Microfluidic cell culture platforms to capture hepatic physiology and complex cellular interactions

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<u>Running title</u>**:** Microphysiological systems capturing *in vivo* hepatic function

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<u>Document Statistics</u>:

Number of text pages: 20
Number of tables: 0
Number of figures: 5
Number of references: 86
Number of words in abstract: 341
Number of words in introduction: 590

<u>Nonstandard abbreviations used in the paper</u>:

Microphysiological Systems (MPS)

Cytochrome P450 (CYP450)

Embryonic Stem Cells (ESCs)

Induced pluripotent stem cells (iPSC)

Cytochrome P450 1A1/2 (CYP1A1/2)

Human endothelial cell line (Ea.hy 926)

Human hepatic stellate Cell line (LX-2)

Human monocyte cell line (U-937)

Cytochrome P450 3A4 (CYP3A4)

Cytochrome P450 2B (CYP 2B)

Cytochrome P450 3A (CYP 3A)

3-Methylcholanthrene (3-MC)

Poly dimethoxysiloxane (PDMS)

Embryonic mouse fibroblast cell line (3T3-J2)

Polymethyl Pentene (PMP)

Cyclic olefin copolymer (COC)

Human epithelial breast cancer cell line (MCF-7)

5-Fluorouracil (5-FU)

Hepatocyte growth factor (HGF)

Epidermal growth factor (EGF)

Insulin-like growth factor (IGF)

Fibroblast growth factor 7 (FGF7)

Transforming growth factor (TGF-β)

Connective tissue growth factor (CTGF)

Abstract

Animal models such as rats and primates provide body-wide information for drug and metabolite responses, including organ-specific toxicity and any unforeseen side effects on other organs. While effective in the drug screening process, their translatability to humans is limited due to the lack of high concordance and correlation between enzymatic mechanisms, cellular mechanisms and resulting toxicities. A significant mode of failure for safety prediction in drug screening is hepatotoxicity, resulting in ~30% of all safety-related drug failures and withdrawals from the market. The liver is a multi-functional organ with diverse metabolic, secretory and inflammatory response roles and is essential for maintaining key body functions. Conventional cell culture platforms (such as multi-well plate cultures) and metabolic enzyme (microsomes, CYP450 enzyme) systems have been routinely utilized to assess drug pharmacokinetics and metabolism. However, current *in vitro* models often fail to recapitulate the complexity and dynamic nature of human tissues, imposing a heavy reliance on *in vivo* testing using preclinical species that have metabolic processes, disease mechanisms and modes of toxicity distinct from humans. Recently, microphysiological systems (MPS) have gained attention as powerful tools with the potential to generate human-relevant information that can supplant and fill the gap of knowledge between preclinical animal models and simpler, conventional *in vitro* cell culture systems. Developments in microfabrication technologies for generating complex microfluidic systems, along with the ability to establish and maintain multi-cellular models to capture dynamic, human-relevant behavior, have provided new avenues to generate such physiologically-relevant systems. These MPS platforms, when designed and developed with *in vivo*-derived design parameters, have the potential to capture key aspects and better mimic organ functionality. In this review, we discuss developments in microtechnologies for fabricating, establishing and maintaining hepatic cell culture systems, with a specific focus on models that aim to capture *in vivo* physiology *in vitro*. By designing microscale systems to impart specific *in vivo* physiological parameters, it is possible to

create a dynamic system that can capture multiple aspects of the hepatic microenvironment,

bringing us closer to a comprehensive *in vitro* testing platform for hepatic responses and toxicities.

Introduction

The liver is a central organ performing critical roles within the human body, with metabolic, storage, synthesis, and filtration functions, as well as mediating inflammatory responses (Lee and Senior, 2005; Godoy *et al.*, 2013; Lauschke *et al.*, 2016). The drug screening process relies heavily on animal models to evaluate drug metabolism and its body-wide influence (Olson *et al.*, 2000; Greaves *et al.*, 2004). Hepatotoxicity accounts for ~ 50% of cases of acute liver failure and remains a major factor responsible for withdrawal or restricted use of approved drugs (Olson *et al.*, 2000; Schuster *et al.*, 2005; Wilke *et al.*, 2007; Kaplowitz, 2013). Apart from drug hepatotoxicity, liver-generated metabolites are transported to other tissues in the human body through the systemic circulation, resulting either in therapeutic effects (e.g., pro-drugs) or unwanted side effects (Bale, Moore, *et al.*, 2016; Hughes *et al.*, 2017).

In vitro cell culture is an attractive alternative to animal models and *ex vivo* organ culture, and is an integral component of biomedical research and drug screening (Guillouzo, 1998; Zguris *et al.*, 2005; Emoto *et al.*, 2006; Ewart *et al.*, 2018). Hepatic platforms with varying complexity and composition have been actively used in the development of therapeutic drugs, providing information regarding hepatic biology, pharmacokinetics and pharmacodynamics (Godoy *et al.*, 2013; Lauschke *et al.*, 2016; Ewart *et al.*, 2018). Current state-of-the-art techniques for assessing human-relevant hepatic responses include *in vitro* models comprising either primary hepatocyte monocultures or co-cultures in 2D and 3D formats (Fourches *et al.*, 2010; Godoy *et al.*, 2013; Lauschke *et al.*, 2016). However, most of these systems are hepatocyte-centric static systems, and fail to capture the dynamic and multi-cellular nature of the liver. Recently, developments in microscale manufacturing technologies have enabled the construction of well-defined microenvironments mimicking native microarchitectures, thereby leading to remarkable advances in recapitulation of niche environments of organs *in vitro* (Bale *et al.*, 2014; Bhatia and Ingber, 2014; Wikswo, 2014; Abbott and Kaplan, 2015; Yoon No *et al.*, 2015; Bale, Moore, *et al.*, 2016;

6

Lauschke *et al.,* 2016; Ewart *et al.*, 2018). These microscale cell culture platforms represent attractive alternatives to animal models, providing easily accessible, highly reproducible and human-relevant information in advance of further pre-clinical and human studies. Key requirements for the development of such MPS platforms for capturing liver functionality are aimed at 1) constructing complex microscale structures suitable for mimicking *in vivo* microarchitecture, cellular composition and interactions, 2) simulating liver pathophysiology under an *in vivo*-like microenvironment, and 3) providing a rapid, easy and high-throughput process for screening of diverse treatment methods and toxic materials using a small number of human cells. Further, capturing hepatic responses in MPS models can drive the generation of multi-organ MPS systems that are capable of capturing inter-organ interactions and assaying for compounds and their metabolites, and drug responses (Bale, Moore, *et al.,* 2016; Hughes *et al.*, 2017).

