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Microengineered cell and tissue systems for drug screening and toxicology applications: Evolution of *in-vitro* liver technologies

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Abstract

The liver performs many key functions, the most prominent of which is serving as the metabolic hub of the body. For this reason, the liver is the focal point of many investigations aimed at understanding an organism's toxicological response to endogenous and exogenous challenges. Because so many drug failures have involved direct liver toxicity or other organ toxicity from liver generated metabolites, the pharmaceutical industry has constantly sought superior, predictive *in-vitro* models that can more quickly and efficiently identify problematic drug candidates before they incur major development costs, and certainly before they are released to the public. In this broad review, we present a survey and critical comparison of *in-vitro* liver technologies along a broad spectrum, but focus on the current renewed push to develop "organs-on-a-chip". One prominent set of conclusions from this review is that while a large body of recent work has steered the field towards an ever more comprehensive understanding of what is needed, the field remains in great need of several key advances, including establishment of standard characterization methods, enhanced technologies that mimic the *in-vivo* cellular environment, and better computational approaches to bridge the gap between the *in-vitro* and *in-vivo* results.

1. INTRODUCTION

The difficulties and increasing costs of drug development and testing faced by the pharmaceutical industry raise questions about the effectiveness and efficiency of current drug screening approaches. The cost of bringing a single compound to market is now estimated at almost a billion US dollars[1–4]. This high cost stems from the large number of failed drugs during both preclinical and clinical studies, where the two major factors for failure are a lack of efficacy and toxicity[5]. According to Adams and Brantner[3] and a study conducted by the Boston Consulting group in 2001[6], a major portion of the drug development costs, 40–70% of the total development cost, is invested during the preclinical

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stages. This necessitates a closer examination of the preclinical screening studies in particular, where the efficacy and safety of new chemical entities in the pipeline are tested.

Animal testing is the most popular form of assessment used during the preclinical, and in some cases clinical, context. However, the success of animal studies in predicting the human physiological response in terms of both efficacy and toxicity is sometimes poor, and this practice has been increasingly questioned[5,7,8]. Moreover, animal models are also hampered by their poor ability to isolate cell-based mechanisms of action and pathways[9]. As a consequence, many drugs that are doomed to fail unnecessarily go through clinical trials, substantially increasing the overall cost of the drugs that make it through the certification processes. There is also a strong push to move away from animal models due to ethical concerns following the 3R approach, i.e. “Reduction, Refinement and Replacement” of animal studies[10,11]. One of the important aims of “Replacement” is to create alternative technologies and particularly *in-vitro* platforms that are less expensive, more predictive, and more time efficient than animal models. One example of this push was the 7th Amendment of the European Union, which banned all animal testing in safety evaluation of cosmetic products and commercial chemicals in 2013[12]. Although the amendment did not include pharmaceuticals, it may be a step in that direction.

Among all organs, the liver plays the most central role in human-drug interactions and is also the most common target for drug-induced toxicity[5,13]. Liver toxicity results in costly, late stage drug failures as 25–40% of drugs are found to cause hepatic injuries by phase III clinical studies[5,14]. Moreover, despite our best efforts to ensure drug safety, a sizeable number of drugs are withdrawn from the market after approval. The primary reason for after-market release is hepatotoxicity[15], which accounts for ~20–30% of all withdrawals in the US and EU over the last 30 years[14,16]. The FDA highlights the importance of liver toxicity and its severe risks during drug development with the following statement: “The presence of even a single case of liver injury from treatment in the premarketing clinical trials database is a signal of a high level of hepatotoxic risk”[17].

Given the overwhelming importance of the liver in drug metabolism and toxicity, there have been a wide range of academic and commercial studies aimed at developing *in-vitro* models to predict liver toxicity associated with therapeutic drugs. These studies primarily examine the enzymatic and synthetic activities of drug uptake and metabolism, as well as drug-drug interactions that affect metabolism. The selection of *in-vitro* platforms ranges from microsomal[18,19] and electrochemical assays[20,21], suspension[22–26] and plate cultures[27–31] of primary cells and cell lines, and macroscopic flow culture systems[32–39] to liver slices[40–43] and whole perfused organs[44]. While liver slices and whole perfused organs provide the most physiologically realistic systems with intact tissue structure and cell proportions, their characterization and long-term maintenance have proven to be very difficult[10]. New technologies such as decellularized and repopulated liver slices[45] and organs[46] can alleviate some of these problems, but still lack the throughput and analytic flexibility for drug screening purposes. In this respect, a newer class of *in-vitro* tools that can potentially provide fine microscopic control of the cellular environment and dynamics, via microfabrication and tissue engineering methods has recently gained more attention. These “on-a-chip” tissue models may be able to mimic the architecture of small tissue sections and

certain characteristics of the dynamic *in-vivo* flow environment, while also offering more precise spatial and temporal control of soluble factors. Moreover, unlike full organ or thick tissue sections, these models can be engineered to be imaging- and analysis-friendly for real-time/near real-time monitoring of the state of cells and their extracellular environment, which is crucial for determining cellular mechanisms of action. Another expected, but not yet fully realized, advantage is the large multiplexing capacity of these systems to make them amenable to high throughput screening approaches.

While there are a variety of other advances that may positively impact the drug development process (such as toxicogenomics, metabolomics, etc.), these microengineered *in-vitro* systems — "the organs-on-chips" (or more correctly defined, "tissues-on-a-chip"), with their improved physiological relevance may serve as an important platform for providing the data for such analytical approaches. While some researchers have been individually championing this idea for over a decade, with the publication of the US National Research Council (NRC)'s 2007 vision report titled "Toxicity Testing in the 21st Century"[47,48], the push towards replacing traditional *in-vitro* models with these tissues on chips has been accelerated both in the US and EU. Notable EU initiatives along these lines are the SEURAT-1[49] and the Body-on-a-Chip (BoC)[50]. With a similar conviction and as set forth in the NRC report, in 2012, the NIH and the FDA, along with DARPA, also invested over one hundred million US dollars to create the "Tissue Chips for Drug Screening"[51,52] program in response to these challenges and in line with the goal of reducing and/or replacing animal studies. This joint effort seeks to create robust, long-term 3D microengineered tissue systems that closely mimic the human physiological and pharmacological response. Cellular systems are required to be viable for at least 28 days to allow testing for chronic as well as acute drug toxicity. Eventually, the goal is to connect these organ systems in a physiologically relevant manner to create a "human-on-a-chip". For example, our lab, in collaboration with investigators at the University of Pittsburgh, is creating a physiologically relevant "liver-on-a-chip" platform for enhanced prediction of the human liver response to exogenous and endogenous challenges. The scope beyond the liver chip involves building connections to other tissue systems (e.g. kidney, heart, gut, etc.) in a deliberate step-by-step fashion.

In this review, we highlight decades of work and ideas that inspired and guided the development of new microengineered tissue systems that focus on the liver. We start the review, in section 2, with a brief recap of the liver structure, importance, and toxicological intricacies. This is followed, in section 3, by a historical perspective on the static and perfused culture systems that paved the road for the current microscopic models that we review in the same section. We then discuss the gaps and opportunities in the field going forward in section 4.

Following this we give a brief review of the ongoing work in our lab towards building a more predictive liver model in section 4.5. We posit here that in order to have better predictive capabilities and better representation of the liver physiology, we should start with the notion of an underlying unit process/unit structure of the liver as opposed to arbitrarily matching one or several scaling parameters as others have done. Because the liver is a complex organ with an enormous array of functions, it is equally important to identify what

functions we need to replicate to achieve our desired goals. This leads to a discussion of the cell types that are needed for the faithful recapitulation of those functions with the conviction that non-parenchymal cells constitute an important part of the liver response both in healthy and diseased states of the organ. We conclude with a summary and future directions, the challenges and opportunities that still need to be addressed, and the need for developing standardized criteria for testing the plethora of new *in-vitro* models in section 5.

2. LIVER

The liver, a complex organ with a multitude of functions, is the largest organ in the body[53,54]. It is located between the digestive tract and the upper body[10] and plays a central role in homeostasis. It is a living factory responsible for synthesis, metabolism, storage, filtration, and removal of vital compounds along with some immune functions. Through the synthesis and secretion of bile, the liver enables digestion and absorption of fatty compounds[55], and many waste products, such as bilirubin, are removed by secretion into bile. The liver, provides the body with nearly all the main plasma proteins including albumin, transferrin, prothrombin, fibrinogen, lipoproteins, and complement proteins[54,56]. One critical function of the liver is the maintenance of blood glucose levels via synthesis (glucogenesis) and storage of glucose (as glycogen). Of all the important liver functions, its metabolism — especially that of pharmaceutical drugs and other xenobiotics — is perhaps the most important. The liver is able to metabolically detoxify xenobiotics and modify them into either waste or non-toxic metabolites for further use. Accordingly, recapitulation of the liver drug metabolism *in-vitro* will be the central focus of this review.

2.1 Structure of the liver

The liver is comprised of a) a stroma that consists of connective tissue (septa), reticular fiber, and portal canals and b) the parenchyma that consists of the cells, the bile canaliculi, and sinusoidal and perisinusoidal spaces. The traditional view is that there are four major, but unequal, lobes identified by external demarcations[57]. Nevertheless, this lobular separation is incomplete[56,58] and there are conflicting views on how best the liver can be segmented for surgical and radiological purposes[57]. We can, however, understand the structure and examine the smallest functional unit of the liver while attempting to create physiologically relevant *in-vitro* liver systems.

The liver has a highly vascularized structure that is perfused by a dual blood supply[58–60]: a) arterial blood via the hepatic artery and b) venous blood via the hepatic portal vein (Fig. 1a). While the arterial blood is supplied directly from the heart at systemic oxygen, hydrostatic pressure, and solute concentrations, the venous blood, which can be as much as 80% of the total supply[57], has already circulated through the gastrointestinal tract and has been enriched with certain hormones, nutrients and toxins, but depleted of oxygen and pressure[58]. The hepatic artery and portal vein travel together and continuously branch into smaller veins in the portal tract to deliver and perfuse all of the cells in the liver. This perfused blood is eventually collected into the central vein, which is then returned to the systemic circulation. The excretion of bile by hepatocytes into bile canaliculi, which form a network independent of the blood circulation, forms a counter circulation where the bile eventually flows into the

bile duct. Anatomically, each branch of the bile duct, the portal vein, and the hepatic artery forms a structure called the portal triad (Fig. 1a).

One striking feature of the liver is the simplicity of its seemingly complex structure, wherein the basic vasculature and the circulatory network are repeated many times akin to fractal structures[61–63]. A unitary functional network structure has been deemed the hepatic acinus, which is shown in Fig. 1a,b by the parallelogram whose corners consist of two neighboring central veins and the portal triads in between. A larger unit in terms of blood circulation is the classical hepatic lobule (Fig. 1a). While the classical lobule has no morphological boundaries[56,58], the center of each lobule is marked by the central vein where blood perfused through the liver mass is collected. This mass is hypothesized to be surrounded and fed by a highly vascularized diamond-like (with a hexagon like cross-section, see Fig. 1a) network of the portal tract system. A remarkable consequence of this organization (the repeating pattern of flow from the portal triads to the central veins), is the zonal gradation of oxygen, nutrients, and toxins and the resulting zones with different cellular specializations along the portal triads to the central veins (see Fig. 1a, section 2.3).

2.2 Cells of the liver

In each acinus structure, hepatocytes, the parenchymal cells of the liver, are organized into plates/laminae (Fig. 1b) forming a continuous 3D lattice[56,58]. While the apical surfaces on this hepatic matrix coordinate to form the bile canalicular system that exports bile into the bile ducts, the basal surfaces project microvilli that face the space of Disse[64] (Fig. 1c). The space of Disse, a semi-fluidic space (thickness ~ 0.2–1 μm[65]) with a complex composition of extracellular matrix (ECM) proteins, is occupied by stellate cells[66,67]. Stellate cells are in close contact with both the hepatocytes and the liver sinusoidal endothelial cells (LSECs) that line the walls of the hepatic sinusoid (Fig. 1c). Hepatic sinusoids are the venous capillaries (diameter ~5–10 μm, length ~250–300 μm)[68,69] between the plates of hepatocytes that are the principle site of exchange between the blood and the perisinusoidal space of Disse[59].

The primary resident cells of the liver, i.e. hepatocytes, LSECs, and Kupffer and stellate cells, form a complex signaling and metabolic environment[70–72] wherein these cells normally function in unison to fend off internal and external challenges, as well as to supply the rest of the body with useful metabolites and proteins. The cells perform liver functions directly and through autocrine and paracrine signaling. Below, we review each cell type and its contributions to liver function along with its importance in the context of toxicity; we summarize the important physical properties of the main liver cells in Table 1. While the liver houses other important cell types such as cholangiocytes, immune cells, and dendritic cells, we will not be reviewing them here, but instead refer readers to several excellent books[57,58,65,73] for more information.

2.2.1 Parenchymal cells

Hepatocytes: Hepatocytes are highly differentiated epithelial cells[31,74] that are responsible for a major portion of the complex liver functions[31]. They constitute the majority (~78% by mass/volume and 60–65% by number[58,69,75,76] of cells in the liver. They are large (20–30

μm[58,69]), rhomboidal with many facets[68], and highly polarized for specialized functions such as bile excretion and extraction from and secretion into the blood stream[58]. With a highly concentrated cytoplasm that possesses a large number of mitochondria (1000–2000/cell), peroxisomes (400–700/cell), lysosomes (~250/cell), Golgi complexes (~50/cell), aggregates of rough and smooth endoplasmic reticulum (~15% of cell volume)58,77,78, hepatocytes are seemingly the most metabolically active cells in the entire body. Accordingly, they have very high oxygen consumption rates (~2.5–5 × 10^{-5} nmol/cell/min[79]) when compared to most other cell types.

