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Special Section – New Models in Drug Metabolism and Transport—Minireview

Microfluidic Cell Culture Platforms to Capture Hepatic Physiology and Complex Cellular Interactions

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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. Although effective in the drug-screening process, their translatability to humans is limited because of the lack of high concordance and correlation among 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 multifunctional organ with diverse metabolic, secretory, and inflammatory response roles and is essential for maintaining key body functions. Conventional cell culture platforms (such as multiwell plate cultures) and metabolic enzyme systems (microsomes, cytochrome P450 enzymes) have been routinely used 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 multicellular 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 physiologic 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 in the human body, performing critical metabolic, storage, synthesis and filtration functions and 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., prodrugs) or unwanted side effects (Bale et al., 2016b; Hughes et al., 2017).

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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 cocultures in two-dimensional and three-dimensional (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 multicellular 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 the recapitulation of niche environments of organs in vitro (Bale et al., 2014,

ABBREVIATIONS: 3D, three-dimensional; COC, cyclic olefin copolymer; MCF-7, Michigan Cancer Foundation-7 human epithelial breast cancer cell line; MPS, microphysiological systems; PDMS, poly(dimethoxysiloxane); PMP, polymethylpentene.

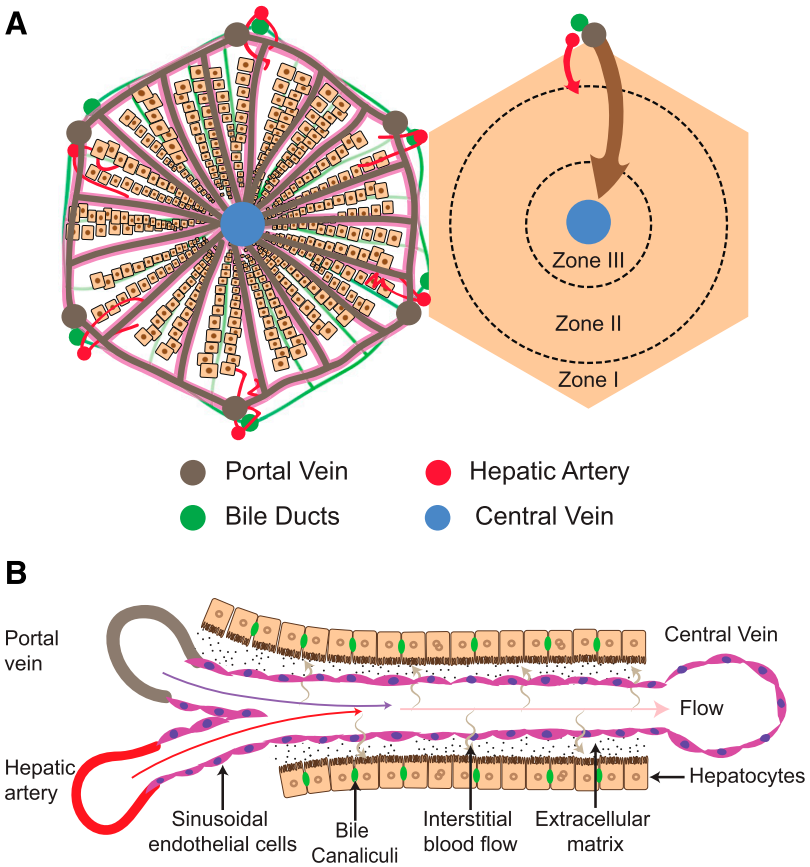

Fig. 1. Structure of the liver. (A) Schematic showing hepatic lobular structure with cells located in a vascularized structure between the portal triad (hepatic artery, portal vein, and bile ducts) and the central vein. Hepatocytes within the lobule have varying functionality, which are roughly identified by the various zones. (B) The liver sinusoid is a dynamic environment receiving blood flow from the 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.

2016b; Bhatia and Ingber, 2014; Wikswo, 2014; Abbott and Kaplan, 2015; Yoon No et al., 2015; 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 preclinical 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 in an in vivo–like microenvironment; and 3) providing a rapid, easy, and high-throughput process for the 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 multiorgan MPS systems that are capable of capturing inter-organ interactions and assaying for compounds and their metabolites, and drug responses (Bale et al., 2016b; Hughes et al., 2017).

