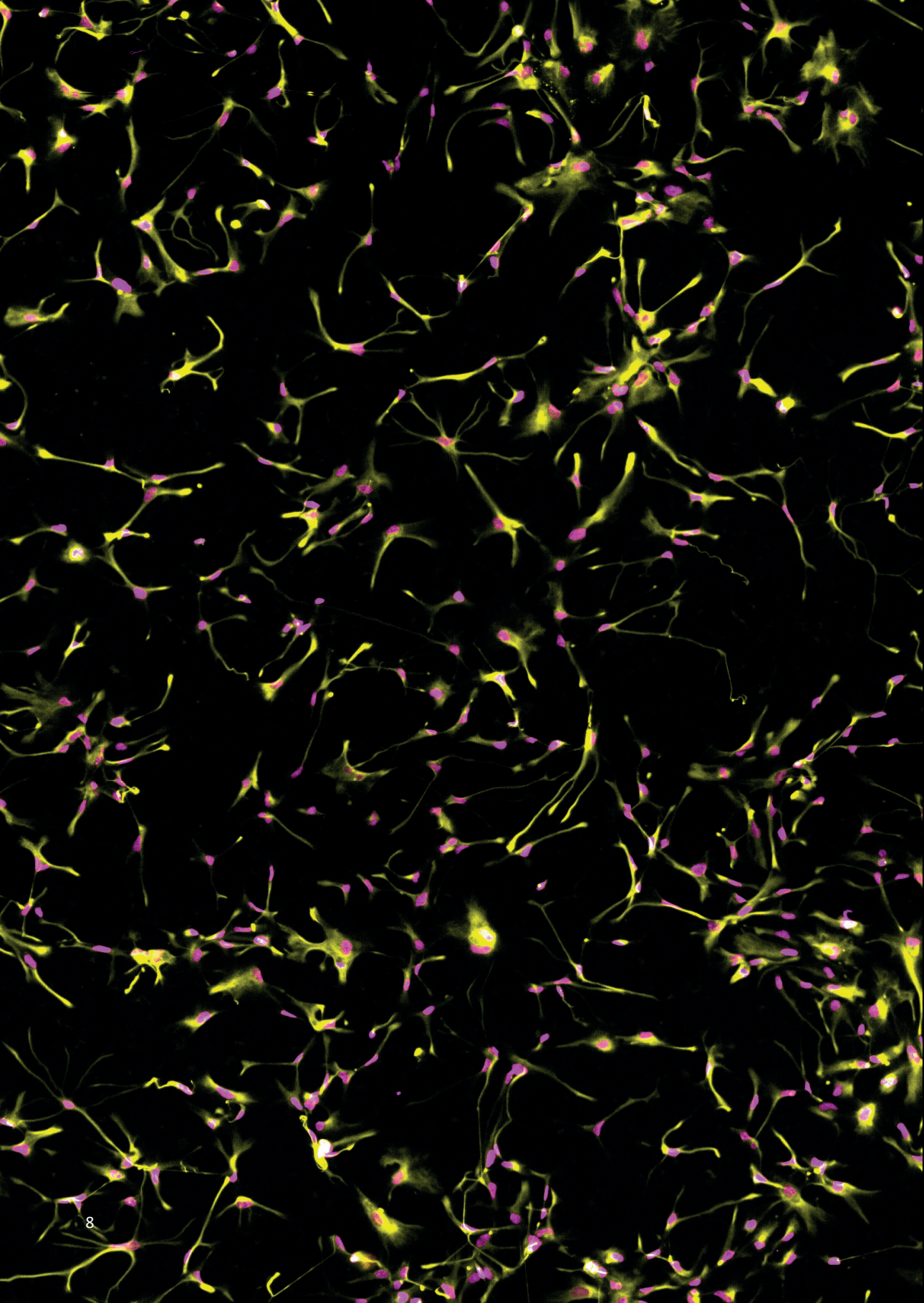
Modeling vascular disease using self-assembling human induced pluripotent stem cell derivatives in 3D vessels-on-chip

Nahon, D.M.



