In vitro models of the blood-brain barrier using iPSC-derived cells

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Cover illustration: Human induced pluripotent stem cell-derived brain endothelial cells stained with a green fluorescent antibody for the glucose transporter Glut-1.

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To Daniel

“In nature, nothing exists alone”

- Rachel Carson

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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, these cell lines and primary cells have failed to recreate physiologically relevant control 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 para-cellular permeability. Given the possibility to generate large amounts of iPSC-derived brain endothelial cells they are a feasible alternative when modelling the BBB in vitro.

This thesis aimed to develop iPSC-derived models of the BBB that display a barrier like phenotype and characterize these models in terms of specific properties. The BBB model development was based on investigations into mechanisms important for barrier formation in iPSC-derived endothelial cells and development of high-quality supporting cells. The possibilities to use the model in drug discovery, and in determination of brain penetrating capacity of drug substances were specifically considered. These studies have increased knowledge of molecular mechanisms behind the restricted permeability across iPSC-derived endothelial cells and identified transcriptional changes that occur in iPSC-derived endothelial cells upon coculture with relevant cell types of the neurovascular unit. Furthermore, high quality iPSC-

derived astrocytic cells were developed, and the biological relevance and model diversity between astrocytic models were evaluated. Both astrocytes and brain endothelial cells have been adapted to xeno-free culture conditions and used in the BBB models, demonstrating a xeno-free BBB model. Finally, a more biologically relevant microphysiological dynamic BBB model was generated. This model demonstrated improved permeability modelling and compatibility with high-throughput substance permeability screening.

Taken together these results show that iPSC-derived BBB models are useful for studying BBB-specific properties in vitro and that both marker expression and functional evaluation of iPSC-derived cells are important in assessing cell identity and cell quality. In addition, these results show that iPSC derived BBB models are feasible for high-throughput permeability studies.

Keywords: Blood-brain barrier, iPSC, in vitro model, permeability

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SAMMANFATTNING PÅ SVENSKA

Blod-hjärnbarriärens (BHB) huvudsakliga uppgift är att skydda det känsliga centrala nervsystemet från potentiellt skadliga substanser som cirkulerar i blodet. Genom att begränsa permeabiliteten av blodkärlen i hjärnan bibehålls den specifika miljön i centrala nervsystemet som krävs för att hjärnan ska fungera optimalt. Eftersom BHB är en vital del av det centrala nervsystemet är det svårt att studera BHB direkt i människokroppen utan att göra skada. Därför behövs modeller.

Modeller av BHB är viktiga för att studera utvecklingen och upprätthållandet av BHB och även för att förutsäga i vilken utsträckning nya medicinska molekyler kommer att ta sig in i centrala nervsystemet. Ofta används cellbaserade BHB-modeller uppbyggda av hjärnendotelceller med eller utan pericyter och nervceller. Immortaliserade cellinjer av humana hjärnendotelceller och primära hjärnendotelceller från djur har använts. Tyvärr uppvisar dessa cellmodeller inte en tät barriär likt den i människa när de odlas i laboratoriemiljö (in vitro). Det finns även bevis för att BHB skiljer sig åt mellan människa och djur, vilket medför att modeller som baseras på djurceller kan vara missvisande. Därtill pågår stora ansträngningar för att reducera djurförsök inom forskningen. Sammanfattningsvis behövs det nya och bättre in vitro-modeller för att ingående kunna studera egenskaper hos den mänskliga BHB i laboratorier.

Mänskliga inducerade pluripotenta stamceller (iPSC) skapas genom att celler från en vuxen person återprogrammeras till ett tidigt utvecklingsstadium där dessa kan bilda alla olika celltyper i kroppen. Hjärnendotelceller som bildats från iPSC har visat sig återskapa en mycket tät barriär i laboratoriemiljö. En av de mest typiska egenskaperna för iPSC är att de har hög delningsfrekvens. Genom att använda iPSC kan stora mängder mänskliga hjärnendotelceller med barriäregenskaper lika de i BHB produceras och användas för att studera specifika egenskaper hos den mänskliga BHB.

Syftet med denna avhandling var att utveckla BHB-modeller från iPSC och undersöka BHB-specifika egenskaper hos dessa modeller. Modellerna har utvärderats med avseende på faktorer som påverkar barriäregenskaper hos hjärnendotelceller. Särskild hänsyn har tagits till modellernas förmåga att användas i läkemedelsutveckling för att studera hjärnexponeringen av nya medicinska molekyler. Specifik identitet för olika celltyper har utvärderats genom att undersöka uttryck av gener och protein som kännetecknar dessa celltyper. Funktionalitet hos cellerna har studerats genom att undersöka deras förmåga att utföra processer som normalt utförs av dessa celler i kroppen. Både passiv och aktiv permeabilitet över BHB-modellen studerades med hjälp av fluorescerande verktygssubstanser och kända läkemedelssubstanser.

När hjärnendotelceller från iPSC odlas tillsammans med pericyter och nervceller reducerades permeabilitet i BHB-modeller. Avhandlingens resultat har ökat förståelsen för vilka molekylära mekanismer som bidrar till denna reducerade permeabilitet. Högkvalitativa astrocyter har skapats från iPSC och jämförts med andra astrocytmodeller för att utvärdera deras relevans som human astrocytmodell samt för att förstå skillnader mellan olika, vanligt förekommande, astrocytmodeller. Produktion av både astrocyter och hjärnendotelceller från iPSC har anpassats till odlingsbetingelser utan användning av animaliska biprodukter. Astrocyter och hjärnendotel celler har sedan använts i BHB-modeller för att skapa en helt human modell. Slutligen har BHB-modellen förbättrats ytterligare genom utveckling av en mer biologiskt relevant modell som återskapar den tredimensionella miljön som råder i hjärnans blodkärl. I denna modell växer hjärnendotelceller i ett artificiellt kärl där de fysiska påfrestningarna av blodflöde simuleras med hjälp av genomströmning av endotelcellskärlet. Modelleringen av specifik transport som framför allt påverkar läkemedelssubstanser förbättrades i denna modell. Denna modell lämpar sig även för storskalig permeabilitetsanalys.

Sammantaget visar resultaten i denna avhandling att BHB-modeller som är uppbyggda av celler från iPSC är mycket användbara för att studera BHB-specifika egenskaper i laboratoriemiljö. Dessutom tydliggörs hur analyser av både proteinuttryck och funktionalitet är viktigt för att utvärdera kvaliteten hos specifika celltyper, samt för analysen av deras förmåga att utföra sina respektive uppgifter. Därtill visas att storskalig analys av BHB-permeabilitet är möjlig med BHB-modeller från iPSC.

LIST OF PAPERS

This thesis is based on the following studies, referred to in the text by their Roman numerals.

I. Delsing L, Dönnes P, Sánchez J, Clausen M, Voulgaris D, Falk A, Herland A, Brolén G, Zetterberg H, Hicks R, and Synnergren J. Barrier Properties and Transcriptome Expression in Human iPSC-Derived Models of the
Blood-Brain Barrier.
Stem Cells. 2018 Dec;36(12):1816-1827.

II. Lundin A, Delsing L, Clausen M, Ricchiuto P, Sanchez J, Sabirsh A, Ding M, Synnergren J, Zetterberg H, Brolén G, Hicks R, Herland A and Falk A. Human iPS-Derived Astroglia from a Stable Neural Precursor State Show Improved Functionality Compared with Conventional Astrocytic Models.
Stem Cell Reports. 2018 Mar;10(3):1030-1045.

III. Delsing L, Kallur T, Zetterberg H, Hicks R, and Synnergren J. Enhanced Xeno-Free Differentiation of hiPSC-Derived Astroglia Applied in a Blood-Brain Barrier Model
Fluids and Barriers of the CNS. 2019 Aug;16(1):27.

IV. Delsing L, Zetterberg H, Herland A, Hicks R and Synnergren J. A Human iPSC-Derived Microphysiological Blood-Brain Barrier Model for Permeability Screening
Manuscript

Contents

ABBREVIATIONS

2D	Two Dimensional
3D	Three Dimensional
Aβ	Amyloid Beta
ABC	ATP-Binding Cassette
AD	Alzheimer's Disease
AJ	Adherens Junction
ALDH1L1	Aldehyde Dehydrogenase 1 Family Member L1
Ang-1	Angiopoietin 1
ANOVA	Analysis of Variance
apoE	Apolipoprotein E
AQP4	Aquaporin 4
BBB	Blood-Brain Barrier
BCRP	Breast Cancer Resistance Protein
BLBP	Brain Lipid-Binding Protein
BM	Basement Membrane
BMP	Bone Morphogenetic Protein
Caco-2	Human Epithelial Colorectal Adenocarcinoma Cells
CCF	CCF-STTG1 Astrocytoma Cell Line
CD31	Cluster of Differentiation 31
CMT	Carrier Mediated Transport

CNS	Central Nervous System
CSF	Cerebrospinal Fluid
EAAT	Excitatory Amino Acid Transporter
ECM	Extra Cellular Matrix
EGF	Epidermal Growth Factor
ELISA	Enzyme-linked Immunosorbent Assay
ESC	Embryonic Stem Cell
FDA	Food and Drug Administration
FGF	Fibroblast Growth Factor
FPKM	Fragments Per Kilobase Million
GDNF	Glia Derived Neurotrophic Factor
GFAP	Glial Fibrillary Acid Protein
GLN	Glutamine
GLU	Glutamate
GLUL	Glutamine synthetase
GW	Gestation Week
HEK	Human Embryonic Kidney Cell Line
HIV	Human Immunodeficiency Virus
ICC	Immunocytochemistry
iCellAstro	Commercially Available iPSC-Derived Astrocytes
IGF1	Insulin Like Growth Factor

IgG	Immunoglobulin G
IL6	Interleukin 6
iPSC	Induced Pluripotent Stem Cell
kDa	Kilo Dalton
L2020	ECM From Murine Sarcoma Cells
LAT	Amino Acid Transporter
LN521	Recombinant Laminin 521
LRP-1	Lipoprotein Receptor-Related Protein 1
Lt-NES	Long-Term Neuroepithelial Stem Cells
LXR	Liver X Receptor
MDCK	Madin-Darby Canine Kidney Cell Line
MS	Multiple Sclerosis
MPS	Microphysiological System
MRP	Multidrug Resistance Protein
NES-astro	Astrocytes Derived from Lt-NES
NG2	Neuron Glia Antigen 2
NVU	Neurovascular Unit
P_{app}	Apparent Permeability
PCA	Principal Component Analysis
PDGFRβ	Platelet-derived Growth Factor Receptor Beta
P-gp	P-Glycoprotein

phaAstro	Primary Human Astrocytes
RA	Retinoic Acid
RNAseq	RNA Sequencing
RMT	Receptor-Mediated Transcytosis
SHH	Sonic Hedgehog
SLC	Solute Carrier
TEER	Trans Endothelial Electrical Resistance
TGF-β	Transforming Growth Factor Beta
TJ	Tight Junction
TNFα	Tumour Necrosis Factor Alpha
VEGF	Vascular Endothelial Growth Factor
ZO	Zonula Occludens

1 THE BIOLOGY OF 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 barrier consists of endothelial cells with properties specific to the central nervous system (CNS) (1). These 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, Figure 1) (1). Together these components make up the BBB and govern its development, maintenance and function. The paracellular tightness of the endothelial cells in the BBB acts as a physical barrier for cells, proteins and water-soluble agents. Transporter 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 and extra cellular matrix (ECM) parts of the NVU. Isolated primary brain endothelial cells rapidly lose their BBB properties when cultured in vitro (2), 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 components of the NVU create together.

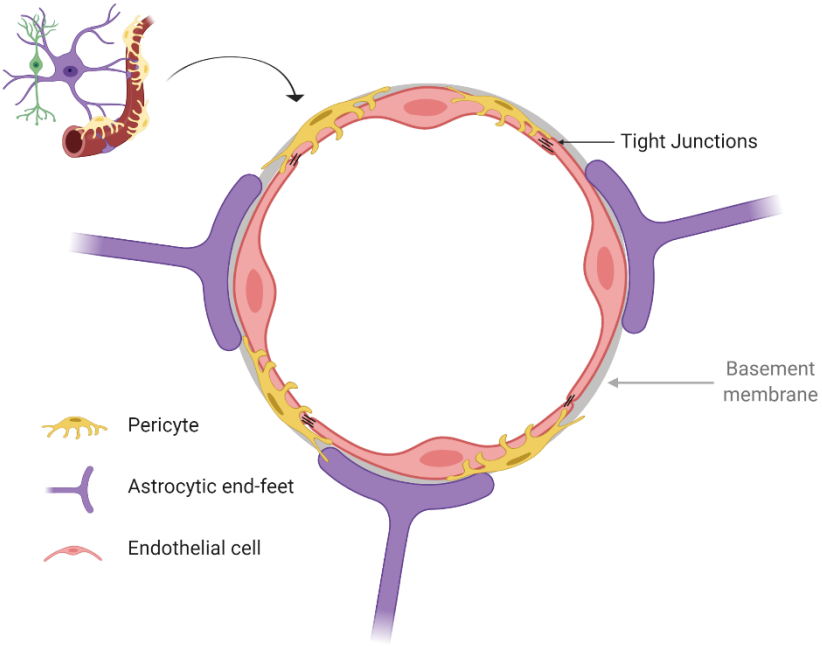

Figure 1. The neurovascular unit. Endothelial cells are linked together via tight junctions. On the brain side of the endothelial cell layer the basement membrane surrounds the endothelial cells and embeds the pericytes. Astrocytic end-feet are in contact with the endothelial cells.

