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Utilizing microphysiological systems and induced pluripotent stem cells for disease modeling: a case study for blood brain barrier research in a pharmaceutical setting

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ABSTRACT

Microphysiological systems (MPS) may be able to provide the pharmaceutical industry models that can reflect human physiological responses to improve drug discovery and translational outcomes. With lack of efficacy being the primary cause for drug attrition, developing MPS disease models would help researchers identify novel targets, study mechanisms in more physiologically-relevant depth, screen for novel biomarkers and test/optimize various therapeutics (small molecules, nanoparticles and biologics). Furthermore, with advances in inducible pluripotent stem cell technology (iPSC), pharmaceutical companies can access cells from patients to help recreate specific disease phenotypes in MPS platforms. Combining iPSC and MPS technologies will contribute to our understanding of the complexities of neurodegenerative diseases and of the blood brain barrier (BBB) leading to development of enhanced therapeutics.

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Contents

1. Improving Translatability: Human Microphysiological Models (MPS) and Inducible Pluripotent Stem Cells (iPSC)

Drug discovery is an arduous process (12 to 15 years) that involves the cohesion of a wide range of scientific disciplines. Furthermore, delivery of a novel drug that is safe and efficacious is estimated to cost $2.6 billion [1–3]. Given that approximately 60% of overall drug attrition is

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due to lack of efficacy in later-stage clinical trials, there is a substantial effort by pharmaceutical companies to adopt technologies and models that can better reflect tissues in human health and disease [2,4,5].

Evidence is abundant that the translatability of preclinical research, including target identification and target validation [5], could be improved by utilizing cell and tissue based systems that model normal physiology as well as disease pathophysiology [6,7]. Current *in vitro* assays are limited in that they often lack multicellular populations, and many are structured in a 2-dimensional format. 3D spheroids or organoids can have multidimensional elements, but lack mechanical stress (such as flow and cyclic stretch) that tissues experience *in vivo*. Moreover, lack of translatability from *in vivo* models to the clinic are attributed to a consequence of differences between human and other species, including the interactome [8], signaling pathways [9], DNA variation and gene expression patterns [10–12].

Microphysiological Systems (MPS), designed to incorporate multicellular tissues, 3D scaffolding and mechanical stress offer a credible solution to these challenges [13,14]. Careful consideration into the bioengineering regarding 3D molds, flow rates, material and microfluidics is given to MPS platform design to mimic organ physiology. As for choices on the right tissue or cells that populate MPS devices, practical challenges often occur when using primary human tissues because of isolation procedures, storage conditions and donor variability, which ultimately can compromise the reproducibility of experiments [15]. Using human iPSC is one way of overcoming these practical issues as well as avoiding ethical concerns associated with access to primary tissue.

iPSC are adult somatic cells, reprogrammed to an embryonic stem cell-like stage by transcription factors, this method was first published by Yamanaka and colleagues in 2007 [16]. iPSC resemble the embryonic stem cells and can be used to generate most cell types of the human body. Reprogramming can be performed by forcing the cells to express transcription factors. Today, the preferred reprogramming methods do not permanently alter the DNA of the cells, such as non-integrating virus mediated expression or mRNA transfection [17]. iPSC lines are commonly validated by expression of pluripotency markers such as, Oct4, klf4 and Nanog, karyotype and their ability to differentiate to cell types of all germ layers. However, iPSC lines can be generated from different donors, using multiple techniques, culture conditions, and different somatic cell types. Consequently, each iPSC line is unique, even though standard requirements for an iPSC line is met. When studying disease, the possibility to use patient specific cells is a great advantage of iPSC technology, but because of variations between iPSC lines it is highly important that results are validated in several lines from different donors. This has also been recognized by the scientific publication community where it is not uncommon for journals to require several donors and clones to be tested in each study. For industry, access to iPSC to populate MPS platforms will be advantageous for gaining better insight into human disease, improving target identification/validation and enabling development of safer, and more efficacious therapeutics. iPSC are highly proliferative and can be generated in very large quantities. They have self-renewal capacity and thus the number of experiments that can be performed with cells from the same donor is close to unlimited [18]. Consequently, iPSC technology allows for studies of human cells in a reproducible fashion that has not been possible before using primary cells or tissues. In recent years, the use of iPSC in preclinical models has gained interest and a wide range of models including lung epithelial cells, cardiac cells, neural cells and liver cells have now been developed and used in research projects [19–22]. Another key advantage to iPSC is that many iPSC derived cell types are now readily available from commercial sources, which come with a basic level of characterization and quality control helping with standardization.