In this review, we provide an overview of current state-of-the-art microtechnologies and strategies aiding the development of liver MPS platforms. We describe the novel technical advances and approaches adapted in microfluidic organ-on-chip systems to extend the longevity of hepatic cultures and to recapitulate the microenvironment of the liver. Studies have shown that recapitulation of physiological levels of mass transport, fluid flow, media-to-cell ratios and oxygen supply to the hepatic cultures enhances hepatic function, and allows for the interrogation of chemicals at a human translatable scale. Advanced liver MPS platforms, both in recapitulating liver physiology and implementation in high-throughput formats represent an attractive option for investigating healthy and disease models of the liver, cellular interactions and therapeutic responses.

Microarchitecture of Liver

The basic structural unit of the liver is the hepatic lobule, a roughly hexagonal unit consisting of parenchymal (hepatocytes) and non-parenchymal (Kupffer, stellate, sinusoidal endothelial, and cholangiocytes) cells between the portal triad and the central vein (Figure 1A). Cells within the liver have well-defined functions with hepatic responses to any external stimuli or perturbation (chronic or acute) being a cumulative response of the constituent cells. In addition to multiple cell types, the liver is a highly vascularized organ perfused by a dual blood supply, with arterial blood via the hepatic artery and venous blood via the portal vein. Spent blood from the liver is collected into the central vein, and the bile ducts collect bile, which is then concentrated in the gall bladder. The functional unit of the liver is the acinus, comprising of sinusoidal capillaries which are defined by the venous blood capillary connecting the portal triad (hepatic artery, hepatic vein and bile ducts) draining into the central vein (Figure 1B). The sinusoid is lined with a layer of fenestrated endothelial cells (Wisse *et al.*, 1996; Braet and Wisse, 2002) that regulate nutrient and xenobiotic transport, and a layer of hepatocytes (major metabolic component). The stellate cells (Friedman, 2008), matrix producing, myofibroblast-like cells, reside in the matrix between sinusoidal endothelial cells and hepatocytes, identified as the space of Disse. Kupffer cells (Wisse *et al.*, 1996; Haubrich, 2004; Bilzer *et al.*, 2006) are the resident macrophages that reside in the sinusoid. The oxygen-rich arterial blood from the hepatic artery mixes with the venous blood via the portal vein that is low in oxygen saturation but rich with hormones and nutrients from the gastrointestinal tract. The mixed blood supply travels along the liver sinusoid to the central vein, generating a unique, complex environment (Figure 1B) (Vollmar and Menger, 2009). Hepatocytes utilize high amounts of oxygen, and are involved in the secretion and metabolism of several molecules, and thus the environment within the sinusoid is dynamic, driven by hepatocyte metabolism. In addition, the transport of nutrients and oxygen from the liver sinusoid occurs through the endothelial cells and the space of Disse, creating a unique environment whose

physiological responses are driven by the mass transport occurring within the micro-architecture of the liver sinusoid.

Micro-technologies for Hepatic culture

Cell types, culture systems and heterotypic interactions: Key hepatic model developments have focused on culturing primary hepatocytes aimed at (1) extending the longevity of hepatocyte cultures (viability, protein secretion and enzymatic activity) and (2) capturing multi-cellular complexity and responses. In addition to precision-cut liver slices, which capture the complex microenvironment of the liver, metabolic enzyme components, and *in vitro* cell-culture systems based on cell lines, primary cells and stem cell-derived cells have been investigated, providing a variety of levels of function and responses. Liver slices comprise multiple cells of the liver and capture the tissue complexity, however they have a relatively short life (several days) in culture (Vickers *et al.*, 2004; van de Bovenkamp *et al.*, 2006; Olinga and Schuppan, 2013). Isolation of purified primary hepatocyte fractions enables their incorporation in suspension and plate cultures, ideal for developing assays for evaluating drug metabolism, and widely used in various culture formats (Godoy *et al.*, 2013; Lauschke *et al.*, 2016). Major advances in extending the longevity of primary hepatocyte culture include sandwich culture (Dunn *et al.*, 1991, 1992), micro-patterned co-cultures (Bhatia *et al.*, 1999; Khetani and Bhatia, 2008), 3D printing (Nguyen *et al.*, 2016; Nguyen and Pentoney, 2017), and spheroid formation (Messner *et al.*, 2013). These models often incorporate extracellular matrix materials, and co-cultures, extending hepatocyte cell cultures for several weeks, and thereby providing a suitable platform for drug testing. Collagen (or matrigel) sandwich primary hepatocyte provide an *in vivo*-like environment, stabilizing and enabling hepatocyte polarization driven by cell-cell contacts and leading to the formation of bile junctions in culture. Such stabilization allows the recovery of several hepatic secretory and metabolic functions in a relatively short time frame (3-4 days) and allowing retention of function for several weeks (Dunn *et al.*, 1991, 1992; Bale, Golberg, *et al.*, 2015). Hepatic co-cultures generated using

micro-patterning methods and co-cultures enable hepatic stabilization, driven by the interactions of secreted matrix, integrins and secreted molecules (Yaakov *et al.*, 2006; Bale, Golberg, *et al.*, 2015; Bale, Geerts, *et al.*, 2016; Lauschke *et al.*, 2016). Micropatterned hepatic co-cultures have been developed with fibroblasts (Bhatia *et al.*, 1999) and hepatic-relevant non-parenchymal cells, (Yaakov *et al.*, 2006) providing an environment that can capture paracrine and autocrine signaling functionality. Spheroid and 3D printing models are driven by the self-assembly of hepatocyte cultures, enhancing hepatic functionality by mimicking the 3D environment and increasing cell-cell contacts (Messner *et al.*, 2013; Nguyen *et al.*, 2016). In addition to hepatocytes, isolation of non-parenchymal cells to obtain pure populations has been challenging, although advances in methods are currently yielding Kupffer and hepatic stellate cells that can be utilized in developing hepatic co-cultures. Hepatocyte co-cultures with non-parenchymal cell fractions (Kostadinova *et al.*, 2013; Esch *et al.*, 2015; Bale, Geerts, *et al.*, 2016; Du *et al.*, 2017), Kupffer cells (Tukov *et al.*, 2006; Zinchenko, Culberson, *et al.*, 2006; Zinchenko, Schrum, *et al.*, 2006), stellate cells (Thomas *et al.*, 2006) and sinusoidal endothelial cells (Hwa *et al.*, 2007; Kim and Rajagopalan, 2010; Bale, Golberg, *et al.*, 2015) are providing valuable information regarding cellular cross-talk and hepatic responses.