1.1 Early brain development

The human brain is an immensely complex structure that consists of more than 100 billion neurons (3). To support these neurons, different types of glia cells are present, mainly astrocytes, oligodendrocytes and microglia. While the functions of oligodendrocytes and microglia are well characterized as myelinating and immune surveillance respectively, the functions of astrocytes are more diverse, and the list of tasks performed by astrocytes are growing continuously. For example, astrocytes maintain brain homeostasis, support accurate synaptic signalling, govern synaptic formation and promote BBB formation and maintenance (4).

Human brain development is a lengthy process that begins in the third gestation week (GW) when gastrulation occurs (3). In the gastrulation phase, the three germ layers;

ectoderm, mesoderm, and endoderm, are formed. The ectodermal cells give rise to the CNS, the mesodermal cells give rise to muscle cells, blood cells and the blood vessels that make up the BBB, and the endoderm give rise to many of the cell types that make up internal organs such as lung cells and gastrointestinal tracts. The neuroectoderm develops through three distinct phases, the development of the neural plate, formation of the neural groove, and finally, folding into the neural tube, which buds of from the ectodermal tissue (3). The neural tube is regionally specified, the rostral part will give rise to the brain and the caudal part will give rise to the hind-brain and spinal tube, in addition the hollow middle part of the neural tube will give rise to the ventricles and is thus referred to as the ventricular zone. Already at this stage, the vascularization of the neuroectoderm and the development of the BBB are initiated.

1.2 Blood-brain barrier development

The development of the BBB begins when vessels start to invade the developing neuroectoderm (1). Neural progenitors secrete the strongly angiogenic factor vascular endothelial growth factor (VEGF), which guides sprouting of vessels into the neural tissue (5). Neural progenitors also secrete WNT, which is necessary for endothelial cell migration and induces expression of BBB associated genes, such as the glucose transporter Glut-1 and tight junction proteins in the endothelial cells (6). Downstream signalling from WNT is essential for vascularization of the CNS but not peripheral tissues (6), suggesting a specific role of WNT signalling in development of the brain vasculature. In addition, WNT-mediated signalling deficits have been identified as a cause of BBB disruption in iPSC-derived endothelial cells from Huntington's disease patients, further emphasizing its importance in BBB development (7). 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 (8). 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 restricts permeability of blood-derived molecules and are immunopositive for claudin-5, suggesting that even the earliest brain blood vessels at GW five have BBB characteristics (9). Cues from astrocytes and pericytes are essential in BBB development, and lack of such signals are linked to severe abnormalities of the BBB (10, 11). Sonic hedgehog (SHH)-signalling is important for BBB formation and SHH knockout mice display embryonic lethality (10). The vascularization of their CNS is complete but expression of tight junction (TJ) proteins is reduced, suggesting that SHH

is important for BBB maturation and tightening. This effect is proposed to be astrocyte-mediated as SHH is produced and released by astrocytes. Furthermore, SHH-signalling is important in both embryonic BBB development and adult BBB immunocompetence (10). Most of the mature astrocytes develop after birth (4). Consequently, astrocyte-dependent changes to BBB function is likely to continue after birth.

1.3 Brain endothelial cells

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 (1). Brain endothelial cells make up the micro-vessels of the brain and have features that differentiate them from endothelial cells in other organs. Brain endothelial cells have longer continuous stretches of TJs, higher number of mitochondria, no fenestrae (small pores) and low pinocytic activity (12-14). All of these features contribute to the brain endothelial cell capacity to restrict permeability and act as a selective barrier. TJs are important structures in brain endothelial cells that separate the blood face from the brain face of the cells. TJ structure and function are further discussed in section 2.1. The different faces of the endothelial cells have distinct properties, making endothelial cells polarized. TJ restriction of water-soluble molecules in the paracellular space cause high trans-endothelial electrical resistance (TEER), a hallmark of brain endothelial cells. Physiological brain TEER is estimated to be above 1000Ohm x cm^2 compared with 2-20Ohm x cm^2 in the majority of the body (15). TJ proteins, such as claudin-5, occludin and specific transporters, such as P-glycoprotein (P-gp) and Glut-1 are often used as markers of brain endothelial cells. 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 bran endothelial cell lines do not maintain barrier restriction capacity in vitro (2). 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 (2, 16). However, the restrictive capacity in vivo and the expression of specific transporters are different between species (17, 18). Hence, to be able to predict and study the human BBB a human model is highly preferable.

1.4 Astrocytes

Astrocytes or astroglia are at least as abundant as neurons in the adult human brain (19), and the number of astrocytes has even been speculated to be one of the features explaining human cognitive abilities (4). Astrocytes are a very diverse cell type, both morphologically, molecularly and functionally (20). During development of the human brain, astrocytes in the cerebral cortex are derived from four distinct progenitor populations: radial glia, subventricular zone progenitors, locally proliferating glia and neuron glia antigen 2 (NG2) glia. To what extent each of these four progenitor populations contribute to the production of glia remains elusive. Although, it is clear that each of these subpopulations of progenitors contributes to the production of astrocytes at distinct stages of development and at different locations (19). The specifics of the astrocytic developmental process are not yet fully understood. One of the underlying reasons for this lack of understanding is the absence of well-characterized markers to study astrocytes and their progenitors during development. Specific functional and molecular profiles of astrocytes depend on signalling from surrounding cells, astrocytic diversity and heterogeneity are defined by both regional identity and functional input from surrounding neurons (21). Microarray analysis has revealed a small number of genes that are expressed by most astrocytes, while expression of other genes is specific to astrocytes in certain brain regions (22).

The understanding of astrocyte functions has evolved over time from being considered as a passive helper cell to the insight that astrocytes play a crucial role in development, maintenance and aging of the CNS. Astrocytes are key players in the CNS and one astrocyte domain can contain many million synapses (20). Astrocytes regulate the formation of synapses, control synaptic signalling (23), and support rapid and accurate synaptic signalling by regulating availability of nutrients and neurotransmitters (24, 25). Glutamate is the most abundant excitatory neurotransmitter. Glutamate uptake by astrocytes serve as a protective mechanism for excitotoxicity in adjacent neurons (25). By removing excess glutamate from the synaptic cleft, the astrocytes maintain homeostasis and signalling fidelity in the synapse. Glutamate is taken up, mainly, via the EAAT1 and EAAT2 transporters and is then converted to glutamine by glutamine synthetase (GLUL) (Figure 2). Glutamine can subsequently be exported from the astrocyte via the glutamine exporters SNAT3, SNAT5, ASCT2 and be reused by the neurons. Glutamate uptake does not only serve as a protective

mechanism for neurons but also activates glycolysis and glucose uptake in astrocytes.

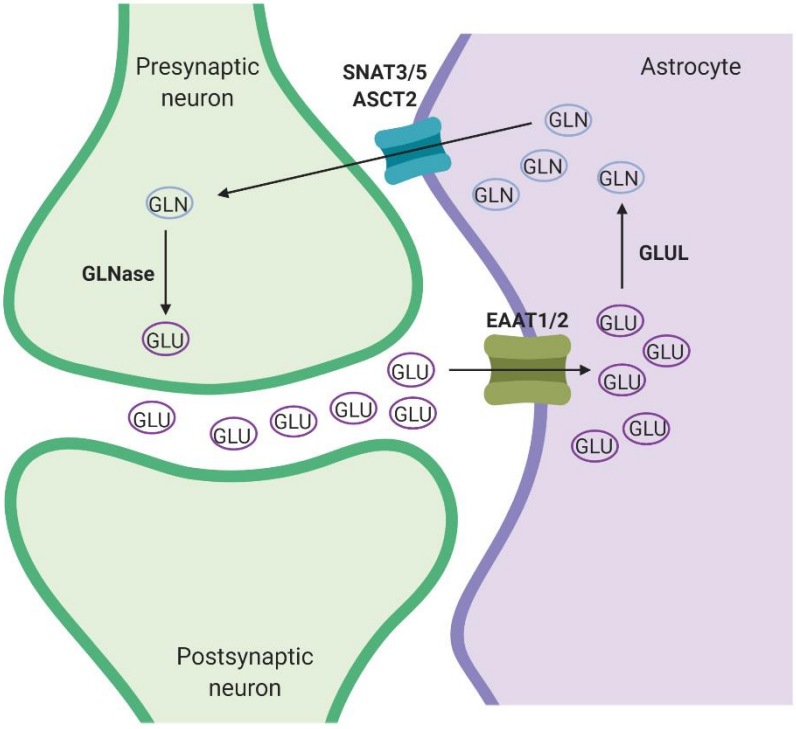

Figure 2. Glutamate metabolism. Excess glutamate (GLU) in the synaptic cleft is taken up into the astrocyte by EAAT1 and EAAT2. In the astrocyte, glutamate is converted to glutamine (GLN) by glutamine synthetase (GLUL). Glutamine can then be transported out of the astrocyte by glutamine exporters SNAT3, SNAT5 and ASCT2 and be reused in the neuron.

Through their end-feet, astrocytes are in close proximity to brain endothelial cells and affect the development and maintenance of the BBB through physical interaction and secretion of signalling molecules (26, 27). Astrocytes produce a range of molecules that are associated with the BBB phenotype and proper differentiation of brain endothelial cells, including components of the BM, glia-derived neurotrophic factor (GDNF), VEGF, apolipoprotein E (apoE), transforming growth factor beta (TGF-β), inter leukin 6 (IL6) and angiopoietin 1 (Ang-1) (1, 27). Furthermore, SHH produced by astrocytes cause upregulation of TJ components and reduced permeability in brain endothelial cells (10). Astrocytes also help regulate the blood flow within the brain through calcium signalling in their end-feet (28). Many pathological changes in the CNS are accompanied by astrocyte activation, commonly identified by increased cell

body volume and upregulation of intermediate filament proteins, such as glial fibrillary acid protein (GFAP) and vimentin (29). Activation of astrocytes and subsequent production of cytokines and pro-inflammatory substances in the brain can be protective, however, there is evidence that prolonged activation has detrimental effects in many CNS trauma and neurodegenerative conditions (29). The production of cytokines and inflammatory molecules from activated astrocytes are likely to influence the closely connected brain vasculature and thus the BBB. Reactive astrocytes have been suggested to increase secretion of both BBB promoting and disrupting factors (10, 30). However, the understanding of how reactive astrocytes influence the BBB in vivo is still poor.

1.5 Pericytes

There is general consensus that pericytes are a highly diverse cell type with different subtypes having different functions and characteristics. Interestingly, while most pericytes are believed to be of mesodermal origin, studies in birds have suggested that CNS pericytes derive from the neural crest (31). Due to the ambiguity of universal pericyte properties, finding a gold standard protein marker to identify these cells has been challenging. As such, the identification of pericytes relies on a combination of morphological criteria and assessment of marker expression. Expression of proteins, such as platelet derived growth factor receptor beta (PDGFRβ), NG2, caldesmon, CD13 and CD146, is commonly used to identify pericytes. Characterization of different subtypes of pericytes are underway. Vitronectin and fork head transcription factors FOXF2 and FOXC1, have been shown to be expressed specifically in brain pericytes (32-34). In addition to their brain pericyte specific expression, FOXF1 and FOXC1 have been shown to influence BBB development.

At the BBB the pericytes ensheath the endothelial cells. Pericytes are believed to play a specific role in the neurovasculature, as neural tissue has higher pericyte coverage of the vasculature compared with other organs. Neural tissue has up to one pericyte per one endothelial cell (35). Additionally, pericyte coverage correlate positively with endothelial barrier properties of different tissues. Pericytes have a diverse set of functions, they aid in angiogenesis and microvasculature stabilization, regulate capillary diameter and phagocyte toxic compounds (35). Pericytes produce many of the BM components and in that way contribute further to the structure of the BBB (36). Large parts of the current knowledge around pericyte function come from studies in pericyte-deficient rodents. These rodents show a number of BBB abnormalities, such

as increased permeability, increased transcytosis and irregular TJs (11). Studies show that developing endothelial cells attract pericytes by PDGFβ-signalling, leading to pericyte proliferation and co-migration with endothelial cells (11). Pericytes then contribute to brain endothelial cell specific maturation such as formation of TJs, reduced vesicle trafficking and reduced permeability (11). Human in vivo studies have shown increased cerebrospinal fluid (CSF) markers of pericyte damage and BBB breakdown in cognitive impairment patients compared to healthy individuals (37). In summary, pericytes play an essential role in both BBB formation and maintenance, but many of the mechanisms behind pericyte influence on the BBB are still unknown.

1.6 Neurons and microglia

Both neurons and microglia affect the BBB, however their contributions are less studied than those of pericytes and astrocytes. Microglia are the innate immune cells of the brain. Despite their name and stellate morphology, they do not share the common neural stem cells origin with neurons, astrocytes and oligodendrocytes. Mouse studies suggest that microglia progenitors originate from the yolk sack and migrate into the CNS during early embryogenesis, later, these cells proliferate to populate the CNS with microglia (38). Microglia invasion of the CNS precedes vascular sprouting into the tissue, but when vessels appear, endothelial cells and microglia are in close proximity to each other. Microglia have been suggested to play a role in both endothelial cell stability and angiogenesis during the development of the brain vasculature (39). Microglia become activated in response to injury and immunological stimulation. Active microglia can affect BBB stability and increase the permeability across the BBB. Studies of rodent in vitro models have suggested that these effects depend on reactive oxygen species (40) and tumour necrosis factor alpha (TNFα) (41) released by activated microglia. Microglia are important players in BBB formation and maintenance in vivo, however, as immunological challenges are kept to a minimum in in vitro BBB models, microglial contributions were not investigated in this thesis.

