Chapter 5
Quantitative and Targeted Proteomics of the Blood-Brain Barrier: Species and Cell Line Differences

Shingo Ito, Takeshi Masuda, and Sumio Ohtsuki

Abstract Proteomics is a powerful tool for comprehensive comparison of protein expression using quantitative proteomics as well as for determining the absolute expression levels of target proteins by quantitative targeted absolute proteomics (QTAP). Such proteomic techniques have been used in blood-brain barrier (BBB) research, and the output of these approaches has yielded substantial information. This chapter introduces two proteomic applications for understanding BBB models. One of them is QTAP, which is used for assessing species differences in a variety of different BBB proteins, including both transporters (e.g., ABC and SLC family members) and receptors. Analysis of protein expression levels of transporters such as MDR1/Mdr1a in isolated brain microvessels has demonstrated significant species-level differences. Quantitative plasma membrane proteomics is another technique used for comparing BBB model cell lines, which also introduces methodologies for plasma membrane preparations. The expression profile of membrane proteins in cultured cells provides helpful information and new insights for assessing such cells, particularly for in vitro BBB model systems.

Keywords Quantitative proteomics · Targeted proteomics · Protein expression · ABC transporter · SLC transporter · Receptor · Plasma membrane · Species differences · Cell line differences

5.1 Introduction

Proteomics is becoming an important and essential approach for the comprehensive identification of proteins in the blood-brain barrier (BBB) research as well as in other areas of life sciences research. Currently, proteomics can be used to obtain

S. Ito · T. Masuda · S. Ohtsuki (✉)
Department of Pharmaceutical Microbiology, Faculty of Life Sciences, Kumamoto University, Kumamoto, Japan
e-mail: sohtsuki@kumamoto-u.ac.jp

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quantitative information on protein expression levels via mass spectrometry and can be classified into two main types: quantitative and targeted proteomics (Veenstra 2007). Quantitative proteomics is an unbiased approach that is used to comprehensively compare the relative protein levels among multiple sample groups. Targeted proteomics is a biased approach that is used to measure only targeted proteins. While quantitative proteomics can provide significantly higher proteomic information than targeted proteomics, the sensitivity of targeted proteomics is better. Furthermore, targeted proteomics can be used to determine the absolute amounts of targeted proteins by introducing internal standard peptides along with the samples, and this method is known as quantitative targeted absolute proteomics (QTAP) (Ohtsuki et al. 2013). There are various applications of these techniques in BBB research. From the drug development point of view, quantitative proteomics has been utilized for predicting drug distribution in the human brain, which is rather challenging (Ohtsuki et al. 2011). In this chapter, we focus on two proteomic applications for understanding BBB models: QTAP for assessing differences in BBB proteins at the species level and quantitative plasma membrane proteomics for comparing protein expression in BBB model cell lines.

5.2 Profile of Transporter and Receptor Proteins in the Microvessels of the Human Brain

It is important to elucidate the molecular basis of transport function at the human BBB to understand the distribution of drugs and endogenous compounds in the human brain and to identify species-level differences. Uchida et al. investigated the expression of ABC and SLC transporter proteins from isolated human brain microvessels using QTAP (Uchida et al. 2011a). Brain microvessels were isolated from six frozen human cerebral cortices obtained from Caucasian patients and one frozen human cerebral cortex from a Japanese patient. The absolute protein expression levels of 34 ABC transporters, 66 SLC transporters, and eight receptors were measured in the microvessels of the isolated human brain using QTAP. Among the ABC transporters, ABCG2 (8.14 fmol/μg protein) was the most abundant ABC transporter, and MDR1/ABCB1 (6.06 fmol/μg protein) was the second most highly expressed ABC transporter. The mRNA expression profile of each brain cell type is available in the Brain RNAseq database (Zhang et al. 2016). The mRNA expression of ABCG2 (FPKM 48.2) in human brain endothelial cells was greater than that of MDR1 (FPKM 18.5), which supports that expression of ABCG2 in human brain microvascular endothelial cells was greater than that in MDR1.

Among the measured SLC transporters, GLUT1/SLC2A1 (glucose transporter 1, 139 fmol/μg protein) was the most abundant SLC transporter in isolated human brain microvessels. High levels of EAAT1/SLC1A3 (excitatory amino acid transporter 1, 24.5 fmol/μg protein) were detected. GLUT3/14/SLC2A3/14 (4.40 fmol/μg protein), 4F2hc/SLC3A2 (3.47 fmol/μg protein), BGT1/SLC6A12 (betaine-GABA

transporter, 3.16 fmol/μg protein), CAT1/SLC7A1 (cationic amino acid transporter 1, 1.13 fmol/μg protein), and MCT1/SLC16A1 (monocarboxylate transporter 1, 2.27 fmol/μg protein) were also detected at levels greater than 1 fmol/μg protein in the isolated human brain microvessels. From the mRNA expression profile obtained from the Brain RNAseq database, the mRNA of GLUT1 (FPKM 79.5), CAT1 (FPKM 15.8), and MCT1 (FPKM 17.1) was found to be predominantly expressed in the brain endothelial cells, suggesting that the amount of these proteins reflected their expression levels in microvascular endothelial cells. In contrast, EAAT1 mRNA was predominantly detected in mature astrocytes (FPKM 972), and its expression in endothelial cells was low (FPKM 17). Since it is possible that the astrocytes in the isolated brain microvessels are contaminated, the EAAT1 levels measured using QTAP are likely to be inclusive of their levels in astrocytes.

The amounts of LAT1/SLC7A5 (large neutral amino acids transporter 1, 0.431 fmol/μg protein), RFC/SLC19A1 (reduced folate carrier, 0.763 fmol/μg protein), and ENT1/SLC29A1 (equilibrative nucleoside transporter 1, 0.568 fmol/μg protein) were below 1 fmol/μg protein. The mRNA of LAT1 (FPKM 22.7) was predominantly expressed in human endothelial cells, but the mRNA of RFC (FPKM 1.81) and ENT1 (FPKM 1.5) was widely expressed in the brain cells. Therefore, it is suggested that although LAT1 mRNA is predominantly expressed in the microvascular endothelial cells of humans, its protein expression is low. Expression of drug transporter proteins such as peptide transporters (PEPTs), organic anion transporters (OATs), organic anion-transporting polypeptides (OATPs), organic cation transporters (OCTs), organic cation/carnitine transporters (OCTNs), and multidrug and toxic compound extrusions (MATEs) could not be detected.

Among the transcytosis receptors, transferrin receptor (TFRC, 2.34 fmol/μg protein) insulin receptor (INSR, 1.09 fmol/μg protein), and low-density lipoprotein receptor-related protein 1 (LRP1, 1.51 fmol/μg protein) were detected in the isolated human brain microvessels. The mRNA expression level of TFRC (FPKM 21.9) was found to be the highest in human brain endothelial cells compared to INSR (FPKM 2.72) and LRP1 (FPKM 1.13), suggesting that among these receptors, TFRC1 is predominantly expressed in the human BBB.