Together with drug metabolism, the synthesis and secretion of proteins — especially albumin — and the excretion of urea are crucial functions, as well as distinct markers of hepatocyte function and health. Although non-parenchymal cells support and contribute to xenobiotic metabolism[80–82], the phase I and II metabolism of exogenous and endogenous compounds are carried out predominantly by hepatocytes[70]. The effect of phase I reactions, predominantly via the cytochrome P450 (CYP 450) enzyme family located on the smooth endoplasmic reticulum[57] is the modification of compounds through oxidation, reduction, and hydroxylation for either excretion or further modification in phase II, or other types of, metabolism[54]. The subsequent phase II modifications are mostly carried out by cytosolic enzymes termed “transferases”, which conjugate the phase I intermediates with charged species such as glycine, sulphate, glucoronate, or glutathione[57]. The overall effect of these two phases is the increased water solubility and acidity and decreased toxicity of most parent compounds[54]. This high metabolic activity of hepatocytes also makes them the prime target for the toxic effects of the compounds that they metabolize. A common route for injury is the toxicity of intermediate metabolites produced by phase I metabolism. We briefly review drug induced liver injuries in section 2.4.

2.2.2 Non-parenchymal cells

Liver sinusoidal endothelial cells (LSECs): LSECs are sessile cells[83], 6–11 μm in cross-section[69]. They make up 50% of all sinusoidal cells[59–76] and constitute 15–20% of the liver by number[84] and ~3% by volume[69]. LSECs can be identified by the expression of SE-1[85] and CD-31[74] surface markers, as well as by their uptake of formaldehyde-treated serum albumin[86,87]. They form a tubular structure that lines the liver sinusoidal wall with the primary function of transvascular exchange, i.e. filtration, between the blood stream and the tissue[59]. This exchange is enabled via fenestrations that are clustered into sieve plates[67,88,89] with 10–50 fenestrae in each plate[84]. These fenestrae, specific to the liver endothelium[90], are 100–170 nm in diameter, but are dynamic structures whose size responds to the luminal blood pressure, vasoactive substances, drugs, and toxins[88,91,92]. LSECs show a high capacity of receptor-mediated endocytosis for different molecules, which forms the basis of their scavenger functions[89,93,94]. Accordingly, together with the Kupffer cells, they form the reticuloendothelial system[72,82], which is responsible for scavenging circulating macromolecules and microorganisms from the systemic circulation[95]. LSECs are also assumed to take part in the regulation of blood flow[84] along with the stellate cells through complex signaling between the two cell types[96,97].

LSECs are an early target for several toxicants[59] because they are directly exposed to blood flow from the portal circulation. In the specific case of acetaminophen/paracetamol, they become swollen and lose their ability to endocytose as early as 30 minutes after administration[59,98]. They are implicated in many different injury mechanisms, including neutrophil-induced liver injury[99] and hepatic fibrosis[100]. Importantly, they also contribute to both phase I and phase II metabolism, although their contribution on a per cell basis is one to two orders of magnitude lower than that of hepatocytes[80,81]. A number of *in-vitro* experiments have now demonstrated that the presence of LSECs has a significant positive effect on the retention of hepatocyte phenotype and metabolic activities[85,101,102] and also leads to more representative models of the drug response of hepatic tissues. For further information about LSECs, we refer the readers to the following references[72,88,103–105].

Kupffer cells: Kupffer cells, named after Karl W. von Kupffer[106,107], are the resident macrophages in the liver, accounting for more than 80% of all macrophages in the body[71] and ~15% of the liver cells[84]. They are 10–13 μm in diameter[105], irregularly shaped[56], and mobile cells[83,107] that adhere to the luminal surface of the sinusoidal wall. They can be identified by their expression of ED-1 and ED-2 surface antigens[57,108,109]. Phagocytosis and endocytosis of toxicants, particulates, and endotoxins such as lipo-polysaccharides (LPS) are important functions of the Kupffer cells. An equally important function is their secretion of mediators, such as cytokines, prostanoids, oxygen radicals, and proteases[71,84], which provide local and long distance cellular signaling cues. While some of these secretions are beneficial for liver regeneration[110,111] and host defense[71], others may be involved in liver injury[56,71]. The activation and involvement of Kupffer cells have been implicated in a) diseases including neoplasia[70], non-alcoholic fatty liver disease[71,112,113], and immunological diseases; b) damage such as ischemia-reperfusion[114] and cold-preservation injury[84,115]; and c) liver toxicity such as acetaminophen[99], copper, and iron toxicity[116], as well as immune-mediated adverse drug reactions[70].

Thus, Kupffer cells play an important role in the acute and chronic responses of the liver to toxic compounds[70]. In the context of xenobiotic metabolism, three different pathways of interaction between Kupffer cells and hepatocytes have been proposed[117]: 1) xenobiotics induce Kupffer cell stimulation in a similar manner to LPS stimulation, which in turn limits several hepatic functions; 2) the intermediates resulting from hepatocyte xenobiotic metabolism induce Kupffer cell activation; and 3) xenobiotics induce primary lesions in hepatocytes that are converted into cytotoxic lesions by contact with factors induced by constitutive levels of LPS. Supporting these hypotheses, Milosevic and co-workers[117] showed that Kupffer cells co-cultured with hepatocytes show distinctly different nitric oxide (NO) and TNF-α release after stimulation with LPS. Recent unpublished work (Life Technologies[118]) suggests that the phase I metabolism of hepatocytes can be greatly reduced with addition of pro-inflammatory factors LPS or IL-2, but only when they are co-cultured with Kupffer cells. These studies, along with many others[70], point to the prominent interactions of Kupffer cells with hepatocytes and other cells in response to drugs and other challenges. For further information on Kupffer cells and their involvement in hepatic metabolism, we refer the readers to excellent reviews by McCuskey[59], Roberts[70], Bilzer[71], and Laskin[72].

Hepatic stellate (Ito) cells: Hepatic stellate cells (HSCs), also called vitamin A-storing cells, lipocytes, interstitial cells, fat-storing cells or Ito cells[66], are distinct, star-shaped[106,119,120] cells specific to the liver. They make up ~1.4% of the liver volume, and there is about 1 stellate cell for every 8 hepatocytes[69]. The stellate cells can be identified by CRBP-1[121], glial fibrillary acidic protein (GFAP) staining[122], or the original gold-chloride method[106,120], among others. They are the primary storage site for vitamin A[57,97,122] and fat droplets[56], and actively participate in controlling the microvascular tone[57,70], and thus the blood flow[96,97].

A very active interaction established through both cell-cell contact and intracellular signaling between HSCs and LSECs exists and contributes to liver homeostasis[66]. For example, the HSCs secrete VEGF, which maintains LSEC phenotype[123], and the LSECs produce and release NO in return[123]. While NO keeps the HSCs quiescent[123] in a healthy liver, a lack of NO in a diseased state may lead to the activation of HSCs, resulting in excessive ECM secretion and subsequent fibrosis[123]. Similar to Kupffer cells, HSCs play an important role in modulating drug induced liver injury and hepatocarcinogenesis via the release of growth factors, inflammatory cytokines, and reactive oxygen species upon activation by exogenous insults[70,124]. For further and more detailed information on the involvement of stellate cells in liver metabolism and injury, we refer readers to excellent reviews by Winau[97], Senoo *et al*.[66], and Friedman[125].

2.3 Zonation

The liver displays a functionally important gradient of nutrients, hormones, and especially oxygen concentration from the portal triad, the periportal zone (PP), to the central vein, the perivenous zone (PV). The oxygen tension in the PP zone is 60–65 mmHg and in the PV zone is 30–35 mm Hg[126–128]. These gradients, along with differences in ECM composition, structure, and soluble factors from the PP to PV zones[74] manifest as differences in cellular metabolism, including that of xenobiotics and secreted molecules, as well as changes in the morphology and number and phenotypic characteristics of all cell types along the PP-PV axis[128–130] (see Fig. 2). This specialization of liver cells is referred to as “zonation”. The acinus structure is generally divided into three different zones from PP (Zone 1) to PV (Zone 3)[131–133], which display heterogeneous cell functions.

While we discuss the zonation of hepatocytes below, one should note that non-parenchymal cells also display important zonal differences. Specifically, the number of LSECs increases from Zone 1 to Zone 3, while the number of Kupffer cells decreases along the same axis[134]. Functionally, LSECs display a lower density of fenestrae with larger diameters (>200 nm) in Zone 1 and higher density with smaller pore diameters (<150 nm) in Zone 3[135]. Kupffer cells, on the other hand, show higher phagocytic activity in Zone 1 compared to Zone 3.

2.3.1 Hepatocyte zonation—Hepatocytes display a distinct heterogeneity of morphology, enzymatic activities, and functional capacity along the three zones of the liver acinus. In contrast to Zone 1 hepatocytes, which are small, Zone 3 hepatocytes are larger, terminally differentiated, and display a much higher number of polyploidy[136]. While Zone 1 hepatocytes are efficient at oxidative metabolism, fatty acid oxidation, gluconeogenesis, bile

acid extraction, ammonia detoxification, and urea and glutathione conjugation, Zone 3 hepatocytes are efficient at glycolysis, liponeogenesis, and CYP 450 biotransformation[133,137–139]. Zone 2 hepatocytes exhibit intermediate function. Zonal distributions have been observed in both gene expression and activities of important enzymes for a variety of hepatic functions.

Within each lobule, the xenobiotic metabolism is not evenly distributed, as the majority of drug metabolism occurs in PV hepatocytes[140]. This is especially true for phase I metabolic activity, but elevated levels for some phase II detoxification activities can also be observed in the PP zone. One explanation for this distribution is that by producing most of the phase I metabolic intermediates, which can be highly toxic/reactive and could lead to cell death, in the PV zone[141], the liver has evolved to protect itself from organ-wide necrosis[142]. Supporting this hypothesis, the detoxification of reactive oxygen intermediates by glutathione and glutathione peroxidase, a phase II metabolic activity, is higher in the PP than in the PV zone[141,143–145], a setup that protects the liver cells in zones I and II from intermediate-induced damage. In line with these results, hepatocytes in the PV zone are more likely to die from hepatotoxicity than PP hepatocytes[140]. This metabolic zonation is usually a missing or poorly controlled feature in most *in-vitro* platforms.

2.4 Liver and drug induced liver injury (DILI)

Because of its central role in drug metabolism, the liver is also one of the main targets for the toxic effects of xenobiotics[5,14]. There are close to 1000 identified pharmaceutical drugs that result in liver disease, even at a low incidence. Moreover, the liver is directly exposed to 25% of the cardiac output, which can contain high concentrations of intravenously-administered drugs that have not been cleared from the circulation, increasing the risk of toxicity in liver cells.

Pharmaceutical drugs and other xenobiotics are often poorly soluble in aqueous solutions, and thus have to be transformed to a more hydrophilic form to be more readily used and eliminated[146]. This is achieved through multi-phase metabolic process, as described in section 2.2. An equally important step in drug transformation by the liver is the transport of drugs into the cells, without which the cellular enzymatic metabolism cannot take place. While some drugs enter passively through the cell membrane, others require an active mode of transport[147]. This is accomplished by “transporter proteins” in the cell plasma membrane that facilitate the transport of chemical substances into and out of cells. Based on the direction of transport, transporters are classified into two broad categories: a) uptake and b) efflux drug transporters. Based on their ontology and sequence, the transporters form two superfamilies named the a) solute linked carrier (SLC) superfamily and b) the adenosine triphosphate (ATP) binding cassette (ABC) superfamily[148,149]. Because the activity of drug transporters has direct implications for all the subsequent enzymatic activities that take place, the characterization and an improved understanding of these transporters is crucial to all the pharmacokinetic and pharmacodynamics studies, in addition to all the work examining the phase I and II drug metabolizing enzymes.

While most intermediates and end products are harmless thanks to liver metabolism, a non-negligible fraction of intermediates can cause injury to the cells of the liver, as well as to

other organ systems. As a result, drug-induced-liver injury (DILI) is the leading (~50%[150]) cause of acute liver failures[151–154], as well as the most important cause of late stage drug failures in clinical trials[154,155]. In fact, from 1992–2002, the percent of clinical trials that failed because of hepatotoxicity at phase I, II, and III were 43, 23, and 35%, respectively. Moreover, about ~20–30% of all drug withdrawals from the US and EU markets from 1975–2005 was due to hepatotoxicity, and most "black box warnings" on common drugs refer to hepatotoxicity more than any other effect.

Drugs are broadly classified into two groups based on their hepatotoxicity: a) intrinsic hepatotoxins and b) idiosyncratic hepatotoxins[155,156]. Intrinsic hepatotoxins induce a predictable and common toxic response with respect to dose in a majority of subjects. On the other hand, idiosyncratic hepatotoxicity, which constitutes a sizeable fraction[155,157] of drugs, is hard to predict and is not always dose-dependent, but instead depends on many factors such as inter-individual genetic differences, metabolic state, inflammation, or other disease states. This makes idiosyncratic toxicity very difficult to study and almost impossible to detect using animal testing and current analysis tools. Nevertheless, both groups most likely metabolize these toxins into large quantities of highly reactive and toxic metabolites[15], which cannot be quickly cleared.

One well-known example of dose-dependent hepatotoxicity is the thoroughly studied case of paracetamol/acetaminophen[152,158–161]. Conventional wisdom states that Paracetamol is metabolized by the CYP2E1 enzyme to form the intermediate metabolite NAPQI, which is followed by a cascade of disruptive and fatal events if a threshold amount is exceeded. These events range from disturbance of cellular homeostasis and mitochondrial dysfunction to the activation of cell death promoting pathways and the release of drug-modified macromolecules and/or danger signals that initiate an innate and/or adaptive immune response[151,154]. Recent work indicates that the dose response of drug toxicity can be modulated by LPS exposure[162,163], which suggests that inflammatory or diseased states can significantly alter the body's metabolic response to drugs[164,165].

As evidenced from the discussion above, the liver has a highly complex set of responses that may involves hepatocytes and the other non-parenchymal cell types. Furthermore, the pathways that lead to toxicity are not necessarily clear and, except for a few toxins like Paracetamol, have not been well studied. Accordingly, a new generation of *in-vitro* technologies that attempt to more faithfully mimic the liver ecosystem will be critical for the better understanding required to help solve some of the problems facing the pharmaceutical industry.

