SUPPLEMENTARY MATERIAL

Accompanying the article entitled

Liver Microphysiological Systems for Predicting and Evaluating Drug Effects

Alexandre J.S. Ribeiro[1*], Xinning Yang[2], Vikram Patel[1]; Rajnikanth Madabushi[2]; David G. Strauss[1,2]

[1] U.S. Food and Drug Administration, Center for Drug Evaluation and Research, Office of Translational Science, Office of Clinical Pharmacology, Division of Applied Regulatory Science.

[2] U.S. Food and Drug Administration, Center for Drug Evaluation and Research, Office of Clinical Pharmacology.

*** Correspondence**:
Alexandre Ribeiro
U.S. Food and Drug Administration
10903 New Hampshire Avenue
Silver Spring, MD 20993
Tel: + 301-796-0126
Email: Alexandre.Ribeiro@fda.hhs.gov

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THREE-DIMENSIONAL CULTURE OF HEPATOCYTES PROLONGS HEPATIC FUNCTION IN VITRO

Preceding the development of microphysiological systems (MPS), the initial studies on three-dimensional (3D) cultures of hepatocytes revealed the development of more physiological properties in these culture systems compared with two-dimensional (2D) cultures (1-3), and improved the capacity for detection of drug-induced toxicity (4). 3D cellular organization is a characteristic of the liver microenvironment (5). However, MPS and other 3D platforms do not fully replicate the architectural complexity of the liver lobule (see **Figure 2b** in the main article) but are designed to enable long-lasting culture of hepatic cells with improved function. Overall, hepatocyte function is prolonged for a greater time in culture when cells are organized in 3D, which is a primary advantage of 3D relative to 2D cultures (6). Different studies with platforms for hepatocyte culture demonstrate the dependence of metabolism, transport, and biomarker expression on the 3D polarized morphology of these cells and on their cell-cell interactions (7). For example, polarity can be induced in hepatocytes that are cultured as 2D sandwich cultures (8), where cells are exposed to a collagen layer on the basal side and to basement membrane proteins on the apical side (**Figure 2c**).

In association with the polarization of cells, protein layers are also thought to enable a level of diffusion around cells that mimics physiological conditions (9). On one hand, sandwich cultures demonstrate how proper hepatocyte function relies on cell polarity, extracellular barrier function, and cell-cell adhesions, but on the other hand it is difficult to maintain functional hepatocytes in sandwich cultures beyond 6 to 9 days (10-13). 3D cultures are a more suitable alternative for assays that rely on prolonging cellular mechanisms of liver function beyond 21 days (6, 12, 14). More than a decade of work on 3D hepatocyte culture platforms demonstrates that cellular shape, extracellular diffusion and adhesions are more physiological when hepatocytes are cultured in 3D, which may define better cellular conditions to prolong the *in vitro* lifetime of their functional activity. This condition is required to study subchronic drug effects (15), drug-drug interactions involving enzyme regulation (16), or disease states or conditions for testing drug efficacy that rely on long-term exposure to a pathogen (17) or on the development of malignant microenvironment conditions (18). In addition to culturing cells in 3D, liver MPS also expose cells to media flow and to interactions with other types of hepatic cells (e.g., Kupffer cells, stellate cells, endothelial cells, and fibroblasts). The contribution of each of these factors on the function of hepatocytes is not clearly understood and is discussed here.

Even with considerable advances in the field of MPS, the complexity of liver microarchitecture and structural organization, as illustrated in **Figure 2b**, is still not fully replicated in the systems represented in **Figures 2d, 2e, and 2f**. However, microfabrication approaches can improve the

complexity of MPS and replicate more *in vivo* settings in these systems. For example, separation of bile from media is absent from most liver MPS, but some systems have already demonstrated the possibility of enabling this type of fluid separation (19). Regarding bile separation from media, future research should evaluate how this feature may affect the performance of MPS and potentially evaluate if the presence or absence of bile counterflow affects the modeling and predictive capabilities of liver systems. However, increasing the complexity of *in vitro* systems often presents the risk of decreasing the capability to control device properties that may affect cell function. Therefore, device changes in MPS that aim to enhance their physiology should naturally be followed by comprehensive evaluations on how they affect MPS robustness and reliability in applications that aim to model or predict drug effects.

