however, further work is needed to determine if zonation can be replicated in the LiverChip.

Microfluidic devices (i.e., miniature bioreactors that induce laminar flow over cells) have also been used to induce zonated phenotypes in hepatocyte cultures. For example, a PDMS-based microdevice utilizes a bilayer system to culture primary mouse hepatocytes within an established O_2 gradient via an air–gas channel.[101] A Pd-meso-tetra (4-

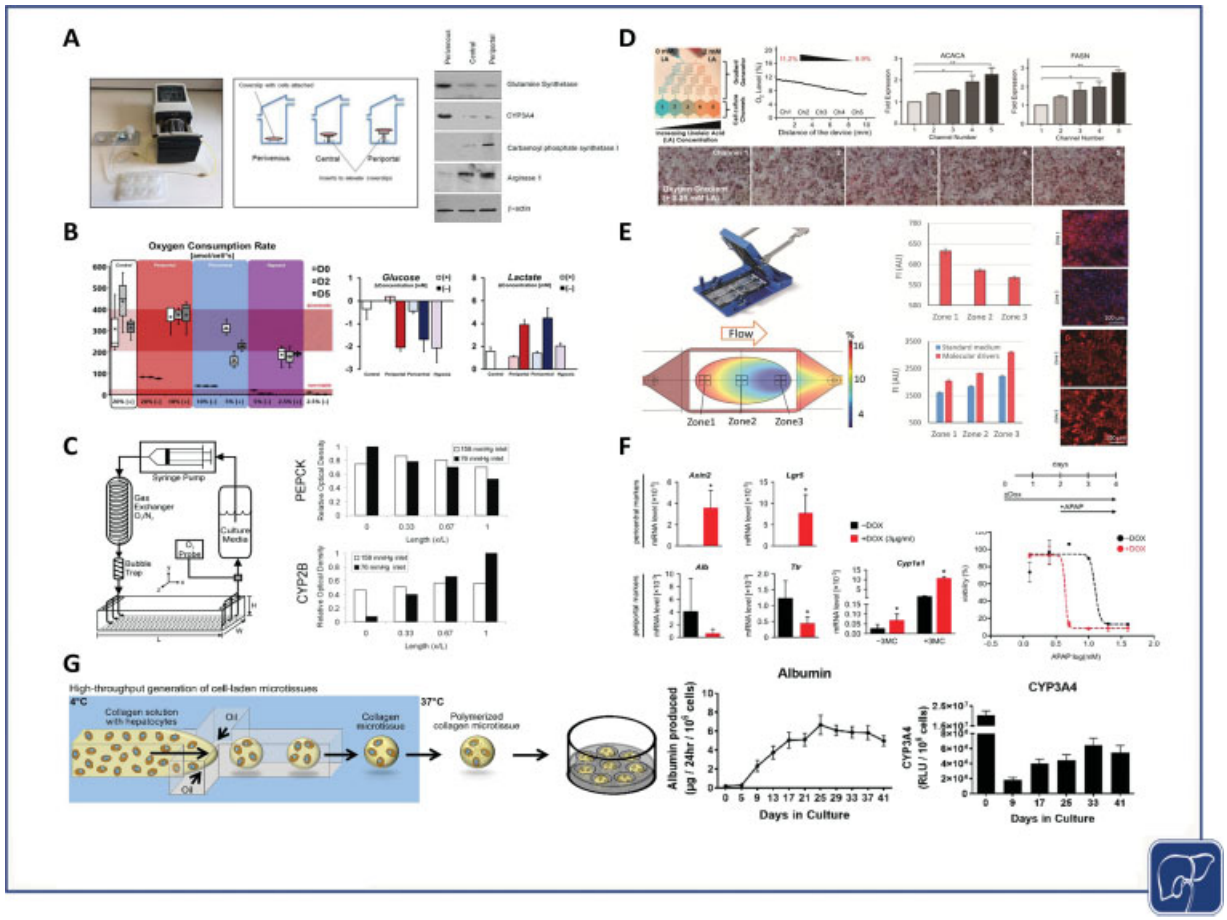


Fig. 3 In vitro liver platforms useful for investigating zonation. (**A**) Schematic of adjustable devices that control the elevation of coverslips for cell culture to modulate the oxygen diffusion within the perfusion chamber; perivenous settings (low oxygen) upregulated glutamine synthetase and CYP3A4, whereas periportal settings (high oxygen) upregulated carbamoyl phosphatase synthetase I and arginase based on Western Blot analysis (adapted from Tomlinson et al[92]). (**B**) Hepatocytes cultured on PDMS [+] gas-permeable plates or gas-impermeable [-] plates were assessed for oxygen consumption rates (amol/cell*s) over 5 days of culture. Extracellular glucose levels and glucose-6-phosphate (G6PC) gene expression were assessed and supported increased gluconeogenesis in periportal-like cultures (high oxygen) (adapted from Scheidecker et al[95]). (**C**) Flat-plate bioreactor to subject hepatocyte cultures to an oxygen gradient via flowing culture medium. Primary rat hepatocytes (PRHs) were assessed along the length of the device and phosphoenolpyruvate carboxykinase (PEPCK) (periportal marker, high at inlet) and CYP2B (perivenous marker, high at outlet) correlated with zonated trends found in vivo (adapted from Allen et al[96]). (**D**) Microfluidic-based mixers can modulate the oxygen tension along the length of the device. A gradient of free fatty acids (FFA) from 0 to 2 mM linoleic acid was established with the higher FFA concentration increasing genes associated with lipogenesis. Oxygen and FFA gradients together increased lipid accumulation in the perivenous-like region based on Oil Red-O staining (adapted from Bulutoglu et al[103,104]). (**E**) The schematic depicts the liver acinus microphysiological system (LAMPS) microfluidic device and its various components, including primary hepatocytes, endothelial cells, stellate cells, and macrophages. Control over oxygen tension via flowing medium created zone 1-like and zone 3-like regions with higher oxidative phosphorylation and glucose levels measured at the inlet (zone 1) (adapted from Li et al[107]). (**F**) Gene expression analysis for key perivenous (e.g., Axin2, LGR5) and