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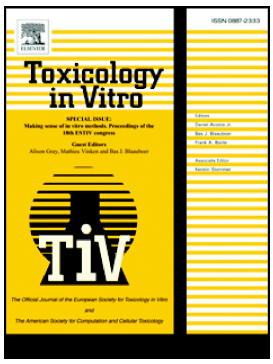

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Challenges and opportunities in the use of transcriptomics characterisation for human iPSC-derived BBB models

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Abstract

The blood-brain barrier (BBB) is localized at the brain microvascular endothelial cells. These cells form a tight barrier, limiting the access of cells, pathogens, chemicals, and toxins to the brain due to tight junctions and efflux transporters. As the BBB plays a role in the assessment of neurotoxicity and brain uptake of drugs, human *in vitro* BBB models are highly needed. They allow to evaluate if compounds could reach the central nervous system across the BBB or can compromise its barrier function. Past decade, multiple induced pluripotent stem cell (iPSC)-derived BBB differentiation protocols emerged. These protocols can be divided in two groups, the one-step protocols, direct differentiation from iPSC to BBB cells, or the two-step protocols, differentiation for iPSC to endothelial (progenitor) cells and further induction of BBB characteristics. While the one-step differentiation protocols display good barrier properties, reports question their endothelial nature and maturation status. Therefore protocol characterization remains important. With transcriptomics becoming cheaper, this may support iPSC-derived model characterization. Because of the constraints in obtaining human brain tissue, good human reference data is scarce and would bear inter-individual variability. Additionally, comparison across studies might be challenging due to variations in sample preparation and analysis. Hopefully, increasing use of transcriptomics will allow in-depth characterization of the current iPSC-BBB models and guide researchers to generate more relevant human BBB models.

Key words

Blood-brain barrier

Brain microvasculature

iPSC

Transcriptomics

In vitro BBB models

Abbreviations

BBB Blood-brain barrier
BLECs Brain-like endothelial cells
CLDN Claudin
hiPSC human induced Pluripotent Stem Cell
hESC human Embryonic Stem Cell
OCLN Occludin
TEER Trans Endothelial Electrical Resistance
ZO-1 Zonula Occludens-1

Introduction: the blood-brain barrier

The blood-brain barrier (BBB) has been localized at the level of the brain microvascular endothelial cells, separating the blood from the brain interstitial fluid (Reese and Karnovsky, 1967; Serlin et al., 2015). These endothelial cells share their basal lamina with pericytes and interact with the astrocyte end-feet (Figure 1). The pericytes and astrocytes play a crucial role in the formation, maturation and maintenance of the BBB and form the neurovascular unit together with the BBB, the basal lamina, neurons and microglia (Daneman et al., 2010; Serlin et al., 2015). The BBB forms a tight barrier and limits the access of chemicals, pathogens and toxins to the other cells of the neurovascular unit thanks to the presence of tight junctions between adjacent endothelial cells. Tight junction proteins seal the paracellular pathway by fusing endothelial membranes together and consist of different polymerized proteins like claudins (CLDN) and occludin (OCLN) (Hashimoto and Campbell, 2020). At the BBB, the intracellular scaffold protein Zonula Occludens-1 (ZO-1) is

responsible for the stabilization of claudin-5 (CLDN5) and its connection to the actin cytoskeleton. Besides the tight junction complexes, more basolateral situated adherens junctions (like VE-cadherin) are also present. Since these junctions drastically limit the paracellular pathway, BBB transport mainly occurs through transcellular pathway (i.e. passive and facilitated diffusion and active transport). Endothelial cells at the BBB express several transporters, belonging to the adenosine triphosphate (ATP) binding cassette (ABC) and the solute carrier (SLC) superfamilies, on the apical and basolateral membranes for the influx or efflux of endogenous substances and xenobiotics. The presence of multiple efflux transporters, localized at the apical (blood vessel) side of the cells, like ABCB1 (P-gp), ABCG2 (BCRP) and ABCC1-5 (MRP1-5), can limit the access of substrate drugs to the brain (Abbott et al., 2010; Daneman and Prat, 2015; Langen et al., 2019; Serlin et al., 2015; Sweeney et al., 2019). Besides these efflux transporters, the transport of circulating nutrients (glucose, amino acids, ketone bodies, choline, and purines) and hormones through the brain endothelial wall can be facilitated by transporters and receptors. A nutrient transporter, part of the SLC transporters, known to be highly expressed at the level of the brain endothelial cells is SLC2A1 or GLUT-1, facilitating the glucose transport from the blood to the brain (Cornford et al., 1994; Daneman and Prat, 2015). Some others are SLC16A1 (monocarboxylic acids), SLC7A1 (cationic amino acids) and SLC7A5 (neutral amino acids) (Abbott et al., 2010; Daneman and Prat, 2015; Langen et al., 2019; Suhy et al., 2017).

