Modeling vascular disease using self-assembling human induced pluripotent stem cell derivatives in 3D vessels-on-chip

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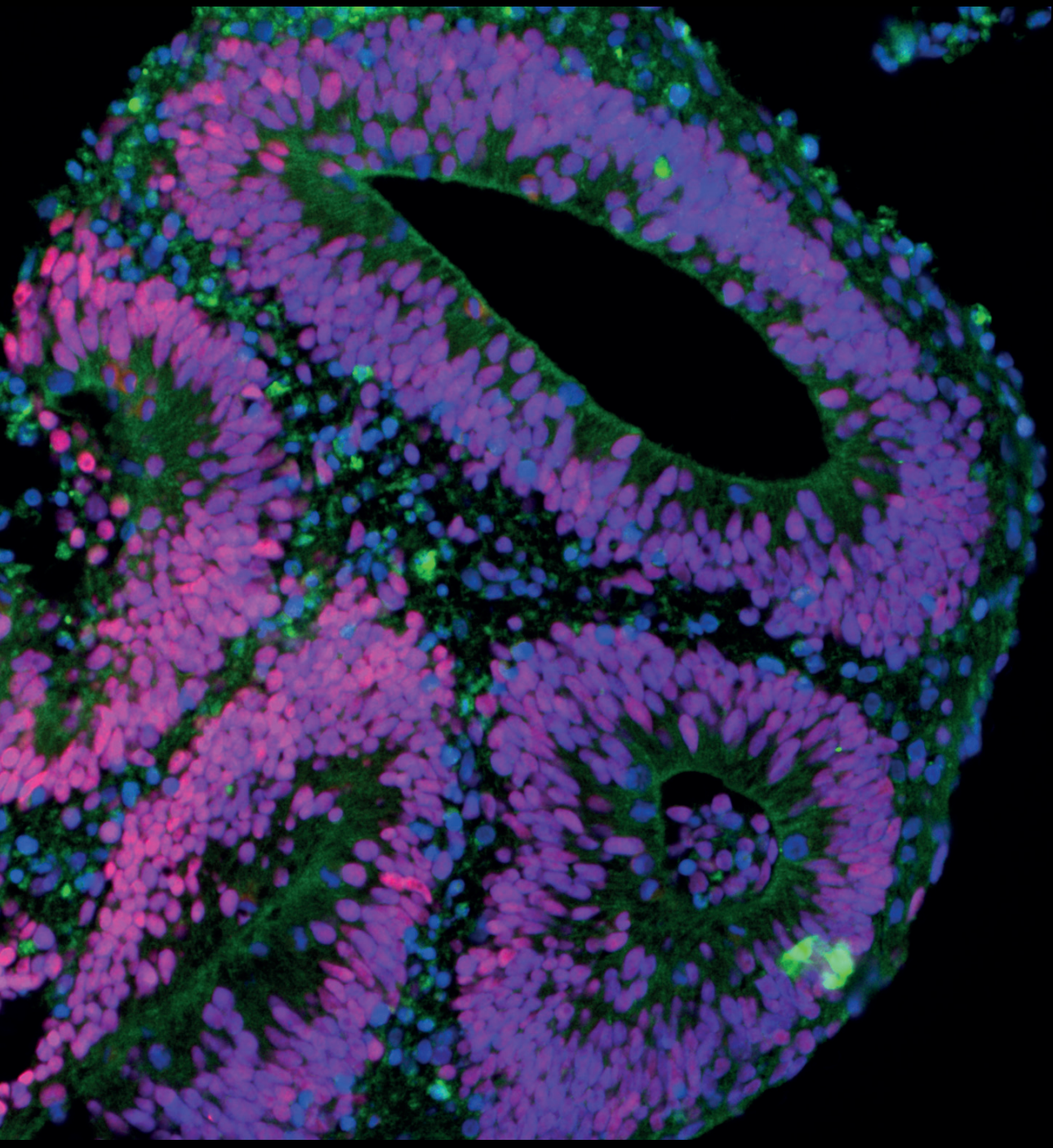