periportal (e.g., Alb, Ttr) markers in immortalized murine hepatocytes genetically modified to upregulate Wnt signaling after doxycycline (+DOX) administration; hepatocytes had a higher induction of Cyp1a1 enzyme expression with co-treatment with 3-methylcholanthrene (3MC) and were more sensitive to acetaminophen toxicity with +DOX treatment (adapted from Wahlicht et al[50]). (**G**) Schematic of a droplet microfluidic device to generate hepatocyte microtissues. The extracellular matrix type and microtissue size can be tuned, and a second cell type (e.g., fibroblasts or endothelial cells) can be coated onto the outside of the hepatic microtissues to generate cocultures. The hepatocyte/fibroblast microtissues display functions for 6+ weeks and can be used to study the effects of soluble factors and their gradients on zonated liver functions in a 3D microenvironment (adapted from Kukla et al[112]).

carboxyphenyl) porphyrin (Pd-TCPP) oxygen-sensitive fluorescent dye is mixed with the PDMS in the devices to enable real-time oxygen sensing. Importantly, a twofold higher expression of PEPCK was observed in the region corresponding to the periportal O_2 tension and fourfold higher expression of glucokinase was observed in the region mimicking the perivenous O_2 tension. An alternative approach to establish zonation in vitro is to couple dual-inlet micromixers with microfluidic devices to establish metabolic gradients across the cell culture surface. The Metabolic Patterning on a Chip (MPOC) platform uses a "Christmas-tree" mixer to induce physiologic-like gradients of hormones (e.g., insulin or glucagon) and can modulate carbohydrate and nitrogen metabolism in hepatocytes in a zonated manner.[102] PHHs subjected to opposing hormone gradients in the MPOC device responded with increased CPS1 staining in the high glucagon region and higher glycogen storage in the high insulin region.[103] Novel microfluidic designs incorporate

multiple methods to establish key gradients in the liver to better recapitulate zonation. Most recently, the MPOC has been adapted for dual O_2 and lipid gradients along the surface of PRHs.[104] A micromixer established a 0 to 2 mM linoleic acid gradient to replicate zonated lipid metabolism (increased Oil-Red-O staining in zone 3-like region) and utilized an O_2 quenching solution (i.e., 0.13% sulfite and 13 μM cobalt) to overlay a chemically induced oxygen gradient across the cell culture surface (►**Fig. 3D**); an increase in transcripts for markers of lipid metabolism (e.g., ChREBP, ACACA, FASN) was observed in zone 3 as compared with the zone 1 regions for PRHs.