5.2.1 Species-Level Differences in Isolated Brain Microvessels: ABC Transporters

In a previous study, species-level differences in mRNA expression of ABC transporters were identified in the brain microvessels of various species, including mice, rats, pigs, cows, and humans (Warren et al. 2009). To date, information on the protein expression of drug transporters at the BBB by QTAP for species such as mice (Kamiie et al. 2008; Uchida et al. 2013), rats (Hoshi et al. 2013), dogs (Braun et al. 2017), marmosets (Hoshi et al. 2013), cynomolgus monkeys (Ito et al. 2011), and

humans (Uchida et al. 2011a) have been published. The protein expression levels for each species are summarized in Table 5.1.

Among the ABC transporters, there is cooperative action of MDR1/ABCB1 and ABCG2 at the brain barrier to prevent the entry of drugs into the brain by pumping them out from the endothelial cells into the circulating blood. The prediction of the drug distribution pattern in the human brain greatly depends on species-level differences in these ABC transporters at the BBB. Furthermore, information on their absolute protein expression levels is essential for choosing appropriate animal models and for interpreting the results obtained using these models in preclinical studies. QTAP revealed species-level differences in the expression of MDR1 and ABCG2 in the isolated brain microvessels. In mouse brain microvessels, the expression of Abcg2 was approximately 70% lower than that of Mdr1a/Abcb1a. In contrast, in humans, the expression of ABCG2 was 1.3-fold greater than that of ABCB1, which was 36% and 32% of Mdr1a in mice and rats, respectively. The expression level of ABCB1 in monkeys is similar to that in humans and is 31% of Mdr1a expression in mice.

It is possible that the lower protein expression of MDR1 in human and monkey brain microvessels leads to the prediction of higher brain distribution of MDR1 substrates in primates than in rodents. In fact, a previous PET analysis study has reported that the brain distribution of [^{11}C]GR205171 and [^{18}F]altanserin, which are MDR1 substrates, was 8.6- and 4.5-fold greater in humans than in rodents, respectively (Syvanen et al. 2009). The extent of penetration of [^{11}C]verapamil and [^{11}C]GR205171 into the brains of monkeys was also 4.1- and 2.8-fold greater than in rodents, respectively (Syvanen et al. 2009). As shown in Table 5.1, the brains of marmosets show similar expression levels of MDR1 and ABCG2 as humans. This suggests that marmosets are an appropriate model to predict drug distribution patterns in the human brain rather than in rodents. In dogs, ABCG2 expression was greater than MDR1 expression as in humans. However, it should be noted that ABCG2 expression in dogs is 5.6-fold greater than in humans.

The compensation of Mdr1a/b and Abcg2 expression was investigated in Mdr1a/b(–/–) double knockout, Abcg2(–/–) knockout, and Mdr1a/b(–/–) Abcg2(–/–) triple knockout mice (Agarwal et al. 2012). There was no significant difference in the expression of Abcg2 between wild-type and Mdr1a/b(–/–) double knockout mice. Similarly, there was no difference in the expression of Mdr1a between wild-type and Abcg2(–/–) knockout mice. Furthermore, a similar trend was observed in the expression of the other transporter and receptor proteins that were measured in the isolated brain microvessels in the Mdr1a/b(–/–) double knockout, Abcg2(–/–) knockout, and Mdr1a/b(–/–) Abcg2(–/–) triple knockout and wild-type mice. Thus, it was concluded that there are no compensatory changes in the protein expression of transporters and receptors in these knockout mice.

Table 5.1 Protein expression levels of transporters, receptors, and tight junction proteins in the isolated brain capillaries of different species

	Protein expression (fmol/μg protein)							
	Mouse (ddy)	Mouse (C57BL/6 J)	Rat (SD)	Rat (Wistar)	Dog	Marmoset	Monkey (Chinese adult cynomolgus)	Human
	Cerebrum	Cerebrum	Cerebrum	Cerebrum	Cerebrum	Cerebrum	Cerebrum	Cerebrum
Gene symbols/alias	Mean ± SEM	Mean ± SEM	Mean ± SEM	Mean ± SEM	Mean ± SEM	Mean ± SEM	Mean ± SEM	Mean ± SEM
ABC transporters								
ABCB1/MDR1 (Mdr1a: mouse and rat)	15.5 ± 0.8	17.8 ± 1.2	19.0 ± 2.0	19.2 ± 1.1	6.71 ± 1.82	6.48 ± 1.31	5.12 ± 0.91	6.06 ± 1.69
ABCC1/MRP1	Not measured	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
ABCC4/MRP4	1.59 ± 0.07	1.51 ± 0.27	1.60 ± 0.29	1.46 ± 0.08	<LOQ	0.320 ± 0.057	0.303 ± 0.008	0.195 ± 0.069
ABCG2/BCRP	4.02 ± 0.29	5.48 ± 0.37	4.15 ± 0.29	5.74 ± 0.50	45.2 ± 10.8	16.5 ± 1.4	14.2 ± 1.4	8.14 ± 2.26
SLC transporters								
SLC2A1/GLUT1	90.0 ± 2.9	101 ± 4	84.0 ± 4.1	98.2 ± 7.0	209 ± 64	145 ± 20	131 ± 22	139 ± 46
SLC3A2/4F2hc	16.4 ± 0.3	Not measured	<LOQ	<LOQ	24.7 ± 5.0	3.69 ± 0.30	Not measured	3.47 ± 0.83
SLC7A5/LAT1	2.19 ± 0.09	1.17 ± 0.36	3.41 ± 0.74	2.58 ± 0.84	<LOQ	not measured	<LOQ	0.431 ± 0.091
SLC15A2/PEPT2	Not measured	Not measured	Not measured	Not measured	1.73 ± 0.50	Not measured	Not measured	<LOQ
SLC16A1/MCT1	23.7 ± 0.9	13.7 ± 0.5	11.6 ± 0.6	13.5 ± 0.8	<LOQ	3.04 ± 0.35	0.755 ± 0.373	2.27 ± 0.85
SLC22A2/OCT2	Not measured	Not measured	Not measured	Not measured	±	Not measured	<LOQ	<LOQ
SLC22A8/OAT3	1.97 ± 0.07	2.29 ± 0.40	2.13 ± 0.49	1.37 ± 0.18	<LOQ	not measured	<LOQ	<LOQ
SLCO1A2/OATP1A2 (Slco1a4: mouse)	2.11 ± 0.12	Not measured	Not measured	Not measured	<LOQ	Not measured	0.725 ± 0.046	<LOQ
SLCO2B1/OATP2B1	Not measured	Not measured	Not measured	Not measured	Not measured	<LOQ	<LOQ	<LOQ

(continued)

Table 5.1 (continued)