In this review, we provide an overview of the 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 the recapitulation of physiologic levels of mass transport, fluid flow, media-to-cell ratios, and oxygen supply to the hepatic cultures enhance hepatic function and allows for the interrogation of chemicals at a human-translatable scale. Advanced liver MPS platforms, both in models that recapitulate of 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 cells (hepatocytes) and non-parenchymal cells (Kupffer, stellate, sinusoidal endothelial, and cholangiocytes) between the portal triad and the central vein (Fig. 1A). Cells within the liver have well-defined functions, where hepatic responses to any external stimuli or perturbation (chronic or acute) are based on 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 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 (Fig. 1B). The sinusoid is lined with a layer of fenestrated endothelial cells (Wisse et al., 1996; Braet and Wisse, 2002), which 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, which 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 (Fig. 1B) (Vollmar and Menger, 2009). Hepatocytes use 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 physiologic responses are driven by the mass transport occurring within the microarchitecture of the liver sinusoid.

Microtechnologies 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 multicellular 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). The isolation of purified primary hepatocyte fractions, which is ideal for developing assays for evaluating drug metabolism and is widely used in various culture formats (Godoy et al., 2013; Lauschke et al., 2016), enabling the incorporation of hepatocytes into suspension and plate cultures. Major advances in extending the longevity of a primary hepatocyte culture include sandwich culture (Dunn et al., 1991, 1992), micropatterned cocultures (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 cocultures, extending hepatocyte cell cultures for several weeks, and thereby providing a suitable platform for drug testing. The collagen (or Matrigel; Corning, Corning, NY) sandwich primary hepatocyte provides 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 the retention of function for several weeks (Dunn et al., 1991, 1992; Bale et al., 2015a). Hepatic cocultures generated using micropatterning methods and cocultures enable hepatic stabilization, driven by the interactions of secreted matrix, integrins, and secreted molecules (Nahmias et al., 2006; Bale et al., 2015a, 2016a; Lauschke et al., 2016). Micropatterned hepatic cocultures have been developed with fibroblasts (Bhatia et al., 1999) and hepatic-relevant nonparenchymal cells (Nahmias 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 micro-environment and increasing cell-cell contacts (Messner et al., 2013; Nguyen et al., 2016). In addition to hepatocytes, the isolation of nonparenchymal cells to obtain pure populations has been challenging, although advances in methods are currently yielding Kupffer and hepatic stellate cells that can be used in developing hepatic cocultures. Hepatocyte cocultures with nonparenchymal cell fractions (Kostadinova et al., 2013; Esch et al., 2015; Bale et al., 2016a; Du et al., 2017), Kupffer cells (Tukov et al., 2006; Zinchenko et al., 2006a,b), stellate cells (Thomas et al., 2005), and sinusoidal endothelial cells (Hwa et al., 2007; Kim and Rajagopalan, 2010; Bale et al., 2015a) are provide key insights regarding cellular cross talk and hepatic responses.

Although significantly extending the life of the cell culture, the limited quantity of primary hepatocytes and nonparenchymal hepatic cells from isolations limits their extensive use, particularly in high-throughput culture systems. Hepatic cell lines [e.g., Hepa-RG (Thermo Fisher Scientific, Waltham, MA) 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 and adult-induced pluripotent stem cells 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-the-art cell development methods require further improvement before their incorporation into mainstream toxicology assays (Shan et al., 2013; Godoy et al., 2015).

Capturing Physiologic 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 using 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 micropatterned 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 multicellular 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 capture not only the cellular complexity, but also aspects of the physiologic exchange of materials among 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 the evaluation of combinatorial effects of soluble factor signaling and cell-cell and cell-matrix interactions. Several physiologic phenomena have been explored as part of the MPS platform design and function.