Another microfluidic device, which incorporated PHHs and liver NPC cell lines in a sequentially layered assembly, supported long-term hepatic functions and showed increased sensitivity to immune-mediated drug responses.[105] The next iteration of this device, the 3D Liver Acinus Microphysiologic System (LAMPS), replicated zone-specific oxygen tensions by modulating the flow rate from 15 μL/h (zone 1-like; higher oxygen due to lower OCR) to 5 μL/h (zone 3-like).[106] Zone 1 devices supported approximately twofold higher oxidative phosphorylation (measured via tetramethylrhodamine, ethyl ester [TMRE] fluorescence) and approximately fourfold higher glucose output, relative to zone 3 devices. In contrast, zone 3 devices had higher CYP2E1 activity, steatosis, and higher toxicity due to APAP. Recently, the vascularized-LAMPS model incorporated primary human LSECs in a glass-based chip with a continuous oxygen gradient along the cell culture surface (►**Fig. 3E**).[107] Using fluorescent labeling to quantify mitochondrial membrane potential and lipid accumulation, the vascularized-LAMPS supported higher oxidative phosphorylation in zone 1 and increased steatosis in zone 3, especially with molecular drivers of NAFLD (e.g., lipopolysaccharide, epidermal growth factors, and transforming growth factor β). Zone-specific activation of transmigration in polymorphonuclear leukocytes was not observed, whereas HSCs displayed higher activation in zone 3 upon incubation with transforming growth factor β.

Direct modulation of molecular drivers (vs. soluble factor gradients) is an alternative technique to model zonal phenotypes of hepatocytes in vitro. For example, regulation of Wnt signaling in vitro is a promising tool to induce zone-specific phenotypes in hepatocyte cultures. Though surrogate Wnt agonists have been utilized in vivo to regulate liver zonation,[108] their utility in vitro has not been established. Due to the instability of recombinant Wnt proteins in culture medium, genetic modifications of immortalized primary murine hepatocytes in conventional cultures have been used to induce Wnt signaling using a doxycycline (DOX) pulse.[50] A 4-day treatment with DOX significantly increased the perivenous genes, Axin2 and LGR5, whereas it downregulated periportal genes such as Alb and Ttr; co-treatment with DOX and 3-methylcholanthrene, a CYP1A inducer, caused a significant increase in Cyp1a1 gene expression (►**Fig. 3F**). Additionally, DOX-treated cultures (i.e., pericentral-like cells) had an increased sensitivity toward APAP toxicity. Alternatively, CHIR99021, which is a GSK inhibitor and causes increased Wnt/β-catenin signaling, has been used to upregulate perivenous-like markers in 3D hepatoma (HepaRG) spheroid cultures.[109] Using a microfluidic device to create a gradient across the HepaRG spheroids, this platform could upregulate CYP450 functions and replicate zonal toxicity (i.e., increased cell death was observed in the high-CHIR99021 region of the device following treatment with 10μM APAP). While direct modulation of Wnt/β-catenin signaling can modulate zonated phenotypes as above, these studies do not provide a natural context in which hepatocytes respond to overlapping gradients of multiple soluble factors in the flowing blood.

While the in vitro models discussed above have recapitulated some aspects of liver zonation, it has not been yet possible to mimic the full complexity of liver zonation. Single cell RNA sequencing has allowed for unprecedented characterization of zonal (spatial) gene expression in rodent livers for hepatocytes[1] and LSECs[110]; these studies provide a robust benchmark for recapitulating zonation in vitro. Moving forward, we anticipate that in vitro models of liver zonation will need to be higher throughput, utilize primary human liver cell types in coculture within a physiological 3D ECM content, and provide the ability to subject cultures to individual and precise combinations of soluble factor gradients (from portal and arterial blood streams) toward decoupling the effects of such gradients on zonated functions of multiple cell types of the liver. Furthermore, many liver tissue culture media formulations contain supraphysiological concentrations of components implicated in zonation (e.g., glucose, insulin, and glucagon), which may lead to dysregulated zonated phenotypes in vitro. Recently, we developed a more physiologic media formulation containing physiologic levels of glucose and insulin, while using human serum, to support PHHs; this media formulation prolonged the functional lifetime, including insulin sensitivity, of PHHs in micropatterned cocultures with 3T3-J2 fibroblasts for 60+ days in culture.[111] In another study, we utilized high-throughput droplet microfluidics to generate 3D PHH-NPC cocultures embedded in a microscale collagen gel (►**Fig. 3G**).[112] These so-called "microtissues" have precisely tuned dimensions to facilitate optimal nutrient and O_2 transport, enable control over homotypic and heterotypic cell–cell interactions via their microscale dimensions, and support hepatic functions (e.g., albumin secretion and CYP3A4 activity) for 40+ days in culture. Importantly, microtissues outperformed bulk collagen gels and self-assembled spheroids with respect to the level and stability of PHH functions over time. While we anticipate that the functional stability and modularity of microtissues with respect to size and homotypic/heterotypic cell–cell interactions may be useful for modeling zonation, further studies are needed with devices that allow control over soluble factor gradients to test our hypothesis.