The value of many promising CNS drug candidates is diminished by their inability to permeate the BBB in sufficient amount to reach therapeutical concentration within the brain parenchyma and to interact with their target. In addition, dysfunction of the BBB has been associated with different central nervous system disorders like multiple sclerosis, stroke, Alzheimer's disease, epilepsy, traumatic brain injuries and brain cancers (Abbott et al., 2010; Daneman and Prat, 2015). The BBB dysfunction in these pathologies or following exposure of the brain vasculature to toxic agents, may result in compromised BBB transport and permeability, and lead to alterations in cerebrovascular regulatory mechanisms of blood flow,

with ensuing perturbed signalling between the brain endothelium and associated cells, such as glia and neurons.

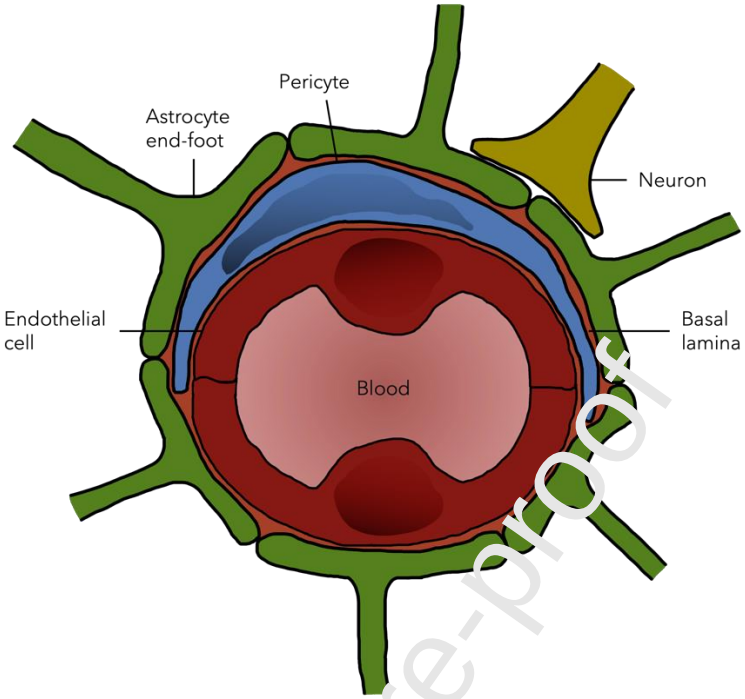

Figure 1 The blood-brain barrier

The blood-brain barrier consists of specialized capillary endothelial cells in the brain, which are in close proximity of pericytes, astrocyte end-feet and perivascular neurons.

By modelling the BBB, it is possible to make predictions about brain uptake of potential drug candidates and to study the molecular mechanisms taking place at the level of the cerebral capillaries in neurodegenerative diseases (Aday et al., 2016; Cecchelli et al., 2007). In line with the 3Rs (Replacement, Reduction and Refinement of animal experimentation), there is also a growing interest, since the last 15 years, to use BBB cell models for toxicological evaluations (Fabulas-da Costa et al., 2013; Wellens et al., 2021). Such data may enhance the value of the toxicological results generated in animals and facilitate the assessment of risk and safety in humans.

Human material was for a long time not considered a feasible option due to ethical reasons and other constraints in obtaining tissue and primary (or low passage) culture of brain capillary endothelial cells. Models from bovine and porcine tissues have received significant

attention as a source for brain endothelial cells, given the brain size and availability (Deli et al., 2005).

Rodent *in vivo* models are commonly used in CNS drug programs as preclinical assessment tools for pharmacokinetic parameters (e.g., absorption, distribution, metabolism and excretion (ADME) properties) and CNS toxicity potential of candidate compounds. But the high failure rate observed in CNS drug development programs to bring drugs market has raised concerns regarding potential important species difference at the BBB between rodents and human.

Several recent studies already highlighted species differences in parameters that could affect CNS drug delivery i.e. transporter expression, receptors and tight junctions (Hoshi et al., 2013; Shawahna et al., 2011; Syvänen et al., 2009). Therefore, the use of human *in vitro* models is expected to facilitate translation of preclinical evaluation to the clinic, as an ideal *in vitro* BBB model should be able to correctly assist the selection of drug candidates based on their expected distribution in the human brain, thereby circumventing potential species differences.

The first human *in vitro* BBB models to emerge consisted of isolated primary human brain microvessel endothelial cells (Biegel et al., 1995; Walker and Coleman, 1995). These cells display BBB characteristics, like the presence of tight junction proteins, apical ABCB1 and a reduced permeability to paracellular markers although with limited paracellular tightness. Use of these models, with a limited lifespan, relies on the availability of human cortical tissue and its extraction and purification method defines the purity of the cell population. In order to overcome this problem, immortalized human brain capillary endothelial cell lines have been developed like the BB19 cell line (Prudhomme et al., 1996), SV-HCEC (Muruganandam et al., 1997), HBMEC (Stins et al., 2001) and hCMEC/D3 (Weksler et al., 2005). Even though these cells display multiple characteristics of the BBB, the tightness of the barrier remains limited. According to the research of Eigenmann in 2013, co-culturing of some immortalized

human brain capillary endothelial cell lines with immortalized human astrocytes or pericytes did not result in an increased tightness (Eigenmann et al., 2013). Some astrocyte co-culture conditions of hCMEC/D3 result in an increased trans endothelial electrical resistance (TEER) (Hatherell et al., 2011), while with another astrocyte lineage no improvement was reported (Hinkel et al., 2019). As the barrier tightness remains limited with these immortalized cell lines, multiple models have been developed with the use of stem cells.

