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Review

Microphysiological systems in early stage drug development: Perspectives on current applications and future impact

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ABSTRACT — Microphysiological systems (MPS) are making advances to provide more standardized and predictive physiologically relevant responses to test articles in living tissues and organ systems. The excitement surrounding the potential of MPS to better predict human responses to medicines and improving clinical translation is overshadowed by their relatively slow adoption by the pharmaceutical industry and regulators. Collaboration between multiorganizational consortia and regulators is necessary to build an understanding of the strengths and limitations of MPS models and closing the current gaps. Here, we review some of the advances in MPS research, focusing on liver, intestine, vascular system, kidney and lung and present examples highlighting the context of use for these systems. For MPS to gain a foothold in drug development, they must have added value over existing approaches. Ideally, the application of MPS will augment *in vivo* studies and reduce the use of animals via tiered screening with less reliance on exploratory toxicology studies to screen compounds. Because MPS support multiple cell types (e.g. primary or stem-cell derived cells) and organ systems, identifying when MPS are more appropriate than simple 2D *in vitro* models for understanding physiological responses to test articles is necessary. Once identified, MPS models require qualification for that specific context of use and must be reproducible to allow future validation. Ultimately, the challenges of balancing complexity with reproducibility will inform the promise of advancing the MPS field and are critical for realization of the goal to reduce, refine and replace (3Rs) the use of animals in nonclinical research.

Key words: Microphysiological systems, Complex *in vitro* models, Organ-chips, Drug safety

INTRODUCTION

Microphysiological systems (MPS) is a wide-encompassing term for transformative novel *in vitro* models which are carefully designed to offer more predictive *in vivo*-like potential by mimicking physiologically relevant functions in living tissues and organ systems. The definition of MPS is frequently equated with the *state-of-the-art* engineered microfluidic organ-chip technology but can also be synonymous with other types of complex *in vitro* models, including static Transwell systems, spheroids, organoids or micropatterned co-culture systems, and frequently implies the trade-off between assay throughput and physiologically relevant *in vivo* functions

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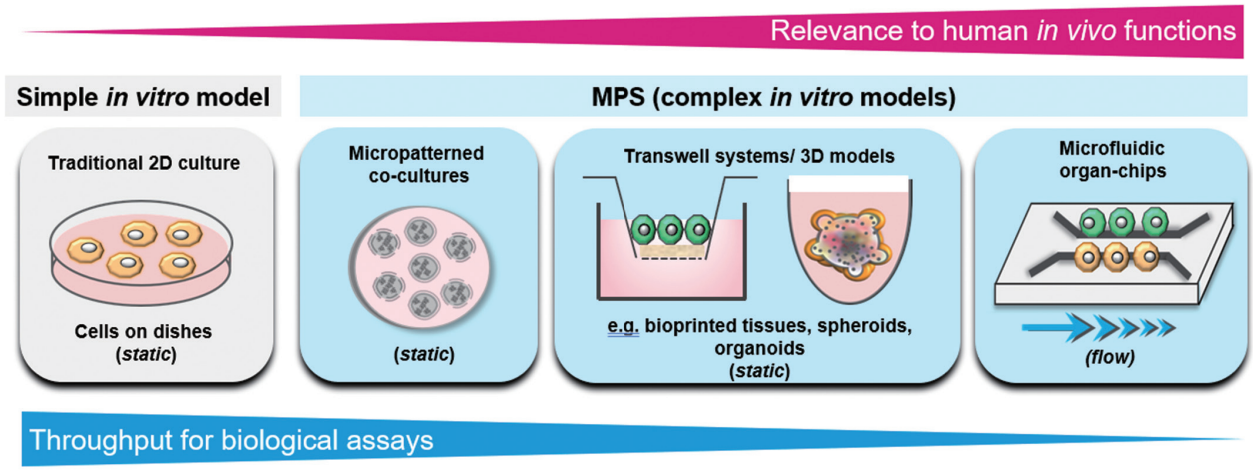

Fig. 1. Graphical representation of *in vitro* model examples demonstrating a range of platform complexity, throughput and relevance to human *in vivo* functions.

(Fig. 1). A recently published definition by the Innovation & Quality (IQ) MPS Affiliate consortium states that MPS refer to systems extending beyond traditional 2D *in vitro* models to include several of the following design aspects: a multi-cellular environment of primary or stem-cell derived cells within a relevant matrix, a 3D structure, flow, incorporate mechanical cues such as stretch for mimicking breathing or peristalsis, and/or include immune system components (Fabre *et al*., 2020).

MPS have gained a lot of attention in biopharmaceutical research in the last 10 years as evidenced by the increasing number of publications highlighting both the rapid development and use of these emerging technologies by MPS developers, academic institutions and the pharmaceutical industry. Because each MPS can support multiple cell types, often using relevant primary or stem-cell derived cells and 3D architecture, MPS are considered more appropriate for answering complex questions than using simple 2D *in vitro* models when studying physiological responses to drugs; they have the potential to become a revolutionary tool to transform the drug discovery process. As a result of improved design features, MPS offer more precise control over cell culture conditions compared with traditional 2D *in vitro* systems by providing relevant cell-to-cell interactions and external environment cues (e.g. shear stress, stretch) to recapitulate *in vivo* conditions and to better predict physiological responses. Furthermore, compared with *in vivo* studies, MPS typically require smaller quantities of test article, reducing the need for synthesizing large batches of compound.

Owing to the unique technological and design advancements, there is a growing potential for MPS to disrupt and eventually transform the drug discovery process. At present, to progress from target identification and validation to clinical assessment, many nonclinical studies including the use of *in vitro* and *in vivo* models must be conducted to profile nonclinical safety and efficacy. Traditional *in vitro* models typically use monocultures of primary cells or cell lines without complex tissue-specific architectures and are generally lacking with respect to *in vivo* relevance and complexity. Nonclinical animal models may offer more predictive power in some cases, but translation of findings to humans frequently remains uncertain. A futuristic vision for MPS, would be to combine *in vitro* human MPS organ data (both normal and disease-derived) from multiple tissues (i.e. human-on-a-chip) with advanced mathematical modeling to allow drug programs to directly progress into the clinic eliminating the need for nonclinical *in vivo* studies. The near-term vision is centered around determining the translation of *in vivo* animal effects to humans by bridging across animal and human MPS and improving *in vivo* study design with potential reduction in research animals and steady progress towards elimination of some nonclinical studies, resulting in a positive impact on reduction, refinement and replacement of animal use (3Rs). It is also theorized that MPS could eventually be used to stratify patients for clinical trials, identify subpopulations of patients that may need drug dosing adjustments and pre-

Table 1. High-level overview of the strengths and limitations of different *in vitro* platforms.