3. *IN-VITRO* PLATFORMS FOR MIMICKING THE LIVER PHYSIOLOGY AND DRUG RESPONSE: A HISTORICAL PERSPECTIVE

A successful *in-vitro* liver platform is expected to replicate the major liver-specific functions over a prolonged period (>28 days) to allow for both acute and chronic studies in normal and diseased liver pathology. This is quite an undertaking and requires a concerted effort from a large and diverse research effort. Past efforts to mimic the liver physiological and toxicological response *in-vitro* span decades of work among thousands of researchers. The

difficulty and complexity of this task along with the still vast amount of unknowns in the field, resulted in a wide variety of approaches ranging from artificial electrochemical experiments[166–168] and use of microsomes[169–172] all the way up-to using whole organs[173,174]. The field is a dynamic one where each advance is adopted slowly and incorporated in the toolbox; this adoption depends on a delicate balance between ease of use, and the longevity and accuracy provided. In the interest of brevity, we only review 1) static suspension and plated culture liver cells and 2) perfused macroscopic and microscopic culture systems. A summary of the platforms we discuss with some of their key success criteria are provided in Table 2.

3.1 Static culture of the liver cells

In this section we discuss 1) systems that only incorporate hepatocytes and 2) systems that feature secondary cells, including the non-parenchymal cells of the liver as well as others such as murine fibroblasts or endothelial cell lines; we conclude with a brief look at commercially available static platforms.

3.1.1 Hepatocyte only static culture platforms—Hepatocytes in culture have provided a first-order approximation of an *in-vitro* liver, for over 40 years. Fresh or cryopreserved primary cells (typically rat or human), hepatoma cell lines (HepG2, Mz-Hep-1, BC2, HepaRG, etc.), and progenitor cell-derived cells (embryonic or induced pluripotent stem cells) each recapitulate certain aspects of *in-vivo* liver physiology with individual strengths and weaknesses. Here, we only discuss systems that use primary cells, since enzymatic expression and activity are typically much lower in cell lines compared to primary cells[175], and only a few toxicology studies using progenitor-derived cells have been published. We refer the readers to recent reviews[74,176,177] for detailed information on cell line and explant culture.

Time scale and system complexity: Extending *in-vitro* culture duration, while maintaining an *in-vivo*-like phenotype is challenging; hepatocytes often lose function over time when isolated from their native environment[30,31,178,179]. Nevertheless, culture methods — that both overcome these challenges and provide a convenient work flow for a various applications — have been developed with time scales ranging from several hours to 6 weeks. Methods that provide an appropriate utilization of topology, ECM components, cell-cell contact and soluble factors to mimic the *in-vivo* liver environment, retain cell phenotype and liver-specific function for a longer time period. Isolated hepatocytes in suspension are widely used for drug clearance[22,23] and toxicity[24–26] studies. Unfortunately, hepatocyte viability in suspension decreases significantly after ~4 hours[180,181] and the method is limited to very short-term studies. Moreover, many cell characteristics lost in the isolated state[182,183], including polarity, junctions, bile production, and zonation, cannot be re-established in suspension culture.

Hepatocytes are adherent cells and depend on anchorage to a suitable substrate for the maintenance of differentiated function[27]. Accordingly, adherent monolayer culture of hepatocytes — on plastic culture dishes[28] or ECM coated surfaces (especially collagen type I)[29] — is able to slightly extend the culture duration (~1–3 days). Gene expression profiling

studies[183] indicate significant changes in hepatocyte phase I P450 and phase II metabolism, glucose metabolism, cytoskeleton and ECM, and cell cycle for monolayer cultures over 72 hours. Despite a reduction in expression of biotransformation genes, selected P450 enzyme activities remain inducible[184], allowing for short-term use in toxicity studies[185].

Spheroid culture methods, usually conducted in suspension, work by inhibiting hepatocyte attachment to vessel walls thereby enforcing cellular aggregation and formation of floating spheroids. Various methods exist for spheroid formation, including mechanical agitation by rotary shaker[186] or spinner flask[187], hanging drop[162,188] or non-adherent surface chemistry[189,190]. In addition to providing anchorage via cell-cell contact, the 3D organization in spheroids also seems to result in cellular polarity and to some extent the retention of ECM that hepatocytes themselves secrete. Spheroid culture typically maintains many of the liver-specific functions[25,177,191], such as albumin, urea, transferrin, and bile secretion, as well as certain phase I and II biotransformation activity for a few weeks[162]. Despite this encouraging picture, several limitations exist in spheroid culture, including difficulty in imaging, a distribution of nutrients, wastes, and test compounds across the aggregates, and difficulty in scaling the system down for microfluidic applications.

Another advance in long-term function came via the sandwich culture or overlay method[30,31,178,179] developed in our labs over two decades ago. Sandwiching hepatocytes between two layers of ECM, typically collagen or Matrigel™ basement membrane matrix, leads to development of stable hepatocyte polarity[30] and hepatocyte “plate” structures similar to the *in-vivo* liver anatomy[30]. Such polarity, including basal surfaces induced by ECM layers and apical surfaces by cell-to-cell contact, leads to bile canalicular network development[192] and bile secretion that resembles *in-vivo* secretion[193]. Ease of microscopic imaging is also a benefit of this planar technique.

Matrix sandwich and matrix immobilization methods for hepatocyte culture have significantly increased the viable culture time period, up to 6–8 weeks and enabled stable albumin, urea, transferrin, fibrinogen, and bile salt secretions[30,31,178]. Moreover, biotransformation activities and induction of many phase I CYP isozymes and phase II enzymes are adequately maintained over at least 2 weeks[194]. Although the sandwich initially uses one or a few ECM components, hepatocytes themselves secrete ECM and alter the local microenvironment over time[195]; thus, an important role of the sandwich structure is to act as a scaffold and retain the secreted ECM components[196]. The success of the sandwich method demonstrates that not only composition but also the topography of the ECM can play an important role in guiding organization and expression of cytoskeletal proteins, cellular polarity and maintaining phenotypic stability[69,195,197].

Although several culture configurations have been developed to preserve hepatocyte function and morphology for varying times, translating those methods to microfluidic dimensions has proven difficult. Encapsulation techniques that employ a wide variety of biomaterials have also been used to create more *in-vivo* like 3D microenvironments for cultured hepatocytes. These biomaterials include alginate[198], hyaluronic acid esters[199], collagen[200,201], and methylated collagen[202], and have been able to maintain the liver-specific functions for varying lengths of time. Such encapsulation with biomaterials has

been translated successfully in microfluidic devices[203] which we also highlight in section 3.3.2, indicating the potential of this approach in efforts towards miniaturization. A promising method, in this context, for creating a thin extracellular matrix is layer-by-layer deposition of polyelectrolyte multilayers[204]. In this technique, alternating layers of cationic and anionic polymers are deposited via electrostatic attraction[205,206] on top of hepatocytes to mimic the space of Disse. While many potential polymers could be utilized for good results in terms of hepatocyte morphology, function, co-culture with LSECs, and CYP 1A1/2 activity have been reported for up to 12 days using chitosan and hyaluronic acid[85,207]. We have recently been able to translate this approach to a microfluidic device for hepatocyte culture using charge modified collagen strands creating a ECM barrier that is on the order of ~100 nm and have also shown long-term stability of important hepatic functions[208].

Hepatocyte stimulation: Soluble factors: Basal medium and supplementary additives for culturing hepatocytes are not standardized and many different formulations are used; the discrepancies in culture configuration and media formulation often make comparisons between different studies and laboratories difficult. Typical basal formulations include DMEM, DMEM/Ham's F12, and William's E and these are commonly supplemented with serum, insulin, EGF, corticosteroids (hydrocortisone or dexamethasone), and glucagon to maintain phenotype. While serum is necessary to improve attachment and some hepatic functions, it also causes a range of deleterious effects on morphology and polarity[148]. In the absence of serum, formulations providing complete amino acids, including proline[209], are necessary to support albumin and collagen synthesis.

Many different additional soluble factors, including cytokines and non-physiologic compounds, have been reported to have various beneficial effects on hepatocyte functions. Exogenous cytokine stimulation is a powerful, though potentially non-physiologic, signal to cells. TGF-β1 typically induces apoptosis[210] and fibrosis, whereas VEGF can help maintain hepatocyte endothelial cell co-cultures[211,212]. Addition of up to 2% dimethylsulfoxide (DMSO) to media helps maintain albumin and plasma protein production, morphology, and cellular junctions in hepatocytes in monolayer[213], though it may decrease certain CYP P450 activities[214]. Other compounds reported to improve aspects of hepatocyte function in culture[10] include isonicotinamide[215], metyrapone[216], nafenopin[217], and transferrin[218].

More complex media formulations have typically maintained hepatocyte function better than basal formulations[74,219], though reduction in concentration of additives is likely possible in culture configurations that maintain ECM contacts and cell-cell interactions[69]. Several attempts have been made to use a factorial design and isolate the effects of specific hormone or cytokine additives on hepatocyte function; in one such example, Zupke *et al.* demonstrated that glucagon supplementation increases glucose and urea synthesis[220]. Translation of media requirements from macro-scale tissue culture applications to microfluidic devices, which may have constant or intermittent perfusion, remains unresolved.

3.1.2 Static co-culture systems—Non-parenchymal cells support and contribute to drug metabolism; they also play a crucial role in modulating intrinsic as well as

idiosyncratic liver injuries via the release of growth factors, inflammatory mediators as well as reactive intermediates[70,71,124]. Moreover, the overwhelming evidence demonstrate that these "secondary" cell types enhance hepatocyte function, and prolong the retention of their phenotypes in long-term *in-vitro* studies[9,221–227]. With this insight, many systems have been devised to co-culture hepatocytes not only with cells of the liver but also cells from other tissues. Below we review these efforts, which shed light on the question of how we can better recapitulate the liver function by multiple cell culture *in-vitro*.

2D co-culture approaches: One of the first examples of 2D (coplanar) hepatic co-culture methods was established by Guillizo and co-workers[221,222]; they demonstrated a significant improvement in both viability and hepatic function in hepatocytes and liver epithelial cells, monolayer co-culture system compared to control group of hepatocyte monolayers. Following this work, it has been shown that by randomly seeding hepatocytes with non-parenchymal cells of liver[228,229] or cells of non-hepatic origin, such as fibroblasts[230], leads to maintenance of differentiated hepatic function for several weeks through a number of mechanisms including cell-cell contact, and secreted factors such as growth factors and extracellular matrix (ECM) components[9,231].

In contrast to random co-cultures, application of microfabrication and patterning approaches has facilitated systematic investigation of the role of homotypic, and heterotypic cell-cell contact on the maintenance of hepatocyte function. By patterning rat hepatocytes and murine 3T3-J2 fibroblasts, our group established that the heterotypic cell-cell contact between hepatocytes and fibroblasts[223] as well as homotypic fibroblast interaction[224] contributed to enhanced synthetic function of hepatocytes in a co-culture system. More recently, the micropatterning approach was extended to culturing primary human hepatocytes in a multi-well format for drug toxicity screening[225] and hepatitis C virus (HCV) infection[232]. Khetani *et al*.[225] demonstrated the utility of hepatocyte/3T3-J2 fibroblast co-cultures by assessing phase I/II xenobiotic metabolism, bile canalicular transport, secretion of the liver specific products, and susceptibility to hepatotoxins. For HCV infection studies, Ploss *et al*.[232] demonstrated sustained replication of virus for several weeks in human hepatocytes, although the infection was limited to 1–3% of hepatocytes. In addition to fibroblasts, micropatterning approaches have also been applied to patterning of hepatocytes with other cell types such as Kupffer cells with a concomitant increase in the synthetic function[233]. A comparison between the effects of cell-cell contact and secreted factors was conducted by Hui *et al*.[234] via micro-machined silicon substrates with moving parts which enabled both spatial and temporal control over cell placement. The authors demonstrated the subtle result that maintenance of hepatocyte phenotype by fibroblasts required direct contact for a few hours followed by sustained soluble signals with a spacing between two cell types that is less than 400 μm[234].

3D co-culture approaches: While coplanar seeding of cells provided invaluable information about the interactive environment of the liver, recent studies have focused on creating 3D layered structures of hepatic cells in order to supposedly better mimic the liver sinusoid. Ito *et al*.[235] observed enhanced albumin function in co-cultures of hepatocyte and endothelial cells, where layering was achieved by labeling endothelial cells with magnetite

cationic liposomes and placing them on top of hepatocytes using a magnet. Another approach relied on growing endothelial cells as a separate sheet and then placing the sheet on top of hepatocytes for creating layered structures[236,237]. Using this approach, Kim *et al*.[237] were able to maintain hepatocyte phenotype for 4 weeks with well-developed bile canaliculi networks.

A novel 3D co-culture approach was presented by Rajagopalan and co-workers[85,204] where they utilized polyelectrolyte layer-by-layer assembly[205,206] to layer endothelial cells on a monolayer of hepatocytes. They demonstrated both higher synthetic function and enzymatic activity in co-cultures compared to a control group of pure hepatocytes. In order to include a somewhat crude model of the space of Disse, Katsuta and co-workers[238] cultured hepatocytes and endothelial cells on the opposite sides of a micro-porous membrane with stellate cells intercalated in the pores of the membrane. This model was used for investigating the role of stellate cells in mediating intercellular communication between hepatocytes and endothelial cells in the context of endothelial cell morphogenesis. Salerno *et al*.[239] used synthetic biodegradable membranes with human umbilical vein endothelial cells (HUVECs) and primary human hepatocytes to create a layered model, which resulted in enhanced albumin production, urea synthesis, and drug transformation due to heterotypic cell-cell interactions.

Layering can also be achieved using natural ECM rather than synthetic materials. Specifically, Jindal *et al*.[201] utilized collagen as the intervening layer for creating layered co-culture of hepatocytes and endothelial cells. They also established that in the layered structure, surprisingly, proline secreted by endothelial cells contributed to the maintenance of hepatocyte function. The utility of this model was further expanded by integrating a fluorescent reporter clone of endothelial cells and assessing the activation state of endothelial cells under inflammatory conditions[240].