Different types of stem cells have been used as a basis for human *in vitro* BBB models. In 2014, BBB models derived from multipotent stem cells, either hematopoietic stem cells (Cecchelli et al., 2014) or endothelial progenitor cells (Boyer-Di Ponio et al., 2014), have been published, achieving a more restricted permeability when co-cultured with bovine brain pericytes and rat astrocytes respectively. Besides these models from multipotent stem cells, also models from embryonic stem cells have been developed (James et al., 2010; Levenberg et al., 2002; Zhang et al., 2001). Human embryonic stem cells (hESCs) display important properties of self-renewal and pluripotency theoretically capable of generating unlimited amounts of any differentiated cell in the human body. However, given the ethical concerns related to the use of embryos, they have largely been abandoned once human induced pluripotent stem cells (hiPSCs) became available (Takahashi et al., 2007).

Nowadays, hiPSCs have been hailed as an effective replacement for hESCs and could be differentiated in all cell types with the same genetic background. This has opened exciting new opportunities for the modelling of the BBB and in recent years, several protocols have been published to differentiate iPSCs into brain-like endothelial cells (BLECs). Here, we will discuss aspects of the plethora of differentiation protocols that exist today and discuss the challenge of the characterisation of these models using transcriptomic methods.

BBB models from human induced pluripotent stem cells

The first BBB differentiation protocol using human pluripotent stem cells (hPSCs) (induced and embryonic), is based on the principle of co-differentiation of hPSCs to both neural and endothelial lineages followed by endothelial cell purification by seeding on a collagen/fibronectin extracellular matrix on transwell inserts (Figure 2) (Lippmann et al., 2012). This co-differentiation reflects the developmental induction of the BBB as the embryonic brain microenvironment provides inductive cues, like Wnt/β-catenin pathway signalling, to the invading endothelial cells from the perineural vascular plexus. The resulting cells contain BBB characteristics like the presence of tight junction(-related) proteins CLDN5, OCLN and ZO-1, endothelial markers PECAM-1, vWF and VE-cadherin, functional efflux transporters (e.g.: ABCB1 and ABCG2) and barrier-forming cells, indicated by a high TEER which further increased by co-culturing with primary rat astrocytes. Improvement of the differentiation protocol by the addition of retinoic acid to the endothelial cell medium led to the enhancement of the BBB phenotype with an improved barrier function, increased VE-cadherin expression and increased multidrug resistance protein efflux activity (Lippmann et al., 2014; Stebbins et al., 2016). From this retinoic acid enhanced protocol, multiple protocols emerged over the years, aiming to further characterize and improve different aspects of the protocol like the differentiation time and the replacement of serum by a chemically defined alternative. As these differentiations are time and cost intensive, an accelerated differentiation protocol was developed using defined iPSC medium (E8), reducing the differentiation time by five days by shortening the iPSC expansion phase (Hollmann et al., 2017). As these protocols still relied on the use of (platelet-poor plasma-derived) serum, a more defined version of the Hollmann protocol was published making use of fully defined components (B27, N2 or a combination of insulin, transferrin and selenium (ITS)) to reduce variability in the differentiation process (Neal et al., 2019). This improved the consistency of the differentiations resulted in BMECs with a high TEER (for more than 14 days), low permeability to sodium fluorescein, functional P-gp and MRPs transporters and expression

of VE-cadherin, tight junction proteins and GLUT-1 across multiple iPSC lines. In 2017, a more developmentally relevant and chemically defined protocol was published using small-molecules for the activation of key signalling pathways (Qian et al., 2017). With the activation of canonical Wnt signalling pathway, the iPSCs were driven to a primitive streak-like stage and further differentiated into intermediate mesoderm, endothelial progenitor cells and finally BBB-like endothelial cells with the use of retinoic acid. As all these aforementioned protocols directly differentiate iPSC to brain-like endothelial cells (BLECs), without a purified endothelial differentiation step in between, we will further refer to them as “one-step protocols”.

