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Models of the blood-brain barrier using iPSC-derived cells

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ABSTRACT

The blood-brain barrier (BBB) constitutes the interface between the blood and the brain tissue. Its primary function is to maintain the tightly controlled microenvironment of the brain. Models of the BBB are useful for studying the development and maintenance of the BBB as well as diseases affecting it. Furthermore, BBB models are important tools in drug development and support the evaluation of the brain-penetrating properties of novel drug molecules. Currently used *in vitro* models of the BBB include immortalized brain endothelial cell lines and primary brain endothelial cells of human and animal origin. Unfortunately, many cell lines and primary cells do not recreate physiological restriction of transport *in vitro*. Human-induced pluripotent stem cell (iPSC)-derived brain endothelial cells have proven a promising alternative source of brain endothelial-like cells that replicate tight cell layers with low paracellular permeability. Given the possibility to generate large amounts of human iPSC-derived brain endothelial cells they are a feasible alternative when modelling the BBB *in vitro*. iPSC-derived brain endothelial cells form tight cell layers *in vitro* and their barrier properties can be enhanced through co-culture with other cell types of the BBB. Currently, many different models of the BBB using iPSC-derived cells are under evaluation to study BBB formation, maintenance, disruption, drug transport and diseases affecting the BBB. This review summarizes important functions of the BBB and current efforts to create iPSC-derived BBB models in both static and dynamic conditions. In addition, it highlights key model requirements and remaining challenges for human iPSC-derived BBB models *in vitro*.

1. Introduction to the blood-brain barrier

The blood-brain barrier (BBB) is the interface between the blood and the brain tissue. Its primary function is to maintain the tightly controlled microenvironment of the brain. The BBB is a microvascular structure composed of the smallest vessels; arterioles, capillaries and venules, which regulate the exchange between the blood and the surrounding tissue. The brain vasculature consists of endothelial cells with properties specific to the central nervous system (CNS) (Abbott et al., 2010; Obermeier et al., 2013). The structure and function of the BBB has been reviewed elsewhere, for detailed reviews see references 1 and 2 by Obermeier et al. and Abbott et al. The brain endothelial cells control the permeability of the barrier. At the brain side of the endothelial cells, the extracellular basement membrane (BM) surrounds the endothelial cells and embeds the pericytes. Astrocytic end-feet are in contact with the basal membrane. This unit of astrocytes, pericytes, basal membrane and endothelial cells is often referred to as the neurovascular unit (NVU, Fig. 1) (Iadecola, 2017; Obermeier et al., 2013). Together these components make up the BBB and govern its development, maintenance and function. The concept of the NVU was first formalized at the 2001 Stroke Progress Review Group meeting of the National Institute of Neurological Disorders and Stroke. For an extensive review on the subject of the NVU see reference 3 by Iadecola. The paracellular tightness of the endothelial cells in the BBB acts as a physical barrier for cells, proteins and water-soluble agents in-between the brain parenchymal and the systemic circulation. Transporter

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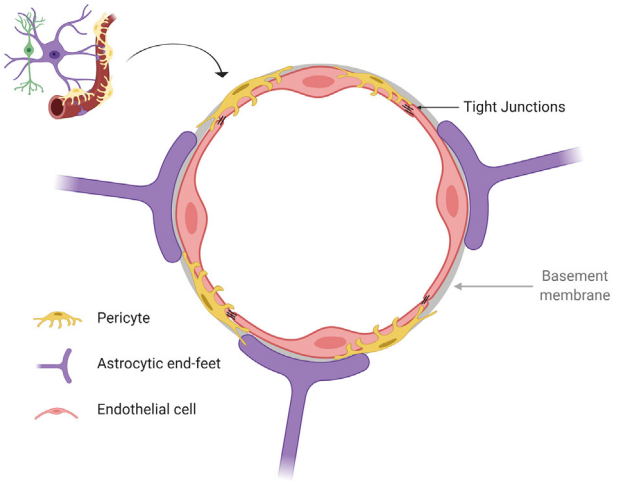

Fig. 1. The neurovascular unit. Endothelial cells are linked together *via* tight junctions. On the brain side of the endothelial cell layer, the basement membrane surrounds the endothelial cells and embeds the pericytes. Astrocytic end-feet are in contact with the endothelial cells.

proteins control nutrient supply and permeability of small molecules in a specific manner. The BBB is a highly dynamic structure, which is regulated by the interactions of the cellular components and extra cellular matrix parts of the NVU. Isolated primary brain endothelial cells rapidly lose their BBB properties when cultured *in vitro* (Urich et al., 2012), consequently it is plausible that the BBB properties are not intrinsic to the brain endothelial cells but rather depend on the specific microenvironment that all the components of the NVU create together. In the zoom-out, a neuron can be seen in green.

The development of the BBB begins when vessels start to invade the developing neuroectoderm (Obermeier et al., 2013). Permeability restriction occurs already early in development and rodent studies show that the early embryonic BBB prevents leakage of proteins from the blood to the brain as early as E14 (Johansson et al., 2008). Similar restriction of blood to brain permeability was recently confirmed in human early embryos. The first vessels penetrating into the brain parenchyma in the human embryo restrict permeability of blood-derived molecules and are immunopositive for the TJ protein claudin-5, suggesting that even the earliest brain blood vessels at gestation week five have BBB characteristics (Møllgård et al., 2017). Cues from astrocytes and pericytes are essential in BBB development. Lack of such signals is linked to severe abnormalities of the BBB seen in both mice and human primary cells (Alvarez et al., 2011; Daneman et al., 2010).