Conclusion

Metabolic zonation of the hepatocytes along the lobule is important for the liver to simultaneously carry out a multitude of functions. Complex networks of underlying molecular gradients and signaling pathways tightly control the zonation of gene and protein expression. Liver zonation can also lead to the compartmental initiation and progression of several liver diseases, which in turn can disrupt the

metabolic features of zonation. The onset of liver regeneration tends to disrupt zonation but once the liver fully regenerates, hepatocytes in specific zones express the proper phenotypes, which suggests a functional plasticity that hepatocytes possess to modulate their functions depending on the zonated microenvironmental signals present in their vicinity.

Almost half a century of research on live rodent models has helped unravel several key molecular modulators of zonation, such as the Wnt/β-catenin and Hh pathways, among several others. These in vivo studies have been complemented by in vitro models containing hepatocytes and liver NPCs that can be used in a higher throughput manner to elucidate the effects of specific soluble factor gradients on zonated phenotypes and perhaps more importantly, allow exploration of the differences in the regulators of zonation in human and animal cells. We have summarized the key features of in vitro models discussed here in ►**Table 1**. In spite of considerable progress, how various soluble factor gradients (both those derived from portal

Table 1 In vitro platforms previously used to model zonation. Platforms are organized as increasing in physiological complexity but reducing in throughput from top to bottom

Model system	Reference	Cell type(s)	Approach to establishing zonation	Aspects of zonation achieved
Bulk O_2 control in multiwell cultures	93	2D monolayer and 3D paper-based platform of HepG2	Hypoxia chamber maintained at different oxygen tensions	Higher sensitivity to hepatotoxins at lower oxygen tension
	91	HepG2 monolayer over thin collagen gels	Adjusted culture media height to control oxygen tension	Ten-fold increase in CYP450 activities in hypoxia, relative to hyperoxic conditions
	95	Monolayer of PRHs	Gas-permeable PDMS multi-well plate cultured in multi-gas incubator	Intracellular localization of active β-catenin and glutamine synthetase and high CYP2E1 expression in low oxygen conditions
Bioreactors	96,97	PRHs with or without coculture with supportive 3T3-J2 fibroblasts	Cell-mediated depletion of oxygen in a flat-plate perfusion bioreactor	Periportal distribution of PEPCK and pericentral distribution of CYP2B and CYP3A; Zonal APAP toxicity
	92	Sandwich culture of PRHs within a millifluidics perfusion system	Adjusting the position of cell culture to manipulate media height	Higher GS and CYP3A4 expression in perivenous conditions; higher CPS1 and Arg1 expression in periportal conditions
	99	Monoculture and bi-layer coculture of PHHs and PRHs with primary human and rat LSECs	Cell-mediated depletion of dissolved oxygen in ExoLiver platform	Expression of periportal genes Gls2 and Aqp1 in inlet and perivenous genes Glul and Oat in outlet region
Microfluidic/ Liver-on-a-Chip Platforms	101	Monolayer of primary mouse hepatocytes	Cell-mediated depletion; linear distance from air channel located next to periportal region	Zone specific expression of PEPCK (high O_2) and GK (low O_2)
	103	Monolayer of PRHs and PHHs	Christmas tree gradient microfluidic device to induce gradients of hormones and chemicals on the cell culture; Metabolic Patterning on a Chip (MPOC)	Zonal induction of CYP1A2 and APAP toxicity
	104	Monolayer of PRH		Increased expression of lipogenesis markers (e.g., ACACA, FASN) in zone 3-like region
	106	PHHs sequentially layered with non-parenchymal cell (NPC) lines within ECM gels	O_2 tension controlled by varying flow rate and cell-mediated O_2 consumption within liver acinus microphysiologic system (LAMPS) and glass-based vascularized LAMPS (vLAMPS)	Higher oxidative phosphorylation and higher glucose output in Zone 1; Higher CYP2E1 activity and higher APAP toxicity at Zone 3
	107	PHHs, primary human LSECs, and cell lines for Kupffer cells and stellate cells		Increased steatosis in Zone 3 with molecular drivers of NAFLD