In vitro platform	Complexity	Throughput	Cost	Considerations
Traditional 2D cultures	+	++++	+	Simple; Not costly; Easy to handle; Amenable to high-throughput screening; May lack relevant phenotypic markers; Short-term viability
Micropatterned co-cultures	+	+++	++	Amenable to screening; Easy to handle; Extended culture longevity; Stromal cells from different species may complicate analysis
Spheroids	++	+++	++	Amenable to screening; Difficult to handle due to size; Can develop necrotic core if too large; Imaging into spheroids can be problematic
Organoids	+++	++	+++	Maintain organ anatomic microstructure; Difficult to culture/perfuse; May require complex hydrogel/scaffolds
Microfluidic organ-chips	++++	+	++++	Most physiologically relevant; Require intense user training; Generally costly with lower throughput

dict responses of susceptible patient populations and their risk of adverse events (Ewart and Roth, 2020).

While the vision for comprehensive application of MPS is looking far into the future, in order to advance it, there are currently several opportunities for MPS to add value across the drug discovery pipeline. For example, MPS can improve target identification and validation by recapitulating relevant disease models and using human MPS can help enhance compound progression in lead compound identification and optimization. Additionally, once the compound reaches clinical trials, MPS can also be applied at the clinical assessment stage to provide human relevant follow-up of clinical safety findings. Moreover, animal cell-based MPS allow for direct comparisons between animal MPS and *in vivo* animal data in turn building confidence in the utilization of human MPS models to inform data translation to humans. Overall, application of MPS in a rigorous and standardized way could enable streamlined and efficient drug discovery and development.

CONTEXT OF USE FOR APPLICATION OF COMPLEX *IN VITRO* MODELS

The current predominant context of use for MPS models is screening of compounds for efficacy or toxicity, identifying mechanisms of action when adverse phenotypes are identified and investigating species differentiation and concordance. Overall, the main areas of MPS utility fall under the umbrella of safety, efficacy and absorption, distribution, metabolism and/or excretion (ADME) considerations. MPS can also be used to predict the translation of nonclinical findings to people, assess advanced disease models, identify and validate targets, improve pharmacology and may even contribute to new biomarker development.

Application of complex MPS models is expected to be strategically driven to evaluate compound profiles regarding safety and efficacy and to further investigate potential mechanisms of action. Additionally, application of MPS models should augment *in vivo* studies to reduce and potentially replace the use of animals via tiered screening of compounds. This could range from rank ordering compound safety in the context of MPS-generated data to a more comprehensive evaluation of *in vitro* data to provide early risk assessment. In addition, MPS platforms may supplement traditional 2D platforms when functional and more physiologic endpoints are necessary and/or beneficial to help address outstanding mechanistic questions.

An overview of general strengths and weaknesses of select MPS models is summarized in Table 1. The overall appeal of physiologically relevant MPS models makes them very desirable for any *in vitro* application. However, given the associated cost and relatively lower throughput of complex MPS models compared with 2D *in vitro* systems, there is a need to demonstrate clear improvement over traditional methodologies when deciding on using MPS to address a specific question for a particular context of use. For example, certain clinical effects are poorly detected by current *in vitro* approaches, such as prediction of glomerular toxicity that is only sufficiently captured by *in vivo* models, or vascular injury that requires organ-specific architecture with organized vessels and appropriate interactions with tissue cells. However, if questions can be answered with simpler 2D *in vitro* models, then investment in MPS models that provide only a moderately improved response may not be recommended.

An important consideration to move complex *in vitro* models from specific targeted use to more consistent and standard use in pharmaceutical development is qualification or validation. An example of an extensive validation effort is the assessment of skin irritation that has been undertaken in reconstructed skin models and published as guidelines by the Organisation for Economic and Cooperation and Development (OECD). Unlike the activities leading to the official qualification of biomarkers used in clinical trials, the validation efforts conducted by the OECD represent a high level of validation that assures the reproducibility of specific skin models to mitigate risk for skin irritation or photosensitivity in humans. However, this type of multisite effort testing of many compounds to ensure reproducibility may not be necessary for the regulatory submission of MPS data for a single program. Qualification for a specific context of use supporting reproducibility and accuracy in a single laboratory would likely be sufficient.

Disclosure of assay development and testing for a specific context of use could support a model/assay for a regulatory filing, if the information could be used by a reviewer to confidently assess the assay and the results. Supporting data should include the assay conditions and format, cell types, analyses, and controls used. The company submitting the data has the responsibility to ensure that the entirety of the generated data, including reliance on MPS results, offers no risk to humans. There is always a risk that when MPS data are submitted to regulators, they could be interpreted differently by the submitting company and the regulator, possibly causing confusion and delaying the progress of a life-saving medicine. Potential hesitancy on the part of sponsors and confusion on the part of regulators will decrease as more reproducible and qualified systems become available.

TARGET ORGANS MOST FREQUENTLY ASSOCIATED WITH ADVERSE FINDINGS

Animal toxicity studies are conducted along the entire continuum of discovery and development of new molecular entities whether in the pharmaceutical or chemical industries. Initially, these studies contribute to de-risking targets or screening potential compound series and later for clinical development and marketing authorizations. When information is combined from two toxicology species, animal data yield 70% concordance in uncovering toxicities seen in humans (Olson *et al*., 2000). The critical data from these animal studies allow timely decisions on the fate of new molecules moving to the next stages of development (Monticello *et al*., 2017). One of the most important pieces of information gained from these studies is the identification of target organs and the characterization of adverse effects, reversibility of these effects, clinical monitorability, and importantly their impact on humans and the environment. Recent progress in early screening has allowed advancing promising molecules to next stages; however, attrition of compounds that have entered pivotal studies can be costly and prevent promising therapeutic candidates from being assessed in humans. Based on compound modality, as many as 30% of drug candidates are lost to emerging toxicology issues (*Pfizer's internal data*). Some of the main target organs of toxicity have remained consistent over the years but do vary by therapeutic area and compound modality. Without a particular order, cardiovascular, hepatobiliary, gastrointestinal, reproductive, renal, hemolymphatic, and central nervous system are among the most affected.

ORGAN-SPECIFIC MPS CONSIDERATIONS

A number of organ-specific MPS are in development and being utilized in biopharmaceutical research including liver, gastrointestinal tract, vascular system, kidney, and lung. These are further discussed below.

Liver

Drug-induced liver injury (DILI) is poorly predicted using animal models (Olson *et al*., 2000) and attrition of therapeutic candidates due to DILI in nonclinical and clinical development remains high despite advancements in predictive DILI model developments. This may be related to the fact that current approaches using immortalized cancer-derived cell lines and monoculture conditions fail to recapitulate the multicellular and complex structural environment found in liver tissue *in vivo*. For example, screening compounds in general cell viability assays along with experiments using liver-specific assays (such as BSEP inhibition) and primary human sandwich-cultured hepatocytes still fail to predict DILI potential in many cases including complex DILI mechanisms e.g., idiosyncratic DILI. Therefore, modeling hepatic responses with complex liver MPS models may help to close the gaps associated with poor prediction potential of DILI in humans.