While only making up 2% of the total body mass, the brain consumes about 20% of the glucose and oxygen. To support this massive claim of energy and oxygen the cerebral blood vessel network is enormous. The blood flow is rapidly increased at sites of activity in the brain to accommodate the high energy demand, this is known as neurovascular coupling (Obermeier et al., 2013). Brain endothelial cells have features that differentiate them from endothelial cells in other organs, longer continuous stretches of TJs, higher number of mitochondria, no fenestrae (small pores) and low transcytosis activity (Abbott et al., 2006; Carvey et al., 2009; Zlokovic, 2008). All of these features contribute to the brain endothelial cell capacity to restrict permeability and act as a selective barrier. TJ restriction of water-soluble molecules in the paracellular space results in high trans-endothelial electrical resistance (TEER), a hallmark of brain endothelial cells. Physiological brain TEER is estimated to be above 1000 Ohm x cm^2 compared with 2–20 Ohm $\times$ cm^2 in the majority of other endothelial barriers in the body (Butt et al., 1990). TJ proteins, such as claudin-5, occludin and specific transporters, such as P-glycoprotein (P-gp) breast cancer resistance protein (BCRP) and Glut-1 are often used as markers of brain endothelial cells (Patabendige et al., 2013). The study of brain endothelial cells has been hampered by the difficulty to obtain human primary brain endothelial cells from healthy individuals and the fact that human primary brain endothelial cells and immortalized brain endothelial cell lines do not maintain barrier restriction capacity *in vitro* (Urich et al., 2012). Primary endothelial cells isolated from animals such as pigs and rats retain fairly tight barriers *in vitro* and can be useful tools to study paracellular permeability (Patabendige and Abbott, 2014; Urich et al., 2012). However, the restrictive capacity *in vivo* and the expression of specific transporters are different between species (Syvanen et al., 2009; Uchida et al., 2011). Hence, to be able to predict and study the human BBB a human model is highly preferable.

1.1. Permeability of the blood-brain barrier

Controlled movement across the BBB involves restriction and facilitated transport of essential substances to supply nutrients. Transport into the CNS occurs through paracellular transport, transcellular diffusion, carrier-mediated transport (CMT), receptor-mediated transcytosis (RMT) and transcytosis. In addition, ATP-dependent efflux transporters and ion pumps are active at the BBB. Small hydrophilic molecules may pass through the paracellular route; however, due to the high density of TJs in brain endothelial cells, this transport route is very restricted. Oxygen and carbon dioxide freely diffuse across the endothelial cell membrane in transcellular transport. Similarly, small lipophilic molecules, such as ethanol, can diffuse across. Larger molecules and nutrients such as glucose and amino acids rely on CMT or RMT. CMT enables molecules such as carbohydrates, amino acids and vitamins to be transported down their concentration gradient through membrane carrier proteins. Clathrin and caveolin mediate the formation of vesicles for RMT and non-receptor-mediated vesicular transport (Ayloo and Gu, 2019). Reduced caveolin-mediated transport has been identified as a differential factor between brain endothelial cells and peripheral endothelial cells. Furthermore, increased caveolin vesicle transport has been implicated as a contributing factor to barrier leakage (Andreone et al., 2017; Reyahi et al., 2015).

The efflux transporter system functions as a second security mechanism in the control of BBB permeability. Some substances may be able to diffuse across the cell membrane or are able to pass into the cell through CMT. However, they will have substantially reduced permeability into the CNS if they are recognized by the efflux transporters. Substrates of efflux transporters are efficiently shuttled back into the blood. The three main efflux transporters are P-glycoprotein (P-gp), breast cancer resistance protein (BCRP) and multidrug resistance proteins (MRPs) (Uchida et al., 2011). Efflux transporters have a broad substrate range, particularly P-gp, and are responsible for the low permeability into the CNS of many endogenous and exogenous molecules circulating in the blood. This protects the CNS from substances such as xenobiotics, pesticides and drugs, that could be harmful to the brain (Choi and Yu, 2014).

1.2. Inter-cellular junctions

Inter-cellular junctions between the endothelial cells at the BBB are made up of TJs and adherens junctions (AJs), in addition, cluster of differentiation 31 (CD31) protein is highly expressed and its connections contribute to cell-cell adhesion (Bauer et al., 2014; Vanlandewijck et al., 2018). Very constricted TJs are a hallmark of the BBB and limit the permeability of polar solutes in the paracellular space. AJs connect the cells through transmembrane cadherins. Cadherins are linked to the cytoskeleton by the scaffolding proteins alpha-, beta- and gamma catenin. In brain endothelial cells, VE-cadherin is the most prevalent cadherin with only low levels of E and N-cadherin (Vanlandewijck et al., 2018). The composition of TJs is more complex; transmembrane proteins; occludin, claudins and junctional adhesion molecules (JAMs) span the junctions between the cells. Occludin and claudins are linked to the cytoplasmic scaffolding proteins zonula occludens-1, 2 and 3