Chapter 1

General Introduction

Introduction

Functional vasculature is essential for maintaining the health of every part of the human body. It delivers oxygen and nutrients, transports immune cells and removes metabolic and other waste products. It consists of connected cylindrical tubes of divergent diameters in an organized network of arteries, arterioles, capillaries, venules and veins, through which blood flows. The vessel wall is composed of a single layer of endothelial cells surrounded by either multiple layers of smooth muscle cells or single pericytes, depending on the location of the vessel within the vascular tree. The exact composition and functionality varies greatly between tissues and along the arteriovenous axis[1,2]. Vascular cell dysfunction plays a key role in many chronic conditions, such as ischemic heart disease and stroke and even the initiation and propagation of severe COVID-19[3,4]. Creating predictive pre-clinical human models to study vascular (patho)physiology is essential if cures or therapies are to be found for the many vascular diseases affecting all age groups. The overall aim of this thesis is to generate the cellular components of the vessels and introduce them into advanced (on-chip) models to improve vascular disease modeling, with a specific focus on vascular pathologies affecting the brain.

The blood-brain barrier

The central nervous system (CNS) consists of a minimal functional entity, known as the neurovascular unit (NVU); it is composed of vascular cells, glial cells and neurons (Figure 1). This intricate assembly is responsible for inducing and maintaining the highly specialized barrier known as the blood-brain barrier (BBB), which separates the blood from the brain parenchyma.

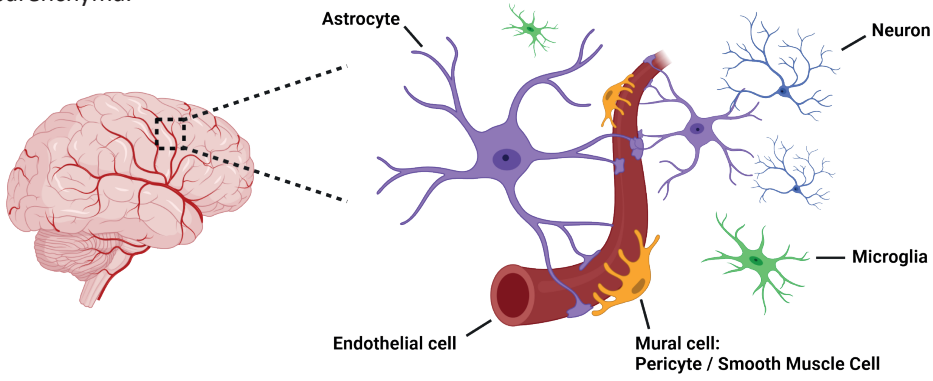


Figure 1: Schematic representation of the composition and organization of the cell types making up the neurovascular unit.

Formation of the BBB is initiated when mesoderm-derived blood vessels of the perineural vascular plexus penetrate the neuro-ectoderm, guided by neuronal and neural progenitor cell (NPC)-derived vascular endothelial growth factor (VEGF)[5]. Subsequent induction of BBB-

identity in ECs is regulated by activation of multiple pathways, with the canonical Wnt/ß-catenin pathway being the most prominent[6].

Within the BBB, the expression of specific tight junction (TJ) proteins strictly regulates the paracellular passage of molecules. Simultaneously, active inhibition of transcellular transport through the vessel wall occurs. Additionally, expression of several influx and efflux transporters tightly regulate active transport in- and out of the CNS. Moreover, (low levels of) adhesion molecules on the ECs limit the entry of immune cells into the brain. These combined characteristics not only prevent the entry of neurotoxic components and pathogens but also allow precise regulation of molecular transport, required for the high metabolic demand[7]. Though the primary BBB properties appear to be EC-specific, their functionality is highly dependent on the intricate interactions and crosstalk with the other cell types present in the NVU. Pericytes and astrocytes are recognized as the most significant contributors to maintaining BBB stability.

Pericytes, located in close proximity to the ECs, share the same basement membrane and play a crucial role in the BBB by regulating the inflammatory response, strengthening tight junctions and secreting laminins[8] and vitronectin[9,10]. The recruitment of pericytes to the BBB is facilitated by the binding of platelet-derived growth factor BB (PDGF-BB), secreted by ECs, to platelet derived growth receptor ß (PDGF-ß) in pericytes[5]. Activation of this pathway leads to pericyte recruitment and proliferation in a concentration-depend manner. Disruption of this and other pathways, leading to abnormal pericyte function, has been associated with various neurological disorders[11,12]. These include diabetic retinopathy and multiple neurodegenerative diseases such as Alzheimer's disease[13].