Combining iPSC and MPS technologies will lead to the next generation research for target identification, disease modeling and drug discovery. While MPS platforms provide an *in vivo*-like microenvironment, iPSC lines offer reliable cell sources, derived from patients. Furthermore, iPSC are amenable to precise genome editing, which can be used to correct for genetic mutations or introduce genetic variants associated with a disease [23]. This offers invaluable tools for deciphering disease mechanisms and identification of new therapeutic approaches [24]. Many primary cell types, such as neurons, are difficult to genetically modify *in vitro*, thus iPSC cells provides a new possibility to create isogenic pairs for any cell type. With the recent discovery of CRISPR/Cas9, genetic modifications of cells can now be performed faster, cheaper and with better precision than before [25]. By combining the targeting of specific sites with cleverly engineered donor DNA, it is possible to create a variety of genetic changes that include knock in, knock out, tagging of a protein, reporter lines, genetic correction of a mutation, introduction of a mutation, conditional knockouts, conditionally expressed proteins and more [26–30]. Indeed, populating MPS platforms with these engineered cells will allow investigation of how genetic alterations modulate physiological and disease processes when compared to corresponding isogenic controls or to healthy tissues [31]. Performing MPS experiments on such isogenic pairs can truly reveal correlation between genotype and phenotype without the uncertainty of having different donors for disease and control lines.

1.1. iPSC-MPS case example: the Blood Brain Barrier (BBB)

Modeling neurodegenerative diseases and disorders for novel target identification or efficacy assessment remains a daunting task because 1) the pharmacokinetics and pharmacodynamics of the blood-brain-barrier is complex and 2) current *in vitro* models often lack the intricacies of neurophysiology such as the architecture [32–34]. And, as previously mentioned, animal models can address some of these issues but often fail to translate into clinical success [32,35,36]. Because of these obstacles, most pharmaceutical companies have reduced research and development in this space. However, according to the World Health Organization, the numbers for disability-adjusted life years (DALYs) for mental disorders/substance abuse, neurodegenerative diseases and brain/nervous system cancers in the Americas are 28.8 M, 14.6 M and 1.41 M, respectively [37]. Data from the Center for Disease Control demonstrates that cerebrovascular disease and Alzheimer's dementia are the 5th and 6th leading causes of death, respectively, accounting for a combined 9% of total deaths in the US alone [38]. Therefore, strategic research and development in this space to improve human quality of life and ease economic burden is needed.

The BBB presents a significant challenge for the development of compounds to treat diseases as it limits the exposure of molecules in the brain. The BBB is made up of endothelial cells, astrocytes and brain-specific pericytes that form the blood-brain capillaries [39]. The multi-cellular complex is tightly packed such that transport of molecules into the brain occurs mainly by transcellular passive diffusion or active uptake. These BBB-specific endothelial cells are also rich in efflux transporters, particularly P-glycoprotein (P-gp), Breast cancer resistant protein (BCRP) and Multidrug resistance-associated protein (MRPs) which are expressed on the luminal membrane and serve to pump molecules from the brain into the blood [39,40]. Multiple uptake transporters such as OATP, GLUT1, LAT1 are also expressed in the BBB and serve to transport endogenous substrates into the brain.

