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Liver 'organ on a chip'

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Introduction/Abstract

The liver plays critical roles in both homeostasis and pathology. It is the major site of drug metabolism in the body and, as such, a common target for drug-induced toxicity and is susceptible to a wide range of diseases. In contrast to other solid organs, the liver possesses the unique ability to regenerate. The physiological importance and plasticity of this organ make it a crucial system of study to better understand human physiology, disease, and response to exogenous compounds.

The purpose of this review is to inform the reader of the significance and available methods for replicating human liver physiology and pathology *ex vivo*. First, the physiologic roles of the liver and its cellular constituents will be discussed. Second, we will discuss the need for developing an *ex vivo* liver system. Third, the advantages and disadvantages of different cell sources used to populate the system will be mentioned. Fourth, the benefits of currently employed *ex vivo* liver culture systems (both commercially available and used in research laboratories) will be discussed. Finally, future directions to advance these systems, including complexing liver culture systems with other organ-based culture systems ("organ chips"), will be proposed.

Keywords

Microphysiologic systems; organoids; 3D culture systems

Conflicts

LG and AW hold a patent position in the LiverChip commercialized by CNBio Innovations. DLT has a patent application in review on the liver MPS.

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Physiology

The liver is a complex organ that is integral to a multitude of whole body functions and is extensively reviewed in standard medical textbooks. For the purposes of this review, a few salient aspects are emphasized. The liver is organized into lobules, functional units of the liver, which are subsequently organized into larger lobes. Lobules consist of multiple hepatic sinusoids, whose structure is illustrated in Figure 1A. The flow of blood from the portal triad to the central vein contributes to a zonation based on decreasing oxygen tension. This affects both the parenchymal (hepatocytes) and non-parenchymal cells (NPC) that will be discussed herein.

The parenchymal cells, mainly hepatocytes, comprise 60% of the cell population and are responsible for the liver's metabolic activity (including the cytochrome P450 (CYP450) isoforms), the production and release of acute phase proteins, production of plasma proteins (such as albumin and clotting factors), and the integration of various components of glucose, lipid, nitrogen, and oxidative metabolism[1,2]. These hepatocytes mature postnatally, causing different CYP450 isoforms and assemblies thereof to be expressed in fetal, neonatal and adult hepatocytes[3]. For example, CYP3A7 is very highly expressed in fetal hepatocytes, whereas this isoform is replaced by CYP3A4 expression in adult hepatocytes. Moreover, pediatric hepatocytes typically exhibit increased clearance of CYP450-metabolized drugs as compared to adult hepatocytes[4], despite no differences in intrinsic CYP450 expression between the two cell sources[5]. Thus, hepatocyte functioning is key to any *ex vivo* system that replicates the *in vivo* liver.

Oxygen tension plays an important role in regulating liver zonation and functionality, with the liver uniquely receiving both arterial (hepatic artery, ~25%) and venous (portal circulation, ~75%) blood. The partial pressure of oxygen drops as one progresses across the liver sinusoid, the functional unit of the liver, from periportal to perivenous hepatocytes[6]. This oxygen differential regulates the response of the liver to metabolic and toxic stimuli by facilitating differential metabolism, termed "liver zonation." For example, the relatively hypoxic perivenous hepatocytes are responsible for the majority of substrate metabolism through the CYP450 system whereas the relatively oxygen-rich periportal hepatocytes boast mainly oxidative metabolic functions[6]. Liver zonation is also observed in *in vitro* cultures[7,8]. Thus, the organization of the parenchyma motivates careful engineering to replicate hepatic function and toxicity and capture the full panoply seen *in vivo*.

Blood flow carried through vessels lined by the fenestrated endothelium of the sinusoids is imperative for liver functions. Hepatic blood flow dictates the metabolic zonation of the liver[6] and alterations in flow can reduce liver function and drug clearance[9]. Blood flow carries oxygen, nutrients, and chemicals that are distributed to the liver tissue. In the absence of adequate blood flow, these factors are consumed by the proximate cells and do not reach more distant populations. Reciprocally, juxtavascular cells can contribute factors to the circulation. Locally, the potency of hepatocyte-secreted factors is a function of the blood-to-cell volume ratio, which is approximately 20.5 mL blood per mL tissue for healthy adult livers[10]. Assuming a hepatocyte volume of 3.4 pL[11], this corresponds to roughly 14 million hepatocytes being supplied by 1 mL of blood. This consideration is particularly important

for *in vitro* and *ex vivo* culture systems, as media flow is used by some to model *in vivo* blood flow (see "Engineered Culture Systems").

The non-parenchymal cells (NPC) compose the remaining 40% of the cell population and play a significant role in tissue architecture and in mediating responses of the tissue to metabolic and toxic stimuli, as well as supporting the hepatocyte function[2,12]. These cell types include liver sinusoidal endothelial cells (LSECs), Kupffer cells (KCs), hepatic stellate cells (HSCs), and pit cells (natural killer cells, NKs). Inclusion of NPCs in hepatocyte culture systems has shown beneficial effects. For example, 3-dimensional (3D) liver tissue models show increased hepatocyte functions when nonparenchymal cells are incorporated[13]. Additionally, KCs play a significant role in the response of the liver to injury through the production of cytokines and reactive oxygen species[14]. Moreover, HSCs respond to injury both by adopting a myofibroblast phenotype that remodels the liver extracellular matrix[15] and increasing the CYP450 activity of hepatocytes[16]. Finally, LSEC proliferation in response to injury has been suggested to aid the liver's potent regenerative capacity[17]. Thus, the NPCs complement the synthetic and metabolic functions of hepatocytes by contributing pro-regenerative, pro-inflammatory, and pro-fibrotic stimuli.

Modeling the Liver Microenvironment

Current preclinical models for hepatotoxicity involve human cell culture and animal models. Recently, efforts to develop *ex vivo* hepatic culture systems, "liver-on-a-chip," have been undertaken by many research groups and biotech companies due to the liver's capacity for drug metabolism, excretion, vulnerability to drug-induced damage, and as a primary organ in many diseases. Drug-induced liver injury remains a major reason for drugs being withdrawn from the market, and causes both morbidity and mortality for patients. Importantly, humans metabolize and respond to agents differently from other mammals; to the point, most all species present unique xenobiotic handling[18]. In fact, one-third of toxicities observed in humans are not predicted in any of the species commonly employed for drug safety testing[19], possibly due to their failure to model reactive metabolites generated through human-specific metabolic pathways[20]. Moreover, individual animal models have a success rate of as low as 40% in predicting hepatotoxic compounds[21], resulting in 26% of clinical trial failures being due to hepatotoxicity[22].