Details on the recommended specifications for evaluating liver MPS models are discussed by Baudy and colleagues in their recent publication (Baudy *et al*., 2020). When selecting liver MPS, regardless of platform complexity, initial assessment of certain characteristics should be performed to assure the best functional performance of the model. As loss of normal hepatocyte func-

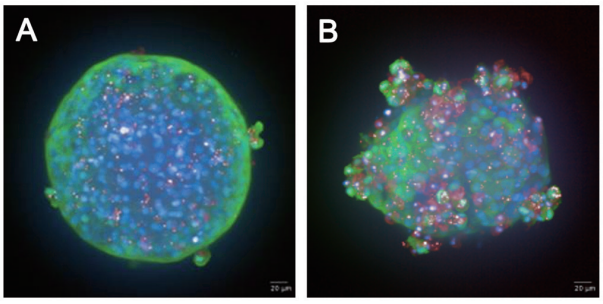

Fig. 2. Visualization of cell death in hepatic spheroids. Human liver microtissues (spheroids, InSphero, Inc.) composed of hepatocytes and Kupffer cells were treated with (A) vehicle with lipopolysaccharide (LPS, 1 µg/mL) or (B) 125 µM diclofenac with LPS (1 µg/mL) for 72 hr to induce hepatic injury. Following treatments, spheroids were stained with fluorescent dyes to visualize plasma membrane (CellMask Green), nuclei (Hoechst, blue) and dead cells (Draq7, red). Spheroids were fixed in 4% paraformaldehyde and cleared in ScaleS reagents for imaging. As expected, treatment with diclofenac and LPS induced morphological changes to the spheroid structure, evidenced by hepatic cell death (increased Draq7 staining), suggestive of drug-induced liver injury. Images were captured with a 20X objective using Operetta CLS High-Content Screening System (Perkin Elmer).

tion is very commonly observed in traditional 2D primary *in vitro* hepatocytes, one way to determine and monitor the overall hepatocyte health status in the MPS platform over time is to evaluate albumin and urea production. In addition to the evaluation of albumin and urea assessment, consideration of the baseline metabolic capacity of hepatocytes in the MPS platform is recommended. Gene/protein expression of key drug metabolizing phase I and phase II enzymes and transporters should be examined over a time course of at least 14 days but can be longer if required by individual liver MPS experiments. Important markers of liver viability, including alanine aminotransferase (ALT) and lactate dehydrogenase (LDH) activities, and transporter function and bile acid homeostasis should also be examined. Furthermore, inclusion of the relevant cell types in liver MPS (i.e. hepatocytes only vs. hepatocytes and non-parenchymal cells such as Kupffer cells and cholangiocytes) should be driven by the specific question and context of use. For example, if liver MPS is to be used for identifying a mechanism of immune-mediated DILI, inclusion of Kupffer cells might be necessary. For the study of bile acid homeostasis and biliary clearance of drugs, inclusion of polarized hepatocytes with adequate bile canaliculi in combination with bile duct epithelial cells (cholangiocytes) should ideally be integrated into liver MPS. Importantly, powerful microscopic imaging offering sufficient resolution of miniature liver MPS models may be necessary when traditional assays evaluating marker release into media lack relevant sensitivity. Examples of high-content imaging of hepatic spheroids following hepatotoxic insult and GFP transduction in liver microfluidic chips are presented in Fig. 2 and Fig. 3, respectively.

Overall, specific research questions being asked will dictate the composition and complexity of liver MPS platforms. As an example, albumin secretion, urea synthesis and cytochrome P450 expression were enhanced by coculturing primary human hepatocytes with stromal cells (Khetani and Bhatia, 2008) or with endothelial cells (Liu *et al*., 2014). This has been explored as liver-on-a-chip MPS using a conventional microfluidic bilayer chip used previously for lung-on-a-chip (Huh *et al*., 2010). Liver sinusoids were mimicked by sequentially seeding sinusoidal endothelial cells and Kupffer cells on one side of a porous membrane, while hepatic stellate cells were seeded on the other side and hepatocytes were cultured on the bottom surface of the channel (Du *et al*., 2017). In this model, flow-induced shear stress enhanced the secretion of hepatocyte growth factors and enzyme activity, and the co-culture enhanced albumin secretion and neutrophil recruitment. In another experimental design, liver spheroids made of HepG2/C3A cells were embedded with gelatin methacryloyl hydrogel in a polydimethylsiloxane/poly (methyl methacrylate) (PDMS/PMMA) chamber (Bhise *et al*., 2016). In that setting, long-term culture (30 days) with media flow rate of 200 µL/hr enabled sufficient oxygen supply (1.0×10^{-4} mol/m^3) by perfusion culture. In a third example, conventional sandwich culture of rat primary hepatocytes and collagen was evaluated in a microfluidic chip, which was also exposed to perfusion flow (Hegde *et al*., 2014). Flow-enabled bile canaliculi formation resulted in higher albumin and urea secretions and induced cytochrome P450 1A1 activity in comparison with static culture (Hegde *et al*., 2014). These examples portray the importance of 3D co-culture in recapitulating adequate structure and function for evaluating physiologic changes in the liver MPS.

Intestine

Gastrointestinal toxicity is one of the leading dose-limiting target organ toxicities predominantly identified in oncology programs. Drug-induced gastrointestinal toxicity hampers clinical development, frequently leading to dose reduction, dose delay and can even pre-

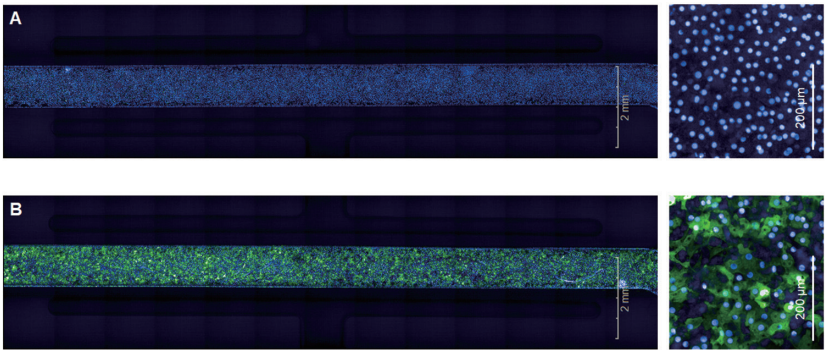

Fig. 3. Assessment of GFP transduction in liver microfluidic chips. Liver chips (Emulate, Inc.) cultured with primary cynomolgus monkey hepatocytes in the top channel and primary cynomolgus monkey liver sinusoidal endothelial cells in the bottom channel, were treated with (A) vehicle or (B) AAV containing an eGFP transgene. Following 5 days post-infection, chips were stained with Hoechst to visualize nuclei and expression of eGFP was assessed. The results demonstrate that microfluidic hepatic co-culture chips are an appropriate model to assess AAV-mediated transduction efficiency. Chips were scanned in their entirety with Opera Phenix High-Content Screening System using a 10X objective (Perkin Elmer). Magnified images are located to the right of full channel scans.