In-vitro models used to assess BBB permeability of molecules in drug discovery are typically epithelial cell culture monolayers such as those formed from human colorectal adenocarcinoma (Caco-2) or Madin-Darby canine kidney (MDCK) cells overexpressing efflux transporters of interest (Pgp, BCRP) [41,42]. In these assays the cells form tight junctions with high trans-epithelial electrical resistances [43,44] and express various human BBB efflux transporters. By determining efflux ratios for compounds in these assays (apparent permeability rate constant in the basolateral (B) to apical (A) direction divided by the apparent permeability rate constant in the A to B direction) an assessment can be made as to whether compounds are substrates for the BBB efflux

transporters, a key determinant of whether a compound will have limited brain exposure.

Complementary *in-vivo* rodent studies are also undertaken in drug discovery as part of BBB permeability assessment. Typically, this involves measurement of free brain and free plasma levels following either an intravenous infusion or an oral dose to a healthy rat. The distribution equilibrium constant, Kpuu, is then derived *i.e.* the ratio of the free brain/free plasma concentration. In a compound with good brain penetration, a value around 1 will be observed.

These *in-vitro* and *in-vivo* BBB models can provide insight into compound permeability in the BBB, but are still only surrogates for the human BBB endothelium. *In-vitro* models do not accurately reflect all the transporters (both active uptake and efflux transporters) seen in the human BBB nor do they reflect their true protein expression levels [45]. Furthermore, 2D mono-culture models are also unable to capture the mechanics of blood flow, which can influence cell function [46]. The rodent *in-vivo* models do represent a more accurate BBB system, but have the limitation of introducing a species difference as well as being an expensive assay typically requiring multiple animals in a study.

1.2. Current BBB MPS models

The iPSC-MPS for BBB model can address challenges of current BBB models by providing the right cell source and the right microenvironment [47,48]. Perfusion promotes formation of a tight barrier, downregulates cell cycle and increases BBB-specific transport protein production [49]. In the context of the BBB platform design, MPS developers bioengineer specific tissue chambers that dictate how cells will be arranged in the device, usually involving a "blood" and "brain" chamber separated by a "barrier" artificial membrane [50–53]. For example, Brown et al. created the Vanderbilt neurovascular unit (V-NVU), and is highlighted here because it is one of the first to use primary human endothelia, pericytes and astrocytes, in addition to iPSC-derived glutamatergic neurons. The device is a Poly-dimethyl siloxane (PDMS) bioengineered platform that contains a "vascular" chamber with inlet channels, coated with endothelial cells and is adjacent to the neural chamber (astrocytes, pericytes, neurons and ECM) [48,54]. The blood/brain compartments are separated by a porous polycarbonate (PC) membrane. Biological readouts showed they could keep cells viable for up to 21 days, with tissues that formed tight junctions (ZO-1 expression). Functional permeability tests included FITC-dextran (10–70 kDa) and trans-epithelial electrical resistance (TEER) assessment. Physiological tests included glutamate exposure, active transport and barrier tightening with ascorbate, cold shock, which increased expression of the tight junction protein ZO-1.

Another approach to building a BBB-MPS is to create a more physiologic BBB architecture, so that the endothelium forms vascular/tubular structures that make direct contact (without an artificial membrane) with neural cells such as astrocytes and pericytes. The reasoning behind this approach is that 3D structure and cell-cell interaction influence transport function, barrier permeability, transcellular passive diffusion and active uptake means of transporters [47]. Furthermore, several BBB models have a rectangular design in the flow chambers, which could expose cells to sheer stress that is not uniform. An example of a physiological-3D BBB model is from Herland et al. from the Wyss Institute, where they designed a 3D BBB microvascular structure from primary human brain endothelia, and was then co-cultured with primary human BBB-specific astrocytes or pericytes [55]. They used this model as a proof-of-concept to show how 3D structure, without an artificial membrane and in co-culture, could identify distinct contributions to the neuro-inflammatory response. When stimulated with tumor necrosis factor-alpha (TNF-α), the presence of astrocytes or pericytes influenced different secretion profiles for granulocyte colony-stimulating factor (G-CSF) and interleukin-6 (IL-6), which was significantly greater than when the same cells were co-cultured in static transwell plates, further supporting that perfusion is needed for BBB function. A similar approach from Phan et al. at the University of California at Irvine created a 3D microenvironment that allowed for biological self-assembly of a functional microvascular network that included stromal cells, endothelia from human endothelial colony forming cell-endothelial cells (ECFC-EC), primary pericytes enriched from human stroma and astrocytes derived from human neural stem cells in enriched ECM [47]. These pharmacokinetic parameters are likely better measured in an MPS containing a physiological co-culture CNS cell types as compared to a simple 2D cell culture. In principle, the presence of relevant neurophysiological architecture in an MPS means that the target engagement of drugs can also be assessed, something that is normally difficult to achieve *in vitro*.