	Protein expression (fmol/μg protein)							
	Mouse (ddy)	Mouse (C57BL/6 J)	Rat (SD)	Rat (Wistar)	Dog	Marmoset	Monkey (Chinese adult cynomolgus)	Human
	Cerebrum	Cerebrum	Cerebrum	Cerebrum	Cerebrum	Cerebrum	Cerebrum	Cerebrum
Gene symbols/alias	Mean ± SEM	Mean ± SEM	Mean ± SEM	Mean ± SEM	Mean ± SEM	Mean ± SEM	Mean ± SEM	Mean ± SEM
SLC29A1/ENT1	Not measured	Not measured	Not measured	Not measured	0.581 ± 0.342	Not measured	0.541 ± 0.072	0.568 ± 0.134
Receptors								
TFRC	Not measured	5.22 ± 0.47	6.74 ± 0.39	8.93 ± 1.16	18.0 ± 0.8	Not measured	Not measured	2.34 ± 0.76
INSR	Not measured	1.13 ± 0.18	0.785 ± 0.111	1.15 ± 0.34	<LOQ	0.656 ± 0.157	1.46 ± 0.22	1.09 ± 0.21
LRP1	Not measured	1.37 ± 0.33	1.09 ± 0.14	1.16 ± 0.21	1.49 ± 0.76	Not measured	1.29 ± 0.05	1.51 ± 0.26
Tight junction proteins								
Claudin-5	Not measured	8.07 ± 1.47	7.91 ± 0.9	7.00 ± 0.80	Not measured	8.03 ± 0.98	7.17 ± 0.77	Not measured
Membrane markers								
Na^+/K^+ ATPase	39.4 ± 1.01	39.0 ± 0.9	68.6 ± 4.5	36.2 ± 5.5	70.5 ± 10.8	31.5 ± 3.5	36.1 ± 8.7	35.1 ± 12.6
Reference	Kamiie et al. 2008	Uchida et al. 2013	Hoshi et al. 2013	Hoshi et al. 2013	Braun et al. 2017	Hoshi et al. 2013	Ito et al. 2011	Uchida et al. 2011

<LOQ: Below the lower limit of quantification

5.2.2 *Species-Level Differences in Isolated Brain Microvessels: SLC Transporters*

Various SLC transporters are expressed in the BBB, and one of their important roles is to supply nutrients, such as glucose and amino acids, to the brain. The glucose transporters GLUT1 (SLC2A1) and GLUT3/14 (SLC2A3/14) were detected in isolated brain capillary endothelial cells (Uchida et al. 2011a). The protein expression level of GLUT1 was 32-fold greater than that of GLUT3/14 (139 vs. 4.4 fmol/μg protein), suggesting that GLUT1 is mainly involved in glucose transport in the human BBB. The maximal velocity of glucose transport across the human BBB was reported to be 0.4–2.0 μmol/min/g of the brain and that across the mouse BBB was 1.42 μmol/min/g of the brain (Pardridge 1983; Gruetter et al. 1996). The similarity in the transport rates in humans and mice is consistent with the GLUT1 protein levels in the BBB (80.4–216 and 82.1–101 fmol/μg protein, respectively) (Uchida et al. 2011a, 2013).

Among the glucose transporters, GLUT3/14 showed a remarkable interspecies difference. Although GLUT3/14 has been quantified in isolated human and monkey brain capillaries, it has not been performed in mouse brain capillaries. Brain RNAseq showed that the mRNA expression of GLUT3 in human endothelial cells (FPKM 19.3) was the same as that in neurons (FPKM 11.6). Furthermore, mRNA expression of GLUT3 was detected in immortalized human brain microvascular endothelial cells (hCMEC/D3 cells) (Meireles et al. 2013). In contrast, mRNA expression of GLUT3 in mouse brain endothelial cells (FPKM 0.983) was much lower than that in mouse neurons (FPKM 32.9). Therefore, GLUT3 is expressed in the primate BBB and might be involved in glucose transport from the blood to the brain.

MCT1 (SLC16A1) is a proton-coupled monocarboxylate transporter that mediates the transport of lactate and ketone bodies across the BBB. Lactate and ketone bodies can also provide energy to the brain like glucose. Although GLUT1 showed similar protein expression levels among species, the protein expression of MCT1 in mouse and rat was more than 3.8-fold greater than that in marmosets, monkeys, and humans (Table 5.1). While the rate of glucose consumption in the mouse brain was estimated to be higher than that of the human brain (van Gelder 1989), the glucose transport rate and GLUT1 expression at the BBB were similar in both humans and mice as mentioned above. Thus, the higher expression of MCT1 in rodents is likely to be responsible for the supply of lactate and ketone bodies as energy sources to the brain to support higher energy demand in the brain.

LAT1 (SLC7A5) and 4F2hc (SLC3A2) can form a heterodimer and function as a transporter for large neutral amino acids, such as leucine, tryptophan, tyrosine, and phenylalanine. Protein expression levels of LAT1 and 4F2hc in the human brain microvessels were 20% and 21% of those in mice, respectively (Table 5.1). PET analysis has indicated that the rate of cerebral protein synthesis in the human brain (0.345–0.614 nmol/min/g) is lower than that in the rodent brain (3.38 nmol/min/g) (Hawkins et al. 1989). The concentration of serotonin, the precursor for which is tryptophan, is lower in the human brain than in the mouse brain (20 ng/g vs. 679 ng/g

brain) (Irifune et al. 1997; Young et al. 1994). Based on these results, it was suggested that the supply of large neutral amino acids across the BBB is slower in humans than in mice due to lower expression of LAT1 and 4f2hc in human brain microvessels.

OAT3 (SLC22A8) is an organic anion transporter involved in the brain-to-blood efflux transport of anionic drugs and neurotransmitter metabolites across the rodent BBB (Mori et al. 2003, 2004). Although OAT3 was detected in mouse and rat brain microvessels, it was found to be under detection limits in human, monkey, and dog brain microvessels (Table 5.1). This species-level difference was supported by the RNAseq data. While the mRNA expression of OAT3 was selectively higher in the microvascular endothelial cells (FPKM 188) in the mouse brain, its expression in human microvascular endothelial cells was lower (FPKM 0.225). The differences in the protein and mRNA expressions suggest that OAT3 plays a weaker role in the brain-to-blood efflux of anionic compounds across the BBB in humans than in mice. Similarly, ASCT2 (SLC1A5), TAUT (SLC6A6), and Oatp2 (Slco1a4) were detected in mouse brain microvessels but not in human brain microvessels.

5.2.3 Species-Level Differences in Isolated Brain Microvessels: Receptors

TFRC, INSR, and LRP1 are expressed at the BBB; therefore, their antibodies and ligand peptides can be considered as BBB-permeable carriers for drug delivery to the brain. The protein expression levels of TFRC in humans are 45%, 30%, and 13% of that in mouse, rat, and dog, respectively (Table 5.1). Although TFRC is a promising target for the delivery of macromolecules across the BBB, differences in its protein expression suggest the possibility that prediction of delivery efficiency using anti-TFRC antibodies to the human brain of animal models based on the delivery efficiency in animal models might be overestimated. The protein expression levels of INSR and LRP1 were similar among the species (Table 5.1).