Astrocytes, have polarized "end-feet" that ensheath the cerebral vasculature; they serve as the essential cellular link between blood vessels and neurons. Their high density and localized expression of the water channel aquaporin 4 (AQP4) and the Kir4.1 K^+ channel ensures tight regulation of ion content in the perivascular space, which is crucial for neuronal function and BBB stability[11,14]. Moreover, astrocytes also secrete basement membrane proteins such as laminins α1 and α2[15] and signaling molecules such as retinoic acid (RA) and sonic hedgehog (SHH), contributing to the induction and stabilization of tight junctions[16]. *In vitro* studies have shown that astrocyte-secreted factors such as glial cell line-derived neurotrophic factor (GDNF)[17] or angiopoietin-1 (ANG-1), acting through the TIE2 EC specific receptor[18], regulate the acquisition and maintenance of EC-BBB identity.

Dysregulation of any of these cellular interactions can compromise integrity of the BBB. These three cell types are thus key players in various (inherited) vascular neurodegenerative disorders, most of which are rare but collectively affect a large group of individuals who develop many pathologies often the result of hemorrhage from unstable vessels.

Inherited vascular disorders with cerebral pathologies

Neurological disorders and neurodegenerative diseases are an increasing burden on society. Accumulating evidence indicates a fundamental role of the dysfunction of the brain vasculature in these pathophysiological conditions[19]. Known links between vascular mediated neuro-pathologies include BBB breakdown, hypoperfusion-hypoxia and EC-derived neurotoxic and inflammatory factors[20]. Three specific vascular disorders associated with cerebral pathologies that we have studied because of their prevalence in the Netherlands are Dutch-type cerebral amyloid angiopathy (D-CAA), also known as hereditary cerebral haemorrhage with amyloidosis-Dutch type (HCHWA-D), Retinal vasculopathy with cerebral leukoencephalopathy and systemic manifestations (RVCL-S) and Hereditary hemorrhagic telangiectasia (HHT).

These pathologies give rise to a spectrum of effects, ranging from mural cell apoptosis and aberrant mural cell-EC cross talk[21] to altered basement membrane composition and structure[22] or more severe arteriovenous malformations (AVMs)[23]. Overall, these phenotypic changes lead to reduced stability and integrity of cerebral blood vessels, that can result in hemorrhagic strokes.

Vascular disease modeling

Our current understanding of these vascular pathologies is primarily based on studies using commercially available cell lines expressing aberrant forms of putatively causative genes, primary patient material or mutant mouse models. While commercially available cell (lines) have proven to be valuable tools in biomedical research, their prolonged culture may lead to the loss of tissue specific characteristics. Additionally, they commonly cannot be obtained from patients as disease specific models. Patient material (from which to derive vascular cells for example) is in principle more relevant to study disease, it may however be limited by its availability, especially in the case of rare diseases. In addition, invasive procedures may be required to obtain the cells and once in culture the lifespan of these cells may be limited. Mouse models by contrast allows studying how a disease might manifest in a living organism but they lack the human genetic background and may differ from human physiology. Blood flow rates or the immune component influencing the vasculature for example may be very different in mice to that in humans. As a result, even mice expressing corresponding human mutations may display different disease phenotypes than those observed in patients. An illustrative example of these differences was demonstrated in a recent study comparing single nuclei sequencing of brain vasculature in Alzheimer's patients and a commonly used Alzheimer's mouse model: this revealed only minimal overlap in the differentially expressed genes in both cases[24]. This further emphasizes the need for humanized models to accurately assess the pathways involved in complex diseases.

In recent years, significant progress in stem cell biology has allowed the increasing use of adult human stem cells or human induced pluripotent stem cells (hiPSCs). So far,

adult tissue sources of vascular stem or progenitor cells have not been described. HiPSCs, by contrast are pluripotent and can form all cell types of the body including vascular cells[25]. They are derived through reprogramming of somatic cells to a pluripotent state using just four transcription factors[26] and have already proven a powerful tool for disease modeling[27]. They can not only be generated from healthy individuals but can also be derived from patients with genetic disorders and can be genetically engineered to introduce- or remove disease-specific mutations or variants for specific types of disease modelling[28]. Additionally, hiPSCs can be genetically modified to incorporate cell type-specific (fluorescent) reporter constructs or phenotypic sensors allowing them to be identified in-, or selected from, mixed cell populations.