Current liver tissue culture systems exist on a spectrum of complexity. Historic hepatocyte culture systems involved collagen-sandwich culture or 2D Micro-Patterned Co-culture (MPCC) systems using primary rat hepatocytes and 3T3-J2 fibroblasts. Systems have progressed to include 3D static spheroid models and perfusion culture devices, which introduce nutrient and oxygen gradients and shear stress that are important for hepatocyte functions[23]. The systems discussed below offer distinct advantages and disadvantages for investigating the response of hepatic micro-tissues to different drugs and other stimuli.

Cell Sourcing

The complex physiology of the liver and need for its accurate representation in engineered systems requires careful selection of cell type(s) and their origin. As previously discussed,

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hepatic tissue is composed of hepatocytes (60% of liver cells) and a complex complement of NPCs (40% of liver cells). Integration of both cell fractions is often needed to adequately reflect pharmacokinetics, pharmacodynamics, toxicity of drugs, and liver disease progression, given the intercommunication between the different liver cell types. Four sources of hepatocytes will be discussed: primary human cells, primary animal cells, immortalized human cell lines, and pluripotent stem cells. Each of these cell sources has its advantages and disadvantages, and each will be discussed below. A qualitative summary of cell selection parameters can be seen in Table 1.

Primary human hepatocytes

Functional primary human hepatocytes obtained through collagenase perfusion and dissociation have been the gold standard in drug discovery for 25 years due to their ability to most accurately reflect *in vivo* metabolism and toxicity[24–26]. Primary human hepatocytes have been isolated from livers with benign (processes not affecting the hepatocytes) and hepatocyte-specific pathologies. Some examples include end stage primary biliary cirrhosis (PBC), primary sclerosing cholangitis (PSC), alcoholic liver disease (ALD), and resections for colorectal metastases or benign growths[27]. Several groups have found patient-specific factors to play a role in the yield of hepatocytes during isolation or in their functionality. While patient gender and type of disease had no effect on hepatocyte yield or functionality[28,29], cholestasis (signified by elevated gamma glutamyl transferase level – GGT)[30,31], severe steatosis[31,32], and older age[29,31,33,34] negatively influenced isolation outcomes. Although some patients have been exposed to chemotherapy, hepatocyte function and viability are not altered[35]. While an ideal source, limited availability of cells and reliance on patient surgeries are major reasons why primary hepatocytes are not more commonly used. Moreover, many donors are pediatric patients, whose hepatocytes may have metabolic dysfunction or exhibit differential clearance when compared to adult human hepatocytes (see “Physiology”).

Advances in cryopreservation have allowed for easier distribution and commercialization of primary human hepatocytes. Previous work has demonstrated cryopreserved primary human hepatocytes to still exhibit 94% of the clearance of model drugs (e.g. diclofenac) after 1 year in liquid nitrogen, as compared to freshly isolated human hepatocytes[36]. With proper cryopreservation, viabilities >90% can be achieved[37]. Additionally, minimal changes in metabolic functions have been demonstrated even after 14 years in liquid nitrogen[38]. While cryopreserved primary human hepatocytes exhibit high viability and comparable CYP450 activity to fresh cells, they exhibit suboptimal attachment to extracellular matrix molecules, due to downregulation and degradation of the adhesion molecules β1-integrin and E-cadherin respectively during cryopreservation[39]. Additionally, decreased expression of uptake transporters such as OATP (organic anion-transporting polypeptide) and NTCP (sodium-taurocholate co-transporting polypeptide) family members results in under-prediction of drug clearance rates[40]. Finally, high viability methods often involve centrifugation to remove dead and damaged hepatocytes, which also reduces the cell yield.

Cryopreserved primary human hepatocytes have been used in numerous studies to investigate CYP450 induction[41], drug clearance[42], and hepatobiliary transport[43]. In contrast

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to human hepatic cell lines or animal primary cells, cryopreserved primary human hepatocytes can probe the influence of genetic background on hepatocyte metabolism or drug clearance. For example, a collection of cryopreserved hepatocytes from 64 donors has been used to investigate gender-specific activities of CYP450 isoforms *in vitro*, which corroborated with *in vivo* findings, namely a higher expression of CYP3A4, the most dominant human isoform, in females[44]. Thus, while human cell lines and animal hepatocytes are more accessible, primary human hepatocytes will likely continue to play an important role in drug development due to their ability to most effectively reflect *in vivo* metabolism, clearance, and response to toxins by patients.

Animal-derived hepatocytes

Animal-based testing has formed the foundation for translating *in vitro* studies to clinical trials. Before moving to humans, the Federal Drug Administration (FDA) requires pharmacokinetic, toxicity, and efficacy studies in at least one rodent (mouse, rat) and one non-rodent (dog, rabbit) species. Such studies are designed to examine integrated organ responses and are able to suggest, at least initially, the influence of genetic diversity on drug responses[45]. Additionally, these species are readily commercially available, thus serving as sources of fresh primary cells. However, animal studies frequently do not elicit the pharmacokinetic behavior seen in humans due to species differences in drug metabolism and clearance, in particular differential CYP450 metabolic activity[46,47]. Differences in CYP450 induction by model drugs between human and animal (rat, dog) are well established[48]. Improvements in predicting human-specific metabolism and clearance from animal studies have been made through retrospective analyses[49]. Yet, sourcing cells from animals may be cost prohibitive, ethically controversial, and futile if interspecies differences prevent human-relevant data from being collected[50]. Moreover, since animal hepatocytes express different CYP450 isoforms than humans, extrapolating of gender-specific differences in drug metabolism is challenging[51].

Human hepatic cell lines

Human hepatic cell lines, either cancer-derived or immortalized hepatocytes, can be effectively propagated in culture over multiple passages. Some of the most commonly used hepatic cell lines include HepG2, Huh7, Hep3B, and SK-Hep-1, all derived from hepatocellular carcinoma (HCC), and HepaRG, an HCC cell line that constitutes a mix of both hepatocytes and biliary-like cells[52,53].

One issue with using human hepatic cell lines is that they exhibit lower and variable CYP450 expression than primary human hepatocytes[53]. For example, CYP3A4 is not expressed in HepG2 or Hep3B cells whereas CYP2D6 is expressed at less than 5% the level of primary human hepatocytes. Studies in HepG2 cells demonstrated that CYP450 expression may also vary according to culture duration and passage number, some isoforms varying up to 200-fold across the first 10 passages[54]. Some cell lines hold more promising results. For example, induction of CYP450 enzymes and the drug transporter MDR1 was seen in a novel hepatic cell line, Fa2N-4, in response to rifampin treatment[55].