vent compounds from achieving desired efficacy. The adverse effects associated with gastrointestinal toxicity can be broken down by individual symptoms and include nausea, vomiting, constipation, diarrhea and abdominal pain. It is important to stress that while *in vivo* modeling of these symptoms is heavily reliant on histopathological assessment of the intestine coupled with functional endpoints like fecal pellet count or motility imaging, recapitulation of these adverse effects *in vitro* has been very challenging.

The required cell types necessary to recapitulate relevant gut function will depend on the specific mechanistic question to be addressed but will generally require major epithelial cell type functions such as stem cell renewal, cell proliferation, Paneth cell support, goblet cell secretion and enterocyte barrier function. Moreover, self-organization into crypt and villus-like domains would provide spatial evidence of relevant cell-to-cell interactions, mimicking intestinal structure and physiology. Ideal gastrointestinal MPS would also carefully replicate the intestinal epithelial cell (enterocyte) interaction with commensal bacteria (microbiome) that is rarely evaluated in traditional gut epithelial monocultures. Finally, incorporation of mechanical factors mimicking peristalsis might further improve *in vivo* predictivity of intestinal MPS. Important considerations for MPS model specifications are discussed in detail by Peters and co-authors in their recent manuscript (Peters *et al*., 2020).

The existence of a well-established human intestinal epithelial cell line, Caco2, has greatly contributed to early evaluation of intestinal MPS models. Prior to the development of a gut-chip, Caco2 cells were traditionally cultured in a static Transwell format. For example, a typical assay was performed by seeding Caco2 on collagen I and a Matrigel-coated porous membrane (Kim *et al*., 2012). As discussed later in lung-on-a-chip case, the application of shear stress exerted by the force of flowing medium over cells along with cyclic mechanical strain imparted by a stretchable PDMS membrane led to the improvement of barrier function and paracellular permeability. The same model was applied to recapitulate 3D villus morphology and damage caused by pathogenic bacterial (enteroinvasive *E. coli*) overgrowth even without mechanical strain (Kim *et al*., 2016). Co-culture with vascular endothelial cells can be used to investigate polarized cytokine release in response to the addition of lipopolysaccharide in the “lumen” channel and human peripheral blood mononuclear cells in the “endothelium” channel. In another example, human intestinal crypts derived from biopsy samples were cultured with intestinal microvascular endothelium to recapitulate intestine-on-a-chip mimicking human duodenum (Kasendra *et al*., 2018). Compared with the Caco2 chip, intestine chips were found to have elevated sucrase activity and mucin 2 levels, demonstrating that key intestinal physiologic functions were present. Other studies recapitulated a hypoxia gradient across the endothelial-epithelial interface by introducing aerobic and anaerobic human gut microbiota onto an intestine-on-a-chip (Jalili-Firoozinezhad *et al*., 2019). In addition to intestine organ-chips, stem-cell derived

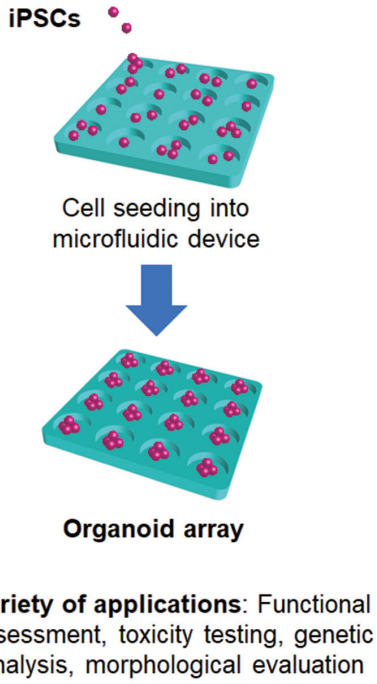

Fig. 4. Idea for massive and parallel organoid evaluation on a chip with many downstream applications.

intestinal organoids have also found routine applications in screening assays and biological modeling of tissues. Recent work described generation of patient-derived gastrointestinal cancer organoids via stem-cell aggregation in microcavity arrays that were used to evaluate efficacy of anti-cancer drugs (Brandenberg *et al*., 2020). The organoid array technology [e.g. using induced pluripotent stem cells (iPSCs)] is likely going to be applicable to other target tissues and organ systems to enable higher throughput scalable screening efforts with many downstream applications (Fig. 4).

Vascular system

Cardiovascular effects including electrophysiological and hemodynamic changes, as well as histopathological findings contribute to as much as 18% of compound terminations in the late discovery phase (Fabre *et al*., 2020). While Fabre and others acknowledge that it will be challenging for *in vitro* MPS to reproduce all potential toxicities, understanding the capabilities and limitations of each system will help to determine the appropriate questions to ask of each system. Among histopathologic changes, drug-induced vascular injury (DIVI) remains a recurring source of nonclinical toxicity-related attrition. In our experience, due to the low incidence and variable location of vascular changes, exploratory animal toxicology studies have not been able to detect DIVI on a consistent basis possibly as a result of low number of animals used in these screening studies. However, in pivotal toxicity studies, DIVI accounted for ~8% of attrition of new therapeutic candidates (*Pfizer's internal data*). Our efforts to identify an *in vitro* screen for DIVI using static 2D preparations tended to lack sensitivity when compared with *in vivo* exposure ranges.

Human umbilical vein endothelial cells (HUVECs) and organ-specific primary human endothelial cells show promise in modeling vascular injury *in vitro* with potentially better clinical translation. It is challenging to create an organ-specific architecture requiring multiple cell types including endothelial cells, pericytes and/or smooth muscle cells, and immune system components and even more difficult to include organ-specific shear flow to mimic physiologic conditions. Many model systems have early promise to overcome one or more of these issues, but few systems have the capability to overcome all of them.

Due to the importance of the vasculature in all tissues *in vivo*, recapitulating vascular connections in a chip format is an important consideration. In recent years, one of the main research directions in the MPS area is to integrate multiple organs-on-a-chip to realize a human/body-on-a-chip. Considering each organ-on-a-chip as a unit component, it is possible to interconnect several components by engineering means, i.e. PDMS microfluidic channels (Schimek *et al*., 2020), PMMA modular devices (Esch *et al*., 2016) and three polymer layers (Edington *et al*., 2018). However, what remains challenging is the incorporation of vessels between individual MPS organs. Given the long history in vascular biology, fundamental studies on angiogenesis and vasculogenesis have been conducted using on-chip vessels even prior to the establishment of MPS technologies.