While significantly extending the cell-culture life, the limited quantity of primary hepatocytes and non-parenchymal hepatic cells from isolations limits their extensive use, particularly in high-throughput culture systems. Hepatic cell lines (such as Hepa-RG™ and HepG2) are attractive alternatives to primary hepatocytes in multiple cell-culture models, but lack many of the active cellular machinery and metabolic components (when compared with primary hepatocytes), limiting their applicability to such *in vitro* screening platforms (Szabo *et al.*, 2013). Emerging alternatives to primary hepatocytes are populations of renewable cells from embryonic stem cells (ESCs) and adult-induced pluripotent stem cells (iPSC) that can be matured into functional, hepatocyte-like cells (Yi *et al.*, 2012; Shan *et al.*, 2013; Subba Rao *et al.*, 2013). Stem-cell-derived

hepatocyte-like cells offer a unique opportunity to revolutionize pharmacological and toxicological assessment by providing a large supply of cells and representing genetic diversity, however, the current state-of-art cell development methods require further improvement before their incorporation into main-stream toxicology assays (Shan *et al.*, 2013; Godoy *et al.*, 2015).

Capturing physiological relevance in MPS platforms

The liver, with its complex architecture and multiple functions, is in many ways an ideal organ for *in vitro* model development utilizing microfabrication techniques to generate models that precisely control the microenvironment, while accommodating cellular complexity to capture heterotypic interactions. Hepatocytes, being the major fraction of the liver and active metabolic component, have been the focus of numerous scientific studies. Key advances in hepatocyte stabilization and culture *in vitro* include sandwich, spheroid and micro-patterned cultures that have extended static hepatocyte cultures for weeks (Godoy *et al.*, 2013; Yoon No *et al.*, 2015; Lauschke *et al.*, 2016). Recent advances in the development of microfluidic systems have focused on translating hepatocyte culture to dynamic cell culture systems, mimicking an *in vivo* environment (Soldatow *et al.*, 2013; Bale *et al.*, 2014; Bhatia and Ingber, 2014).

The liver sinusoid comprises a complex microenvironment with multi-cellular composition, capillary fluid flow, and dynamic responses to external stimuli (Reilly *et al.*, 1981, 1982; McCuskey, 2008). The biochemical microenvironment consists of growth factors, hormones, signaling molecules, and reaction products that combine to produce complex signaling pathways contributing to the fate of the cells. Further, chemical and hormonal gradients exist within the microenvironment due to diffusion through the matrix materials (space of Disse), modulated by cellular secretion, enzymatic functions and flow. For MPS platforms, it is important to not only capture the cellular complexity, but also aspects of physiological exchange of materials between the sinusoid blood flow, hepatocytes, and multiple cells in the liver. In addition to mimicking liver physiology, MPS platforms need to capture the cellular interactions and associated feedback responses that modulate hepatic behavior. Recently, microscale technologies have become capable of generating physical structures that enable assaying the coupling between biochemical gradients and physical cues, for evaluation of combinatorial effects of soluble factor signaling and

cell-cell and cell-matrix interactions. Several physiological phenomenon have been explored as part of the MPS platform design and function.

1. Nutrient transport: Hepatocytes *in vivo* are arranged in monolayer plate structures enclosed by the extracellular matrix of the space of Disse, and are faced on both sinusoidal surfaces by blood (Figure 1 A,B). Nutrient transport to hepatocytes in the sinusoid primarily occurs through (1) sinusoidal blood flow, and (2) diffusion through the space of Disse, generating a continuous nutrient gradient parallel to the axis of blood flow (Figure 1b). Hepatocytes in the sinusoid are surrounded by extracellular matrix in the space of Disse, protecting them from any direct contact with blood flow (Reilly *et al.*, 1981, 1982; Wisse *et al.*, 1996; Vollmar and Menger, 2009; Géraud *et al.*, 2010), and thus any fluid shear imparted by a perfusion flow rate on the culture medium becomes the limiting factor for designing MPS systems. Several microfluidic systems have overcome this limitation by designing models that incorporate a physical separation between regions of flow and cells, in the form of endothelial cell-like barriers with dedicated hepatocyte culture channels (Lee *et al.*, 2007) or microfluidic bilayer devices with tissue culture membrane separating flow and cell-culture channels (Bader *et al.*, 1998; Borenstein *et al.*, 2003; Dash *et al.*, 2013; Hegde *et al.*, 2014; Ljupcho *et al.*, 2015; Du *et al.*, 2017)

In their work, Lee and co-workers have utilized microfabrication techniques to generate a two-channel microfluidic device with an endothelial cell-like barrier that physically separates the cell culture and nutrient transport compartments (Lee *et al.*, 2007). The utilization of a endothelial cell-like barrier separating the cell culture chamber from the media flow chamber enables independent manipulation of flow to precisely control, and thus optimize mass transport to hepatocytes (Figure 2A). Flow in the endothelial cell-like barrier is defined by the channel thickness, providing a diffusion-dominated nutrient exchange and by designing the channels to mimic mass transport in the space of Disse, and manipulating flow to mimic the mass transport of proteins, such as matching the Péclet number using *in vivo*-derived parameters. Utilizing this system, Lee and co-

workers demonstrated hepatic stabilization and functional maintenance for up to 7 days; and hepatic response to diclofenac as a test compound. Similar endothelial-cell like barrier strategies have been developed to culture hepatocytes and cell lines long-term (Toh *et al.*, 2007; Goral *et al.*, 2010).