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Another issue with human hepatic cell lines is that they exhibit reduced expression of both sinusoidal and canalicular transporters[56]. These transporters function to shuttle drug compounds from the blood to the bile canaliculi. While hydrophobic compounds can diffuse across the hepatocyte plasma membrane, hydrophilic compounds require active transport. Reduced transport activity and decreased CYP450-catalyzed metabolism may result in inaccurate pharmacokinetic and toxicity predictions.

The HepaRG cell line is a promising substitute for primary human hepatocytes. When seeded at a low density, HepaRG cells are capable of proliferating and differentiating to confluency to form colonies of hepatocytes surrounded by biliary epithelial cells that exhibit CYP450 expression levels comparable to primary human hepatocytes[57]. HepaRG cells have also been found to functionally express both sinusoidal and canalicular transporters[58,59]. Additionally, HepaRG cells can identify drugs likely to induce liver injury, albeit using doses up to 100-fold higher than would be seen clinically[60]. Moreover, HepaRG cells exhibit a more robust response to inflammatory stimuli (e.g. IL-6, TNFα) on liver drug metabolism and elimination than primary human hepatocytes, potentially due to genetic variation in the latter[61].

While hepatocyte cell lines are effective in predicting the metabolism and elimination of some drugs, their overall sensitivity to detect toxic compounds is lower than primary human hepatocytes (13% for HepaRG compared to 44% for PHHs)[52]. Further, being derived from a single female donor, these cells do not account for genetic variation in drug responses that often uncover limiting toxicities in a subset of persons. This limitation is also seen with primary hepatocytes, as testing is often performed on a limited number of donors. Importantly, since human hepatic cell lines are derived from primary hepatic tumors, they may not accurately represent normal cellular responses. These weakness ultimately require the use of complementary approaches (i.e. normal primary cells)[23].

Induced and Embryonic Pluripotent Stem Cells (iPSC and ESC)

Stem cells are defined as cells capable of self-renewal and differentiation into mature cells of a particular tissue type[62]. Stem cells encompass both those derived from blastocysts (ESC) and also those derived from mature cells through reprogramming (iPSC) using viral vectors, Cre-lox expression cassettes, or mRNA/miRNA transfection[63]. While iPSC generation and differentiation is time consuming, it allows for an easily sharable cell population with theoretically limitless growth potential that can mimic preliminary clinical trials *in vitro*, hopefully improving the success in subsequent human studies.

Many protocols demonstrate differentiation of iPSC[64,65] or ESC[66–68] into hepatocyte-like cells. Protocols that try to replicate liver embryogenesis can achieve hepatocyte-like cell yields of up to 80%[68]. Additionally, the expression of microRNAs (mir), specifically mir-122, during the differentiation process can further improve hepatocyte fidelity[69]. However, many differentiation protocols result in hepatocytes that express immature markers (e.g. alpha-feto protein; AFP), secrete less albumin, and exhibit dramatically reduced CYP450 activity than primary human hepatocytes[70–72]. Indeed, hepatocyte-like cells differentiated from iPSC using multiple sources and two different protocols demonstrated a phenotype akin to fetal hepatocytes[73].

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An advantage of iPSCs is the potential to study a broad spectrum of hepatocyte lines with different genetic, epigenetic backgrounds[71,73] and disease backgrounds. Additionally, iPSCs exhibit sensitivity comparable to primary human hepatocytes for detecting drugs that cause drug induced liver injury (65% vs 70%)[74]. While a promising technology, current iPSC technical limitations include epigenetic memory, genomic instability, state of maturation to adult cells and variability among cell lines. Strategies to address these weaknesses include using small molecule treatment, avoiding targeting p53 during reprograming, and use of multiple iPSCs from different genetic backgrounds, respectively[75]. Namely, platforms aimed to analyze the influence of small molecules on hepatocyte expansion and differentiation of iPSCs to mature hepatocytes have shown promising results[76]. However, a steady supply of hepatocyte-like cells derived from iPSC is often not available to many researchers, which limits consistent use to labs with experience in their differentiation. Encouragingly, some companies are starting to commercialize iPSC-derived hepatocytes.

Non-parenchymal Cells (NPCs)

The NPC component of the liver is critical to recreating physiologic liver functioning and response to injurious stimuli. As such, inclusion of NPCs is desirable to replicate metabolism and signaling pathways observed *in vivo*. Addition of NPCs in hepatic culture systems has shown to alter signaling networks present in the microenvironment, enhance hepatocyte synthetic functions, prevent hepatocyte de-differentiation in culture, and enhance hepatocyte metabolic response to drug treatment[1,77,78]. Both primary and immortalized cell types are available and will be discussed below.

Primary NPCs are isolated in a similar manner to primary hepatocytes, by liver enzymatic perfusion. These cells can either be harvested alone or simultaneously with primary hepatocytes. When harvested alone, the NPCs of the liver can be enriched, at the expense of hepatocyte recovery, by using a pronase-collagenase perfusion method[79,80]. For isolating both cell components, collagenase perfusion followed by density centrifugation are used to collect and separate the hepatocytes from the NPCs, respectively. Several methods have been used to purify the NPCs, which include iodixanol or percol density gradients and magnetic activated cell sorting[81–83]. Importantly, the disease state of the human donor tissue can greatly influence the quality and quantity of cells obtained[84,85]. To avoid the need for donor tissue, others have demonstrated generation of NPCs from human iPSCs[86].

While primary NPCs are the most effective at reproducing the microenvironment seen physiologically, their main disadvantages are non-specific activation and availability. Using primary NPCs requires having surgical specimens. Cryopreserved NPCs can be purchased commercially either as a collective or for specific NPC types. Due to the high cost of primary cells, various cell lines or alternative cell types have been employed in co-cultures with hepatocytes to provide similar trophic signals as primary NPCs. The next several sections will illustrate approaches to incorporate each NPC type into hepatic culture systems.

Endothelial Cells—Liver sinusoidal endothelial cells (LSECs) are a unique endothelial cell type displaying abundant fenestrations, high endocytotic ability, and ability to provide

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ideal trophic support to hepatocytes. These cells are often unstable *in vitro*[87–90] and are difficult to cryopreserve[91]. As such, some groups have instead used human umbilical vein endothelial cells (HUVECs) that, in co-culture with hepatocytes, improve hepatocyte specific function, such as CYP450 activity and albumin synthesis[92–94]. However, as HUVECs are a unique cell type that lack appreciable levels of key cell surface receptors CXCR3[95] and EGFR[96], other primary human endothelial cells have been employed with variable results[94,97].