There are two major vessel bioengineering methods, which include the "pre-designed method", where a hollow structure for the vascular network is prefabricated and the "self-organizing method", which relies on spontaneous formation of endothelial cells in tubes as occurs with vasculogenesis or angiogenesis (Miura and Yokokawa, 2016). Prefabrication requires seeding of endothelial cells in extracellular matrix (ECM) or polymer materials using photolithography (Galie *et al*., 2014; Song *et al*., 2012; Song and Munn, 2011), needles (Chrobak *et al*., 2006; Sadr *et al*., 2011; Usuba *et al*., 2019; Yoshida *et al*., 2013) or sacrificial molding (Golden and Tien, 2007; Miller *et al*., 2012). ECM structures enable facile embedding of

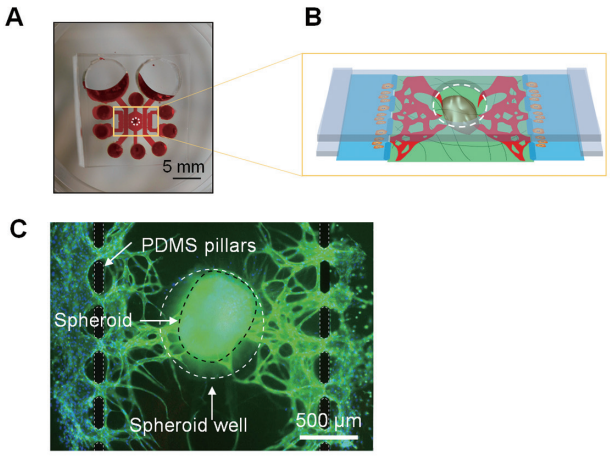

Fig. 5. The on-chip vascular network with a 3D spheroid tissue. (A) Photograph of a PDMS microfluidic device with red ink-filled channels. (B) Schematic of co-culture of vascular network structure with the spheroid in a microfluidic device. (C) Fluorescent micrograph of a vascular network composed of human umbilical vein endothelial cells (HUVECs) connected to a spheroid generated by co-culturing HUVECs with human lung fibroblasts. Additional model details are described in Nashimoto *et al*. (2017) publication.

endothelial cells (Chrobak *et al*., 2006; Galie *et al*., 2014; Golden and Tien, 2007; Usuba *et al*., 2019) when polymer channels are coated with ECM prior to cell seeding (Miller *et al*., 2012; Sadr *et al*., 2011; Song *et al*., 2012). Once endothelial cells, HUVECs in most cases, are cultured inside of the structures, effects of fluid shear stress (Galie *et al*., 2014; Song and Munn, 2011) and growth factors (Usuba *et al*., 2019) on angiogenesis can be studied. The "pre-designed method" is also used for co-culture with organ-specific cells for tissue engineering applications (Zhang *et al*., 2016). Owing to the stable and robust features of the method, vascular networks can be pre-designed to interconnect multiple organ components to create multi-organ MPS. Moreover, it is possible to quantitatively evaluate the behavior of endothelial cells at the single-cell level in response to biomechanical and biochemical cues, because the method precisely defines the structure of the vascular network, flow rate and gradient of growth factors in the microfluidic environment. The drawbacks of this approach are poor permeability of vascular walls and inability for vascular remodeling, both very important for the evaluation of DIVI *in vitro*.

The "self-organizing method" overcomes some of the disadvantages of the "pre-designed method", as it relies on spontaneous pattern formation of endothelial cells, as seen with vasculogenesis or angiogenesis, which is induced using Matrigel (tube formation assay) (Lawley and Kubota, 1989), fibroblast-coated fibrin beads (Nakatsu and Hughes, 2008) or direct application of recombinant VEGFs (Gerhardt *et al*., 2003). These culture dish-based methods are easily accessible in conventional drug discovery settings; however, the method is limited in the ability to evaluate intraluminal flow that is essential for assessing the efficacy of compounds. The advantage of microfluidic devices is to provide access to the intravascular space to reconstitute 3D perfusable vascular networks *in vitro*. In early models, human lung fibroblasts were co-cultured with HUVECs in microfluidic channels and VEGF and other angiogenic factors were introduced in a fibrin-collagen gel, in which HUVECs autonomously invaded the gel structure to create a hollow lumen, while leaving an opening at the interface of microfluidic channels (Kim *et al*., 2013; Yeon *et al*., 2012). Advantages of the "self-organizing method" include *in vivo* relevance such as vascular morphology, permeability and dynamic morphogenetics. A significant number of studies with co-culturing target cells in the vascular network have been reported for evaluating cancer cell extravasation (Boussommier-Calleja *et al*., 2019; Chen *et al*., 2017; Jeon *et al*., 2015) and permeability of the blood-brain barrier (Bang *et al*., 2017; Campisi *et al*., 2018). More recently, *in vivo* relevance of the method promoted not only the co-culture with target cells but also the connection of vessels with 3D tissues including spheroids (Nashimoto *et al*., 2017; Nashimoto *et al*., 2020) (Fig. 5) and organoids (Park *et al*., 2019). The self-organizing model also provides a platform to evaluate the effect of blood flow on drug efficacy (Nashimoto *et al*., 2020). One major drawback of the method is the design capability of the structure of the vascular network, which might make it challenging to interconnect multiple on-chip organs to create a human-on-a-chip.

Cell-derived microparticles or microvesicles are a type of extracellular vesicle (typically between 100 and 1000 nm in diameter) which have been reported to have roles in intercellular communication in addition to other biologic functions such as immunity and tissue regeneration (Hromada *et al*., 2017). In vascular MPS models with a microporous membrane separating two microfluidic channels (e.g. an endothelial channel and pericyte channel), when the membrane pores are 1-3 µm in diameter, cell-derived microparticles (< 1 µm) could travel from one channel to the other. Flow cytometry for pericyte-specific (NG2) or endothelial-specific (CD31) markers might have utility in exploring the movement of microparti-