Microfluidic bilayer models provide a similar microarchitecture for hepatocyte culture by separating the media flow chamber from the cell culture chamber and protecting hepatocytes from any flow-induced shear stresses (Borenstein *et al.*, 2003; Hegde *et al.*, 2014; Ljupcho *et al.*, 2015; Du *et al.*, 2017). The basic structure of microfluidic bilayer systems is the overlap of two independently accessible microfluidic channels, with a tissue-culture membrane separating the two channels. By incorporating primary rat hepatocytes in a collagen-sandwich in the bottom channel, and providing media by flow in the top channel, Hegde and coworkers demonstrate the importance of achieving optimal flow rates for hepatic stabilization and long-term hepatic function (Figure 2B) (Hegde *et al.*, 2014). By optimizing the media flow, the authors demonstrated increased secretions, metabolic activity, and bile junction formation, and increased collagen production by the hepatocytes, suggesting a level of hepatocyte stabilization that mimics an *in vivo*-like environment. This results in increased secretion (albumin, urea), metabolic function (CYP1A1/2) and formation of bile junctions within the hepatocyte monolayer culture. A key aspect of the collagen sandwich model is the stabilization and polarization of hepatocytes, driven by the collagen secreted by hepatocytes *in situ* (Dunn *et al.*, 1989, 1991). These authors demonstrated collagen-driven stabilization as a driving factor for hepatic culture, showing increased expression of Collagen 1A1, 4A1 and 5A1 in flow when compared with static culture conditions. Further, by incorporating cis-Hydroxyproline (an isomer of proline essential for collagen synthesis) in the media the authors demonstrate the loss of hepatocyte monolayer integrity (driven by the disruption of triple helix structure of collagen by cis-Hydroxyproline), and subsequently loss of function (Uitto *et al.*, 1975). Using a similar bilayer model, Prodanov and co-workers incorporated

multiple hepatic cells, including primary hepatocytes, cell lines representing endothelial (Ea.hy 926), Stellate (LX-2) and Kupffer (U-937) cells capturing the major cell types present in the liver sinusoid (Ljupcho *et al.*, 2015). Hepatocytes and stellate cells were cultured in one chamber, while the flow channel was comprised of endothelial cells as a mono-layer (exposed to flow) and Kupffer cells in the flow channel mimicking the architecture of the sinusoid (Figure 2C). Mass transport in the device (between flow channel and hepatocytes) is optimized using Péclet number estimates, generating a hepatic culture with optimal functions (secretions, bile canaliculi formation and CYP3A4) for up to 4 weeks.

Dash and co-workers have utilized a combination of a spinning cone and perfusion flow to achieve controlled hemodynamics, mimicking the sinusoidal and interstitial blood flow to hepatocytes in culture (Dash *et al.*, 2013). The system used a standard transwell plate, with hepatocytes cultured in a standard sandwich culture format on the underside of the membrane, and a spinning cone producing shear conditions on top of the membrane (Figure 2D). In combination with media perfusion in both the well and transwell, the authors demonstrated recovery of hepatic function, measured by albumin and urea secretions and polarization

2. In vitro zonation: A key physiological feature of the liver sinusoid is zonation, identified with cells of varying metabolic and enzymatic functionality along the capillary (Jungermann and Katz, 1982; Lindros, 1997). Immunohistochemical staining of tissue sections reveals this signature variation in hepatic function, presenting as compartmentalization of oxidative energy, carbohydrate, lipid and nitrogen metabolism, bile conjugation and xenobiotic metabolism (Giffin *et al.*, 1993). This change in functionality occurs over the length of the sinusoid, which is approximately 25 hepatocytes long. For instance, zone 1 hepatocytes are efficient in glucose uptake, urea formation, amino acid breakdown and phase II conjugation of molecules, while zone 3 hepatocytes are efficient at glucose uptake, glutamine formation, alcohol degradation and phase I metabolism (Figure 3A). This variation along the length of the liver sinusoid contributes to the

overall function of the liver as a glucose regulator and process several environmental agents and xenobiotics.

Methods to isolate location-specific hepatocytes from the liver using micro-dissection have met with limited success and do not provide cells with significant quantity for extensive use in *in vitro* models (Teutsch, 1986; Bars *et al.*, 1992). Several studies have utilized mixed populations of isolated hepatocytes in culture to generate an *in vitro*-like zonation in a continuous hepatocyte culture by varying oxygen and environmental cues, such as hormones and chemicals. In an effort to capture zonal features of the liver, Allen and co-workers developed a biomimetic flat-plate bioreactor with either hepatocyte monoculture or hepatocyte-fibroblast co-cultures generating an oxygen gradient along the axis of flow (Allen and Bhatia, 2003; Allen *et al.*, 2005). The custom flat-plate bioreactor is manufactured from oxygen impermeable polysulfone and designed to receive a microscope slide seeded with hepatocyte cultures, as well as integrated with a media oxygenator upstream and oxygen monitor downstream of the bioreactor (Figure 3B). Cells are seeded onto a microscope slide and stabilized in static culture for 5-7 days prior to incorporation into the bioreactor. By introducing oxygenated media and flowing through the length of the cell culture, an oxygen gradient is generated within the bioreactor, driven by the balance of oxygen content in the media, consumption by cells and the flow rate of media. Spatial expression of metabolic enzymes (CYP2B, CYP3A) showed a location-dependent expression along the length of the flow, suggesting that oxygen-dependent (and location-dependent) function is generated along the length of media flow within the bioreactor. Further, the authors demonstrate location/zone-dependent toxicity of acetaminophen, a compound known to target zone 3 hepatocytes specifically.

In a different study, McCarty and co-workers developed a microfluidic device to generate a continuous gradient across a hepatocyte culture to capture hepatic zonation using chemical and hormonal gradients (McCarty *et al.*, 2016). The model generates spatially-controlled zonation

across multiple hepatocyte metabolism levels through controlled application of hormonal and chemical gradients (Figure 3C). A key difference in the device is generation of "zonation" perpendicular to the flow, while the gradient exists in the direction of flow *in vivo*. Utilizing this model, the authors demonstrate variations in carbohydrate and nitrogen metabolism in a glucagon-insulin gradient; and enzymatic variations using chemical (3-Methylcholanthrene, 3-MC) gradient. Further, variations in enzymatic activity within the chemical-driven zonation are revealed by assaying for acetaminophen toxicity zone-dependent response. In a recent work, similar devices have been developed to capture zonation using hormones and inducers in both rat and human hepatocyte cultures (Kang *et al.*, 2018).