Immortalized endothelial cell lines have also been used in hepatocyte co-culture. HMEC-1 (human foreskin endothelium) cells have been shown to enhance hepatocyte albumin secretion[98] and can provide inflammatory cues that affect both the hepatocytes as well as metastatic cancer cells[99]. TMNK-1 cells are an immortalized LSEC cell line that has been used in hepatocyte co-culture to investigate paracrine signaling[100], influence on cancer cell phenotype[99], and promote hepatocyte-specific functions in ESC-derived hepatocytes[101]. However, immortalized LSECs phenotypically resemble activated endothelial cells implicated in pathologic vessel formation in chronic liver disease[102], and may not emulate normal physiology, in particular due to their lack of fenestrations.

Kupffer Cells—KCs are implicated in host defense and are important producers of cytokines in inflammatory responses and liver diseases. Immortalized KCs have been generated in mouse and rat[103], but not human. THP-1 monocytes have been differentiated into macrophages and used in co-culture applications with both primary hepatocytes and human hepatic cell lines. Inflammatory responses were noticed in hepatocytes as a result of macrophage-secreted products[104] and direct co-culture[105]. To demonstrate the importance of KC inclusion in hepatocyte cultures, LPS stimulation of a primary hepatocyte and KC perfusion co-culture system demonstrated no effect on hydrocortisone metabolism, similar to that seen clinically[106]. However, immortalized KCs or activated macrophage cell lines do not replicate physiologic KC behavior. KCs are typically quiescent and tolerogenic due to high basal expression of TGFβ and PD1 and expression of IL-10 upon LPS stimulation[107]. As such, alternatives to primary KCs may promote more inflammatory-related changes than seen *in vivo*. Of benefit to labs without access to fresh primary cells, cryopreserved KC are commercially available for use.

Stellate Cells—Hepatic stellate cells (HSCs) are important cells for the storage of retinoids and other lipids and are implicated in hepatocyte proliferation after partial hepatectomy, hepatic fibrosis, and portal hypertension. Freshly isolated primary HSCs quickly activate when cultured on plastic[108] and their gene expression *in vitro* does not fully reproduce that observed *in vivo*[109]. HSC quiescence can be preserved by culturing on laminin-rich gels or in suspension on a non-adherent surface[110]. Several immortalized HSC lines exist for study, including hTERT-HSC, GREF-X, LI90, TWNT-1 and more recently, LX-1 and LX-2. However, only the LX-2 cell line can be maintained in serum-free media and transfected with relatively high efficiency (30%)[111]. Importantly, while immortalized cell lines express many of the same markers as primary HSCs, their response to stimuli and basal level of activation differ from that observed *in vivo*[112]. Co-culture of HSCs with

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hepatocytes has been used to maintain hepatocyte differentiation *in vitro* by cell contact mediated signals and transfer of lipids[113].

NPC Considerations

It is clear that including NPCs in culture systems influences hepatocyte functions and the tissue microenvironment. ECs can enhance albumin synthesis and CYP450 activity in co-culture with hepatocytes. KCs can elicit inflammatory changes in response to stimuli that resemble those seen *in vivo*. Finally, HSCs are important for storing lipids that can be utilized by the hepatocytes and preserving hepatocyte differentiation. While primary non-parenchymal cells are the gold standard, they are difficult to obtain in sufficient numbers. NPC yield is low when isolated concurrently with hepatocytes and subsequent purification steps can alter NPC function. Several immortalized cell lines can serve comparable (though not equivalent) roles for each NPC type, and also offer the benefits of being easily distributable and homogeneous.

Engineered Culture Systems

The overarching goal of all liver culture systems is to provide either a high throughput, low cost, easy to operate, and reliable system that recapitulates a reasonable fraction of human physiology, or a medium to low throughput system that reliably recapitulates liver structure and function (liver biomimetic). This depends on the ability of the system to generate an environment that reflects the *in vivo* phenotype of the hepatocytes and NPCs, which is highly dependent on both chemical and mechanical cues. A critical issue with routine 2D primary human hepatocyte culture systems is that hepatocyte functionality rapidly declines within days[114]. To overcome this, hepatocytes have been cultured using the collagen-sandwich method. This involves seeding a monolayer of hepatocytes on a gelled layer of rat tail collagen and overlaying another gelled collagen layer on top. This culture method allows hepatocytes to maintain synthetic function (as determined by albumin mRNA) for at least six weeks[115]. However, collagen sandwich cultures do not replicate the complex multi-cellular nature of the liver and does not incorporate fluid flow, which is important for preserving *in vivo* hepatocyte function[116]. Several of the most recent engineering advances to improve hepatocyte function and viability in culture will be discussed in this review. These systems are demarcated into the following categories: 2D Micro-Patterned Systems, 3D Spheroid Culture Systems, and Perfusion Culture Systems. For a comprehensive review of all advancements made in the realm of hepatocyte culture, the reader is referred to other references[25,117,118].

Non-Perfusion Systems

Both static and perfusion systems exist for hepatocyte culture. Static culture systems include 2D micro-patterned systems and 3D spheroid culture systems. A summary of the properties of these systems can be found in Table 2.

2D Micro-Patterned Systems—The use of 2D micro-patterned culture systems are an improvement upon standard sandwich culture as they fine-tune tissue architecture by controlling the size, geometry, and functionality of culture chambers. One commercialized

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system, HepatoPac™ by Hepregen, Corp. consists of micro-patterned 2D plates seeded with either rat or human hepatocytes and 3T3-J2 stromal cells. Cultures maintain hepatocyte specific functions for 4–6 weeks[119–121], notably albumin and urea secretion, phase I/II metabolism, and formation of canalicular networks. Publications have also reported sensitivities of 66% and 100% for corroborating hepatotoxins when tested with one or at least two cell donors, respectively[120]. The extended hepatocyte functionality may allow for accurate predictions of *in vivo* drug transporter activity[122]. Additionally, this system can be used to model CYP450 enzyme induction[121,123]. However, this system's low throughput make it less suitable than traditional monolayer culture for toxicity screening studies[124].

Similar systems have used micro-patterned hepatocyte islands to enhance hepatocyte functioning in 2D culture. For example, Cho and colleagues used polydimethylsiloxane (PDMS) stencils to co-culture rat hepatocytes and 3T3 fibroblasts and demonstrated enhanced hepatocyte functions when co-cultured in a layered format (hepatocytes seeded on an island of fibroblasts) as compared to a co-planar format[125]. Ware and colleagues used micro-patterned hepatocyte-murine fibroblast co-cultures to predict hepatotoxicity using both primary human hepatocytes and iPSC-derived hepatocyte-like cells[74]. However, these systems do not take advantage of the positive influence of LSECs on hepatocyte functions, as previously shown with hepatocyte co-culture with HUVECs[126].