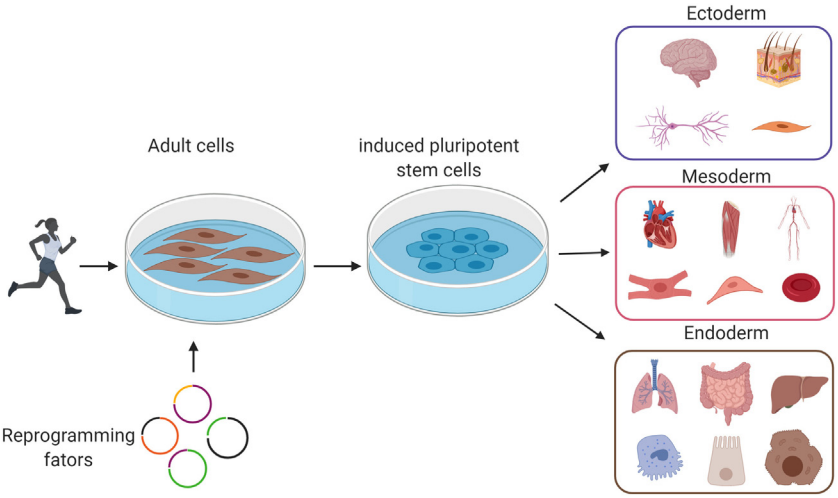

Fig. 2. Induced pluripotent stem cells are reprogrammed adult human cells from patients or healthy individuals. Once reprogrammed to an early development stage, induced pluripotent stem cells can self-renew and be differentiated into all cell types of the human body.

(ZO-1, ZO-2, ZO-3). Claudin-5 is commonly identified as the most abundant claudin in brain endothelial cells (Vanlandewijck et al., 2018), however other claudins are also present at the BBB. Both claudin-5 and claudin-3 have been shown to localize at TJs in the brain endothelium (Wolburg et al., 2003). Occludin expression is higher in brain endothelial cells than in peripheral endothelial cells and occludin expression levels have been shown to correlate with barrier tightness (Hirase et al., 1997; Liu et al., 2014; Urich et al., 2012). However, the specific contributions of occludin and claudins to barrier function, reviewed elsewhere (Zihni et al., 2016), are highly complex and not yet fully understood.

2. iPSC-derived cells for blood-brain barrier modelling

Induced pluripotent stem cells (iPSC) are somatic cells reprogrammed to a pluripotent state using overexpression of defined transcription factors (Takahashi et al., 2007) (Fig. 2). iPSCs are similar to embryonic stem cells (ESC) and can be differentiated to all cell types of the human body. iPSCs do not suffer from the same ethical obstacles as ESCs because they can be generated from cells obtained from an adult individual, without destruction of human embryonic tissue. The generation of iPSCs from adult cells allows for a number of new applications. Some cell types, such as neural cells and brain endothelial cells, have been difficult to study *in vitro* due to the challenges in obtaining these primary cells from healthy individuals.

The iPSC technology provides great possibilities to generate large amounts of these cell types for *in vitro* studies without invasive sampling of healthy humans or use of animals for research purposes (Takahashi et al., 2007). Furthermore, iPSCs can be generated from patients to provide patient-specific cell lines, for modelling of diseases and putative assessment of individual drug response. Undifferentiated iPSCs have a high proliferation capacity making them suitable for large-scale production of cells as well as genetic manipulation. In theory, the amounts of cells that can be produced are unlimited, however in practice the number of cells is limited by cell culture capacity. To unleash the potential of iPSCs, robust and reliable protocols for differentiation are required. The development of differentiation protocols for directing iPSCs to a specific cell type generally relies on recreating the signalling processes that govern the development of the desired cell type during embryogenesis. This review will focus on iPSC-derived brain endothelial cells, astrocytes and pericytes for BBB models.

2.1. iPSC-derived endothelial cells

Among well-defined signalling pathways, bone morphogenetic protein (BMP), FGF, and VEGF-signalling are most commonly modified for endothelial differentiation (Xu et al., 2019). The BMP family modulates early vascular development *via* the downstream SMAD family proteins, as demonstrated by studies in human embryonic stem cells (Bai et al., 2010). Addition of BMP early in the differentiation process has been shown to significantly induced endothelial differentiation (Goldman et al., 2009). Notably, the VEGF family members were among the first secreted molecules observed to be specific to endothelial differentiation. VEGF receptors that are specific to the endothelial lineage, contribute to endothelial differentiation (Carmeliet et al., 1996). This suggests that VEGF is not an early endothelial signalling cue but rather a later specification factor.

The brain endothelial cells have different properties than the peripheral endothelial cells. Hence, the differentiation of brain endothelial cells from iPSCs may require specialized protocols different from those used to derive peripheral endothelial cells. In 2012, Lippman et al. published a protocol for differentiation of iPSCs to brain endothelial cells (Lippmann et al., 2012). During the years after 2012 several improvements of the protocol have been proposed, including the addition of retinoic acid (RA) (Lippmann et al., 2014), optimizing of seeding density (Wilson et al., 2015), hypoxia stimulation (Park et al., 2019) and use of more defined medium components (Hollmann et al., 2017; Neal et al., 2019; Qian et al., 2017). The protocol relies on spontaneous co-differentiation of endothelial cells with neural cells and subsequent purification of the endothelial cells by passage on-to collagen/fibronectin in an endothelial cell medium containing FGF and RA. Particularly the RA treatment at the end of the differentiation has proven important for the cells to develop a mature BBB phenotype, with high tightness and increased expression of several TJ proteins and transporters (Lippmann et al., 2014; Qian et al., 2017). Additionally, the codifferentiation with neural cell types in this protocol is believed to be important for the development of a brain specific phenotype in the endothelial cells. Endothelial cells generated with this protocol display high TEER 500-4000Ohm x cm^2, low permeability and expression of claudin-5, occludin, ZO-1, CD31, VE-cadherin and Glut-1 (Canfield et al., 2017; Hollmann et al., 2017; Lippmann et al., 2014). Most recently, fully defined versions of this protocol that eliminate the use of serum have been reported (Neal et al., 2019; Qian et al., 2017). Furthermore, endothelial cells produced with this protocol can be produced in large amounts and can be cryopreserved in an assay ready state (Wilson et al., 2016). Hence, hundreds of millions of brain