Chapter 7

Discussion and future perspectives

Introduction

Organ-on-Chip (OoC) models have emerged in the recent years as possible solutions to the substantial hurdle faced in drug development, where only a small fraction of candidate drugs advances to clinical approval, largely due to the inability to predict responses (and effectivity) in humans. Simple models are adequate in most primary compound screens for drug discovery but for more complex (secondary) screens *in vitro* models are needed that faithfully recapitulate human physiology[1]. Cardiovascular diseases are an increasing burden on society and thus present a prime target for the development of advanced (secondary) *in vitro* models. In this thesis, several steps have been taken to enable modeling of vascular diseases. Firstly, multiple cellular tools were generated from human pluripotent stem cells (hPSCs): endothelial cells, vascular smooth muscle cells and astrocytes. Some of these cells were employed successfully to study vascular defects in traditional 2D vasculogenesis assays. Secondly, Vessel-on-Chip (VoC) systems were developed to increase complexity and provide the appropriate stimuli to uncover cellular defects as seen *in vivo.* Ultimately, these complex secondary screen could be used to determine the effectivity of drug treatments in patients. The foundation of disease modeling in this thesis lies in the utilization of human induced pluripotent stem cells (hiPSCs) and their derivatives.

hiPSC-derived cells for disease modeling

hiPSCs have found applications across various model types for disease modeling and drug screening in traditional 2D cultures, microfluidic chips, tissue engineering or organoids[2,3]. While the pursuit for the most complex models may be academically intriguing, it is essential to adhere to the principle of seeking as much as simplicity as the research question allows. If a disease phenotype can be observed with a 2D assay, it offers a more cost-effective, potentially high throughput platform for future mechanistic and therapeutic studies. In this thesis, hiPSC lines and/or their isogenic controls were generated from patients with three distinct genetic vascular diseases in Chapter 3, Chapter 4 and Chapter 5. These studies yielded varying results in demonstrating disease phenotypes, some assays showing expected outcomes in relation to the patient phenotype, others being more challenging to interpret. This underscored the importance of several key considerations in hiPSC-based disease modeling.

Among the most basic issues, the first consideration regards the verification of the hiPSC lines and determination of the number of lines and clones to use when modeling diseases. hiPSC disease modeling often demands a large cohort size due to the greater genetic variability between lines compared to traditional inbred animal models or standard cell lines[4]. Recent studies have focused on understanding the statistical power and optimal study design for hiPSC-derived disease models. These studies have highlighted the increased power of using isogenic paired- over non-isogenic (familiarly related or independent) hiPSCs, particularly in modeling monogenic disorders, by minimizing the variation stemming

from individual genetic differences[5]. This aligns with the experimental design employed in the disease modeling in both Chapter 4 and Chapter 5. However, to further enhance the generalizability of findings to broader patient groups, inter-individual variation needs to be incorporated in the study. The most cost-effective way to achieve this is by including additional donors rather than increasing the number of hiPSC clones in the study design[6]. An innovative and potentially effective approach to do this is by combining different lines in a single dish, referred to as "village in a dish" model systems. These model systems have demonstrated the ability to significantly increase throughput while maintaining consistency in genetic, epigenetic or line-dependent effects underlying gene expression variation, comparable to cells cultured separately[7].

The second consideration in hiPSC disease modeling is the potential influence of the X-chromosome inactivation status (XCI) when working with female lines. As discussed in Chapter 4, aberrant XCI status in female hiPSCs could lead to changes in the cellular protein content and differentiation potential[8–10]. While erosion of XCI is primarily seen in human embryonic stem cells (hESCs) and only to a lesser extent in hiPSCs[11], significant variation remains between hiPSC lines. Interestingly, in Chapter 4, differences in XCI status between clones did not appear to directly correlate with hiPSC-ECs differentiation potential, as evidenced by variation in both gene and protein expression of EC-specific markers. This is consistent with some other reports demonstrating XCI status independent differentiation efficiencies into germ cells[12]. Future investigations should aim to elucidate the underlying mechanisms, potentially by exploring strategies to prevent aberrant XCI, as demonstrated with hESCs through changes in media composition using lithium chloride or inhibitors of GSK-3 proteins[13].

The final consideration is the recognized immaturity of most hiPSC-derived cells, which can limit the *in vitro* replication of disease phenotypes observed *in vivo*[3,14]. Immaturity as a limitation may also explain the absence of EC-defects seen in RVCL patient-derived hiPSC-ECs, as demonstrated in Chapter 4. Several strategies could be employed to enhance the maturity of hiPSC-derived cells, ranging from manipulation and improvement of the extracellular matrix (ECM), metabolism (e.g. altering the energy substrate), 3D culture, or biochemical stimulation of the cell culture using a multiplicity of reagents[15]. OoCs, and specifically VoC devices used in this thesis, offer unique abilities to alter these aspects in a controlled fashion by including microfluidic flow for factor delivery as well as shear stress over the endothelial cells lining the vessel wall.