Since these systems involve a monolayer of cells, they are amenable to high content image analysis without confocal optics. However, these systems have not been fully characterized for co-culture with liver-specific NPCs and are patterned using rat tail collagen I rather than liver specific matrix proteins[23]. Previous studies have shown that culturing hepatocytes on liver-specific ECM leads to higher attachment efficiency and lower expression of the dedifferentiation markers vimentin and cytokeratin 18[127]. Moreover, supplementation of liver-specific ECM digests in hepatocyte cultures showed improved albumin synthesis and CYP450 activity[128,129]. Enhanced albumin secretion and cellular connectivity is also observed with less stiff culture substrates, such as heparin gels, with the least physiological morphology observed on collagen coated glass[130]. These findings suggest culture materials that mimic physiologic liver extracellular matrix promote maintenance of *in vivo* hepatocyte functions.

3D Spheroid Culture Systems—Three dimensional culture systems add an additional layer of complexity by more accurately representing the tissue architecture of a whole liver. Micro-patterning and functionalizing surfaces can facilitate the formation of 3D hepatocyte spheroids. For example, Fukuda and colleagues micro-patterned cylindrical culture wells with collagen and polyethyleneglycol, which facilitated rat hepatocyte spheroid formation. These spheroids demonstrated enhanced albumin secretion and ammonia detoxification compared to monolayer culture for up to 14 days in culture[131]. They also used a photo-crosslinkable chitosan hydrogel to incorporate 3T3 fibroblasts into their spheroids[132].

Liu *et al* micro-patterned electrospun fibrous mats for the co-culture of rat hepatocytes, HUVECs, and 3T3 fibroblasts lasting up to 15 days[93]. They observed the formation of hepatocyte spheroids and noted co-culture with both fibroblasts and endothelial cells enhanced albumin secretion, urea synthesis, and CYP450 expression as compared to

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monoculture or co-culture with either fibroblasts or endothelial cells alone. While this system incorporates all three cell types on the same culture surface, each cell type is seeded in a specific area, limiting direct contact but allowing paracrine communication[93].

Alternatively, primary rat hepatocytes and 3T3 fibroblasts can initially be co-cultured on 2D micro-patterned surfaces and the resulting cell aggregates detached and encapsulated in PEG to form 3D spheroids[133]. These spheroids are capable of showing dose-dependent acetaminophen toxicity responses, as well as CYP450 induction by model compounds, such as rifampin and phenobarbital. Additionally, the small size of these spheroids (~100μm) prevents the formation of a necrotic core. However, this system has not yet been characterized using human hepatocytes.

Bell and colleagues have generated spheroids from primary human cryopreserved hepatocytes and NPCs that demonstrate preserved metabolic function and viability up to five weeks in culture[134]. Beneficially, this system uses all human cells, is highly scalable (made in 96 well plates), and demonstrates long term response to hepatotoxins. However, the cell-to-media ratio is quite low, which may reduce the impact of paracrine factors secreted by the spheroids.

There are two 3D spheroid systems commercially available. First, RegeneTox™ marketed by Regenemed Inc. allows for the co-culture of NPCs and hepatocytes through use of a transwell insert. NPCs are seeded above two interconnected nylon scaffolds and cultured for a week prior to hepatocyte isolation and seeding. The media are changed three times a week and the co-cultures can last more than three months in the system, as demonstrated by albumin, urea, transferrin, and fibrinogen secretion, and stable CYP1A1, 3A4, and 2C9 activity. Moreover, the liver tissue was responsive to inflammatory stimuli by releasing mediators such as TNFα and IL-8[135]. However, this system is relatively low throughput (24-well plate equivalent) and utilizes a culture surface orders of magnitude stiffer than native liver tissue.

GravityTRAP™ marketed by InSphero AG uses a hanging drop platform to seed cells in a concentrated suspension to grow liver tissue in 3D. These tissues can be transferred into a specialized 96-well plate for culture and analysis, remaining viable for at least 4 weeks. Spheroids demonstrate robust CYP3A4 expression and albumin production compared to 2D cultures. Spheroid size is highly reproducible but may be time consuming without automation. Additionally, spheroid size is limited, as spheroids larger than 200μm develop central necrosis due to hypoxia[136].

While the dimensionality of these systems allow for more complex intercellular communication than 2D culture systems, they do not incorporate flow, which is known to be important for maintaining liver-cell specific functions.

Perfusion Culture Systems

Perfusion culture systems allow for the incorporation of physiological “blood” flow (media), which is an important factor for hepatocyte detoxification of drugs[137] and establishment of the physiological oxygen and chemical gradients. Such systems will be grouped by their size

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(both volume and cell number), designated as either macroscale or microscale. A summary of the properties of the discussed systems can be found in Table 3.

Macroscale—A few groups have applied large scale perfusion bioreactor devices to the culture of hepatocyte spheroids for drug discovery applications. For example, Tostoes and colleagues demonstrated the culture of primary human hepatocyte spheroids in a spinning perfusion bioreactor for up to 3–4 weeks[138]. The working volume was 300mL and 20% of this was replaced daily through medium perfusion. They demonstrated CYP450 induction and the metabolism of a model substrate, 7-methoxycoumarin. While albumin synthesis increased and stabilized over the culture period, urea synthesis significantly decreased with culture, likely due to spheroid hypoxia. Additionally, this culture system has only been tested for hepatocyte monoculture.

Another system uses a radial flow bioreactor (RFB), where the bed of matrix housing cells separates the media inlet and outlet, which facilitates nutrient and oxygen diffusion across the tissue. Park and colleagues co-cultured rat hepatocytes and 3T3-J2 fibroblasts on a series of stacked glass substrates, with microfabricated grooves and a collagen coating, to improve oxygen delivery to the tissue[139]. However, while RFBs culture large number of hepatocytes ($2–5\times10^7$), the high flow rates used cause loss of hepatic-specific functions after a few days due to unphysiological shear stresses[139].

Vinci and colleagues cultured primary human hepatocytes on coverslips for 7 days then transferred the coverslips into either their multichamber bioreactor or a new dish for continued static culture. They found that the expression of metabolic (CYP450) and transport (OATP, MDR1) genes were much higher under perfusion conditions and that higher flow rates affected this enhanced expression[140]. The use of primary human hepatocytes makes this system amenable to the investigation of human-specific drug interactions. However, the working volume (5mL) is quite large compared to the cell seeding number (200k), which reduces the influence of secreted factors.