hiPSC-derived ECs

There are multiple protocols to differentiate ECs from hiPSCs. These vary from embryoid body (aggregate)-formation, direct 2D monolayer cultures using cytokines, directed genetic modification, and most recently formation of vascular organoids. Arguably the most commonly used are the monolayer protocols, wherein developmental cues are simulated by the sequential addition of molecules and growth factors. Initially, hiPSCs are patterned towards mesoderm using either Glycogen synthase kinase i3 (GSK3) inhibitor (CHIRS99021)[29] either alone or in combination with Bone morphogenetic protein 4 (BMP4)[30]. Subsequently, EC specification is achieved by using Vascular Endothelial Growth Factor (VEGF) in combination with an ALK5 inhibitor (SB431542)[29] or protein kinase A activation via cyclic AMP using Forskolin[30]. More recently, specialized protocols have been developed to differentiate tissue specific endothelial cells[31].

Generation of brain endothelial cells has been of particular interest to enable study of brain pathologies and facilitate improved drug treatments through the BBB. This is important because many drugs (for example to treat neurodegenerative diseases or brain tumors) cannot cross the BBB and reach its potential cellular target within the brain. Various approaches have been taken to establish authentic 'BBB-ECs'[32]. One of the most well-known and widely used are hiPSC-derived brain microvascular endothelial cells (iBMECs Differentiation of iBMECs was based on the initial protocol from[33] which was later adapted to enhance the yield and improve BBB properties further[34,35]. However, while these cells typically exhibit EC properties such as high transendothelial electrical resistance (TEER) and expression of efflux transporters, they lack proper expression of vascular lineage genes and instead express markers for the neuroectodermal epithelial lineage[36,37].

An alternative approach involves co-culturing of hiPSC-ECs with tissue-specific cells. This is an approach which has previously been shown to induce an EC tissue-specific molecular identity for several organ systems[38,39], including the brain[40]. This co-culture strategy underscores the plasticity of ECs and the significance of the environment and cellular interactions in creating more *in vivo*-like systems, essential for studying complex

diseases.

Organ-on-chip

One of the more recent approaches which enables closer simulation of the native environment are microphysiological systems (MPS). MPS are distinct from conventional cell cultures in that they include features such as gas- and fluid flow; for this reason, some are referred to as microfluidic Organs-on-Chip (OoCs)[41,42]. These advanced models can provide mechanical cues to cells as in real tissues, creating systems with the potential to impact understanding of human physiology and disease, toxic effects of the environment or drugs and the identification of novel therapeutics. Each OoC aims to replicate one or more specific aspects of organ and tissue function *in vitro* and thus can be different in design depending on the application.

Vessel-on-chip

Numerous Vessel-on-Chip (VoC) models have been developed to facilitate the study of various functional aspects of the vasculature (Figure 2)[43,44]. Broadly, these models can be categorized into two types: those in which vessels are engineered to have specific dimension, and those where vessels form themselves (self-organize) through vasculogenesis or angiogenesis. Engineered models with vessels of specific sizes, offer the advantage of more tightly regulated architecture, which aids in recapitulating uniform flow patterns and functional readouts influenced by local vessel dimension and flow pattern, such as permeability. On the other hand, models utilizing the self-assembling capacity of vascular cells into 3D lumens have the advantage of better mimicking the formation and dynamics of complex vascular bed, resembling what is seen *in vivo*. Moreover, the direct interaction between the different cell types incorporated in the device can be investigated more accurately as these cells are not “forced” into specific configurations. Lastly, self-assembling models tend to be easier to handle in comparison to other systems.