Vessels-on-Chip

The potential of complex humanized models, such as VoCs, has been recognized for over a decade[16]. However, examples of studies actually demonstrating their true benefit over traditional models to uncover disease pathologies remained limited. In this thesis, we provided such an example in Chapter 5 by unveiling a disease phenotype within a VoC

device that was absent in standard 2D assays. Furthermore, we extended our VoC model towards a brain-specific variant in Chapter 6. The extensions to model these brain-specific interactions hold particular significance due to the urgent need for complex humanized models, specifically in the context of vascular pathologies of the human brain for which access to primary tissue (biopsies) is mostly not an option. Recent studies have emphasized this urgency, by clearly showing only minimal overlap between *in vivo* mouse and human transcriptomic changes induced by stroke in the brain microvasculature[17].

The VoCs models in this thesis make use of the self-assembly ability of the vascular cells introduced into the chips. While they demonstrated their value, our work has also highlighted certain general considerations in future exploration of these models. For discussion of these considerations, it is helpful to regard the model as a concept of *designed* and *emergent* features which have to be compared to *in vivo* physiology as far as possible, this was described and considered at length in the review in Chapter 2. For an overview and distinction of designed versus emergent features, particularly relevant in self-assembling VoCs, see Figure 1.

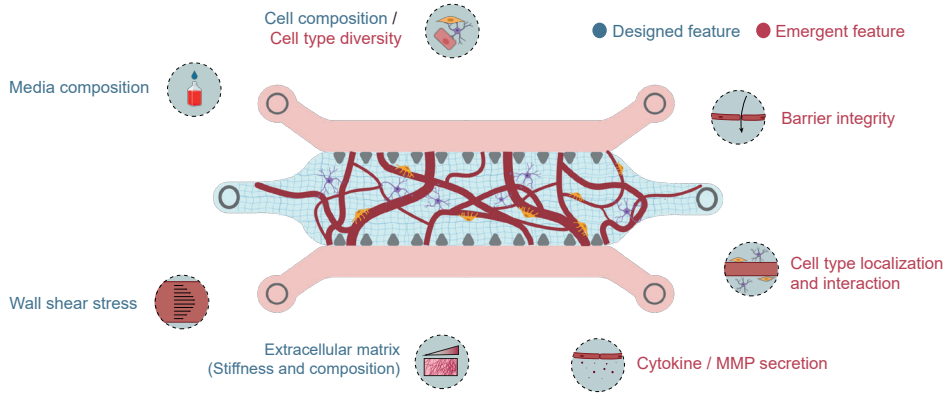

Figure 1: Schematic of the essential designed and emergent features in self-assembling Vessel-on-Chip models. *MMP = matrix metalloproteinases. (Icons of the features were adapted from Nahon, Moerkens et al 2023, in press)*

Designed features in self-assembling VoCs

In Chapter 5, we successfully uncovered a disease phenotype using hiPSC-ECs derived from hereditary hemorrhagic telangiectasia (HHT) patients, using a commercially-available self-assembling VoC model. While this underscores the model's current utility, several facets offer room for refinement to achieve a closer resemblance to clinical manifestations. We were able to replicate the reduced mural cell – EC interactions as observed *in vivo*[18]; however, we simultaneously observed a decrease in the vessel diameter where in HHT patients, the average vessel diameter is increased, culminating in the formation of arteriovenous

malformations (AVMs). This occurs due to increased EC proliferation, EC enlargement and reduced EC migration[19–22]. One plausible explanation for this *in vivo*/*in vitro* difference is that the *in vivo* HHT phenotype is significantly influenced by secondary factors such as inflammation[23] and shear stress[19,24–26]. Consequently, incorporating inflammation as an additional trigger, either in the form of cellular source of inflammatory cells or via media supplementation with inflammatory cytokines, could be considered as a designed feature. Notably, we have proof-of-concept data illustrating an enhanced inflammatory response of HHT1 hiPSC-ECs when exposed to TNFα and BMP9 (Xu Cao, unpublished work). This was demonstrated at both a transcriptional level through RNA-sequencing and functionally via impaired barrier function. Future work could also include the inflammatory component in a cellular form by introducing HHT1 mutant and wildtype hiPSC-derived monocytes in the system. Previous reports have demonstrated that self-assembling models like these enable the functional characterization of immune cell recruitment, interaction and transmigration within the microvascular network[27].