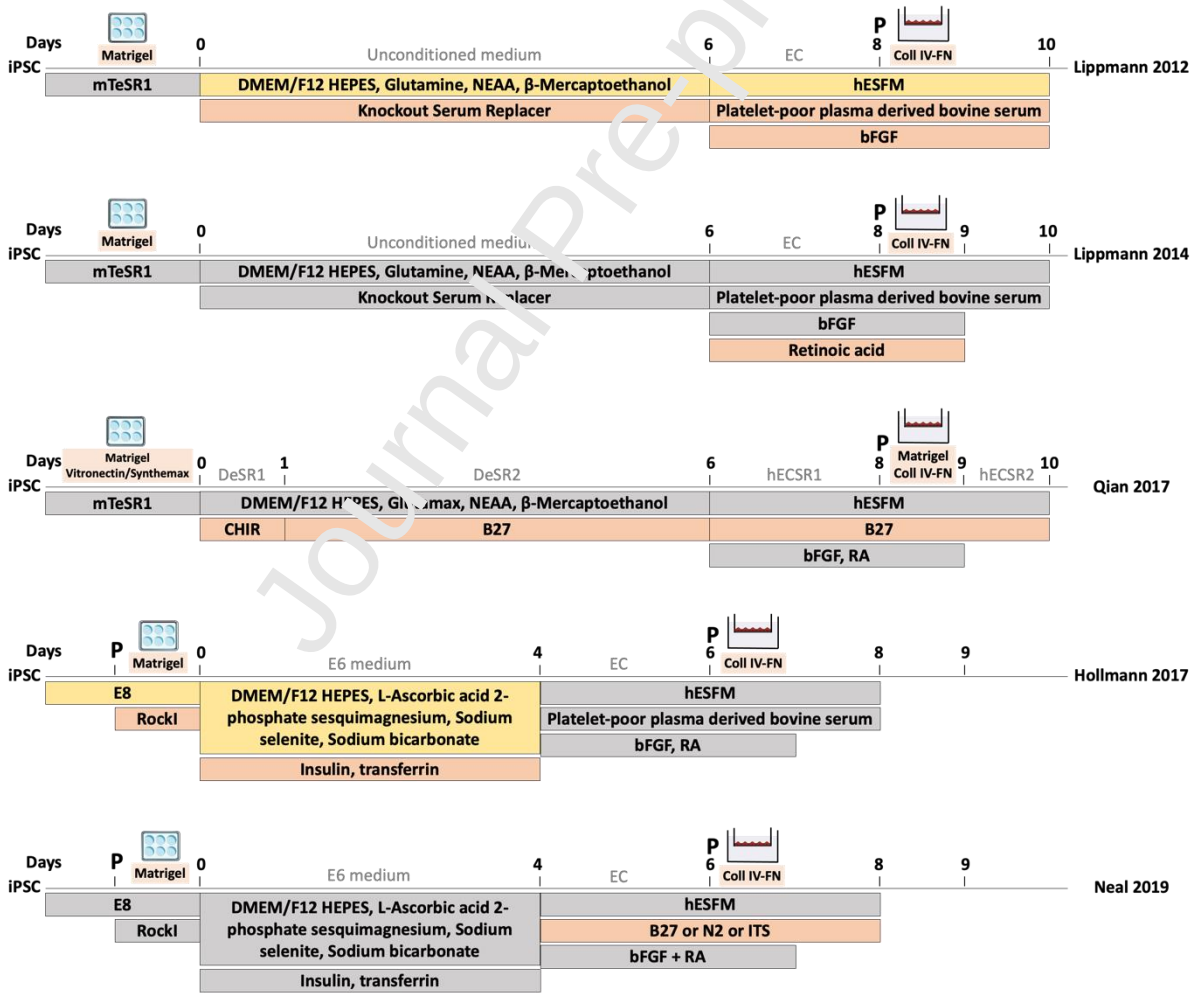

Figure 2 One-step differentiation protocols

This figure displays the evolution of the main “one-step” differentiation protocols developed during the past years. New aspects of a specific protocol are indicated in yellow (basic medium composition) and orange (growth factors and particular supplements). Abbreviations: bFGF = basic Fibroblast Growth Factor; Coll IV = Collagen

IV; EC = Endothelial cell medium; FN = Fibronectin; hESFM = human Endothelial Serum Free Medium; ITS = Insulin, Transferrin and Selenium; NEAA = Non-Essential Amino Acids; RA = Retinoic Acid; RockI = Rock Inhibitor Y-27632. Six-well plates were included from Servier Medical Art, licensed under a Creative Common Attribution 3.0 Generic License. http://smart.servier.com/ Note: mTeSR is a defined medium for human pluripotent stem cells (Ludwig and A Thomson, 2007).

An alternative approach, to development of a BBB model from iPSC, is the differentiation of iPSC to endothelial (progenitor) cells and further maturation to BBB cells. These differentiation protocols will be referred at as "two-step protocols" (Figure 3). In 2015, Minami *et al.* published a protocol in which iPSC were differentiated to endothelial progenitor cells and BLECs were formed by co-culture with C6 rat glioma cells or its conditioned medium (Minami et al., 2015). The endothelial progenitor cells, expressing VEGFR2, CD31, TIE2 and vWF, were purified using CD34 antibodies. These cells, with an endothelial-like morphology and positively stained for CD31 and vWF, displayed tube formation in Matrigel and were able to take up acetylated LDL. Maturation to BLECs resulted in an increased TEER (although still relatively low compared to other models), decreased dextran transport, presence of functional efflux transport and up-regulation of tight junction and transporter related genes. Another two-step protocol, in which VEGF, Wnt3a and retinoic acid signalling pathways were used for a time-dependent induction of CD31+ sorted endothelial progenitor cells to BLECs, was reported by Praça *et al.* (Praça et al., 2019). The resulting cells formed a ZO-1 and CLDN5 expressing monolayer with a lucifer yellow permeability of 1 x 10^{-3} cm/min and functional efflux pump activity only when co-cultured with bovine brain pericytes. The main difference between the one-step protocols, based on the Lippmann protocols (Lippmann et al., 2014, 2012), and the two-step differentiation protocols, is the barrier forming capacity of these cells. Even though comparing TEER measurements between different laboratories is difficult because of confounding factors like temperature, measurement device, and porosity of the insert, the TEER achieved using the two-step protocols is roughly 100 times less than the one-step protocols (Santa-Maria et al., 2020; Srinivasan and Kolli, 2019; Vigh et al., 2021). More information on the factors interfering with TEER measurements can be found in recent articles (Srinivasan and Kolli, 2019; Vigh et al., 2021).