endothelial cells can be produced in a standard cell laboratory. Other protocols for derivation of brain endothelial cells have been proposed, but without successful adoption in the iPSC BBB community (Campisi et al., 2018; Minami et al., 2015; Praça et al., 2019; Rieker et al., 2019). The protocol developed by Lippmann et al., and subsequent optimizations of it, remains the most widely used methods for derivation of brain endothelial cells from iPSCs for *in vitro* BBB models.

2.2. iPSC-derived pericytes

The development of differentiation protocols for pericytes has been hampered by the lack of detailed knowledge of the pericyte characteristics. Pericyte marker proteins, functional characteristics and even their origin have been debated. Before brain pericyte-specific protocols were developed, pericytes were mainly differentiated through mesodermal intermediates (Orlova et al., 2014). Recently, an in-depth analysis of the cell types in the brain vasculature has provided new insight into the brain pericyte phenotype, and new markers that differentiate brain pericytes from peripheral pericytes were proposed, such as a higher abundance of SLC, ABC and ATP transporters (Vanlandewijck et al., 2018). Brain pericytes have been shown to develop from neural crest stem cells (Korn et al., 2002), and recently a protocol for derivation of brain pericytes from iPSCs *via* neural crest stem cells was published (Stebbins et al., 2019). However, pericyte differentiation through both neural crest stem cells and mesodermal intermediates has given similar results (Faal et al., 2019).

2.3. iPSC-derived astrocytes

There are several published protocols for astrocyte differentiation from iPSCs, and iPSC-derived astrocytes have been used to study many different aspects of astrocyte biology including inflammatory response (Lundin et al., 2018; Oksanen et al., 2017; Perriot et al., 2018; Santos et al., 2017), glutamate uptake (Lundin et al., 2018; Santos et al., 2017; Shaltouki et al., 2013), apoE biology (Lundin et al., 2018; Zhao et al., 2017) and genome wide expression studies (Lundin et al., 2018; Perriot et al., 2018; Santos et al., 2017). Astrocyte development and maturation occurs late in the embryonic development and continues after birth (Robertson, 2014). As such, mimicking the *in vivo* astrocyte development is a very lengthy process, often spanning several months. Many protocols for astrocyte differentiation rely on long-term culture of neural stem cells in FGF and epidermal growth factor (EGF) and/or serum (Krencik et al., 2011; Zhang et al., 2016). iPSC-derived astrocytes are commonly characterized by their expression of GFAP, CD44, EAAT1/2, S100B, and vimentin, and their ability to perform astrocyte specific tasks, such as glutamate uptake and inflammatory response to treatment with inflammation regulators (Shaltouki et al., 2013; Zhang et al., 2016). There have been numerous efforts to shorten the differentiation time required for astrocyte development, for example, through remodelling of the chromatin structure (Majumder et al., 2013) and using genetic techniques to overexpress transcription factors (Li et al., 2018). Even with recent efforts to shorten protocols for astrocyte differentiation the process is still labour-intensive and long, typically more than a month. However it has been shown that differentiated astrocytes can be cryopreserved for extended use (Lundin et al., 2018). The understanding of heterogeneity in human astrocytes is increasing and there is a growing interest in generating subtype-specific astrocytes from iPSCs. Both major astrocytic subtypes, protoplasmic and fibrous astrocytes, are in contact with the blood vessels *in vivo* (Tabata, 2015). However, it remains unknown if astrocyte subtype influences the effects that astrocytes have on the brain vasculature and the BBB.

3. Blood-brain barrier models

Models of the BBB serve as important tools in drug development and support evaluation of chemical properties and brain penetrating capacities of novel drug molecules. Regardless if the brain is the intended target or not, it is central to understand the permeability of a drug candidate into the CNS. Although many drug candidates appear promising in animal models, as many as 80% of them later fail in clinical trials (Perrin, 2014). This clearly demonstrates the need for better pre-clinical models with higher translatability to the human *in vivo* situation. At the same time, large efforts are being made to reduce the use of animal testing in research. The three Rs ethical principle to reduce, replace and refine animal-based science is widely accepted and implemented throughout the research community. In many countries, the three Rs principle is explicit legislation. Human cell-based models are important alternatives to *in vivo* animal models and *in vitro* models using animal cells.

Current models of the BBB span from *in vivo* animal models to more complex cocultures of several primary cell types and *in silico* modelling (Cecchelli et al., 2007; Garberg et al., 2005; Helms et al., 2016). *In vivo* animal models, using techniques such as brain perfusion, are considered some of the most accurate ways of determining BBB penetration. However, these techniques require animals to be subject to research, are time consuming, expensive and have low throughput, compared to cellular models (Cecchelli et al., 2007). A wide range of cellular models of the BBB have been described, including primary cells and cell lines from both human and animal origin. Primary cells from animals have proven to have suitable barrier integrity and relatively low permeability (Abbott, 2004; Garberg et al., 2005), but disadvantages linked to the use of animal cells include resource demanding isolation procedures, batch-to-batch variability and incompatibility with the three R's principle. An important aspect of BBB modelling using animal cells is the differences between the human BBB and the BBB in other species. For example, there is evidence of species differences in the expression of BBB transporters, including the important efflux transporter P-gp, and in permeability of P-gp substrates (Syvanen et al., 2009; Uchida et al., 2011). By using immortalized cell lines from both human and animal origin, issues with reproducibility and batch variability can be circumvented. However, many of the immortalized brain endothelial cell lines available fail to form tight barriers with low permeability, which questions their usability. iPSC-derived models provide a feasible option for overcoming many of these limitations by creating a human origin model with readily available cell types.