5.3 Necessities of Plasma Membrane Proteome Analysis for BBB Research

BBB transport functions are mainly regulated by plasma membrane proteins, such as transporters, receptors, and tight junction proteins. To maintain CNS health, selective permeability of endogenous metabolites by the BBB is required, and these various plasma membrane proteins cooperate with each other for this purpose. In case of the tight junction as the physical barrier of the BBB, a study has shown that knockout of the claudin-5 gene in mice showed only a minor effect on the BBB

physical barrier, despite the fact that claudin-5 is considered to be an essential protein at the tight junctions (Nitta et al. 2003). This indicates that the tight junctions might be formed of other proteins in addition to claudin-5. Moreover, for the transport functions of the BBB, the concentration of small compounds in the CNS is regulated by multiple transporter proteins that are responsible for efflux or influx actions.

The subcellular localization of plasma membrane proteins is dynamically changed, which affects their functions. It has been reported that the protein level of ABCG2 on the plasma membrane is highly correlated with its transporter activity (Liu et al. 2017). The expression level of plasma membrane proteins is relatively low within the proteome. The mRNA levels do not correlate completely with protein expression levels (Ohtsuki et al. 2012). Therefore, to understand the role of the expression of plasma membrane proteins in the BBB functions, it is important to enrich the plasma membrane fraction from the cells and comprehensively analyze the membrane proteins by quantitative and targeted proteomic approaches.

5.4 Methodologies of Plasma Membrane Preparation

To perform plasma membrane proteomics, it is necessary to collect the plasma membrane fraction using a compatible sample preparation method for proteomic analysis. To date, several plasma membrane enrichment protocols have been reported. Sucrose density gradient centrifugation was reported by Boone et al. (Boone et al. 1969) and has been applied to analyze various cell types and tissues. This protocol has been established since long, and it is possible to obtain other cellular organelles at the same time. However, it requires a gradient maker as well as an ultracentrifuge. Affinity chromatography-based purification protocols use antibodies or lectins to target cell surface proteins (Lee et al. 2008; Pahlman et al. 1979; Lawson et al. 2006). The enrichment efficiency of plasma membranes by these protocols was greater than that of sucrose density centrifugation. However, the plasma membrane fractions include several contaminants of antibodies or lectin proteins, which affect the accurate quantification of proteins in the subsequent steps. In addition, these traditional separation protocols require a large number of cells (at least 10^9 cells as the starting material). On the other hand, large-scale proteome profiling methods require lesser amounts of starting materials owing to the continual advancements in LC-MS techniques.

Recently, we have demonstrated that the Plasma Membrane Extraction Kit (BioVison, USA) with a modified protocol is effective for the enrichment of the plasma membrane fraction from as low as 5×10^6 cells using HEK293 cells and human BBB model cell lines, and it is a proteomics compatible approach (Fig. 5.1) (Masuda et al. 2019). In this method, Na^+/K^+ ATPase, which was used as a plasma membrane marker protein, was highly enriched in the plasma membrane fraction, whereas GM130 and COX4 proteins, which are organelle markers, were effectively eliminated from the plasma membrane fraction. One of the tips for using this kit is

to use a fully thawed cell pellet. It is important to uniformly break the cells to obtain a highly pure plasma membrane fraction. A partially frozen cell pellet results in a lack of uniformity in cell disruption. Moreover, this kit does not require ultracentrifugation and yields a few micrograms of plasma membrane proteins, which is sufficient for recent large-scale quantitative proteomics (Fig. 5.1). In addition, the plasma membrane fraction can be collected as a pellet, which is directly applied to sample preparation for LC-MS/MS-based proteomics.

5.5 Comparison of Plasma Membrane Proteome Between Two BBB Cell Lines

In a previous study by Kubo et al., luminal-rich and abluminal-rich fractions of the plasma membrane of brain microvessels were prepared from 100 g of porcine brain using Ficoll density gradient centrifugation (Kubo et al. 2015). From the values quantified for each fraction by QTAP, they successfully determined the luminal- and abluminal-distribution ratios of membrane transporter proteins in brain microvessels comprehensively. Although plasma membrane proteomics of intact brain microvessels can provide important information, large amounts of brain are necessary for sample preparation. Due to this limitation in analyzing the human BBB,

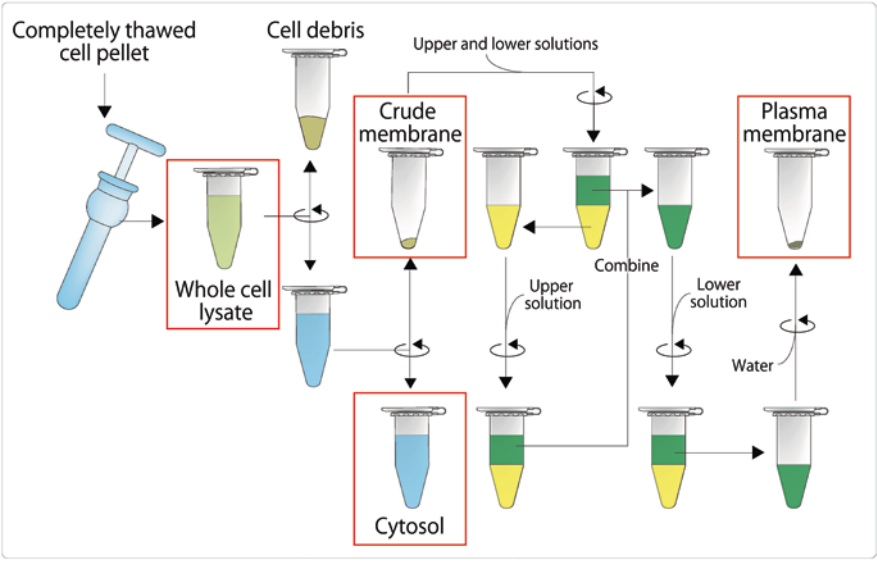

Fig. 5.1 Schematic representation of plasma membrane preparation procedure using the Plasma Membrane Extraction Kit
The subcellular fractions, including the cytosol, crude membranes, and plasma membrane fractions, were prepared from cultured cells. Detailed information regarding the preparation procedure has been described in our previous report (Masuda et al. 2019)

in vitro human BBB cell models have been established and used not only for the development of drugs targeting the CNS but also for studying BBB biology. Several groups have reported different approaches to study the BBB model cell line, such as immortalizing the human brain microvascular endothelial cells (HBMEC) (Kamiichi et al. 2012; Stins et al. 2001; Weksler et al. 2005) and deriving them from human embryonic, pluripotent, or cord blood hematopoietic stem cells (Boyer-Di Ponio et al. 2014; Cecchelli et al. 2014; Lippmann et al. 2012). The expression of major signature proteins of BBB, such as GLUT1, LAT1, MCT1, MDR1, ZO-1, and occludin, was confirmed by mRNA and/or protein expression analysis. In addition, it was validated that all these models have selective permeability and physical barriers, which are observed in the in vivo BBB. The uptake and efflux activities were measured using marker compounds, such as acetylated low-density lipoproteins, rhodamine, and verapamil. Interestingly, stem cell-derived BBB models show relatively high transendothelial electrical resistance values (TEER) compared to immortalized BBB model cell lines (Helms et al. 2016). However, MDR1 expression was insufficient in stem cell-derived models (Kurosawa et al. 2018; Ohshima et al. 2019). To assist researchers in selecting the most appropriate cell line for specific purposes, information on the large-scale plasma membrane protein expression profile of these BBB model cell lines would be beneficial.