3. Oxygen transport in in vitro systems: Hepatocytes are metabolically active cells requiring high amounts of oxygen to perform various enzymatic processes. *In vivo*, the liver receives two-thirds of its blood supply from partially oxygen-depleted venous blood and one-third from fully oxygenated arterial blood, and active consumption of oxygen from the blood results in the formation of zonation. Development of cell culture systems and microfluidic systems in particular requires the careful consideration of requirements for oxygen transport to the hepatocytes. *In vitro*, oxygen requirements by hepatocytes vary depending upon the stage of the culture (seeding, stabilization and continued culture), and it is essential to provide sufficient oxygen at all stages. Depending on the ability to interact with oxygen in the incubator environment (maintained at 21% ambient oxygen), *in vitro* cell culture systems can be broadly classified as open and closed cell culture systems. For example, conventional multi-well cell culture platforms have an open air-liquid interface surface that interacts directly with the incubator environment and provides adequate oxygen to hepatocytes. In case of closed bioreactor systems and microfluidic systems (Bale *et al.*, 2014), oxygen replenishment in the media is accomplished by either (1) in-line oxygenation of the media in the fluidic circuit, or (2) utilization of materials with high oxygen diffusivity (e.g., Poly(DiMethylSiloxane), PDMS). Media oxygenation systems are large-volume

systems, increasing the quantity of media utilized for cell culture and leading to an apparent reduction of secreted factors. PDMS is an attractive material for microfluidic systems from fabrication, ease-of-use, and optical transparency perspectives. As a result, PDMS has found extensive use in several microscale platforms for organ-on-chip systems. However, recent studies have shown the significant loss of drugs within PDMS based microfluidic systems due to absorption; and have highlighted the incompatibility of soft polymeric materials with fabrication of high-throughput multi-chip systems (Halldorsson *et al.*, 2015; Shirure and George, 2017).

To generate a microscale model that utilizes non-PDMS materials (such as thermoplastics), an alternate strategy is to incorporate active oxygen transport systems within the microfluidic device as active structural elements. Bader and co-workers demonstrated the incorporation of gas-permeable films in the construction of microscale bioreactors to provide oxygen to collagen-sandwich hepatocyte cultures (Bader *et al.*, 1998). The active oxygen transport layer used in the system is a collagen-coated, gas-permeable Teflon layer that not only supports oxygen transport but also provides an active attachment surface for hepatocytes, enabling the generation of a collagen sandwich layer in later stages (Figure 4A). Media and nutrients were introduced through a channel between a microporous film and a glass on top of the collagen sandwich. Utilizing this model, rat hepatocytes were cultured for 14 days, maintaining albumin and urea secretions. Further, the influence of serum, fibronectin and collagen in cell culture media are evaluated, suggesting a 5% Serum and 0-30 µg/mL fibronectin proved higher levels of albumin and urea secretions over a period of 28 days. Active oxygen transport to hepatic cultures can be achieved by incorporating oxygen-transport layers using PDMS as a structural element, as shown by Kane and coworkers (Kane *et al.*, 2006). Micropatterned co-culture of hepatocytes and 3T3-J2 fibroblasts were seeded on a glass substrate followed by capping the top surface using a PDMS-oxygen permeable layer as a composite lid. Media was introduced above the cells in culture and perfused, demonstrating maintenance of hepatic functions (Figure 4B).

18

In a different study, Ochs and co-workers determined the oxygen consumption by hepatocytes in thermoplastic devices by directly measuring oxygen concentrations in the cell culture (Ochs *et al.*, 2014). The device comprised of an oxygen sensing foil forming the bottom of the microfluidic device with the top channel formed using either (1) PDMS, which has high oxygen diffusivity, or (2) Polymethyl Pentene (PMP), with high oxygen diffusivity and excellent processability and biocompatibility and (3) Cyclic Olefin Copolymer (COC), which is oxygen-impermeable. Oxygen content within 1 hour of cell seeding for hepatocyte cultures in the device decreased to ~4% in case of COC and ~ 10% in case of PMP, while it remained at ~18% in the case of PDMS, demonstrating high oxygen consumption by hepatocytes. In comparison, endothelial cells seeded in similar devices did not show any appreciable loss in oxygen content in the case of PDMS and PMP chips, and a decrease to ~13% in the case of COC chips.

4. Small-volume effects in microfluidic cell culture devices: Spatial confinement in the *in vivo* microenvironment is a less studied component in *in vitro* model systems, particularly in microscale models designed to capture the responses of endogenous signals, secreted molecules, drugs and their metabolites (Mehling and Tay, 2014; Wikswo, 2014). Conventional cell culture methods such as standard multi-well platforms and bio-reactors incorporate large fluid volumes per unit surface area, resulting in the dilution of secreted molecules (Mehling and Tay, 2014; Wikswo, 2014). Further, these systems require complete medium exchange providing renewed media and nutrient components; however, consistent media exchange results in the removal of any autocrine, paracrine factors and, particularly in case of the liver, metabolites that have accumulated over the course of exposure. In comparison, microscale manufacturing techniques generate models with fixed dimensions (length, breadth and height), providing large areas for cell attachment with constrained volumes, and fixed media-to-cell ratios (Mehling and Tay, 2014; Wikswo, 2014; Bale, Moore, *et al.*, 2016). This constrained microenvironment allows for the

precise control over the volume from which nutrients/compounds are consumed; and molecules, including hormones, signaling molecules, and reaction products are excreted.