While macroscale perfusion culture systems allow for the influences of shear stress and nutrient supply on drug detoxification to be extrapolated, they carry several weaknesses. First, the number of cells required is substantially higher than smaller-scale culture systems, which may prove challenging given the limited availability of hepatocytes. Second, the media volume to cell volume ratio is much higher than is seen *in vivo*. Third, many of these systems replace the circulating media with fresh media on a continuous basis. As such, these systems may demonstrate reduced signaling from soluble factors released by the hepatic tissue.

Microscale—In contrast to macroscale (5–300mL) perfusion culture systems, the use of microscale (0.1–3mL) systems allows for higher cell-to-media volume ratios, which approach those seen *in vivo*. The LiverChip by CNBio Innovations (originally Zyoxel) models the liver tissue with a perfusion system on a larger volume scale than most micro-perfusion systems (1.6mL)[2,77,141,142]. Earlier generations of this system utilized alternative well geometry[143,144]. Hepatocytes and a full complement of donor matched NPCs are seeded on polystyrene (mechanically stiff) scaffolds at a 1:1 ratio. Fluid flow is driven by

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pneumatic pumps at a rate of 60 μL/min through the scaffolds, providing direct, but physiologically representative shear stresses and oxygen gradients on the resident tissue cells (Figure 1B). Tissues are established and remain functional up to one month, as measured by blood urea nitrogen levels, fibrinogen, alpha-1 antitrypsin (A1AT), and CYP450 activity. The culture chamber allows for a 3D hepatic tissue to form, which provides an advantage over 2D culture systems by facilitating cell junction formation. This culture system has been used to study metastasis of cancer cells in the context of the liver microenvironment[2,77,141,142] and probe the influence of LPS on hydrocortisone metabolism[145]. This system offers a unique opportunity to study cancer drug efficacy and hepatotoxic effects in a single system. However, there is currently no effective way to physically monitor tissue formation during the culture period.

Choucha-Snouber and colleagues designed a liver biochip to investigate metabolic responses to the anticancer drug flutamide[146]. Their chip is fabricated from PDMS and contains fibronectin-coated microstructures for cell seeding. HepG2/C3a cells are seeded in the system and kept in culture for a total of 48 hours, 24 hours of which is for perfusion and drug treatment. In comparison to the LiverChip, this system uses a lower flow rate of 10μL/min. The low volume of the biochip (40μL) is desirable to achieve a physiologic ratio of media supply to cell volume. However, this system has not tested co-culture with NPCs. Additionally, using PDMS as the fabrication substrate allows for gasses to readily equilibrate between the external environment and the cell culture chamber, which may limit modeling of a physiologically hypoxic liver microenvironment. Additionally, small hydrophobic compounds adsorb to PDMS, which limits the drugs this system can accurately assess without extensive calibration and testing. This design was extended to investigate kidney-liver co-culture systems[147].

Hoffmann and colleagues designed a hollow-fiber based microscale perfusion culture system, with a volume of 0.5mL[148]. A unique feature of this system is the incorporation of three different supply channels to the cultured tissue – two media circuits and one gas circuit. The authors demonstrate robust tissue formation, which includes NPCs. Moreover, NPCs were noted to form vascular-like structures. While the culture volume is low, the flow rate and fresh media replenishment rate are quite high in comparison (3mL/min and 0.5mL/hour respectively). This may prove a limiting factor for analyzing long-term effects of secreted factors. Additionally, the expression and activity of several CYP450 (3A4 and 2B6) isoforms decreases dramatically between days 3 and 10 of culture[148]. Since 50% of currently tested drugs are metabolized by CYP3A4[149], the decrease in its activity over the culture period may make this system not suitable for long term toxicity or metabolic assays.

CellAsic designed a hepatic sinusoid cord-like culture system, in which a central cord of cells is enveloped by a fluid transport channel. Communication between the cells and fluid channel was accomplished by small (1μm by 2μm) canals, limiting transport to diffusion. The system is primed with fluid and cells are subsequently seeded. After cell seeding, the chip is placed in the incubator on an incline to facilitate gravity-driven continuous flow, at rates of 10–20nL/min, to supply the tissue. The system can maintain hepatocytes for over 1 week, as determined by albumin secretion, and exhibit dose-dependent hepatotoxicity of diclofenac as seen *in vivo*[150,151]. Thirty two independent culture systems can be run

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simultaneously on a device the size of a 96-well plate. While this system generates 3D tissue, an improvement over 2D culture systems, it has not been investigated for culture periods longer than 7 days.

HμREL® biochips by Hurel Corp. allow up to 8 microfluidic circuits to operate in parallel[152]. These chips contain two cell culture compartments connected in series, one of which was designed to house liver tissue. Cells are seeded onto the polystyrene biochip either in monoculture or co-culture, using human hepatocytes and NPCs. Flow was generated by use of a peristaltic pump with a flow rate of 4.5μL/min/chip. Hepatocyte co-cultures in the system subjected to flow demonstrated increased metabolic activity when compared to static monocultures or co-cultures and remained viable for up to 6 days[116]. One advantage of this system is that the HμREL tubing exhibits limited adsorption of hydrophobic drug molecules. Additionally, the polystyrene housing reduces gas permeability of the device, facilitating formation of a hypoxic microenvironment. However, the tissues formed in this system are not 3D and the maximum culture period is limited compared to other systems. Moreover, CYP450 isoforms exhibit varying activities in the system – while CYP3A4 activity increases over the 6 day culture duration, CYP2D6 activity decreases after 2 days of culture[116].

Finally, the liver acinus microphysiology system (LAMPS) is a microfluidic platform to investigate hepatic physiology, drug safety, and disease models[8] that evolved from the first generation liver MPS (SQL-SAL)[153]. The LAMPS incorporates either fresh or cryopreserved human hepatocytes, as well as a full complement of non-parenchymal cells: human endothelial (primary or TMNK-1), immune (THP-1 or U937), and stellate (LX-2) cells in a Nortis, Inc microfluidic device. A porcine liver extracellular matrix (LECM), ca. 10μm thick, is layered between the endothelial cells and the hepatocytes. Approximately 20% of the hepatocytes and stellate cells contain fluorescent biosensors that measure apoptosis and reactive oxygen species for real-time fluorescence readouts[154], along with analysis of the secretome and metabolic activity. Distinct zone 1 and zone 3 microenvironments are created that allow the direct determination of zone-specific physiological and disease functions[155]. This system is viable for at least 28 days under continuous perfusion flow and exhibits concentration- and time-dependent toxicity profiles to drugs (e.g. troglitazone) in a similar manner to that seen clinically[156]. Structurally, the endothelial cells are separated from the hepatocytes by a biomimetic of the Space of Disse forming a three-dimensional layer of cells and LECM. The immune cells permit the system to be LPS responsive, releasing TNFα upon stimulation and in combination with some drugs causes immune-mediated hepatotoxicity. Moreover, the LX-2 cells can be activated in response to methotrexate treatment, resulting in the expression of smooth muscle actin (SMA) and the production of collagen 1A2, reflecting a fibrosis phenotype. Most importantly, this system is amenable to real-time transmitted light and fluorescence imaging, allowing for investigating toxicity kinetics and disease progression.