hCMEC/D3 and HBMEC/ciβ cells are brain microvascular endothelial cell lines and were established by immortalization through transduction of a human telomerase reverse transcriptase (hTERT) subunit and a simian virus 40 large T antigen (SV40T) (Weksler et al. 2005) or temperature-sensitive SV40T (tsSV40T) (Kamiichi et al. 2012). Both cell lines showed similar TEER values (5–20 Ω cm^2) and sodium fluorescein permeability values (1–3 × 10^{-5} cm/s) (Eigenmann et al. 2013; Furihata et al. 2015). Recently, to assist researchers in selecting the most appropriate cell line, large-scale quantitative proteomic data of the plasma membrane fractions was reported (Masuda et al. 2019). The plasma membrane fractions enriched by the Plasma Membrane Extraction Kit were subjected to comparative proteomics, and 2350 proteins were quantified. This dataset contains 345 plasma membrane proteins, and the expression levels of 100 and 35 out of the 345 proteins were significantly increased or decreased in hCMEC/D3 to HBMEC/ciβ, respectively.

As shown in Fig. 5.2, hCMEC/D3 cells expressed higher levels of amino acid transporters (SNAT1, SNAT2, SNAT5, ASCT1, CAT1, and LAT1), ABC transporters (BCRP, MDR1, and MRP4), and GLUT1 than HBMEC/ciβ. The expression level of TFRC was also 4.56-fold greater in hCMEC/D3 cells. In another report, the absolute amounts of plasma membrane proteins were compared between hCMEC/D3 cells and human brain microvessels (Ohtsuki et al. 2013). MDR1 levels in hCMEC/D3 cells were similar to those in human brain microvessels, indicating that hCMEC/D3 cells are more suitable for efflux assays than HBMEC/ciβ cells. In addition to P-gp, the protein expression profile suggested that the sensitivity and dynamic range of the ABC transporter-mediated efflux assay and TFRC-mediated uptake assay is higher in hCMEC/D3 cells than in HBMEC/ciβ cells. The internalization of plasma membrane proteins was comprehensively identified in hCMEC/D3 cells by the combination of surface biotinylation and quantitative proteomics

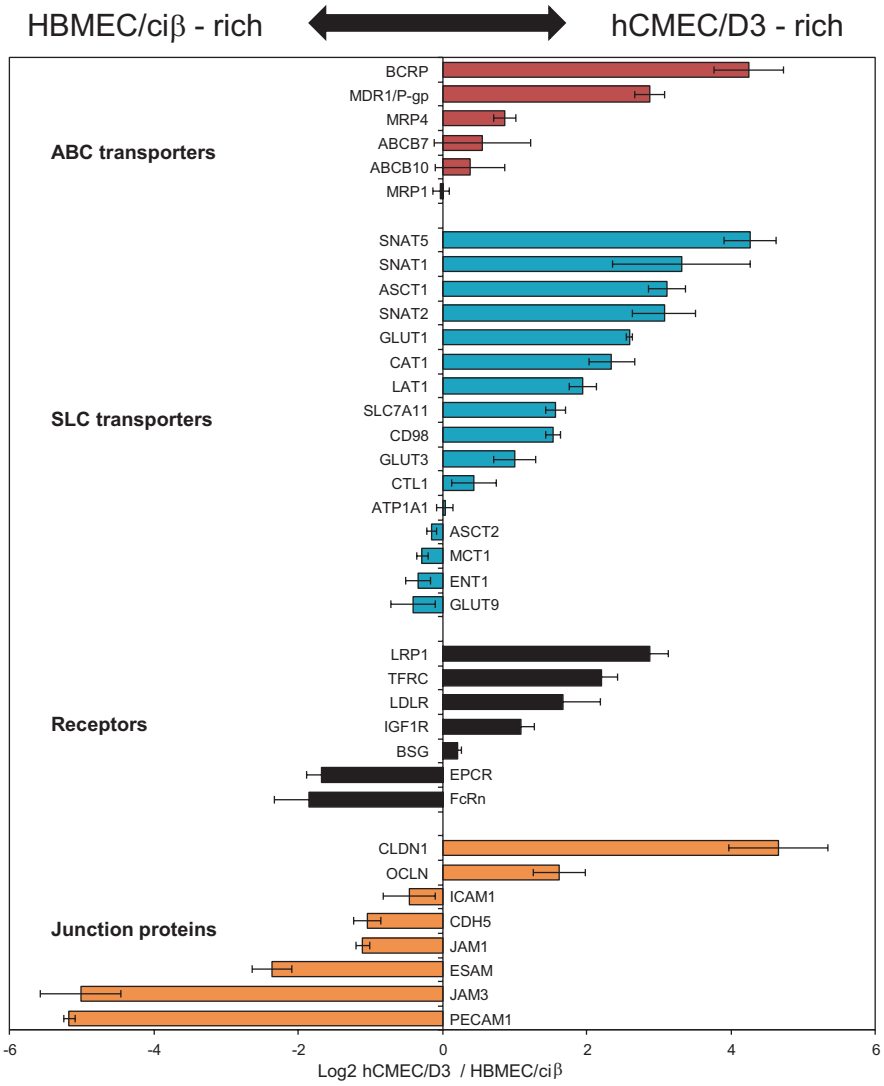

Fig. 5.2 Comparison of protein expression levels between two types of cells used as BBB models The ratios of the proteins expressed in hCMEC/D3 cells were compared to that in HBMEC/ciβ cells. The original protein expression data have been reported in our previous paper (Masuda et al. 2019)

(Ito et al. 2020). Among the identified internalized proteins, TFRC showed the most abundant levels in the internalization fraction.

In contrast, junction proteins, such as PECAM1, JAM1, JAM3, and ESAM as well as neonatal Fc receptor (FcRn), were highly expressed in HBMEC/ciβ cells compared to hCMEC/D3 cells. FcRn mediates the clearance of IgG from the CNS

to the blood across the BBB (Zhang and Pardridge 2001). For efficient pharmacological treatment with antibodies, it is important to understand the FcRn-mediated clearance of IgG from the brain, which might indicate that HBMEC/ciβ cells are a more suitable model cell line for antibody retention assays. In addition, HBMEC/ciβ cells might form better tight junctions than hCMEC/D3 cells as tight junction protein levels in HBMEC/ciβ cells were higher (2.19-fold on an average of eight proteins) than in hCMEC/D3 cells. From these results, it was concluded that HBMEC/ciβ cells might be a more suitable BBB model cell line than hCMEC/D3 for IgG-clearance assay and for integrity assays of tight junctions. Similarly, plasma membrane proteome analysis is also an effective technique for assessing the effect of culture conditions on cellular functions as a BBB model.