In a recent study, Bale and co-workers demonstrated the importance of such dilution effects in capturing a short-lived therapeutic molecule in a liver- breast cancer model, utilizing the reduced dilution effects in a microscale bilayer device when compared with standard transwell cultures (Bale, Sridharan, *et al.*, 2015). Initially, the authors compare the metabolic performance of hepatocytes in a microfluidic device (100 μm height) with a standard 24-well plate culture with a 0.1 nL/hepatocyte and 1 nL/hepatocyte media dilution levels respectively. Making a simplistic comparison, there are ~60 hepatocytes per 1 nL of blood in the human body (Wikswo *et al.*, 2013; Bale, Sridharan, *et al.*, 2015; Hughes *et al.*, 2017). By comparing the products of a CYP3A4 assay (Luciferin-IPA), and reduced dilution of metabolites in the microfluidic device, the authors demonstrated increased accumulation and increased concentration (3-4 times higher) of products in the microfluidic device when compared with standard plate cultures. The authors extended these findings by co-culturing rat primary hepatocytes and breast cancer (MCF-7) cells in a microfluidic bilayer device, resulting in a low combined volume of 0.35 nL/hepatocyte (Figure 5A). In comparison, a typical 12 well transwell culture, primary hepatocytes and MCF-7 cells require 1,500 μL of cell culture medium, resulting in an increased volume of 3 nL/hepatocyte. Utilizing the membrane bilayer model with a liver-cancer system, the authors demonstrated the metabolism of Tegafur, a chemotherapeutic pro-drug, and the formation of its metabolite 5-Fluorouracil (5-FU), by the metabolic functionality of hepatocytes and its toxic effect on cancer (MCF-7) cells. A key observation was a low but measurable concentration of 5-FU detected in the microscale system, which is not detectable in case of multi-well plate cultures. This suggests the need for careful consideration of platform design for drug metabolism studies, particularly in the case of short-lived therapeutic metabolites. Similarly, confinement of endogenous signals in small volumes in microfluidic devices can influence the phenotype and longevity of hepatocyte cultures, as

demonstrated by Haque and co-workers (Haque *et al.*, 2016). Hepatic performance was evaluated in both microscale chambers and multi-well plates; and influence of culture dimensions on protein synthesis, metabolic activity and epithelial morphology of hepatocytes are evaluated (Figure 5B). Hepatocytes in small volume culture showed higher albumin secretory functions, and upregulation of hepato-inductive signals (growth factors such as HGF, EGF, IGF and FGF7) and downregulation of hepato-disruptive signals (TGF-β and CTGF) when compared with multi-well plates.

5. Fluidic flow in microfluidic systems:

Dynamic cell culture systems, such as bioreactors and MPS models, utilize various strategies to introduce and remove media from cell culture environment at a controlled rate, including pressure-driven pumping and gravity. Pressure-driven systems include syringe pumps, peristaltic pumps and custom-built pumping systems while gravity-driven flow systems utilize height differential in channels either by pumping media or tilting the platform to drive media flow. A key advantage of microfluidic MPS platforms is the relative reduction in media-to-cell ratio in the cell culture chamber in comparison with the overall media in the system, which includes connecting tubing and reservoirs. Accumulation of signaling molecules, cellular secretions and reaction products plays an important role hepatic functionality and capturing such mechanisms via media flow is essential. For instance, any influence on active enzymatic and metabolic components due to cellular secretions (e.g., cytokine response from Kupffer cells in inflammatory conditions) in a hepatic cell culture results in a regulated hepatic function and response (Bale, Geerts, *et al.*, 2016). In addition, modulating media flow is critical in maintaining and extending the longevity of hepatocyte cultures by optimizing flow to match *in vivo* mass transport parameters, as discussed earlier. While providing media flow at a rate intended to enhance hepatic function, careful consideration should be given to the "residence time" of the media within the cell culture system to capture the hepatic secretions, metabolites and feedback. While systems that incorporate flow

21

in a single-pass format are capable of generating flow-dependent physiology, systems that re-circulate media within a reasonable time frame with reduced dilution effects hold the promise of capturing enzymatic products, secretions and feed-back responses, particularly for studies dealing with chronic exposure and stimuli. Development of low-volume and preferably on-board pumping systems is an important step in this direction to (1) conserve the reduced media-to-cell ratios that are achieved by the microfluidic systems and (2) generate hepatic systems with media recirculation, allowing the interrogation of active feedback responses that arise from cellular secretions and cellular interactions. The reduced media-to-cell ratio in the microfluidic systems open the possibilities of capturing short-lived metabolites, multi-cellular interactions and feedback (Bale, Sridharan, *et al.*, 2015; Haque *et al.*, 2016) and aid in the generation of multi-organ systems (Bale, Moore, *et al.*, 2016) with better *in vitro-in vivo* correlations.

High-throughput platforms for drug screening

High-throughput MPS platforms are an attractive option for pharmaceutical industry, allowing their adaptation as advanced cell-culture models for pre-clinical drug evaluation. Several static hepatic cell culture systems have already been modified for high-throughput format, including micro patterning - Hepregen, bio printing - Organovo, Solidus, mixed co-cultures – Regenemed and spheroids - Insphero to name a few. Standalone, individual, microfluidic systems are currently available as options for hepatic culture, such as Hurel, Emulate and Hemoshear. Case studies and proof-of-concept demonstrations utilizing these systems have yielded some results validating their applicability and human-translatability. Several of these models have been tested in the industry setting and are currently in active collaboration with pharmaceutical companies for drug screening and validation studies. With advances in microscale manufacturing techniques, several microfluidic systems and MPS platforms have been adapted to, and manufactured in a high-throughput format, notably Cell-ASIC Pearl and Mimetas systems.

22

In addition, adaptation of high-throughput MPS systems into mainstream drug screening process requires the development of analytical tools for molecular and genetic analyses, and imaging tools for rapid assessment of cellular function. High-throughput imaging systems are already in use for multi-well plate systems, requiring minimal development for deployment to MPS systems. Analyte measurement in MPS systems with limited volume and low concentrations (in comparison with traditional multi-well plate systems) is now possible with commercially available systems, such as Luminex and Mass Spectrometry analysis. In addition to the suite of analytical capabilities, development of novel tools incorporating genetic, proteomic and metabolomics will aid in the generation of human-relevant data to accelerate drug-screening process.

Conclusion

In vitro models capable of more accurately predicting human hepatotoxicity and mechanisms involved in liver diseases are urgently needed to address gaps in the drug development process. Enhancing early detection capabilities of compound toxicity would provide a major advancement in drug discovery and screening processes. The role of MPS technologies in generating human-relevant, preclinical data is evolving with major advancements in understanding the native microenvironment and utilizing microscale fabrication methods to generate *in vitro* mimics. Emerging capabilities in microfabrication technologies, microfluidic control systems, biomaterials and multi-cell culture formats are converging to provide an opportunity to address these gaps. The major current challenge is the validation of these systems in establishing *in vitro – in vivo* correlations to build confidence in these tools for drug development. Once validation is achieved, the focus will shift toward the development of practical higher throughput systems that can be implemented in pharmaceutical laboratories.