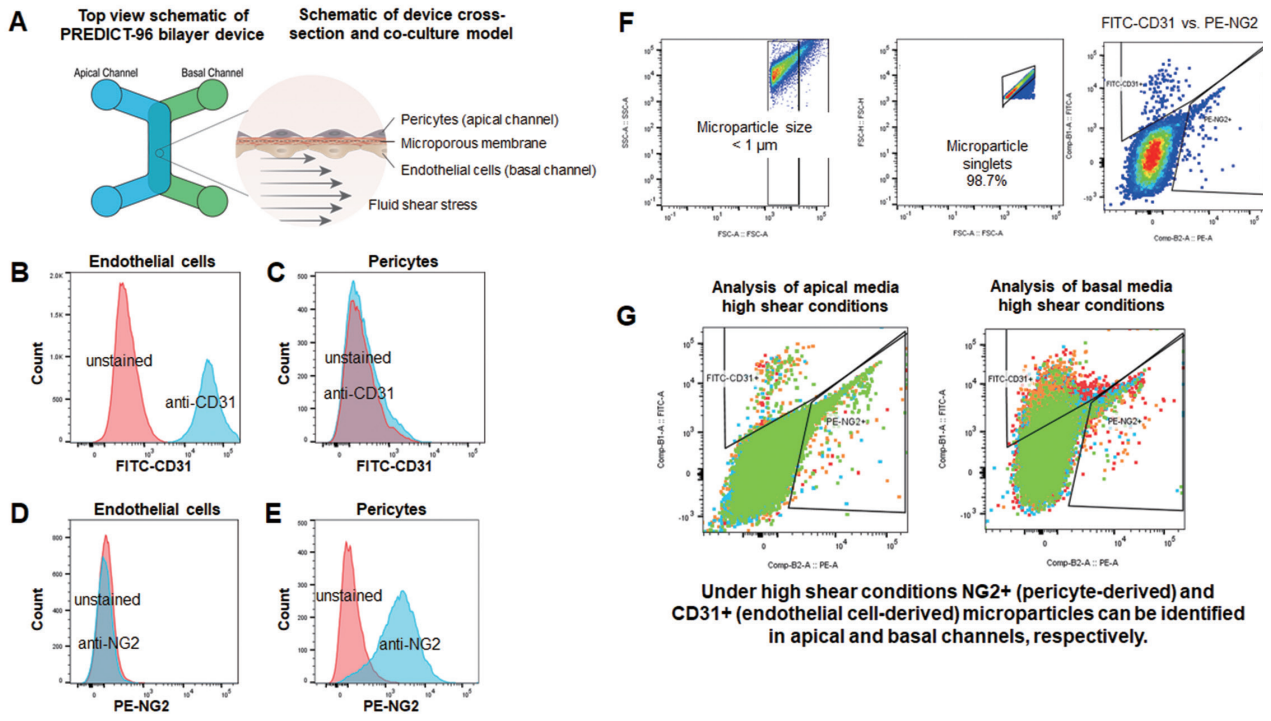

Fig. 6. Media microparticle evaluation from a microfluidic vascular-chip platform. (A) Human retinal microvascular pericytes and human microvascular endothelial cells were seeded in the apical and basal channels of the PREDICT-96 platform (Draper), respectively, to establish an indirect co-culture on either side of a microporous membrane separating the channels. Media were collected from each channel and cellular microparticles specific to each channel were evaluated. (B-C) Antibody against CD31 (labeled with FITC) was shown to be specific to endothelial cells only, while (D-E) antibody against NG2 (labeled with PE) showed unique specificity to pericytes. (F) Gating strategy was set to evaluate endothelial and pericyte-derived microparticles that were less than 1 µm in diameter. (G) Analysis of media from co-culture under high shear conditions demonstrated evidence of NG2+ and CD31+ microparticles in the apical and basal channels, respectively.

cles to allow investigation of intercellular communication in a co-culture system (Fig. 6). In-house flow cytometry analysis of media samples from a vascular microfluidic device (PREDICT-96 platform described in Tan *et al*. (2019)) demonstrated presence of cellular microparticles in both channels under high shear conditions (Fig. 6). While we cannot exclude the possibility that small cell fragments were also captured in the analysis (gating captured all particles < 1 µm), the preliminary results indicate that the majority of identified microparticles were found in the channel consistent with their cell of origin. Further exploration of high shear effects and TGF-β activation on the number of cell-derived microparticles in a vascular-chip is ongoing. In our experience, multichannel co-culture systems that incorporate pressurized fluid flow are capable of measuring the response to perturbations in a complex system such as cellular damage, barrier disruption, cytokine stimulation, and fluid shear stress. While initial results indicate improved robustness of these co-culture models, additional qualification for context of use is required. When considering qualification for context of use, specific criteria must be utilized to ensure adequate accuracy and reproducibility.

Kidney

Drug-induced nephrotoxicity is among the top 5 most frequently observed adverse effects reported during drug development. The development of *in vitro* models to successfully predict drug-induced kidney toxicity requires solid understanding of the specific cellular targets, mode of action of nephrotoxicants and reliable biomarkers of nephrotoxicity. In standard *in vitro* models, kidney epithelial cells exhibit poor apical-basal polarization and have low or no expression of transporter proteins, resulting in poor predictivity for kidney toxicity screening. The majority of kidney MPS models utilize the proxi-

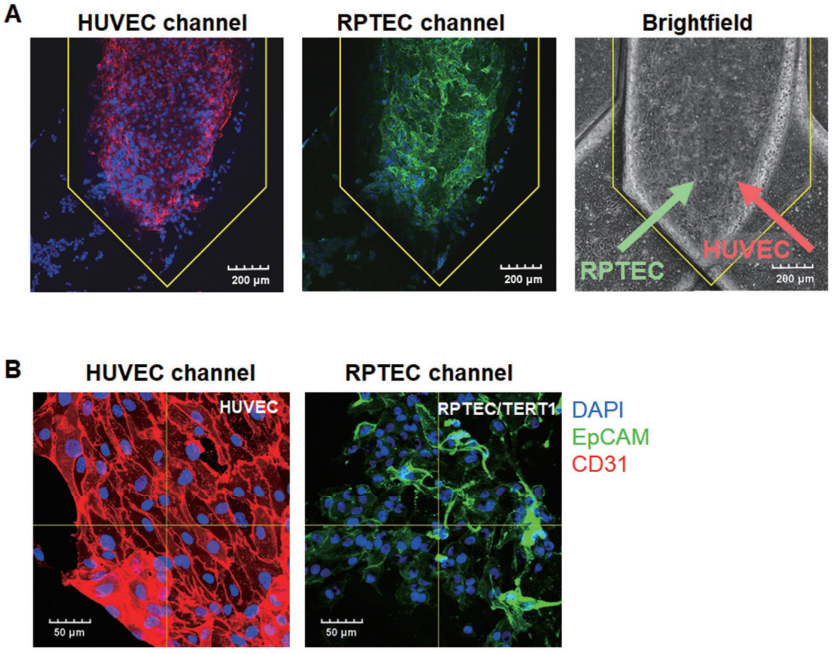

Fig. 7. Staining in a proximal tubule-on-a-chip. (A) Human umbilical vein endothelial cells (HUVECs) and renal proximal tubule epithelial cells (RPTECs) were cultured on the bottom and top sides of a porous membrane, respectively, in a microfluidic chip (Emulate, Inc.). Crosstalk, i.e. paracrine signaling, between the two channels leading to improved functionality of the epithelial layer is achieved through the membrane micropores. (B) Magnified images show expression of characteristic proteins: CD31 (red) for HUVECs and EpCAM (green) for RPTECs; nuclei are stained with DAPI (blue).