FLOWING MEDIA IN HEPATOCYTE-BASED MICROTISSUES SETS LEVELS OF OXYGENATION THROUGH CONTROLLED OXYGEN TRANSPORT

Given the microfluidic component of MPS, hepatic microtissues in these systems are exposed to flowing media. The use of flow or stirring to culture hepatocytes has followed pragmatic experimental goals of mass transfer to set culture conditions, and only recently has the field become concerned with understanding the mechanistic changes induced by flow in hepatocytes (2, 20-22). Hepatocytes in 2D exposed to flow have higher metabolic activity and secrete higher levels of albumin and urea for a longer culture time (23). These results clearly show an effect of flow characteristics on hepatocyte function that is independent of other microenvironment properties, such as 3D, extracellular adhesion proteins, the presence of other cell types, and specialized culture media. Overall for adherent cells, flow can directly affect cellular properties through mechanical activation of biological pathways (24, 25) or indirectly affect them through improved mass transfer that enables controlling the levels of oxygen and media nutrients around cells (23, 26). In the case of hepatocytes, few studies have focused on the potential mechanotransduction effects of fluid shear flow on their mechanobiology (27). Therefore, instead of flowing media to directly activate biological pathways via mechanosensing of extracellular shear flow (28), the main target for flowing media in advanced hepatocyte cultures is to replicate levels of oxygen as they physiologically occur in different zones along each sinusoidal unit of hepatic lobules (**Figure 3**) (29).

There are three different zones within the *in vivo* hepatic lobule that differ in oxygen concentration, metabolic activity, and gene expression (30). The sinusoid regions closer to the central vein (Zone 3) have an oxygen tension of 30 to 35 mm Hg (42 to 49 μM), while this value is closer to 60 to 65 mm Hg (84 to 91 μM) in the terminal end of the sinusoids (Zone 1), where the bile ducts, hepatic

arterioles, and portal venules are located (31). Zone 2 of the hepatic sinusoid is between these two regions and contains intermediate levels of oxygen between Zone 1 and Zone 3. The characteristics of the metabolic zones of the hepatic sinusoid in relation to oxygen concentration, regulation of gene expression, and other markers of hepatic function have been well reviewed by Kietzmann (30). In addition, Soto-Gutierrez and colleagues (29) reviewed in detail how defects in the metabolic zones lead to pathophysiological scenarios of liver disease and cancer. These authors further described the potential of MPS and other hepatic cellular systems to model the role of liver zones in the development of disease.

Wnt/β-catenin signaling is one of the main targets of modulating oxygen concentrations in hepatocytes, which is most active in the central vein region and reduced in the extremity of the hepatic sinusoid (29, 30, 32, 33). Understanding this regulation is important for modeling drug hepatic effects because the Wnt/β-catenin pathway plays a strong role in regulating liver function (34-36). Therefore, the ability to modulate this pathway by varying oxygen tension with different flow rates clearly illustrates the potential of MPS to enable mechanistic understanding of drug effects.

Of general interest for regulating Wnt/β-catenin signaling *in vitro*, cellular studies have shown a potential for modulation of this pathway with varied profiles of extracellular fluid flow (37-39). Furthermore, mechanical stimulation of cells generally affects Wnt/β-catenin signaling (40-43), which raises the possibility of having an effect of flow on hepatocytes that involves mechanical forces exerted on cells, independently of oxygen regulation. In addition to modeling more physiological cellular settings in drug development, regulating this signaling pathway is also important for evaluating drug efficacy, mechanisms, or side effects that depend on it (44-47).

RECREATING THE EFFECTS OF MULTICELLULAR INTERACTIONS WITH LIVER MPS

Hepatocytes are the units of liver function and contain the molecular machinery that metabolizes and transports drugs and metabolites (8, 48, 49). However, nonparenchymal hepatic cells [e.g., Kupffer cells (50), stellate cells (51, 52), and sinusoidal endothelial cells (53)] also regulate liver function and hepatic drug effects. Beyond setting a more physiological microenvironment, the inclusion of nonparenchymal cells further allows studying the role in drug responses of distinct biological pathways that regulate immune responses. For this context of use of liver MPS, further research should define immune mechanisms that solely depend on liver cells and differentiate these from effects that depend on other organs or adaptive immunity (54).

Overall, MPS are developed to co-culture different cell types, which is achieved through the establishment of intercellular interactions in 3D. In general, co-culturing cells in standard 2D plates/wells does not lead to physiological-like remodeling of the extracellular microenvironment and of intercellular interactions that occur in 3D systems (55-57). However, studies with co-cultures of hepatic cells in 2D show the ability to better evaluate the biological effects of other liver cell types on hepatocyte function, and a prolongation of hepatocyte physiology in co-cultures relative to monocultures (58-60).

It is natural to assume that liver MPS can further maintain a functional co-culture of hepatic cells beyond one month because cells are also cultured in 3D and under flow. However, different uses of MPS with relevance to drug development have demonstrated the importance of including multiple cell types in these systems to better evaluate specific mechanistic effects of drugs. In general, studies on MPS with co-cultures tend to use physiological ranges of ratios of different cell types in cell combinations, but further work should evaluate how varying the percentage of different cell types in a co-culture impacts cellular function and drug responses.

The next section summarizes the relevance of including different cell types for regulatory applications, where the presence of specific cell types dictates the ability to replicate physiological settings *in vitro*.