Shear stress is another secondary factor influencing HHT phenotypes *in vivo*[28]. Ways to modulate this could be introduced to the model as designed feature, specifically as fluid flow under controlled and variable flow rates. The most straight forward way, as implemented in Chapter 6, is to use a "rocker" platform that can induce continuous (albeit bidirectional) fluid flow in the microfluidic chips. This method is easy to scale for large numbers of samples; however, we estimate that it yields an average shear rate in the microvascular networks of ~0.01 Pa, an order of magnitude lower than seen in the human body[29]. It is worth noting, that the use of a rocking platform induces bidirectional flow, which has been shown to induce distinct cellular behavior and transcriptional responses compared to the physiological unidirectional flow seen *in vivo* [30–33]. This divergence in behavior is likely influenced by the ECs' known directional response to flow, inducing cellular rearrangements through cytoskeletal remodeling to migrate in the opposite direction of the flow[34]. To circumvent this, unidirectional flow in self-assembling systems has been established by use of large 3D printed reservoirs on the inlets allowing application of a substantial hydrostatic pressure[35–37], or by connecting the microfluidic chip to a pressure pump[38,39]. Implementing similar strategies in our model could potentially enhance the physiological relevance and facilitate the exploration of additional disease mechanisms.

In Chapter 6 we established a self-assembling VoC model using hiPSC-ECs, human brain vascular pericytes (HBVPs) and hiPSC-derived astrocytes (iAstros), replicating cellular interactions that would occur in the blood brain barrier (BBB). iAstros and primary human astrocytes (pAstro) exhibited similar behavior within the VoC cultures and we subsequently demonstrated that we could further improve the culture by application of continuous flow or activation of the cAMP pathway. Nevertheless, the model did not consistently exhibit an upregulation of BBB-specific EC markers, so that even though the correct cell types were present, features seem to be still missing. To optimize the model further, exploration of

additional alterations in designed features, such as cell source, media composition and extracellular matrix (ECM), is needed.

The generation of hiPSC-ECs with BBB characteristics has been a dynamic and debated field over the past decade. As outlined in Chapter 1, one of the most used protocols to generate these cells, yields ECs with a strong resemblance to epithelial-like cells[40]. To enable establishment of genuine ECs with BBB characteristics, in Chapter 6 we chose to use hiPSC-ECs generated from a mesodermal state, aligning with known developmental pathways. While we previously demonstrated the capacity of hiPSC-ECs to acquire tissue-specific characteristics through co-culturing with tissue-specific cells[41], the co-culture with iAstros did not consistently induce tissue-specific transcriptomic changes. *In vitro* studies have highlighted the role of astrocyte-secreted factors such as sonic hedgehog and angiotensin in supporting and inducing BBB function[42–44]. Future work should investigate whether our iAstros produce these proteins in the VoC model. Alternatively, supplementing the culture media with these factors could potentially enhance the BBB-phenotype in hiPSC-ECs.

Furthermore, the choice of matrix in the self-assembled model could be fine-tuned to enhance astrocyte culture and EC-brain specification. Currently, the OoC model uses a fibrin hydrogel for embedding the cells in the microfluidic device. Although hiPSC-ECs eventually deposit their own basement membrane, modifying the hydrogel to more closely mimic the brain's molecular composition and stiffness is a potential avenue for future research. Fibrin is favored for its ease of use, EC compatibility and relatively low batch-to-batch variability. However, it lacks similarity to the native brain ECM, which is composed of glycosaminoglycans (particularly hyaluronan), proteoglycans, glycoproteins, and small amounts of fibrous proteins (e.g. collagen, fibronectin, and vitronectin)[45–47]. Supplementing the fibrin hydrogel with some of these factors could increase the similarity with *in vivo* brain tissue, potentially inducing a more faithful EC-BBB phenotype. Alternatives are designed defined matrices, such as crosslinked networks of polyethylene glycol (PEG) linked to RDG peptides, which could target and adhere to integrins on cell surfaces, minimizing variability associated with biological matrices[48].

Emergent features in self-assembling VoCs

To accurately model a disease and relate *in vitro* to *in vivo* observations, a model system must enable accurate quantification of the relevant emergent features. In Chapter 5 and Chapter 6, three emergent features of interest were barrier integrity in the form of apparent permeability, cell type diversity and cytokine and matrix metalloproteinases (MMP) secretion.