mal tubules, as this region is considered the major site of drug-induced toxicity, although other systems have also emerged that focus on different regions of the kidney. To this end, prediction of glomerular injury remains incredibly difficult as glomerular physiology has yet to be accurately represented *in vitro*. Moreover, models incorporating distal tubule or collecting ducts may be developed to capture renal segment-specific toxicants. Ideally, a future kidney MPS would integrate an entire nephron-on-a-chip containing all relevant units in the proper succession to allow screening and characterization of all renal toxicants in a single platform. Since it is recognized that this is a high bar to reach, current kidney models require proper characterization to support specific context of use for generation of decision-making data.

When selecting a kidney MPS, assessment of metabolizing enzymes and renal transporters that play a key role in disposition of drugs and toxicity is necessary. For example, in renal proximal tubule cells (RPTECs), there are multiple basolateral transporters necessary for uptake of metabolic products and drugs into the proximal tubule epithelium (e.g. OAT1, OAT3, OCT2) (Nieskens *et al*., 2016), as well as efflux transporters localized on the apical membrane (e.g. MRP2, MRP4). Examples of fluorescent staining and brightfield images of proximal tubule-on-a-chip are shown in Fig. 7. The epithelial (RPTEC) and endothelial (HUVEC) sides are identified by EpCAM and CD31 markers, respectively. Frequently, the cell lines and even primary cells used in kidney toxicity screening lack the appropriate transporter expression, and engineered cells overexpressing certain transporters are linked with dramatic increase in screening sensitivity. Given these considerations, careful cell sourcing for kidney MPS models is crucial. In addition to enzyme and transporter evaluation at the gene or protein level (and/or via immunohistochemistry), structural and functional readouts, like assessment of active transport capabilities is necessary for kidney MPS characterization. Finally, the ability to detect biomarkers in culture media (e.g. kidney injury molecule-1 [KIM-1], clusterin and neutrophil gelatinase-associated lipocalin [NGAL]) using commercial ELISA assays would further strengthen the performance and effectiveness of kidney MPS models in screening efforts. Additional kidney MPS qualifications and current gaps are discussed in detail by Philips and coauthors in their recent manuscript (Phillips *et al*., 2020).

In one of the reported examples of the glomerulus-on-a-chip which aimed to recapitulate glomerular barrier function, chips were created by seeding rat podocytes onto an ECM in a microfluidic device (Wang *et al*., 2017). In that model, authors demonstrated an increase in filtered albumin which was linked with increased glucose concentrations (from 5.5 mM to 35.5 mM), recapitulating a model of diabetic nephropathy. Other types of human podocytes, including primary, immortalized and amniotic fluid-derived podocytes, have been evaluated in a commercialized glomerulus-chip (Petrosyan *et al*., 2019). In that setting, immortalized podocytes did not deposit enough components of glomerular basement membrane such as collagens and laminins (specifically COL4A3 and LAMA5), resulting in less retention of albumin compared with primary and amniotic fluid-derived podocytes. In another study, glomerulus-chip was reconstituted with human iPSCs, which were differentiated to podocytes and expressed relevant phenotype markers (e.g. WT-1 and NPHS1) (Musah *et al*., 2017). Evaluation of adriamycin-induced toxicity demonstrated an increase in albumin filtration and podocyte damage, effects consistent with *in vivo* nephrotoxicity. These examples highlight models that are moving towards demonstration of barrier function and toxicity; however, glomerular physiology has yet to be fully reconstituted.

Significant efforts have been made in proximal tubule-on-a-chip in the MPS community owing to the above-mentioned importance of nephrotoxicity and transporter assays which are already being employed in biopharmaceutical research. When primary human proximal tubule epithelial cells were cultured under a perfusion flow, ZO-1 expression and albumin/glucose reabsorption increased compared with the static culture condition (Jang *et al*., 2013). In addition, P-glycoprotein ATP-binding cassette membrane transporter activity was elevated by the perfusion culture condition and the inhibition of its activity was demonstrated with verapamil. Co-culture with normal human microvascular endothelial cells resulted in enhanced proliferation of proximal tubule cells and mitochondrial activity compared with the epithelial monolayer culture condition. Another commercial chip recreated 3D proximal tubules (Tourovskaia *et al*., 2014; Weber *et al*., 2016), in which expression of γ-glutamyl transpeptidase and sodium/glucose cotransporter 2, ammoniagenesis and lower levels of KIM-1 were assessed as markers of cellular function. Importantly, secretory transport of the prototypical organic anion, para-aminohippurate, was measured with and without an inhibitor, probenecid, which typically cannot be assessed using a Transwell system. Long-term culture produced *in vivo*-like morphological characteristics of RPTECs such as physiologically relevant cell height, microvilli length and density (Homan *et al*., 2016), for which a sacrificial patterning method of tubular structure was developed using 3D printing. The result was applied to a co-culture model with an adjacent channel for endothelial cells (Lin *et al*., 2019). Glucose reabsorption was enhanced 5- to 10-fold in comparison with Transwell experiments. Owing to the capability of long-term culture, the MPS models described have the potential to be applied for evaluation of glucose filtration and reabsorption.

Lung

Modeling lung responses *in vitro* is met with multiple challenges due to the complex architecture and diverse functions of the lung. Desirable characteristics for lung models should at minimum include alveolar epithelium with air interface as well as pulmonary endothelium with access to a media/blood compartment to best mimic *in vivo*-like lung functions. Lung was the first organ-on-a-chip model reported in 2010 that led to the development of the MPS community (Huh *et al*., 2010). As with any MPS system, sources of pulmonary cell types and their functional capabilities were closely examined before selecting a relevant model. Multiple lung *in vitro* models have been described to date for drug discovery applications, including lung organoids, air-liquid interface cultures using Transwell systems (Harney *et al*., 2021), bioprinted human lung tissues and lung-chip devices, with the latter exhibiting the greatest complexity. PDMS microfluidic devices allow for the cyclic stretch to be applied to reproduce physiological breathing movements. In that model, human alveolar epithelial cells and pulmonary microvascular endothelial cells can be seeded in the top and bottom channel of the device, respectively. Air and media flow can be independently set for each channel with cyclic vacuum applied to the sides of the channels to imitate breathing.