Neurons make up the main signalling networks of the brain. Neuronal signalling requires large amounts of energy and signalling from the neurons affects the blood flow through the brain vasculature to ensure enough energy supply. TJ stabilization was observed in cocultures of brain endothelial cells and neurons. Brain endothelial cells in the coculture were also induced to synthesize and sort occludin to the surface (42), indicating that the stabilization of TJs in coculture with neurons may be an effect of increased occludin production. Neurons and astrocytes are tightly coupled,

astrocytes support correct signalling environment for neurons and neuronal presence aids in astrocyte maturation (21, 23). Hence, the effects that neurons have on the BBB are likely to be both direct signalling from the neurons to the endothelium and secondary effects that arise via changes that neurons promote in the astrocytes. In this thesis, neuron cocultures were performed in the in vitro BBB models, however their specific contributions were not examined in detail.

1.7 The basement membrane

The BM is the non-cellular component of the BBB, it is a specific extracellular matrix that surrounds the endothelial cells. The BM contains highly conserved proteins, and consists mainly of laminin, collagen IV, perlecan, and nidogen (43, 44). The BM contributes to structural support and signalling by binding growth factors and neurotrophic factors such as fibroblast growth factor (FGF), VEGF and GDNF (27, 45, 46). Endothelial cells, pericytes and astrocytes secrete the proteins, which together, make up the BM (36, 44, 47, 48). Laminins are the most abundant component of the BM. In addition to their structural functions, laminins play an essential role in the organization of the BM and the regulation of cell behaviour (47-49), hence the BM modulation in this work has focused on laminins. Laminins are multidomain, heterotrimeric glycoproteins, composed of three different subunits; an α-chain, β-chain and γ-chain, combined and expressed in at least 16 different isoforms in the human body (50). The physical, topological, and biochemical expression of the different laminin isoforms in the BM is heterogeneous and laminin expression changes during development. Without the right combination of laminin isoforms, cells and tissues become dysfunctional. Brain endothelial cells generate laminins 411 and 511 (47) whereas astrocytes produce laminins 111, 211 (48). Furthermore, mouse studies suggest that laminin alpha 5 is more highly expression in brain than in the periphery and that it is important in protecting the brain vasculature from mononuclear infiltration (47). Laminin 521 has been shown to be specifically important for astrocyte migration and vascularization in the retina (51). All the above laminin isoforms are also expressed by the primary brain capillary pericytes (47, 49, 52). Effects of culture on de-cellularized ECM from pericytes, astrocytes and brain capillary endothelial cells on brain endothelial cell differentiation from iPSC was recently investigated (53). It was concluded that the de-cellularized ECM from astrocytes had the most beneficial effects on brain endothelial cell differentiation, however, this was not significantly different from using fibronectin. iPSC-derived brain endothelial cells did not adhere to

de-cellularized pericyte ECM suggesting that ECM produced by pericytes alone is not sufficient to support brain endothelial cell cultures. However, the de-cellularized ECM used in these experiments were derived from animal sources and it cannot be excluded that ECM from human sources would give a different result. The BM is a complex mixture of ECM from several cell sources and synergistic effects may be at play in the BM that are not accurately modelled using ECM from individual cell types. Consequently, the BM is highly important for normal BBB formation and function, but it remains unclear exactly how individual components contribute.

1.8 The blood-brain barrier and disease

This thesis focuses on modelling of the healthy BBB. However, to appreciate possible future applications for iPSC-derived BBB models it is helpful to understand how the BBB is linked to disease. The BBB is of major clinical relevance as dysfunction of the BBB is observed in many neurological diseases, and the efficacy of drugs designed to treat neurological disorders is often limited by their inability to cross the BBB. BBB disruption is observed in many pathological conditions such as, stroke, multiple sclerosis (MS), human immunodeficiency virus (HIV) encephalitis, and Alzheimer's disease (AD) (12), both as a cause of disease and as a symptom. BBB disruption refers to a decrease in the tightness of the barrier, resulting in less controlled transport across the barrier and higher permeability from the blood into the CNS. The connection between BBB permeability and AD is of increasing interest as the BBB is suggested to play a role in the accumulation of the AD hallmark amyloid β (Aβ) peptides. It has been hypothesized that a deficiency in the efflux of Aβ from the CNS contributes to accumulation and toxicity (54). This has also been shown in mouse models with mutations known to cause age-dependent Aβ accumulation and cognitive impairment (55). Efflux of Aβ is suggested to be mediated by the low-density lipoprotein receptor-related protein 1 (LRP-1). In a mouse model, where LRP-1 was knocked down by antisense, brain Aβ levels increased by 40% and cognitive function declined. In addition, efflux of Aβ1-42 was more significantly lost than efflux of Aβ1-40, favouring retention of the more toxic form with aging (55). There is an increased uptake of Aβ into pericytes in AD and Aβ overload could explain the loss of pericytes seen in AD. Mouse models suggest that pericyte loss influences disease progression in AD by diminishing clearance of Aβ (56).

Another example of a neurological disease with prominent BBB contributions is MS (57). MS is initiated by activation of myelin autoreactive T-cells that drive

inflammatory response against myelin antigens in the CNS. In response to the inflammation, several pathways leading to loss of BBB tightness are activated, for example metalloproteases degrading the ECM and basement membrane. As BBB integrity is compromised, T-cells infiltrate the CNS and there is activation of macrophages and microglia. This collectively leads to demyelination, plaque formation, and ultimately neurodegeneration. Clearly, the BBB plays a major role in several diseases and consequently therapeutic targeting of the BBB is likely to increase.

1.9 The blood-brain barrier in drug discovery

Diseases of the CNS are affecting an increasing number of individuals worldwide as the populations of most countries are aging. Furthermore, for many common diseases, such as neurodegenerative disorders, autism spectrum disorders and schizophrenia, there are no treatments affecting the disease and only few and inadequate options for treating the symptoms (58). The unmet medical need in neurological disorders is significant. A contributing factor is the many challenges in drug development of CNS active drugs. It has been estimated that almost all large molecule therapeutics and the majority of all small molecule drugs fail to penetrate the BBB (59). At the same time, major pharmaceutical companies are decreasing their investment in neuroscience research compared to other therapeutic areas (58). One plausible reason for the decrease in investment is the disproportionate failure rate in later stage clinical trials for CNS-targeting drugs compared to non-CNS-targeting drugs (60). Major challenges in CNS drug discovery include the multifactorial nature of many CNS diseases, difficulties in modelling the complexity of the CNS in vitro, and predicting BBB permeability. Consequently, there is a need for reliable models of the human BBB with high precision predictions of BBB permeability of novel drug molecules.

2 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 (Figure 3). 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.

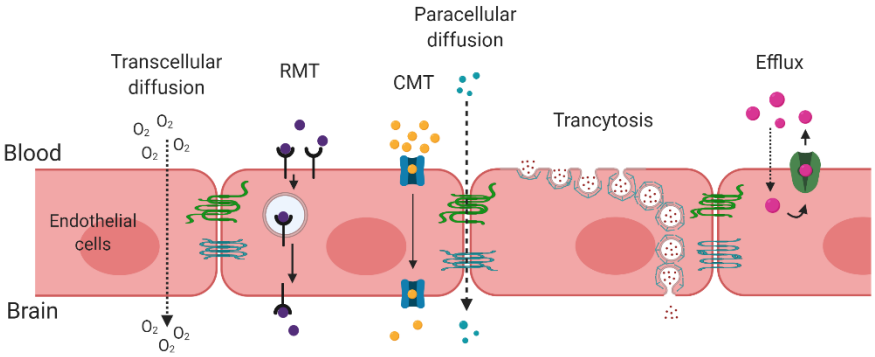

Figure 3. Transport across the blood-brain barrier. Small molecules such as oxygen and carbon dioxide can diffuse across the endothelial cell membrane in transcellular diffusion. Selective mediated transport occurs via receptor-mediated-transcytosis (RMT) or carrier-mediated-transport (CMT). In RMT the transported substance binds to a receptor which is subsequently internalized in a vesicle, transported across the cytosol and released on the other side of the cell by fusion of the vesicle with the membrane. CMT-specific transporters allow substances to pass through the cell down their concentration gradient. Diffusion in the paracellular space is very restricted at the BBB due to the high density of tight junctions, however, some molecules can still pass the barrier via paracellular diffusion. Transcytosis occurs via endocytosis, transport across the cell and exocytosis similar to RMT but without depending on receptors to bind the transported molecules. Efflux transport is polarized and occurs through pumping of substances from the cytosol back into the blood.

Larger molecules and nutrients such as glucose and amino acids rely on CMT or RMT. Moreover, the BBB is polarized, which means that the blood-facing side and the brain-facing side of the endothelial cells have different compositions of transporters. The TJs between the endothelial cells function as boundaries restricting diffusion of transporters between the blood and the brain sides of the endothelial cells, maintaining the polarization of transporters.

2.1 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 (Figure 4) (61, 62). 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, which reach from the cytoplasm to the extra-cellular space between the cells. 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 (62). The composition of TJs is more complex; transmembrane proteins; occludins, claudins and junctional adhesion molecules (JAMs) span the junctions between the cells. Occludins and claudins are linked to the cytoplasmic scaffolding proteins zonula occludens-1, 2 and 3 (ZO-1, ZO-2, ZO-3). The claudin family is large and diverse, with more than 20 known subtypes (63). Claudin-5 is commonly identified as the most abundant claudin in brain endothelial cells (62), and both claudin-5 and claudin-3 have been shown to localize at TJs in the brain endothelium (64). WNT-signalling has been suggested to play an important role in stabilizing TJs and enhancing barrier properties, at least partly, through the regulation of claudin-3 (65, 66). The TJs are responsible for restricting the permeability of soluble molecules and ions, which gives rise to the high electrical resistance often used to characterize highly impermeable cell layers. In addition to anchoring the claudins and occludins to the cytoskeleton, ZO-1, ZO-2 and ZO-3 function as regulatory elements by interacting with cytoplasmic proteins, signalling molecules and transcriptional regulators (67). 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 (2, 68, 69). The importance of occludin in the brain vasculature is further reinforced by reports of rare mutations in the occludin gene causing a disorder with severe malformations in cortical

development (70). TJ-associated proteins play an important part in creating the unique phenotype of brain endothelial cells and are frequently used to identify and visualize brain endothelial cells.

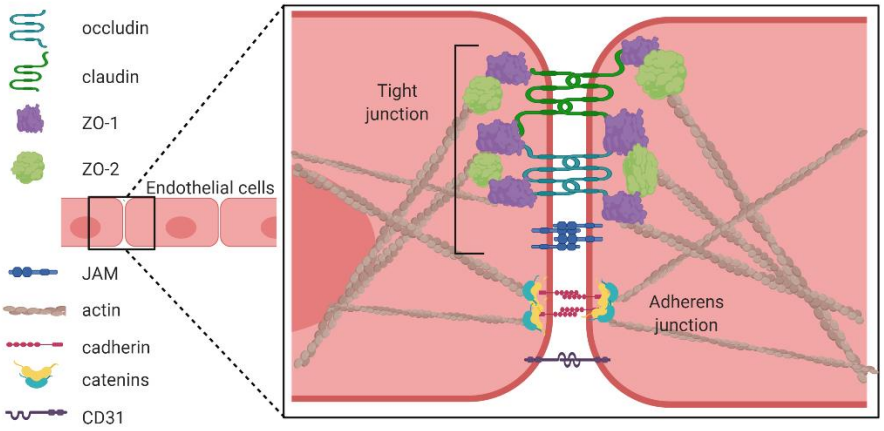

Figure 4. Inter cellular junctions. Tight junctions contain occludins, claudins and JAMs which span the paracellular space. These are linked to the cytoskeleton via zonula occludes. Adherens junctions contain cadherins which span the paracellular space and are linked in the cytoskeleton via catenins. In addition, CD31 contributes to intercellular connections.

2.2 Transport proteins

Brain endothelial cells control the ability of molecules and ions to diffuse from the blood into the brain. To supply the brain with required energy and nutrients, specific proteins at the BBB facilitate the transport (Figure 3). Selective mediated transport occurs through either CMT or RMT. CMT enables molecules such as carbohydrates, amino acids and vitamins to be transported down their concentration gradient through membrane carrier proteins. Examples of CMT include the solute carrier (SLC) transporters and the amino acid transporters (LATs). The energy supply to the brain is facilitated in this manner; the SLC transporter Glut-1 transports glucose down its concentration gradient from the blood into the CNS (71). In addition to Glut-1, the SLC transporter family contains numerous other transporters essential to the BBB, such as nucleoside and peptide transporters. RMT mediates transport of proteins and peptides through the binding of these to specific receptors. The receptors are subsequently internalized with the protein or peptide attached, shuttled across the

cytoplasm and released on the other side. RMT is responsible for the transport of nutrients and hormones such as iron, leptin and insulin across the BBB. Clathrin and caveolin mediate the formation of vesicles for RMT and non-receptor-mediated vesicular transport (72). 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 (32, 73). As caveolin, but not clathrin, has been identified as a differential factor in BBB permeability, caveolin expression level is commonly investigated as an indicator of vesicular trafficking.

2.3 Efflux transporters

The efflux transporter system works 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. Efflux transporters are ATP-binding cassette (ABC) transporters, they hydrolyse ATP to provide energy for transport of substances across the blood-side endothelial membrane. The three main efflux transporters are P-glycoprotein (P-gp), breast cancer resistance protein (BCRP) and multidrug resistance proteins (MRPs) (18). 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. Chemical properties of many drug molecules allow them to diffuse across cell membranes, however, they are also common substrates for efflux transporters, reducing their transport across the BBB (74).