Spheroids also provide an alternative for co-cultivating hepatocytes in a 3D configuration. Wong *et al*.[241] exploited a concave micro-well array platform for creating spheroids of hepatocytes and stellate cells. They observed that albumin secretion and drug metabolizing activity was superior in co-cultured spheroids; and also demonstrated that stellate cells played an important role in the formation of stable and uniformly sized spheroidal aggregates. In a similar study, spheroids of hepatocytes and fibroblasts were formed and then cultured in either a bioreactor or a spinner flask[242]. This study demonstrated significantly improved longevity, albumin production and phase I/II drug metabolizing activity.

Commercial 2D and 3D co-culture platforms: The sandwich method and monolayer culture of pure hepatocytes have been de-facto standards in drug screening and drug discovery studies in the pharmaceutical industry; nevertheless, a few of the aforementioned co-culture approaches have now also reached a commercially viable stage and are available for wider use by academic and industrial establishments. While some of these platforms have been initially cultivated in academic settings, others have been developed directly via commercial efforts. Below we review four of these systems that claim better physiological relevance as well as improved predictive capabilities.

One example of an academic-born technology making its way into the commercial environment is the “Hepatopac” platform developed by Hepregen (Fig. 3a). This platform, which was initially developed in our labs in the late 1990s[223,224,231,243,244], was further developed by Khetani and Bhatia at MIT and then by Hepregen[225,245–248]. This 2D coculture system, as discussed earlier, has two important features: a) use of micropatterning to make rat or human hepatocyte islands of roughly ~100–500 μm and b) use of 3T3-J2 fibroblasts to stabilize and enhance the function of hepatocytes in long-term cultures (> 4 weeks). In its latest iterations, the model has been successfully translated into a multi-well culture plate platform amenable to higher throughput experiments. While the physiological relevance of flbroblasts — of mouse origin — in this system is unclear, recent studies[245] on panels of drugs (~40) show improved sensitivity and specificity especially when human hepatocytes were used. The Hepregen platform has been characterized in terms of its enzymatic and functional capabilities and retention of such capabilities.

Another successful academia industry collaboration, in this realm, is between ETH Zurich and Insphero AG (Schlieren, Switzerland). Their platform (Fig. 3c) features a reincarnation of the hanging drop model combined with a multi-well plate format to reliably produce and culture spherical aggregates (spheroids) of hepatocytes and non-parenchymal cells[162]. The use of a multi-well plate format allows the easy integration of the abundantly used imaging and automation systems in the industry, thus a seamless integration into current workflows. By tightly controlling the size (~200 μm) of the hepatic aggregates, they have been able to demonstrate high viability and stable function of these spheroids for up to 5 weeks and have conducted several drug toxicity studies with or without inflammatory stimulation[162]; the system also allows spontaneous zonation within the spheroid. Unpublished data by the company shows significant effect of NPCs on the IC50 values of different compounds highlighting once again the importance of NPCs on drug metabolism. Despite the success and advantages of this platform a notable drawback is the difficulty in imaging a thick tissue construct. Additionally, full characterization and predictive capabilities of this promising platform on a large panel of drugs still remain to be demonstrated. Published[249] and unpublished data[250] shows high stability of enzymatic expression and activity for long culture periods (~28 days).

Another important static co-culture platform which also originated our labs, and then was transferred to an industrial startup, is the “HμREL-hepatic co-culture” platform from the Hμrel Corporation (North Brunswick, New Jersey). While the company also provides microfluidic “flow” culture products (reviewed in section 3.3.2), their static platforms (i.e. a well-mixed and optimized co-culture of cryopreserved hepatocytes (human, rat, dog and primate origin) with a proprietary stromal cell cohort, has shown longevity (>30 days) and competency in terms of CYP 450 and synthetic functions according to published[251] and unpublished data[252]. Most recently in a large drug panel study (~50) in collaboration with UCB Pharma, the dog co-culture version of this platform has been shown to have high sensitivity (78%) and the highest specificity (73%) among all groups[251]. Their successful use of cryopreserved dog (canine) hepatocytes also marks an important step towards closing the species gap in drug testing where all species used in animal studies can be compared to *in-vitro* model results.

The Regenemed (San Diego, CA) platform (Fig. 3b), also features co-culture of human or rat hepatocytes with a full complement of the non-parenchymal liver cells in a 24-well plate format[124]. It makes use of a removable transwell as well as porous (d ~140 μm) nylon scaffolds to culture a matured NPC fraction on top of hepatocytes that are cultured in the outer well; a cell number ratio of 60% hepatocytes and 40% NPC fraction was deemed optimal. In one report, the platform was demonstrated to be viable and functionally stable for up to 11 weeks as inferred by albumin, transferrin and fibrinogen secretion and urea synthesis[124]. The feasibility of this platform for drug screening studies was also demonstrated, albeit using a small panel of drugs; a species specific response was observed during the studies and they also noted that specificity of the system is improved when the NPC fraction was included[124].

3.2 Flow-based *in-vitro* liver platforms

Optimal liver function is presumably not only dependent on the coordinated function of the parenchymal and non-parenchymal cells within the hepatic acinus, but also dependent on hepatic blood microcirculation. Aspects of the microcirculation can be simulated *in-vitro*, via perfusion models, to create a dynamic *in-vivo* like environment. Through perfusion, nutrient, oxygen, and soluble factors can be replenished in a controlled way so as create a pseudo-steady state of those parameters; similarly secreted factors, biliary secretions, and intermediate or end metabolites can be cleared. This is unlike a static culture where nutrients are consumed in an exponential decay; and end or intermediate products are secreted in an inverse exponential manner; eventually everything is reset to the initial state via media changes. This media cycle is clearly not representative of the physiological state, and also may be stressful for the cells. Spikes in media may be representative of drug injections; however, if the rest of the system is stressed by a lack or overabundance of nutrients and waste, the metabolic response to the drug challenges may be misleading. This problem has long been recognized and researchers have incorporated perfusion in their culture systems to allow for a better *in-vivo* mimicry.

3.2.1 Macroscopic flow platforms—The early works on macroscopic perfused *in-vitro* liver systems were primarily motivated by creating a bioartificial liver as an extracorporeal assist device akin to the dialyzers for kidney; however, the bioartificial liver devices went beyond the filtering function by the inclusion of hepatocytes for metabolic functions. Three prominent types of such devices exist 1) flat-plate bioreactors as developed by our group[32,34,253,254] and others[255,256], 2) hollow fiber[37–39] liver assist devices, and 3) macroscopic liver perifusion devices[257,258]. Although we consider these devices macroscopic, some incorporate microscopic features, for example via SU-8 micropatterning[253] in the flat-plates. Some of these platforms were the precursors to today's microfiuidic devices, and in general, demonstrated enhanced hepatic function over static embodiments[32]. They also served as the initial proof of concept for critical technologies such as membrane oxygenation[32,34,254], and shear reducing microgrooves[253,259]. While these platforms were initially not intended for drug screening purposes, a recent reincarnation of a scaled down version of the hollow fiber reactor by Zeilenger *et al.*[260] demonstrated such feasibility using human hepatocytes. However, even in their smallest (2 ml) and most successful version of the device, the albumin synthesis and urea excretion

declined considerably after a week of perfusion; enzymatic activity, demonstrated via metabolite formation, showed a similar decline.

Other macroscopic devices of note, for their accessibility, longevity and possible commercial success, respectively are: 1) multicompartmental modular bioreactor (MCmB) by Ahluwalia and co-workers[261–265], 2) the work of De Bartolo[266,267] and that of Gebhardt[268], and 3) the Hemoshear platform[269]. The MCmB[261] (Fig. 3f) involves a simple macroscopic low shear environment which uses regular peristaltic pumps, a cylindrical flow chamber on the bottom of which a cell-laden cover slip can be introduced with ease. The MCmB platform was used with co-culture and multi-tissue cultures[261,264,265], and enzymatic gene expression was demonstrated for 21 days. The fluidic chambers used in the MCmB platform are commercially available in an easy to use format for any type of perfused cell culture. The work of De Bartolo stands out with respect to longevity of the system, an important requirement for chronic drug response studies. Their platform[266] consists of a disk like bioreactor with a 25 μm thick gas permeable membrane for gas exchange (Fig. 3d); they have demonstrated 33 days of stable secretion for culture of cryopreserved human hepatocytes although no direct viability measurement was conducted. They also demonstrated drug clearance studies in addition to IL-6 stimulation of the entire system in intervals. Gebhardt *et al.*[268] showed that addition of RL-ET-14 cells, a rat liver cell line resembling LSECs, to their perifusion system of rat hepatocytes can improve longevity of the system to 14 days in addition to higher stable enzymatic activity and inducibility; this work also demonstrated the potential utility of co-cultured cells for drug clearance as well as drug-drug interactions.

Another platform, the Hemoshear system (originally developed to study endothelial cell hemodynamics[267]), features a cylindrical macro-perfusion chamber for cells (Fig. 3e) where the hemodynamic environment, i.e. flow, is created in a fashion similar to a cone-and-plate viscometer. The liver version of the platform features a synthetic membrane over the confluent layer of rat hepatocytes in a collagen sandwich configuration; this membrane protects both the cells and the gel structure form the high shear rate (0.6 dynes/cm^2) used in their recent study. This recent work[267] demonstrates the operation of the system for 14 days with stable secretory function and improved enzymatic activity compared to static cultures; further data with large drug panels are still needed to establish the success of this platform.

3.2.2 Microfluidic platforms—The macroscopic platforms described above provide some evidence that perfusion can improve longevity and function in cultured hepatic systems. An important design consideration when developing tissue analogues is the size of the device and most importantly media volume/height which directly affects the key parameter of “media volume to cell number ratio”. In a typical liver sinusoid (diameter ~5–10 μm), this ratio is about 0.03 nL of blood per hepatocyte[270]. While achieving this ratio is critical if one desires precise *in-vivo* mimickry, it is currently not feasible because of: a) technological limitations with regard to fabrication and tissue engineering and b) the lack of appropriate media which must have oxygen carriers in order to deliver sufficient oxygen given the reduced volume. Nevertheless, approaching this ratio is of utmost importance, especially in multicellular tissue constructs and/or multi-organ systems, where one wants to study interactions between two or more cell types through secreted factors and intermediate

drug metabolites. Most conventional systems such as traditional culture well plates or current macrofluidic systems[262,263,271–273] are at least two orders of magnitude off in their "media volume to cell ratios", and thus are not suited to capture interactions signaled through the fluid at the physiologically relevant doses of stimulation or drug application. As a rule of thumb, device heights that are 100 μm or smaller provide a much more relevant signaling environment for multicellular systems and can capture interactions among cells that would otherwise be missed in larger systems[274]. Thus, microfabrication and microfiuidic models appear essential for approaching media volume/cell ratios characteristic of *in-vivo* values[270,275].

Below we discuss microfluidic liver platforms that aim to recapitulate different aspects of the liver taking advantage of technologies that enable precise machining and control of microenvironments[276]. A brief comparison of these microfluidic platforms along with few macroscopic ones can be found in Table 2 and Figs. 3,4.

Hepatocyte only microfluidic platforms: A common approach towards liver mimicry *in-vitro*, via microfabrication, is to create cellular assemblies that resemble hepatic cord/sinusoid like structures. One example is the work by Lee and co-workers[277,278] at UC Berkeley that gave rise to a commercial platform via the CellAsic Pearl and Onix systems (EMD Millipore, Billerica, MA). The building block of this platform is a perifusion system where hepatocytes are densely seeded into a narrow, high aspect ratio pocket (Fig. 4d) with a microfabricated artificial barrier featuring fenestrae like structures (2 μm wide); the perifusion is established outside this barrier region thus protecting the cells from mechanical stress. Although the barrier channels are about an order of magnitude larger than endothelial fenestrae, they do create an effective barrier to convective transport which only allows diffusion — a factor that makes the platform amenable to mathematical modeling since the characteristics of the transport barrier are known. Like other commercial platforms this model has also been translated into a culture plate format with 32 individually addressed sinusoid arrays on one plate, and gravitational flow is employed to negate the need for pumps. While the earlier work[277,278] on this platform demonstrated high viability of only about a week, recent work indicates hepatocyte culture for over 30 days[279].

In a similar fashion, Nakao and co-workers[280] cultured primary rat hepatocytes in a 37 μm by 30 μm microfluidic channel, surrounded by an array of 2 μm wide slits. They demonstrated that such narrow and high aspect ratio channels induce hepatocytes to align and form an *in-vivo* like bile canalicular structure. However, the scalability and long-term viability of the system was not demonstrated. A recent, innovative yet underdeveloped effort in this respect is the creation of sinusoid like structures using dielectrophoretic assembly of primary human hepatocytes and endothelial cells in microfluidic channels[281]; while the authors demonstrated cord-like structures that can then be perfused in the same channel they were created, they have not yet shown the stability, viability and functionality of these structures.

A recurring theme in the microfluidic work cited so far is either the dilute use[280,281] or lack[277,278] of ECM proteins which highlights the difficulty of translating some of the successful macroscopic technologies, such as the collagen sandwich method[31], to the

microfluidic realm. The sandwich configuration relies on hydrogels with typical thicknesses of 100 s of microns that are too bulky to situate easily in enclosed PDMS devices with features as small as ~10 μm and channel heights around ~100 μm. The ECM free approach relies on the premise that the ECM components secreted by hepatocytes and other supporting cells might be able to sustain the long-term hepatic function[196]; however, the retention of these secreted ECM components is questionable especially in open-loop perfusion systems. One approach that takes this ECM-free approach is the work of Goral *et al*.[282] where they use a dense array of micro-pillars (15 μm tall, pitch is less than a single cell diameter[282]) both to hold human hepatocytes together and induce a cord like structure. While these investigators demonstrated viability for 14 days, along with formation of 3D bile canalicular structures and healthy expression of connexin 32 and MRP-2, they have not yet investigated broader functions of this system.