3.1. iPSC-derived blood-brain barrier models

A model with iPSC-derived cell types could overcome important challenges with reproducibility and availability of human cells, and provide the possibility for an isogenic model with all cell types originating from the same human individual. In addition, using iPSC-derived cells would reduce the need for animals and animal derived tissues to be used. The establishment of an iPSC-derived BBB model requires robust and reliable differentiation protocols for derivation of several cell types of the CNS. As described above, differentiation protocols for endothelial cells, astrocytes and more recently pericytes are available. Hence, an iPSC-derived BBB model is feasible, and during recent years, there has been a rapid increase in iPSC-derived BBB models. The establishment of the brain endothelial cell differentiation protocol by the Shusta lab in 2012 (Lippmann et al., 2012) served as an accelerator for iPSC-derived BBB model work. Most of the published iPSC-derived BBB models used variations of that differentiation protocol. Several of these iPSC-derived BBB models have rapidly developed into tools for investigation of drug permeability studies (Appelt-Menzel et al., 2017; Delsing et al., 2018; Li et al., 2019; Lippmann et al., 2012; Mantle et al., 2016; Ribecco-Lutkiewicz et al., 2018), disease modelling (Faal et al., 2019; Katt et al., 2019; Lee et al., 2018a; Lim et al., 2017; Vatine et al., 2017) and modelling of BBB disruption (Al-Ahmad et al., 2018; Martinez and Al-Ahmad, 2018; Page et al., 2019; Patel et al., 2018). Both monoculture models of the BBB using only iPSC-derived endothelial cells and coculture models with endothelial cells and other

cell types of the NVU have been established. Coculture models contained endothelial cells and different combinations of astrocytes, pericytes, neurons and neural stem cells (Appelt-Menzel et al., 2017; Canfield et al., 2019, 2017; Stebbins et al., 2019) and iPSC-derived coculture models of the BBB form monolayers with highly restricted permeability in the paracellular space. TEER values for coculture models have been reported to be higher than 6000 Ohm × cm^2 (Canfield et al., 2019), however the variability in maximum TEER reported between iPSC-derived models is quite high and others have reported TEER values of ~1000–4000 Ohm × cm^2 (Appelt-Menzel et al., 2017; Canfield et al., 2017; Delsing et al., 2018; Qian et al., 2017). Permeability of passively diffused soluble substances such as fluorescein have been reported to be in the range of ~1–5 × 10^{-7} cm/s in iPSC-derived BBB models (Canfield et al., 2017; Hollmann et al., 2017). This is substantially lower than the permeability achieved in brain endothelial cell line cultures of ~12–15 × 10^{-6} cm/s (Eigenmann et al., 2013) which can be compared to *in vivo* measurements in rat of 2.7 × 10^{-6} cm/s (Yuan et al., 2009). Efflux by P-gp is commonly investigated and found to be active in iPSC-derived BBB models, however, the activity of P-gp is not affected by coculture (Appelt-Menzel et al., 2017; Canfield et al., 2019; Canfield et al., 2017). Across iPSC-derived BBB models coculture with astrocytes appears to increase the barrier restriction capacity of the endothelial cells. Results from pericyte cocultures are more conflicting, with some studies showing improved barrier restriction with coculture (Canfield et al., 2019; Hollmann et al., 2017; Lippmann et al., 2014; Stebbins et al., 2019) and other reporting no differences (Appelt-Menzel et al., 2017; Jamieson et al., 2019). Interestingly, these conflicting results have been reported for both cocultures with iPSC-derived pericytes and primary pericytes. One of these studies demonstrated that even though pericyte coculture had no effect on endothelial cells under normal conditions, pericyte coculture had the ability to rescue barrier properties in stressed endothelial cells and allowed endothelial cells to maintain high TEER over longer culture time. This suggested that the pericyte contribution in *in vitro* BBB models was maintenance rather than induction (Jamieson et al., 2019). These discrepancies highlight the numerous factors contributing to variability in complex multicellular models, such as the iPSC-line background, culture conditions and assay conditions. Even though some differentiation protocols have proven to be highly robust and transferrable between different labs and applications, variability is an issue when comparing models. This was exemplified in a study comparing the differentiation capacity of four different iPSC lines to isogenic BBB models including endothelial cells and astrocytes (Patel et al., 2017). Even though many iPSC-derived BBB models have been developed, characterized and used in different applications, several questions remain. Specifically, with regard to stability and reproducibility. Investigating the reproducibility of iPSC derived BBB models is challenging as different parental iPSC lines introduces genetic variability in addition to variabilities in protocols and handling. To facilitate investigation of stability and reproducibility in iPSC-derived BBB models standardization of the characterization and validation of models is needed. Such standardization may include establishing analytical performance standards for models and a defined set of reference compounds for permeability assays that can demonstrate desired outcome and be compared across different models and labs. Such standardization would also facilitate the use of iPSC-derived BBB models in disease modelling and drug discovery. Furthermore, the mechanism of BBB induction by coculturing cell types and the expression and functionality of more brain-specific transporters need to be thoroughly investigated. Additional studies of drug permeability prediction are needed.