The platform is continually being evolved to replace the NPCs with either primary human cells or iPSC-derived cells, since the use of immortalized NPCs may not reflect full physiological capabilities. This platform contains PDMS which requires the presence of carrier proteins for hydrophobic drugs and pre-measurement of the loss of drugs to the

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device before making any interpretations[157]. Finally, the media in this system is not recirculated in the current format. However, the small volumes and low flow rates allow the detection of paracrine signaling mediators secreted by the cells, although there are other advantages to having the media recirculated (see above).

Conclusions and Future Directions

Advanced liver culture systems hold the potential to transform drug discovery and development, through more accurate modeling of human *in vivo* pharmacokinetics and pharmacodynamics, as well as understanding mechanisms of disease progression and toxicity. Several factors affect the accuracy of these model systems in predicting pharmaceutical toxicity. The cells used in the culture system and the culture surface (e.g. plastic, hydrogel) allow for modeling cell-cell and cell-ECM interactions respectively. Systems that include media flow ("blood flow") provide mechanical stimuli that benefit tissue function. Inclusion of cells derived from human tissues allows for human-specific pathways to be modeled. Special attention should be paid to the use of primary versus immortalized cells, as the latter may elicit signals not seen in physiologic conditions. The use of animal cells instead of human cells may encounter similar intractable weaknesses. Importantly, the expression levels of CYP450 isoforms and drug transporters over the culture period may influence toxicity predictions.

Future directions for improving these systems include incorporating lentiviral-induced fluorescent biosensors for an automated readout of various cellular functions[154] and finer architectural control of liver microstructure. Importantly, the ability to image the devices in real time is highly advantageous. Fabrication of devices using materials other than PDMS – which adsorbs hydrophobic molecules and biologics – will improve the utility of these devices as pharmacokinetic models for drug discovery. Moreover, procurement of a universal, readily available cell source (potentially iPSC-derived hepatocytes) will improve the ease with which these systems are used for drug characterization studies. Networking of additional organ chips, such as gut and kidney, will contribute additional signals to the liver tissue, allow for modeling of drug absorption, and disposition after hepatic metabolism. Finally, further development and usage of quantitative systems pharmacology as a new paradigm in drug discovery and development involving the integration and iteration of experimental and computational models, in parallel with advanced culture systems will facilitate complex data analysis and may allow for predictive models to be generated[158,159].

It is hoped that the development of validated human experimental model systems can be used in place of animal models to improve preclinical drug selection. By implementing more accurate human experimental model systems, it is expected that there will be increased efficacy and safety in clinical trials leading to improved therapeutics at a lower cost.

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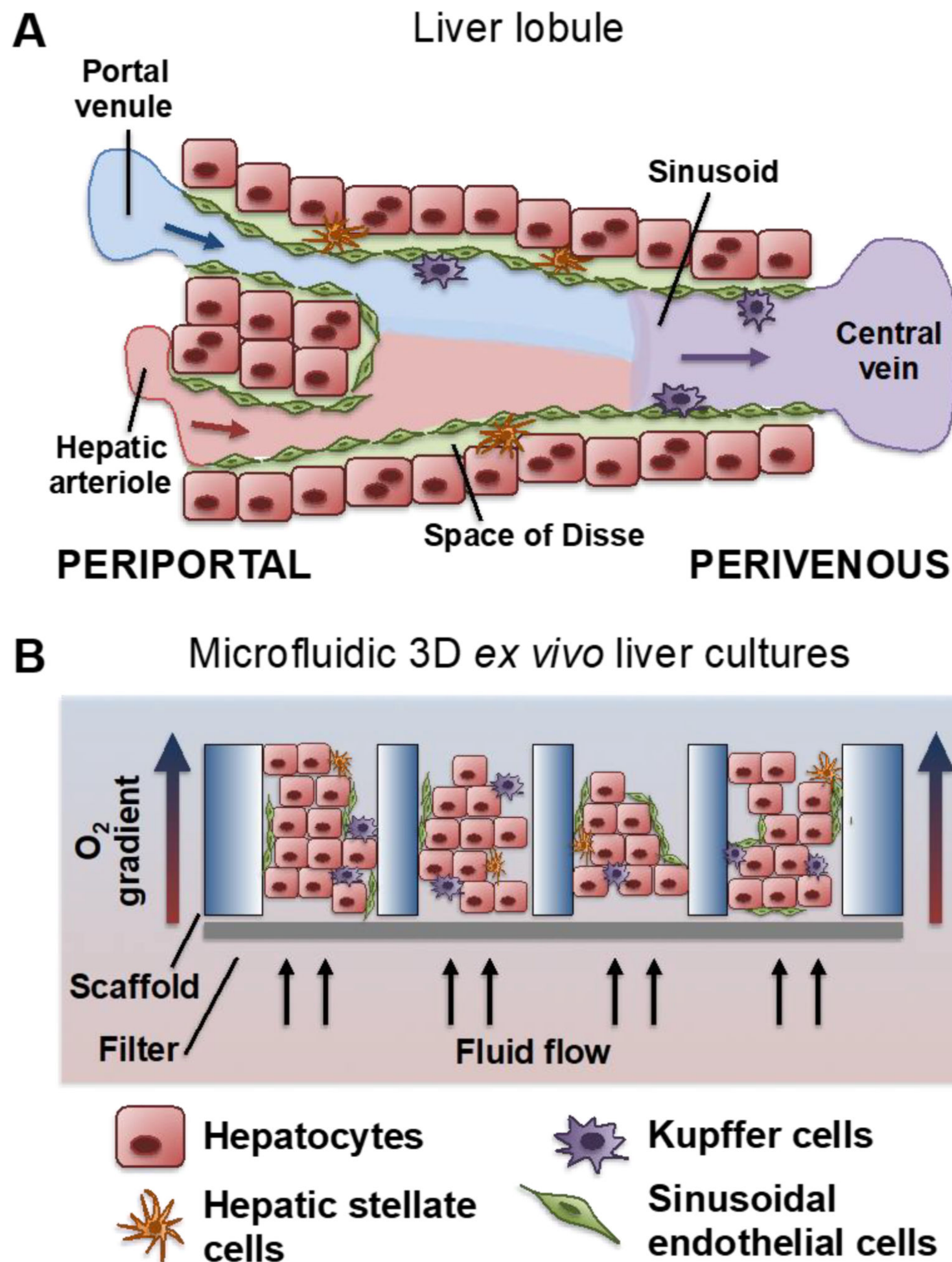

Figure 1.