Another microfluidic liver platform by Toh and co-workers[203,283], dubbed the 3D-μFCCS or the 3D HepaTox Chip, also makes use of a microfabricated endothelial barrier but with 20 μm fenestrae (Fig. 4e). Additionally they use a complex coacervation method using polyelectrolytes to create a 3D matrix for the cultured hepatocytes addressing the need for an initial ECM environment. They demonstrated the versatility of this platform by culturing HepG2, MCF7 and bone marrow stem cells[283] initially, and then by using primary rat hepatocytes[203,283]. The second study[203] also demonstrated multiplexing by incorporating a gradient generator for drug dosing purposes. In the same study they also showed higher enzymatic inducibility as well as metabolic activity for up to 72 hours of perfusion culture compared to static plate cultures. Finally, they also conducted a toxicity study for a panel of 5 drug compounds and showed a slightly improved correlation to *in-vivo* toxicity compared to multi-well plates (R^2 = 0.84 vs. R^2 = 0.8).

An example of high throughput capability of hepatocyte microfluidic systems comes from our lab which developed a scalable experimental platform that combines microfluidic addressability with quantitative live cell imaging of fluorescent protein transcriptional reporters (the “living cell array”)[284–290]. The platform uses microvalve arrays to achieve reliable seeding and orthogonal stimulation of multiple fluorescent reporter cell lines while enabling automated time-lapse microscopy to continuously monitor dynamic responses from a 2D matrix of experiments. Using this platform with eight different reporter cells and 8 different stimulating agents, we were able to monitor a remarkable 5000 single time points in a 36-hour period. While this proof of concept work used the H35 hepatoma cell line to create stable GFP reporter cells and was aimed at examining short-term gene expression, the extension to primary hepatocytes for longer periods of time is eminently doable.

Microfluidic co-culture systems: One example of integrating technologies for hepatic tissue engineering and microfabrication comes from our lab where Kane *et al*.[291] combined several previous ideas such as the gas permeable membrane[254] as well as a micropatterned co-culture system of hepatocytes with 3T3-J2 fibroblasts[224,243] (see Fig. 4g). Cultures were miniaturized and integrated into an 8 × 8 microfluidic well array, similar to the living cell array described above[284] (Fig. 4h). Each row or column could be perfused independently with regard to both media and oxygen through separate fluidic manifolds. Stable albumin

synthesis and urea excretion was demonstrated for 32 days in this microfluidic array of co-cultures but no enzymatic or toxicity related studies were reported.

A liver platform that has been long in development both academically and in a commercial setting is the Zyoxel platform (see Fig. 4i). This platform stems from early work at MIT[292,293] and features cross-flow micro-wells (300 μm wide, 235 μm high), manufactured using silicone or polycarbonate, for perfusion of 3D aggregates of hepatic cells. The cross-flow across hepatocytes is established via a micro-porous membrane at the bottom of the micro-wells. Their early work featured only hepatocytes and demonstrated stable secretory function for up to 15 days. Later incarnations employed co-cocultures with LSECs[294,295] and then a full complement of an LSEC enriched parenchymal cell fraction[295] for improved stability and mimicry; they also demonstrated spontaneously formed oxygen gradients[295] in their scaffolds akin to the *in-vivo* zonation. These newer incarnations also showed convergence towards a culture plate format complete with pneumatics and sensors, allowing 12 experiments on each plate. Unpublished data on this platform claims longevity of 21 days, and recent publications demonstrate CYP activity[296] and drug clearance[226] with good *in-vitro* to *in-vivo* correlation. While this platform has been around for long time, several issues remain. For example, 1) although the aim of 3D aggregate formation is to create sinusoid like plate structures, the architecture of these aggregates are completely random and reproducibility is relatively poor[69,74], 2) the height of the tissue structure interferes with high content methods that rely on optical methods, and 3) the current system appears too complex for widespread adoption[124].

Hμrel*flow*™ (Fig. 4j), a perfusion-driven, microfluidic *in-vitro* liver platform geared from its inception towards drug and toxicant screening, has been developed by Hμrel Corporation[227,297,298]. Hμrel*flow*™'s academic predecessor platform was developed by Michael Shuler and Gregory Baxter of Cornell University (see the discussion on μCCAs). Hμrel*flow*™ devices are comprised of multiple, fluidically interconnected microscale cell culture compartments which enable simulation of the interaction of test substrates with 2 or more organs, so as to provide enhanced prediction of human physiological response based on PBPK models[299,300]. Made entirely of a type of plastic selected for its optical clarity and minimized absorption of test substrates[301], the platform features flat-bottomed, microscale tissue culture compartments capable of holding adherent cellular materials; the tissue culture compartments are interconnected by microscale channels that enable the device's culture media to flow in a recirculating pathway throughout the course of the experiment. A recent initiative by the company has specifically focused on integration of small, independently controlled actuation (i.e. pumping) mechanisms into the substrate of each device to reduce the overall culture medium in the device, shortening the circulatory cycle time and enabling Hμrel*flow*™ to better mimic actual *in-vivo* recirculation. Maintenance of the platform has been demonstrated up to 6 days with respect to its enzymatic and synthetic function in published work[227], and recent unpublished data[302] claims over 14 days of stable maintenance. More importantly in the same studies[227] clearance data of six drugs showed high *in-vitro in-vivo* correlation especially in perfused co-culture models of human hepatocytes and stromal cells and the same system also showed a superior metabolite generation compared to all other controls. While more work is needed on this platform to

show its retention of enzymatic and functional capacities over longer durations as well as to demonstrate its multi-organ/tissue interaction capabilities, their most recent static co-culture results[251] together with their technological advances in micropumping look promising for wider adoption of the system.

With regard to multi-organ interactions in microfluidic systems, with the liver as the primary organ, the field has been advanced by Shuler and co-workers and their micro cell culture analog (μCCA) systems[299,303–307]. These are, as in the Hurel system, connected microfluidic compartments of different tissue/organ systems with stated physiologically relevant volume ratios as well as *in-vivo* like residence times for the recirculating media flow. These systems were designed with the notion and premise that they are the microscopic analogues of the *in-vivo* visceral system and that modeling their response to drug challenges via PBPK approaches can provide important insights and predictions about the real system. These investigators have demonstrated the feasibility of such systems first with a liver-lung[299] and a liver-lung-fat tissue model[307] using cell lines for all cultures. These early models also demonstrated the clearance of naphtelene and the toxicity of its liver metabolized intermediates on the lung chamber[307]. More recent examples of the μCCAs demonstrated similar studies for liver-uterus cancer-colon-colon tumor with integrated optical *in-situ* imaging[306], liver-tumor-bone marrow[305] and a GI tract model complete with liver, kidney, bone marrow and fat tissues while modeling the intricacy of poorly perfused and well perfused medium flow[304]. While these μCCAs have been important in establishing the idea of PBPK in the *in-vitro* microfluidic realm as well as demonstrating multi-organ interactions for toxicity, two obvious drawbacks of this work have been the regular use of cell lines rather than primary cells and the usually short duration (several days) of the studies.

Counterparts of this work in Europe where multiple organ/tissue chips are connected — with the liver as the focal point of the system — has been predominantly developed by Ahluwalia and co-workers and their McMB (Multicompartmental Modular Bioreactor) model[261,308,309]; and more recently by the body-on-a-chip (BoC) initiative that is led by InSphero AG. While the McMB system consists of fluidically macroscopic modules that are easily connected and disconnected with more common interconnects, the BoC initiative uses a microfluidic translation of the hanging drop spheroid formation and the microfluidic connection of eventual spheroids again in a custom multi-well format[310]. The McMB model has been commercialized or under the process of commercialization by two companies Kirkstall Ltd. (UK) and IVTech Srl (Italy) where the companies provide off-the-shelf compartments for researchers to develop their own tissue models.

4. A CRITIQUE OF THE HISTORY AND GOING FORWARD: CHALLENGES AND OPPORTUNITIES

As stated earlier one of the major goals of current governmentally supported research initiatives is to develop robust 3D microfluidic tissues that provide improved predictive capabilities of the human response to drugs. While some of platforms claim to meet the arbitrary 28-day duration criterion they fall short in some other category; i.e. they are poorly characterized and/or their physiological relevance is in question or completely unknown.

Below we discuss key issues that exist currently as well as ones involve heavy use of microfabricated platforms.

4.1 The need for standardized characterization

In Table 2 we compare various current microfluidic as well as few commercial static culture platforms and provide information about their outputs; there is clearly a significant discrepancy among these platforms in terms of their assessment criteria even amongst the more mature ones. While some groups focus primarily on secretory functions and cell viability as a first indicator of success others march onto drug studies without these more basic validations; few have performed complete characterization of the general health, phenotypic retention and drug response of the platform. This partially stems from the fact that the complete characterization is a long, costly, and challenging task that might not be feasible without adequate support. Nevertheless, in order for the field to move forward as a whole there should be a logical and common checklist to assess the success and guide the evolution of new *in-vitro* liver models. While the current NIH/DARPA/FDA initiatives pose a challenge of 28 days of viability in perfused organ systems (presumably to match the common practice of 28 animal toxicity studies used by the pharmaceutical industry), the functional and physiological relevance — especially for the liver — warrants deeper discussion. Below we briefly discuss a three-stage characterization strategy and summarize it in Table 3.

4.1.1 Stage 1: Cell viability and functional stability—Viability and cell health are the first success indicators of any tissue model. These assessments can be conducted either via fluorescence imaging techniques that rely on a combination of nuclear and cytosolic dyes or via resazurin based transformation assays[311,312]. Although the functional secretion assays, discussed below, provide some information regarding cell health they provide an incomplete picture where one cannot isolate individual effects. Viability assays should be conducted at short intervals (every day to several days) to demonstrate initial success of any *in-vitro* liver platform.

Albumin synthesis and urea excretion, are two of the most important markers to deduce the functional capacity of both the liver and its parenchymal cells, the hepatocytes. A consistently high albumin production (~1–5 μg/hr/10^6 cells) not only makes sure of the synthetic function but is also an indicator of the overall metabolic health of the cells; urea plays a similar role for general metabolic capacity. Recent drug screening studies in micropatterned hepatic co-culture systems show that albumin and urea levels can also be a reasonable but indirect indicator of toxicity[245].

4.1.2 Stage 2: Drug metabolism and transport—In addition to the indirect assessment of synthetic and detoxifying capacities, retention of *in-vivo* like and stable phase I and phase II metabolic activity has possibly become the most important goal. We suggest that every platform should demonstrate stable phase I enzymatic activity, proper inducibility and expression for enzymes from the CYP family; assess phase II conjugative activity, such as glucuronidation (UGT) and sulfation (SULT), at regular intervals; and establish the preliminary toxicological relevance. CYP1A2, CYP3A4, CYP2C9, CYP2C19, CYP2D6,

and CYP2B6 have been deemed to be the most import[313] phase I enzymes relevant to human drug response; expanded lists can be found in literature[150,314,315] and FDA guidance documentations. While full characterization of all enzymatic activity might be cost prohibitive in a purely academic setting, it could very well be established if the platform moves towards heavy commercialization.

A more recent trend in the industry is the push towards better understanding of drug transporter activity in addition to the characterization of the phase I and II enzymatic activity; this is a crucial change since the availability of a drug for metabolism is highly dependent on its kinetics and ability to be transported inside and outside of a cell. Drug transporters play a prominent role in absorption, distribution and elimination of drugs[149]; thus, their failure or reduced function *in-vitro* would result in seemingly reduced or impaired metabolism even if the drug metabolism enzymes themselves are intact. Moreover, when a patient takes multiple drugs, they compete for transport pathways resulting in a completely different kinetic and dynamic metabolic picture for both drugs; accordingly an understanding of the way drug transporters handle such interactions has become a crucial piece of the puzzle towards predicting human drug response[149]. It's for these reasons that we suggest that a successful *in-vitro* liver analog should aim to maintain high and stable drug transporter activity; testing for these transporters (Table 3) can be performed by measuring drug uptake and secretion[316] as well as by measuring inhibition[149]. FDA provides general guidance documents for such transporter activity measurements especially in the context of drug-drug interactions.

The four criteria we describe 1) viability, 2) secretory capacity, 3) toxicologically-relevant enzymatic activity, and 4) drug transporter activity should take place in vetting an *in-vitro* platform for drug based-studies. These seem minimally appropriate before moving on to the next important and final stage, the demonstration of predictive capabilities, in establishing a successful *in-vitro* liver platform for drug and toxicity testing.

4.1.3. Stage 3: Specificity and sensitivity of in-vitro liver systems—The most important challenge for any platform is to test its' sensitivity and specificity with a large panel (>100) of diverse drugs of different categories, over long culture terms. These panels should ideally include multiple known drug examples from four categories which are a) safe and efficacious (SE), b) safe but not efficacious (SNE), c) not safe but efficacious (NSE), and d) not safe and not efficacious (NSNE) drugs. Once these panels are established, the platform can be challenged systematically with a range of doses of these drugs; short-term (acute) IC50/LD50 tests as wells as long-term functional marker (albumin, urea) and enzymatic assays can then be performed to assess the dose-response and metabolism of each compound. The next step is then to establish the predictive capabilities of the given platform based on the toxicity correlation between the *in-vitro* dose response and the *in-vivo* data available for these known compounds. In this context, two important measures of the success of each platform are a) sensitivity, fraction of correctly predicted positives to all positives *in-vivo* and b) specificity, the fraction of correctly predicted negatives to all negatives *in-vivo* where positive or negative is defined in terms of a compounds toxicity. Three examples of such studies with large drug panels (N~45) are demonstrated by Xu *et al.*[317], Khetani *et al.*[245] and Atienzar *et al.*[251]

The establishment of a standardized procedure/checklist falls onto the shoulders of the general research community as a whole, funding and overseeing agencies, as well as the end users — the pharmaceutical industry. The FDA, in this regard, provides crucial guidelines which can form the basis of a starting point.