DIFFERENT HEPATIC CELL TYPES TO BE CO-CULTURED WITH HEPATOCYTES IN MPS

Kupffer cells reside within the lumen of liver sinusoids (**Figure 2**) and are nonparenchymal specialized macrophages with roles in regulating innate immunity, constituting up to 90% of the overall number of macrophages in the body (61). These cells constitute approximately 15% of the total population of cells in the liver (62), and occupy 2% of the liver volume/mass (63). The role and abundance of these cells reveals the pivotal role of the liver in systemic immunity, a topic that has been reviewed elsewhere (64). The primary interest of co-culturing Kupffer cells with hepatocytes in MPS for *in vitro* prediction of clinical drug effects relates to the roles of this cell type in inflammatory-induced liver toxicity or damage (16-18, 65). The hepatic properties of MPS are not altered with the presence of Kupffer cells, but the role of inflammatory pathways in drug-induced liver injury or other liver functional properties can only be evaluated when these cells are present. For example, co-culture of Kupffer cells with hepatocytes in MPS does not affect CYP activity, albumin production, or infection rates of hepatitis B virus. However, the activation of Kupffer cells suppresses the replication of hepatitis B virus in MPS, replicating levels of inflammatory clinical response (17).

Liver sinusoidal endothelial cells account for 15 to 20% of cells in the liver but represent only 3% of the total organ volume (53). These cells compose the permeable barrier that enables the active uptake, degradation, and exchange of drugs and metabolites along the sinusoid (66), as represented in **Figure 2b**. Co-culturing hepatocytes with these cells improves hepatocyte metabolic function and increases the expression of albumin, which relates to physiological liver function (67). Therefore, including these cells in MPS will prolong and increase the physiological relevance of hepatic function of these systems. However, drug development may also benefit from testing specific mechanistic properties that are regulated by endothelial cell activity. Beyond their barrier function, liver sinusoidal endothelial cells have additional and distinct roles in liver development, regulation of sinusoid blood flow, clearance of toxic molecules, inflammation, and liver regeneration after injury or hepatectomy (53). Therefore, the presence of these cells in MPS can enable the investigation of different functional properties of the liver, but also presents difficulties in clearly defining their contexts of use in drug development.

By setting media flow and gradients of oxygen tension, MPS can facilitate the tuning of optimal microenvironment conditions for maintaining functional liver sinusoidal endothelial cells in culture (68). Oxygen tension affects the size, localization, and function of liver sinusoidal endothelial cells, playing clear roles in liver development and regeneration (69-71). As opposed to the effects on hepatocytes, the pattern of shear flow along the sinusoid directly affects endothelial cell function via defined mechanotransduction pathways (72, 73). For example, most of the vasodilating agent nitric oxide in the liver is produced by liver endothelial cells due to activation by shear force of endothelial nitric synthase (74). In conclusion, the effects of oxygen and shear stress in the physiology of liver endothelial cells can be tuned with MPS due to their microfluidic nature (68).

Of interest to drug development, liver sinusoidal endothelial cells differ from other types of endothelial cells in terms of endocytic activity and absence of the basement membrane that regulates the barrier function of endothelial tissues (53). Liver endothelial cells have a high endocytic activity for clearance of toxic molecules, and are one of the known human cell types with the highest endocytic activity (75). Other types of endothelial cells lack this functional property. In addition, basement membrane is a hallmark of endothelial barriers (76, 77), which is absent from the barrier formed by sinusoid endothelial cells. Therefore, the use of other types of endothelial cells in liver MPS may not yield a physiologically relevant liver-specific activity induced by liver sinusoidal endothelial cells. Given the endocytic activity of these cells, it is reasonable to assume that they may be involved in hepatic drug response and required for specific contexts of use in drug evaluation. Different studies show that

sinusoidal endothelial cells play roles in the effects of drugs in liver function (78). For example, endothelial cells are targeted by metabolites from the bioprocessing of drugs and secreted by hepatocytes (e.g., acetaminophen, cyclophosphamide) (79, 80) and metabolically activate compounds that lead to cellular damage and toxicity (e.g., acetaminophen, monocrotaline, dacarbazine) (80-82).

Stellate cells account for 5 to 8% of cells in the liver, occupy 13% of the cellular volume of the sinusoid, and are located in the space of Disse (**Figure 2b**) that separates the endothelial layer from interconnected hepatocytes (83). Hepatic stellate cells play different physiological roles, such as retinoid storage and release (84), regulation of sinusoidal microcirculation (83), remodeling of the extracellular matrix (85), liver development (86), and maintaining the microenvironment homeostasis of paracrine factors via the uptake and release of cytokines and other regulatory factors (87). In theory, the use of stellate cells in liver MPS should aim to partially recreate a set of these functions *in vitro*. Non-controlled activation of stellate cells from a quiescent state occurs easily *in vitro* or upon cryopreservation, and it is difficult to revert these cells back to a physiologically quiescent state (88, 89). For this reason, it has been difficult to incorporate these cells in MPS (90).