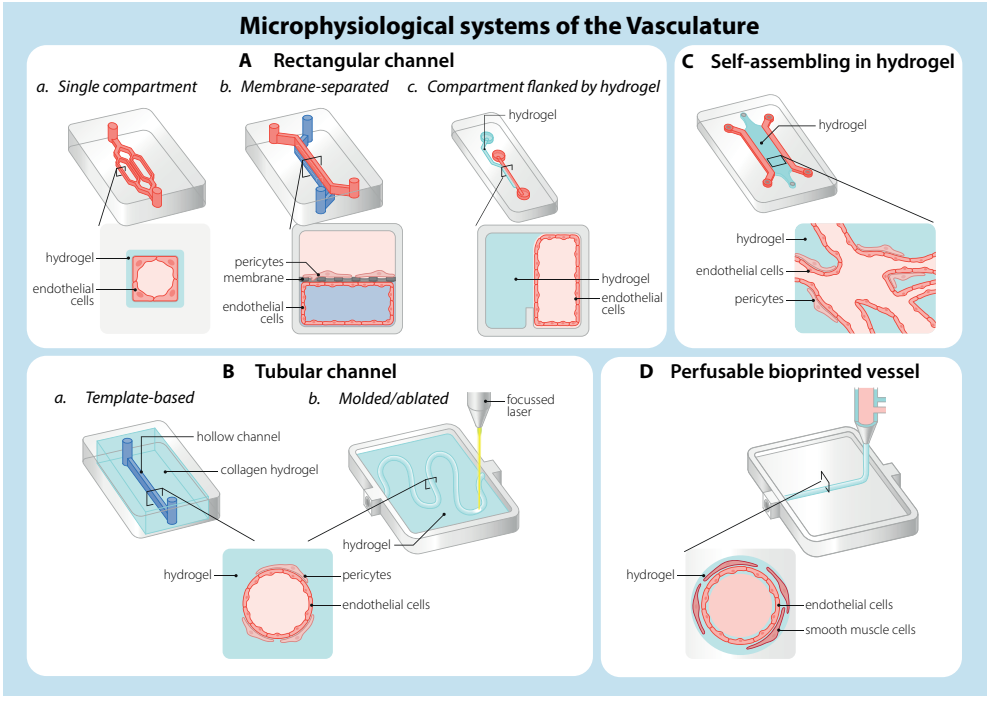


Figure 2: Design principles of different microphysiological systems of the human vasculature (adapted from Nahon, Moerkens et al 2024, in press)

Aim and scope of this thesis

In the past decade, a wide range of complex models have been developed to study neuropathologies. However, most of these have been either technically intricate and hard to reproduce, or have only been described in proof-of-concept formats using primary cells or commercially available cell lines. In this thesis, the objective was to enhance the biological relevance of the existing models by creating modular and robust models that incorporate hiPSCs to study disease phenotypes (Figure 3).

In **chapter 2** we first discuss the current status and future prospects of MPS and emphasize the need for measurable standards that allow quantitative comparisons of MPS outcomes with physiological observations in humans so that their *in vivo* relevance and predictive value can be properly assessed as fit-for-purpose in specific applications. Moving to **chapter 3**, we describe the genetic repair of a human induced pluripotent cell line obtained from a patient with D-CAA. This provided isogenically paired hiPSC lines, as a resource for further study of the underlying disease mechanism. In **chapter 4** we use similarly generated isogenically paired disease and corrected hiPSC lines from a patient with RVCL-S to study the EC defects in these cells. Continuing to **chapter 5** we employed isogenic HHT diseased and healthy hiPSCs, to produce ECs and investigate vascular defects using both 2D assays

and a more complex 3D VoC model. Here, the significance of the more complex VoC model becomes evident, as disease phenotypes were not evident in conventional 2D assays, but were clearly revealed in the VoC model. In **chapter 6**, we further enhanced the complexity of the VoC model towards a BBB model by incorporating hiPSC-derived astrocytes into the vascular networks. Detailed characterization of the VoC was carried out, and the networks are further improved through the application of continuous flow or activation of the cyclic AMP pathway. Finally, in **chapter 7**, the work described in this thesis is discussed and the future outlook for the field of complex vascular disease modeling described based on the present state-of-the-art.

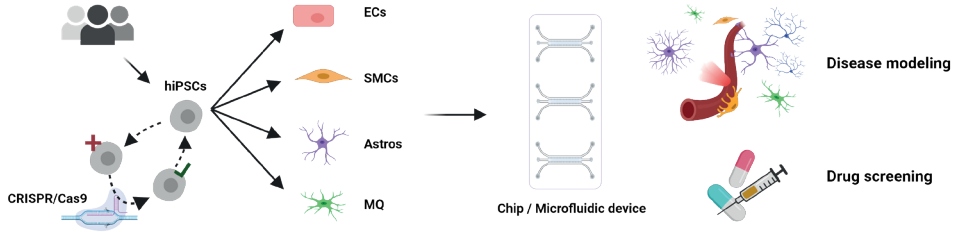


Figure 3: The use of hiPSC-derived vascular cells inside a microfluidic device for generating robust vessel-on-chip systems to study disease modelling or drug screening. *hiPSCs of either healthy donors or patients can be used. Disease specific mutations in patient hiPSC lines can be corrected for example, using CRISPR/Cas9 technology, to create isogenically paired cells. This way creating a modular vessel-on-chip system to investigate the cell-type specific contribution of each cell type. hiPSCs = human induced pluripotent stem cells, ECs = Endothelial cells, SMCs = Smooth Muscle Cells, Astros = Astrocytes, MQ = Macrophages.*