4.2 Zonal recapitulation

As we have discussed above, the liver cells can display a distinct heterogeneity in their morphology, enzymatic activities, and functional capacity along the liver sinusoid[38–41]. Such heterogeneity in metabolizing and detoxifying capabilities may have significant ramifications in drug testing and screening but this heterogeneity is seldom considered or carefully reproduced in current *in-vitro* models[9]. One example that explicitly focuses on recapitulation of zonation is that by Allen and Bhatia[318,319] where they use the flat plate bioreactor geometry and active media oxygenation system to induce oxygen gradients across the length of the device. They confirmed the formation of these gradients both through measurements as well as theoretical modeling approaches. They were able to induce a heterogeneous distribution of PEPCK and CYPS similar to *in-vivo* and demonstrated varying APAP toxicity akin to an *in-vivo* zonal response. Nevertheless, even this study relies on a passive gradient formation and it is unclear how stable the gradients would be if the system is perturbed by random or deterministic effects.

Most other studies focus on the activity of a single zone which usually ends up being Zone 3, the zone with highest phase I activity and lower phase II detoxification. This means that the generation of intermediate metabolites might be exaggerated while detoxification is underestimated; conversely if Zone 1 conditions are induced, toxicity can be minimized due to high phase II activity. One must consider this to be a potential cause of limited prediction capability of many existing *in-vitro* liver models that could lead to false negatives and positives and accordingly suggest an active strategy towards recapitulation of zonation as an important avenue of further improvement for *in-vitro* liver models.

4.3 The need for better detection and instrumentation

The microfluidic realm, which offers good control and precision to recapitulate certain aspects of organ systems *in-vitro*, comes with its own problems. The micro/nano liter volumes used are both a blessing and a burden in that while these techniques minimize the cost of reagents, it also poses a new problem for the biologists and engineers who routinely handle milliliter volumes both in handling and assaying. Where milliliter volumes can be forgiving, microliter volumes can penalize one for the smallest error.

4.3.1 Instrumentation—Pumping and controlling microliter volumes, and distributing the flow at critical junctions require specialized equipment with high degree of precision. The current solution of pumping via controlled syringes or pressure driven pumps are neither inexpensive nor flexible enough in their operation; moreover they occupy an unwarranted large physical space, hampering the portability and ease of use of these otherwise miniature platforms; commercially available high precision pump systems also fail at the task of handling high throughput systems[320]. These problems put a high cost of entry and a barrier to wide scale use of microfluidic tissue engineering approaches, currently limiting it to

specialized labs. Thus, the field would greatly benefit from new, improved, and integrated technologies that can pump, partition and control the flow of microliter volumes which do not add to the size of the platforms. Some potential solutions and new platforms have been proposed, used, or are under development; these include active miniaturized pumping[321,322] and valve solutions, integrated microcontrollers on a tissue culture plate sized platform[323] as well as passive pumping solutions[279,303,324,325] and resistance based flow splitting[307]. The accelerated development of these solutions will be important in the quest to minimize the cost and foster large scale adoption of microfluidic drug screening platforms.

4.3.2 Detection—Detection, in the microfluidic realm, is an important problem but also an avenue of great opportunity. On the one hand, most biological assays are optimized and geared towards milliliter volumes of the tissue culture plate and the liters of perfusates from organs or tissue sections which cannot be obtained easily in the microfluidic realm; on the other hand the highly controlled aspect of microfluidics present exciting opportunities for automated and real time detection which can offer new insights and a better mechanistic understanding of metabolic processes. Moreover, microfluidic detection methods, once fully realized, can offer higher sensitivity, minimal invasiveness, and more economical and high throughput/high content assays.

The most popular assays that can be somewhat readily transferred to the microfluidic studies from the macroscopic realm are the optical assays that rely on fluorescent staining. While these are extremely useful end-point studies they generally do not provide continuous real-time data. In this respect, reporter cells[284,285,326] provide great promise; such cells natively fluoresce upon stimulation or under certain stress conditions giving specific information about the cell state. Sparse use of such cells with different target signals can provide real time information via optical interrogation of the system. The development of microfluidic small volume analyses such as bead based rolling circle amplification[327], and microfluidic ELISAs[328,329] and enzymatic assays[330,331]; and their integration onto the microfluidic tissue culture platforms may bring us closer to near real-time analyses of *in-vitro* microengineered tissue platforms.

Another avenue of development in the low volume detection comes via the translation of traditional analytical chemistry approaches to the microfluidic world; an additional contribution of such approaches is to free up optical channels that would otherwise be used for a similar analyses. Two examples in this come via two different approaches: a) printed microelectrode arrays for microfluidic integration and[322,332] b) droplet based analytical chemistry approaches[333–338]. We refer the interested readers to a series of reviews by Wikswo and colleagues[322,332] in which they discuss the importance of new and improved detection methods in the new microfluidic tissue culture realm.

4.4 The need for better materials

Since its adoption by the Whitesides group for micro-molding techniques[102,339] in the 1990s, PDMS has become the staple of microfluidic research as well as other platforms requiring small features. PDMS provides low cost and rapid prototyping capabilities, and upon plasma oxidization, can adhere to itself or other materials without adhesives[340].

Because PDMS is non-toxic, optically transparent, and oxygen permeable, it seems ideal for culturing and imaging of live cells as in the tissue-on-a-chip models. However, PDMS constructs can suffer from water evaporation, resulting in osmolarity shifts in culture media[341], reversal back to a hydrophobic state aft er the initial plasma treatment, and the fact that the material itself displays a small amount of background fluorescence. Another limitation of PDMS, oft en cited as an advantage, is its high gas-permeability, i.e. allowing free diffusion of oxygen and CO_2; this makes it hard to control oxygen tension required to induce oxygen-based zonal behavior. Nevertheless, the most significant drawback, for the purposes of drug screening, is the problem of absorption[301,342]; small hydrophobic molecules, a good fraction of new molecular entities current being developed by the pharmaceutical companies, can easily penetrate and perhaps bind to PDMS.

While strategies to overcome some of these problems have been developed, there still remains a void and an urgent need for PDMS alternatives where absorption is a non-issue. Machined glass and hard plastics are costly alternatives that can fulfill this need; a good strategy might be to consider these for late stage production and not prototypes. The research community has also been working on producing and using alternative polymers such as fluorinated PDMS[343], photocurable perfluoroethers (PFPE)[344], thermoset polyester[345], cyclo-olefin polymer[342], the common labware polystyrene (PS)[342], and many more; yet, these also oft en come with their own drawbacks and the whole issue becomes one of trade-offs[340]. Therefore, PDMS is still a suitable material for developing an early platform granted one is aware of its limitations, takes these limitations into account for proper analyses, and eventually replaces PDMS with alternative materials depending on the application. A promising candidate in this regard is the development of novel biocompatible styrenic elastomers such as styrene-ethylene/butylene-styrene (SEBS) which retains the ease of micro-molding similar to PDMS but also provides favorable properties such as low adsorption of hydrophobic molecules like PS[346]. For more detailed accounts of use of PDMS in biological applications we refer the readers two reviews by Mukhopadhyay[340] and Berthier *et al*.[347]

4.5 Making sense of the data: Scaling of physical and physiological parameters and predictive models of drug response and toxicity

Translating an *in-vivo* organ system to an *in-vitro* screening platform and successfully correlating the output back to the response of the real organ, pose several technical challenges and engineering decisions. While the static culture systems are easier to handle, the recent knowledge base[270,275,297] indicates that flow systems have an advantage in not only improving the predictivity of the *in-vitro* liver systems but also help in retaining hepatic phenotype in long-term experiments. When one settles on using a perfused tissue model, the next question is how to scale the dynamic as well as static parameters so as to create the best mimicry in terms of drug clearance or other outcomes that one is interested in.

For device dimensions most scientists would agree that devices which have a height of 100 μm or less are preferable since they start approaching diameters of the liver sinusoids (on the order of 5–10 um), and thus reduce the signal and/or metabolite dilution problems that many macroscopic platforms face. For dynamic parameters (volumetric flow rate, shear rate, drug

dosing and clearance) many integrated approaches such as allometric scaling relying on power law scalings across different mammalian species[348–350], integrated physiologically based pharmacokinetic (PBPK) modeling approaches[275,351–353] and IVIVE approaches[354,355] have been proposed both to guide the parameters as well as work around their limitations when trying to make sense of data.

A very important parameter which controls the adsorption, distribution, metabolism, excretion and toxicity (ADME-TOX) in physiological systems, is the exposure time of the tissue to drugs and other xenobiotics; this is called the "residence time" (~10–20 seconds for the liver[356]). Accordingly a good first approximation, to mimic *in-vivo* ADME characteristics in *in-vitro* fluidic systems is expected by using fluidic residence times similar to the physiological values. Some success in improved PBPK modeling and better predictivity has been claimed[299]; however, a recent study shows that simply matching residence time can be misleading, for example, in predicting drug clearance[275]. A concern that arises when focusing on matching residence times is with respect to shear stress; while some liver cells such as the LSECs tolerate shear stress well and do actually need it for proper function, hepatocytes can be injured beyond a shear stress of 0.3 dynes/cm^2 [33]; this hampers the longevity in such systems unless engineered solutions such as microgrooves or indirect perfusion of hepatocytes are used. Perhaps an even more important consideration with residence time matching is the fact that, *in-vivo*, the parenchymal cells are not directly exposed to flow and actually reside in the perisinusoidal space where the concentrations of nutrients and oxygen are most likely different than the sinusoidal space. We have recently conducted both theoretical and computational analysis of matching drug clearances across different geometries where metabolically active cells are directly exposed to flow. Our preliminary results in very simple theoretical models indicate that matching drug clearance might require matching a combination of Peclet number and the aspect ratio (height vs. length) of the two geometries in question.

In addition to meaningful scaling of dynamic and static parameters, an even more important part in the effort to predict human response to drugs, is the computational and analytical models that can allow us to make sense of the data. While the micro and macro *in-vitro* platforms aim to act as surrogates for human physiology by providing relevant information on ADME-TOX related parameters, the data they provide cannot be of immediate use except maybe for some cases related to toxicity. For example, studies of clearance of a drug in some static platforms might under-predict the real values by as much as an order of magnitude[357]; opposite cases of over-prediction is just as likely if the data is taken at face value. Therefore, the field is in need of models with predictive capabilities which connect the dots and bridges the gap between the *in-vitro* platform realm and the *in-vivo* reality. In this respect there are several promising approaches discussed in the following reviews[353,358–360].

4.6 Towards a more predictive *in-vitro* microfluidic liver platform

For every *in-vitro* platform there usually is a trade-off between the cost, complexity, robustness, ease of use of and predictive capabilities. The complexity can arise from the geometry, number of handling steps, intricacy of cell seeding and number of cell types.

While the more complex models pose a barrier of entry in terms of their ease of use and cost, they may offer better physiological relevance, longevity, and ultimately higher *in-vivo in-vitro* correlation of drug response. These are three goals that we are embracing in developing a new 3D microfluidic liver platform where we combine advanced microfabrication and culture of four liver cell types in physiologically relevant ratios.

We posit that in order to best mimic the liver physiological response we need to think from the inside out and identify the smallest representative unit of the liver. The sinusoid (Fig. 1c) is possibly the smallest "functional unit", and our approach is to linearize this 3D tubular structure into a flat design (Fig. 5), where we have two chambers separated by a PET membrane to simulate the barrier function of an endothelial lining. In one recent publication[361], we have described a PDMS on PDMS version of this device and demonstrated long-term stability of function with primary hepatocytes. The flat nature of the design was motivated by the need to incorporate optical readouts using reporter cells as well as conducting regular imaging analyses. In more current embodiments, we have been able to modify our design to a PDMS on glass (Fig. 5) and in preliminary studies we have been able to sustain primary human hepatocytes co-cultured with human cell lines that represents stellate, endothelial and Kupffer cells for 28 days. The unique aspect of this approach is that cells in our device are layered similar to the liver sinusoid: LX-2s (a stellate cell line) are introduced in a pre-gel solution between hepatocytes on glass and the membrane that separates the two chambers; EA.hy926 cells (a LSEC cell line) along with Kupffer cell line U937 cells are seeded on the membrane. This layering resembles the separation between space of Disse and the sinusoidal space. We have also developed methods for primary non-parenchymal cell isolation to replace the cell lines.

The perfusion in our device is conducted on the top layer and further a layer of gel between the hepatocytes and this perfusion provides shear protection for the hepatocytes, akin to the shear protection by endothelium in the liver sinusoid. A guiding principle in our studies is to reduce the media/volume to cell ratio towards that of physiological values; this is driven by the desire to alleviate problems of large fluidic systems which are not well suited to capturing cell-cell or tissue-tissue interactions in connected systems due to dilution of signaling factors. Despite our push towards a more physiological volume/cell ratio, the volume of our device is still relatively large (100 μm in the fluidic top compartment) compared to the volume in a sinusoid with a diameter of 5–10 μm. While further reducing the dimensions would be useful in terms of better capturing interactions this is prohibited by two factors: 1) difficulties in manufacturing and ease of use and 2) more importantly the difficulty of oxygen delivery in such systems without significant advances in artificial oxygen carriers[362–364]. In terms of drug clearance, as we have discussed above, we have theoretically analyzed this problem to assess if it's possible to translate clearance values between different geometries such as a device and a sinusoid and found that a combination of channel aspect ratio and Peclet number can be used to guide the flow parameters. As a first approximation we have been using Peclet numbers to primarily guide our flow parameters. Additional computational tools and theoretical analyses will be used in this context to allow us to create a concentration field of nutrients and target drugs that comes as close to the physiological scenario as possible.

Although the initial material used in our platform is PDMS, we are currently testing thermoplastic alternatives that are much better suited for drug screening. We have also developed a new ultrathin collagen coating[208] solution for microfluidic applications which circumvents problems that arise when translating hydrogel methodology to the microfluidic realm. We will use this technique to further reduce dimensions in our device and approach more realistic scenarios for interaction between different cell types. While the proposed platform can seem complex, the ultimate aim is to provide both longevity and the best possible representation of the liver physiology resulting in high predictive capability for both acute and chronic drug challenges. As the platform matures and demonstrates its capability, it will be engineered towards more robust handling and ease of use for wider adoption. Further improvements will be made so that it is also more compatible with high-throughput and high-content imaging systems which will be useful tools for drug screening studies.