3.2. Microfluidic models

Recently several microfluidic BBB models containing iPSC-derived endothelial cells have been reported (Campisi et al., 2018; DeStefano et al., 2017; Jamieson et al., 2019; Linville et al., 2019; Park et al., 2019; Vatine et al., 2019; Wang et al., 2017). These models aim to mimic the 3D morphology of vessels and allow the cells to interact under more physiological conditions. In these models, the cells are subject to shear forces introduced by flow, similar to the *in vivo* conditions in brain blood vessels. The shear forces that affect the cells are determined by the vessel diameter, the viscosity of the flowing liquid and the flow rate. Human micro-vessels and shear stress have been studied in the eye where diameters ranged between 6 and 24 μm. The shear stress was measured to be between 2.8 and 95 dyne/cm^2, the calculated average shear stress was 15.4 dyne/cm^2 (Koutsiaris et al., 2007). These findings can be compared to measurements of vessel diameters in the human motor cortex where the perforating capillaries have a diameter ranging from 5 to 8 μm, the arterioles have a diameter ranging from 10 to 15 μm and the venules have a diameter ranging from 16 to 20 μm (Marín-Padilla, 2012). Brain post capillary venules are characterized by diameters of around 100 μm, a relatively thick basement membrane, and a wall shear stress of 1–4 dyne/cm^2 (DeStefano et al., 2018; Marín-Padilla, 2012). Compared to the human *in vivo* brain vasculature, most BBB microphysiological systems (MPS) recreate an environment, which is similar to the vessel diameter and shear forces of post capillary venules. Similarly to the static models, the majority of iPSC-derived MPS models have used variants of the protocol proposed by Lippmann et al. (2014) to derive brain endothelial cells and cultured them as monocultures or cocultures.

Recently, several iPSC-derived BBB models in MPS have been reported, both monoculture systems (DeStefano et al., 2017; Faley et al., 2019; Linville et al., 2019) and coculture systems with pericytes and/or neural cell types (Campisi et al., 2018; Jamieson et al., 2019; Park et al., 2019; Vatine et al., 2019; Wang et al., 2017). Several coculture models used primary sources for pericytes and astrocytes (Campisi et al., 2018; Park et al., 2019; Wang et al., 2017). However, two fully iPSC-derived models have been reported, one using iPSC-derived pericytes (Jamieson et al., 2019) and one using iPSC-derived neural cells for coculture (Vatine et al., 2019). In these studies, coculture with only pericytes did not affect permeability (Jamieson et al., 2019), but coculture with astrocytes, with or without pericytes and neurons, resulted in reduced permeability (Campisi et al., 2018; Vatine et al., 2019; Wang et al., 2017) or increased impedance (Park et al., 2019). Permeability measurements using fluorescently labelled dextrans show that these models have very low permeability, several models reported a permeability of 2–4 × 10^{-7}cm/s (Campisi et al., 2018) or below detection limit for 10 kDa dextrans (Jamieson et al., 2019; Linville et al., 2019). Permeability for 4 kDa dextran and 3 kDa dextran was reported to be in the range of single digit 10^{-7}cm/s and 1–3 ×10^{-7}cm/s. Permeability for 4 kDa dextrans across *in vivo* rat cerebral microvasculature has been reported to be the 9.2×10^{-7}cm/s (Yuan et al., 2009). Additionally, iPSC-derived BBB MPS models have reported P-gp activity (Linville et al., 2019; Park et al., 2019; Vatine et al., 2019) and BCRP activity (Park et al., 2019). Taken together permeability data from these models show that it is possible to create iPSC-derived BBB models with high paracellular tightness similar to that *in vivo*.

Comparing 2D and 3D models to elucidate effects of more physiological culture conditions and addition of shear stress is challenging. Consequently, effects of introducing shear stress on iPSC-derived brain endothelial cells are not well studied. Evaluation of permeability between static and dynamic models will have inherent differences in physical prerequisites. Furthermore, elucidating what differences flow creates is difficult because medium volumes in many MPS are low and therefore flow is necessary to supply the cells with oxygen and nutrients. Hence, creating a static culture in these systems is often not possible and direct comparisons between static and dynamic conditions are not feasible. Despite these difficulties, comparisons of MPS cultures with and without shear stress have been reported (DeStefano et al., 2017; Faley et al., 2019). It was concluded that introducing shear stress on iPSC-derived endothelial cells had other effects than introducing shear stress on primary endothelial cells. iPSC-derived endothelial cells

subjected to shear stress had lower apoptosis, lower proliferation and lower cell mobility, however no change in TJ proteins were found, even though shear stress served to increase contact area between cells (DeStefano et al., 2017). Another recent study comparing iPSC-derived brain endothelial cells under shear stress and static conditions showed that iPSC-derived brain endothelial cells that were subject to flow had lower passive paracellular permeability but no difference in efflux transporter activity (Faley et al., 2019). However, expression levels of several TJ and endothelial cell markers have been found to depend on the flow rate in a fully iPSC-derived model (Vatine et al., 2019). Studies of primary brain endothelial cells revealed interesting effects of shear stress in culture. Under flow, these cells went from a mostly anaerobic metabolism producing lactate to a mixed aerobe and anaerobe metabolism producing both lactate, H_2O and CO_2 (Cucullo et al., 2011). There have been speculations that, as the BBB tightens more active and energy demanding transport is necessary and hence the endothelial cells make use of a more aerobic metabolism, which is more efficient in generating ATP. Another speculation was that the metabolism is dependent on blood flow and thus oxygen levels. When the blood flow is low and hence the oxygen availability is low, a more anaerobic metabolism can be utilized.