5.6 Conclusion

This chapter summarizes the application of quantitative and targeted proteomics for assessing animal and cultured cell models for human BBB. However, this is only one aspect of the applications, and proteomics can also be used for identifying novel targets for drug development and brain delivery at the BBB. The unique characteristics of the protein are due to their posttranslational modifications, such as phosphorylation. Phospho-proteomics has also been conducted to understand the posttranslational regulation of MDR1 activity at the BBB (Hoshi et al. 2019). Currently, highly sensitive proteomic methods are available, and the expression levels of a number of proteins can be compared from samples whose amounts are as low as 1 μg. For the progress of BBB proteomics, one of the important challenges is the purification of brain microvessels. As mentioned above, contamination of other brain cells cannot be excluded in the proteomic data from isolated brain microvessels. Furthermore, at least five mouse brains are necessary to isolate brain microvessels. To overcome this, protein expression can be confirmed using immunohistochemical analysis, but it is difficult to obtain antibodies for specific modifications in the target proteins. Recently, we developed a new method to isolate brain microvessels from single frozen mouse brains with higher purity than the standard isolation method (Ogata et al. 2021). This method can produce multiple sample preparations in parallel using a bead homogenizer. Despite higher purity, contaminants from other brain cells were still observed. Therefore, purification methods need to be improved for brain microvessels to obtain better omics data, including proteomics.

The absolute amounts of transporters obtained using QTAP are also important information for predicting drug distribution in the brain. Such prediction studies have been reported in terms of in vitro to in vivo extrapolation from the liver and intestine using the relative expression factor method. Uchida et al. reported that the transporter activity of MDR1/Mdr1a in the mouse BBB and the drug distribution in the mouse and monkey brain can be reconstructed using the absolute amounts of MDR1/Mdr1a in vitro and in vivo (Uchida et al. 2011b, 2014a, b). Since proteins are functional molecules in our body, proteomics will help us to promote BBB research.

Points of Discussion

- How do the function and molecular expression differ in the BBB of different brain regions?
- How does the BBB proteome change as a function of age and sex?
- How is the BBB proteome altered in disease conditions, and what are the species differences in the changes?
- What kind of transporters are involved in organic anion and cation transport across the human BBB?
- Which subtypes of ABCC/MRP are expressed in the human BBB, and which one has the highest contribution to BBB transport?
- What types of membrane proteins are involved in macromolecular transport across the BBB?
- How can the purity of isolated brain microvessels for omics analysis be improved?
- How can the polarized proteome in brain microvascular endothelial cells be addressed?

References

Agarwal S, Uchida Y, Mittapalli RK, Sane R, Terasaki T, Elmquist WF (2012) Quantitative proteomics of transporter expression in brain capillary endothelial cells isolated from P-glycoprotein (P-gp), breast cancer resistance protein (Bcrp), and P-gp/Bcrp knockout mice. Drug Metab Dispos 40(6):1164–1169. https://doi.org/10.1124/dmd.112.044719

Boone CW, Ford LE, Bond HE, Stuart DC, Lorenz D (1969) Isolation of plasma membrane fragments from HeLa cells. J Cell Biol 41(2):378–392. https://doi.org/10.1083/jcb.41.2.378

Boyer-Di Ponio J, El-Ayoubi F, Glacial F, Ganeshamoorthy K, Driancourt C, Godet M, Perriere N, Guillevic O, Couraud PO, Uzan G (2014) Instruction of circulating endothelial progenitors in vitro towards specialized blood-brain barrier and arterial phenotypes. PLoS One 9(1):e84179. https://doi.org/10.1371/journal.pone.0084179

Braun C, Sakamoto A, Fuchs H, Ishiguro N, Suzuki S, Cui Y, Klinder K, Watanabe M, Terasaki T, Sauer A (2017) Quantification of transporter and receptor proteins in dog brain capillaries and choroid plexus: relevance for the distribution in brain and CSF of selected BCRP and P-gp substrates. Mol Pharm 14(10):3436–3447. https://doi.org/10.1021/acs.molpharmaceut.7b00449

Cecchelli R, Aday S, Sevin E, Almeida C, Culot M, Dehouck L, Coisne C, Engelhardt B, Dehouck MP, Ferreira L (2014) A stable and reproducible human blood-brain barrier model derived from hematopoietic stem cells. PLoS One 9(6):e99733. https://doi.org/10.1371/journal.pone.0099733

Eigenmann DE, Xue G, Kim KS, Moses AV, Hamburger M, Oufir M (2013) Comparative study of four immortalized human brain capillary endothelial cell lines, hCMEC/D3, hBMEC, TY10, and BB19, and optimization of culture conditions, for an in vitro blood-brain barrier model for drug permeability studies. Fluids Barriers CNS 10(1):33. https://doi.org/10.1186/2045-8118-10-33

Furihata T, Kawamatsu S, Ito R, Saito K, Suzuki S, Kishida S, Saito Y, Kamiichi A, Chiba K (2015) Hydrocortisone enhances the barrier properties of HBMEC/cibeta, a brain microvascular endothelial cell line, through mesenchymal-to-endothelial transition-like effects. Fluids Barriers CNS 12:7. https://doi.org/10.1186/s12987-015-0003-0

Gruetter R, Novotny EJ, Boulware SD, Rothman DL, Shulman RG (1996) 1H NMR studies of glucose transport in the human brain. J Cereb Blood Flow Metab 16(3):427–438. https://doi.org/10.1097/00004647-199605000-00009

Hawkins RA, Huang SC, Barrio JR, Keen RE, Feng D, Mazziotta JC, Phelps ME (1989) Estimation of local cerebral protein synthesis rates with L-[1-11C]leucine and PET: methods, model, and results in animals and humans. J Cereb Blood Flow Metab 9(4):446–460. https://doi.org/10.1038/jcbfm.1989.68

Helms HC, Abbott NJ, Burek M, Cecchelli R, Couraud PO, Deli MA, Forster C, Galla HJ, Romero IA, Shusta EV, Stebbins MJ, Vandenhaute E, Weksler B, Brodin B (2016) In vitro models of the blood-brain barrier: an overview of commonly used brain endothelial cell culture models and guidelines for their use. J Cereb Blood Flow Metab 36(5):862–890. https://doi.org/10.1177/0271678X16630991

Hoshi Y, Uchida Y, Tachikawa M, Inoue T, Ohtsuki S, Terasaki T (2013) Quantitative atlas of blood-brain barrier transporters, receptors, and tight junction proteins in rats and common marmoset. J Pharm Sci 102(9):3343–3355. https://doi.org/10.1002/jps.23575