Earlier versions of the lung-chip model have been utilized in several experiments including drug toxicity-induced pulmonary edema (Huh *et al*., 2012), evaluation of metabolic activity and cytokine secretion by physical stimuli (Stucki *et al*., 2015), asthma and chronic obstructive pulmonary disease models (Benam *et al*., 2016b), and effects of smoking including e-cigarettes (Benam *et al*., 2016a). These applications demonstrated applicability of the lung-on-a-chip to disease models and to other organs as discussed in previous sections. Additional details regarding desirable characteristics of lung MPS models have been carefully reviewed by Ainslie *et al*. (2019).

The respiratory viral pandemic caused by SARS-CoV-2 (COVID-19) makes the lung-on-a-chip an attractive platform to evaluate the efficacy of existing drugs. In a publication preprint, an airway model with human lung epithelial cells expressing ACE2 and TMPRSS2 and endothelium were prepared for evaluation of FDA-approved drugs, demonstrating the significant decrease of viral entry by amodiaquine, toremifene, and clomiphene (Si *et al*., 2020). Although the study employed a pseudotyped SARS-CoV-2 virus, the model may provide potential value for the current pandemic. Another publication preprint effort recently reported that the native SARS-CoV-2 infection induced lung injury and an immune response (Zhang *et al*., 2020). The infection on the chip was demonstrated by immunostaining of viral Spike protein and evaluation by transmission electron microscopy. Further, *in vivo* infection was modeled by circulating immune cells caused by removal of endothelial cells and an increase of inflammatory cytokines. Suppression of viral replication and damage of the alveolar-capillary barrier was significant with the use of remdesivir, despite the clinical findings reporting mixed evidence (Eastman *et al*., 2020; Singh *et al*., 2020). The acute demand during the pandemic significantly accelerates the use of and need for MPS models in nonclinical evaluation not only of existing drugs but also for the evaluation of new drugs in the future. MPS will likely enable understanding of the infection mechanism by monitoring infection and immune responses using a route of infection model from upper to lower airway and alveoli-on-a-chip. Manifestation of COVID-19 symptoms depends on the age, race, and concomitant diseases and additionally on potential mutation of the SARS-CoV-2 proteins. Such diversification of COVID-19 infection can likely be recapitulated using MPS models with a variety of viruses and patient-derived iPSCs in contrast to the cost- and time-intensive development of new humanized mouse models.

CELL SOURCING FOR MPS MODELS

Primary and immortalized cells are mainly used in traditional 2D *in vitro* models in the pharmaceutical industry due to the stability of cell supply, expression of functional genes/proteins and reproducibility of outcomes in assays. Recent advances in stem-cell technology are enabling the use of human iPSCs and embryonic stem cells in the development of relevant tissue MPS models. The stable supply of iPSCs and their differentiation protocols to organ-specific cells may provide advantageous benefits for conducting reproducible experiments. The use of stem cells can also improve physiological functions of MPS by the appropriate expression of target genes and functional proteins, which are often lacking in primary/immortalized cells used in the majority of current MPS models enabling more human *in vivo*-like functions compared with conventional 2D *in vitro* models.

FUTURE OF COMPLEX *IN VITRO* MODELS AND IMPACT ON 3RS

Despite the immense potential of MPS technology, there are still multiple challenges to the widespread adoption of these models by the pharmaceutical industry, with the main challenges linked to model qualification, clinical translation and acceptance by regulators. The exuberance of the industry to adopt these models will need to be tempered by the ability to reproduce the results in different laboratories. This will be accomplished through execution of proof-of-concept studies, more cross-pharma consortia level qualifications of organ-specific MPS platforms, and regulatory acceptance of specific systems enabled by collaborations and internal learnings. All of these will enable the adoption and acceleration of MPS usage. The universal adoption and acceptance of the models as the new *status quo* is crucial for the short-term success and industry-wide implementation of this technology. Once the predictive capacity of MPS models is recapitulated with confidence across organ models, MPS could be used to rank order compounds with a known toxicity to start to decrease the need for multiple exploratory toxicology studies with different chemotypes. The benefit of such an application would have an immediate impact on the 3Rs and increase the use of MPS platforms without the need to submit the drug candidate screening data to regulatory agencies.

With regards to a long-term focus on the application of MPS, pairing traditional 2D *in vitro* models with MPS for more complex readouts may be the best way to leverage the tools that have had reproducibility for early compound risk assessment while incorporating more physiologically relevant systems. Using a combined strategy, one could envision a head-to-head comparison with traditional exploratory toxicology studies in order to move towards reducing animal usage within studies. With new drug modalities, more complex formulations, and the desire to get medicines to patients sooner, programs strive to streamline active pharmaceutical ingredient (API) manufacturing alongside risk assessment, often reducing the availability of API in early stage drug development. This may make the determination of a maximum tolerated dose in early toxicity studies less likely, which increases the potential of an *in vitro* approach to compensate for

the lack of these early *in vivo* studies. Selection of specific organs to be modeled with MPS could be derived from target knowledge reviews, chemical class knowledge, and *in silico* readouts partnered with standard *in vitro* platforms. Ultimately, the functional readout from an MPS model could drive understanding of the risks of a chemotype prior to *in vivo* evaluations and could enable a program to move forward even with limited API availability.

Although eliminating *in vivo* animal studies from drug research and development is a high bar for the foreseeable future, application of MPS has already shown potential to significantly impact the 3Rs. Addressing mechanistic toxicity questions with MPS models instead of conducting additional *in vivo* studies can yield a positive step towards the refinement and reduction of animal use in research.

CONCLUSION

There is an ongoing collaboration between regulators and industry on the development of alternative models to *in vivo* animal experiments which is critical to future success of MPS. These models are being discussed and evaluated by multiple disciplines (pathology, toxicology, pharmacology, ADME) and on multiple continents (e.g. North America, Europe, and Asia). One of the largest barriers to adoption is the hesitancy of pharmaceutical companies to submit data to regulatory authorities that may initially slow down the progress of getting medicines to patients. Without understanding of how regulators will assess the data, there will continue to be concerns with reliance of MPS-focused submissions. The frequent usage of human cell lines in the MPS models and platforms promises better translatability but also a lot of discussion on producing data that are yet to be fully understood. A "safe harbor" for data submissions, such as has been utilized for other initiatives e.g. toxicogenomics, could overcome some of these barriers.

The conversation about the potential for and future use of MPS is becoming more grounded as the hype surrounding the initial promises of these technologies is tempered with additional understanding of the limitations and qualification efforts required to allow widespread usage. The US-FDA has focused on reproducibility of assays (Ribeiro *et al*., 2019; Rubiano *et al*., 2020) to allow adoption in different laboratories and uniformity of data produced to enable consistent interpretation of findings. The improving capabilities of these complex models will continue to move the field towards a reduction in animal usage and better translatability to human endpoints.

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Conflict of interest---- The authors declare that there is no conflict of interest.

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