After establishment of these “classic” protocols, different adaptations and optimisations emerged, investigating the use of other cell lines, cell seeding density, co-culture, extracellular matrices and 3D cultures.

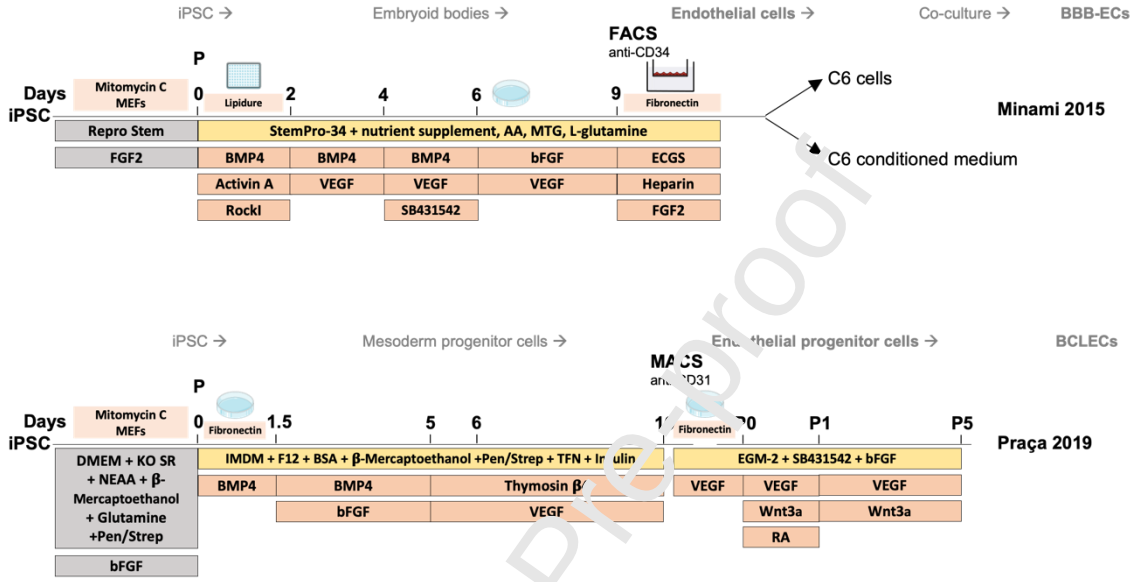

Figure 3 Two-step differentiation protocols

This figure displays the “two-step” differentiation protocols described in this article. Abbreviations: AA = Ascorbic acid; BCLECs = Brain Capillary-Like Endothelial Cells; bFGF = basic Fibroblast Growth Factor; BMP4 = Bone Morphogenetic Protein 4; BSA = Bovine Serum Albumin; C6 = C6 rat glioma cells; DMEM = knockout Dulbecco’s Modified Eagle Medium; ECGS = Endothelial Cell Growth Supplement; EGM-2 = Endothelial Cell Growth-Medium-2; FACS = Fluorescence activated Cell Sorting; IMDM = Iscove’s Modified Dulbecco’s Medium; KO SR = KnockOut Serum Replacer; MACS = Magnetic-Activated Cell Sorting; MEFs = Mouse Embryonic Fibroblasts; MTG = 1-thioglycerol; NEAA = Non-Essential Amino Acids; P = Passage; RA = Retinoic Acid; RockI = Rock Inhibitor Y-27632; TFN = Transferrin; VEGF = Vascular Endothelial Growth Factor Petri-dishes and 96-well plates were included from Servier Medical Art, licensed under a Creative Common Attribution 3.0 Generic License. http://smart.servier.com/

iPSC cell line and seeding density

In 2015, the effects of cell seeding on the one-step differentiation protocols were investigated (Wilson et al., 2015). A singularized cell seeding protocol was used which allowed a more precise control of the starting iPSC seeding density. For some cell lines a

high TEER could be achieved at a wide range of early seeding densities while other iPSC cell lines displayed a restrictive barrier only for a specific optimal seeding density. These results demonstrated that the iPSC seeding density and cell line play an important role in the formation of a restrictive barrier. Optimization of these factors to every new differentiation protocol is needed, which might influence the differentiation results.