Acknowledgments:

Authorship contribution:

Wrote or contributed to the writing of the manuscript: Bale, Borenstein.

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Figure Legends:

Figure 1. Structure of the liver. A) Schematic showing hepatic lobular structure with cells located in a vascularized structure between the portal triad (Hepatic artery, portal vein and bile ducts) and the central vein. Hepatocytes within the lobule have varying functionality, roughly identified by the various zones. B) The liver sinusoid is a dynamic environment receiving blood flow from portal vein and hepatic artery and draining into the central vein. Nutrients and oxygen are transported through the sinusoidal endothelial cells and extracellular matrix to the hepatocytes.

Figure 2. Capturing hepatic mass transport in *in vitro* models. A) Device with endothelial-like barrier, separating flow from cell culture area, regulating nutrient flow through the intervening barrier (Lee *et al.*, 2007). B) Microfluidic bilayer model for culturing hepatocytes in collagen gel and flow, demonstrating *in situ* production of collagen for hepatic stabilization (Hegde *et al.*, 2014). C) Incorporation of multiple cells within the microfluidic bilayer model to mimic the liver sinusoid, and extending hepatic culture to 4 weeks (Prodanov *et al.*, 2016). D) Cone and plate model for hepatocyte culture, capturing aspects of interstitial flow and hemodynamics (Dash *et al.*, 2013).

Figure 3. Hepatic zonation in *in vitro* models A) Hepatic zonation in the liver sinusoid results in hepatocytes with distinct enzymatic and metabolic functionality along the length of the sinusoid. B) Flat-plate bioreactor model to generate zonation with active oxygen consumption in the direction of media flow (Allen *et al.*, 2005). Gas exchanger (O_2, CO_2, N_2) upstream oxygenates the media and consumed along the length of the bioreactor. C) Microfluidic device with a gradient generator to create hormonal/chemical gradients across multiple hepatocytes (McCarty *et al.*, 2016).

Figure 4. Oxygen transport for optimal hepatocyte culture. A) Microfluidic device for hepatocyte culture in a collagen gel incorporating a gas permeable membrane (Bader *et al.*, 1998). B) Cross section of a microfluidic device with a gas perfusion channel sandwiched within

36

PDMS layers for optimal hepatocyte function (Kane *et al.*, 2006).