Abbreviations: ECM, extracellular matrix; LSEC, liver sinusoidal endothelial cell; NAFLD, non-alcoholic fatty liver disease; 2D, two dimensional; 3D, three dimensional.

blood and hepatic artery) act in isolation and in combinations to regulate/interact with key molecular pathways in liver zonation has not been fully elucidated. We anticipate that continued advances in both in vivo and in vitro models will help facilitate a deeper understanding of liver zonation, in not just hepatocytes, but also the different liver NPC types, across both physiological and disease scenarios. We anticipate that such a deeper understanding of the regulators and functional outcomes of liver zonation will have far reaching implications for the development of better therapeutics against liver diseases and zonated liver tissue surrogates useful for treating patients suffering from end-stage liver failure.

Main Concepts and Learning Points

- Liver zonation leads to the compartmentalization of functions in hepatocytes along the sinusoid.
- Gradients of soluble factors, such as oxygen, hormones, and nutrients, interact with key molecular pathways (e.g., Wnt/β-catenin, hedgehog) to regulate liver zonation.
- Several liver diseases, including alcoholic and non-alcoholic fatty liver diseases, hepatitis B/C virus infections, and hepatocellular carcinoma, display zone-specific initiation.
- Transgenic mouse models have provided key insights into the regulators of liver zonation, including demonstrating that midlobular hepatocytes contribute the most to hepatocyte proliferation in homeostasis and regeneration.
- Specialized plates and microfluidic devices are useful to investigate the effects of soluble factor gradients on hepatic functions in vitro.
- Further research can help elucidate how precise combinations of diverse soluble factor gradients regulate zonation in primary human hepatocytes and nonparenchymal cells.
- Obtaining a deeper understanding of regulators of liver zonation may lead to the development of better therapeutics for liver diseases, microphysiological systems, and cell-based therapies.

Abbreviations

2D	two-dimensional
3D	three-dimensional
AA	allyl alcohol
AAV	adeno-associated virus
ADH	alcohol dehydrogenase
ALD	alcoholic liver disease
APAP	acetaminophen
APC	adenomatous polyposis coli
CCL4	carbon tetrachloride
CTNNB1	catenin β 1
CYP450	cytochrome P450
DDC	3,5-diethoxycarbonul-1,4-dihydrocollidine
Dhh	Desert hedgehog
DOX	doxycycline
ECM	extracellular matrix
GLI	glioma-associated oncogene
GS	glutamine synthetase
GSH	glutathione
HBV	hepatitis B virus
HCC	hepatocellular carcinoma
HCV	hepatitis C virus
HH	hedgehog
HIF	hypoxia-inducible factor
HNF4α	hepatocyte nuclear factor-4 α
HSC	hepatic stellate cell
IGF	insulin-like growth factor
IGFBP	insulin-like growth factor binding protein
Ihh	Indian hedgehog
IRS	insulin receptor substrate
KO	knockout
LGR	leucine-rich repeat-containing G-protein coupled receptor
LSEC	liver sinusoidal endothelial cell
NAFLD	nonalcoholic fatty liver disease
NASH	nonalcoholic steatohepatitis
NPC	nonparenchymal cell
OCR	oxygen consumption rate
PDMS	polydimethylsiloxane
PEPCK	phosphoenolpyruvate carboxykinase
PHH	primary human hepatocyte
PRH	primary rat hepatocyte
R26R	Rosa26-Rainbow
ROS	reactive oxygen species
RSPO	R-spondins
Shh	Sonic hedgehog
Smo	smoothened
TCF	T-cell factor

Data Availability
Data sharing is not applicable to this article as no new data was created or analyzed in this study.

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Conflict of Interest
None declared.

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