Hoshi Y, Uchida Y, Tachikawa M, Ohtsuki S, Couraud PO, Suzuki T, Terasaki T (2019) Oxidative stress-induced activation of Abl and Src kinases rapidly induces P-glycoprotein internalization via phosphorylation of caveolin-1 on tyrosine-14, decreasing cortisol efflux at the blood-brain barrier. J Cereb Blood Flow Metab 271678X18822801. https://doi.org/10.1177/0271678X18822801

Irifune M, Fukuda T, Nomoto M, Sato T, Kamata Y, Nishikawa T, Mietani W, Yokoyama K, Sugiyama K, Kawahara M (1997) Effects of ketamine on dopamine metabolism during anesthesia in discrete brain regions in mice: comparison with the effects during the recovery and subanesthetic phases. Brain Res 763(2):281–284. https://doi.org/10.1016/s0006-8993(97)00510-6

Ito K, Uchida Y, Ohtsuki S, Aizawa S, Kawakami H, Katsukura Y, Kamiie J, Terasaki T (2011) Quantitative membrane protein expression at the blood-brain barrier of adult and younger cynomolgus monkeys. J Pharm Sci 100(9):3939–3950. https://doi.org/10.1002/jps.22487

Ito S, Oishi M, Ogata S, Uemura T, Couraud PO, Masuda T, Ohtsuki S (2020) Identification of cell-surface proteins endocytosed by human brain microvascular endothelial cells in vitro. Pharmaceutics 12(6). https://doi.org/10.3390/pharmaceutics12060579

Kamiichi A, Furihata T, Kishida S, Ohta Y, Saito K, Kawamatsu S, Chiba K (2012) Establishment of a new conditionally immortalized cell line from human brain microvascular endothelial cells: a promising tool for human blood-brain barrier studies. Brain Res 1488:113–122. https://doi.org/10.1016/j.brainres.2012.09.042

Kamiie J, Ohtsuki S, Iwase R, Ohmine K, Katsukura Y, Yanai K, Sekine Y, Uchida Y, Ito S, Terasaki T (2008) Quantitative atlas of membrane transporter proteins: development and application of a highly sensitive simultaneous LC/MS/MS method combined with novel in-silico peptide selection criteria. Pharm Res 25(6):1469–1483. https://doi.org/10.1007/s11095-008-9532-4

Kubo Y, Ohtsuki S, Uchida Y, Terasaki T (2015) Quantitative determination of luminal and abluminal membrane distributions of transporters in porcine brain capillaries by plasma membrane fractionation and quantitative targeted proteomics. J Pharm Sci 104(9):3060–3068. https://doi.org/10.1002/jps.24398

Kurosawa T, Tega Y, Higuchi K, Yamaguchi T, Nakakura T, Mochizuki T, Kusuhara H, Kawabata K, Deguchi Y (2018) Expression and functional characterization of drug transporters in brain microvascular endothelial cells derived from human induced pluripotent stem cells. Mol Pharm 15(12):5546–5555. https://doi.org/10.1021/acs.molpharmaceut.8b00697

Lawson EL, Clifton JG, Huang F, Li X, Hixson DC, Josic D (2006) Use of magnetic beads with immobilized monoclonal antibodies for isolation of highly pure plasma membranes. Electrophoresis 27(13):2747–2758. https://doi.org/10.1002/elps.200600059

Lee YC, Block G, Chen H, Folch-Puy E, Foronjy R, Jalili R, Jendresen CB, Kimura M, Kraft E, Lindemose S, Lu J, McLain T, Nutt L, Ramon-Garcia S, Smith J, Spivak A, Wang ML, Zanic M, Lin SH (2008) One-step isolation of plasma membrane proteins using magnetic beads with immobilized concanavalin A. Protein Expr Purif 62(2):223–229. https://doi.org/10.1016/j.pep.2008.08.003

Lippmann ES, Azarin SM, Kay JE, Nessler RA, Wilson HK, Al-Ahmad A, Palecek SP, Shusta EV (2012) Derivation of blood-brain barrier endothelial cells from human pluripotent stem cells. Nat Biotechnol 30(8):783–791. https://doi.org/10.1038/nbt.2247

Liu H, Huang L, Li Y, Fu T, Sun X, Zhang YY, Gao R, Chen Q, Zhang W, Sahi J, Summerfield S, Dong K (2017) Correlation between membrane protein expression levels and transcellular transport activity for breast cancer resistance protein. Drug Metab Dispos 45(5):449–456. https://doi.org/10.1124/dmd.116.074245

Masuda T, Hoshiyama T, Uemura T, Hirayama-Kurogi M, Ogata S, Furukawa A, Couraud PO, Furihata T, Ito S, Ohtsuki S (2019) Large-scale quantitative comparison of plasma transmembrane proteins between two human blood-brain barrier model cell lines, hCMEC/D3 and HBMEC/cibeta. Mol Pharm 16(5):2162–2171. https://doi.org/10.1021/acs.molpharmaceut.9b00114

Meireles M, Martel F, Araujo J, Santos-Buelga C, Gonzalez-Manzano S, Duenas M, de Freitas V, Mateus N, Calhau C, Faria A (2013) Characterization and modulation of glucose uptake in a human blood-brain barrier model. J Membr Biol 246(9):669–677. https://doi.org/10.1007/s00232-013-9583-2

Mori S, Takanaga H, Ohtsuki S, Deguchi T, Kang YS, Hosoya K, Terasaki T (2003) Rat organic anion transporter 3 (rOAT3) is responsible for brain-to-blood efflux of homovanillic acid at the abluminal membrane of brain capillary endothelial cells. J Cereb Blood Flow Metab 23(4):432–440. https://doi.org/10.1097/01.WCB.0000050062.57184.75

Mori S, Ohtsuki S, Takanaga H, Kikkawa T, Kang YS, Terasaki T (2004) Organic anion transporter 3 is involved in the brain-to-blood efflux transport of thiopurine nucleobase analogs. J Neurochem 90(4):931–941. https://doi.org/10.1111/j.1471-4159.2004.02552.x

Nitta T, Hata M, Gotoh S, Seo Y, Sasaki H, Hashimoto N, Furuse M, Tsukita S (2003) Size-selective loosening of the blood-brain barrier in claudin-5-deficient mice. J Cell Biol 161(3):653–660. https://doi.org/10.1083/jcb.200302070

Ogata S, Ito S, Masuda T, Ohtsuki S (2021) Efficient isolation of brain capillary from a single frozen mouse brain for protein expression analysis. J Cereb Blood Flow Metab 41(5):1026–1038. https://doi.org/10.1177/0271678X20941449

Ohshima M, Kamei S, Fushimi H, Mima S, Yamada T, Yamamoto T (2019) Prediction of drug permeability using in vitro blood-brain barrier models with human induced pluripotent stem cell-derived brain microvascular endothelial cells. Biores Open Access 8(1):200–209. https://doi.org/10.1089/biores.2019.0026

