● PERSPECTIVE

Human induced pluripotent stem cell based *in vitro* models of the blood-brain barrier: the future standard?

There is an urgent and tremendous need for human disease models in drug development in order to improve preclinical predictability. In the case of brain disorders drugs have to cross the blood-brain barrier (BBB) to enter the central nervous system (CNS). It was estimated that more than 95% of the drugs cannot cross the BBB. In the case of biopharmaceutics, it seems to be even more difficult for them to overcome the BBB and reach their target sites. The major tasks of the BBB are to maintain CNS homeostasis and prevent entrance of pathogens and toxins, but also to be a part of the brain's waste disposal system. In a simplistic view, the BBB could be understood as a kind of bidirectional, active filter system. The tightening component of the BBB is the brain capillary endothelial cells (BCECs) which differ from peripheral endothelial cells by forming tight junctions sealing the intercellular gaps, possessing no fenestrae and exhibiting strongly reduced transcytosis (Avdeef et al., 2015).

Changes of the BBB functionality have been reported for a myriad of diseases, chronic as well as acute ones such as Alzheimer's disease, Parkinson's disease, multiple sclerosis, amyotrophic lateral sclerosis (ALS), epilepsy, pain, brain tumor, stroke, and traumatic brain injury. More and more data suggest that alterations of the BBB functionality are not only disease's symptoms, they are contributing to disease progressions and targeting the BBB can milden the adverse outcomes. This probably relates to the fact that the functionality of the BBB is strongly related to its microenvironment. It has been shown that cells from the CNS such as astrocytes, pericytes and neurons can modulate BBB functionality and *vice versa*. This collaboration of various cell types at the BBB is summarized in the term neurovascular unit (NVU) and it seems probable that disturbances in this communication involving a changed BBB might also effect the functionality of astrocytes (probably also of oligodendrocytes or microglia) and neuronal cells. Shear stress applied by the blood flow has to be considered as another factor regulating the BBB phenotype. All these facts underline that the need for proper, complex BBB models is enormous, because it is of essential interest not only to screen molecules for their BBB permeability, but also to understand the biology and the biology of the diseases involving the BBB. The idea could be to elucidate the specific changes during diseases and then either to consider or target these changes in the treatment strategies. Moreover, complex models might clarify not only the reasons why a drug cannot enter the CNS, they might also lead to the discovery of novel delivery routes which are upregulated during the diseases and could be utilized for drug delivery systems especially for biopharmaceutics. This knowledge then could be incorporated in simpler screening models being more feasible for drug development processes.

To study the BBB *in vitro*, a huge array of different models has been developed and characterized. They are based on immortalized, tumor as well as primary brain endothelial cells from different species and cultivated as mono – or co-cultures together with mainly astrocytes or pericytes. There are well established models based on porcine, bovine or rat primary brain endothelial cells reaching high values of the transendothelial electrical resistance (TEER) over 1,000 $\Omega \cdot cm^2$ as a measure for high paracellular tightness similar to *in vivo* ranges. Also several cell lines – although exhibiting mainly lower paracellular tightness - have proven their value as screening tools to study drug permeability, signaling pathways or to develop disease models. However, reflecting the fact that high paracellular tightness is especially important for cellular polarity and correct localization of transporter proteins, current human models need to be essentially improved. Most human BBB *in vitro* models are based on immortalized BCECs such as hCMEC/D3, hBMEC, TY10 and BB19 and lack significant paracellular tightness. Use of primary human BCECs is critical because of their limited access *via e.g.* biopsy or autopsy and ethical issues. Biopsy obtained cells from *e.g.* surgery of epilepsy or tumor patients retain the risk of impurities with diseased cells. Interestingly, there are some commercial sources for human BCECs. However, most of the models applying primary human BCECs from these sources show low paracellular tightness challenging their advantages in relation to their cost. Some attempts to improve the barrier properties of human immortalized cell lines were somehow successful such as including shear stress in microfluidic or hollow-fiber models. Furthermore, cultivation on hydrogels simulating the soft CNS tissue also enhanced localization of tight junction associated proteins. However, although these approaches confirmed the importance of mimicking the *in vivo* environment in order to come closer to a human *in vivo* like phenotype, finally the cell source still seems to be very critical.