Co-culture

Since the first iPSC-derived BBB protocol, co-culturing iPSC-derived BLECs (from the one-step protocols) with other cells of the neurovascular unit has been used for its positive influence on the restrictive barrier properties (increase in TEER) and has evolved over time (Lippmann et al., 2012). While the first co-cultured cells consisted of primary rat astrocytes, the following protocols already included primary human brain pericytes and human neural progenitor-derived astrocytes and neurons (Lippmann et al., 2014, 2012). The different cell types of the neurovascular unit, astrocytes, pericytes and neuronal (stem) cells from different sources were co-cultured with the use of permeable inserts and evaluated for their ability to improve the BBB phenotype, to increase the TEER and to modulate the expression of efflux transporters and tight junction proteins (Appelt-Menzel et al., 2017). The inclusion of iPSC-derived cells from the neurovascular unit, lead to the development of isogenic BBB models, co-culturing iPSC-derived cells from the same cell line (Appelt-Menzel et al., 2017; Canfield et al., 2019, 2017; Motallebnejad et al., 2019; Ribecco-Lutkiewicz et al., 2018). These isogenic models allow disease modelling using patient-derived iPSC and further investigation of interindividual variability for personalized medicine, however there is still a need for reliable and stable iPSC differentiation protocols (Patel et al., 2017; Vatine et al., 2019). The most commonly reported effect of co-culture is an increased TEER. While other research also focuses on more in-depth analysis of the effects of co-culture by transcriptomic analysis using RNA-Seq or a BBB and endothelium-specific microarray (Delsing et al., 2018; Li et al., 2019; Ribecco-Lutkiewicz et al., 2018). In 2018, Delsing *et al.*

investigated the effect of co-culture with iPSC-derived astrocytes, neurons and pericytes on two different, previously published, protocols with the use of transcriptomics (Delsing et al., 2018). The first protocol consisted of the one-step retinoic acid-enhanced Lippmann protocol while the second one consisted of a protocol for the generation of endothelial and pericytes from iPSC (Lippmann et al., 2014; Orlova et al., 2014). Besides investigating the transcriptional changes in transporter and junctional associated genes between these two protocols in mono- and co-culture, this study reported an unchanged expression of OCLN, CLDN3, CLDN4, CLDN5, CDH5 and THP1 after co-culture, while TJP3 (ZO-3) and SLC6A15 were significantly upregulated in both protocols. The extensive variety of cells used for co-culture together with the different *in vitro* platforms with diverse levels of cell-cell contact and spatial orientations, leads to a wide diversity in the iPSC-derived BBB models (Appelt-Menzel et al., 2017; Jamieson et al., 2019). Even the slightest change, like another extracellular matrix, in the differentiation of the co-cultured cells, can influence the expression levels of BBB markers (Delsing et al., 2019).

Extracellular matrix and 3D models

Different incentives, like 3D model development, lead to the investigation of the use of alternative extracellular matrices besides Matrigel, which is used during the differentiation, and the collagen IV/ fibronectin combination, used at the end of the differentiation, in most of the one-step protocols (Figure 2) (Hollmann et al., 2017; Lippmann et al., 2014, 2013; Qian et al., 2017). As Matrigel contains different basement membrane components (laminin, collagen and entactin) and is sensitive to supplier and batch variations, replacing this coating by defined alternatives can improve the differentiation and reduce variation (Aoki et al., 2020; Patel and Alahmad, 2016; Qian et al., 2017). BBB differentiation performed on defined laminin 221 enhanced the barrier integrity (Aoki et al., 2020). While the use of the more developmentally relevant laminin 511 instead of collagen IV/ fibronectin at the end of the differentiation lead a more sustained barrier stability over time combined with an improved

junctional protein expression, reduction of stress fibers and response to shear stress (Motallebnejad and Azarin, 2020). Most differentiations are performed in 2D, using transwells under static flow conditions. To make more physiologically relevant cylindrical models, which allow the use of flow to induce shear stress, multiple 3D models have been developed like microvessels, microfluidic devices and BBB-on-a-Chip (Katt et al., 2018). Often these 3D models make use of collagen I gels as this proves to be a stable and modifiable structural matrix, allowing to mimic the brain stiffness even though collagen I is not present in the brain (Grifno et al., 2019; Jamieson et al., 2019; Katt et al., 2018; Linville et al., 2019). Other possibilities explored are the use of gelatin and fibrin hydrogels (Campisi et al., 2018; Faley et al., 2019).

Challenges and perspectives

Recently, with the use of transcriptomic approaches, more and more research reported the presence of epithelial characteristics using the one-step differentiation protocols (Delsing et al., 2018; Hollmann et al., 2017; Lippmann et al., 2014, 2012; Lu et al., 2021; Qian et al., 2017; Vatine et al., 2019). In the previously mentioned research from Delsing *et al.*, two differentiation protocols were compared, the first one according to the one-step retinoic acid-enhanced Lippmann protocol and the second one making use of an endothelial and pericyte co-differentiation protocol with CD31 magnetic sorting (Lippmann et al., 2014; Orlova et al., 2014). Analysis of these cells in mono and co-culture lead to the conclusion that the one-step protocol exhibited better barrier properties (i.e. higher TEER, functional efflux transporters and the ability to discriminate between CNS permeable and non-permeable drugs). However, the expression and staining for endothelial markers like CD31, VE-cadherin and vWF were reported to be much lower in comparison with the second differentiation protocol and the hCMEC/D3 cell line. Whole transcriptome analysis also revealed the low expression of CLDN5 and VE-cadherin while the expression of, among others, the epithelial associated CLDN7 was higher (Ding et al., 2012; Farkas et al., 2015).