Regarding the cell selection for the BBB MPS platform, it has been shown that co-culture of endothelial, astrocytes and pericytes enhances BBB function by improving tightening barrier functions [47,55,56], enhances neuro-inflammatory responses [55] and increases expression of several key BBB genes [47]. Therefore, a robust BBB MPS platform should incorporate key features in a variety of ways to model an *in vitro* physiological microenvironment using co-cultures of endothelial, astrocytes and pericytes plus perfusion. For the BBB endothelia, many of the current models contain rodent immortalized cells (bEnd.3) or RBE4, immortalized human (hCMEC/ D3), human primary or human iPSC-derived [47]. Co-cultured BBB cells include immortalized mouse astrocytes, primary rat astrocytes, primary human brain pericytes and primary human astrocytes [47]. Based on the review of the present BBB MPS landscape, it is evident that incorporating iPSC-derived BBB models are only now beginning to emerge. Careful consideration must be taken into account in selecting the right cell source and should be fully characterized to ensure the cells possess correct BBB function. For example, the presence of the various cell types in an BBB-MPS along with vascular flow should not only induce induces genes shared by all endothelia, such as Tie2, PECAM1 and claudin 5, but also those transcripts that are expressed almost exclusively in the BBB endothelium, such as glut-1, p-glycoprotein, occluding and the transferrin receptor [57,86].

1.3. BBB iPSC-MPS for disease models

Evidence is clear that populating BBB MPS platforms with iPSC-derived cells from healthy and diseased patients will enhance our understanding BBB biology and lead to the identification of novel targets. iPSC BBB cells can be derived from somatic tissue (skin, blood) and differentiated into most cell types such as astrocytes, pericytes, neurons, oligodendrocytes and brain endothelial cells [58–64]. Indeed, significant work has been done in the iPSC neurological space [60,65], with a range of applications involving patient/control panels, disease models, 3D organoids, chemical screening and personalized therapy [66]. Phenotypes from monogenic brain-related rare diseases can be recapitulated using iPSC, providing a "limitless" cell resource. [18,67]. Complex neurodevelopmental diseases have also been studied using iPSC, including schizophrenia where they were able to replicate diminished neuronal connectivity, low neurite numbers, PSD95-protein levels and glutamate receptor expression known to occur with the disease [18,68]. Autism spectrum disorder with macrocephaly was also modeled using iPSC-derived neural organoids (including GABAergic), which showed upregulation of genes involved in cell proliferation, neuronal differentiation and synaptic assembly when compared to control tissue from the parents [69].

Supplementing the model with additional cell types like innate immune cells will allow the study of the role of inflammation in neurodegenerative diseases, such as with Alzheimer's Disease (AD). For AD, there are two factors that underline the pharmacological importance of the BBB. Firstly, inflammatory mediators, generated centrally, may increase the permeability of the BBB locally, predictably helping therapeutic access to the brain. Secondly the BBB likely gates the important interaction between peripheral inflammatory signals, generated by

systemic infection, and the pace of central neuro-inflammation. Supporting such a link are mouse data showing peripheral inflammatory responses accelerating central neurodegenerative pathology, particularly in prion disease but also in AD models [70]. Accordingly, elderly people with repeated systemic infections are at increased risk of developing AD [71]. The interaction between immunity and the BBB has been underlined by genome-wide association studies (GWAS) and whole genome sequencing studies in AD [72]; these show that polymorphisms in neuro-inflammatory genes, specifically those linked to the innate immune response, are strong predictors of AD risk. Important examples of such risk genes are TREM2 and CD33. With the advent of iPSC technologies each cellular component (endothelial, astrocytes, pericytes, neurons and innate immune cells) of the BBB may be replaced by cells generated from patients with AD *vs* control individuals, allowing the MPS to encompass a greater range of disease pathophysiology.