There are multiple reasons why Transwell cultures are replaced by microfluidic systems. MPS provides a more physiologically relevant culture in several aspects, a 3D spatial organization of the cells allows for the endothelial cells to form tubes and for the coculturing cells to interact physically with the basolateral face of the endothelial tube. The importance of shear stress in BBB and vasculature development has been emphasized in several studies (Cucullo et al., 2011; DeStefano et al., 2017; Vatine et al., 2019) and MPS allows for the addition of flow and shear stress. Moreover, there are technical aspects that favour the MPS, for example, the number of cells needed to create an MPS model is substantially lower and continuous live cell imaging is greatly facilitated in some MPS models compared with the Transwell models. However, MPS systems similarly have inherent technical drawbacks, the setup of MPS is often very complicated and requires special laboratory equipment, TEER measurements are difficult to perform which means that more laborious assays are needed to assess paracellular permeability. Furthermore, current solutions are very expensive compared to Transwells and the throughput of MPS is typically very low.

4. Future perspectives

The iPSC technology has allowed human cells to be used to a larger extent within BBB modelling, overcoming issues with limited availability and variable quality of primary cells. Primary brain endothelial cells and cell lines have not been demonstrated to create restrictive barriers *in vitro* (Eigenmann et al., 2013; Urich et al., 2012), as such primary cell models have not served as the gold standard to which iPSC-derived models can be compared. Extensive research has been performed to improve primary BBB models, for example, through coculture (Paradis et al., 2016), overexpression of microRNAs (Rom et al., 2015) and chemical stimuli (Hoheisel et al., 1998) with varying improvements in barrier restriction potential. Interestingly, iPSC-derived brain endothelial cells are able to create restrictive barriers with paracellular permeability similar to that seen *in vivo*. It is still unclear why the iPSC-derived brain endothelial cells are able to recreate the phenotype of their primary counterparts *in vitro*. Recently, the endothelial identity of iPSC-derived endothelial cells has been questioned, and claims have been made that these cells are actually neuroepithelium (Lu et al., 2019). However, these claims are still debated and under investigation. Even though the protein expression signature of brain endothelial cells derived from iPSCs with the brain endothelial cell specific protocol may be mixed, these cells display exceptionally high tightness and expression of BBB specific transporters. As such, they are a very relevant human model system that can be used for permeability assessments. *In vitro* cell models will never be able to recapitulate the full complexity of the *in vivo* biology and in the interest of usability, models need to be simplified versions of the modelled process or structure. The BBB is a complex multi-component structure that is likely to have several critical requirements for *in vitro* culture to correctly model its functions. Enhancing the brain endothelial cell phenotype of iPSC-derived endothelial cells and optimization of iPSC-derived BBB models is far from complete. Improvement of models is an ongoing process. Likely, there are opportunities for optimization of the differentiation and culture processes, which could be exploited to further improve the BBB phenotype of the model and produce iPSC-derived brain endothelial cells with more similar transcription signature to brain endothelial cells *in vivo*. In addition, there is a great need to further characterize the capacity of iPSC-derived BBB models to be used in permeability assessments. More information about activity and expression is required for many of the transporter proteins active at the BBB.

4.1. Brain permeability prediction in drug discovery

The current strategy for CNS permeability assessment in drug discovery relies on, first, determining if the substance is an efflux transporter substrate and second, determining the *in vivo* brain exposure in rodents. This is commonly preceded by *in silico* modelling of BBB permeability used in the lead generation process. Efflux transporter assays are generally performed using the low permeability human epithelial colorectal adenocarcinoma cell line (Caco-2) and Madin-Darby canine kidney cells overexpressing efflux transporter P-gp (MDCK-MRD1) line in Transwell systems relatively early in the drug discovery process. Later, *in vivo* rodent permeability assessments are performed. The ratio of the total brain concentration to total plasma concentration at equilibrium combined with the fraction unbound in brain and fraction unbound in plasma is determined. After infusion the concentrations in blood and in whole brain homogenate are analysed, brain binding is typically assessed by incubating rat brain slices with a compound cocktail. These methods are very low throughput and require several animals per data point. Hence, they are performed at the last stages of drug development before clinical trials.

iPSC-derived BBB models could replace the Caco-2 and MDCK-MDR1 lines in efflux transporter assays. In contrast to Caco-2 and MDCK cell lines, these iPSC-derived BBB models contain human brain-specific cells with expression of many BBB transporters lacking in the Caco-2 and MDCK-MDR1 lines, which originate from colon and kidney respectively. Using human brain-specific cells provides an opportunity to evaluate other transport routes in addition to efflux transport. Consequently, an iPSC-derived BBB model has the possibility to provide important information about several mechanisms of drug transport earlier in drug discovery process. Earlier prediction of brain exposure by a combination of mechanisms rather than efflux only would generate better translatability to the rodent *in vivo* models, causing fewer undesired compounds to make it as far as the *in vivo* assay. If fewer substances require *in vivo* model testing it would both reduce the number of animals needed for testing and provide a more cost-efficient process. Even though it would be desirable to replace *in vivo* animal testing completely, that is not likely to transpire in the near future. Due to the inability of present *in vitro* cell models to estimate brain-binding and metabolism, which govern the unbound drug concentration. These models are not able to predict the amount of substance which exerts the physiological function, *i.e.* the unbound fraction. Furthermore, there are regulatory requirements for animal testing before human trials, and human data to verify an *in vitro* model to a satisfactory extent is lacking. Consequently, CNS permeability assessment in drug discovery would benefit from the use of an iPSC-derived BBB model in efflux assays. However, for the added benefit of modelling additional transport processes a more extensive analysis of what transport routes are accurately modelled in the iPSC-derived BBB model would need to be performed beforehand. Such analysis should include gene and protein expression of transporters together with functional analysis of transport compared