Assessing BBB permeability of novel drug candidates is of high importance, both for drugs targeting the CNS and other organs. For drugs targeting the CNS, there is a need to assess if penetration of the BBB is sufficient for the drug to be active and reach its target in the CNS. For example, many anti-cancer agents have very limited effects on cancers of the CNS due to low penetration of the BBB (75). On the contrary, a drug with a target outside of the CNS should preferably not enter the CNS as that may cause additional side effects. For example, the beta-blocker propranolol has been shown to readily cross the BBB and generate side effects, such as hallucinations, nightmares and

sleep disturbance, at a higher incidence than other beta-blockers with lower BBB permeability (76). Furthermore, it is important to evaluate drug-drug interactions, which can cause one drug to affect the clearance of another drug. One common way through which this occurs is when a drug affects efflux transporter activity. Interactions between drugs need to be carefully evaluated in drug discovery, and guidelines for how to perform these evaluations have been suggested by the US Food and Drug Administration (FDA). One of the key recommendations for evaluating drug-drug interaction is to investigate if the drug has interactions with efflux transporter proteins P-gp and BCRP. The expression levels of BCRP and P-gp are substantially higher than expression of MRPs in brain endothelial cells (62). Hence, evaluation of efflux activity in the models developed in this thesis has focused on P-gp and BCRP. Clinically significant interactions with P-gp and BCRP have been reported for several drugs. For example, digoxin, which is used to treat various heart conditions, has been shown to interact with P-gp and is often co-administered with other P-gp interacting substances. Upon co-administration the availability of digoxin changes to correspond to a higher intake, which results in increased risk of over-dosing and generating side effects (77). Furthermore, at the BBB, common antidepressants such as selective serotonin re-uptake inhibitors, have been suggested to reduce the activity of P-gp, hence reducing its capacity to act as a protector of the CNS. Consequently, efflux transporter interaction studies are highly important in predicting BBB permeability and side effects of novel drug candidates.

2.4 Modulating blood-brain barrier transport for therapeutic purposes

Entry into the CNS is a major challenge for many novel drug substances and one of the major hurdles in drug development for neurological disorders. Studies of how pathogens enter the CNS have revealed that interaction with surface proteins on the brain endothelial cells facilitate the process. For example, *E. coli* interacts with a glycoprotein on the brain endothelial cell surface, which facilitates its penetration of the BBB (78). This observation provoked ideas of adopting similar strategies for drug delivery. Several approaches to increase the permeability of drugs to the CNS are under investigation. Current strategies include transient opening of the BBB and using drug carriers or ligands that facilitate penetration (79, 80). For example, cancer drugs have been linked to amino acid sequences, recognized by the RMT system, to increase their BBB permeability (79). Furthermore, exploiting the RMT system can be used to target

specific regions of the brain with high expression of certain receptors. Other strategies have focused on reducing the activity of efflux transporters, specific inhibitors, such as elacridar and tariquidar, have been developed. Clinical trials to increase CNS availability of efflux transporter substrates using these inhibitors are ongoing (81). Modulating BBB permeability may be beneficial in increasing permeability of some drugs to the CNS, however, changing the general permeability of the BBB may have very severe side effects. Selective modulation of BBB permeability is preferable but clearly more difficult to accomplish.

3 iPSC-DERIVED CELLS FOR BLOOD-BRAIN BARRIER MODELING

Induced pluripotent stem cells (iPSC) are somatic cells reprogrammed to a pluripotent state using overexpression of defined transcription factors (82) (Figure 5). iPSCs are similar to embryonic stem cells (ESC) and can be differentiated to most 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. 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, are difficult to study in vitro due to the challenges in obtaining these cells from healthy individuals.

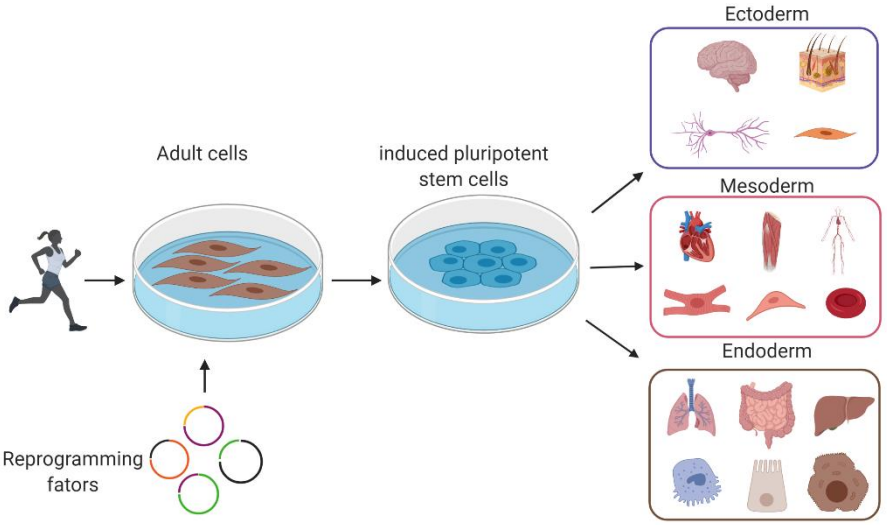

Figure 5. Induced pluripotent stem cells are reprogrammed adult human cells from patients or healthy individuals. Once reprogrammed to an early development stage induced pluripotent stem cells can self-renew and be differentiated into most cell types of the human body.

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. Furthermore, iPSCs can be generated from patients to provide patient-specific cell lines, which can be used to produce and regenerate damaged tissues. iPSCs have a high proliferation capacity making them suitable for

large scale production of cells as well as genetic manipulation. 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.

3.1 iPSC-derived endothelial cells

Among well-defined signalling pathways, bone morphogenetic protein (BMP), FGF, and VEGF-signalling are most commonly modified for endothelial differentiation (83). The BMP family modulates early vascular development via the downstream SMAD family proteins, as demonstrated by studies in human embryonic stem cells (84). Treating stem cells with BMP early in the differentiation process has been shown to significantly induced endothelial differentiation (85). 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 (86). 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 (87). During the years after 2012 several improvements of the protocol have been proposed, including the addition of retinoic acid (RA) (88), optimizing of seeding density (89) and use of more defined medium components (90-92). 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 (88, 91). 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 (88, 90, 93). Most recently, fully defined versions of this protocol that eliminate the use of serum have been proposed. These protocols give similar results as the original versions and rely on sequentially activating WNT- and RA-signalling, or spontaneous

differentiation followed by RA-signalling (91, 92). Other protocols for derivation of brain endothelial cells have been proposed, but without successful adoption in the iPSC BBB community. A proprietary method for deriving brain endothelial cells has been used in an investigation of apoE4 mediated endothelial cell toxicity, and more recently in a self-organizing microphysiological model (94, 95). Other protocols have used directed differentiation by sequential BMP and VEGF treatment to initiate differentiation to endothelial cells. Although these cells displayed upregulation of brain endothelial cell markers, they did not form tight monolayers and showed TEER values of approximately 50Ohm x cm^2 (53, 96). 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.

3.2 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. Differentiation of the pericytes used in this thesis was performed before brain-specific pericyte differentiation protocols were developed. As such, the pericyte differentiation approach used in this thesis depends on mesodermal induction and further differentiation of sorted cells that lack CD31 expression (97). Mesodermal differentiation was induced through activating TGF-β signalling by BMP4 and Activin, activation of WNT signalling and VEGF treatment, subsequent vascular specification through inhibition of TGF-β-signalling and continued VEGF treatment. After sorting of CD31-positive cells, the CD31-negative cells were further differentiated to pericytes by treatment with FBS, PDGFβ and TGF-β. These pericytes were characterized through expression of caldesmon, SM22 and smooth muscle actin. 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 (62). Brain pericytes have been shown to develop from neural crest stem cells (31), and recently a protocol for derivation of brain pericytes from iPSCs via neural crest stem cells was published (98). Initiation of neural crest differentiation was performed by WNT activation and TGF-β inhibition, neural crest stem cells were enriched through sorting, and pericytes

were generated through serum treatment. However, pericyte differentiation through both neural crest stem cells and mesodermal intermediates has given similar results (99). Mesodermal pericytes were derived via mesodermal induction and subsequent pericyte differentiation through culture in a proprietary medium promoting pericyte growth. Neural crest pericytes were derived through activation of WNT to derive neural crest cells and subsequent culture in the same pericyte growth medium. Development of well-defined differentiation protocols to derive brain pericytes from iPSCs is still in its initial stages. However, recent developments in generating brain-specific pericytes hold great promise for their use in iPSC-derived BBB models. iPSC-derived brain pericytes have been shown to reduce permeability of iPSC-derived brain endothelial cells at similar levels as induction by astrocytes and neurons (98). Since the role of pericytes may be primarily structural it is possible that Transwell BBB models benefit less from pericyte cocultures than microfluidic models where endothelial cells form vessel-like structures.

3.3 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 (100-102), glutamate uptake (101, 103), apoE biology (104) and genome wide expression studies (100, 101). Indeed, iPSC-derived astrocytes have proven to be a powerful tool for understanding human astrocyte biology in health and disease. Astrocyte development and maturation occurs late in the embryonic development and continues after birth (4). 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 (105, 106). 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 (103, 106). Long-term differentiation protocols often require repeated passaging, selecting the proliferating population, which is likely to contribute to the long differentiation time as maturation of astrocytes generally reduces proliferation. There have been numerous efforts to shorten the differentiation time required for astrocyte development, for example, through remodelling of the chromatin structure (107) and using genetic techniques to overexpress transcription factors SOX9

and NF1A that govern the gliogenic switch in which neural stem cells switch from neurogenesis to gliogenesis (108).

In this thesis, neural stem cells were derived using spontaneous differentiation and manual isolation of neural rosette forming cells (109). These were subsequently cultured in FGF and EGF and maintained as long-term neuroepithelial stem cells (lt-NES) expressing the neural stem cell markers SOX1, SOX2 and Nestin. The protocol used to derive astrocytes from lt-NES is a slightly modified version of the protocol first published by Shaltouki et al. (103). It relies on 4 weeks culture of neural stem cells in FGF, heregulin, insulin-like growth factor 1 (IGF1) and activin A to drive astrocyte differentiation. It is still unclear exactly how these factors drive astrocyte differentiation, however, heregulin has been shown to play a particularly important role. Heregulin is a splice variant of neuregulin. It interacts with the EGF family of receptors and is able to induce astrocyte differentiation (103). Even with recent efforts to shorten protocols for astrocyte differentiation the process is still labour-intensive and long. Furthermore, 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 (20). However, it remains unknown if astrocyte subtype influences the effects that astrocytes have on the brain vasculature and the BBB.

4 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 (110). 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 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 (111, 112). 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 (111). 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 (112, 113), but disadvantages linked to the use of animal cells include resource demanding isolation procedures, batch-to-batch variability and incompatibility with reducing the use of animals for research. 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 (17, 18). 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.

Availability of primary human brain cells is very limited, and samples are typically residual tissue from patient biopsies or post mortem brains. In addition, isolated

primary brain endothelial cells rapidly lose their BBB properties when cultured in vitro (2). Considering this loss of functionality in vitro, it is plausible that the BBB properties are not intrinsic to the brain endothelial cells but rather depend on the specific microenvironment that all components of the NVU create together. This suggests that a more complex model with coculture of multiple cell types is needed to recapitulate important functions of the BBB. Several coculture models have been described that demonstrate improved barrier properties compared to endothelial cells alone. There is a need for models that recapitulate the combined barrier functions of the BBB. A reproducible model from a continuous human cell source would increase the possibility of the model to be used in high-throughput screening experiments.

A recent review identified five groups of criteria for benchmarking in vitro BBB models; structure (ultrastructure, wall shear stress, geometry), microenvironment (basement membrane and extracellular matrix), barrier function (TEER, permeability, efflux transport), cell function (expression of BBB markers, turnover), and coculture with other cell types (astrocytes and pericytes) (114). Historically, in vitro BBB models have used static two-dimensional (2D) cultures in Transwell plates, but recently perfused three-dimensional (3D) models have gained increasing interest (Figure 6). 2D static models have been very useful in providing new understanding of mechanisms of transport across the BBB and are still widely used to assess CNS transport in drug discovery processes. In 3D models the structure of the brain vasculature can be more accurately modelled and flow can be added to introduce shear stress, which more closely recapitulates the in vivo conditions. Even though most of our knowledge about BBB in vitro models come from static systems, shear stress arising from flow is thought to regulate several BBB specific properties, for example permeability, metabolism and expression of transporters (115). In an in vitro vessel with cylindrical geometry, there are relatively few cells that form tight interactions with each other around the vessel. This limits the motility of the cells more than in the 2D sheet of cells present in the Transwell models, and more accurately reflects the in vivo conditions.

The basement membrane generates a specific microenvironment for the brain endothelial cells and its composition has been shown to effect BBB specificity of endothelial cells (36, 43, 44, 53). ECM proteins, such as collagen 1, are widely used in in vitro models but are not present at the healthy human BBB. Consequently, in vitro models need to provide a mix of ECM proteins carefully selected based on the composition of the in vivo BM. An alternative approach for creating a suitable ECM

would be to coculture brain endothelial cells with astrocytes and pericytes, which secrete many of the specific BM proteins that endothelial cells rely on in vitro.