5. CONCLUSION AND OUTLOOK

It's evident from this review of *in-vitro* liver technologies that despite a large body of work conducted by a talented group of scientists and engineers, the field is still somewhat in its infancy in terms of clear standards, procedures and methods for translating *in-vitro* results to reliable *in-vivo* predictions. For this very reason funding and direction by government agencies such as in the US and EU are needed to accelerate advances in the field; especially in terms of establishing good practices, standardized challenges, overall characterization of new proposed systems. Despite the excitement currently felt by many in the field, there are critics from both industry and academia who view the current push as headlong and not very thoughtful. Whether it be the "top-down" 10 tissue mandate of DARPA or the "bottom up" tissue integration push of the NIH, critics claim that sub-par systems are being created and connected, whereas more emphasis should be placed on getting individual tissue chips to demonstrate utility and real value first. The pharmaceutical industry views these projects as interesting but sees little possibility of implementation in an industry that wants and needs robust, cost-effective, simple solutions to their problems. The current focus on human cells, although laudatory, may also be misplaced as the bulk of the toxicity data that exists is rat and other animal data and not human data. To some the smarter approach would be to develop all rat tissue models that provide excellent correlations with *in-vivo* results. Finally, there seems to be a disconnect between some the major problems that exist in drug development and what these systems may provide. It is true that if there is conflicting data among the safety results obtained from different animal species, that a human surrogate might help in deciding whether to pursue further drug development or not. In this case the most predictive, cost-effective system will win out. However, if the problem being considered is the late recognition of toxicity as larger and larger groups of clinical trial phase populations are treated, then these systems may never yield a solution, unless they are developed to represent very large populations of individuals. In theory, thousands of different tissues developed from thousands of IPS cells might be considered a pathway towards a solution, albeit cost-prohibitive in development, and questionable as to its utility until rigorously tested for its predictivity.

On the scientific front, which continue to excite academics and others for their utility in understanding health and disease, many improvements are still needed in terms of tissue engineering materials, and fabrication to engineer more physiologically relevant cellular environments; detection and analytical schemes to improve measurements and better dissect mechanisms of action; theoretical and computational approaches to better bridge the results between *in-vitro* experiments and *in-vivo* observations. This means a generation of fantastic opportunities and exciting challenges for scientists and engineers trying address these gaps with innovative ideas and solutions.

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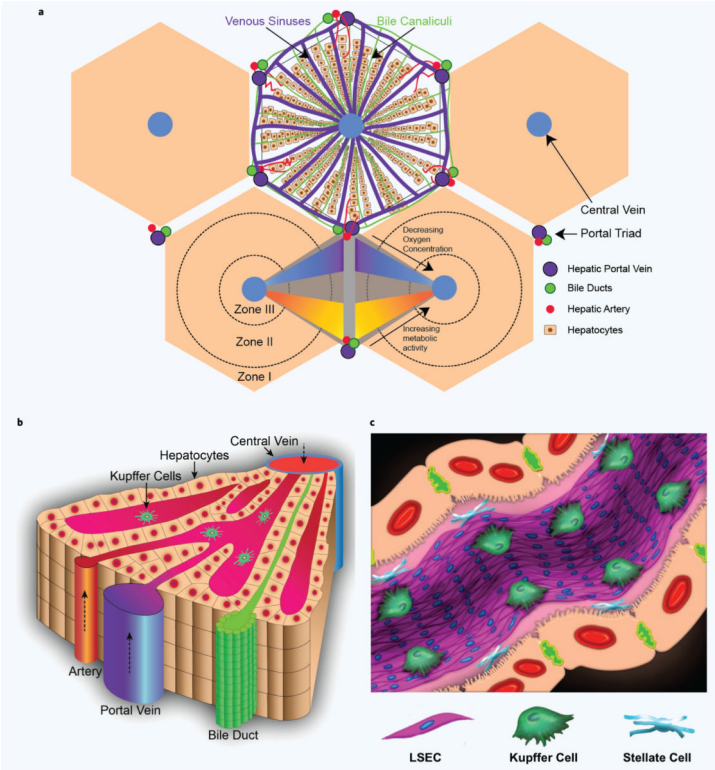

Figure 1.

Structure of the liver (**a**) A 2D cutout of the liver structure, showing the hexagonal organization of the theoretical classical lobules. At the center of each hexagon is a central vein that collects the all the blood from the sinusoids while the corners of the hexagon represent the main portal triad — comprised of the hepatic artery, portal vein and the bile duct. The acinus, the smallest functional unit of the liver, is usually described as the parallelogram whose corners consist of two neighboring central veins and the portal triads in between. (**b**) 3D illustration of half of the acinus structure. (**c**) Illustration of the liver sinusoid with the four prominent cells we discuss in this review.

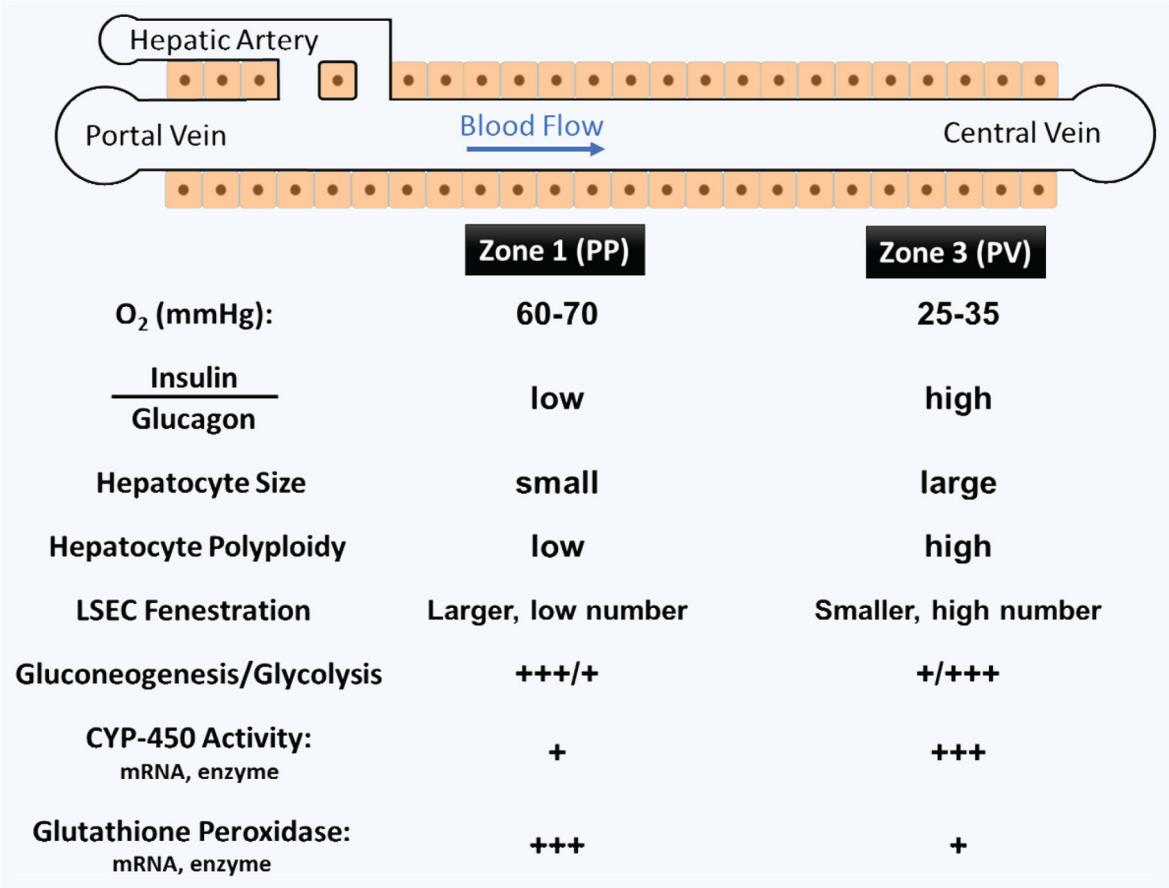

Figure 2.

Summary of zonation in a liver acinus. Gradients in oxygen, nutrients, hormonal and other factors lead to morphological as well as functional changes from the portal triad to the central vein.

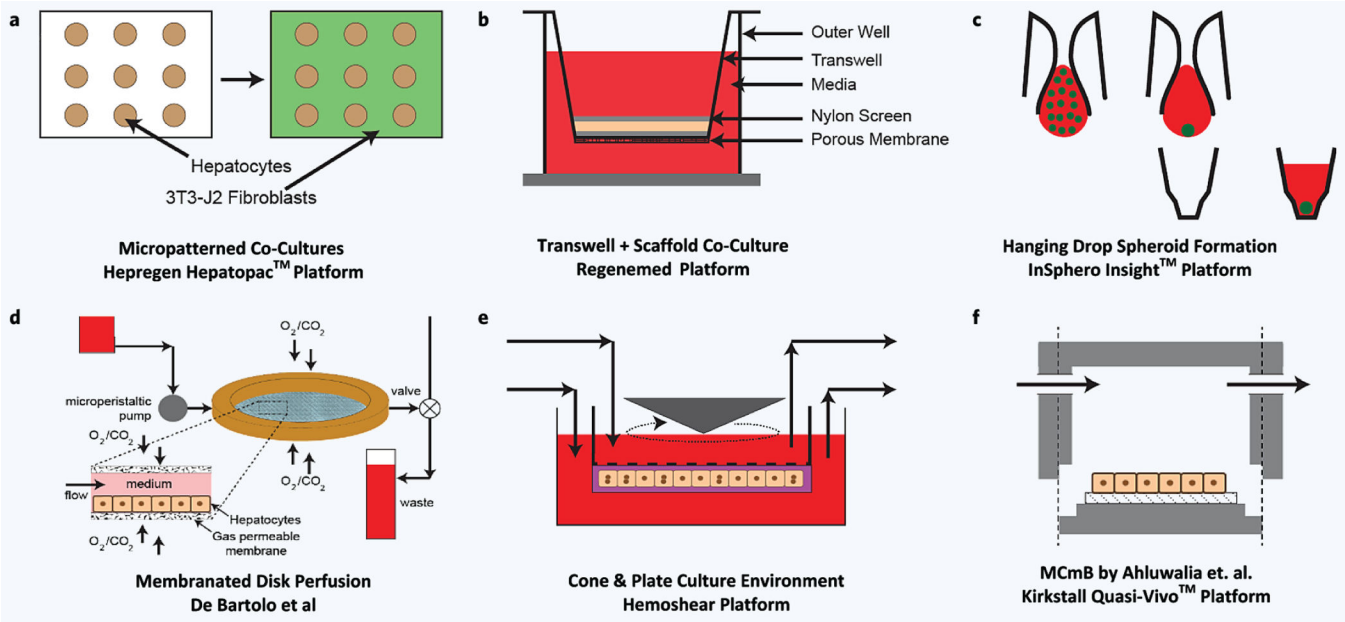

Figure 3.

Selection of macroscopic or static *in-vitro* liver models. On top row we share three important platforms which are all designed for static plate like culture while the bottom row show macroscopic perfusion culture systems. (**a**) Hepregen Hepatopac™ micropatterned co-culture. (**b**) Regenemed co-culture platform for hepatocyte and NPC co-culture. (**c**) InSphero Insight platform taking advantage of hanging drop method and concave culture wells. (**d**) Work of De Bartolo with disk like geometry and membrane oxygenation. (**e**) Hemoshear platform for hepatocyte culture with ECM for shear protection. (**f**) Multicompartmental Modular Bioreactor system (MCmB).

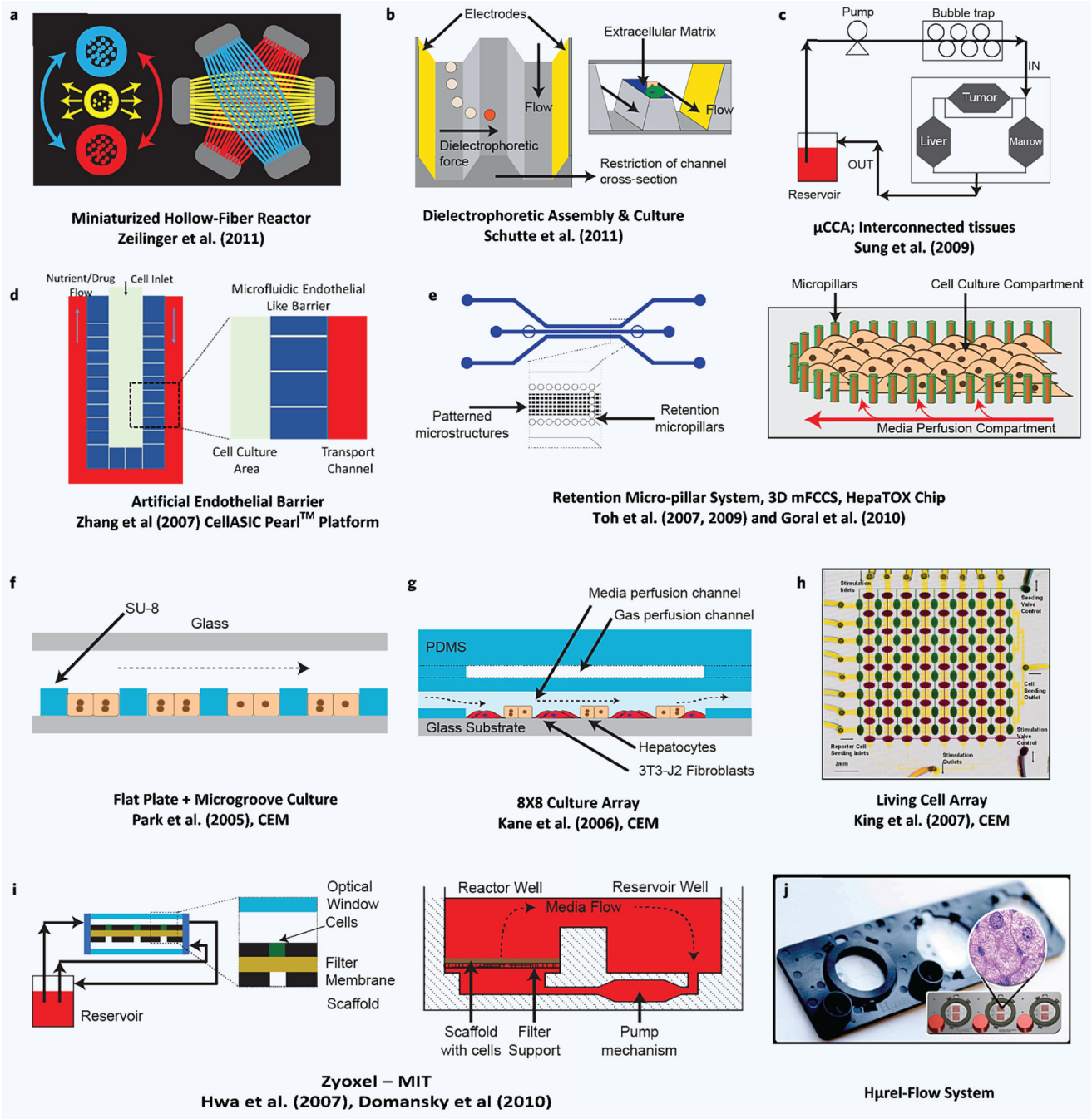

Figure 4.