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 (Fig. 1). 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 (Fig. 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

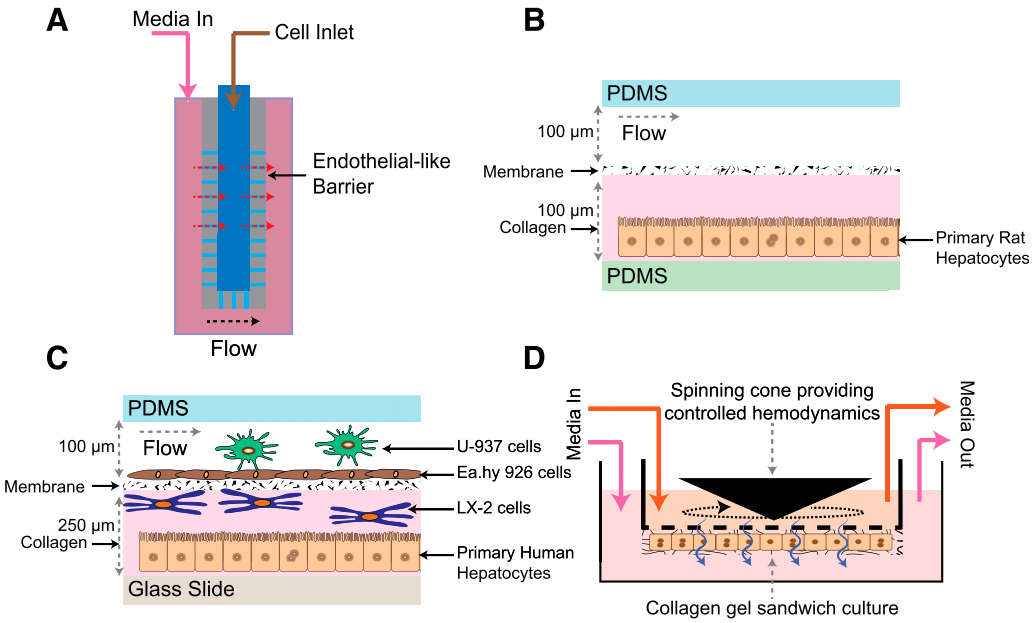

Fig. 2. Capturing hepatic mass transport in in vitro models. (A) Device with endothelial cell–like barrier, separating flow from cell culture area, regulating nutrient flow through the intervening barrier (Lee et al., 2007). (B) Microfluidic bilayer model for culturing hepatocytes in collagen gel and 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).

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; Prodanov et al., 2016; Du et al., 2017).

In their work, Lee et al. (2007) have used 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. The use of an 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 (Fig. 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. Using this system, Lee et al. (2007) 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 in the 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; Prodanov et al., 2016; 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 et al. (2014) demonstrate the importance of achieving optimal flow rates for hepatic stabilization and long-term hepatic function (Fig. 2B). 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) and metabolic function (CYP1A1/2), and the formation of bile junctions within the hepatocyte monolayer culture. A key aspect of the collagen sandwich model is the stabilization and polarization of hepatocytes, which are 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 the triple helix structure of collagen by *cis*-hydroxyproline) and subsequently the loss of function (Uitto et al., 1975). Using a similar bilayer model, Prodanov et al. (2016) incorporated multiple hepatic cells, including primary hepatocytes, cell lines representing the human endothelial cell line (Ea.hy 926), the human hepatic stellate cell line (LX-2), and Kupffer human monocyte cell line (U-937) capturing the major cell types present in the liver sinusoid. Hepatocytes and stellate cells were cultured in one chamber, while the flow channel was composed of endothelial cells as a monolayer (exposed to flow) and Kupffer cells in the flow channel mimicking the architecture of the sinusoid (Fig. 2C). Mass transport in the device (between flow channel and hepatocytes) is optimized using Péclet number estimates, generating an hepatic culture with optimal functions (secretions, bile canaliculi formation, and CYP3A4) for up to 4 weeks.

Dash et al. (2013) have used a combination of a spinning cone and perfusion flow to achieve controlled hemodynamics, mimicking the sinusoidal and interstitial blood flow to hepatocytes in culture. 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 (Fig. 2D). In combination with media perfusion in both the well and transwell, Dash et al. (2013) demonstrated the recovery of hepatic function, measured by albumin and urea secretions and polarization.