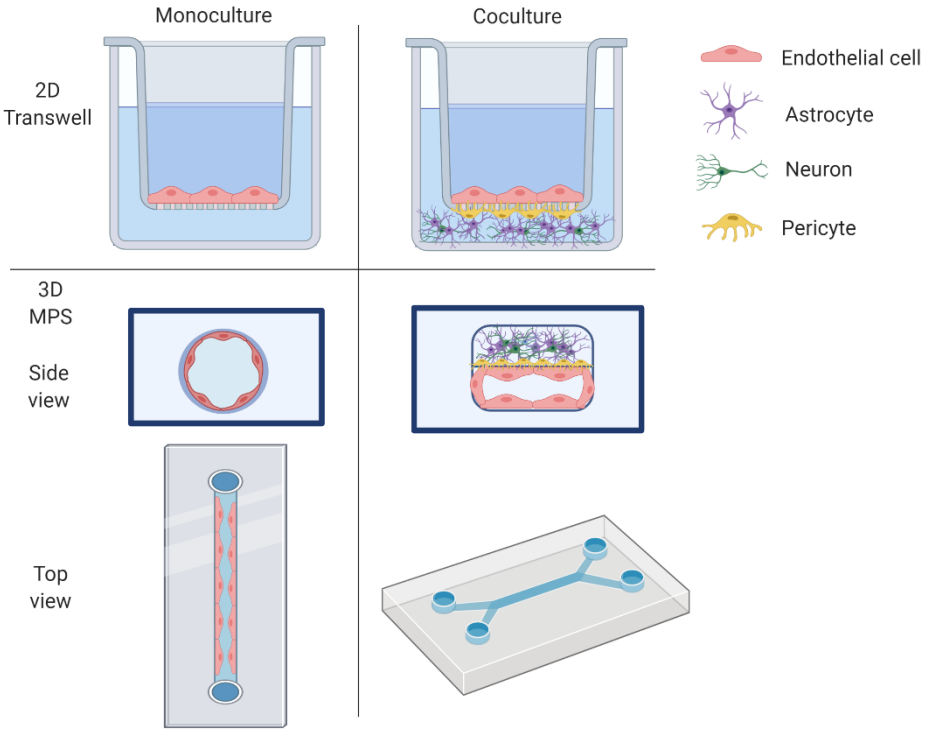

Figure 6. Schematic of common 2D and 3D monoculture and coculture BBB models. In 2D Transwell cultures, endothelial cells are seeded on a porous membrane hanging inside a culture well. In coculture Transwell models, other NVU cell types can be added to the bottom of the membrane and/or to the bottom of the well. In 3D microphysiological systems (MPS), endothelial cells can be seeded in cylindrical hollow channels. Systems can contain single or multiple channels separated by porous membranes. In multiple channel systems, one channel can be used to culture the endothelial cell tube, and an adjacent tube can be used to culture other NVU cell types.

4.1 Characterization of in vitro BBB models

Expression and localization of endothelial cell marker proteins are frequently used to characterize BBB models. Specifically, the expression of TJ proteins, such as occludin, claudin-5 and ZO-1, are used to verify that endothelial cells form TJs. TJs between brain endothelial cells create a physical barrier, which reduces permeability and

sustains polarization of transport proteins. Furthermore, the tightness of the barrier needs to be sufficient in order for any measurement of efflux activity to be meaningful. If the endothelial cells express efflux proteins but do not physically restrict the paracellular space, which is the case with many primary cell models, it is difficult to verify the activity of the efflux transporters. TEER measurements are the most common way of assessing how well the cells limit the transport in the paracellular space. High TEER represents a low paracellular transport and a tight monolayer. TEER measures the conductance of the cell membranes, a measurement that is relatively straightforward to perform in 2D Transwell cultures but can be very difficult to perform in microfluidic 3D models. TEER measurements are not feasible in vivo but in vivo TEER values have been estimated to be above 1000Ohm x cm^2. Even though systems with physiological TEER will have negligible paracellular transport, the permeability across a monolayer is not linearly correlated with the TEER. In addition, TEER is affected by other parameters such as the composition of solutes in the medium, temperature and handling of the equipment during the measurement. Hence, permeability measurements using small molecular tracers should be performed in addition to TEER measurements. Fluorescent tracers provide an opportunity to follow permeability in real time and assessment of permeability with a fast and easy plate reader analysis. Small fluorescent substances are commonly used for such analyses, for example, fluorescent dextrans of different sizes, sodium fluorescein and Lucifer yellow.

Evaluations of expression and functionality of efflux transporters are important to understand the translatability of BBB models when determining entry of novel therapeutics into the CNS. The two most studied transporters are P-gp and BCRP. In 2D Transwell systems where bi-directional permeability measurements are possible, efflux ratio is often used to determine if a substance is affected by efflux transporter activity. As efflux transporter location is polarized and mostly located in the apical (blood facing) membrane the transport of an efflux transporter substrate is lower in the apical to basolateral direction (blood to brain) than in the basolateral to the apical (brain to blood) direction. The efflux ratio is the ratio between the apical to basolateral and the basolateral to the apical transport. Consequently, the efflux ratio of a substance with equal permeability in both directions not affected by efflux is 1, while substances affected by efflux typically has efflux ratios of 2-10. Generally, a substance with efflux ratio greater than 2 is considered an efflux transporter substrate. Investigation of efflux transporter functionality can also rely on analysing the permeability of known efflux

transporter substrates with and without inhibition of the efflux transporters. For example, rhodamine 123 is a known substrate of P-gp and its permeability can be monitored with or without the addition of the P-gp inhibitor verapamil. In 3D dynamic systems bi-directional transport measurements are often difficult to perform, hence the investigation of efflux functionality usually relies on permeability studies of known substrates with and without inhibition of the efflux transporters.

Astrocyte cocultures have shown to improve barrier properties in many in vitro BBB models (93, 96, 116). These models use non-contact cocultures, suggesting that the astrocyte contribution occurs mainly via soluble factors. However, in vivo, astrocytes have a significant structural contribution to the barrier with astrocytic end-feet wrapping around the endothelial cells. Due to the difficulties in recreating the astrocyte-endothelial cell structural connections in vitro, the significance of the physical interaction of these two cell types remains elusive. Pericyte cocultures have similarly been reported to be beneficial to BBB models (98, 99, 117), and many of these have the pericytes in close proximity to the endothelial cells. Pericytes surround endothelial cells and help regulate capillary diameter and blood flow, which suggest that the contact between pericytes and endothelial cells is important. In addition, both pericytes and astrocytes contribute structural components of the BBB by secreting proteins that constitute the basement membrane.

In summary, for improved translatability to the in vivo situation, a BBB model should adopt a 3D tubular morphology and the endothelial cells should be subjected to shear forces. Endothelial cells should express TJ markers and efflux proteins. The permeability across the endothelial cell layer should be low. BBB model can benefit from coculture with astrocytes and pericytes, however the intended use for the model ought to dictate what level of complexity is needed.

4.2 iPSC-derived blood-brain barrier models

A model with iPSC-derived cell types could overcome the challenges with reproducibility and availability of human cells and would 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 (87) 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 (87, 116, 118-120), disease modelling (7, 99, 121-123) and modelling of BBB disruption (124-127). 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 (93, 98, 116, 128). iPSC-derived coculture models of the BBB formed monolayers with highly restricted permeability in the paracellular space. TEER values for coculture models have been reported to be higher than 6000Ohm x cm^2 (128), however the variability in maximum TEER reported between iPSC-derived models is quite high and others have reported TEER values of ~1000-4000Ohm x cm^2 (91, 93, 116). Permeability of passively diffused soluble substances such as fluorescein have been reported to be in the range of ~1-5 x 10^{-7}cm/s in iPSC-derived BBB models (90, 93). This was substantially lower than the permeability achieved in brain endothelial cell line cultures of ~12-15 x 10^{-6}cm/s (129) and can be compared to in vivo measurements in rat of 2.7 x 10^{-6}cm/s (130). Efflux by P-gp is commonly investigated and found to be active in iPSC-derived BBB models, however, the activity of P-gp was not affected by coculture (93, 116, 128). 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 (88, 90, 98, 128) and other reporting no differences (116, 117). 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 (117). 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 (131). Even though many iPSC-derived BBB models have been developed, characterized and used in different applications, several questions remain. To facilitate the use of iPSC-derived BBB models in disease modelling and drug discovery, the mechanism of BBB induction by coculturing cell types and the expression and functionality of more brain-specific transporters need to be thoroughly investigated. Furthermore, additional studies of drug permeability prediction are needed.

4.3 Microfluidic models

Recently several microfluidic BBB models containing iPSC-derived endothelial cells have been reported (95, 117, 132-136). These models aim to recreate 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 24µm. The shear stress was measured to be between 2.8 and 95dyne/cm^2, the calculated average shear stress was 15.4dyne/cm^2 (137). 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 – 8µm, the arterioles have a diameter ranging from 10 – 15µm and the venules have a diameter ranging from 16 – 20µm (138). Brain post capillary venules are characterized by diameters of around 100µm, a relatively thick basement membrane, and a wall shear stress of 1–4dyne/cm^2 (114, 138). 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. (88) to derive brain endothelial cells and cultured them as monocultures or cocultures. Several coculture models used primary sources for pericytes and astrocytes (95, 132, 134). However, recently two fully iPSC-derived models have been reported, one using iPSC-derived pericytes (117) and one using iPSC-derived neural cells for coculture (133). iPSC-derived BBB models in MPS

formed tight barriers and showed low permeability, below $5x10^{-7}$cm/s for 10kDa dextran in both monoculture of brain endothelial cells (135, 139) and coculture models (95, 132, 133). Similarly to static 2D models, MPS BBB models showed active P-gp efflux (132, 135, 139) and improvements in barrier phenotype after coculture (133, 134).

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 (136, 139). 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 (136). 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 (139). However, expression levels of several tight junction and endothelial cell markers have been found to depend on the flow rate in a fully iPSC-derived model (133). 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 (115). 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 aerobic metabolism can be utilized.

In addition, MPS models have been suggested to be more compatible with permeability screening as continuous sampling is possible in dynamic systems. However, many MPS models require complex laboratory setups that are incompatible with high-throughput screening. There is evidence that MPS may be useful for providing a physiologically more relevant culture environment for iPSC-derived BBB models. However, many questions remain regarding how adding flow and a 3D culture environment benefit iPSC-derived BBB models.

5 AIMS

The thesis aims were to develop human iPSC-derived models of the BBB that display barrier phenotype and characterize these models in terms of brain endothelium-specific properties. The model development was based on investigations of mechanisms important for barrier formation in iPSC-derived endothelium and development of high-quality cells to use in the model. The possibilities to use the model in drug discovery, and in determination of brain-penetrating capacity of drugs were specifically considered.

The specific aims were:

- To increase the knowledge of the molecular mechanisms behind the restricted permeability across iPSC-derived endothelial cells and to identify transcriptional changes that occur in iPSC-derived endothelial cells upon coculture with relevant cell types of the neurovascular unit. (Paper I)

- To develop and identify high-quality astrocytic cells, and to evaluate the biological relevance and model diversity between astrocytic models. (Paper II)

- To evaluate a xeno-free differentiation process of iPSCs to astrocytes, and subsequently evaluate the capacity of xeno-free-derived astrocytes to induce BBB properties in iPSC-derived endothelial cells. (Paper III)

- To generate a biologically more relevant iPSC-derived BBB model and to improve compatibility with high-throughput substance permeability screening by developing a microphysiological dynamic model. (Paper IV)

6 METHODOLOGICAL CONSIDERATIONS

The following section describes the reasoning behind method choices and discusses advantages and limitations of the methods used in this thesis. Detailed methods for specific experiments can be found in Paper I-IV.

6.1 Ethics

Human iPSC lines C9 and C1 used in these studies were derived with written informed consent by the donors or their parents. Generation of iPSCs were reviewed and supported by regional ethical consent boards. R-iPSC1j were generated from BJ fibroblast (CRL-2522) purchased from ATCC, Manassas, Virginia, USA, in compliance with the ATCC materials transfer agreement. AF22 were generated from primary fibroblast purchased from Cell Applications Inc., San Diego, CA, USA and were used in compliance with vendor agreements. ChiPSC22 iPSC line were purchased from Takara Bio, Kusatsu, Japan and used in compliance with vendor agreements. SFC-SB-AD2-01 iPSC line were obtained through the Innovative Medicines Initiative Joint Undertaking StemBancc and used in compliance with user agreements. Primary brain endothelial cells (Cell Systems, Kirklans WA, USA), astrocytoma cell line CCF-STTG1 (ATCC, Manassas, VA, USA), human embryonic kidney cell line HEK293 (ATCC, Manassas, VA, USA), human brain astrocytes HBA (Neuromics, Edina, MN, USA), immortalized brain endothelial cell line CMEC/D3 (Merck, Kenilworth, NJ, USA), and iPSC-derived iCell Astrocytes (FUJIFILM Cellular Dynamics, Inc, Madison WI, USA) used are commercially available and used in compliance with vendor agreements.

6.2 Cells

Even though iPSC lines generally are of high quality and exhibit the gold standard requirements for stem cells, there are inherent differences between cell lines. These differences have previously been investigated in isogenic BBB models and subtle variances in marker expression and maturation state were observed (131). Observed differences may depend on multiple factors, such as genetic makeup of the donor, the cell type and method that was used to derive the iPSCs, and under what culture conditions the iPSCs have been maintained. Due to the inherent differences between lines they may respond differently to differentiation, coculture or other treatments.

Thus, it is important to include several iPSC lines in the experiments to be able to draw general conclusions. In this work, the aim has been to include 2-3 lines per experiment. In some cases, one line has been used in parts of the analyses after sufficient verification that similar outcome for other lines is to be expected.

6.3 Differentiation of iPSC-derived endothelial cells

Differentiation of iPSC-derived endothelial cells was evaluated using one protocol generating brain-specific endothelial cells through a differentiation process that includes spontaneous neuroectodermal coculture (88, 90) and one general endothelial cell differentiation protocol (97), in which cells are generated via mesenchymal induction. Most of the work was performed using cells derived with the brain-specific endothelial cell differentiation protocol. This protocol was first published in 2012 (87), modifications and improvements of the protocol have since then been implemented (88, 90). Until very recently this protocol was the only published brain endothelial cell differentiation protocol generating endothelial cells capable of forming tight monolayers. It has proven to be robust and has been successfully implemented with many different iPSC lines and labs for numerous applications. iPSC-derived endothelial cell characterization is based on both expression of endothelial cell markers and ability to form a restrictive barrier, further discussed below.