Recent developments of *in vitro* models of the BBB based on stem cells are very promising. Beginning with the first protocol from the group of Eric Shusta (together with first author Ethan Lippmann and Abraham Al-Ahmed) published in *Nature Biotechnology* in 2012, human induced pluripotent stem cells (hiPSCs) have been differentiated into BCECs exhibiting BBB marker expression as well as major BBB properties such as high paracellular tightness (TEER > 1,000 $\Omega \cdot cm^2$) or a distinct transport barrier (Lippmann et al., 2012). It was shown that these cells expressed endothelial markers von Willebrand factor (vWF), CD31 and Tie-2 as well as BBB markers such as amino acid transporter SLC1A1, glucose transporter SLC2A1 or efflux transporters ABCB1, ABCG2 and ABCCs. In

addition, the functionality of ABC-transporters such as ABCB1 was confirmed. Especially, the expression of vWF seemed to be crucial as a marker for a mature endothelium (Lippmann et al., 2012; Appelt-Menzel et al., 2017). Until now, some protocol developments with regard to medium composition (*e.g.*, addition of retinoic acid, usage of medium E6), timing and co-cultivations have been published yielding to maximum TEER values of > 6,000 $\Omega \cdot cm^2$ and shortened differentiation durations to eight days (Hollmann et al., 2017). In the meantime, we and others were able to confirm the reproducibility of the protocols and reported the improved barrier properties yielding distinct tight junction formation shown by freeze fracture electron microscopy (Katt et al., 2016; Appelt-Menzel et al., 2017). A comprehensive overview about the development of hiPSCs-based *in vitro* BBB models was recently given in a review by Lauschke et al. (2017). Alternatively, BBB *in vitro* models based on stem cells derived from cord blood have been developed. While having the potential of forming also a distinct paracellular barrier, these models have not achieved such high TEER values as hiPSCs-based models did (~175 $\Omega \cdot cm^2$ *vs.* 2,000–5,000 $\Omega \cdot cm^2$), but might be stable for a longer period (2–3 weeks). However, in general hiPSCs-based models seem to possess currently significant advantages with regard to their broader applicability. hiPSCs protocols are available to reprogram hiPSCs from several sources such as skin fibroblasts or cells isolated from the amniotic fluid or urine enabling non-invasive personalized collection. Moreover, there is a vast number of protocols to cultivate and propagate hiPSCs with very little ethical concerns and numerous hiPSC lines are available. For example, recent projects in the EU led to the installation of stem cell banks (StemBANCC, EBiSC) collecting and characterizing a significant number of hiPSC lines from patients. These patient-derived hiPSCs can also be used to establish BBB disease *in vitro* models which might recapitulate the disease phenotype more closer. A remarkable example for this is the recently published work of Vatine et al. (2017) who formed a human BBB model based on hiPSCs from a patient with a mutation in the transporter MCT8. MCT8 is a thyroid hormone transporter, and mutations in MCT8 cause neuropsychomotor impairments. They showed that the hiPSCs-BBB model from patients with specific MCT8-mutations revealed a decreased transport of L-3,30,5-triiodothyronine (T3). Controls with isogenic hiPSCs-based BBB models with CRISPR/Cas9 induced MCT8 malfunction and its subsequent rescue confirmed their results. The authors hypothesized that lower CNS concentration of T3 due to restricted BBB permeation caused the observed neuronal dysfunction. Another recent example is the study from Lim et al. (2017) who generated a hiPSCs-BBB model from patients suffering from Huntington's disease (HD). They demonstrated that HD-hiPSCs BCECs exhibited intrinsic abnormalities in barrier and angiogenesis properties and linked

this to underlying pathways such as Wnt-signaling. In this context, we are currently involved in a project funded by the BMBF in Germany ("HiPSTAR"-project) to generate several Alzheimer's disease hiPSCs-BBB models based on different mutations and to investigate relevant functional differences.