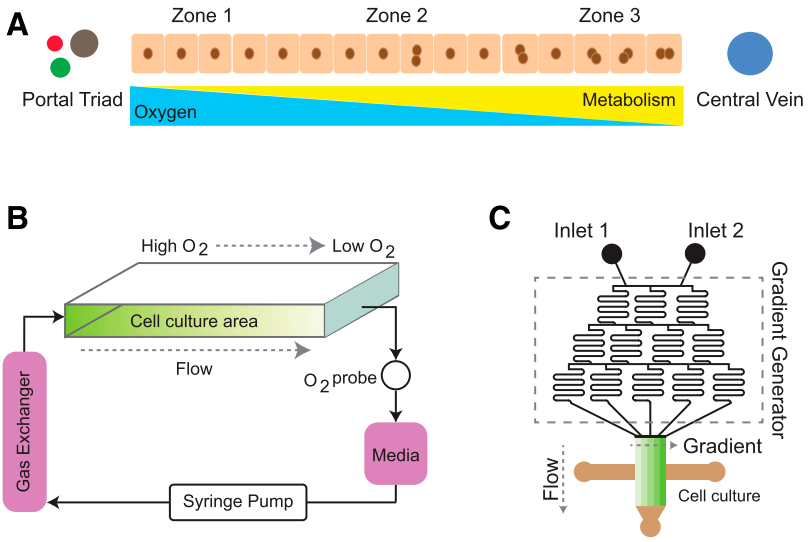

Fig. 3. Hepatic zonation in in vitro models. (A) Hepatic zonation in the liver sinusoid results in hepatocytes with distinct enzymatic and metabolic functionality along the length of the sinusoid. (B) Flat-plate bioreactor model to generate zonation with active oxygen consumption in the direction of media flow (Allen et al., 2005). Gas exchanger (O_2, CO_2, N_2) upstream oxygenates the media and is 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).

In Vitro Zonation. A key physiologic feature of the liver sinusoid is zonation, which is 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 the 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, whereas zone 3 hepatocytes are efficient at glucose uptake, glutamine formation, alcohol degradation, and phase I metabolism (Fig. 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 microdissection 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 used 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 the zonal features of the liver, Allen and coworkers (Allen and Bhatia, 2003; Allen et al., 2005) developed a biomimetic flat-plate bioreactor with either hepatocyte monoculture or hepatocyte-fibroblast cocultures generating an oxygen gradient along the axis of flow. The custom flat-plate bioreactor is manufactured from oxygen-impermeable polysulfone and is designed to receive a microscope slide seeded with hepatocyte cultures, as well as to be integrated with a media oxygenator upstream and an oxygen monitor downstream of the bioreactor (Fig. 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, and driven by the balance of oxygen content in the media, the 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 et al. (2016) developed a microfluidic device to generate a continuous gradient across an hepatocyte culture to capture hepatic zonation using chemical and hormonal gradients. The model generates spatially controlled zonation across multiple hepatocyte metabolism levels through controlled application of hormonal and chemical gradients (Fig. 3C). A key difference in the device is the generation of "zonation" perpendicular to the flow, whereas the gradient exists in the direction of flow in vivo. Using this model, McCarty et al. (2016) demonstrate variations in carbohydrate and nitrogen metabolism in a glucagon-insulin gradient, and enzymatic variations using a chemical (3-methylcholanthrene) gradient. Further, variations in enzymatic activity within the chemical-driven zonation are revealed by assaying for an acetaminophen toxicity zone-dependent response. In recent work (Kang et al., 2018), similar devices have been developed to capture zonation using hormones and inducers in both rat and human hepatocyte cultures.

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. The 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 multiwell cell culture platforms have an open air-liquid interface surface that interacts directly with the incubator environment and provides adequate oxygen to hepatocytes. In the 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) the use of materials with high oxygen diffusivity [e.g., poly(-dimethylsiloxane) (PDMS)]. Media oxygenation systems are large-volume systems, increasing the quantity of media used 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.

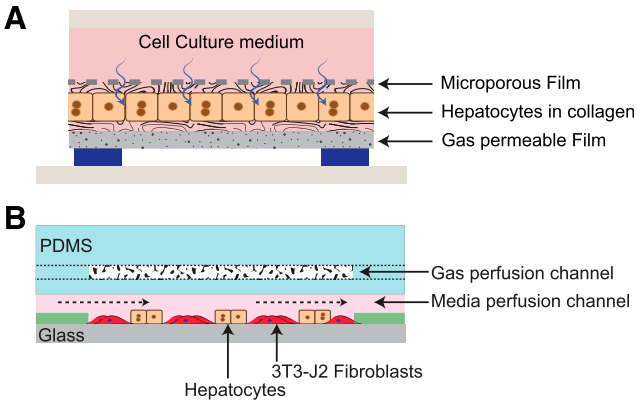

Fig. 4. Oxygen transport for optimal hepatocyte culture. (A) Microfluidic device for hepatocyte culture in a collagen gel incorporating a gas-permeable membrane (Bader et al., 1998). (B) Cross section of a microfluidic device with a gas perfusion channel sandwiched within PDMS layers for optimal hepatocyte function (Kane et al., 2006).