6.4 Differentiation and functional characterization of iPSC-derived astrocytes

Differentiation of iPSC-derived astrocytes was performed using a modified version of a previously published protocol (103), which provides the possibility to generate astrocytes from neural progenitors within a month. Astrocytes are a very diverse cell type, and as such, finding a common marker to identify astrocytes has proven difficult. GFAP is commonly used as an astrocytic marker, but not all astrocytes in the human brain express GFAP (140), and GFAP expression is commonly recognized as a marker for reactive astrocytes (29). Preferably, expression of several astrocyte associated proteins, in combination with functional testing, should be used to characterize astrocytes. In these studies, glutamate uptake, inflammatory response and calcium signalling were used to evaluate important functionality of astrocytes. Glutamate uptake analysis was performed by measuring the decrease of glutamate in the culture over time using a colorimetric assay, with or without the addition of glutamate

transporter inhibitors. To avoid interference with the glutamate detection process, non-substrate inhibitors were used. All glutamate uptake measurements were normalized to number of cells in each well through either nucleus counting or double stranded DNA content. Astrocytes are active in the immune response of the CNS and inflammatory response assays were performed by measuring secretion of cytokines indicating reactive activation with or without stimulation with the proinflammatory factors TNFα and IL-1β, known to be produced by microglia, leukocytes and astrocytes. Calcium signalling analysis was performed using a neutral calcium indicator that can cross the cell membrane. Once inside the cell, the indicator is cleaved by esterases activating its calcium-binding properties and rendering it charged, which prevents transport out of the cells. When calcium is released, it is bound by the indicator and increases its fluorescence. Calcium signalling was measured in response to ATP and glutamate and was compared to an injection control as changes in shear stress has been shown to influence calcium signalling.

6.5 Characterization of protein and mRNA expression

Protein expression was analysed using immunocytochemistry (ICC) for intracellular proteins and enzyme-linked immunosorbent assay (ELISA) for secreted proteins. ICC provides the possibility to visualize the location and pattern of expression of the investigated protein that other methods, such as western blot, do not provide. When studying important structural components such as TJs it is helpful to be able to assess if TJ proteins locate to the cell junctions and if there are continuous TJs between cells, which is a requirement for permeability restriction across the cells. Expression analysis through qPCR is a useful tool to detect changes in expression of certain genes of interest. A change in mRNA may indicate that there are changes in protein expression, even if the relationship between mRNA and protein expression is not perfectly correlated. Equally important, a protein can be present in a cell without exhibiting its function. Hence, the characterization of cells should contain analysis on mRNA level, protein level and functional analysis. Complementary mRNA and ICC analyses are highly desirable, but not always feasible when quality antibodies to detect the protein of interest are not available. Developing specific antibodies against membrane proteins has been a long-standing challenge due to the difficulties in delivering enough protein in pure enough forms with intact tertiary structure to immunize. This can be a particular challenge when studying the BBB where many of the proteins and transporters of

interest are membrane-bound. qPCR analysis also comes with the need to carefully consider methods for normalization of samples. For experiments in this thesis three housekeeping genes were tested and the most stable one was selected using the NormFinder algorithm (141). Adding shear stress to a BBB model system has been shown to cause major shifts in fundamental processes such as metabolism and protein production (115), because of this five housekeeping genes were evaluated with NormFinder for experiments in Paper IV.

6.6 Barrier integrity assays

Barrier integrity assays are performed using TEER and permeability analyses of fluorescent tool compounds and drug substances. TEER measures the conductance of the cell membranes: high TEER represents a low paracellular transport and a tight monolayer. TEER is a fast, easy and effective method of assessing tightness of a cell monolayer. However, it is important not to rely solely on TEER for permeability measurements as this method is quite variable and exact values can be problematic to compare between labs. A universal permeability measurement such as apparent permeability (P_{app}) is preferable when comparing models and hence both of these methods can be used together to get comprehensive information on permeability across a BBB model. Efflux transporters are an important aspect of permeability restriction across the BBB. In this work, efflux transporter analyses have focused on the efflux transporters P-gp and BCRP as they are both highly expressed in the BBB and have been found to limit the brain permeability of many drug substances. Analysis of efflux protein activity and polarization was performed through directional permeability analyses and permeability studies with and without inhibiting the efflux transporters. Fluorescence-based assays where the permeability of a fluorescent substrate is monitored provides significantly simpler analyses compared with other methods of determining substrate concentrations such as mass spectrometry or radiolabelling of substances. Even though these assays are generally very useful they have certain limitations. For example, any assay using a P-gp substrate to detect the interaction with P-gp of another substance will be limited by the affinity of the substrate to P-gp. It will be difficult to detect effects of substances with a lower affinity to P-gp than the chosen substrate. In addition, non-substrate inhibitors will be difficult to separate from substrates with high affinity having prolonged attachment to P-gp, which slows down its activity rate.

6.7 RNA sequencing and pathway analysis

RNA sequencing (RNAseq) is a powerful method for identifying and characterizing cell cultures by investigating the expression of all genes under certain conditions. It allows for analysis of the similarity and differences between samples and of larger groups of genes related to a specific functionality or a specific signalling pathway. An important limitation of the technique is that it gives cross-sectional data and does not capture dynamic changes. Signalling pathways regulated by genes identified as differentially expressed by RNAseq can be detected with pathway enrichment analysis. Such pathway enrichment analyses depend on lists of genes annotated in databases to belong to certain pathways. Overrepresentation of the members of such gene lists among the differentially expressed genes is then investigated. Even though signalling is a dynamic process, evaluating expression of most of the components of a signalling pathway can provide insight into differences between samples. However, experimental verification is still required. RNAseq experiments in this thesis examines bulk RNA, the collected RNA from a pool of cells. Another RNAseq method is single cell RNAseq, where data is collected for each cell separately. This kind of analysis would be interesting to perform in future analysis of iPSC-derived BBB model as it can provide further insight about heterogeneity within the cell population. However, it requires a more complex and time-consuming analysis compared to bulk RNAseq.

6.8 Microphysiological culture systems

To create a more physiological culture setting for brain endothelial cells efforts are under way to adapt cultures to MPS. Most MPS use tubing and pumps to create a dynamic flow culture, which provides shear stress and flow that are unidirectional and adjustable. However, these systems require complicated laboratory setups that are very difficult to run in high-throughput. The Organoplate used in this thesis provides 40 units within one 384 well plate creating a system which is suitable for automated high-throughput screening, but has a bi-directional flow driven by gravity on a tilting platform. Thus, the Organoplate system provides a compromise in which a unidirectional flow system is sacrificed in favour of a system with bi-directional flow suitable for high-throughput screening. However, rat brain capillary endothelial cells in vivo experience flow fluctuations, extended stalls and even reversals of direction under physiological conditions (142). Even though the Organoplate clearly has the potential to be used in high-throughput screening, this has not yet been reported for any BBB models. The Organoplates' parallel membrane free channels are suitable for

barrier integrity assays relevant specifically to the BBB, vasculature and renal models, which represent the majority of the developed models in this system. The Organoplate endothelial tube compartment is 400 x 220µm, when plates are perfused at a 7° angle the cells are subject to a shear stress of 1.2dyne/cm^2, which is comparable to the shear stress in post capillary venule in the brain. Consequently, the Organoplate system is a feasible option for creating a perfused, 3D, high-throughput compatible iPSC-derived BBB model.

6.9 Statistical analysis

Most of the statistical tests in this thesis were performed using an ANOVA or double-sided Student's t-test. Student's t-tests are used to compare means of two groups e.g. coculture vs. monoculture, L2020 differentiation vs. LN521 differentiation or Transwell vs. MPS. In this work such comparisons were only made within iPSC lines. To perform multiple comparisons ANOVA was used and p-values were corrected for multiple comparisons using Tukey's or Dunnett's methods if all groups were compared to each other or if treatments were compared to a control, respectively.

Experiments in this thesis generally rely on three independent replicates and three technical replicates for each analysis. Technical replicates are included to control for variability in the testing protocol. Cell culture data brings controversy with regards to what is considered a biological replicate and what is simply a technical replicate. In iPSC-derived models in this thesis, replicates represent different batches of differentiation. However, one could argue that since these cells all come from the same parent iPSC line using three differentiation batches are only technical replicates of the differentiation protocol. Hence multiple iPSC lines have been used as each iPSC line can be viewed as a biological replicate. The statistical comparisons were typically made within the same line and subsequent conclusions were made based on what trends were observed for all lines. Similar issues can be raised for immortalized cell lines, whether a new batch of frozen cells is a biological replicate or if a new isolation is needed to obtain a new biological replicate. For cell lines, such as the CMEC/D3 used in this thesis, replicates come from individual cultures prepared separately but originating from the same stock. One possible solution to this problem would be to include several different brain endothelial cell lines. In this thesis, the brain endothelial cell line was used as a reference and was not the primary the focus of the work, hence only one line was included.

RNAseq data is not normally distributed; it has a negative binomial distribution. Transcript abundance is estimated by read counting, so the measured variable is discrete. In addition, RNAseq data is heteroscedastic, meaning that variance in expression depends on the mean. All of these features have to be considered in visualization and statistical testing of the data. RNAseq data was analysed using R and the DESeq2 package. To perform visualizations such as a principal component analysis (PCA), the data was normalized using the regularized log2 method to correct for heteroscedasticity and sequencing depth. Statistical analysis was performed with a generalized linear model and the Wald test was used for significance testing. Corrections for multiple comparisons were done using the Benjamini-Hochberg method. The threshold for false discovery rate was set to 5%. For visualization in bar plots, the data was normalized for sequencing depth and gene length using the fragments per kilobase million (FPKM) method for paired-end sequencing. Pathway analysis was performed with the DAVID tool using Fisher's exact test to investigate if any pathways were enriched among the differentially expressed genes. The Benjamini-Hochberg correction for multiple comparisons were used

7 SUMMARY OF FINDINGS

7.1 Paper I: Barrier Properties and Transcriptome Expression in Human iPSC-Derived Models of the Blood-Brain Barrier

There is a need for better in vitro models of the BBB as immortalized cell lines and primary brain endothelial cells have limited capacity to recreate barrier restriction when cultured in vitro. iPSC-derived brain endothelial cells have been shown to exhibit high barrier function in vitro. This work was designed to set up a fully iPSC-derived model of the BBB containing endothelial cells, pericytes, astrocytes and neurons that recapitulate barrier functions of the BBB in vitro. It investigated how different methods of deriving endothelial cells from iPSC affected their ability to serve as BBB models. Furthermore, the BBB specification of endothelial cells in coculture with other cell types of the NVU, was investigated. Both the functional changes in barrier restriction and the transcriptional changes induced by coculture were evaluated. Two published protocols for generation of endothelial cells were compared, one which generates an unspecified subtype of endothelial cells and another which generates brain-specific endothelial cells. The endothelial cells in monoculture and coculture with iPSC-derived pericytes, astrocytes and neurons were then compared. The results showed that the brain endothelial cell-specific protocol generated a BBB model with highly selective permeability. These cells had markedly improved barrier properties compared with the cells derived using the other protocol, which generated unspecified endothelial cells. The brain-specific endothelial cells had high TEER and low passive permeability of fluorescein. Coculture of brain specific endothelial cells with iPSC-derived astrocytes, pericytes and neurons improved barrier properties and the cocultured brain endothelial cells showed higher TEER and lower fluorescein permeability compared to the monocultured brain endothelial cells. Efflux transporters, such as P-gp and BCRP regulate the brain penetration of many drug molecules and the activity of these efflux transporters are very important for correct modelling of permeability, especially of drug-like substances, which often are small and able to diffuse across cell membranes. The brain-specific endothelial cells in monoculture and coculture showed active efflux by P-gp, while only the cocultured endothelial cells showed active efflux by BCRP. No activity of P-gp or BCRP, could be detected in the nonspecific endothelial cells in either monoculture or coculture. The

apparent permeabilities of six drug substances were tested and the brain specific coculture model could distinguish between CNS permeable and non-permeable substances. To understand molecular mechanisms behind the improvement seen in the coculture, the transcriptome of iPSC-derived endothelial cells in monoculture and coculture were compared. Coculture increased the expression of both junction-associated mRNAs and brain specific transporter mRNAs. A pathway analysis revealed enrichment of changed genes in the WNT, TNF and Pi3K-Akt pathways. These data suggested that differentiation towards brain-specific endothelial cells is needed to obtain endothelial cells with the capacity to form a tight barrier in vitro. Furthermore, non-specific endothelial cells derived from iPSCs did not develop brain-specific endothelial cell properties by coculture with NVU cell types. Our results suggested that the coculturing cell types exerted an influence on the brain endothelial cells through the WNT, TNF and Pi3K-Akt pathways, ultimately leading to a more highly restricted barrier in coculture. This work highlighted the plasticity of the iPSC-derived brain endothelial cells and the ability of coculture with other cell types of the NVU to enhance their barrier phenotype.