In both Chapter 5 and Chapter 6, we evaluated barrier integrity by quantifying the apparent permeability of cells in the self-assembling VoC using a dextran permeability assay. The quantified apparent permeabilities for 70 kDa were in the range of 10^{-4} cm/s. This

value is orders of magnitude lower than seen *in vivo* (~10^{-7})[49]. Interestingly, other similar *in vitro* self-assembling models, using primary astrocytes, have reported permeability values more in line with physiological norms[35,39]. The differences between these values could may be attributed in part to the inherent variability in the technical challenges associated with conducting permeability measurements in self-assembling models: the ECs may not form an entirely intact monolayer or the junctions may be deficient. Fluid flow rates inside the microvascular networks directly influence the availability and passage of the fluorescent dextran across the vasculature. The published studies each employs varying hydrostatic pressure when conducting the permeability assay and investigates microvascular networks with different average diameters, both factors directly affecting the flow rate within the system. Additionally, the density of the microvascular network can influence the fluorescent signal of the dextran detected within the 'extracellular matrix compartment'. Therefore, to facilitate meaningful comparisons of EC permeability, it would be advantageous to use a more standardized system offering control over both the flow rate and the vessel diameter.

In the development of a novel models, such as in Chapter 6, a comprehensive understanding of the exact cell type diversity and identity is essential for assessing the similarity of cultured cells to their *in vivo* counterparts. Chapter 6 uses a co-culture approach to induce a BBB identity in the ECs. This is one of the many different identities that ECs can adopt, each associated with the tissue in which they reside[50]. Due to the challenges associated with collecting sufficient high-quality RNA from the microfluidic devices, our comparisons between conditions have been limited to a select list of key BBB-EC markers. While informative, a preferred and less biased approach would be to perform single cell RNA sequencing (scRNAseq) on the VoCs. This would allow comparison with recently established scRNA seq databases containing data from both mouse and human brain vasculature[51–53].

Lastly, to gain a comprehensive understanding of cellular responses, particularly under diseased conditions, accurate analysis of secreted components within the VoC culture is of great importance. In Chapter 6 we investigated the secretion of the matrix metallopreinase 2 (MMP2) by measuring its concentration in the conditioned media using a cytokine bead assay. This assay offered quantitative detection of several proteins in a multiplexed fashion, using media samples collected at specific time points. Implementation of real-time sensors inside the microfluidic devices would allow the monitoring of cell state throughout the experiment. In this thesis, commercially available AIM Biotech chips were used, limiting the ability to incorporate such technological advances where needed. Future research using in-house microfluidic chip production, particularly optimization chip design through methods such as maskless photolithography (e.g. Alvéole Lab PRIMO)[54], could facilitate the development of advanced platforms tailored to specific needs.

Future perspectives

VoCs are beginning to prove to be a powerful tools for modeling various aspects of vascular

biology that has long been promised. In particular, the self-assembling VoCs, as used in this thesis, have demonstrated their value in uncovering biological processes associate with diseases. However, like all OoC models, a single model cannot address all biological questions. Future disease modeling efforts will benefit from the integration and application of multiple VoC models, each suited for the study of specific physiological features. Among the most complementary models to self-assembling VoCs are single lumen models, which offer superior control of fluid flow rates due to their uniform diameter[55,56]. Alternatively, more complex approaches are emerging, enabling the generation of engineered vascular networks using methods such as laser ablation, laser cavitation or UV-hydrogel patterning[57,58]. These networks facilitate the standardization of fluid flow profiles and permeability analysis, while allowing for some self-organization of the vascular structures to establish relevant cell-cell interactions as seen *in vivo*.

OoC in general, particularly in combination with hiPSCs and their derivatives, have also gained significant interest from both academic and industrial perspectives, most notably during the duration of this thesis research. A prime example of this growing interest and confidence in the models is the approval of the FDA modernization act 2.0 in December 2022, which authorizes the use of alternatives to animal testing, such as OoC technology. However, to fully realize their potential and completely integrate in the workflows of end-users, such as major pharmaceutical companies, several considerations, as evidenced by the work in this thesis, must be addressed. Firstly, reproducibility should be more thoroughly accounted for and reported. When working with hiPSCs and their derivatives, understanding and reporting the line-to-line variation would enhance our comprehension of the model and ultimately contribute to confidence in their use. Well-characterized and widely distributed standard hiPSC lines could facilitate comparisons between and within models, i.e. including those related to lab-to-lab variability. Additionally, descriptions of OoC models should be provided in a more structural fashion, establishing connections between the implemented and measured physiological features and relevant *in vivo* data whenever possible. Lastly, increased utilization and integration of continuous monitoring, coupled to artificial intelligence, can enable more and direct improvements to cellular models.

In conclusion, OoC technology as carried out in this thesis represents the convergence of cutting-edge stem cell biology and technical advancements. While the considerations mentioned are essential for achieving widespread application and adoption, there is an exciting future ahead for complex disease modeling using these innovative approaches.

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