However, recent studies (Halldorsson et al., 2015; Shirure and George, 2017) 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 the fabrication of high-throughput multichip systems.

To generate a microscale model that uses non-PDMS materials (e.g., thermoplastics), an alternate strategy is to incorporate active oxygen transport systems within the microfluidic device as active structural elements. Bader et al. (1998) demonstrated the incorporation of gas-permeable films into the construction of microscale bioreactors to provide oxygen to collagen sandwich hepatocyte cultures. 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 (Fig. 4A). Media and nutrients were introduced through a channel between a microporous film and a glass on top of the collagen sandwich. Using 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 that 5% serum and 0–30 μg/ml fibronectin proved to yield 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 et al. (2006). Micropatterned coculture of hepatocytes and fibroblasts from an embryonic mouse fibroblast cell line (3T3-J2) were seeded onto a glass substrate followed by capping of the top surface using a PDMS-oxygen–permeable layer as a composite lid. Media were introduced above the cells in culture and perfused, demonstrating the maintenance of hepatic functions (Fig. 4B).

In a different study, Ochs et al. (2014) determined the oxygen consumption by hepatocytes in thermoplastic devices by directly measuring oxygen concentrations in the cell culture. The device was composed of an oxygen-sensing foil forming the bottom of the microfluidic device with the top channel formed using the following: 1) PDMS, which has high oxygen diffusivity; 2) polymethylpentene (PMP), which has 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 the case of COC and to ~10% in the 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.

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 multiwell platforms and bioreactors incorporate large fluid volumes per unit of 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 and paracrine factors and, particularly in the 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 et al., 2016b). This constrained microenvironment allows for 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 et al. (2015b) demonstrated the importance of such dilution effects in capturing a short-lived therapeutic molecule in a liver- breast cancer model, using the reduced dilution effects in a microscale bilayer device when compared with standard transwell cultures. Initially, Bale et al. (2015b) compare the metabolic performance of hepatocytes in a microfluidic device (100 μm height) with that of a standard 24-well plate culture with 0.1 nl/hepatocyte and 1 nl/hepatocyte media dilution levels, respectively. Making a simplistic comparison, there are ~60 hepatocytes/1 nl of blood in the human body (Wikswo et al., 2013; Bale et al., 2015b; 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 to 4 times higher) of products in the microfluidic device when compared with standard plate cultures. Bale et al. (2015b) extended these findings by coculturing rat primary hepatocytes and breast cancer cells [MCF-7 (Michigan Cancer Foundation-7)] in a microfluidic bilayer device, resulting in a low combined volume of 0.35 nl/hepatocyte (Fig. 5A). In comparison, a typical 12-well transwell culture, primary hepatocytes and MCF-7 cells require 1500 μl of cell culture medium, resulting in an increased volume of 3 nl/hepatocyte. Using the membrane bilayer model with a liver-cancer system, Bale et al. (2015b) demonstrated the metabolism of tegafur, a chemotherapeutic prodrug, and the formation of its metabolite 5-fluorouracil in the microscale system by the metabolic functionality of hepatocytes and its toxic effect on cancer (MCF-7) cells. A key observation was the detection of a low but measurable concentration of 5-fluorouracil, which is not detectable in the case of multiwell plate cultures. This suggests the need for careful consideration of the platform design for drug metabolism studies, particularly in the case of short-lived therapeutic metabolites. Similarly, the confinement of endogenous signals in small volumes in microfluidic devices can influence the phenotype and longevity of hepatocyte cultures, as demonstrated by Haque et al. (2016). Hepatic performance was evaluated in both microscale chambers and multiwell plates, and the influence of culture dimensions on protein synthesis, metabolic activity, and epithelial morphology of hepatocytes was evaluated (Fig. 5B). Hepatocytes in a small-volume culture showed higher albumin secretory functions, and the upregulation of hepatoinductive signals (growth factors such as hepatocyte growth factor, epidermal growth factor, insulin-like growth factor, and fibroblast growth factor 7) and the downregulation of hepatodisruptive signals (transforming growth factor-β and connective tissue growth factor) when compared with multiwell plates.