7.2 Paper II: Human iPS-Derived Astroglia from a Stable Neural Precursor State Show Improved Functionality Compared with Conventional Astrocytic Models

This work was aimed at developing iPSC-derived astrocytes and to evaluate their usefulness as an astrocyte model compared to conventional astrocytic models. Models were compared in terms of transcriptome, protein expression and functionality. Astrocytes were derived from lt-NES, a homogeneous and stable neural precursor, which provides shorter differentiation time compared to starting from iPSCs. In this work it was established that lt-NES can acquire gliogenic potency by expression of key gliogenic transcription factors SOX9 and NFIA. Astrocytes derived from NES (NES-astro) expressed many key glia marker proteins such as brain lipid-binding protein (BLBP), S100B, CD44, SLC1A3 and several astrocyte related mRNAs such as GFAP, aldehyde dehydrogenase 1 family member L1 (ALDH1L1) and aquaporin 4 (AQP4). NES-astro were compared to primary human astrocytes (phaAstro), the astroglioma cell line CCF-STTG1 (CCF) and the commercially available iPSC-derived astrocytes (iCellAstro). In addition, the neural stem cell lt-NES and human embryonic kidney cell line (HEK) served as neural and non-neural controls respectively.

Transcription and protein expression analysis revealed large differences between the models. As there is no reliable marker or transcription identity that fully specifies astrocyte biology, several important functional properties were compared between the different models. Removal of excess glutamate in the synaptic cleft is a critical astrocytic function that ensures reliable synaptic signalling and prevents excitotoxicity in neurons. The glutamate uptake mainly occurs through the sodium dependent glutamate uptake transporters EAAT1 (SLC1A3) and EAAT2 (SLC1A2). NES-astro showed active glutamate uptake through SLC1A3 that was not observed in any of the other models. Another important function of astrocytes in vivo is to produce an inflammatory response. This capacity was assessed by stimulation with inflammatory cytokines and subsequent evaluation of the response by measuring secreted IL6 and IL8. Dose dependent inflammatory responses were detected in NES-Astro, these responses were significantly different from the response in NES. Inflammatory responses were detected in the other models as well, however the baseline IL6 and IL8 levels were high in phaAstro, CCF and iCellAstro while no base line secretion was detected from NES-astro. This suggests a completely inactive inflammatory state of NES-astro at baseline. Calcium responses to ATP and glutamate were evaluated and both phaAstro and NES-astro showed response to ATP. Only NES-astro showed calcium response to glutamate. Next, the ability of the models to serve as screening platforms for apoE secretion were evaluated. Cholesterol and lipid metabolism in the brain is regulated by astrocytes, and the lipoprotein transporter protein apoE is predominantly produced by astrocytes. Given the strong genetic link between apoE isoform and Alzheimer's disease, apoE is a highly interesting target in drug development. As such, a high-throughput compatible pilot screen was set up to evaluate apoE secretion after treatment with known apoE inducers. Results showed that none of the substances tested induced apoE secretion in all models, highlighting that hit-finding depends on the cellular model used. For example, liver X receptor (LXR) agonists are well documented apoE enhancers that produced very high responses in CCF but did not produce uniform increased apoE secretion in NES-astro or phaAstro. Interestingly, substances acting on the cholesterol biosynthesis pathway were identified to increase apoE secretion in both phaAstro and NES-astro, while no effect was seen in CCF. These results suggested that caution needs to be taken when choosing an astrocyte in vitro cell model for screening, especially if primary and secondary screens are undertaken with different cell types. In summary, NES-astro showed high similarity to phaAstro, demonstrated several functional characteristics

and astrocytic markers and represented an astrocytic model with high biological relevance.

7.3 Paper III: Enhanced Xeno-Free Differentiation of hiPSC-Derived Astrocytes Applied in a Blood-Brain Barrier Model

To improve astrocyte differentiation and adapt astrocyte differentiation to xeno-free conditions, a comparative study was made between differentiating astrocytes on murine sarcoma derived laminin (L2020) and human recombinant laminin 521 (LN521). We showed in Paper I that iPSC-derived BBB models could benefit from coculture with iPSC-derived astrocytes, pericytes and neurons. In addition, our comparison in Paper II of astrocyte models shows that NES-astro is a more reliable astrocyte model than iCellAstro, which was used in the BBB model in Paper I. Consequently, the BBB model may benefit from coculture with NES-astro rather than iCellAstro. Furthermore, several published models have achieved improvements in BBB models using only astrocyte coculture. Our previous data suggest that NES-Astro can be differentiated on laminin 521 with similar transcriptional profile to L2020-differentiated astrocytes, but no functional comparison of xeno-free and conventional NES-astro differentiation was performed. Hence, this work was aimed at comparing functionality and barrier inducing capacity of astrocytes differentiated from lt-NES on L2020 and LN521. Laminins are the most abundant component of the BM, which lines the brain blood vessels. In addition to their structural functions, laminins play essential roles in the organization of the BM and in the regulation of cell behaviour. Several in vitro cell models have shown enhanced functional development when cultured on specific laminins. Astrocytes derived on L2020 and LN521 showed expression of astrocyte-related proteins and mRNAs such as BLBP, GFAP and S100B. In addition, astrocytes differentiated on LN521 had higher expression of GFAP, S100B, ALDH1L1, Ang-1 and GDNF mRNAs. Glutamate uptake analysis showed that both L2020- and LN521-differentiated astrocytes have functional uptake of glutamate through EAAT1 (SLC1A3). However, LN521-differentiated astrocytes had higher expression of EAAT1 on both protein and mRNA levels. The reduction in glutamate uptake upon inhibition of EAAT1 were greater in two of the biological replicates of astrocytes differentiated on LN521 compared to astrocytes differentiated on L2020, this could be an effect of the increased protein and mRNA levels of EAAT1. Further investigating expression of mRNA involved in the glutamate metabolism revealed that

astrocytes differentiated on LN521 had higher expression of the glutamine exporter SNAT3. Together with the increased levels of EAAT1 this suggested that the glutamate metabolism was affected by differentiation on LN521. Proteins secreted by astrocytes have many important functions in the CNS and a large part of the influence that astrocytes have on brain endothelial cells is via secreted proteins. Astrocytes differentiated on LN521 secreted more Ang-1 and S100b compared to astrocytes differentiated on L2020. This is in agreement with increased mRNA levels of Ang-1 and S100B in astrocytes differentiated on LN521. The capacity of the astrocyte to induce barrier properties in a xeno-free BBB model was subsequently investigated. iPSC-derived brain endothelial cells were derived using a xeno-free version of the protocol used in Paper I and cocultured with astrocytes differentiated on LN521 or L2020. Both astrocytes differentiated on LN521 and L2020 improved the barrier properties of brain endothelial cells in coculture. No differences were observed in TEER, but a slightly lower passive permeability was observed in coculture with L2020 differentiated astrocytes. Interestingly, brain endothelial cells cocultured with LN521 astrocytes showed higher expression of VE-cadherin, one of the improvement points previously identified for the BBB model in Paper I. Furthermore, the increased expression of ABCB1 mRNA and the decreased expression of caveolin 1 (Cav1) in brain endothelial cells appeared to depend on coculture with differentiated astrocytes and was not observed in coculture with NES. This suggested that coculture with more mature astrocytes is beneficial. In conclusion, these results show that astrocytes can be derived on LN521 and used in a xeno-free iPSC-derived BBB model in vitro with similar results as L2020 derived astrocytes. In addition, astrocytes differentiated on LN521 may display a more mature phenotype with a higher secretion of factors important for BBB formation in iPSC-derived endothelial cells such as Ang-1.

7.4 Paper IV: An iPSC-Derived Microphysiological Blood-Brain Barrier Model for Permeability Screening

In vitro BBB models have been hampered by lack of physiological structural arrangement of the cells and inability to recreate the physical forces that affect brain endothelial cells in vivo. To create a more physiological culture setting for brain endothelial cells this work was aimed at adapting the BBB model to an MPS. In these systems, the cells will be subject to both a 3D culture environment and dynamic flow introducing shear stress, both of which have been proven important for functional

development of the neurovasculature. Furthermore, to address the need to develop BBB models that are compatible with high-throughput screening, the MPS used in this study is well-suited for high-throughput applications. An MPS BBB model was created by culturing iPSC-derived brain endothelial cells in the Organoplate MPS, which has the same format as a regular 384 well culture plate and contains 40 microfluidic units per plate. The flow through the Organoplate was bidirectional and gravity-driven. Culture conditions of iPSC-derived brain endothelial cells in the Organoplate were optimized and BBB-specific marker expression and barrier integrity were analysed. The data was compared with data obtained from cultures in the commonly used Transwell system (used in Paper I). The iPSC-derived brain endothelial cells showed expression of the brain endothelial cell marker proteins ZO-1, Glut-1, occludin and claudin-5, and formed leak-tight tube structures in the MPS, which had negligible permeability to 4.4kDa dextran and very low permeability to the P-gp substrate rhodamine 123. Both the 3D MPS and the static 2D Transwell cultures had low permeability of 4.4kDa dextran and rhodamine 123. Comparison of mRNA expression of brain endothelial cell-associated markers revealed that mRNA levels of VE-cadherin, CD31 and BCRP were increase in the MPS culture compared with the Transwell culture. Intracellular accumulation of the BCRP substrate Hoechst was inhibited by the BCRP inhibitor ko143 in the MPS model but not in the Transwell model, suggesting that BCRP activity was dependent on the more physiological culture environment of the MPS. To facilitate high-throughput screening, cryopreservation and direct seeding of frozen iPSC-derived brain endothelial cells into the MPS were evaluated. Brain endothelial cells were cryopreserved and seeded directly into the Organoplate without effects on barrier integrity or mRNA marker expression. Finally, a pilot screen to identity substances that interact with the efflux transporters P-gp and BCRP was performed. Known P-gp and BCRP substrates and inhibitors were evaluated in a fluorescence-based assay where the changes in permeability of the known P-gp substrate, rhodamine 123, and the known BCRP substrate Hoechst were evaluated. The MPS BBB model was able to detect all the P-gp and BCRP inhibitors. In addition, it was able to detect two out of three BCRP substrates. Taken together the results from this study showed that it was possible to generate a 3D microphysiological iPSC-derived BBB model with barrier function similar to Transwell models. Even though the permeability was generally higher in the MPS model the permeability values were very low for both models. Several brain endothelial cell specific functions were enhanced in the MPS model compared with the Transwell model. mRNA expression of junction-associated proteins and BCRP were increased. Interestingly,

functional BCRP activity was observed in the MPS but not in the Transwell, and functional P-gp activity was observed in both MPS and Transwell. Furthermore, thanks to the 384wp format of the Organoplate and because it was possible to cryopreserve the iPSC-derived brain endothelial cells in an assay-ready state, this MPS model was found compatible with high-throughput screening. Consequently, this microphysiological model of the BBB provides a promising starting point for using iPSC-derived MPS for predicting brain permeability of novel therapeutics.

8 DISCUSSION

8.1 iPSC-derived blood-brain barrier models

This thesis follows the trajectory of the BBB modelling field in general. Static Transwell models are being replaced by more complex 3D models. There are multiple reasons why Transwell cultures are replaced by microfluidic systems. MPS provides a more physiologically relevant culture in several aspects, a three-dimensional spatial organization of the cells allows for the endothelial cells to form tubes and 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 (115, 133, 136) 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. Finally, the throughput of MPS is typically very low.

The iPSC technology has allowed human cells to be used to a larger extent within BBB modelling, overcoming issues with availability and quality of primary cells. Primary brain endothelial cells and cell lines are not able to create restrictive barriers in vitro (2, 129), 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 (143), overexpression of microRNAs (144) and chemical stimuli (145) with only small 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. In Paper I, we performed gene expression profiling to elucidate underlying mechanisms of endothelial cell ability to restrict permeability. The analysis showed significant changes in the WNT, AKT-Pi3K and TNF pathways, and a differential expression of occludin and claudins. Specifically, claudin-8 and -19 mRNA expression, which were found to be upregulated

by coculture, are associated with reduced permeability (63). Interestingly, we show in Paper I that the brain endothelial cell line CMEC/D3 and the iPSC brain endothelial cells derived with the non-brain-specific protocol have higher mRNA expression of VE-cadherin, CD31 and claudin-5 compared to iPSC brain endothelial cells derived with the brain endothelial cell specific protocol. Yet, the non-specific endothelial cells and the CMEC/D3 had higher paracellular permeability, hence our results were not able to corroborate the previously suggested notion that claudin-5 expression levels are a determinant of paracellular permeability (2, 116, 146, 147). When investigating occludin, differences in protein expression correlating with paracellular permeability were detected, and occludin expression has previously been suggested as a determinant of TJ permeability in BBB endothelial cells (2, 68, 69). Furthermore, occludin was recently suggested to have a potential role in increasing TEER levels (128). Taken together, our results suggest that occludin plays an important role in the iPSC derived endothelial cells ability to restrict paracellular permeability.

Of note, in Paper II the baseline secretion of cytokines was found to be substantially higher in iCellAstro compared to NES-astro. Interestingly, it was recently reported that addition of inflammatory cytokines reduced ZO-1 expression and increased permeability in an iPSC-derived BBB model (133). Consequently, it is reasonable to speculate that the BBB models in Paper I and Paper III are affected accordingly, as iCellAstro was used in Paper I and NES-astro was used in Paper III, however this was not directly compared.