1.4. Challenges with using iPSC-MPS BBB in drug discovery – How can they be overcome?

Although the MPS and iPSC field is developing at breathtaking speed, there are key requirements relating to characterization and validation that would enhance their application and adoption within drug discovery. This includes real-time sampling of liquid and cellular components to characterize and assess biomarkers, the ability to perform morphological assessments and the ability to relate more mechanistic and molecular endpoints to their likely clinical manifestations or outcomes. Analytical performance standards for these models also need to be established which would include throughput capability, biological platform stability, drug-biomaterial interactions, intra- and inter-laboratory reproducibility, integration and compatibility with existing laboratory processes and feasibility of shipping these delicate systems between vendors and users. Models should also be exposed to a set of reference compounds and demonstrate they can produce the desired outcome. A more formal qualification may be required if data from these models are to be included as part of a regulatory submission document. This will require close partnership with key regulatory bodies that is likely to involve intensive data sharing. Of course, for these models to be successfully adopted by the pharmaceutical industry they will need to be manufactured at scale and deployed using control units that are robust and easy to use. Each of these points is covered in detail in a recent manuscript published by a consortium of pharmaceutical companies [14].

In the context of the BBB MPS, cellular protein expression profiling, focusing on the components of the tight intercellular junctions that characterize the BBB, allow a first characterization of an MPS. Furthermore, looking for the presence of proteins that are found in the BBB endothelium is also important to exclude the presence of proteins that are not normally seen in the brain endothelium. Likewise, single cell RNA sequencing of single endothelial cells should have an expected transcriptional profile and exclude other transcript such as those more characteristic of epithelial cells. At a more functional level the administration of a series of small molecules, and biologics such as antibodies, to the vascular compartment, with recovery in the "interstitial fluid or tissue (parenchyma)" compartment may be informative. *In vivo* brain Kpuu experiments (from rodent PK, monkey PET and human PET studies) will provide estimates of the penetration across the BBB and comparison with the MPS will test physiological relevance and translatability. In practice these validation experiments are not trivial, not least because the polymer materials used in many MPS models may absorb small molecules non-specifically, and also because the fate of antibodies when crossing the BBB remains uncertain [73].

While using iPSC-MPS in drug discovery holds great possibilities, working with iPSC and iPSC derived cells is not without its challenges. Generation, culture and maintenance of iPSC is very laborious and some organ tissues are difficult to differentiate from iPSC. For example, it is difficult to establish differentiation protocols towards cell types for which the understanding of function and expression pattern is low, such as the pericyte [74]. In addition, many differentiation protocols, for example towards astrocytes and oligodendrocytes, require very long culturing times increasing the risk for contaminations. Furthermore, the lack of standardized protocols can lead to large heterogeneity in phenotype and immaturity of differentiated cells. In fact, iPSC derived cells often display a fetal phenotype [75–77]. However, culturing cells in an MPS model can help promote maturity, physiological response and long-term culture stability of iPSC derived cells, which has been demonstrated in cardiac, hepatic and neural cell cultures [78–80]. MPS culturing may be beneficial for the maturity of iPSC cells as it provides a more *in vivo* like culture setting and better supports cell-cell interactions than 2D cultures [81]. In addition, the maintenance of the cell cultures can be improved in MPS as it permits tighter control of temperature, pH and oxygen [82].

1.5. Future directions for iPSC-MPS in pharmaceutical industry

As the industry goes beyond the development of small molecules, particularly for drug development involving the CNS and BBB, there will be an increased need for *in vitro* model systems that are therapeutic-agnostic because the new therapeutic modalities will likely come from bio-therapeutics such as proteins, antibody drug conjugates and modified RNA. While these complex molecules hold much promise, they often rely on specialized drug delivery mechanisms to ensure target engagement that can only be adequately be tested *in vivo*. iPSC-MPS models allow for a viable alternative to not only testing efficacy, but also pharmacokinetics/pharmacodynamics of these cutting-edge therapeutic approaches in the patient-derived tissue models. Outcomes from these experiments will better inform industry on preclinical investments. iPSC-MPS will also play a key role in understanding idiosyncratic, or patient-dependent response to treatment.