Figure 5. Dilution effects in *in vitro* systems. A) Microfluidic device with reduced media-to-cell ratio to capture primary metabolite toxicity in a hepatocyte – breast cancer model system (Bale, Sridharan, *et al.*, 2015). B) Effect of small volumes in maintaining the differentiated phenotype of hepatocytes in micro chamber culture (Haque *et al.*, 2016).
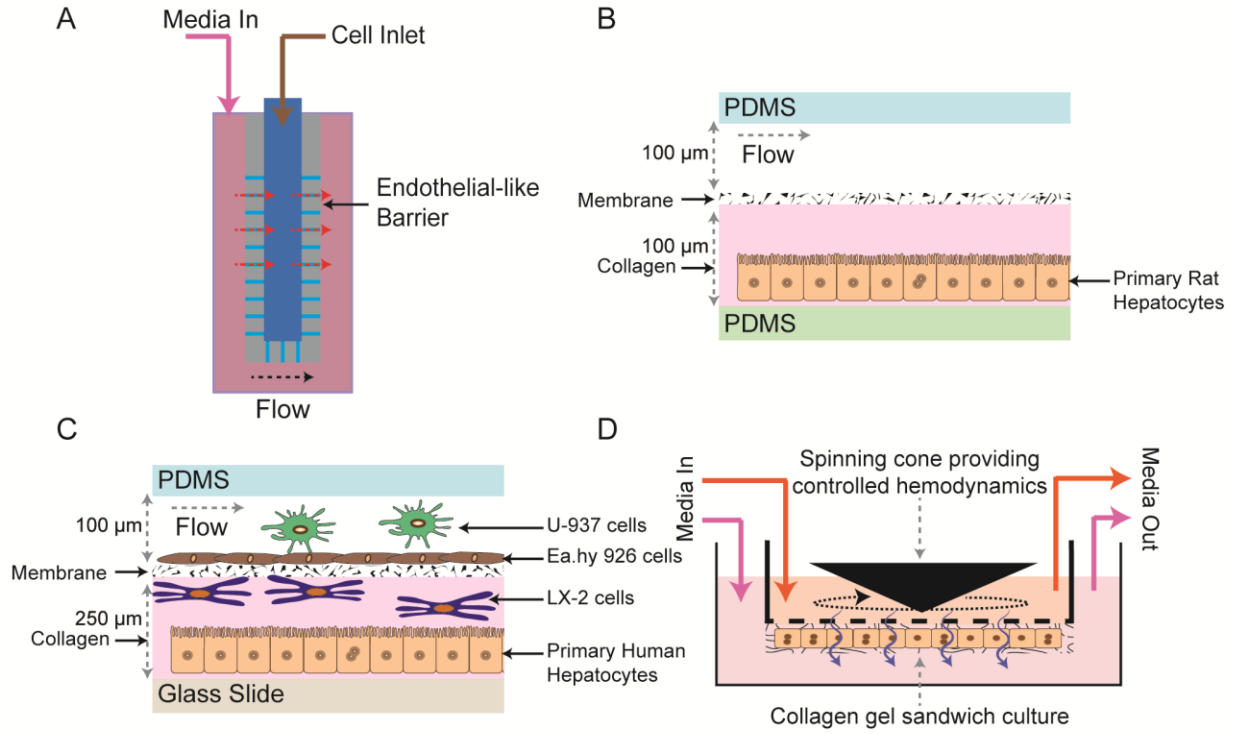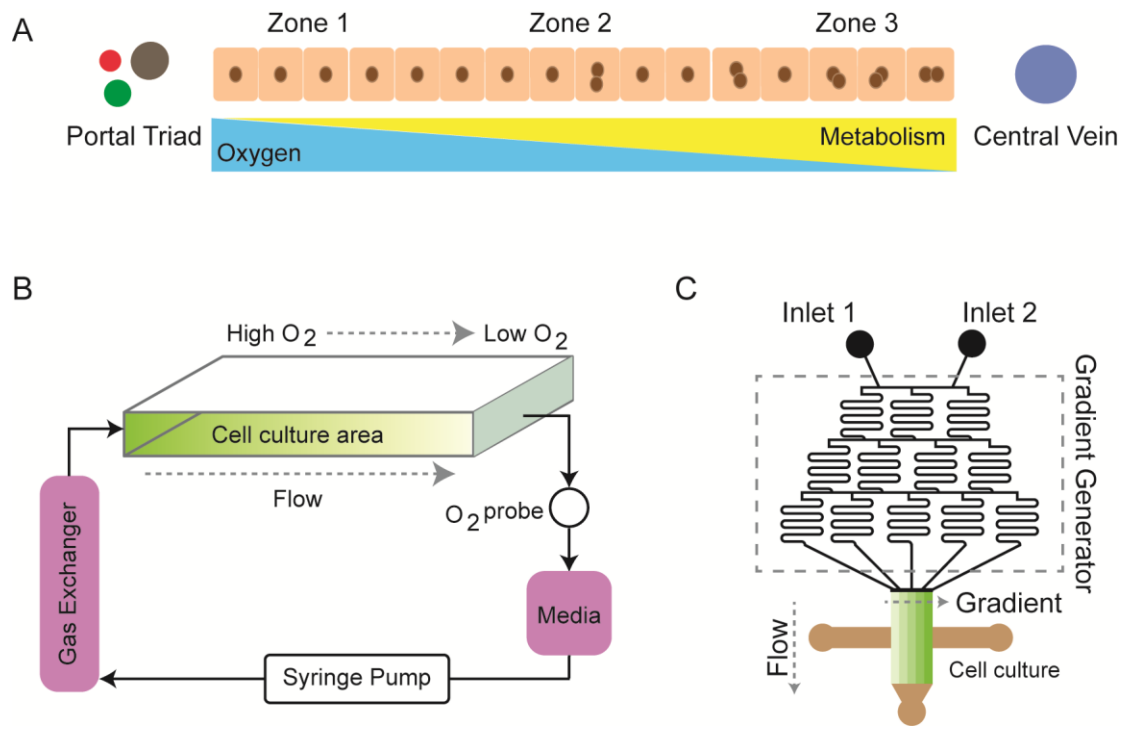
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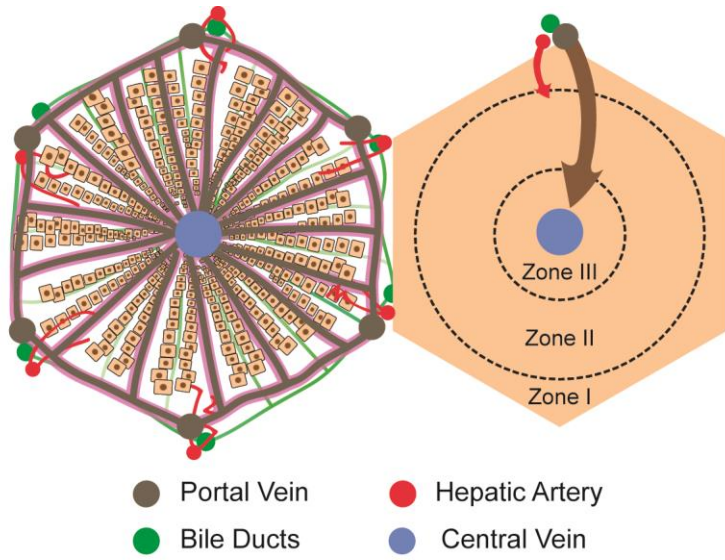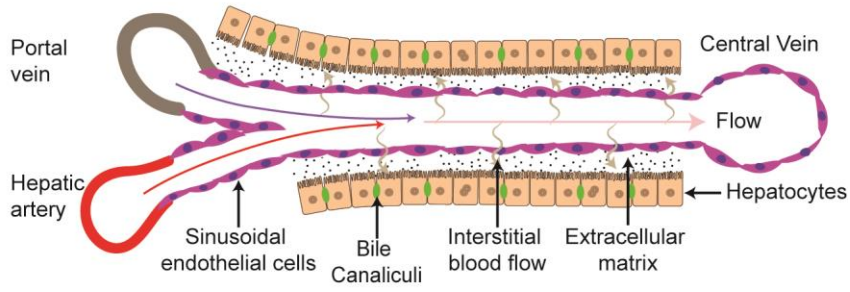
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3 **Figure 1**

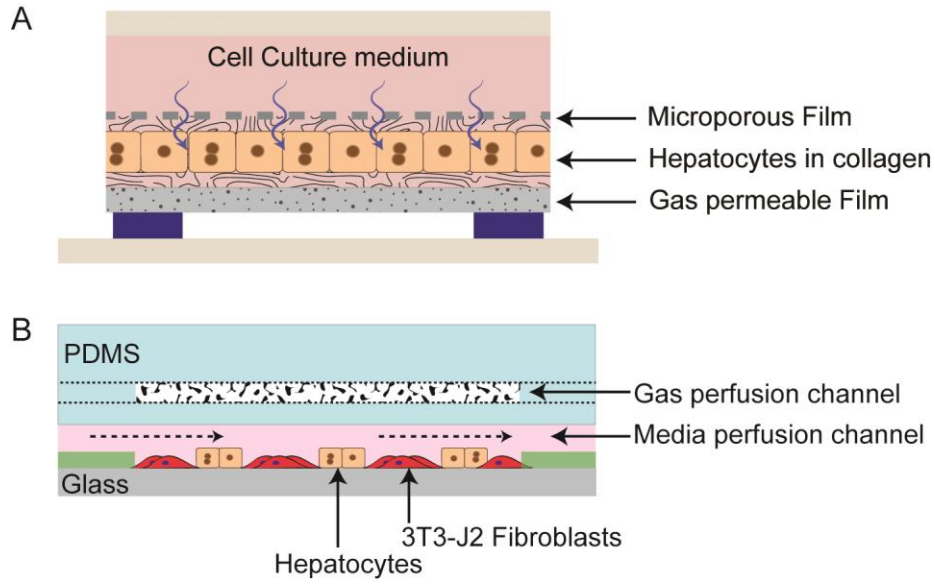1

2   **Figure 2.**

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Figure 3.

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2 **Figure 5.**