Several data were obtained from hiPSCs-BCECs mono-cultures without considering cells from the microenvironment. Protocols are available to differentiate astrocytes, pericytes and neural stem cells from hiPSCs. Thus, isogenic models with several cell types of the NVU derived from the same hiPSC clone seem to be feasible. Canfield et al. (2017) showed a first perspective example of isogenic, multicellular human BBB models. This could become a very important aspect in the future when considering the already known, significant role of NVU cells for the BBB breakdown in animal *in vitro* disease models. In this regard, recently Yamamizu et al. (2017) demonstrated in a hiPSCs-BBB model the role of neural cells for BBB property induction *via* the Notch-signaling pathway. The general, pivotal role of astrocytes, pericytes and neural stem cells for the improvement of the paracellular tightness was already highlighted in studies from the group of Eric Shusta and Ethan Lippmann. Beyond that, co-cultivation of hiPSC-BCECs with astrocytes, pericytes and neural stem cells resulted in a significant change of the transcellular permeability of caffeine (Appelt-Menzel et al., 2017). In summary, these results indicate that – although the paracellular barrier is already very tight in hiPSC-BCEC mono-culture models – inclusion of further cells of the NVU might uncover the regulation of transport pathways and BBB properties which are possibly relevant for the translation of the data to the *in vivo* situation.

Future developments might comprise novel protocols for BCEC differentiation without the currently necessary co-differentiation of BCECs with neural stem cells and the subsequent cell separation by different matrix proteins after cell reseeding. This would improve the stability and robustness of the protocols. In this context, Katt et al. (2016) already increased the amount of BCECs after hiPSC-differentiation to > 90%. Another point to be developed is the longevity of the models. The application of shear stress could improve the life-time of BBB models from days to several months. Longevity might pave the way to study chronic processes such as mild inflammation or nutritional changes. In this context, we are currently establishing models culturing hiPSC-BCECs in hollow-fiber devices (This project is funded by SET, a foundation to promote research leading to the replacement, reduction or refinement of animal studies). In addition to the intended long-term models, hiPSC-based BBB models of ischemic insults such as stroke and traumatic brain injury are currently under development within this project. A first study of an *in vitro* BBB ischemia model based on hiPSCs-BCECs was published in the journal *Fluids and Barriers of the CNS*

in 2016. Major, remaining questions deal with the fact of species differences and how far the hiPSC-BBB models really reflect the human BBB or how artificial the found barrier properties are. For example, the expression of some barrier forming claudins were found in hiPSC-BBB models by us (claudin-4, Appelt-Menzel et al., 2017) and others (claudin-6, -8 and -9, Lim et al., 2017) which have not been found yet to be relevant in animal BBB models. A comprehensive analysis of the tight junction protein expression in human brain tissue as well as brain capillaries is still missing and highly needed in order to classify the data obtained with the hiPS-BCECs. The comparison of transporter proteins showed distinct differences of the expression of *e.g.* ABCB1 or ABCG2 in brain capillaries of rodents, marmosets and human. Also the total protein amount of claudin-5 was significantly altered in these samples indicating that the findings in hiPSC-BCECs could also be due to species differences. In this regard, data of the MCT8 deficiency model are an excellent example for the usage of hiPSCs-based BBB models to elucidate species differences (Vatine et al., 2017). Within their studies they found out that mice express a different set of transporters responsible for thyroid hormone delivery into the CNS in comparison to humans.

With respect to the future, pre-differentiated hiPSC-BCECs could be applied in the construction of 3D models such as spheroids or organoids in order to support the development of a brain vasculature within these models. Especially current brain organoids lack a proper vascular system possessing no access points for intravenous drug administration making it difficult to study BBB permeability. Other future 3D models based on hiPSC-BCECs might use cultivation in or on plastic scaffolds or hydrogels with defined 3D structures and incorporated luminal channels for BCECs culture. Other basic questions still have to be answered in relation to, for example, the role of epigenetic or gender influences or how complex will a future hiPSC-BBB assay have to be for drug screening applications.

In summary, hiPSCs-based BBB models are the first human BBB models with *in vivo* like paracellular barrier properties. These models possess an enormous potential for preclinical disease models especially to elucidate and reflect disease and species dependent differences.

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