Because of the expression of endothelial specific markers and quite a few brain endothelial specific transporters, it was suggested that these cells might have a mixed endothelial and epithelial phenotype. In line with these observations, Vatine *et al.* reported that one-step differentiated BLECs on a Chip resembled endothelial cells but had some epithelial characteristics (Vatine et al., 2019). In 2020, Nishihara *et al.* also reported a lack of expression of key adhesion molecules important for immune cell migration across the BBB in two different one-step differentiation protocols and introduced an extended endothelial cell culture method as alternative (Nishihara et al., 2020). In 2021, Lu *et al.* performed a meta-analysis of previously published transcriptomic data from one-step protocols (Lu et al., 2021). They reported a lack of endothelial cell makers like CDH5, PECAM1 and KDR while gene clusters related to the neuroectodermal epithelial lineage were expressed. Overexpression of endothelial transcription factors ETV2, FLI1 and ERG, reprogrammed the cells to true endothelial cells. The authors suggested this approach for the generation of true BBB endothelial cells, although these cells exert limited barrier properties. As a result of the multiple studies reporting epithelial characteristics, Lippmann and colleagues confirm these reports but underlined that still multiple independent labs reported endothelial characteristics (Lippmann et al., 2020). They advise to call the cells hPSC-derived brain microvascular-*like* cells and stress the fact that applications of a model should be matched to its capability and serve as a complement with other *in vitro* and *in vivo* assays. Furthermore, other factors like differences in culturing conditions (e.g.: incubation time for cell detachment and cell counting techniques) and analytical methods, can influence the differentiation and performance of these models across laboratories. The application of Good Cell Culture Practice (GCCP), with in depth characterisation, consistent recording and reporting of the data, can aid the reproducibility and reliability of these models (Bal-Price and Coecke, 2011; Pamies et al., 2022, 2018, 2017). Additional care must be taken when working with iPSCs as "spontaneous" differentiation might be observed. It is essential to confirm the pluripotency of the iPSC lines at regular time points. In conclusion, both the one and two-step differentiation protocols have their strengths and weaknesses and the better a model is characterized, the

better it will allow decisions on its applicability. An alternative approach to develop iPSC-derived BBB models is, like suggested by Lu and colleagues, the use of transcription factors. Roudnicky *et al.* investigated which transcription factor combination, which are not necessarily endothelial specific, could be used to increase barrier resistance in native iPSC-derived endothelial cells (Roudnicky et al., 2020). Transduction with the transcription factors ETS1, SOX7, SOX18 and LEF1 (or TAL1) induces barrier resistance and mRNA and protein expression of endothelial barrier relevant genes, although still containing relatively low barrier properties in comparison with the one-step protocols. Transduction with BBB relevant transcription factors holds a promising approach for the development iPSC-derived BBB models. Identification of these transcription factors and developmental trajectories might greatly benefit from the use of single cell RNA sequencing in the understanding of developmental trajectories (Pokhilko et al., 2021; Schiebinger et al., 2019).

Transcriptomic characterization

Every single study is focused on some specific markers depending on the intended application of the model but fail to cover other potentially important characteristics, like, for example, a broader characterization of the tight junction network (Berndt et al., 2019). Ideally, each differentiation protocol should be evaluated through a complete multi-omics characterization. Even in this "ideal" situation, an additional challenge would be to compare it to the human *in vivo* counterpart. However, given the obvious constraints in obtaining fresh human brain tissue, this data is scarily available and would necessarily bear inter-individual variability. Transcriptomics analysis recently became more accessible and cheaper, as illustrated by the use of TempO-Seq in our studies, transcriptomic profiling can be a more realistic approach for a better detailed characterization of the iPSC-derived models (Wellens et al., 2021).

The limited human brain transcriptomic data available, is derived from different brain regions of patients undergoing neurological surgery for diseases like epilepsy and glioblastoma

(Darmanis et al., 2015; Spaethling et al., 2017; Zhang et al., 2016). Different processes have been applied to these tissues, like sorting of individual cell types by immunopanning followed by RNA-sequencing, immediate single cell RNA-sequencing or single cell RNA-sequencing after long-term primary culture of the cells (Darmanis et al., 2015; Spaethling et al., 2017; Zhang et al., 2016). A recent review provides guidelines for the experimental design of brain barrier RNA sequencing (Francisco et al., 2020). The use of these different processes, like RNA isolation, sample preparation, RNA sequencing method and bioinformatic analysis, influences the transcriptomic results. Implementation of external RNA controls provides the possibility to estimate technical variability (Francisco et al., 2020; Pine et al., 2016). The transcriptomic results can also be confirmed by using qPCR (requiring proper characterization and testing of primers) or on protein level (Bustin et al., 2009). When validating on protein level, it has to be taken into account that there is a discrepancy between the mRNA and protein levels (Liu et al., 2016). While immediate single cell RNA-sequencing does not allow any morphological or functional investigation of the cells, it permits identification of the individual cells type and limits transcriptome changes caused by culturing of cells (Darmanis et al., 2015)