Despite this difficulty, co-culture of stellate cells with parenchymal and nonparenchymal hepatic cells in MPS has been reported in different studies (65, 91, 92). In these, stellate cells seemed to enhance metabolic activity of hepatocytes and general hepatic properties, as well as prolonging the time for which hepatocytes lasted in culture as functional cells. In addition, these co-culture systems have shown improved performance for detecting drug-induced hepatic effects, demonstrating the potential for the use of stellate cells in MPS for drug development purposes (91-93). However, improving the ability to control the activation of stellate cells into matrix-remodeling and contractile fibroblast-like cells remains the major challenge for leveraging their potential in improving the use of MPS.

Related to the physiological roles of quiescent stellate cells, their activation is linked to different pathophysiological mechanisms, such as liver fibrosis (94), cancer progression and mitigation (86, 95), and liver regeneration (86). Given the pathophysiological roles of stellate cells, different pharmacological interventions targeting them have been researched with the intent of developing therapeutic strategies to treat disease settings involving liver fibrosis (96). Therefore, MPS with stellate cells could be used to model the efficacy of such interventions.

In the context of cancer treatment, 3D spheroids have been shown to model interactions between cancer cells and stellate cells that affect tumor chemoresistance (95), further demonstrating the potential of using stellate cells to predict drug efficacy in specific contexts of use. Inclusion of stellate cells in MPS for evaluating drug safety could potentially screen for drugs with side effects that

target stellate cells and affect their physiological roles or improve the prediction of drug inflammatory effects (97). In conclusion, replicating the different physiological roles of stellate cells in MPS could set different contexts of use for MPS related to drug hepatic effects.

CELL SOURCES AND THE POTENTIAL OF DIFFERENTIATING HUMAN INDUCED PLURIPOTENT STEM CELLS

Overall, establishing reliable cell sources and ensuring the quality of cellular materials to integrate MPS is one of the major roadblocks for using these systems in drug development (98). Given the known differences between humans and other species in metabolism, transport, and other hepatic properties (99-102), cells in MPS must be of human origin and have human-specific function to predict clinical drug effects. In general, cells isolated from human livers (18, 65, 103) or cells differentiated from human iPSCs (104, 105) have been used in the liver MPS field. So far, with the most recent differentiation protocols, the function, drug metabolism, gene expression, and protein-protein interactions of iPSC-differentiated hepatocytes do not fully resemble what is observed in primary hepatocytes (106-108). Primary hepatocytes and primary hepatic cells in general are still the most mature and physiologically relevant cell types to use in liver MPS due to these differences and variable efficiency of iPSC differentiation (109). However, as occurs generally for most tissue-specific primary cells, the quality of cells isolated from human liver depends on the quality of tissue origin and experimental variables inherent to isolation and cryopreservation techniques (110). In addition, iPSC-differentiated cells have a potential for replicating genetic backgrounds that may represent different populations, minorities, and rare disease states, which is not trivial to achieve by banking primary hepatic cells (111). Therefore, iPSC-differentiated cells can overcome several limitations associated with the use of primary hepatocytes, which are still the gold standard for cellular materials to use in liver MPS.

More reliable iPSC-differentiated models can originate from further research in improving differentiation and maturation protocols (112-115), as well as in setting standards for cell handling and use (98). The liver consists of endoderm-derived hepatocytes, biliary lineages, and various mesoderm-derived cells (116-118), and the differentiation of all different cell types in the liver must involve different specialized differentiation protocols. Therefore, inducing simultaneous differentiation of all cell types in the same culture microenvironment may be difficult given the need for different protocols for each cell type (119-121).

Most importantly, iPSC-hepatocytes differ biologically from primary hepatocytes in properties that define hepatic maturity (119). For example, dramatic differences exist in biomarker expression (e.g.,

albumin and urea), binucleation, gene expression, metabolism, and transport (119, 122, 123). Maturity of cells differentiated from iPSCs is still the main limitation of this technology, but the field is progressing quickly towards enhancing the maturity of iPSC-differentiated hepatic-like cells and evaluating their potential contexts of use (122) despite the biological disparities between these cells and primary hepatic lineages. Beyond improvement of differentiation protocols based on small molecules or transcription factors, more physiological culture conditions have been shown to enhance the functional maturity of iPSC-derived hepatocytes. Some of these conditions include micropatterning cells, co-culturing with other cell types, controlled oxygenation, and 3D cultures (104, 106, 114, 124). These results strongly suggest that MPS can also be used and designed to improve the maturity of iPSC-derived hepatocytes and the physiological relevance of drug assays with these cells.

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