References

1. Winkler, E. A. et al. A single-cell atlas of the normal and malformed human brain vasculature. Science (1979) 7377, (2022).
2. Vanlandewijck, M. et al. A molecular atlas of cell types and zonation in the brain vasculature. Nature 554, 475–480 (2018).
3. Trimm, E. & Red-Horse, K. Vascular endothelial cell development and diversity. Nat Rev Cardiol 0123456789, (2022).
4. Teuwen, L. A., Geldhof, V., Pasut, A. & Carmeliet, P. COVID-19: the vasculature unleashed. Nat Rev Immunol 20, 389–391 (2020).
5. Zhao, Z., Nelson, A. R., Betsholtz, C. & Zlokovic, B. V. Establishment and Dysfunction of the Blood-Brain Barrier. Cell 163, 1064–1078 (2015).
6. Langen, U. H., Ayloo, S. & Gu, C. Development and cell biology of the blood-brain barrier. Annu Rev Cell Dev Biol 35, 591–613 (2019).
7. Sweeney, M. D., Zhao, Z., Montagne, A., Nelson, A. R. & Zlokovic, B. V. Blood-brain barrier: From physiology to disease and back. Physiol Rev 99, 21–78 (2019).
8. Gautam, J., Cao, Y. & Yao, Y. Pericytic Laminin Maintains Blood-Brain Barrier Integrity in an Age-Dependent Manner. Transl Stroke Res 11, 228–242 (2020).
9. Ayloo, S. et al. Pericyte-to-endothelial cell signaling via vitronectin-integrin regulates blood-CNS barrier. Neuron 110, 1641-1655.e6 (2022).
10. He, L. et al. Analysis of the brain mural cell transcriptome. Sci Rep 6, (2016).
11. Armulik, A. et al. Pericytes regulate the blood-brain barrier. Nature 468, 557–561 (2010).
12. Bell, R. D. et al. Pericytes Control Key Neurovascular Functions and Neuronal Phenotype in the Adult Brain and during Brain Aging. Neuron 68, 409–427 (2010).
13. Winkler, E. A., Bell, R. D. & Zlokovic, B. V. Central nervous system pericytes in health and disease. Nature Neuroscience vol. 14 1398–1405 Preprint at https://doi.org/10.1038/nn.2946 (2011).
14. Abbott, N. J., Rönnbäck, L. & Hansson, E. Astrocyte-endothelial interactions at the blood-brain barrier. Nat Rev Neurosci 7, 41–53 (2006).
15. Yao, Y., Chen, Z. L., Norris, E. H. & Strickland, S. Astrocytic laminin regulates pericyte differentiation and maintains blood brain barrier integrity. Nat Commun 5, 1–12 (2014).
16. Alvarez, J. I. et al. The hedgehog pathway promotes blood-brain barrier integrity and CNS immune quiescence. Science (1979) 334, 1727–1731 (2011).
17. Igarashi, Y. et al. Glial cell line-derived neurotrophic factor induces barrier function of endothelial cells forming the blood-brain barrier. Biochem Biophys Res Commun 261, 108–112 (1999).
18. Lee, S.-W. et al. SSeCKS regulates angiogenesis and tight junction formation in blood-brain barrier. Nat Med 9, (2003).
19. Sweeney, M. D., Kisler, K., Montagne, A., Toga, A. W. & Zlokovic, B. V. The role of brain vasculature in neurodegenerative disorders. Nat Neurosci 21, 1318–1331 (2018).
20. Zlokovic, B. V. Neurovascular pathways to neurodegeneration in Alzheimer's disease and other disorders. Nat Rev Neurosci 12, 723–738 (2011).
21. Kamp, J. A. et al. Amyloid β in hereditary cerebral hemorrhage with amyloidosis-Dutch type. Rev Neurosci 25, 641–651 (2014).
22. Stam, A. H. et al. Retinal vasculopathy with cerebral leukoencephalopathy and systemic manifestations. Brain 139, 2909–2922 (2016).
23. Letteboer, T. G. W. et al. Hereditary hemorrhagic telangiectasia: ENG and ALK-1 mutations in Dutch patients. Hum Genet 116, 8–16 (2005).
24. Yang, A. C. et al. A human brain vascular atlas reveals diverse mediators of Alzheimer's risk. Nature 603, 885–892 (2022).
25. Cochrane, A. et al. Advanced in vitro models of vascular biology: Human induced pluripotent stem cells and organ-on-chip technology. Adv Drug Deliv Rev (2018) doi:10.1016/j.addr.2018.06.007.
26. Takahashi, K. & Yamanaka, S. Induction of Pluripotent Stem Cells from Mouse Embryonic and Adult Fibroblast Cultures by Defined Factors. Cell 126, 663–676 (2006).
27. Sharma, A., Sances, S., Workman, M. J. & Svendsen, C. N. Multi-lineage Human iPSC-Derived Platforms for Disease Modeling and Drug Discovery. Cell Stem Cell 26, 309–329 (2020).
28. Van Den Berg, A., Mummery, C. L., Passier, R. & Van der Meer, A. D. Personalised organs-on-chips:

functional testing for precision medicine. Lab Chip 19, 198–205 (2019).
29. Orlova, V. V. et al. Generation, expansion and functional analysis of endothelial cells and pericytes derived from human pluripotent stem cells. Nat Protoc 9, 1514–1531 (2014).
30. Patsch, C. et al. Generation of vascular endothelial and smooth muscle cells from human pluripotent stem cells. Nat Cell Biol 17, 994–1003 (2015).
31. Nguyen, J., Lin, Y.-Y. & Gerecht, S. The next generation of endothelial differentiation: Tissue-specific ECs. Cell Stem Cell 28, 1188–1204 (2021).
32. Workman, M. J. & Svendsen, C. N. Recent advances in human iPSC-derived models of the blood–brain barrier. Fluids Barriers CNS 17, 1–10 (2020).
33. Lippmann, E. S. et al. Derivation of blood-brain barrier endothelial cells from human pluripotent stem cells. Nat Biotechnol 30, 783–791 (2012).
34. Lippmann, E. S., Al-Ahmad, A., Azarin, S. M., Palecek, S. P. & Shusta, E. V. A retinoic acid-enhanced, multicellular human blood-brain barrier model derived from stem cell sources. Sci Rep 4, 1–10 (2014).
35. Qian, T. et al. Directed differentiation of human pluripotent stem cells to blood-brain barrier endothelial cells. Sci Adv 3, (2017).
36. Lu, T. M. et al. Pluripotent stem cell-derived epithelium misidentified as brain microvascular endothelium requires ETS factors to acquire vascular fate. Proc Natl Acad Sci U S A 118, (2021).
37. Lu, T. M. et al. Human Induced Pluripotent Stem Cell-Derived Brain Endothelial Cells: Current Controversies. Front Physiol 12, (2021).
38. Cao, X. et al. Tissue microenvironment dictates the state of human induced pluripotent stem cell-derived endothelial cells of distinct developmental origin in 3D cardiac microtissues. Biorxiv (2022).
39. Palikuqi, B. et al. Adaptable haemodynamic endothelial cells for organogenesis and tumorigenesis. Nature 585, (2020).
40. Campisi, M. et al. 3D self-organized microvascular model of the human blood-brain barrier with endothelial cells, pericytes and astrocytes. Biomaterials 180, 117–129 (2018).
41. Vunjak-Novakovic, G., Ronaldson-Bouchard, K. & Radisic, M. Organs-on-a-chip models for biological research. Cell 184, 4597–4611 (2021).
42. Ingber, D. E. Human organs-on-chips for disease modelling, drug development and personalized medicine. Nat Rev Genet (2022) doi:10.1038/s41576-022-00466-9.
43. Mandrycky, C. J., Howard, C. C., Rayner, S. G., Shin, Y. J. & Zheng, Y. Organ-on-a-chip systems for vascular biology. J Mol Cell Cardiol 159, 1–13 (2021).
44. Pollet, A. M. A. O. & den Toonder, J. M. J. Recapitulating the vasculature using Organ-on-Chip technology. Bioengineering 7, 1–18 (2020).