In 2015, Darmanis *et al*. investigated the human brain transcriptome diversity by the use of single cell RNA-seq (Darmanis et al., 2015). With the use of unsupervised clustering, ten distinct cell groups were identified. Based on the presence of cell-type specific markers in the top 20 enriched genes, eight out of the ten clusters were assigned to known brain cell types, out of which one group consisted of brain endothelial cells. This unsupervised clustering was in good agreement with the clustering method based on cell-type enriched genes and can be used when no cell-type specific expression information is available. McKenzie *et al.* used this RNA expression dataset together with four other human and murine brain datasets to investigate specific expression patterns of brain cell types (astrocytes, oligodendrocytes, neurons, microglia and endothelial cells) (Darmanis et al., 2015; McKenzie et al., 2018; Zhang et al., 2016). Multiple human brain endothelial cell specific genes were identified like APOLD1, SDPR, DC34 and TGM2. Specificity was

defined as a minimum fold change between a certain cell type in comparison with each of the other cell types and as a result these specific genes could potentially be used as human brain endothelial marker genes. In this study, and many other brain RNA-sequencing studies, the transcriptome of brain endothelial cells are compared to one or more of the other cells of the neurovascular unit (neurons, glia, pericytes, etc.). Therefore, no discrimination is made between the different subtypes of endothelial cells of the brain vasculature, this could lead to potential bias in the analysis by comparing endothelial cells to non-endothelial cells and assuming the marker to be specific for brain endothelial cells. Although the BBB is assumed to reside at the brain capillaries, its properties vary along the brain microvasculature (Vanlandewijck et al., 2018). To uniquely assess the microvasculature transcriptome, Song *et al.* used laser capture microdissection to extract brain microvessels with a diameter smaller than 10 µm of snap-frozen human and mouse brain tissue (Song et al., 2020). As endothelial cells and pericytes share the same basal lamina, the sequenced microvascular cells consisted of both cell types and matched whole brain samples were used to identify microvascular enriched genes. While mouse microvessel biological triplicates clustered closely together, the three human samples displayed low similarity which might be a result of interindividual variability, effects of their neurological condition or differences in brain region where the sample was taken. Even though the human microvessel transcriptomes were enriched for known pericyte and brain microvascular endothelial markers, larger sampling groups are needed and would allow to investigate the influence of brain region, age or disease on the microvasculature transcriptome. Furthermore, Song *et al.* investigated species-specific differences with the use of the matched transcriptomic data. They found the gene VTN, vitronectin, to be highly expressed mouse brain pericytes while undetectable in human pericytes. Noteworthy, vitronectin is used as coating in some human iPSC-derived BBB differentiation protocols (Aoki et al., 2020; Jamieson et al., 2019; Patel and Alahmad, 2016; Qian et al., 2017). As the extracellular matrix is an important factor in the differentiation, it might be important to take

these species-specific differences into account for the establishment of differentiation protocols.

While it is important to remain aware of possible species-specific differences, given the paucity of human transcriptomic studies on the BBB, animal studies can provide a much more detailed molecular atlas of the brain vasculature. In 2018, Vanlandewijck *et al.* investigated the gradual cellular phenotypic changes, called 'zonation', along the arteriovenous axis using single-cell transcriptomics. This allowed the identification of capillary specific genes and transcription factors in mouse which might aid to distinguish brain capillaries from the whole brain vasculature.

Different online tools, like the human protein atlas, provide a convenient overview of gene and protein expression in multiple tissues. Unfortunately, the BBB is almost never identified as an individual tissue and it is not possible to investigate the expression of specific genes and proteins at the BBB. Different research groups made their brain transcriptome data available on websites such as the brain RNA sequence website (www.brainrnaseq.org), providing expression levels in both mice and human, and the brain cell types website (www.celltypes.org/brain/), providing a user friendly way of assessing the gene expression from five different studies (McKenzie et al., 2018; Zhang et al., 2016). Additionally, RNA and microRNA sequencing data from the human cerebrovascular endothelial cell line hCMEC/D3 are available on the BBBomics website (www.bioinformaticstools.mayo.edu/bbbomics/) (Kalari et al., 2016). The ongoing BBB Carta Project from the Canadian National Research Council is harvesting multiple experimental omics (including RNA sequencing, proteomics, glycoproteomics, glycomics, metabolomics) from hundreds of internal, publicly available, or curated datasets with the objective of generating comprehensive molecular "maps" of the BBB from a number of mammalian species (including human) (Haqqani and Stanimirovic, 2019).

Conclusion

High-throughput sequencing for transcriptome profiling is an increasingly accessible and important tool for biological research. To date, the paucity of human transcriptomic databases on the BBB makes it difficult to use transcriptomic to efficiently characterize iPSCs derived BBB models. Hopefully, the increasing use of transcriptomic and the development of such databases will in a near future allow an in-depth characterization of the current protocols to derive BBB models from iPSCs and guide researchers in the implementation of changes in those protocols to generate more relevant BBB models. This will be particularly important, for the successful use and implementation of these iPSC-derived BBB models.

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Declaration of interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Highlights

- Modelling of the blood-brain barrier (BBB)
- BBB models from human pluripotent stem cells
- Challenges of iPSC-derived BBB models
- Use of transcriptomics for iPSC-derived BBB model characterization