And to further extend how we build the right therapeutic for the right patient, another area of drug discovery where MPS together with iPSC will make impact is with phenotypic drug discovery (PDD). In contrast to target based drug discovery, PDD does not rely on the knowledge of specific targets and mechanisms. Instead, PDD relies on screening of pharmaceutical candidates in a biologically relevant model and measuring an applicable endpoint. The low productivity of the drug discovery industry has been suggested to be one of the reasons for the increased interest in PDD during recent years [83,84]. However, one of the major challenges with PDD is to provide biologically relevant models in which the readout can accurately translate to the *in vivo* situation. Given that MPS models have been designed with biological relevance in mind they are perfectly suited to be involved in PDD. But, these models need to be provided in a format which can be screened with sufficient throughput [84]. Incorporation of iPSC into MPS models can enhance the creation of the disease phenotype either because these cells can be genetically modified to capture relevant genetic variants that drive disease phenotype or because iPSC taken from individuals with the required disease phenotype have been shown to retain a disease "memory" [85]. Consequently, the increased maturity and *in vivo* resemblance of MPS and iPSC systems will be very useful in the space of PDD.

Indeed, the future of iPSC-MPS will make impact in drug development, disease modeling and patient-specific therapies. Both iPSC and MPS technologies are still novel, with much validation still needing to be done. However, we recognize the importance of these models, especially for BBB and new modality therapeutics that have such limited options for human-based physiological-relevant models. All of these advantages mean that pharmaceutical companies need to decide whether to "buy" or "build" MPS expertise that involves skill sets which are not traditional pharmaceutical company roles. Establishing partnerships with MPS designers, engineers and manufacturers will be essential, but will also rely on partners from industry and MPS

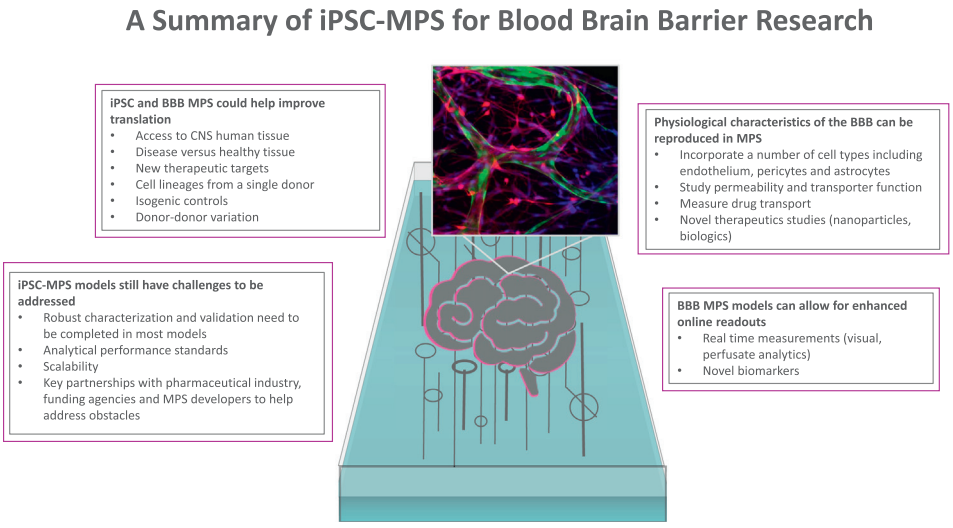

Fig. 1. Example of a bio-engineered BBB model: perfusable vessels (green; GFP) and astrocytes (red; GFAP) that make direct contact with the vascular structure (blue, DAPI for nuclear stain). Courtesy of Kino Biosciences.

developers, clearly articulating needs and challenges [14] to ensure success in adopting iPSC-MPS in R&D Fig. 1.

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