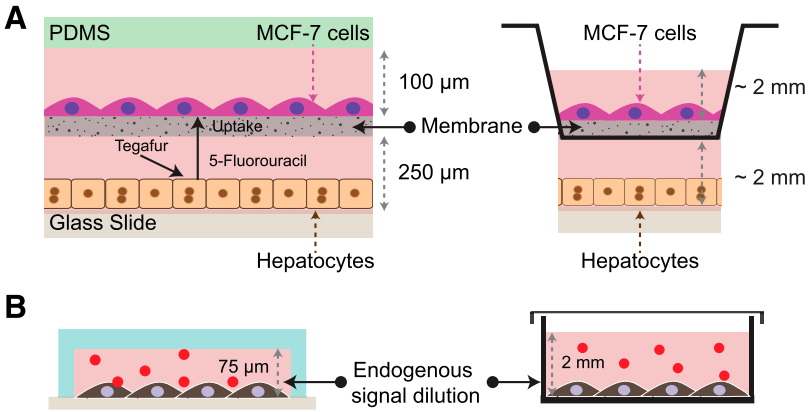

Fig. 5. Dilution effects in in vitro systems. (A) Microfluidic device with reduced media-to-cell ratio to capture primary metabolite toxicity in an hepatocyte-breast cancer model system (Bale et al., 2015b). (B) Effect of small volumes in maintaining the differentiated phenotype of hepatocytes in a microchamber culture (Haque et al., 2016).

Fluidic Flow in Microfluidic Systems. Dynamic cell culture systems, such as bioreactors and MPS models, use various strategies to introduce and remove media from the 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, whereas gravity-driven flow systems use 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. The accumulation of signaling molecules, cellular secretions, and reaction products plays an important role in 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 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. Although systems that incorporate flow in a single-pass format are capable of generating flow-dependent physiology, systems that recirculate media within a reasonable time frame with reduced dilution effects hold the promise of capturing enzymatic products, secretions, and feedback responses, particularly for studies dealing with chronic exposure and stimuli. The development of low-volume and preferably on-board pumping systems is an important step in this direction: 1) to conserve the reduced media-to-cell ratios that are achieved by the microfluidic systems and 2) to 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 ratios in the microfluidic systems open the possibilities of capturing short-lived metabolites, multicellular interactions, and feedback (Bale et al., 2015b; Haque et al., 2016), and aid in the generation of multiorgan systems (Bale et al., 2016b) with better in vitro-in vivo correlations.

High-Throughput Platforms for Drug Screening

High-throughput MPS platforms are an attractive option for the pharmaceutical industry, allowing their adaptation as advanced cell culture models for preclinical drug evaluation. Several static hepatic cell culture systems have already been modified for the high-throughput format, including micropatterning (BIOIVT, Westbury, NY); bioprinting (Organovo, San Diego, CA); mixed cocultures (RegeneMed, San Diego, CA); and spheroids (InSphero, Brunswick, ME), to name a few. Stand-alone individual microfluidic systems are currently available as options for hepatic culture, such as those of the Hμrel Corporation (North Brunswick Township, NJ), Emulate, Inc. (Boston, MA), and HemoShear (Charlottesville, VA). Case studies and proof-of-concept demonstrations using these systems have yielded initial results regarding their applicability for human clinical translation. 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 the CellASIC Pearl Perfusion Liver System (Millipore Sigma, Burlington, MA) and the Mimetas (Leiden, The Netherlands) system.

In addition, the adaptation of high-throughput MPS systems into mainstream drug-screening processes 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 multiwell 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 multiwell plate systems) is now possible with commercially available systems, such as Luminex (Austin, TX) and mass spectrometry analysis. In addition to the suite of analytical capabilities, the development of novel tools incorporating genetics, proteomics, and metabolomics will aid in the generation of human-relevant data to accelerate the 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 are evolving with major advancements in understanding the native microenvironment and using microscale fabrication methods to generate in vitro mimics. Emerging capabilities in microfabrication technologies, microfluidic control systems, biomaterials, and multicell culture formats are converging to provide an opportunity to address these gaps. The current major 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.

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