(A) The architecture of the hepatic sinusoid. Blood enters the portal triad region of the liver through a hepatic arteriole and portal venule and traverses the hepatic sinusoid to the central vein, whereby it is drained into the larger hepatic central veins. The sinusoidal endothelial cells mediate blood flow and are fenestrated to allow rapid diffusion of nutrients, signaling factors, and drug compounds. The hepatocytes compose the parenchyma of the liver and sit deep to the endothelium. The hepatic stellate cells reside in the Space of Disse, a zone between the endothelium and hepatocytes. Finally, the Kupffer cells line the inside of the sinusoid and mediate antigen sensing and intercellular communication. (B) A schematic of

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the three dimensional liver tissues as presented within the LiverChip, that are subject to fluid flow in micro-scale pores of a scaffold, which facilitates shear stresses and establishment of a physiological oxygen gradient.

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Table 1

A summary of properties from different hepatocyte sources: animal, human cell line, primary human, and iPSC-derived.

Hepatocyte Source	Animal	HepG2	HepaRG	Primary Human	Cryopreserved Human	iPSC-derived
Viability	Medium to High *	High	High	Medium to High *	Medium	High
Attachment to ECM	High	High	High	High	Medium	High
CYP450 Activity	Medium	Low	Medium	High	High	Low to Medium
Longevity in Culture	Medium	High	High	Medium	Low to Medium	Low
[1]OATP/NTCP	High	Low	Medium	High	Medium	Low to Medium
Cost	Medium	Low	Low	High	High	High
Availability	Medium	High	High	Low	Medium	Medium
References	45–47, 49	52, 53, 55	53, 55, 56, 58–60	23–25	35, 36, 39, 41	69–71

* Dependent on efficiency and time of isolation procedure.

[1] OATP (organic anion transporting polypeptide), NTCP (sodium taurocholate co-transporting polypeptide).

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Table 2

A summary of properties of different non-perfusion hepatocyte culture systems.

System	Reference	System Type	Cell Type(s)	Culture Time	CYP450	Urea	Albumin	Throughput
HepatocPac™ by Hepregen, Corp.	119	2D	PHH or PRH + 3T3	4–6 Weeks	Stable	Stable	Stable	Low
PDMS Stencil	125	2D	PRH + 3T3	7 Days	N.D.	Stable	Stable	Low
Micropatterned 96-well Plate	74	2D	PHH or iHep + 3T3	4 Weeks	Stable	Stable	Stable	High
Spheroid Microarray Chip	131	3D Spheroid	PRH + 3T3	14 Days	N.D.	Stable	Stable	High
Micropatterned Fibrous Mat	93	3D Spheroid	PRH + 3T3 + HUVEC	15 Days	Stable	↓	Stable	Low
PEG-encapsulated Spheroid	133	3D Spheroid	PRH + 3T3	50 Days	Stable	N.D.	Stable	High
Human Microspheroids	134	3D Spheroid	PHH + HNPC	35 Days	Stable	N.D.	Stable	High
RegeneTox™ by Regenemed, Inc.	135	3D Spheroid	PHH or PRH + HNPC or RNPC	90 Days	Stable	Stable	Stable	Medium
GravityTRAP™ by InSphero AG	136	3D Spheroid	HepaRG	21 Days	Stable	↑	Stable	High

PHH = Primary Human Hepatocyte, PRH = Primary Rat Hepatocyte, 3T3 = 3T3 Fibroblasts, iHep = iPSC-derived human hepatocytes, HUVEC = Human Umbilical Vein Endothelial Cells, HNPC = Human Non-Parenchymal Cells, RNPC = Rat Non-Parenchymal Cells, N.D = Not determined.

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Table 3

A summary of properties of different perfusion hepatocyte culture systems.

System	References	System Type	Cell Type(s)	Culture Time	Flow Rate (μL/min)	Total Volume	CYP450	Urea	Albumin	Throughput
Spinning Bioreactor Spheroids	138	Macroscale Perfusion	PHH	3–4 Weeks	41.6	300mL	Stable	↓	↑	High
Radial Flow Bioreactor	139	Macroscale Perfusion	PRH + 3T3	5–11 Days	830	54mL	N.D.	↓	↓	Medium
Multichamber Modular Bioreactor	140	Macroscale Perfusion	PHH	7–21 Days	250–500	5mL	Stable or ↓	Stable	Stable	Low
LiverChip™ by CN Bio Innovations Ltd.	77	Microscale Perfusion	PHH + HNPC	15–29 Days	60	1.6mL	Stable	Stable	Stable	High
Metabolomics on a Chip	147)	Microscale Perfusion	HepG2/C3A	48 Hours	10	3mL	Stable	N.D.	Stable	Low
Hollow Fiber Perfusion Bioreactor	148	Microscale Perfusion	PHH + HNPC	10 Days	3000	0.5mL	↓	↓	N.D.	Low
Artificial Liver Sinusoid	150, 151	Microscale Perfusion	PHH or PRH or HepG2/C3A	7–8 Days	0.01 – 0.02	0.075mL	N.D.	N.D.	Stable	Low
HμREL® biochips by HμREL Corp.	116, 152	Microscale Perfusion	cPHH + HNPC	6 Days	4.5	0.1mL	Stable or ↓	N.D.	N.D.	Medium
Human Liver Sinusoid	153, 157	Microscale Perfusion	PHH & cPHH + EA.hy926 + U937 + LX-2	28 Days	0.08 to 0.25	100 μl	Stable	Stable	Stable	Medium

PHH = Primary Human Hepatocyte, cPHH = cryopreserved Primary Human Hepatocyte, PRH = Primary Rat Hepatocyte, 3T3 = 3T3 Fibroblasts, HNPC = Human Non-Parenchymal Cells, N.D = Not determined, EA.hy926 = human endothelial cell line, U937 = human monocyte cell line, LX-2 = human hepatic stellate cell line