8.2 Comparison to other iPSC-derived blood-brain barrier models

During recent years, there has been a rapid increase in iPSC-derived BBB models. iPSC-derived BBB models have developed into tools for investigation of drug substance permeability studies similar to the one in Paper I (87, 116, 118, 119, 133), disease modelling (7, 99, 121-123) and modelling of BBB disruption (124-127). BBB models developed in this thesis are comparable to previously published models, both in marker expression and barrier restriction. As discussed in Paper I, TEER values obtained of ~ 1250Ohm x cm^2 in the coculture were lower than TEER values reported for similar models of up to ~2500Ohm x cm^2 (116) and ~3000Ohm x cm^2 (90, 117) but higher than some other reported TEER values of ~500Ohm x cm^2 (93). The inverse relationship between TEER value and permeability has been shown to disappear at

higher TEER levels in iPSC-derived endothelial cells. Threshold values where a higher TEER no longer corresponds to a lower permeability were found to be 500Ohm x cm^2 for the small molecule sodium and 900Ohm x cm^2 for a large molecule IgG (119). A similar study in an animal-derived BBB in vitro model concluded that a TEER value of above 500Ohm x cm^2 only had marginal effects on mannitol permeability (148). Small differences in TEER were observed between different iPSC lines and variations between lines in the same experiment are expected, likely due to different donors and methods of deriving the iPSCs. The TEER measurement is an important tool for assessing paracellular tightness and restricted permeability in the paracellular space is of the highest importance for accurate modelling of any transport across the BBB. However, above approximately 1000Ohm x cm^2 the actual TEER value is of little importance. In vivo TEER values are very difficult to measure due to the invasive nature of the techniques and requirement of putting electrodes on either side of the BBB without disrupting it. Despite this, TEER values for vertebrates have been obtained and values across rat and frog brain endothelial cells have been measured in the range of 1200–1900Ohm x cm^2 (15, 149). In summary, TEER values obtained in iPSC-derived BBB models are similar to the in vivo TEER and exceed the critical value for paracellular permeability restriction.

Only a handful of studies have investigated the permeability of drug substances across iPSC-derived BBB models, but many different substances have been tested in permeability studies. Some studies display a significant distinction between CNS permeable and non-CNS permeable substances similar to that in Paper I (116, 118, 119, 133). Substances that have been tested in more than one model include atenolol, propranolol, caffeine and cimetidine. Atenolol was reported to have a permeability of approximately 5, 8 and 11×10^{-6}cm/s (Paper I, (118, 119)), propranolol approximately 20 and 40 x 10^{-6}cm/s (Paper I, (119)), caffeine approximately 60 and 100 x 10^{-6}cm/s (116, 119) and cimetidine approximately 1 and 10 x 10^{-6}cm/s (118, 119). In summary, permeability values reported for drug substances are comparable between published iPSC-derived BBB models. However, there is a need to further investigate the permeability of drug substances in iPSC-derived BBB models and to standardize permeability assays to facilitate comparisons between models.

Recently, several iPSC-derived BBB models in MPS have been reported, both monoculture systems (135, 136, 139) and coculture systems with pericytes and/or neural cell types (95, 117, 133, 134). In these studies coculture with only pericytes did not affect permeability (117), but coculture with astrocytes, with or without pericytes

and neurons, resulted in reduced permeability (95, 133, 134) or increased impedance (132). Permeability measurements using fluorescently labelled dextrans show that these models have very low permeability, several models reported a permeability of 2-4 x 10^{-7}cm/s (95) or below detection limit for 10kDa dextrans (117, 135). Permeability for 4kDa dextran and 3kDa dextran was reported to be in the range of single digit 10^{-7}cm/s and 1-3 x10^{-7}cm/s. These permeability assessments correspond well to the ~5 x 10^{-7}cm/s permeability of 4,4kDa observed in Paper IV. Permeability for 4kDa dextrans across in vivo rat cerebral microvasculature has been reported to be the 9.2 x 10^{-7}cm/s (130). Additionally, experiments in Paper IV showed P-gp and BCRP activity in the MPS, similarly, other iPSC-derived BBB MPS models have reported P-gp activity (132, 133, 135) and BCRP activity (132). A rhodamine 123 permeability of approximately 0,2-5 x 10^{-7}cm/s was observed for the MPS in Paper IV, very similar rhodamine123 permeabilities of 1-2 x 10^{-7}cm/s have been observed in other iPSC-derived BBB MPS (135). Notably, the expression of several brain endothelial cell markers has previously been shown to depend on flow (133). Similarly, the expression of several endothelial cell markers was upregulated in the MPS model compared to the Transwell model in Paper IV, suggesting that this effect may indeed be flow dependant. Consequently, the MPS model developed in Paper IV showed very similar permeability properties to other iPSC-derived BBB MPS and lower permeability compared with in vivo rat data. However, the model may still benefit from an astrocyte coculture. An important difference between the MPS model reported in Paper IV and other iPSC-derived MPS BBB models is the compatibility with high-throughput screening.

8.3 Efflux assessment in iPSC-derived blood-brain barrier models

A major requirement for any BBB model to be used in permeability assessments for safety evaluation of novel drug molecules is the expression and function of the two main efflux transporters BCRP and P-gp. Evaluation of P-gp and BCRP interactions are needed for safety studies and are important assessments of drug-drug interactions. P-gp and BCRP expression and activity were measured in the BBB models in Papers I, III and IV. Paper I show that both P-gp and BCRP mRNA levels increased after coculture. No differences in P-gp activity could be detected between monoculture and coculture, however, BCRP activity was only detected in coculture. Paper III shows that P-gp mRNA increases when endothelial cells are cocultured with differentiated

astrocytes compared to coculture with the neuro epithelial stem cells (lt-NES). No change was observed in the mRNA expression of BCRP between cocultures with differentiated astrocytes and NES. Paper IV, examining iPSC-derived endothelial cell monocultures in MPS and Transwell, showed that BCRP mRNA expression increased in MPS compared to Transwell and that BCRP activity was detectable in MPS but not in Transwell. P-gp mRNA expression and activity was not found to be different between Transwell and MPS. In summary, P-gp is expressed and functional in iPSC-derived endothelial cells and does not require coculture or culture in MPS for its activity to be detectable. However, BCRP activity is not detectable in iPSC-derived endothelial cells in static monoculture. Coculture and MPS culture both increased BCRP mRNA expression and either coculture or MPS culture is required for detection of functional BCRP efflux. This is in agreement with studies concluding that iPSC-derived brain endothelial cells display active efflux by P-gp which is unaffected by coculture (93, 116, 128). Most published iPSC-derived BBB models do not examine BCRP activity, but similarly to our results one study reported active BCRP in coculture (87). Other studies reported both active P-gp and BCRP in monocultures of iPSC-derived brain endothelial cells. However, these results were obtained using a different BCRP substrate (131) and after using an adjusted differentiation protocol (91). Similarly to the results in Paper IV, other iPSC BBB models have shown both active P-gp (132, 135) and active BCRP (132) in monoculture MPS. However, no direct comparisons to BCRP activity in 2D culture were made in these studies. In conclusion, our data suggest that any assay of BCRP-mediated permeability requires a more complex model with either astrocyte coculture or culture in an MPS.

8.4 Blood-brain barrier phenotype of iPSC-derived endothelial cells

Recently, the endothelial identity of iPSC-derived endothelial cells has been questioned, and claims have been made that these cells are actually neuroepithelium (150). From the gene expression analysis in Paper I, it was concluded that iPSC-derived brain endothelial cells may display a mixed endothelial epithelial phenotype. However, as exemplified in Paper I and IV, the in vitro barrier restriction capacity of these cells is still highly superior to other brain endothelial cells from immortalized cell lines, primary cells and iPSC endothelial cells derived with other protocols. In addition, we showed in Paper I that the expression of the important efflux transporter BCRP was higher in iPSC-derived brain endothelial cells compared to iPSC

endothelial cells derived with a non-specific protocol. 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. As discussed in the introduction, 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 model 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.

8.5 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 described in this thesis 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 routs in addition to efflux transport. Additionally, using microfluidic iPSC-derived BBB models allows for higher throughput analysis than a Caco-2 or MDCK-MDR1 Transwell assay. Consequently, a microfluidic iPSC-derived BBB model have the possibility to provide important information earlier in the 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 provides possible strategies to overcome these challenges.

8.6 Limitations

There are differences between iPSC lines depending on, for example, their genetic background, what method was used for generation and culture conditions. In addition, commercially available iPSC lines originate mostly from Caucasian male individuals. This limits the ability to draw general conclusions. In this work, multiple iPSC lines have been used in experiments to allow for more general conclusions. However, the diversity of the genetic background in the lines used was still limited. There are further limitations to the use of iPSC-derived cell types as they generally display an immature or foetal-like phenotype. Hence, characterization of the function investigated needs to be thorough. In this work complementary analysis of RNA, protein and function increases confidence in cell type identity of iPSC-derived cell types. However, analyses on all levels were not performed for all experiments, which constitutes a limitation of what conclusions can be drawn. Specifically, the pathway analysis and gene expression analysis in Paper I needs experimental verification to allow more definite conclusions. Investigating differences in pathways and processes by evaluation of annotated mRNAs can provide indication of changes because most of the components of the pathway or process are evaluated, but experimental verification is still needed.

Primary brain endothelial cells do not retain BBB phenotype in vitro, hence, they are not suitable for comparison. Additionally, BBB permeability has historically required invasive sampling and human data is very rare. The difficulties in comparing results with both human primary cell culture and human in vivo data are major limitations of this work. Many of the results from experiments in this work need further experimental verification and confirmation via in vivo studies.

A large part of CNS permeability and availability assessment of drug substances are measurements of unbound drug concentrations in the brain. The unbound drug concentration is essentially the active concentration available to exert its physiological effect. The permeability measurements performed in the in vitro models in this thesis do not consider the binding and metabolism that may occur in the CNS affecting the concentration of substance available in the CNS in vivo.

Models in this work were evaluated based on BBB-specific phenotype, however, no challenges to the BBB were performed. Cocultures have been shown to increase the tolerance of BBB models to challenges that could alter their permeabilities, but no such

affects were investigated in these studies. Consequently, coculture may have additional effects only detectable under stress conditions not detected in these studies.

9 CONCLUSIONS

In this thesis, iPSC-derived models of the BBB were developed and evaluated. Model improvements were generated by evaluation of different methods to derive endothelial cells and astrocytes from iPSC as well as evaluating different culture systems. A microfluidic model was developed, which is compatible with efflux transporter assays in high-throughput. The BBB models displayed a barrier phenotype in expression of proteins and mRNA associated with brain endothelial cells. Functional testing showed that the model exhibits selective permeability of both passively diffused substances and substances that interact with brain-specific transporters. These properties were comparable to other iPSC-derived models and in vivo permeability restriction. The outcomes provide new insight into molecular mechanisms that influence iPSC-derived BBB models' ability to restrict permeability and to model requirements for permeability assessments. The models developed in this thesis provide a promising starting point for the use of iPSC-derived BBB models in drug discovery and permeability assessments. In addition, the work highlights remaining questions and challenges for iPSC-derived BBB models that need to be addressed for the models to be useful in a wider range of applications. In particular, there is a need for standardizing permeability assessment assays across models and to increase the number of comparisons to in vivo human data.

10 FUTURE PERSPECTIVES

Building on the work in this thesis there are several opportunities to further characterize and improve iPSC-derived BBB models. In Paper IV, we showed that the microfluidic model has some improved barrier restriction properties and can be implemented in high-throughput assays. However, many questions remain about what changes can be observed in the microphysiological model compared to the static Transwell culture. A deeper investigation of how the culture environment and the shear stress in the microphysiological model affect the cells would be highly interesting. A genome wide expression profiling of mRNA and protein comparing the two models would allow for an analysis of what BBB properties are affected by MPS culture and could help direct further mechanistic studies. Additionally, establishing a coculture model in the MPS system may further improve the BBB model in a similar manner seen in many Transwell static models. Indeed, reduced permeability was observed in a recent iPSC-derived BBB MPS model after coculture with astrocytes, neurons and neural progenitors (133). By applying pericytes and astrocytes in the gel compartment of the MPS system, established in Paper IV, a contact coculture, similar to that in vivo could be formed. An isogenic BBB model could be created using the recently published protocol for derivation of brain-specific pericytes (99) and using the xeno-free derivation of astrocytes described in Paper III. The collagen gel used for gel casting in the MPS and the brain pericyte protocol contain some animal components and needs to be modified to xeno-free conditions before a fully defined, isogenic iPSC BBB model can be derived. Recently, a serum free protocol for differentiation to brain endothelial cells was published which could be adopted in future BBB models (91). A xeno-free BBB model would likely reduce variability and be an important optimization step for the coculture model to be used in high-throughput permeability assays.

The number of iPSC-derived models of the BBB is rapidly increasing, but the use of iPSC-derived BBB models in drug discovery is still limited. While using iPSC-derived cells allow for studying models with a diseases background and comparing to genetically modified isogenic controls it is still likely that the first large-scale use of these models will be permeability assessment. 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. 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. The lack of human in vivo permeability data for comparison is a major hurdle in model development. To establish a reference set of compounds for permeability testing, human in vivo data needs to be obtained so that comparisons can be made. In recent years, quantitative imaging permeability assessments in humans have increased and provide a non-invasive way of measuring brain penetration of substances. The increasing amount of human in vivo data generated with this method may facilitate human in vivo to in vitro comparisons in the future. After all, for these models to be successfully adopted by the pharmaceutical industry they need to have good predictive capacity, sufficient throughput and compatibility with automated handling, low variability and ease of use.

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 (151), 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 (152). 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, not examined in this work, 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 (71, 153). As shown in Papers I, III and IV the high Glut-1 level in the BBB are recapitulated in the iPSC-derived model and thus it may be a good candidate model for studies of the Glut-1 mediated transport. Indeed, active glucose uptake through Glut-1 has been shown in iPSC-derived brain endothelial cells derived with similar methods (154). 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 (155). Reduction in Glut-1 levels and glucose transport have been observed in animal models of both aging and AD (156, 157) and in AD patients (158). Furthermore, glucose uptake is reduced in individuals with genetic risk for AD (159). Glucose metabolism is reduced in individuals with a family history of AD (160) and cognitively normal individuals who later develop AD (161). Consequently, reduced glucose transport has been suggested to precede AD onset and affect the progression, BBB stability and pathology in AD (162). 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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