Selection of recent microfluidic *in-vitro* liver models. Depiction of various microfluidic *in-vitro* liver technologies. (**a**) Zeilinger's work[260] with a miniaturized version of hollow fiber perfusion environment. (**b**) Assembly of hepatocytes and endothelial cells using dioelectrophoresis and their perfusion in the same channel by Schutte *et al.*[281]. (**c**) Micro cell culture analog systeme and multi-tissue interaction by the Cornell group. (**d**) Sinusoid like perifusion environment with an artificial (microfabricated) endothelial barrier by Lee and co-workers[278] that gave rise to the CellASIC Pearl platform. (**e**) Use of micro-pillars for

forced assembly of hepatocytes in works of Toh and Goral.[282] (**f–h**) Various technologies developed in our labs (CEM) featuring various different technologies and demonstrating high throughput capabilities. (**i**) Zyoxel — MIT culture platform for mono and co-culture of hepatic cells. (**j**) Hμrel–flow system.

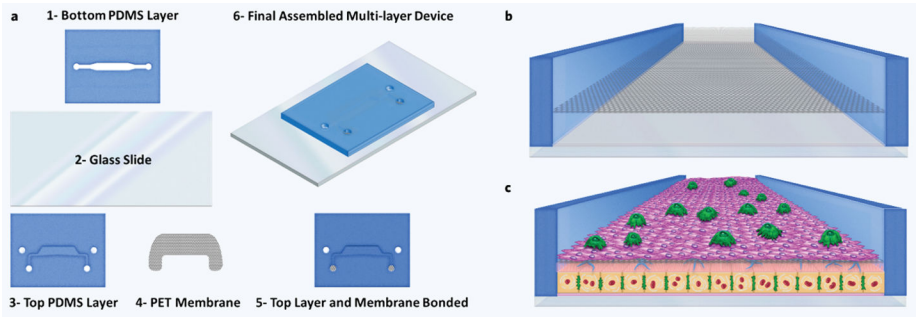

Figure 5.

The 3D microfluidic sinusoid analogue developed at our laboratories. (**a**) Parts and assembly of the current version of our device. Bottom layer (1) is first bonded to the glass slide and then the top section (5) is aligned and bonded to the (1) to create a multi-layer microfluidic device. (**b**) Cross-sectional view of final assembled device; a PET membrane separate the top and bottom fluidic channels. (**c**) Final assembled liver sinusoid like tissue in a multi-layer microfluidic device. While human hepatocytes are seeded on an ECM coat on glass slide, stellate cell line is introduced in a pre-gel solution below the membrane. Endothelial cells are introduced on top of the membrane after ECM coating and Kupffer cells are introduced on top of the endothelial lining. The layered structure of this microengineered tissue is similar to the liver sinusoid as depicted in Fig. 1.

Table 1

Approximate physical properties of important human liver cells (adapted from Ref. 69).

Cell	Type	Diameter (μm)	Volume (% of total)	Number (% of total)
Parenchymal				
Hepatocytes	Epithelial	20–30	~78	~60–65
Non-parenchymal				
LSECs	Endothelial	6.5–11	2.8	16
Kupffer Cells	Macrophages	10–13	2.1	~15
Stellate Cells	Fibroblastic	10.7–11.5	1.4	8

Table 2

Comparison table of historical *in-vitro* platforms.

Platform name	Description and notes	Cell types PC, NPC NPC types	Longevity/viability (days/% live)	Albumin/urea (stability and % control)	Enzymatic activity (enzymes and % efficacy)	Drug screening sensitivity and specificity	Figure [references]
Perfused Platforms							
Micro Hollow Fiber Reactor	Macroscale 3D perfusion-liver bioreactor with microscopic hollow fiber arrangement	Primary human hepatocytes	23 days, but no direct viability measurement at the end of the study	Comparable albumin, urea, and glucose production in 800 mL, 8 mL, and 2 mL bioreactors	Stable CYP1A, CYP2C9, and CYP3A activity in 2 mL bioreactor for up to 23 days. Comparable (LDH) and (AST) release in 800 mL, 8 mL, and 2 mL bioreactors	No data	Fig. 4a [260]
Dielectrophoretic 3D liver assembly device	Dielectrophoretic assembly of hepatocytes and endothelial cells	Human hepatocytes and human liver endothelial cells	No data	No data	No data	No data	Fig. 4b [281]
µCCA (micro cell culture analog) cornell group	3D hydrogel culture in connected microfluidic chambers. Multiple incarnations	Human hepatocarcinoma cell line HepG2/C3A	3 days, >90% viability	No data	No data	No data	Fig. 4c [303, 305]
Pearl liver sinusoid system (Millipore, formerly from CellAsic Inc.)	Microfluidic perfusion with microfabricated artificial endothelial barrier	**1** Human/rat hepatocytes **2** Human hepatocarcinoma cell line, HepG2 **3** Human/rat hepatocytes	**1** 7 days, > 90% viability **2** 8 days **3** Upto 30 days of operation	**1** No data **2** Day 4 albumin data, 3 fold more albumin secreted in microdevice compared with on a static plate culture	**1** No data **2** 8 days of albumin secretion **3** No data	**1** No data **2** No data **3** No data	Fig. 4d **1** [277] **2** [278] **3** [279]
3D-µFCCS (3D microfluidic cell culture system) Later renamed to 3D HepaToX Chip	3D microfluidic channel-based cell culture system	Co-culture of rat hepatocytes and rat BMSCs and co-culture of human hepatocarcinoma cell line (HepG2) and a human breast cancer cell line (MCF7)	3 days, no direct viability measurement	Albumin production for 3 days. Comparable albumin in 3D-µFCCS and 2D monolayer cultures	UDP glucuronyltransferase (UGT) activity measurement for 3 days based on 4-methylumbelliferyl glucuronide (4-MUG) formation. Hepatocytes in the 3D-µFCCS maintain UGT activity at significantly higher level than in 2D monolayer cultures	No data	Fig. 4e **1** [283] **2** [203]
Micro-pillar Array Liver Sinusoid Device	Microfluidic, no ECM, micro-pillar array	Cryopreserved human hepatocytes	14 days constant viability	No data	No data	No data	Fig. 4e [282]
LiverChip from MIT and ZYOXEL Inc.	3D perfused microreactor culture system later translated to perfused multiwell plate Multiple incarnations	Rat hepatocytes and spheroids Co-culture of rat hepatocytes and LSEC Co-culture of rat hepatocytes, LSEC, stellate, and Kupffer cells 4. Hepatocyte spheroids 5. Rat hepatocytes, LSEC, stellate, and Kupffer cells	**1** 15 days **2** 13 days, no direct viability measurement at the end of the study **3** 7 days for hepatocytes, 6 days for LSEC's in co-culture model	**1** 15 days of stable albumin and urea secretion **2** No data **3** No data **4** 14 days of stable albumin and urea secretion	**1** No data **2** No data **3** No data **4** Thorough comparative evaluation of important phase I and II metabolism and inducibility of some enzymes	**1** No data **2** No data **3** No data **4** No data **5** 6 day screening of 9 drugs with/ without LPS	Fig. 4i **1** [292, 293] **2** [294] **3** [295] **4** [296] **5** [226]

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Platform name	Description and notes	Cell types PC, NPC NPC types	Longevity/viability (days/% live)	Albumin/urea (stability and % control)	Enzymatic activity (enzymes and % efficacy)	Drug screening sensitivity and specificity	Figure [references]
			4 No quantified direct evaluation (14 days) **5** LDH release characterized on day	**5** No data	**5** No data		
HμREL®-Flow Liver Culture Device	Microfluidic microscale cell culture analogue (CCA) system (HμREL® chip)[15,16]	**1** Cryopreserved human hepatocytes **2** Cryopreserved human hepatocytes and NPC's	**1** >95% viability data shown for 24-hour culture **2** 6 days under flow and 9 days in static culture, no direct viability measurement with time	**1** No data **2** No data	**1** No data **2** CYP2B6, CYP2D6, CYP3A4, CYP2C19, CYP1A2, CYP2C8/9 activity in day 1 cultures was higher in flow-based co-culture systems compared to static plate co-culture systems	**1** No data **2** Tested metabolic clearance rates of six drugs in flow and static cultures. Showed superior metabolite formation in flow co-culture systems	Fig. 4j **1** [297] **2** [227]
Membrane Based Perifusion Bioreactor	Perifusion of multiple cell types using a membrane	Hepatocytes and epithelial cell line RL-ET-14	14 days, LDH leakage, no direct viability measurement data	No data	Higher EROD activity and inducibility when perfused. Even higher when a co-culture is established. Activity is stable only when both are used	Tested several drugs for cytotoxicity and metabolism. No large scale screening	[257]
Oxygen-permeable Membrane Liver Bioreactor	Macroscopic device 25 μm membrane	Cryopreserved human hepatocytes	33 days; no direct viability measurement	Stable urea (33 days), stable albumin (22 days), protein secretion	No direct enzymatic activity measurement	Tested effects of diclofenac and interval IL-6 stimulations	Fig. 3d [266]
Hemoshear Liver Platform	Macroscopic transwell sandwich culture based perfusion device	Rat hepatocytes	14 days, no direct viability measurement	More albumin and urea production in perfused culture for 14 days compared to static cultures	CYP1A1, CYP1A2, CYP2B1, CYP3A2 expression levels higher in perfused cultures compared with non-flow cultures on day 7	Tested the effects of 3-methyl cholanthrene and dexamethasone in perfused and non-flow cultures on day 7	Fig. 3e [269]
Modular Bioreactor System, McMB	Macroscopic perfusion chambers in series and parallel	Human hepatocytes	7–21 days, no direct viability data, shows morphologically healthy cells on day 14	No data	Shows that detoxification genes are downregulated in static culture conditions but are well maintained in phase I, CYP, enzymes are highly upregulated under flow conditions	No data	Fig. 3f [261–265]
Static Platforms							
Hepregen Hepatopac™	Micropatterned static culture	Rat/human hepatocytes with 3T3-J2 mouse fibroblasts	> 30 days, various methods	Thorough characterization of albumin, urea secretion	Thorough characterization of phase I, II and II activities in different publications	Large scale drug screening (~45 drugs) towards specificity and sensitivity[21]	Fig. 3a [223–225, 231, 234, 243–248]

Platform name	Description and notes	Cell types PC, NPC NPC types	Longevity/viability (days/% live)	Albumin/urea (stability and % control)	Enzymatic activity (enzymes and % efficacy)	Drug screening sensi- tivity and specificity	Figure [references]
Regenemed Static Scaffold Total Liver Culture	Transwel + nylon scaffolding co-culture	Rat and human hepatocytes and NPC fractions	~77 days	Stable albumin, urea, fibrinogen and transferrin secretion over the culture period	Characterization of basal and induced activity of CYP3A4, CYP1A1 and CYP2C9	7 drug screening. Demonstrated species differences as well as effect of inflammation of drug response	Fig. 3b [124]
InSphero Insight Liver Spheroid Culture System	Hanging drop/spheroid culture co-culture	Rat/human hepatocytes, Kupffer or LSECs	~28 days, ATP measurement	No data	Characterization of some phase I and phase II enzymes at gene expression and activity level (workshop, website and few publications)	Some information on specific drug toxicities using the model is available. LPS stimulation of the co-culture spheroids and improved response has been shown	Fig. 3c [162, 249, 250]
Hμrel Human (Hμrel Static) Liver Culture Model	Coplanar mixed co-culture	Canine hepatocytes and a mixture of non-parenchymal cells compared to primary human hepatocytes and HepG2 cell line	31 days	No data	Characterization of basal enzymatic activity	50 drug screening towards specificity and sensitivity	[251]

Table 3

Proposed three-stage characterization of *in-vitro* liver models.

Stage 1 — Viability and functional stability		
Cell viability	Imaging via complementary fluorescent dyes	Metabolic assays i.e. MTT, Presto Blue
Functional assays	Synthetic (albumin, fibrinogen)	Detoxification (urea)
Stage 2 — Drug transport and metabolism		
Enzymatic activity, expression	Phase I CYP 450s (1A2, 3A4, 2C9, 2C19, 2D6, 2B6) activity, inducibility and expression	Phase II UGT, SULT and others
Drug transporter activity	SLC superfamily OATPs NTCP, OCTs	ABC superfamily MRP2, BCRP, MDR1 and MDR2
Stage 3 — Sensitivity and specificity		
Drug panel studies	Efficacious drugs (safe, non-safe)	Non-efficacious drugs (safe, non-safe)