to human *in vivo* data. The major challenges would be finding validated substrates for all transporters and the large amount of work it would take to generate the corresponding human *in vivo* data. However, recent advances in integrated transcriptomic and proteomic analysis and non-invasive brain PET imaging provide possible strategies to overcome these challenges. Standardizing the characterization and validation of models would enhance their application and adoption within drug discovery. Such standardization may include establishing analytical performance standards for models and a defined set of reference compounds that can demonstrate desired outcome and be compared across different models and labs.

It is likely that the first large-scale use of these models will be permeability assessment rather than disease modelling. Recreating a disease phenotype, *in vitro*, in a complex multicellular system such as the BBB is still a great challenge. Major issues include recreating the specific structure of the BBB that is needed for its function, optimizing culture conditions to several cell types, variability in cell culture and differentiation, and providing a biologically relevant model in a usable screening format. Creating a model for permeability assessment may still require complex *in vitro* cultures, but quality control standardization could more easily be adopted for one functional readout than a complex multifaceted disease phenotype.

4.2. Studying disease using iPSC derived blood-brain barrier models

There are many potential future applications of iPSC-derived BBB models, especially in modelling the complex cellular cross talk between different cell types at the BBB. There have been substantial investments in research on how neuronal cells and pericytes influence the BBB formation, function, and maintenance. However recent literature suggests that endothelial cells at the BBB may play a significant role in the communication between the peripheral organs and the CNS, both *via* the proteins secreted by the endothelial cells into the CNS and regulation of the controlled transport across the BBB. It has been shown, in an iPSC-derived system, that the vasculature has specific maturation effects on spinal motor neurons (Sances et al., 2018), and in the adult central nervous system the vasculature regulates neural stem cell behaviour by providing circulating and secreted factors. Age-related decline of neurogenesis and cognitive function is associated with reduced blood flow and decreased numbers of neural stem cells. Therefore, restoring the functionality of the CNS vasculature could counteract some of the negative effects of aging. It has been shown that factors found in young blood induce vascular remodelling, culminating in increased neurogenesis and improved olfactory discrimination in aging mice. Remarkably, one of the identified substances contributing to these effects does so without entering the CNS itself (Katsimpardi et al., 2014). Remodelling of the brain vasculature may function as a mediator in providing benefits such as increased neurogenesis and improved cognition and hence, brain endothelial cell secreted proteins may be of high importance. iPSC-derived BBB coculture models could be a useful tool to further explore how signalling from the brain endothelium affects neurons and other CNS components.

Another highly interesting feature of the BBB is how the nutrient supply to the brain across the BBB is affected in aging and neurodegenerative disorders. The brain accounts for 20% of all energy consumption at rest. Glut-1 is responsible for a majority of the glucose uptake from the blood to the brain and brain glucose uptake correlates with Glut-1 levels (Choeiri et al., 2005; Zeller et al., 1997). As the high Glut-1 level in the BBB is recapitulated in the iPSC-derived models it may be a good candidate model for studies of the Glut-1 mediated transport (Al-Ahmad, 2017). Ideally, iPSC-derived cells from a disease background and their isogenic controls could be used for such studies. Mutations in the Glut-1 gene *SLC1A2*, also known as Glut-1 deficiency syndrome, cause seizures, delayed development and microencephaly, due to low CSF glucose levels (Seidner et al., 1998). Reduction in Glut-1 levels and glucose transport have been observed in animal models of both aging and AD (Ding et al., 2013; Lee et al., 2018b) and in AD patients (Simpson et al., 1994). Furthermore, glucose uptake is reduced in individuals with genetic risk for AD (Ossenkoppele et al., 2013). Glucose metabolism is reduced in individuals with a family history of AD (Mosconi et al., 2013) and cognitively normal individuals who later develop AD (Mosconi et al., 2009). Consequently, reduced glucose transport has been suggested to precede AD onset and affect the progression, BBB stability and pathology in AD (Winkler et al., 2015). Increasing the understanding of glucose transport deficits in healthy and diseased individuals could be useful both in terms of earlier diagnosis and exploration of new therapeutic strategies.

In conclusion, the iPSC-derived BBB model systems are still in their early development, this is especially true for MPS. These systems have great capacity to advance into highly sophisticated models and there will indubitable be many new applications for these systems in the future. However, many challenges still remain, particularly with respect to reproducibility and recreation of multifaceted phenotypes *in vitro* with increasing complexity in the models. An important first step towards improved BBB models would be to establish analytical performance standards that can be compared with *in vivo* human data and across model systems.

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Declaration of competing interest

HZ has served at scientific advisory boards for Roche Diagnostics, Wave, Samumed and CogRx, has given lectures in symposia sponsored by Biogen and Alzecure, and is a co-founder of Brain Biomarker Solutions in Gothenburg AB, a GU Ventures-based platform company at the University of Gothenburg. RH is an employee of AstraZeneca.

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