Ohtsuki S, Uchida Y, Kubo Y, Terasaki T (2011) Quantitative targeted absolute proteomics-based ADME research as a new path to drug discovery and development: methodology, advantages, strategy, and prospects. J Pharm Sci 100(9):3547–3559. https://doi.org/10.1002/jps.22612

Ohtsuki S, Schaefer O, Kawakami H, Inoue T, Liehner S, Saito A, Ishiguro N, Kishimoto W, Ludwig-Schwellinger E, Ebner T, Terasaki T (2012) Simultaneous absolute protein quantification of transporters, cytochromes P450, and UDP-glucuronosyltransferases as a novel approach for the characterization of individual human liver: comparison with mRNA levels and activities. Drug Metab Dispos 40(1):83–92. https://doi.org/10.1124/dmd.111.042259

Ohtsuki S, Ikeda C, Uchida Y, Sakamoto Y, Miller F, Glacial F, Decleves X, Scherrmann JM, Couraud PO, Kubo Y, Tachikawa M, Terasaki T (2013) Quantitative targeted absolute proteomic analysis of transporters, receptors and junction proteins for validation of human cerebral microvascular endothelial cell line hCMEC/D3 as a human blood-brain barrier model. Mol Pharm 10(1):289–296. https://doi.org/10.1021/mp3004308

Pahlman S, Ljungstedt-Poahlman I, Sanderson A, Ward PJ, Grant A, Hermon-Taylor J (1979) Isolation of plasma-membrane components from cultured human pancreatic cancer cells by immuno-affinity chromatography of anti-beta 2M sepharose 6MB. Br J Cancer 40(5):701–709. https://doi.org/10.1038/bjc.1979.250

Pardridge WM (1983) Brain metabolism: a perspective from the blood-brain barrier. Physiol Rev 63(4):1481–1535. https://doi.org/10.1152/physrev.1983.63.4.1481

Stins MF, Badger J, Sik Kim K (2001) Bacterial invasion and transcytosis in transfected human brain microvascular endothelial cells. Microb Pathog 30(1):19–28. https://doi.org/10.1006/mpat.2000.0406

Syvanen S, Lindhe O, Palner M, Kornum BR, Rahman O, Langstrom B, Knudsen GM, Hammarlund-Udenaes M (2009) Species differences in blood-brain barrier transport of three positron emission tomography radioligands with emphasis on P-glycoprotein transport. Drug Metab Dispos 37(3):635–643. https://doi.org/10.1124/dmd.108.024745

Uchida Y, Ohtsuki S, Katsukura Y, Ikeda C, Suzuki T, Kamiie J, Terasaki T (2011a) Quantitative targeted absolute proteomics of human blood-brain barrier transporters and receptors. J Neurochem 117(2):333–345. https://doi.org/10.1111/j.1471-4159.2011.07208.x

Uchida Y, Ohtsuki S, Kamiie J, Terasaki T (2011b) Blood-brain barrier (BBB) pharmacoproteomics: reconstruction of in vivo brain distribution of 11 P-glycoprotein substrates based on the BBB transporter protein concentration, in vitro intrinsic transport activity, and unbound fraction in plasma and brain in mice. J Pharmacol Exp Ther 339(2):579–588. https://doi.org/10.1124/jpet.111.184200

Uchida Y, Tachikawa M, Obuchi W, Hoshi Y, Tomioka Y, Ohtsuki S, Terasaki T (2013) A study protocol for quantitative targeted absolute proteomics (QTAP) by LC-MS/MS: application for inter-strain differences in protein expression levels of transporters, receptors, claudin-5, and marker proteins at the blood-brain barrier in ddY, FVB, and C57BL/6J mice. Fluids Barriers CNS 10(1):21. https://doi.org/10.1186/2045-8118-10-21

Uchida Y, Ohtsuki S, Terasaki T (2014a) Pharmacoproteomics-based reconstruction of in vivo P-glycoprotein function at blood-brain barrier and brain distribution of substrate verapamil in pentylenetetrazole-kindled epilepsy, spontaneous epilepsy, and phenytoin treatment models. Drug Metab Dispos 42(10):1719–1726. https://doi.org/10.1124/dmd.114.059055

Uchida Y, Wakayama K, Ohtsuki S, Chiba M, Ohe T, Ishii Y, Terasaki T (2014b) Blood-brain barrier pharmacoproteomics-based reconstruction of the in vivo brain distribution of P-glycoprotein substrates in cynomolgus monkeys. J Pharmacol Exp Ther 350(3):578–588. https://doi.org/10.1124/jpet.114.214536

van Gelder NM (1989) Brain taurine content as a function of cerebral metabolic rate: osmotic regulation of glucose derived water production. Neurochem Res 14(6):495–497. https://doi.org/10.1007/bf00964908

Veenstra TD (2007) Global and targeted quantitative proteomics for biomarker discovery. J Chromatogr B Analyt Technol Biomed Life Sci 847(1):3–11. https://doi.org/10.1016/j.jchromb.2006.09.004

Warren MS, Zerangue N, Woodford K, Roberts LM, Tate EH, Feng B, Li C, Feuerstein TJ, Gibbs J, Smith B, de Morais SM, Dower WJ, Koller KJ (2009) Comparative gene expression profiles of ABC transporters in brain microvessel endothelial cells and brain in five species including human. Pharmacol Res 59(6):404–413. https://doi.org/10.1016/j.phrs.2009.02.007

Weksler BB, Subileau EA, Perriere N, Charneau P, Holloway K, Leveque M, Tricoire-Leignel H, Nicotra A, Bourdoulous S, Turowski P, Male DK, Roux F, Greenwood J, Romero IA, Couraud PO (2005) Blood-brain barrier-specific properties of a human adult brain endothelial cell line. FASEB J 19(13):1872–1874. https://doi.org/10.1096/fj.04-3458fje

Young LT, Warsh JJ, Kish SJ, Shannak K, Hornykeiwicz O (1994) Reduced brain 5-HT and elevated NE turnover and metabolites in bipolar affective disorder. Biol Psychiatry 35(2):121–127. https://doi.org/10.1016/0006-3223(94)91201-7

Zhang Y, Pardridge WM (2001) Mediated efflux of IgG molecules from brain to blood across the blood-brain barrier. J Neuroimmunol 114(1–2):168–172. https://doi.org/10.1016/s0165-5728(01)00242-9

Zhang Y, Sloan SA, Clarke LE, Caneda C, Plaza CA, Blumenthal PD, Vogel H, Steinberg GK, Edwards MS, Li G, Duncan JA 3rd, Cheshier SH, Shuer LM, Chang EF, Grant GA, Gephart MG, Barres BA (2016) Purification and characterization of progenitor and mature human astrocytes reveals transcriptional and functional differences with mouse. Neuron 89(1):37–53. https://doi.org/10.1016/j.neuron.2015.11.013